Environmental modulation of mucosal immunity

Opportunities in respiratory viral infections

Marcel Alfons Schijf

Cover Design: Juan Osborne "a picture is worth a thousand words"
Thesis Design and Printing: Optima Grafische Communicatie, Rotterdam

ISBN: 978-94-6169-456-0

Environmental modulation of mucosal immunity
Opportunities in respiratory viral infections

Modulatie van mucosale immuniteit door omgevingsfactoren
Mogelijkheden in virale luchtweg infecties

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties,
in het openbaar te verdedigen op
woensdag 11 december 2013 des middags te 12.45 uur

door

Marcel Alfons Schijf

geboren op 11 juni 1974 te De Meern

Promotor: Prof. dr. J. Garssen

Co-promotoren: Dr. Ir. G.M. van Bleek
Dr. B. van't Land

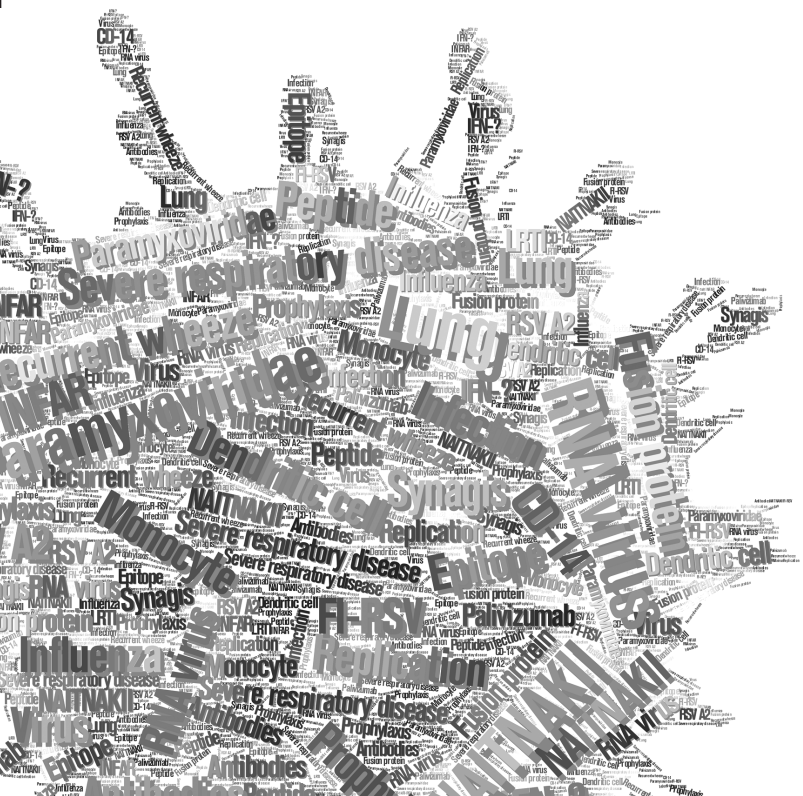
The research presented in this thesis was funded by the Dutch Top Institute Pharma (TIPharma, www.tipharma.com, grant D1.101-0) and Danone Research - Centre for Specialised Nutrition.

Printing of this thesis was kindly supported by Danone Research, Gilles Hondius Foundation and BD Biosciences.

CONTENTS

Chapter 1

General Introduction



I. RSV

1. Virology

Acute lower respiratory tract infections (ALRI) are a great burden of disease worldwide. In children less than 5 years of age, respiratory syncytial virus (RSV) is accountable for 20% of all ALRI diagnosed [1] and RSV induced bronchiolitis is the major cause of respiratory failure in children under 1 year of age [2,3]. Studies published on RSV originate from 1956 when Morris *et al* isolated this pneumovirus from chimpanzees [4]. Only one year later Chanock discovered that this virus was also pathogenic to humans and that infection of pulmonary epithelial cells typically resulted in the formation of syncytia [5]. Now 57 years later, many epidemiologic and fundamental studies on the immunologic and pathogical aspects RSV disease have not led to a safe vaccine against this virus.

RSV is a 120-300nm pneumovirus that belongs to the order *Mononegavirales* and family of *Paramyxoviridae*. In general, 2 major antigenic subgroups of this enveloped, single stranded, negative sense RNA virus are recognized. These serogroups, classified as A and B, are not associated with disease severity [6,7]. With a total of 11 distinct viral proteins on 10 separate mRNAs, RSV is one of the most complex members of *Paramyxoviridae*. Its virion is made up of a symmetrical helix-shaped nucleocapsid enclosed by a lipid bilayer envelope derived from the host cell plasma membrane (**Figure 1**). The envelope contains three surface glycoproteins: G (attachment protein), F (fusion protein), SH (small hydrophobic protein) and the matrix proteins M and M2, essential for viral budding, assembly and RNA regulation [8,9]. Cellular attachment and fusion is mediated via RSV G and F glycoproteins in a pH independent process of clathrin mediated endocytosis or macropinocytosis [10–12]. The F protein mediates fusion of the virus to the host cell membrane and fusion of infected cells with surrounding uninfected cells to form syncytia. Although RSV-G is the major attachment

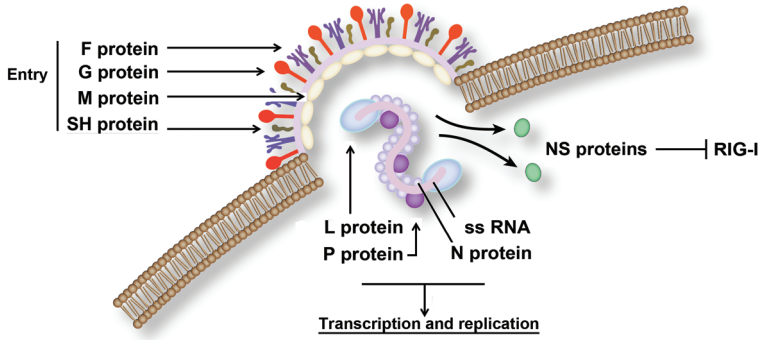


FIGURE 1: Respiratory syncytial virus and its (glyco) proteins involved in viral entry, replication and host immune suppression.

protein, natural strains with extensive mutations in the G protein or even lacking the G protein have been described, indicating that the G protein is not essential for infection, whereas the F protein seems to be indispensable [13,14]. Antibodies to F prevent infection if administered prior to infection [15]. Due to the location of F and G on the viral and cell surface, these antigenic proteins are major targets for vaccine development. The function of the third membrane protein, the minor short trans-membrane SH glycoprotein is not exactly known. Studies have shown that SH deletion mutants are fully viable in tissue culture and mice [16]. The SH protein shares structural features with a class of small hydrophobic proteins, the viroporins, that induce permeability of the cell membrane to ions and small molecules [17], indicating that SH might play a role in cell permeabilisation. Others suggested a role for SH in TNF-α mediated apoptotic signaling pathways [18].

A key step in the virus life cycle is the process of cell entry. Binding of RSV to glycosaminoglycans (GAGs) like heparin sulphate and chondroitin sulphate present on the membrane is important for viral attachment [19,20]. However, *in vitro* studies have shown that infection does not entirely depends on GAGs [21] suggesting that another receptor must be involved. Candidate receptors include intracellular adhesion molecule (ICAM)-1 [22], fractalkine (FKN) receptor CX3CR1 [23,24], annexin II [25] and Toll like receptor (TLR)-4 [26–28]. Furthermore, nucleolin has recently been added to the list of viral receptors binding to RSV F protein [29], however the exact receptor/mechanism for RSV entry remains unidentified.

RSV RNA synthesis depends on five proteins N, L, P, M2-1 and M2-2 that form the nucleocapsid. Genomic RNA is encapsulated by the N protein. RNA synthesis is carried out by the large L polymerase protein, polymerase co-factor Phosphoprotein P and the RNA synthesis factors M2-1 and M2-2. The M2-1 protein is a co-factor involved in RNA transcription whereas the M2-2 protein shifts RNA synthesis from transcription to RNA replication [30]. The amount of both M2 proteins present in infected cells determines the rate of viral genome replication and transcription.

The other two proteins encoded by the virus, NS1 and NS2, are not packaged in the virion, but are expressed early in infection. These accessory proteins are involved in the inhibition of type I IFN production and signalling and are believed to be involved in controlling RNA transcription [31–34]. *Paramyxoviridae* use accessory proteins to suppress transcription and RNA replication to avoid excessive production of unencapsidated genomes and dsRNA and in this manner decrease the risk of recognition by RIG-I, MDA-5 and other pattern recognition receptors (PRR) involved in innate immune responses [35–37]. Suppression of type I IFN signalling in the host cell by NS1 and NS2 proteins involves multiple inhibition strategies, where they exploit their inhibitory activities individually, as well as cooperatively. At least three key signaling molecules of the cellular type I IFN induction and response pathways are targets of these proteins namely: TRAF3 and IKKε (downstream signal proteins from both the RIG-I and TLR pathways) and STAT2 (induction of IFN−α, −β via the IFNAR signaling pathway) allowing RSV to evade both viral RNA and viral F protein-induced recognition

pathway's. Furthermore, the loss of STAT2 activation abrogates IFNAR signaling and the cellular response to type I IFN [38].

2. Epidemiology and Pathogenesis

RSV causes seasonal outbreaks of respiratory disease. Epidemiologic studies show that RSV infections occur during winter or rainy seasons depending on the climate, with 1 or 2 clear peak infections in moderate climates. In tropical regions, the patterns are less predictable. Spreading of viral particles occur through contaminated surfaces and aerosol formation. Humans are the only know natural reservoir of RSV. Where the virus stays between epidemics and what factors regulate the epidemic cycle remains unknown. A possible explanation could be the influence of a cyclic pattern in the circulating level of protective RSV-specific antibodies to the seasonal pattern of RSV infections in temperate climates. An epidemiologic study conducted in Denmark showed that mean titers of maternally derived RSV neutralizing antibodies declined in consecutively hospitalized infants <6 months of age and reached their lowest point shortly before the peak of the following epidemic cycle [39].

Pneumonia induced by RSV is a major cause of morbidity in elderly and immunocompromised individuals [40] and RSV is the most important causative pathogen of bronchiolitis in children under 1 year of age [1,3,41–43]. Approximately 70% of all children are infected by RSV in their first year of life. By the age of 2 years nearly all children have been infected once and 50% have experienced a secondary RSV infection [44]. Primary infections are rarely asymptomatic. Children with RSV induced upper respiratory tract infections (URTI) develop mild symptoms like low grade fever, cough and rhinorrhea [44]. In approximately 10% of the children, RSV spreads to the lower respiratory tract via cell-cell contact leading to infection of epithelial cells in the bronchioles and alveoli. Characteristics of lower respiratory tract infections (LRTI) are severe cough, wheezing and dyspnea. In 1-3% of diagnosed LRTI, hospitalization is required due to nutritional problems or the need for mechanical ventilation [45]. After recovery of LRTI, 42%-75% of infants develop prolonged episodes of wheezing and have an increased risk for developing asthma later in life [46,47]. The mechanism and potential risk factors behind the development of RSV induced severe disease are still under debate. Beside viral strain and load [7,48–51] risk factors are: younger than 3 months of age, male gender, preterm birth, congenital heart disease, Down syndrome, T cell immune deficiency, older siblings, daycare attendance, indoor tobacco smoke and abbreviated breast feeding [52–54]. However, most children hospitalized with severe LRTI do not belong to any of these groups. Involvement of host genetic factors has been suggested as one other possible explanation. Genetic association studies for RSV LRTI have shown several candidates with single-nucleotide polymorphisms (SNPs) in innate immunity genes VDR (Vitamin D receptor), JUN (Jun prot-oncogene), IFNA5 and NOS2A (Inducible nitric oxide synthase) at the allele and genotype level [55]. Other genetic associations were found between the gene

for chemokine receptor CCR5 and disease severity [56]. Some studies have shown correlations between specific patterns in cytokine and chemokine expression and disease severity. Therefore another explanation could be the involvement of immune status. As severe LRTI peaks at 2-3 months of age [57,58], inappropriate responsiveness of the immature immune system present during infection may lead to the development of severe disease, due to a lack of immune maturation caused by different environmental factors.

3. Models to study RSV infection and disease

Modeling human RSV (hRSV) disease is a crucial step in the search for novel therapies and preventive measures against RSV induced disease [59]. In the last 50 years different approaches have been used to develop strategies for improved protective immunity against RSV. Beside the use of epidemiological databases and models to predict populations to minimize the spread of RSV in both industrialized and developing countries, fundamental studies on the understanding of human immunity against RSV has also been extensively conducted. Human healthy volunteers, patient material and a spectrum of animal models using human (hRSV), bovine (bRSV), ovine (oRSV) and pneumonia virus of mice (PVM) have led to many discoveries in the virology, transmission and pathogenesis of RSV disease. Experimental RSV infections in healthy volunteers are useful to study species-specific contributions of the immune response; however adults lack the unique physiology and immunology that makes infants more susceptible to respiratory infection. The use of placenta, amniotic fluid, adult and cord blood are useful tools to study the genetic factors involved in the susceptibility to RSV infections and to study mechanisms and differences in immune responsiveness to RSV in pre-matures, children and adults, but lack the influence of tissue specific conditions present upon natural infection. These specific conditions involve the chemical and physical interaction between different cell types and organs like lung and draining mediastinal lymphnodes, conditions impossible to mimic *in vitro* and therefore *ex vivo* and *in vitro* based studies will always be subjugated to *in vivo* models. Animal models form a crucial link between mechanistic tissue culture studies and phase I human trials [59]. The variety in animal models present, demonstrates that there is no animal model that completely mimics all aspects of RSV infection and RSV induced severe lower respiratory disease. Therefore animal study design must be focused on a specific characteristic of human disease to be useful in answering a specific research question [59]. A simple rationale in the choice of animal models can be based on similarities in anatomy, replication or disease parameters.

Anatomy: The discovery of "chimpanzee coryza agent", as RSV was first named, in naturally infected chimpanzees and the genetic and anatomical similarities to humans makes the chimpanzee and other primates highly relevant to study hRSV disease. However, limited viral replication and high demanding economical, logistic and ethical issues are a disadvantage of working with this model. Anatomically the perinatal (preterm and term) lamb is also a

useful model to study lung histopathology in infant RSV disease. In this model hRSV and bRSV replicates well and lambs have similar pulmonary structure to humans, including airway branching, Clara and type II cells, submucosal glands and prenatal alveologenesis [60].

Replication: Viral replication heavily depends on the animal model used. Sheep and ferrets are both animals highly permissive to viral replication. Interestingly, in ferrets RSV replicates only in the lung of infant animals and rapidly decreases permissiveness to infection when full-grown, making this model ideal for dissecting the mechanisms of age-dependent disease in humans [61]. The cotton rat (*Sigmodon hispidus*) is semipermissive for hRSV replication but remains uniformly susceptible to pulmonary infection through adulthood, thus establishing it as a useful model for long-term studies. A poor approach for viral replication studies is the use of mouse, Syrian hamster and guinea pig models. Viral replication in these models is approximately 100-fold less compared to the cotton rat although there are differences in the susceptibility to infection between different inbred mouse strains, with viral titers varying 100-fold from the most permissive DBA/2N to the least permissive strain [62].

Disease: Clinical and pathologic features of disease upon viral exposure or challenge are important parameters to study RSV induced disease. In general the animal models used can be divided into two groups: 1: to mimic severe lower respiratory tract infections and 2: to mimic vaccine-enhanced illness. Interestingly, not all animal models show clinical signs or symptoms upon infection. In Chimpanzees there are no documented studies that show pulmonary disease following primary infection or manifestations of severe disease upon re-infection and in mice only a high inoculum of virus evokes disease in some susceptible mouse strains [62]. Primary infection in mice susceptible to viral replication results in mild to moderate bronchiolitis, pulmonary inflammation and a Th1 driven immune response depending on strain used. RSV infection induces pulmonary histopathology in the cotton rat similar to that observed in humans, without clinical symptoms. RSV infection induces enhanced disease in both formalin inactivated (FI)-RSV vaccinated mice and cotton rats upon challenge. However, extensive pulmonary inflammatory response present in the lung of cotton rats contains neutrophils and lymphocytes whereas mice show a more diverse infiltration of monocytes, lymphocytes, neutrophils and large amounts of eosinophils. Despite the lack of replication in bronchioles, differences in anatomy and the high inoculum needed to evoke disease, the mouse is by far the most popular animal for experimental modeling of hRSV specific immune responses. The economic advantage above most other species, the immunological opportunities from inbred and genetically engineered mice and a broad spectrum of immunological reagents and tools available for this species, makes the mouse a popular species to unravel cellular and humoral pathway's involved in the innate and adaptive immune responses towards RSV.

Although all these models have helped to better understand RSV infections and host immunity against this virus, none of these heterologous (nonhuman) hRSV models provide a good model to study severe LRTI. The use of respiratory viral pathogens (genetically) related

to hRSV infecting their natural host provides a good alternative to hRSV animal models. Pneumonia virus of mice is closely related to RSV and belongs to the same subfamily of pneumovirusses. The group of Rosenberg showed in several studies that infection with the natural rodent pathogen PVM induces clinical signs of severe LRTI in mice. Depending on the inoculum used, robust viral replication, significant weight loss, labored breathing and airway inflammation were present [63]. Another alternative to study severe disease is the use of bovine RSV. Although bRSV differs genetically from hRSV, infection of calves with bRSV evokes disease characteristics similar to RSV infection in children, like severe pulmonary disease primarily in neonates and an incomplete immune response to primary infection resulting in repeated infections throughout life [64].

In summary, the numerous heterologous hRSV models and cognate host-pneumovirus models available provide a spectrum of tools to study each aspect of RSV induced disease, with the bRSV model most closely related to RSV infection in children with respect to disease parameters. However, animal studies do not allow evaluation of species-specific contributions. The use of adult healthy human volunteers may add extra host-specific information but lacks the age specific variation in immune responsiveness present in children. For future research, the combination of both human studies and animal models is essential to ensure progress in the development of a safe vaccine against RSV.

II. IMMUNITY AGAINST RSV

1. The innate immune response to RSV

Clearance of inhaled virus is essential for the maintenance of lung function and homeostasis. Alveolar epithelial cells form an important physical and chemical barrier between host and external pathogens to preserve the delicate balance between self and non-self. In naturally infected individuals, epithelial cells from alveoli and small airways together with phagocytes like alveolar macrophages, neutrophils and dendritic cells (DC) are the first cells that encounter RSV. Activation of these cells is a robust non-antigen specific first step of host innate immunity for successful removal of virus and on the contrary, also a main target for viral immune evasion strategies [65–68]. In contrast to the highly specific adaptive immune system, with its variety in receptors and endless capacity to recognize foreign antigens, innate immunity is specialized in detection of a restricted set of pathogen specific patterns. The recognition of these pathogen associated molecular patterns (PAMPs) depends on cell type and location of recognition. Pathogen recognition receptors located on the cell membrane are specialized in the recognition of highly glycosylated components like RSVs glycoproteins F and G or bacterial cell wall components such as peptidoglycan (PGN) and endotoxin (LPS). Evolutionary these patterns are not shared with mammalian cells and thereby enable pattern recognition receptors (PRR) to discriminate between self and non-self. The Toll like receptor

superfamily (TLR) represents a set of transmembrane glycoproteins; each with a specific number of leucine rich repeat (LRR) motifs in its extracellular domain, which recognize a specific pattern.

Surface bound PRR: From the 13 TLRs identified in man and mouse, TLR 1, 2, 4, 5, 6 and probably TLR10 (human), TLR11, TLR12 (mice) are surface receptors present on the membrane of cells involved in innate immunity. Interestingly, TLR1 and TLR2 ligands present in bacterial wall components of Gram^{+} and Gram^{-} bacteria facilitate enhanced binding of RSV to heterodimers of TLR1/2 without activation of this complex, leading to increased binding and infection of human target cells [69]. Bacterial lipoproteins could therefore be a co-factor enhancing RSV infection and increased hospitalization of children with RSV LRTI, diagnosed with respiratory bacterial co-infections. Knockout studies in mice have shown that RSV activates heterodimers of TLR2/6 and TLR4, leading to neutrophil recruitment, DC activation and reduced viral titers [70]. In C57BL/6 mice, TLR3 activation does not affect viral clearance, but it is important in maintaining the inflammatory response against RSV [71]. TLR4, the receptor for lipopolysaccharide from Gram^{-} bacteria, forms a complex with MD2 and its co-receptor CD14. In mice and human PBMC the interaction of RSV-F with TLR4/CD14 complex was described for the first time in 2000 by Kurt-Jones *et al* [26]. Now 13 years later, different approaches to study the mechanism behind the involvement of RSV in TLR4 signalling and the subsequent cellular response have led to debate and inconclusive answers. In human PBMC and epithelial cells, RSV infection leads to translocation and upregulation of TLR4 [72,73]. Furthermore, the presence of two mutant TLR4 alleles (Asp299Gly and Thr399Ile) were associated with hypo-responsiveness to LPS and an increased risk of severe RSV bronchiolitis in previously healthy infants [74–76]. In line with these findings, Tulic *et al* showed that the presence of these two TLR4 single nucleotide polymorphisms (SNPs) in human broncho-epithelial cells contained normal levels of intracellular TLR4 but failed to efficiently translocate the receptor to the cell surface upon activation, leading to a decreased cellular immune response [77]. In contrast to these findings, other groups could not confirm the correlation between SNP Asp299Gly and RSV induced severe disease in previously healthy children or in risk populations with a history of respiratory dysfunction [78–81]. Adding to the debate of TLR4 polymorphisms are several *in vivo* studies conducted in TLR4-deficient mice. In these studies impaired viral clearance, a lack of NFκB activation and RSV-specific immunity measured by different groups showed conflicting results. However, possible explanations for the differences seen between TLR4 knockout compared to control mice could involve: the use of whole virus or constructs with viral proteins, background of the mouse strain used and time point of measurement post infection [27,28,82]. In addition to polymorphisms in TLR4 that suggested TLR4 involvement there is no evidence of interaction of RSV with the LPS co-receptor CD14. However recently MD-2 has been shown to interact with RSV-F, leading to NFκB activation in transfected HEK293T cells [83]. In contrast, Marr N *et al* showed with the same reporter cell line a lack of necessity of a functional human

TLR4/MD-2/CD14 complex in viral entrance, replication and NF-κB activation [84]. These studies show that an interaction between RSV and the TLR4 complex might exits, though the mechanism remains inconclusive.

Intracellular PRR: The detection of intracellular pathogen depends on TLRs, nucleotide-binding oligomerization domain (NOD) like receptors (NLR) and retinoic acid-inducible gene (RIG) like receptors (RLR). Detection of intracellular virus is based on the recognition of nucleic acids by cytoplasmic RLR or endosomal TLRs. Intracellular nucleic acid detection is divided into dsRNA; recognized via TLR-3 and RLRs like Melanoma Differentiation-Associated protein (MDA)-5 and RIG-I, ssRNA; detection via TLR7, TLR8 (human) and TLR-9 for the detection of CpG rich DNA. The evolutionary rationale behind this strategy is that intracellular replication of viral pathogen occurs in the cytoplasm. Detection of a variety of replication intermediates within the cytoplasm would therefore be a successful strategy for detection and elimination of pathogen. A fourth group of innate sensing receptors are the C-type lectin receptors (CLRs), specialized in the recognition of glycan structures. Although many antigens are glycosylated the CLRs also recognize self-glycoproteins and therefore not only detect pathogen but also facilitate processes like cell adhesion and phagocytosis [85]. Cytosolic detection of RSV virus-specific patterns in infected alveolar epithelial, alveolar macrophages and DC is mediated via RIG-I/MDA5 pathway [86,87]. In contrast to VSV and influenza virus, RSV is known to enter target cells via membrane fusion at the cell surface. However, in plasmacytoid dendritic cell populations (pDC) phagocytosis or autophagy mediated detection of ssRNA via TLR7 has been described in studies comparing WT with KO mice for PVM [88] and for RSV [89,90]. Paramyxoviruses PVM and RSV suppress TLR-9 mediated IFN-α production in pDC *in vitro* [91,92]. However, mice exposed to TLR-9 ligands during RSV infection or prior to RSV challenge could induce both protective immune responses and enhanced disease. Local mucosal immunization in the lung of (neonatal) mice with CpG ODN during infection reduces disease severity upon viral challenge [93,94] and intra muscular administration of CpG ODN during formalin inactivated (FI-)RSV vaccination reduces FI-RSV vaccine enhance disease [95]. However the same group showed that local administration of CpG ODN as a therapeutic administered 1 day post primary infection, enhanced disease severity upon re-infection.

Activation of PRR triggers a signaling cascade that involves different adaptor proteins. In all TLRs, MyD88 is the first adaptor protein activated, except for TLR3 which activates TRIF and TLR4 which signals via both adaptor proteins, depending on the location present (surface/endosomal). RLRs activate adaptor protein MAVS upon detection of ss- and dsRNA. In pDC, endosomal TLR7, TLR9 mediated MyD88 signaling leads to the translocation of IRF-7 and NFκB to the nucleus and subsequent transcription of IFN-α and pro-inflammatory cytokines like IL-6, IL-8 and TNF-α [96]. Endosomal TLR3, TLR4 mediated TRIF and cytoplasmic RLR mediated MAVS signaling leads to the translocation of IRF-3 and NFκB to the nucleus and subsequent transcription of type I IFNs and pro-inflammatory cytokines. Triggering of

surface TLR results in NFκB activation and a pro-inflammatory cytokine response [97]. The involvement of PRR in the immune response against RSV or as targets for immune evasion strategies is summarized in **Table 1**:

TABLE 1: Receptors reported to be involved in RSV recognition and infection.

	Receptor	Ligand	Location	Adaptor(s)	Involvement in RSV infection
TLR	1+2	Multiple triacyl lipopeptides	Cell surface Mo, MΦ, DC, B	TIRAP, MyD88 Mal	Enhances binding of RSV to primary cells and cell lines, **human** [69]. Mucosal immunization with RSV-virosomes merged with TLR1+2 ligands initiates a protective Ab and cellular response upon infection in BALB/c **mice** [98].
	2+6	Diacylated lipoproteins	Cell surface Mo, MΦ, DC, B	TIRAP, MyD88 Mal	Involved in RSV recognition and stimulation inhibits viral replication *in vivo*, **mice** [70].
	3	Double-stranded RNA	Endosomal B, T, NK, DC, EC	TRIF	Intranasal Poly ICLC prior to RSV or influenza infection reduces viral load but increases lung inflammation in **cotton rats** [99] Intranasal Poly ICLC prior to RSV infection reduces viral load and lung inflammation in BALB/c **mice** [100]
	4	Lipopolysaccharide	Cell surface/ Endosomal Mo, MΦ, DC, MC, EC	TRAM, TRIF TIRAP, MyD88 Mal	Binding of RSV-F, induction of inflammatory cytokines and Type I IFN, **human and mice**. [26,28,72–75,83]
	7	Single-stranded RNA	Endosomal MΦ, DC, pDC, B	MyD88	PVM and RSV infection in **mice** results in a TLR7 dependent antiviral and inflammatory response [88,89]. RSV inhibits the TLR 7 and 9 mediated type I IFN response, **human** [92]
	8	Single-stranded RNA	Endosomes Mo, MΦ, DC, N	MyD88	Inhibition of neutrophil apoptosis, **human** [101].
	9	Unmethylated CpG rich DNA	Endosomes B, MΦ, DC, pDC	MyD88	I.M. administered TLR9 in combination with FI-RSV protects against enhanced disease upon infection, BALB/c **mice** [95]**.** I.N. immunization with CpG ODN/RSV reduces viral load and increases immunity upon re-infection. BALB/c **mice** [94].
RLR	RIG-I	Single-stranded RNA	Intracellular Mo, MΦ, DC, EC	MAVS	RIG-I induced type I IFN is inhibited by RSV NS1, -2 proteins via MAVS, STAT2 and TRAF inhibition, **human** [33]
	MDA-5	Double-stranded RNA	Intracellular Mo, MΦ, DC, EC	MAVS	RSV infection results in increased induction of Type I IFNs and pro inflammatory cytokines via MAVS, **mice** [102].

TABLE 1 (continued)

	Receptor	Ligand	Location	Adaptor(s)	Involvement in RSV infection
NLR	NOD1	Meso-diaminopimelic acid (meso-DAP)	Intracellular Mo, MΦ, DC, EC	RIP2	Inhibition of NOD1 transcription and Type 1 IFN production in macrophages, **mice** [103].
	NOD2	Muramyl dipeptide (MDP)	Intracellular Mo, MΦ, DC, EC	RIP2, CARD9	Respiratory syncytial virus infection increases NOD2 signaling in an IFN-β dependent manner, **human** [104].
	NLRP3	Whole pathogen	Intracellular Mo, MΦ, DC, EC	CARD8, ASC, Pro-Casp-1	Formation of viral ion channels by RSVs SH glycoprotein in lipid bilayers of host cells activates the inflammasome, **human** [105].
CLR	DC- / (L-SIGN)	Mannose containing glycoproteins	Cell surface MΦ, DC	RAS, Raf-1	RSVs G-protein suppresses DC activation and Type I IFN induction via DC-SIGN, **human** [66].
	Collectins SP-A, -D	Mannose containing glycoproteins	Cell surface Enhanced uptake by Alv MΦ, DC, N and EC	---	RSV suppresses SP-A expression in pulmonary epithelial cells, human [106] Binding of SP-A to RSV G protein enhances attachment and viral entry, **human** [107].

B; B-cell. T; T-cell, NK; NK-cell, DC; dendritic cell, pDC; plasmatoid DC, EC; Aleveolar epithelial cell, MΦ; alveolar macrophage. N; Neutrophil.

The variety in PRR and complexity of pathogens like RSV, demonstrate that viral recognition in innate immunity is far more complex than detection by a single receptor. The combination of infectious, non-infectious or opsonized virus particles, the different cell types exposed to the virus, the cellular location and whether the virus can access the cells together with the panel of receptors triggered upon infection are all factors that contribute to the outcome of the innate response that in turn sets the stage for the subsequent antigen specific adaptive response.

2. The adaptive immune response to RSV

Upon primary infection, alveolar epithelial cells and phagocytes that are exposed to virus in the airway lumen excrete cytokines and chemokines that contribute to the recruitment of (inflammatory) cells involved in the innate response. They also cause antigen presenting cells to re-locate to the draining mediastinal lymphnodes and their maturation into efficient antigen presenting cells that activate T-cell and B-cell responses. In contrast to the rapid and robust innate response triggered upon primary viral infection, development of an adaptive immune response against RSV takes 3-11 days and leads to an antigen specific effector response and memory cells that quickly respond to subsequent re-infections [108]. Furthermore, primary and repeated infections lead to high titers of neutralizing antibody in children and adults. After birth, maternally derived antibodies are present in neonates, but

titers decline during the first 3-6 months [109]. Breast feeding ads to stable antibody titers present in serum. High antibody titers in serum are protective during RSV infections because upon (re-)infection virus is quickly neutralized, leading the lower viral loads and less tissue damage induced by the infection [110]. However, re-infections with RSV are common. This shows that immunity against RSV is incomplete and host immunity might contribute to the pathology seen upon re-infection. Differences in response between neonates and adult individuals may be explained by reduced responses upon PPR triggering in innate immune cells upon virus exposure. Suppressed or altered innate immune responses might impact on RSV disease. Virus specific (neutralizing) antibodies might affect disease by lowering viral load but also by altering innate immune responses and routes of viral antigen presentation [110].

Antigen presentation: Pathogen exposure in locations such as the lung and gastro-intestinal tract requires continuous instruction of mucosal immunity to maintain the balance between immune homeostasis and effective pathogen elimination. Antigen presentation is a central component in the formation of adaptive immunity. Important antigen presenting cells (APC) are dendritic cells (DC), sentinels underneath the epithelium that scan for pathogens. Upon contact with RSV, immature DC acquire antigen through infection or sample viral particles via phagocytic [111,112] or receptor mediated endocytic pathways like, macropinocytosis [10], caveolae [12] and clathrin-mediated endocytosis [113]. The subsequent transformation of immature DC into a mature phenotype involves the change into an antigen processing and presentation phenotype, characterized by an increase in surface CD40, -80, -83 and -86 expression and the presence of MHC class I and II molecules loaded with antigenic peptides. The processing of viral antigen in APC like DC follows 3 different pathways resulting in MCH class I presentation, MHC class I cross-presentation and MHC class II presentation. In RSV infection, cytoplasmic viral proteins are cleaved into peptidic fragments by proteasomes and translocated into the endoplasmic reticulum (ER) where they are loaded onto major histocompabillity complex (MHC) class-I molecules [114]. The process of MHC class-I presentation is present in every nucleated cell. Exogenous viral material acquired via pinocytosis is directed into the lysosome and cleaved into peptides. MHC class-II molecules processed in the ER are translocated into MHC class-II compartments where they are merged with lysosomal derived antigenic peptides [115]. However when phagocytosed material in the endosome/phagosome becomes cytoplasmatic, proteasomes cleave exogenous material into peptides and translocate these fragments into the endoplasmic reticulum (ER) where they are loaded onto MHC class-I molecules, a process called cross-presentation [116,117]. Matured DC migrating into the draining mediastinal lymphnodes present their antigen to naive $CD8^+$ or $CD4^+$ T cells. DC are dived into conventional DC (cDC) and plasmacytoid DC (pDC) based on their origin, myeloid or lymphoid precursors and the expression of a combination of specific surface markers. Human pDC are identified by the absence of lineage specific surface markers and the expression of MHC class-II, $CD11c^{dim}$, BDCA-4^+ and $CD123^+$ in combination with

the detection of type I interferons (IFN), cytokines typical for this cell type. Human cDC are myeloid derived cells divided into CD11c^{high} : CD141^{+} DC and CD1c^{+} DC. In mice, different DC subtypes can be distinguished depending on their location and origin. In secondary lymphnodes, DCs derived from bone marrow precursors are separated into 2 groups by the presence or absence of CD8α and subdivided for their CD4^{+} expression. Furthermore, in the murine lung 3 blood monocyte derived DC subtypes are identified as: CD11c^{high}, CD11b^{+}, CD103^{-} DC (CD11b^{+} cDC) located in the lung parenchyma, CD11c^{high} CD11b^{-} CD103^{+} DC (CD103^{+} cDC) located underneath the epithelium and CD11c^{int} B220^{+} pDC [118,119]. The exact location of pDC in steady state conditions is unknown.

For RSV it has been shown in a mouse primary infection model that CD103^{+} cDC localized underneath airway epithelium can be infected by RSV. Furthermore, within 48 hours after infection both CD103^{+} cDC and CD11b^{+} cDC migrate from the lung to the draining mediastinal lymphnodes where they present viral antigen to CD4^{+} and CD8^{+} T cells [120]. Interestingly, isolation of uninfected DC resident in the mediastinal lymphnodes showed that these cells acquired RSV antigen in a noninfectious process and were fully capable to initiate a CD8^{+} RSV-specific T cell response *in vitro*. As CD103^{+} cDC have the unique capacity to cross present exogenous antigen *in vitro* [121], cross presentation could be an important pathway of T cell priming in RSV infections. Although primary infection with RSV induces a strong CD8^{+} T cell response *in vivo* [122], there is evidence that RSV infected DC are impaired in their capacity to activate T cells [123,124]. This has been demonstrated using human monocyte derived DC *in vitro*. Both CD8^{+} T cell and CD4^{+} T cell activation is inhibited when monocyte derived (Mo)DC are RSV infected. This inhibition is observed as a decrease in proliferation but also in an impaired ability of activated T cells to produce cytokines. A possible contact mediated inhibitory mechanism has been suggested by Gonzales *et al* [125,126]. This group showed abrogated synapse formation between RSV infected DC and T cells. However, also soluble factors [127,128] and inhibition of an antigen presenting phenotype in APC [123] might be involved in this suppression of T cell activation. Although the majority of these studies investigating the suppression of T cell activation has been conducted with *in vitro* infected MoDC, Guerrero-Plata *et al* showed that murine *in vivo* infected cDC were also poor inducers of T cell activation, due to impaired capacity of cDC to present antigen to CD4^{+} T cells [91]. Beside the effect of RSV on DC populations in reducing T cell activation via soluble factors or impaired antigen presentation, local ratio in cDC / pDC populations has also been a factor that might influence the outcome of the subsequent T cell response and severity of disease. In infected BALB/c mice, expansion of pDC before infection results in a more Th1 type of response and enhanced viral clearance, factors that might reduce disease severity [129,130]. These examples indicate that RSV has various ways to interfere with antigen presentation by APC, which might contribute to inefficient immune responses to RSV and could be part of the explanation why RSV immunity wanes [131].

Cellular immune response to RSV: Initiation of the adaptive immune response by APC involves a cell mediated response characterized by T cells and a humeral response dominated by B cells and antibodies. Antigen presentation to naïve $CD4^+$ and CD8 T cells via respectively MHC class I and II molecules in the draining lymphnodes, leads to antigen specific T cell clonal expansion and differentiation. Depending on the local cytokine milieu present, naive $CD4^+$ T cells can differentiate into four $CD4^+$ T helper cell subtypes defined by their key transcription factor: DC derived IL-12 differentiates naive $CD4^+$ T cells into T-box expressed (Tbet) Th1 cells; IL-4 (origin under debate, probably T cells) is necessary for differentiation into GATA binding protein (GATA-3) Th2 cells; the inflammatory cytokines IL-1β, IL-6, TGF-β and DC derived IL-23 for retinoid related orphan receptor (RORγT) Th17 cells and IL-2, TGF-β for differentiation into forkhead box protein 3 (FOXP3) regulatory T cells (Treg) [132]. Differentiation into a specific Th subtype is not always permanent, especially at early stages of Th cell differentiation reprogramming one Th cell type to another has been described, demonstrating the flexibility of T cell immunity [133]. After the initial inflammatory response, activated $CD8^+$ T cells respond to RSV infection by producing large amounts of the antiviral effector cytokine IFN-γ and destroy infected cells recognized by viral peptides presented on MHC class I molecules via the granzyme/perforin pathway [122]. Effector cytokines and chemokines produced by activated $CD4^+$ T cells help the antiviral $CD8^+$ and B cell response, depending on the subset triggered [134]. In mice, primary RSV infections result in expansion of T helper (Th) 1 cell phenotype and the production of IFN-γ, 4-8 days post infection [135,136]. Activation of Th2 cells results in the production of the IL-4, -5 and -13, cytokines associated with broncho-hyperresponsiveness, eosinophil recruitment and B cell responses. This "asthma like" Th2 phenotype is seen in FI-RSV vaccine models used to mimic the FI-RSV vaccination trial from the 1960s [137]. The role of Th cells and Th17-related cytokines, such as IL-17A, IL-22 in RSV disease is not clear. Supernatant of human bronchial epithelial cells (HBECs) infected with RSV induce Th0 differentiation into Th2 and Th17 subsets [138]. In mice, RSV infection leads to IL-17 production and IL-17 might play a pathogenic role in RSV disease [139]. IL-17 knockout mice or depletion of IL-17 resulted in increased RSV specific $CD8^+$ T cell responses upon infection and decreased mucus production, viral protein and Th2 cytokines in an RSV induced allergic airway disease model [89,140]. Interestingly, in children recovering from primary RSV bronchiolitis levels of IL-17 in nasopharyngeal aspirates were higher after recovery from infection compared to the levels at initial hospital admission [141]. Despite the fact that $CD4^+$ and $CD8^+$ T cell responses contribute to viral clearance, there is still much debate about the contribution of these T cell subsets in immune pathology evoked by RSV [142–144]. In mice, several studies have shown that depletion of $CD4^+$ or $CD8^+$ T cell prior to primary infection leads to increased viral replication in the lung but also reduced illness [144]. As shown by adoptive transfer studies of RSV primed T cells into naive mice, $CD8^+$ T cell were the major cell type responsible for inducing enhanced lung pathology in mice during primary infection due to production of TNF-α [145,146]. However, lung

tissues of children suffering from a fatal case of severe RSV LRTI showed an almost complete absence of CD8-positive lymphocytes and natural killer cells [147,148]. Furthermore, in children hospitalized with severe primary infection, Heidema and Lukens *et al* both showed that numbers of RSV specific $CD8^+$ T cells in peripheral blood did not correlate with peak viral titers and clinical manifestations [122,149]. In these children, peak $CD8^+$ T cell responses were found in peripheral blood in the recovery phase when children were discharged from the hospital, suggesting a more protective role rather than a pathological role in RSV disease.

Regulatory T cells (Treg) are an important group of $CD4^+$ T cells that can control mucosal tolerance and excessive inflammation by suppression of the function of APC and different T cell subsets [150]. Most studies on the role of Treg in RSV mediated disease have been performed in mice. Regulatory T cells numbers rapidly accumulate in the broncho-alveolar space and draining lymphnodes 0-4 days post RSV infection [151]. Mice depleted of Treg show increased recruitment of $CD8^+$ T cells to the lung after RSV infection, increased viral clearance and disease severity, measured by weight loss, and elevated levels of IFN-γ and TNF-α, cytokines that may contribute to enhanced pathology [151,152]. Granzyme B producing Treg, a population only found in the lung, suppress disease enhancement and enhance viral clearance after primary RSV infection [153]. However in FI-RSV-vaccinated mice depletion of Treg does not cause additional enhancement of disease upon live virus infection [154], illustrating the importance of T cell priming and the inability of local Treg to control disease upon infection in this model.

Humoral response to RSV: The production of antibodies by plasma cells is an important arm of adaptive immunity to control viral spread, facilitate antibody-mediated phagocytosis of viral particles or antibody-dependent cell-mediated cytotoxicity (ADCC) of infected cells by complement and innate immune cells. Upon primary infection, pentameric IgM, dimer (s) IgA, IgG and IgE antibodies against RSV can be detected on mucosal surfaces and in serum of humans [155–159]. Upon infection a part of the protection against invading pathogens on mucosal surfaces like the respiratory tract is the rapid secretion of the IgA. Levels of IgA can be detected 2-12 days and IgM 5-10 days post infection and both decline within 2-3 months post infection to pre-infection levels [160]. For IgG and IgE antibody, responses are slower and peak in serum 3 to 4 weeks after infection. Memory B cells respond quickly upon re-infection by the production of massive amounts of IgG within 5-7 days [161–163]. (Re-) infections with RSV in humans result in high titers of RSV specific antibodies and relatively stable antibody titers throughout life [40,164]. In adults and especially in children with increased risk to develop severe LRTI, high antibody titers present in serum are believed to be protective [165,166]. Antibody titers present in neonates in the first months of life are maternally derived via placenta and breast milk. Several studies that investigated correlations between antibody titers in infants and hospitalization with diagnosed RSV bronchiolitis show that low serum antibody titers correlate with increased risk of RSV disease. Maternally

derived antibodies rapidly decline after birth; peak hospitalization of infants diagnosed with RSV bronchiolitis occurs in the first 6 months of life and in this same time period, a correlation between a low incidence in hospitalization of children with RSV diagnosed disease and high serum RSV-specific IgG titers present in mothers exists [109,167–169]. These indications of increased protection by high antibody titers led to the development of a passive immunity strategy against RSV. Initial prophylaxis started with the use of conventional intra venous immune globulins (IVIG). Success in lowering severity of disease and decrease hospitalizations [170] led to the development of the more specific human polyclonal RSV-IG (RespiGam) and later the 50 times more potent humanized, murine, monoclonal antibody palivizumab (Synagis). In a phase III study prophylactic administered palivizumab resulted in a 55% reduction of hospitalization upon RSV infection in children less than 35 weeks of age [171]. Furthermore, Simoes *et al* showed that in patients with no family history of asthma or recurrent wheezing prophylactic treatment with palivizumab could reduce the risk of recurrent wheeze by 80%, whereas the effects in groups with a history of atopic disease were minor [172]. This and other studies showed that prophylactic palivizumab treatment is most beneficial to premature infants, children with chronic lung disease or children with a family with no history of atopic disease [173,174]. The development of high affinity neutralizing antibodies trough affinity maturation of Palivizumab resulted in the monoclonal Ab Motavizumab [175,176], however currently no solid clinical studies exist to demonstrate its superiority above palivizumab and adverse cutaneous hypersensitivity reactions have been described for this antibody [177].

Despite the clearly beneficial role of high titers and/or high affinity RSV specific IgG antibodies, some aspects must be considered. IgG antibody are mostly present in the lower airways where they can easily enter the lumen, however most children hospitalized with RSV infection suffer from upper respiratory tract infections, a location where high amounts of IgA and low amounts of IgG antibodies are present. Currently it is not known if viral replication or high viral loads are needed to produce high antibody titers in human e.g. are attenuated viruses limited in their replication also effective? In addition, the formation of (poorly neutralizing) RSV immune complexes (IC) does not necessarily limit replication as enhanced infection due to increased binding of virus-IC to FcR on target cells has been described [178–180]. Another aspect is the change in viral pattern recognition by host immunity due to IC. In complement component C3 and B cell-deficient mice it is demonstrated that RSV-IC can mediate FI-RSV induced enhanced disease [181]. RSV-IC could provide a lack in danger signals and thereby suppress innate immunity or alter antigen presentation via FcR mediated signaling and or endosomal uptake [88] and a lack of intracellular infection mediated innate signaling.

III. PROTECTION AGAINST RSV INDUCED DISEASE.

Vaccination strategies against RSV: Since the discovery of RSV in 1957 development of a safe and effective vaccine against RSV remains a challenge. The straight forward approach to vaccinate children with a (formalin)-inactivated virus in combination with the common adjuvant aluminum hydroxide had dramatic consequences when children were infected with the natural virus. The trial conducted in 1960 resulted in 5% hospitalization of children vaccinated with FI-PIV (an equivalent *Paramyxovirus* preparation) and 80 % hospitalization of children vaccinated with FI-RSV, from whom 2 children died [137,182,183]. Examination of the hospitalized patients and post mortem analysis of the deceased revealed that the children suffered from high viral titers, bronchiolitis and a characteristic inflammatory response that differs from RSV (re-)infections e.g. cellular infiltrates containing high numbers of eosinophils and neutrophils and a strong proliferative T cell response [184].

Depletion studies in murine FI-RSV vaccination models for enhanced disease have demonstrated that IL-4, IL-5 and IL-13 producing $CD4^+$ T were mainly responsible for the immune pathogenesis evoked upon re-infection with live virus [185–187]. Depletion of $CD8^+$ T cells did not contribute to immune pathogenesis upon viral challenge. On the contrary, adoptive transfer of RSV primed $CD8^+$ cells prior to RSV challenge could inhibit development of respiratory disease in FI-RSV vaccinated mice [145,146,188]. From analogy with animal models, the mechanism of enhanced disease suggested a poor $CD8^+$ T cell priming and poorly neutralizing antibody response that resulted presumably from the lack of an appropriate innate immune response during immune priming [189]. This is supported by a study where TLR9 stimulation during FI-RSV vaccination could protect against severe disease upon challenge [95]. However, the translation of immunological mechanisms from the FI-RSV animal model to human vaccine enhanced disease should be done with caution. The use of *in vitro* cultured virus to vaccinate mice might mimic the vaccination phase in humans, the challenge with *in vitro* cultured virus differs from community acquired RSV infections, resulting in a mixed adaptive response not only against the virus, but also against cell culture proteins[190].

Due to the substantial evidence that maternally or actively derived antibody titers protect against severe RSV infections [39,173], new vaccination strategies are still focused on the production of high affinity and neutralizing antibodies. Pre-fusion structures of RSVs F glycoprotein and high conserved antigenic regions on F and G glycoproteins are main targets in vaccine research. Strategies involve chemically and genetically modified viruses [191,192] and the use of viral and bacterial vectors [161,193,194]. Furthermore, the use of virus like particles (VLPs), like viral protein attached to bacterial fragments, or the use of single epitopes on scaffold proteins, gives the opportunity to design structures that evoke a specific antiviral immune response [94,195,196]. Alternatively, passive immunization through maternal vaccination or vaccination of specific adult populations are also promising strategies in the protection of neonatal and infant immunity against RSV.

The host microbiome and mucosal immunity: An important mechanism to ensure successful pregnancy includes biased maternal and fetal immune responses. The presence of high numbers of maternal Treg and immunity biased towards a Th2 phenotype in both mother and child avoids the induction of pro-inflammatory/Th1-cell-polarizing cytokines and allogeneic reactions that increase the risk of fetus rejection [197,198]. After birth, the transition between relative sterile intra-uterine environment to the full colonization of mucosal surfaces with microorganisms and foreign proteins is challenging to neonatal immunity and a process that is still not fully understood. Colonization of skin, lung and intestinal tract with substantial amounts of microorganisms, does not result in extensive pro-inflammatory responses. Furthermore, beside the reduced capacity to produce pro-inflammatory Th1 like cytokines, poor responses to vaccination [199] and infection are also present, suggesting that neonatal immune status should be characterized as immature or impaired. However, several studies have shown that, depending on time point of sampling, *ex vivo* re-stimulation of isolated neonatal monocytes with TLR3, TLR4, TLR5 or TLR9 agonists resulted in robust IL-6, IL-10, IL-23 and TNF-α cytokine responses that were similar to adults [200–202]. The type of immune responses seen in neonates might therefore be not a result of impairment but a highly specialized tolerogenic mechanism that regulates immune adaption of the immune system to microorganisms in the first months of life. Bacterial colonization of mucosal surfaces provides signals for PRR and material for DC to present antigen in mucosa-associated lymphoid tissues (MALT) like gut-associated lymphoid tissue (GALT), bronchial/tracheal-associated lymphoid tissue (BALT) and nose-associated lymphoid tissue (NALT). The importance of bacterial populations on host innate and adaptive immunity and the susceptibility to develop disease has been demonstrated in several studies by the use of germ free, restricted flora or pre-, pro-, syn- or anti-biotic treatment animal models. Studies with antibiotic treated mice have shown that commensal microbiota can influence local and systemic immunity by targeting specific cell types like pDC, invariant NKT cells, virus-specific $CD8^+$ memory cells and marginal zone B cells [203–206]. Due to modern molecular techniques identification of bacterial DNA with Denaturing Gradient Gel Electrophoresis (DGGE) and 454 pyrosequencing of the 16S rRNA gene [207], limitations to identify only culturable bacterial populations have disappeared. Furthermore, the interest of human microbiome in health and disease have led to the start of the human microbiome project in 2007 and will provide essential data on microbiome composition in health and disease in the near future [208]. Factors that influence microbiome composition early in life are: dietary intake (breast or formula feeding), mode of delivery (caesarean section or natural birth) [209], environmental factors (difference in exposure to pathogens in Westernized countries) and genetic variation [210]. Moreover, due to the interaction between bacteria and mucosal immunity it is likely that disease also has an impact on microbiome formation [211,212]. The influence of dietary products on intestinal homeostasis of microbial communities and mucosal immunity is a continuous process. In neonates, several studies have described the importance of breast

milk in the protection against infection, bacterial colonization and neonatal immunity [213]. In breast fed children microbial populations are influenced by milk components like: bacteria [214], macronutrients (carbohydrates, fats and protein), macro/microminerals, vitamins and numerous other biologically active substrates.[215,216]. Several immune stimulatory components present in human milk are: immune cells like macrophages [217,218], polyunsaturated fatty acids (PUFAs) [219,220], immunoglobulin secretory (s)IgA and antigen specific IgG's [221], cytokines (especially IL-6, TNF-α) and chemokines (IL-8, GM-CSF) [222], soluble PRRs like TLR2 and CD14 [223,224] and Vitamin A and D [225–227]. This suggests that breast milk components have the potential to contribute directly and indirectly via microbiome formation and pathogen elimination, to early neonatal immune development.

The third most abundant component in human milk are oligosaccharides (HMOS). HMOS are not only versatile in their structure, 100 structurally and functionally distinct HMOS have been identified [228]. Beside an energy source, their non-digestible properties make them available in high concentrations in the gastrointestinal tract where they can have direct effects by inhibiting attachment of bacteria and pathogens to the surface of gut epithelial cells preventing infections [229,230]. They can also stimulate the growth of specific health promoting bacterial species like Bifidobacteria [231,232]. Furthermore, small amounts of specific prebiotic short chain galacto oligosaccharides (scGOS), long chain Fructo oligosaccharides (lcFOS) and pectin derived acidic oligosaccharides (pAOS) that share characteristics with human oligosaccharides can cross a human intestinal epithelial cell monolayer (CaCo-2) *in vitro* [233]. This suggests that *in vivo* orally supplied oligosaccharides can cross the gut epithelial barrier and may become available to a range of immune cells [234]. Several studies in human and mice have shown that these prebiotic oligosaccharides are capable of modulating local mucosal immunity and even systemic immune responses. The combination of scGOS/lcFOS with specific strains of *Bifidobacteria Breve* (Bb) or the combination of scGOS/lcFOS with TLR9 ligands upregulates galectin-9 secretion by intestinal epithelial cells. In addition, the nutritional interventions can result in alteration of allergic responses in humans [235–237]. Not only bacterial populations but also bacterial derived products are important in immune maturation and mucosal homeostasis. Potential stimulatory components are the synthesis of short chain fatty acid (SCFA) acetate, propionate and butyrate but also vitamin B by bacteria [238,239]. Furthermore, the release of microbial products like polysaccharide A (PSA) can promote intestinal immunologic tolerance to *Bacteroides fragilis* through TLR2 activation on Foxp3$^+$ regulatory T cells in mice [240]. Peptidoglycan, translocated from the gut into the systemic circulation, is sensed by NOD1 receptors and can enhance neutrophil mediated innate immunity [241]. These interactions indicate that at birth when mucosal surfaces are being colonised by microbiome communities an essential cross-talk is initiated.

The effect of microbiota composition on mucosal immune responses in local and systemic disease: Intestinal dysbiosis as a result of defective perinatal bacterial colonization is

frequently correlated to metabolic, autoimmune and allergic disease later in life [242,243]. A fundamental question in the research correlating disease susceptibility and microbiome composition is the importance of a single bacterial strain e.g. is the presence of a specific bacterial strain necessary for disease development or maintenance of health? Or is bacterial specificity of minor importance and is susceptibility of developing a specific disease dependent on the presence of a diverse gut microbiome? Several human have demonstrated that a correlation between high bacterial turnover rates or bacterial diversity in the gut and diseases like eczema, ulcerative colitis, HIV and Crohn's disease exist [244–247]. In mice, pharyngeal and intestinal microflora disruption with single or broad spectrum antibiotics both result in severe reduction in bacterial communities and defective immune responses against respiratory viral infections. Antibiotic treatment resulted in increased airway hyperresponsiveness and decreased numbers of regulatory T cells upon RSV infection [248]. Furthermore, antibiotic treatment results in increased viral titers and impaired antigen specific $CD8^+$ responses against influenza [249]. Interestingly, a single oral or rectal administration of TLR ligands 3, 4 or 9 could restore immunity upon challenge, suggesting that reduced microbial pressure impairs/altered immunity to subsequent viral infections and furthermore, that non-specific PRR triggering is sufficient to restore immunity against pathogen. The importance of PRR triggering in respiratory viral infections is also demonstrated in a PVM mouse model were disease severity was almost completely diminished when different *Lactobacillus* species were administered prior to viral infection. However, in this study the authors showed that bacterial protection against PVM infection was not dependent on a MyD88 mediated signaling pathway [250]. Therefore, some caution is warranted, because the data could also be explained by a competition of bacterial and viral binding to epithelium, limiting viral infection. In summary, these studies show that correlations between infection, inflammatory disease and bacterial colonization of mucosal sites are present. Therefore, further investigation may provide information about the consequences of diet and antibiotic use on both local and systemic diseases. Furthermore, optimal stimulation of mucosal immunity through diet or pre-, pro- and synbiotics may add to next generation (vaccination) strategies to improve protection against viral infections.

IV. SCOPE AND OUTLINE OF THIS THESIS

Reducing the risk to develop severe RSV bronchiolitis by prophylactic Palivizumab is currently, due to high costs, only available to high risk children. Despite many achievements in understanding viral infection and RSV-specific immune (evasion) mechanisms and pathology, a safe and efficacious vaccine candidate is not available yet. The use of inactivated virus or viral subunits for vaccination has proven to be a successful strategy against polio and influenza viruses [251]. On the contrary, the dramatic FI-RSV vaccination trial showed that inactivated RSV vaccines might cause increased disease upon later natural viral exposure

[183,252]. The mechanism behind the development of enhanced disease in the FI-RSV vaccination trial is still not completely understood. Many animal models have been used to investigate role of formalin disruption of RSV epitopes, the alum adjuvant and inefficient priming of B and T cell responses [189,253,254]. The enhanced disease phenotype presented in these animal FI-RSV vaccination experiments resembles some aspects from the human situation. However, infection with *in vitro* cultured virus differs from community acquired RSV infections, resulting in a mixed adaptive response not only against the virus, but also against cell culture proteins. We investigated the different components in the FI-RSV vaccine in the murine C57BL/6 mouse model and the components in the challenge virus preparation for their contribution to the biased Th2 response associated with severe disease. This work described in **chapter 2** shows that the different nature of the innate response induced by influenza virus and RSV challenge virus locally in the airways determined the outcome of the Th1/Th2 balance in the lung. Furthermore, priming of the $CD4^+$ T cell response by *in vitro* matured DC during the vaccination phase reduced enhanced disease upon RSV challenge. These data show that viral pattern recognition and the nature of the innate immune response are important factors that determine the outcome of the subsequent T and B cell response and establishment of proper immune memory. Moreover, this study challenges earlier work that suggested a specific Th2 skewing role of RSV proteins or the formalin activation process, because it was clearly shown that FCS priming caused similar Th2 biased immunity during challenge with RSV grown in tissue culture.

In **chapter 3** we investigate the innate immune response elicited upon RSV exposure. We used human epithelial cells and PBMC to determine binding and infection characteristics of RSV A2, Long and clinical isolates with specific cells in a complex mixture (PBMC) and demonstrated the cross talk of different cell types on the inflammatory and type I IFN response elicited against RSV. Additionally, we show that virus specific antibodies altered innate immune responses against RSV. In this process monocytes played an important regulatory role. RSV specific IgGs facilitated IFN-α production by pDC, a process that was inhibited in the presence of $CD14^+$ monocytes.

Neonatal protection through maternally derived RSV specific antibodies depends on the transfer of IgG via the placenta or breast milk. The function of the neonatal Fc receptor (FcRn) is to transport IgG across the placenta from mother to infant and the transfer of IgG across epithelial layers in gut and airways to the lumen [255–257]. In addition to innate immune responses evoked by RSV-IC, we investigate in **chapter 4** whether FcRn played a role in RSV immune responses, either by facilitating viral antigen delivery across the lung epithelium to antigen presenting cells, or by affecting antigen presentation in dendritic cells, because FcRn is highly expressed also in DC. We show that virus specific antibody paluvizumab indeed had a role in antigen processing / presentation and the onset of adaptive immune responses. However, we were unable to find a role of FcRn in the induction of adaptive T cell or B cell responses to RSV.

The second part of the thesis (**chapters 5, 6, 7 and 8**) investigates the interaction between the microbiome and mucosal immunity and explores the potential to improve vaccination strategies against respiratory viral infections trough microbial manipulation. Mucosal immunity plays an important role in the susceptibility to infectious diseases. Not only the initial exposure of mucosal surfaces to bacteria at birth, but also the continuous exposure to different microorganisms and nutritional components contributes to the balance between (self) tolerance and the elimination of pathogen [258,259]. We discuss recent findings on the association between microbial colonization and mucosal immunity and the therapeutic potential of nutritional pharmacology in **chapter 5**.

Breast milk contributes to neonatal protection by providing pathogen specific antibodies. In addition, other immune modulation components in breast milk also add to protection through specific immune modulation. We investigate the possible effect of specific prebiotic dietary oligosaccharides that share characteristics with human milk oligosaccharides (HMOS), on the influenza specific Type IV hypersensitivity vaccination response induced by a human influenza subunit vaccine in a Balb/c mouse model (**chapter 6)**. We show that systemic immunity can be influenced the specific dietary oligosaccharides, resulting in an increase in the local influenza specific delayed type hypersensitivity (DTH) response evoked upon challenge. We further show evidence that a possible role for regulatory T cells within this diet induced immune modulation might exist.

Accumulating data shows that adaptations in immunity to pathogen in one compartment could affect immunity in distal mucosal areas. For example, for many years correlations between asthmatic disease and the lack of (intestinal) microbial stimuli in human and animals [260–262] and a correlation between specific gut bacteria in diseases like atopic eczema and recurrent wheeze have been described [263]. Therefore, we investigate if these orally applied specific GFA oligosaccharides that affected systemic immune responses in the influenza vaccination model can also affect local lung RSV specific T cell responses in a primary RSV infection model and in a vaccination model for FI-RSV induced enhanced disease (**chapter 7)**. Specific dietary oligosaccharides reduced the allergic/Th2 immune response seen in the C57BL/6 FI-RSV vaccination model and increased Th1 responses in the primary infection models. Furthermore, investigation of specific components of the adaptive response evoked upon infection shows that the balance between lung $CD103^+$ and $CD11b^+$ DC and the number of granzyme B producing regulatory T cells are altered by this diet.

In **chapter 6 and 7** we show that dietary GFA could affect adaptive immunity in the host systemically. These specific oligosaccharide mixtures have prebiotic properties. Our preliminary studies [264,265] show altered microbiota composition in mice on a GFA diet. In neonates hospitalized with RSV infections, antibiotics are only administered when a suspected bacterial co-infection is present [266,267]. However, the impact of early antibiotic use on the susceptibility to subsequent RSV hospitalization, recurrent wheeze or the development of atopic disease later in life is poorly understood. Our work in chapters 6 and 7 suggests

that also antibiotic manipulation, since it can dramatically alter microbiota composition, might affect immune responses to RSV. To address this issue, we investigate in **chapter 8** the impact of microbiome disruption induced by broad spectrum antibiotics during FI-RSV vaccination, on the development of Th2 biased immune responses in the C57BL/6 mouse model. We show that a cocktail of broad spectrum antibiotics consisting of Ampicillin, Vancomycin, Neomycin sulfate and Metronidazole in drinking water dramatically decreases gut microbiota composition, increases lung eosinophilia and the intensifies Th2 bias present in the lung of RSV infected mice.

Based on the possible role of Treg in the immune modulation observed after microbiota manipulation by the GFA diet described in **chapters 6, 7 and 8**, we further investigate in **chapter 9** some characteristic and functional aspects of red fluorescent protein (RFP) expressing regulatory T cells in GFA affected influenza vaccination responses. We show that GFA-induced improved Th1 responsiveness was accompanied with a reduced population of CXCR3 /T-bet expressing Tregs in the mesenteric lymphnodes (MLN). Furthermore, Tregs from GFA treated animals could enhance T effector IFN-γ release and the Treg/Th17 balance by suppressing IL-17 production.

Our findings are summarized and discussed in relation to recent literature in **chapter 10**. In addition, concluding remarks and implications for future research are suggested.

REFERENCES

1. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 2010;375:1545–55.
2. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The burden of respiratory syncytial virus infection in young children. The New England Journal of Medicine 2009;360:588–98.
3. Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-Associated Hospitalizations Among US Children, 1980-1996. JAMA: The Journal of the American Medical Association 1999; 282:1440–6.
4. BLOUNT RE, MORRIS JA, SAVAGE RE. Recovery of cytopathogenic agent from chimpanzees with coryza. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY) 1956;92:544–9.
5. CHANOCK R, ROIZMAN B, MYERS R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. American Journal of Hygiene 1957; 66:281–90.
6. Stokes KL, Chi MH, Sakamoto K, Newcomb DC, Currier MG, Huckabee MM, et al. Differential pathogenesis of respiratory syncytial virus clinical isolates in BALB/c mice. Journal of Virology 2011;85:5782–93.
7. DeVincenzo JP, El Saleeby CM, Bush AJ. Respiratory syncytial virus load predicts disease severity in previously healthy infants. The Journal of Infectious Diseases 2005;191:1861–8.
8. Henderson G, Murray J, Yeo RP. Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins. Virology 2002;300:244–54.
9. Teng MN, Collins PL. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. Journal of Virology 1998;72:5707–16.
10. Krzyzaniak MA, Zumstein MT, Gerez JA, Picotti P, Helenius A. Host cell entry of respiratory syncytial virus involves macropinocytosis followed by proteolytic activation of the f protein. PLoS Pathogens 2013;9: e1003309.
11. Kolokoltsov AA, Deniger D, Fleming EH, Roberts NJ, Karpilow JM, Davey RA. Small interfering RNA profiling reveals key role of clathrin-mediated endocytosis and early endosome formation for infection by respiratory syncytial virus. Journal of Virology 2007;81:7786–800.
12. Werling D, Hope JC, Chaplin P, Collins RA, Taylor G, Howard CJ. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. Journal of Leukocyte Biology 1999;66:50–8.
13. Agoti CN, Mbisa JL, Bett A, Medley GF, Nokes DJ, Cane PA. Intrapatient variation of the respiratory syncytial virus attachment protein gene. Journal of Virology 2010;84: 10425–8.
14. Elliott MB, Pryharski KS, Yu Q, Parks CL, Laughlin TS, Gupta CK, et al. Recombinant respiratory syncytial viruses lacking the C-terminal third of the attachment (G) protein are immunogenic and attenuated in vivo and in vitro. Journal of Virology 2004; 78:5773–83.
15. Huang K, Incognito L, Cheng X, Ulbrandt ND, Wu H. Respiratory syncytial virus-neutralizing monoclonal antibodies motavizumab and palivizumab inhibit fusion. Journal of Virology 2010;84:8132–40.
16. Bukreyev A, Whitehead SS, Murphy BR, Collins PL. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture

and exhibits site-specific attenuation in the respiratory tract of the mouse. Journal of Virology 1997;71:8973–82.

17. Gonzalez ME, Carrasco L. Viroporins. FEBS Letters 2003;552:28–34.
18. Fuentes S, Tran KC, Luthra P, Teng MN, He B. Function of the respiratory syncytial virus small hydrophobic protein. Journal of Virology 2007;81:8361–6.
19. Hallak LK, Kwilas SA, Peeples ME. Interaction between respiratory syncytial virus and glycosaminoglycans, including heparan sulfate. Methods in Molecular Biology (Clifton, NJ) 2007;379:15–34.
20. Crim RL, Audet SA, Feldman SA, Mostowski HS, Beeler JA. Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity. Journal of Virology 2007;81:261–71.
21. Kwilas S, Liesman RM, Zhang L, Walsh E, Pickles RJ, Peeples ME. Respiratory syncytial virus grown in Vero cells contains a truncated attachment protein that alters its infectivity and dependence on glycosaminoglycans. Journal of Virology 2009;83: 10710–8.
22. Arnold R, König W. Respiratory syncytial virus infection of human lung endothelial cells enhances selectively intercellular adhesion molecule-1 expression. Journal of Immunology (Baltimore, Md: 1950) 2005; 174:7359–67.
23. Tripp RA, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nature Immunology 2001;2: 732–8.
24. Choi Y, Mason CS, Jones LP, Crabtree J, Jorquera PA, Tripp RA. Antibodies to the central conserved region of respiratory syncytial virus (RSV) G protein block RSV G protein CX3C-CX3CR1 binding and cross-neutralize RSV A and B strains. Viral Immunology 2012;25:193–203.
25. Malhotra R, Ward M, Bright H, Priest R, Foster MR, Hurle M, et al. Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. Microbes and Infection / Institut Pasteur 2003;5:123–33.
26. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nature Immunology 2000;1:398–401.
27. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. Journal of Virology 2001;75:10730–7.
28. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich H-J, Van Rooijen N, et al. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. The Journal of Infectious Diseases 2002;186:1199–206.
29. Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. Nature Medicine 2011;17:1132–5.
30. Bermingham A, Collins PL. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. Proceedings of the National Academy of Sciences of the United States of America 1999; 96:11259–64.
31. Schlender J, Bossert B, Buchholz U, Conzelmann KK. Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta interferon-induced antiviral response. Journal of Virology 2000;74:8234–42.
32. Bossert B, Conzelmann K-K. Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent

bovine cells. Journal of Virology 2002;76: 4287–93.
33. Ling Z, Tran KC, Teng MN. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. Journal of Virology 2009;83:3734–42.
34. Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hellermann G, et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nature Medicine 2005;11:56–62.
35. Atreya PL, Peeples ME, Collins PL. The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication. Journal of Virology 1998;72:1452–61.
36. Boonyaratanakornkit J, Bartlett E, Schomacker H, Surman S, Akira S, Bae Y-S, et al. The C proteins of human parainfluenza virus type 1 limit double-stranded RNA accumulation that would otherwise trigger activation of MDA5 and protein kinase R. Journal of Virology 2011;85:1495–506.
37. Takeuchi K, Komatsu T, Kitagawa Y, Sada K, Gotoh B. Sendai virus C protein plays a role in restricting PKR activation by limiting the generation of intracellular double-stranded RNA. Journal of Virology 2008;82:10102–10.
38. Swedan S, Musiyenko A, Barik S. Respiratory syncytial virus nonstructural proteins decrease levels of multiple members of the cellular interferon pathways. Journal of Virology 2009;83:9682–93.
39. Stensballe LG, Ravn H, Kristensen K, Meakins T, Aaby P, Simoes EAF. Seasonal variation of maternally derived respiratory syncytial virus antibodies and association with infant hospitalizations for respiratory syncytial virus. The Journal of Pediatrics 2009;154: 296–8.
40. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. The New England Journal of Medicine 2005;352: 1749–59.
41. Fishaut M, Tubergen D, McIntosh K. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. The Journal of Pediatrics 1980;96:179–86.
42. Glezen P, Denny FW. Epidemiology of acute lower respiratory disease in children. The New England Journal of Medicine 1973;288: 498–505.
43. Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC, et al. Respiratory syncytial viral infection in children with compromised immune function. The New England Journal of Medicine 1986;315: 77–81.
44. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. American Journal of Diseases of Children (1960) 1986; 140:543–6.
45. Tissing WJ, Van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. European Journal of Pediatrics 1993;152:125–7.
46. Pullan CR, Hey EN. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. British Medical Journal (Clinical Research Ed) 1982;284:1665–9.
47. Ermers MJJ, Rovers MM, Van Woensel JB, Kimpen JLL, Bont LJ. The effect of high dose inhaled corticosteroids on wheeze in infants after respiratory syncytial virus infection: randomised double blind placebo controlled trial. BMJ (Clinical Research Ed) 2009;338: b897.
48. Devincenzo JP. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatric Research 2004;56: 914–7.
49. Hornsleth A, Klug B, Nir M, Johansen J, Hansen KS, Christensen LS, et al. Severity of respiratory syncytial virus disease related to

type and genotype of virus and to cytokine values in nasopharyngeal secretions. The Pediatric Infectious Disease Journal 1998; 17:1114–21.

50. Walsh EE, McConnochie KM, Long CE, Hall CB. Severity of respiratory syncytial virus infection is related to virus strain. The Journal of Infectious Diseases 1997;175:814–20.
51. Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytical virus correlates with disease severity in hospitalized infants. The Pediatric Infectious Disease Journal 2000;19:113–7.
52. Meissner HC. Selected populations at increased risk from respiratory syncytial virus infection. The Pediatric Infectious Disease Journal 2003;22:S40–4; discussion S44–5.
53. Bloemers BLP, Van Furth AM, Weijerman ME, Gemke RJBJ, Broers CJM, Van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics 2007;120:e1076–81.
54. Simoes EAF. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. The Journal of Pediatrics 2003;143:S118–26.
55. Janssen R, Bont L, Siezen CLE, Hodemaekers HM, Ermers MJ, Doornbos G, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes. The Journal of Infectious Diseases 2007;196:826–34.
56. Hull J, Rowlands K, Lockhart E, Moore C, Sharland M, Kwiatkowski D. Variants of the chemokine receptor CCR5 are associated with severe bronchiolitis caused by respiratory syncytial virus. The Journal of Infectious Diseases 2003;188:904–7.
57. Parrott RH, Kim HW, Arrobio JO, Hodes DS, Murphy BR, Brandt CD, et al. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. American Journal of Epidemiology 1973;98:289–300.
58. Bont L, Van Vught AJ, Kimpen JL. Prophylaxis against respiratory syncytial virus in premature infants. Lancet 1999;354:1003–4.
59. Bem RA, Domachowske JB, Rosenberg HF. Animal models of human respiratory syncytial virus disease. American Journal of Physiology Lung Cellular and Molecular Physiology 2011;301:L148–56.
60. Derscheid RJ, Ackermann MR. Perinatal lamb model of respiratory syncytial virus (RSV) infection. Viruses 2012;4:2359–78.
61. Clyde WA. Experimental models for study of common respiratory viruses. Environmental Health Perspectives 1980;35:107–12.
62. Prince GA, Horswood RL, Berndt J, Suffin SC, Chanock RM. Respiratory syncytial virus infection in inbred mice. Infection and Immunity 1979;26:764–6.
63. Rosenberg HF, Domachowske JB. Pneumonia virus of mice: severe respiratory infection in a natural host. Immunology Letters 2008; 118:6–12.
64. Sow FB, Gallup JM, Krishnan S, Patera AC, Suzich J, Ackermann MR. Respiratory syncytial virus infection is associated with an altered innate immunity and a heightened pro-inflammatory response in the lungs of preterm lambs. Respiratory Research 2011; 12:106.
65. Swedan S, Andrews J, Majumdar T, Musiyenko A, Barik S. Multiple functional domains and complexes of the two nonstructural proteins of human respiratory syncytial virus contribute to interferon suppression and cellular location. Journal of Virology 2011;85:10090–100.
66. Johnson TR, McLellan JS, Graham BS. Respiratory syncytial virus glycoprotein G interacts with DC-SIGN and L-SIGN to activate ERK1 and ERK2. Journal of Virology 2012;86:1339–47.
67. Boyapalle S, Wong T, Garay J, Teng M, San Juan-Vergara H, Mohapatra S, et al. Respiratory syncytial virus NS1 protein colocalizes

with mitochondrial antiviral signaling protein MAVS following infection. PloS One 2012;7:e29386.
68. Zhao D-C, Yan T, Li L, You S, Zhang C. Respiratory syncytial virus inhibits interferon-alpha-inducible signaling in macrophage-like U937 cells. The Journal of Infection 2007;54:393–8.
69. Nguyen DT, De Witte L, Ludlow M, Yüksel S, Wiesmüller K-H, Geijtenbeek TBH, et al. The synthetic bacterial lipopeptide Pam3CSK4 modulates respiratory syncytial virus infection independent of TLR activation. PLoS Pathogens 2010;6:e1001049.
70. Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, Tripp RA, et al. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. Journal of Virology 2009;83:1492–500.
71. Rudd BD, Smit JJ, Flavell RA, Alexopoulou L, Schaller MA, Gruber A, et al. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. Journal of Immunology (Baltimore, Md: 1950) 2006; 176:1937–42.
72. Gagro A, Tominac M, Krsulović-Hresić V, Baće A, Matić M, Drazenović V, et al. Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis. Clinical and Experimental Immunology 2004;135:267–72.
73. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. The Journal of Biological Chemistry 2003;278:53035–44.
74. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. The Journal of Infectious Diseases 2004;189: 2057–63.
75. Mandelberg A, Tal G, Naugolny L, Cesar K, Oron A, Houri S, et al. Lipopolysaccharide hyporesponsiveness as a risk factor for intensive care unit hospitalization in infants with respiratory syncitial virus bronchiolitis. Clinical and Experimental Immunology 2006;144:48–52.
76. Awomoyi AA, Rallabhandi P, Pollin TI, Lorenz E, Sztein MB, Boukhvalova MS, et al. Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children. Journal of Immunology (Baltimore, Md: 1950) 2007;179:3171–7.
77. Tulic MK, Hurrelbrink RJ, Prêle CM, Laing IA, Upham JW, Le Souef P, et al. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. Journal of Immunology (Baltimore, Md: 1950) 2007;179:132–40.
78. Paulus SC, Hirschfeld AF, Victor RE, Brunstein J, Thomas E, Turvey SE. Common human Toll-like receptor 4 polymorphisms--role in susceptibility to respiratory syncytial virus infection and functional immunological relevance. Clinical Immunology (Orlando, Fla) 2007;123:252–7.
79. Löfgren J, Marttila R, Renko M, Rämet M, Hallman M. Toll-like receptor 4 Asp299Gly polymorphism in respiratory syncytial virus epidemics. Pediatric Pulmonology 2010;45: 687–92.
80. Douville RN, Lissitsyn Y, Hirschfeld AF, Becker AB, Kozyrskyj AL, Liem J, et al. TLR4 Asp299Gly and Thr399Ile polymorphisms: no impact on human immune responsiveness to LPS or respiratory syncytial virus. PloS One 2010;5:e12087.
81. Kresfelder TL, Janssen R, Bont L, Pretorius M, Venter M. Confirmation of an association between single nucleotide polymorphisms in the VDR gene with respiratory syncytial virus related disease in South African children. Journal of Medical Virology 2011;83: 1834–40.
82. Ehl S, Bischoff R, Ostler T, Vallbracht S, Schulte-Mönting J, Poltorak A, et al. The role of Toll-like receptor 4 versus interleukin-12

in immunity to respiratory syncytial virus. European Journal of Immunology 2004;34: 1146–53.

83. Rallabhandi P, Phillips RL, Boukhvalova MS, Pletneva LM, Shirey KA, Gioannini TL, et al. Respiratory syncytial virus fusion protein-induced toll-like receptor 4 (TLR4) signaling is inhibited by the TLR4 antagonists Rhodobacter sphaeroides lipopolysaccharide and eritoran (E5564) and requires direct interaction with MD-2. mBio 2012;3.
84. Marr N, Turvey SE. Role of human TLR4 in respiratory syncytial virus-induced NF-κB activation, viral entry and replication. Innate Immunity 2012;18:856–65.
85. Robinson MJ, Sancho D, Slack EC, Leibund-Gut-Landmann S, Reis e Sousa C. Myeloid C-type lectins in innate immunity. Nature Immunology 2006;7:1258–65.
86. Yoboua F, Martel A, Duval A, Mukawera E, Grandvaux N. Respiratory syncytial virus-mediated NF-kappa B p65 phosphorylation at serine 536 is dependent on RIG-I, TRAF6, and IKK beta. Journal of Virology 2010;84: 7267–77.
87. Sasai M, Shingai M, Funami K, Yoneyama M, Fujita T, Matsumoto M, et al. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. Journal of Immunology (Baltimore, Md: 1950) 2006;177:8676–83.
88. Davidson S, Kaiko G, Loh Z, Lalwani A, Zhang V, Spann K, et al. Plasmacytoid dendritic cells promote host defense against acute pneumovirus infection via the TLR7-MyD88-dependent signaling pathway. Journal of Immunology (Baltimore, Md: 1950) 2011; 186:5938–48.
89. Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, Lindell DM. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. Journal of Immunology (Baltimore, Md: 1950) 2010;185:2231–9.
90. Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood 2007;110:1578–86.
91. Guerrero-Plata A, Kolli D, Hong C, Casola A, Garofalo RP. Subversion of pulmonary dendritic cell function by paramyxovirus infections. Journal of Immunology (Baltimore, Md: 1950) 2009;182:3072–83.
92. Schlender J, Hornung V, Finke S, Günthner-Biller M, Marozin S, Brzózka K, et al. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. Journal of Virology 2005;79:5507–15.
93. Yamaguchi Y, Harker JA, Wang B, Openshaw PJ, Tregoning JS, Culley FJ. Preexposure to CpG protects against the delayed effects of neonatal respiratory syncytial virus infection. Journal of Virology 2012;86:10456–61.
94. Shafique M, Wilschut J, De Haan A. Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. Vaccine 2012;30:597–606.
95. Johnson TR, Rao S, Seder RA, Chen M, Graham BS. TLR9 agonist, but not TLR7/8, functions as an adjuvant to diminish FI-RSV vaccine-enhanced disease, while either agonist used as therapy during primary RSV infection increases disease severity. Vaccine 2009;27:3045–52.
96. Aoshi T, Koyama S, Kobiyama K, Akira S, Ishii KJ. Innate and adaptive immune responses to viral infection and vaccination. Current Opinion in Virology 2011;1:226–32.
97. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011; 34:637–50.
98. Shafique M, Meijerhof T, Wilschut J, De Haan A. Evaluation of an intranasal virosomal vaccine against respiratory syncytial virus in mice: effect of TLR2 and NOD2 ligands on induction of systemic and mucosal immune responses. PloS One 2013;8:e61287.

99. Boukhvalova MS, Sotomayor TB, Point RC, Pletneva LM, Prince GA, Blanco JCG. Activation of interferon response through toll-like receptor 3 impacts viral pathogenesis and pulmonary toll-like receptor expression during respiratory syncytial virus and influenza infections in the cotton rat Sigmodon hispidus model. Journal of Interferon & Cytokine Research: the Official Journal of the International Society for Interferon and Cytokine Research 2010;30:229–42.
100. Guerrero-Plata A, Baron S, Poast JS, Adegboyega PA, Casola A, Garofalo RP. Activity and regulation of alpha interferon in respiratory syncytial virus and human metapneumovirus experimental infections. Journal of Virology 2005;79:10190–9.
101. Lindemans CA, Coffer PJ, Schellens IMM, De Graaff PMA, Kimpen JLL, Koenderman L. Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. Journal of Immunology (Baltimore, Md: 1950) 2006;176:5529–37.
102. Bhoj VG, Sun Q, Bhoj EJ, Somers C, Chen X, Torres J-P, et al. MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. Proceedings of the National Academy of Sciences of the United States of America 2008;105:14046–51.
103. Senft AP, Taylor RH, Lei W, Campbell SA, Tipper JL, Martinez MJ, et al. Respiratory syncytial virus impairs macrophage IFN-alpha/beta- and IFN-gamma-stimulated transcription by distinct mechanisms. American Journal of Respiratory Cell and Molecular Biology 2010;42:404–14.
104. Vissers M, Remijn T, Oosting M, De Jong DJ, Diavatopoulos DA, Hermans PWM, et al. Respiratory syncytial virus infection augments NOD2 signaling in an IFN-β-dependent manner in human primary cells. European Journal of Immunology 2012;42: 2727–35.
105. Triantafilou K, Kar S, Vakakis E, Kotecha S, Triantafilou M. Human respiratory syncytial virus viroporin SH: a viral recognition pathway used by the host to signal inflammasome activation. Thorax 2013;68:66–75.
106. Bruce SR, Atkins CL, Colasurdo GN, Alcorn JL. Respiratory syncytial virus infection alters surfactant protein A expression in human pulmonary epithelial cells by reducing translation efficiency. American Journal of Physiology Lung Cellular and Molecular Physiology 2009;297:L559–67.
107. Hickling TP, Malhotra R, Bright H, McDowell W, Blair ED, Sim RB. Lung surfactant protein A provides a route of entry for respiratory syncytial virus into host cells. Viral Immunology 2000;13:125–35.
108. Welliver RC. The immune response to respiratory syncytial virus infection: friend or foe? Clinical Reviews in Allergy & Immunology 2008;34:163–73.
109. Brandenburg AH, Groen J, Van Steensel-Moll HA, Claas EC, Rothbarth PH, Neijens HJ, et al. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. Journal of Medical Virology 1997; 52:97–104.
110. Crowe JE. Influence of maternal antibodies on neonatal immunization against respiratory viruses. Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America 2001;33:1720–7.
111. Guerrero-Plata A, Ortega E, Gomez B. Persistence of respiratory syncytial virus in macrophages alters phagocytosis and pro-inflammatory cytokine production. Viral Immunology 2001;14:19–30.
112. Morris S, Swanson MS, Lieberman A, Reed M, Yue Z, Lindell DM, et al. Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses. Journal of Immunology (Baltimore, Md: 1950) 2011;187:3953–61.

113. Gutiérrez-Ortega A, Sánchez-Hernández C, Gómez-García B. Respiratory syncytial virus glycoproteins uptake occurs through clathrin-mediated endocytosis in a human epithelial cell line. Virology Journal 2008;5: 127.
114. Rock KL, York IA, Goldberg AL. Post-proteasomal antigen processing for major histocompatibility complex class I presentation. Nature Immunology 2004;5:670–7.
115. Watts C. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nature Immunology 2004;5:685–92.
116. Grommé M, Neefjes J. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Molecular Immunology 2002;39:181–202.
117. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunological Reviews 2005; 207:145–57.
118. Beaty SR, Rose CE, Sung S-SJ. Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. Journal of Immunology (Baltimore, Md: 1950) 2007; 178:1882–95.
119. Sung S-SJ, Fu SM, Rose CE, Gaskin F, Ju S-T, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. Journal of Immunology (Baltimore, Md: 1950) 2006;176: 2161–72.
120. Lukens M V, Kruijsen D, Coenjaerts FEJ, Kimpen JLL, Van Bleek GM. Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. Journal of Virology 2009;83: 7235–43.
121. Del Rio M-L, Rodriguez-Barbosa J-I, Kremmer E, Förster R. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. Journal of Immunology (Baltimore, Md: 1950) 2007; 178:6861–6.
122. Lukens M V, Van de Pol AC, Coenjaerts FEJ, Jansen NJG, Kamp VM, Kimpen JLL, et al. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. Journal of Virology 2010;84: 2374–83.
123. Guerrero-Plata A, Casola A, Suarez G, Yu X, Spetch L, Peeples ME, et al. Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. American Journal of Respiratory Cell and Molecular Biology 2006;34:320–9.
124. Rothoeft T, Fischer K, Zawatzki S, Schulz V, Schauer U, Körner Rettberg C. Differential response of human naive and memory/effector T cells to dendritic cells infected by respiratory syncytial virus. Clinical and Experimental Immunology 2007;150: 263–73.
125. González PA, Prado CE, Leiva ED, Carreño LJ, Bueno SM, Riedel CA, et al. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proceedings of the National Academy of Sciences of the United States of America 2008;105:14999–5004.
126. González PA, Carreño LJ, Figueroa CA, Kalergis AM. Modulation of immunological synapse by membrane-bound and soluble ligands. Cytokine & Growth Factor Reviews n.d.;18:19–31.
127. De Graaff PMA, De Jong EC, Van Capel TM, Van Dijk MEA, Roholl PJM, Boes J, et al. Respiratory syncytial virus infection of monocyte-derived dendritic cells decreases their capacity to activate CD4 T cells. Journal of Immunology (Baltimore, Md: 1950) 2005; 175:5904–11.
128. Chi B, Dickensheets HL, Spann KM, Alston MA, Luongo C, Dumoutier L, et al. Alpha and lambda interferon together mediate

suppression of CD4 T cells induced by respiratory syncytial virus. Journal of Virology 2006;80:5032–40.
129. Smit JJ, Rudd BD, Lukacs NW. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. The Journal of Experimental Medicine 2006;203:1153–9.
130. Smit JJ, Lindell DM, Boon L, Kool M, Lambrecht BN, Lukacs NW. The balance between plasmacytoid DC versus conventional DC determines pulmonary immunity to virus infections. PloS One 2008;3:e1720.
131. Mella C, Suarez-Arrabal MC, Lopez S, Stephens J, Fernandez S, Hall MW, et al. Innate immune dysfunction is associated with enhanced disease severity in infants with severe respiratory syncytial virus bronchiolitis. The Journal of Infectious Diseases 2013; 207:564–73.
132. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science (New York, NY) 2010; 327:1098–102.
133. Zhu J, Paul WE. Heterogeneity and plasticity of T helper cells. Cell Research 2010;20: 4–12.
134. Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunological Reviews 1997;159:105–17.
135. Schijf MA, Kruijsen D, Bastiaans J, Coenjaerts FEJ, Garssen J, Van Bleek GM, et al. Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. Journal of Virology 2012;86:11472–82.
136. Durbin JE, Johnson TR, Durbin RK, Mertz SE, Morotti RA, Peebles RS, et al. The role of IFN in respiratory syncytial virus pathogenesis. Journal of Immunology (Baltimore, Md: 1950) 2002;168:2944–52.
137. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. American Journal of Epidemiology 1969;89:422–34.
138. Qin L, Hu C, Feng J, Xia Q. Activation of lymphocytes induced by bronchial epithelial cells with prolonged RSV infection. PloS One 2011;6:e27113.
139. Kallal LE, Hartigan AJ, Hogaboam CM, Schaller MA, Lukacs NW. Inefficient lymph node sensitization during respiratory viral infection promotes IL-17-mediated lung pathology. Journal of Immunology (Baltimore, Md: 1950) 2010;185:4137–47.
140. Mukherjee S, Lindell DM, Berlin AA, Morris SB, Shanley TP, Hershenson MB, et al. IL-17-induced pulmonary pathogenesis during respiratory viral infection and exacerbation of allergic disease. The American Journal of Pathology 2011;179:248–58.
141. Faber TE, Groen H, Welfing M, Jansen KJG, Bont LJ. Specific increase in local IL-17 production during recovery from primary RSV bronchiolitis. Journal of Medical Virology 2012;84:1084–8.
142. Graham BS, Johnson TR, Peebles RS. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology 2000;48:237–47.
143. Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. The Journal of Experimental Medicine 1988;168:1163–8.
144. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. The Journal of Clinical Investigation 1991;88:1026–33.
145. Olson MR, Varga SM. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. Journal of Immunology (Baltimore, Md: 1950) 2007;179:5415–24.
146. Olson MR, Hartwig SM, Varga SM. The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the

lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia. Journal of Immunology (Baltimore, Md: 1950) 2008;181:7958–68.

147. Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, et al. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. The Journal of Infectious Diseases 2007; 195:1126–36.
148. Welliver TP, Reed JL, Welliver RC. Respiratory syncytial virus and influenza virus infections: observations from tissues of fatal infant cases. The Pediatric Infectious Disease Journal 2008;27:S92–6.
149. Heidema J, Lukens M V, Van Maren WWC, Van Dijk MEA, Otten HG, Van Vught AJ, et al. CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. Journal of Immunology (Baltimore, Md: 1950) 2007; 179:8410–7.
150. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. Immunity 2013;38:414–23.
151. Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. Journal of Immunology (Baltimore, Md: 1950) 2010;185:2382–92.
152. Ruckwardt TJ, Bonaparte KL, Nason MC, Graham BS. Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. Journal of Virology 2009;83:3019–28.
153. Loebbermann J, Thornton H, Durant L, Sparwasser T, Webster KE, Sprent J, et al. Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. Mucosal Immunology 2012;5:161–72.
154. Loebbermann J, Durant L, Thornton H, Johansson C, Openshaw PJ. Defective immunoregulation in RSV vaccine-augmented viral lung disease restored by selective chemoattraction of regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America 2013; 110:2987–92.
155. Welliver RC, Wong DT, Sun M, Middleton E, Vaughan RS, Ogra PL. The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. The New England Journal of Medicine 1981;305:841–6.
156. Gimenez HB, Keir HM, Cash P. Immunoblot analysis of the human antibody response to respiratory syncytial virus infection. The Journal of General Virology 1987;68 (Pt 5): 1267–75.
157. Ogra PL. Respiratory syncytial virus: the virus, the disease and the immune response. Paediatric Respiratory Reviews 2004;5 Suppl A:S119–26.
158. Vainionpää R, Meurman O, Sarkkinen H. Antibody response to respiratory syncytial virus structural proteins in children with acute respiratory syncytial virus infection. Journal of Virology 1985;53:976–9.
159. Ward KA, Lambden PR, Ogilvie MM, Watt PJ. Antibodies to respiratory syncytial virus polypeptides and their significance in human infection. The Journal of General Virology 1983;64 (Pt 9):1867–76.
160. Valosky J, Hishiki H, Zaoutis TE, Coffin SE. Induction of mucosal B-cell memory by intranasal immunization of mice with respiratory syncytial virus. Clinical and Diagnostic Laboratory Immunology 2005; 12:171–9.
161. Van Drunen Littel-van den Hurk S, Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Reviews in Medical Virology n.d.;17:5–34.
162. Welliver RC, Kaul TN, Putnam TI, Sun M, Riddlesberger K, Ogra PL. The antibody

response to primary and secondary infection with respiratory syncytial virus: kinetics of class-specific responses. The Journal of Pediatrics 1980;96:808–13.
163. Meurman O, Ruuskanen O, Sarkkinen H, Hänninen P, Halonen P. Immunoglobulin class-specific antibody response in respiratory syncytial virus infection measured by enzyme immunoassay. Journal of Medical Virology 1984;14:67–72.
164. Falsey AR, Singh HK, Walsh EE. Serum antibody decay in adults following natural respiratory syncytial virus infection. Journal of Medical Virology 2006;78:1493–7.
165. Walsh EE, Peterson DR, Falsey AR. Risk factors for severe respiratory syncytial virus infection in elderly persons. The Journal of Infectious Diseases 2004;189:233–8.
166. Falsey AR, Walsh EE. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. The Journal of Infectious Diseases 1998;177:463–6.
167. Ogilvie MM, Vathenen AS, Radford M, Codd J, Key S. Maternal antibody and respiratory syncytial virus infection in infancy. Journal of Medical Virology 1981;7:263–71.
168. Prince GA, Horswood RL, Chanock RM. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. Journal of Virology 1985; 55:517–20.
169. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. Vaccine 2003;21:3479–82.
170. Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. The PREVENT Study Group. Pediatrics 1997;99:93–9.
171. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics 1998; 102:531–7.
172. Simões EAF, Carbonell-Estrany X, Rieger CHL, Mitchell I, Fredrick L, Groothuis JR. The effect of respiratory syncytial virus on subsequent recurrent wheezing in atopic and nonatopic children. The Journal of Allergy and Clinical Immunology 2010;126:256–62.
173. Shadman KA, Wald ER. A review of palivizumab and emerging therapies for respiratory syncytial virus. Expert Opinion on Biological Therapy 2011;11:1455–67.
174. Hu J, Robinson JL. Treatment of respiratory syncytial virus with palivizumab: a systematic review. World Journal of Pediatrics: WJP 2010;6:296–300.
175. Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, et al. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. Journal of Molecular Biology 2007; 368:652–65.
176. Kwakkenbos MJ, Diehl SA, Yasuda E, Bakker AQ, Van Geelen CMM, Lukens M V, et al. Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nature Medicine 2010;16:123–8.
177. Carbonell-Estrany X, Simões EAF, Dagan R, Hall CB, Harris B, Hultquist M, et al. Motavizumab for prophylaxis of respiratory syncytial virus in high-risk children: a noninferiority trial. Pediatrics 2010;125: e35–51.
178. Suhrbier A, La Linn M. Suppression of antiviral responses by antibody-dependent enhancement of macrophage infection. Trends in Immunology 2003;24:165–8.
179. Linn ML, Aaskov JG, Suhrbier A. Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis. The Journal of General Virology 1996;77 (Pt 3):407–11.

180. Olsen C, Scott F. Evaluation of antibody-dependent enhancement of feline infectious peritonitis virus infectivity using in situ hybridization. Microbial Pathogenesis 1993; 14:275–85.
181. Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, et al. A role for immune complexes in enhanced respiratory syncytial virus disease. The Journal of Experimental Medicine 2002;196:859–65.
182. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. American Journal of Epidemiology 1969;89:435–48.
183. Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. American Journal of Epidemiology 1969;89:449–63.
184. Kim HW, Leikin SL, Arrobio J, Brandt CD, Chanock RM, Parrott RH. Cell-mediated immunity to respiratory syncytial virus induced by inactivated vaccine or by infection. Pediatric Research 1976;10:75–8.
185. Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC, Sotnikov A V, et al. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. Journal of Virology 1992;66: 7444–51.
186. Connors M, Giese NA, Kulkarni AB, Firestone CY, Morse HC, Murphy BR. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. Journal of Virology 1994; 68:5321–5.
187. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. Journal of Immunology (Baltimore, Md: 1950) 1993; 151:2032–40.
188. Stevens WW, Sun J, Castillo JP, Braciale TJ. Pulmonary eosinophilia is attenuated by early responding CD8(+) memory T cells in a murine model of RSV vaccine-enhanced disease. Viral Immunology 2009;22:243–51.
189. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nature Medicine 2009;15:34–41.
190. Kruijsen D, Schijf MA, Lukens M V, Van Uden NO, Kimpen JL, Coenjaerts FE, et al. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. Vaccine 2011;29:2730–41.
191. Karron RA, Wright PF, Belshe RB, Thumar B, Casey R, Newman F, et al. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. The Journal of Infectious Diseases 2005;191:1093–104.
192. Crowe JE, Bui PT, Davis AR, Chanock RM, Murphy BR. A further attenuated derivative of a cold-passaged temperature-sensitive mutant of human respiratory syncytial virus retains immunogenicity and protective efficacy against wild-type challenge in seronegative chimpanzees. Vaccine 1994; 12:783–90.
193. Bueno SM, González PA, Cautivo KM, Mora JE, Leiva ED, Tobar HE, et al. Protective T cell immunity against respiratory syncytial virus is efficiently induced by recombinant BCG. Proceedings of the National Academy of Sciences of the United States of America 2008;105:20822–7.

194. Olszewska W, Suezer Y, Sutter G, Openshaw PJM. Protective and disease-enhancing immune responses induced by recombinant modified vaccinia Ankara (MVA) expressing respiratory syncytial virus proteins. Vaccine 2004;23:215–21.
195. Quan F-S, Kim Y, Lee S, Yi H, Kang S-M, Bozja J, et al. Viruslike particle vaccine induces protection against respiratory syncytial virus infection in mice. The Journal of Infectious Diseases 2011;204:987–95.
196. McLellan JS, Correia BE, Chen M, Yang Y, Graham BS, Schief WR, et al. Design and characterization of epitope-scaffold immunogens that present the motavizumab epitope from respiratory syncytial virus. Journal of Molecular Biology 2011;409:853–66.
197. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nature Immunology 2004;5: 266–71.
198. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nature Reviews Immunology 2007;7: 379–90.
199. Siegrist CA. Vaccination in the neonatal period and early infancy. International Reviews of Immunology 2000;19:195–219.
200. Belderbos ME, Van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clinical Immunology (Orlando, Fla) 2009;133:228–37.
201. Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA, et al. Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo. Pediatric Research 2006;60:205–9.
202. Vanden Eijnden S, Goriely S, De Wit D, Goldman M, Willems F. Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns. European Journal of Immunology 2006;36: 21–6.
203. Fujiwara D, Wei B, Presley LL, Brewer S, McPherson M, Lewinski MA, et al. Systemic control of plasmacytoid dendritic cells by CD8+ T cells and commensal microbiota. Journal of Immunology (Baltimore, Md: 1950) 2008;180:5843–52.
204. Tanaka K, Sawamura S, Satoh T, Kobayashi K, Noda S. Role of the indigenous microbiota in maintaining the virus-specific CD8 memory T cells in the lung of mice infected with murine cytomegalovirus. Journal of Immunology (Baltimore, Md: 1950) 2007;178: 5209–16.
205. Wei B, Su TT, Dalwadi H, Stephan RP, Fujiwara D, Huang TT, et al. Resident enteric microbiota and CD8+ T cells shape the abundance of marginal zone B cells. European Journal of Immunology 2008;38:3411–25.
206. Wei B, Wingender G, Fujiwara D, Chen DY, McPherson M, Brewer S, et al. Commensal microbiota and CD8+ T cells shape the formation of invariant NKT cells. Journal of Immunology (Baltimore, Md: 1950) 2010; 184:1218–26.
207. Wu GD, Lewis JD, Hoffmann C, Chen Y-Y, Knight R, Bittinger K, et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiology 2010;10:206.
208. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature 2007;449: 804–10.
209. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences of the United States of America 2010; 107:11971–5.

210. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nature Immunology 2011;12:5–9.
211. van't Land B, Schijf MA, Martin R, Garssen J, Van Bleek GM. Influencing mucosal homeostasis and immune responsiveness: the impact of nutrition and pharmaceuticals. European Journal of Pharmacology 2011; 668 Suppl :S101–7.
212. Gori A, Tincati C, Rizzardini G, Torti C, Quirino T, Haarman M, et al. Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis. Journal of Clinical Microbiology 2008;46:757–8.
213. Belderbos ME, Houben ML, Van Bleek GM, Schuijff L, Van Uden NOP, Bloemen-Carlier EM, et al. Breastfeeding modulates neonatal innate immune responses: a prospective birth cohort study. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2012;23:65–74.
214. Jost T, Lacroix C, Braegger C, Chassard C. Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. The British Journal of Nutrition 2013:1–10.
215. Tackoen M. [Breast milk: its nutritional composition and functional properties]. Revue Médicale De Bruxelles 2012;33:309–17.
216. Jeurink P V, Van Bergenhenegouwen J, Jiménez E, Knippels LMJ, Fernández L, Garssen J, et al. Human milk: a source of more life than we imagine. Beneficial Microbes 2013;4: 17–30.
217. Rudloff HE, Schmalstieg FC, Mushtaha AA, Palkowetz KH, Liu SK, Goldman AS. Tumor necrosis factor-alpha in human milk. Pediatric Research 1992;31:29–33.
218. Skansén-Saphir U, Lindfors A, Andersson U. Cytokine production in mononuclear cells of human milk studied at the single-cell level. Pediatric Research 1993;34:213–6.
219. Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. The British Journal of Nutrition 2008;99:91–9.
220. Laitinen K, Hoppu U, Hämäläinen M, Linderborg K, Moilanen E, Isolauri E. Breast milk fatty acids may link innate and adaptive immune regulation: analysis of soluble CD14, prostaglandin E2, and fatty acids. Pediatric Research 2006;59:723–7.
221. Mabuka J, Nduati R, Odem-Davis K, Peterson D, Overbaugh J. HIV-specific antibodies capable of ADCC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads. PLoS Pathogens 2012;8:e1002739.
222. Srivastava MD, Srivastava A, Brouhard B, Saneto R, Groh-Wargo S, Kubit J. Cytokines in human milk. Research Communications in Molecular Pathology and Pharmacology 1996;93:263–87.
223. Holmlund U, Amoudruz P, Johansson MA, Haileselassie Y, Ongoiba A, Kayentao K, et al. Maternal country of origin, breast milk characteristics and potential influences on immunity in offspring. Clinical and Experimental Immunology 2010;162:500–9.
224. LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M, et al. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. Journal of Immunology (Baltimore, Md: 1950) 2003;171:6680–9.
225. Belderbos ME, Houben ML, Wilbrink B, Lentjes E, Bloemen EM, Kimpen JLL, et al. Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis. Pediatrics 2011;127:e1513–20.
226. Brandtzaeg P. Food allergy: separating the science from the mythology. Nature Reviews Gastroenterology & Hepatology 2010;7: 380–400.

227. Wagner CL, Taylor SN, Hollis BW. Does vitamin D make the world go "round"? Breastfeeding Medicine: the Official Journal of the Academy of Breastfeeding Medicine 2008;3:239–50.
228. Kobata A. Structures and application of oligosaccharides in human milk. Proceedings of the Japan Academy Series B, Physical and Biological Sciences 2010;86:731–47.
229. Cravioto A, Tello A, Villafán H, Ruiz J, Del Vedovo S, Neeser JR. Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. The Journal of Infectious Diseases 1991;163:1247–55.
230. Ramphal R, Carnoy C, Fievre S, Michalski JC, Houdret N, Lamblin G, et al. Pseudomonas aeruginosa recognizes carbohydrate chains containing type 1 (Gal beta 1-3GlcNAc) or type 2 (Gal beta 1-4GlcNAc) disaccharide units. Infection and Immunity 1991;59: 700–4.
231. Yu Z-T, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, et al. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology 2013;23:169–77.
232. Donovan SM, Wang M, Li M, Friedberg I, Schwartz SL, Chapkin RS. Host-microbe interactions in the neonatal intestine: role of human milk oligosaccharides. Advances in Nutrition (Bethesda, Md) 2012;3:450S–5S.
233. Oozeer R, Van Limpt K, Ludwig T, Ben Amor K, Martin R, Wind RD, et al. Intestinal microbiology in early life: specific prebiotics can have similar functionalities as human-milk oligosaccharides. The American Journal of Clinical Nutrition 2013.
234. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, et al. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2010;21:1179–88.
235. De Kivit S, Saeland E, Kraneveld AD, Van de Kant HJG, Schouten B, Van Esch BCAM, et al. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012;67: 343–52.
236. De Kivit S, Kraneveld AD, Knippels LMJ, Van Kooyk Y, Garssen J, Willemsen LEM. Intestinal Epithelium-Derived Galectin-9 Is Involved in the Immunomodulating Effects of Nondigestible Oligosaccharides. Journal of Innate Immunity 2013.
237. Van de Pol MA, Lutter R, Smids BS, Weersink EJM, Van der Zee JS. Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics. Allergy 2011;66:39–47.
238. Sunkara LT, Jiang W, Zhang G. Modulation of antimicrobial host defense peptide gene expression by free fatty acids. PloS One 2012;7:e49558.
239. Andresen L, Hansen KA, Jensen H, Pedersen SF, Stougaard P, Hansen HR, et al. Propionic acid secreted from propionibacteria induces NKG2D ligand expression on human-activated T lymphocytes and cancer cells. Journal of Immunology (Baltimore, Md: 1950) 2009;183:897–906.
240. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science (New York, NY) 2011;332:974–7.
241. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nature Medicine 2010;16:228–31.
242. Sartor RB, Muehlbauer M. Microbial host interactions in IBD: implications for pathogenesis and therapy. Current Gastroenterology Reports 2007;9:497–507.
243. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy,

intestinal colonization and human disease. Nature Reviews Gastroenterology & Hepatology 2012;9:565–76.

244. Ismail IH, Oppedisano F, Joseph SJ, Boyle RJ, Licciardi P V, Robins-Browne RM, et al. Reduced gut microbial diversity in early life is associated with later development of eczema but not atopy in high-risk infants. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2012;23: 674–81.
245. Nishikawa J, Kudo T, Sakata S, Benno Y, Sugiyama T. Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. Scandinavian Journal of Gastroenterology 2009;44:180–6.
246. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 2006;55:205–11.
247. Matricardi PM, Bonini S. High microbial turnover rate preventing atopy: a solution to inconsistencies impinging on the Hygiene hypothesis? Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology 2000;30: 1506–10.
248. Ni K, Li S, Xia Q, Zang N, Deng Y, Xie X, et al. Pharyngeal microflora disruption by antibiotics promotes airway hyperresponsiveness after respiratory syncytial virus infection. PloS One 2012;7:e41104.
249. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proceedings of the National Academy of Sciences of the United States of America 2011;108:5354–9.
250. Gabryszewski SJ, Bachar O, Dyer KD, Percopo CM, Killoran KE, Domachowske JB, et al. Lactobacillus-mediated priming of the respiratory mucosa protects against lethal pneumovirus infection. Journal of Immunology (Baltimore, Md: 1950) 2011;186: 1151–61.
251. Breman JG, De Quadros CA, Gadelha P. Smallpox eradication after 30 years: lessons, legacies, and innovations. Introduction: meeting objectives, summary and final statement. Vaccine 2011;29 Suppl 4:D3–5.
252. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nature Medicine 2006;12:905–7.
253. Piedra PA, Faden HS, Camussi G, Wong DT, Ogra PL. Mechanism of lung injury in cotton rats immunized with formalin-inactivated respiratory syncytial virus. Vaccine 1989;7: 34–8.
254. Openshaw PJ, Culley FJ, Olszewska W. Immunopathogenesis of vaccine-enhanced RSV disease. Vaccine 2001;20 Suppl 1:S27–31.
255. Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC, et al. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 2004;20:769–83.
256. Israel EJ, Patel VK, Taylor SF, Marshak-Rothstein A, Simister NE. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. Journal of Immunology (Baltimore, Md: 1950) 1995;154:6246–51.
257. Junghans RP, Anderson CL. The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor. Proceedings of the National Academy of Sciences of the United States of America 1996;93:5512–6.
258. Wu H-J, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes n.d.;3:4–14.
259. Stockinger S, Hornef MW, Chassin C. Establishment of intestinal homeostasis during the neonatal period. Cellular and Molecular Life Sciences: CMLS 2011;68:3699–712.

260. Russell SL, Finlay BB. The impact of gut microbes in allergic diseases. Current Opinion in Gastroenterology 2012;28:563–9.
261. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. The Journal of Allergy and Clinical Immunology 2011;128: 646–52.e1–5.
262. Sobko T, Schiött J, Ehlin A, Lundberg J, Montgomery S, Norman M. Neonatal sepsis, antibiotic therapy and later risk of asthma and allergy. Paediatric and Perinatal Epidemiology 2010;24:88–92.
263. Penders J, Thijs C, Van den Brandt PA, Kummeling I, Snijders B, Stelma F, et al. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut 2007;56:661–7.
264. Moro GE, Mosca F, Miniello V, Fanaro S, Jelinek J, Stahl B, et al. Effects of a new mixture of prebiotics on faecal flora and stools in term infants. Acta Paediatrica (Oslo, Norway: 1992) Supplement 2003;91: 77–9.
265. Fanaro S, Jelinek J, Stahl B, Boehm G, Kock R, Vigi V. Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: effect on intestinal flora, stool characteristics, and pH. Journal of Pediatric Gastroenterology and Nutrition 2005;41: 186–90.
266. Peltola V, Reunanen T, Ziegler T, Silvennoinen H, Heikkinen T. Accuracy of clinical diagnosis of influenza in outpatient children. Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America 2005;41:1198–200.
267. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clinical Microbiology Reviews 2010;23:74–98.

Chapter 2


Prophyla
RSVA
Synagis
Fusion
Severe respirator
Palivizumab
Peptide
Treatment
Infection

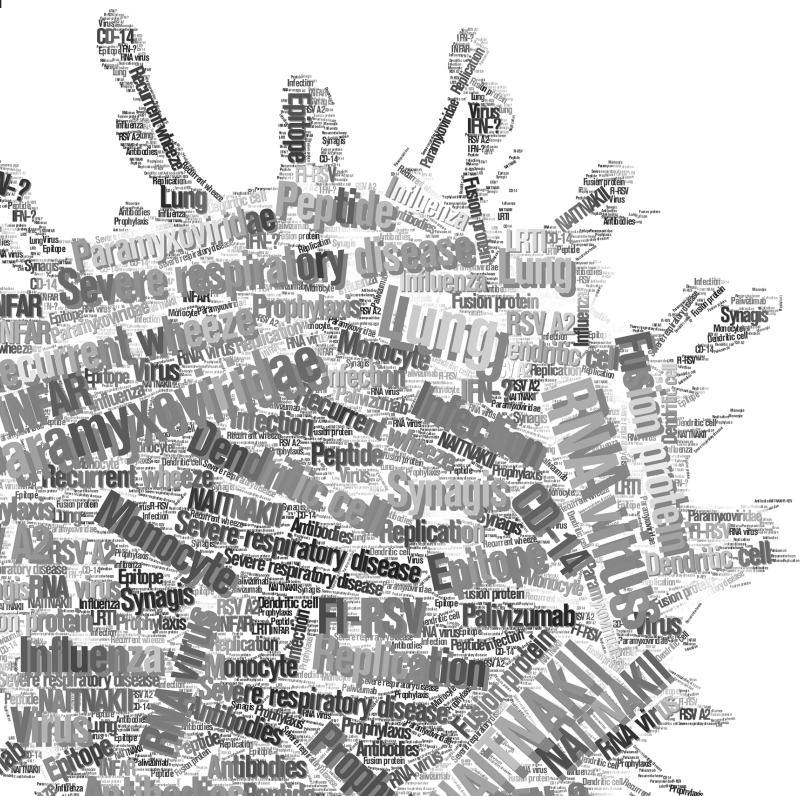
Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV

Debby Kruijsen[a], Marcel A. Schijf[a,b], Michaël V. Lukens[a], Nathalie O. van Uden[a], Jan L. Kimpen[a], Frank E. Coenjaerts[c] and Grada M. van Bleek[*a]

[a]Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Centre, Utrecht, The Netherlands,

[b]Department of Immunology, Danone Research - Centre for Specialised Nutrition, Wageningen, The Netherlands,

[c]Department of Medical Microbiology, University Medical Centre, Utrecht, The Netherlands.



Vaccine (2011): 29:2730-2741.

ABSTRACT

Inactivated Respiratory Syncytial Virus (RSV) vaccines tend to predispose for immune mediated enhanced disease, characterized by Th2 responses and airway hypersensitivity reactions. We show in a C57BL/6 mouse model that the early innate response elicited by the challenge virus (RSV versus influenza virus) influences the outcome of the Th1/Th2 balance in the lung after intramuscular priming with inactivated vaccine. Priming of $CD4^+$/IFN-γ^+ T cells by mature dendritic cells administered intravenously and/or priming of a virus specific $CD8^+$ T cell response ameliorated the Th2-mediated inflammatory response in the lung, suggesting that vaccination procedures are feasible that prevent vaccine induced immune pathology.

INTRODUCTION

Respiratory syncytial virus (RSV) is the most frequent cause of severe lower respiratory tract infections in infants, and can cause severe morbidity in elderly people and patients with an immunodeficiency [1, 2]. By 3 years of age, most children have been infected with RSV at least once. The rate of hospitalization for primary infection is approximately 0.5%, but can vary by situation and ethnic group and can be as high as 25% [3]. There is no licensed vaccine against the virus. Development of a vaccine has been set back due to a dramatic vaccine trial in the 1960's, with a formalin-inactivated alum-precipitated RSV (FI-RSV) [4-7]. FI-RSV vaccinated children who were naturally infected with RSV experienced increased frequency and severity of RSV lower respiratory tract infection compared to children immunized with control vaccine preparations. About 80% of the vaccine recipients required hospitalization and two vaccine recipients died following RSV infection. Post-mortem examination of lung tissue of the diseased children showed an intense inflammatory cellular infiltrate in the lungs composed of mononuclear cells, eosinophils and polymorph nuclear cells [6, 8]. FI-RSV-vaccinated children had high RSV specific serum antibody titers. However, a high proportion of these antibodies was non-neutralizing [4, 5, 9].

FI-RSV-enhanced disease has been reproduced in several animal models [10-13]. Mice vaccinated with FI-RSV and challenged with RSV develop airway hyper-reactivity and a Th2-type immune response measured by enhanced interleukin (IL)-4, IL-5 and IL-13 responses and reduced IL-12 production [12]. Poorly neutralizing antibody responses and enhanced tissue eosinophilia were also described [12, 14, 15]. A central role for T cells in augmented lung pathology was highlighted by Connors et al. who showed that in mice $CD4^+$ T cells were crucial to the immune pathogenesis of FI-RSV-mediated enhanced disease. The $CD4^+$ T cell response outnumbered the $CD8^+$ T cell response, which dominates during a natural RSV infection [16].

The exact mechanism behind the enhanced disease is currently still unclear. Structural alterations by formalin treatment have been held responsible for poor antibody recognition of viral F protein in intact RSV [15, 17]. Poorly neutralizing antibodies might be ineffective in lowering viral load or even cause enhanced infection, while immune complex deposition and complement activation in the lung might cause bronchoconstriction [18]. Inactivated virus particles might be less efficiently processed by APC for presentation by MHC class I molecules, resulting in lower $CD8^+$ T cell responses. $CD8^+$ T cells have been shown to ameliorate RSV disease in mice primed by inactivated virus preparations or viral proteins [19-22]. Moreover, inactivated virus might be less efficient in the activation of innate immune receptors [23]. A role for the adjuvant aluminum hydroxide in the FI-RSV vaccine which characteristically tends to induce Th2-type responses might also contribute to the CD4-Th2 shifted recall immune response [10, 12, 24-27].

In previous studies it has been shown that a-specific contaminations (FI-mock vaccine) caused similar enhanced disease upon RSV challenge as the FI-RSV vaccine itself [10, 24, 28-30]. Moreover, vaccination with purified viral proteins or vaccinia recombinants expressing RSV-G protein, also induced similar Th2-mediated immune pathology [30, 31]. Inactivated or subunit vaccines are successfully used for different viral infections (e.g. poliovirus, influenza virus). However, for several paramyxoviruses (hRSV, pneumovirus of mice (PVM), bovine RSV (bRSV), human metapneumovirus (hMPV) and measles virus) these approaches induce enhanced disease, characterized by respiratory hypersensitivity [32-36].

In the present study we present data showing that the Th2-mediated inflammatory responses in the lung could be ameliorated either at the time of priming, by activation of primary $CD4^+$ T cells by mature dendritic cells administered intravenously and/or priming of a virus specific $CD8^+$ T cell response, or at the time of challenge whereby the nature of the early innate response of the challenge virus influences the outcome of the Th1/Th2 balance locally.

RESULTS

FI-RSV mediated Th2 skewed immune responses in C57BL/6 mice

FI-RSV vaccination studies are routinely performed in BALB/c mice that are prone to develop Th2 responses [41]. To determine whether we were able to analyze FI-RSV-induced Th2 responses accompanied by eosinophilia in C57BL/6 mice with our FI-RSV vaccine, mice were vaccinated intramuscularly with FI-RSV or FI-mock (i.e. formalin treated culture supernatant of HEp-2 cells) at day 0. A third group of animals was intranasally infected with RSV to compare the immune response in vaccinated mice with a secondary RSV infection. All mice were challenged with RSV at day 36. Six days after challenge lung single cell suspensions were stimulated *in vitro* with RSV infected dendritic (D1) cells or untreated D1 cells to measure T cell responses. Production of cytokines by $CD4^+$ and $CD8^+$ T cells was determined by intracellular staining. As expected, mice vaccinated with FI-RSV and challenged with RSV developed a strong Th2 response, characterized by the production of IL-4, IL-5 and IL-13, and lower levels of IFN-γ, compared to a regular secondary RSV infection where $CD4^+$ T cells predominantly produced IFN-γ (**Figure 1A**). Less IFN-γ producing $CD8^+$ T cells were observed in FI-RSV-vaccinated mice compared to a secondary RSV infection. In both the FI-RSV and FI-mock groups, the $CD4^+$ T cells responded to *in vitro* restimulation with RSV-infected and uninfected D1 cells (**Figure 1A**).

In addition to measuring T cell responses in lung tissue, cytospins were performed on BAL samples and the cell composition was analyzed by standard morphologic analysis (**Figure 1B**). BAL of mice with a secondary RSV infection mainly consisted of lymphocytes and macrophages. Only a few neutrophils and eosinophils were observed. In contrast, BAL of FI-RSV-vaccinated and RSV-challenged mice contained >60% eosinophils. Similar cellular influxes

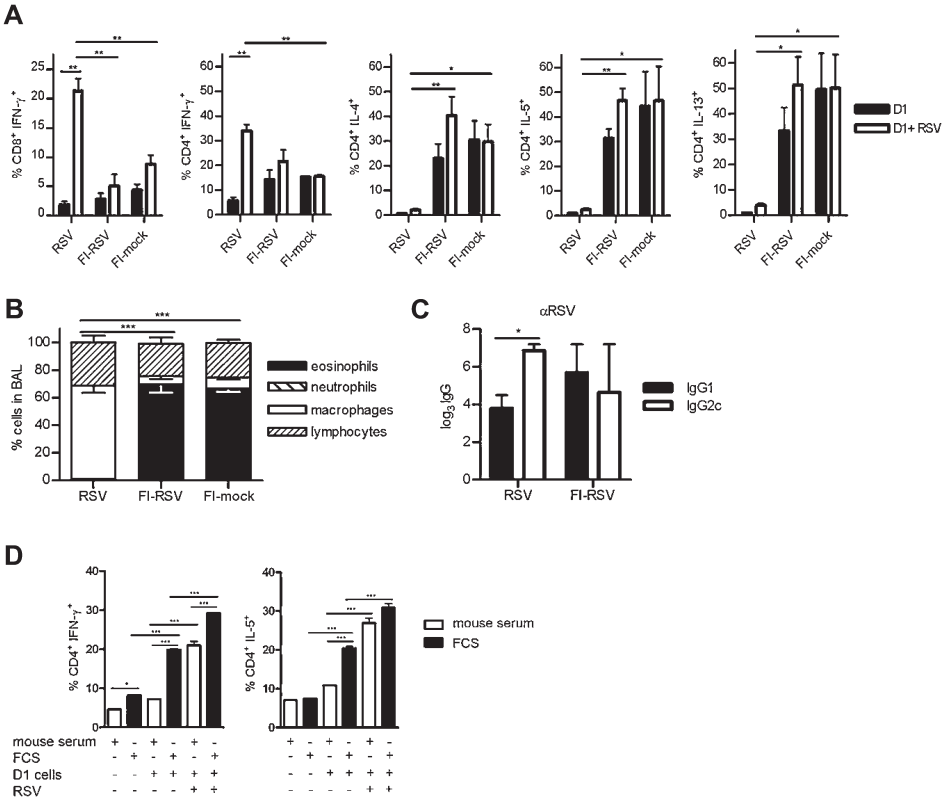
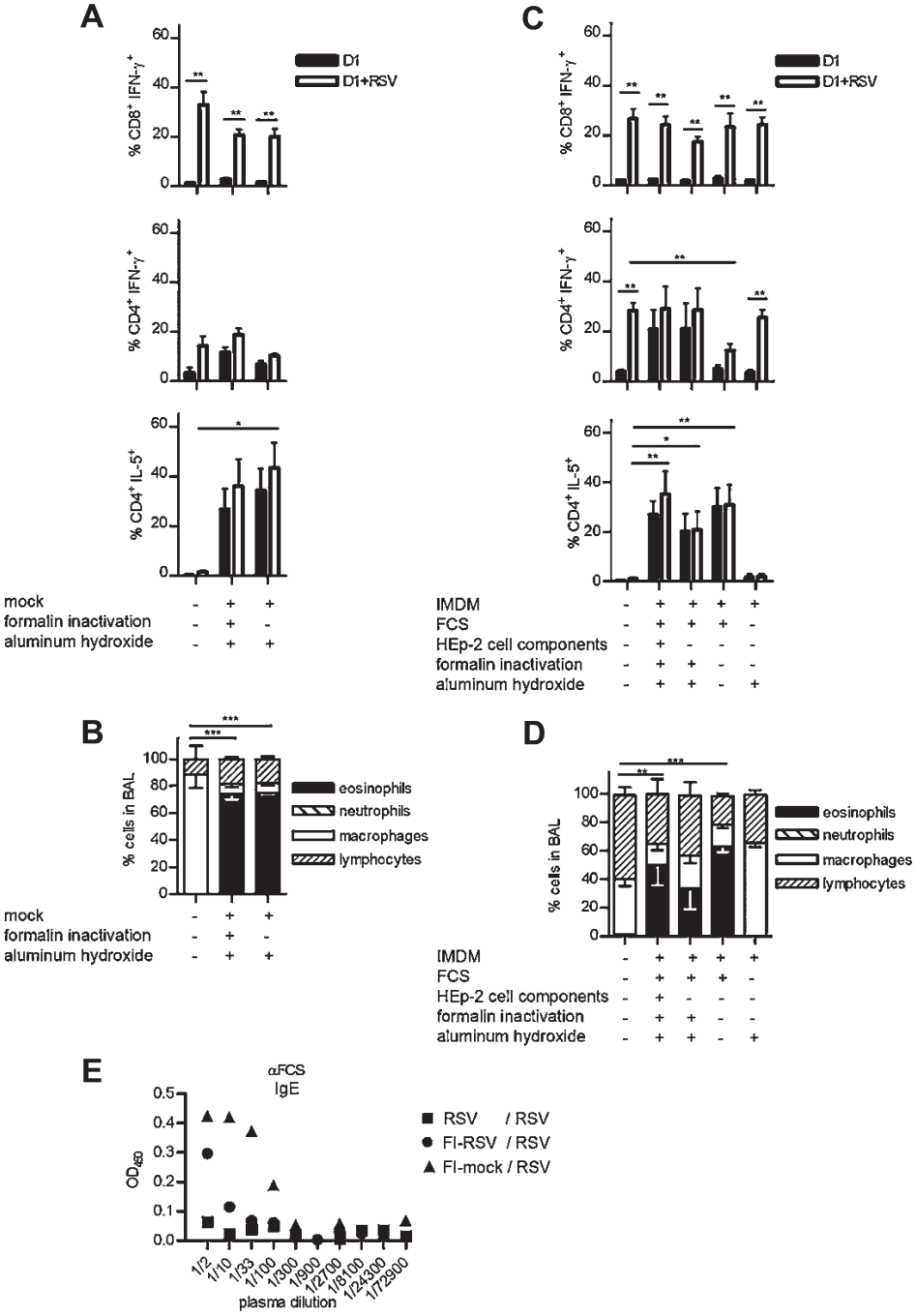


FIGURE 1. FI-RSV vaccination induces strong Th2 responses in lung tissue upon RSV challenge in C57BL/6 mice. Two groups of mice were vaccinated i.m. with either FI-RSV or FI-mock and a third group was i.n. infected with RSV. All groups were i.n. challenged at day 36 with RSV. Six days after challenge lung T cell responses were analyzed *in vitro* after re-stimulation with RSV infected or uninfected D1 cells. T cell responses were measured by intracellular cytokine staining. (**A**). The percentage of $CD8^+$ or $CD4^+$ T cells producing Th1 or Th2 cytokines. (**B**). BAL was analyzed for the percentage of neutrophils, eosinophils, macrophages and lymphocytes. (**C**). Serum was analyzed for RSV specific IgG1 and IgG2a/c. Error bars represent the standard error of the mean (SEM) of 5 mice per group. In A. significance was calculated using a Two-Way ANOVA. In B. and C. significance was calculated with a students *t* test (in B. for the eosinophil population). All results are representative for three individual experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. **(D)** D1 cells cultured in medium with FCS restimulate FCS specific $CD4^+$ T cell responses in lung tissue of FI-RSV vaccinated mice. Mice were vaccinated with FI-RSV i.m. (a different vaccine batch than used in A-C) and i.n. challenged with RSV at day 36. Six days after challenge lung T cells were restimulated with RSV infected or uninfected D1 cells. D1 cells were either cultured in medium with serum of naïve C57BL/6 mice or in medium with FCS. In addition, lung cells were restimulated with medium containing 5 % mouse serum or 5 % FCS in the absence of D1 cells. T cell responses were measured by intracellular cytokine staining and the percentage of $CD4^+$ T cells producing Th1 or Th2 cytokines are shown. Error bars represent the standard error of the mean (SEM) of 3 mice per group. Significance was calculated with a One-Way ANOVA. The results are representative for two individual experiments. * $p<0.05$ and *** $p< 0.001$.

were observed in FI-mock-vaccinated mice. Therefore, viral components were not essential for induction of eosinophilia. Vaccine-specific antibodies in serum were detected by Elisa in mice vaccinated with FI-RSV and compared to the antibody response during secondary RSV infection. In FI-RSV-vaccinated mice the IgG1/IgG2a/c ratio was twofold higher compared to RSV primed mice (**Figure 1C**). In conclusion, these data confirmed the onset of Th2 immune responses in FI-RSV-vaccinated mice in our C57BL/6 model. Although the RSV-specific component during the recall response varied between different FI-RSV preparations we always observed a strong CD4/Th2 cytokine response against non-viral components of the vaccine. Because D1 cells, used for *in vitro* restimulation of lung T cells, were maintained in medium supplemented with FCS we checked whether FCS derived antigens were recognized by lung derived T cells. This would be a plausible explanation because FCS was also present in the FI-RSV and FI-mock preparations used for vaccination. We confirmed that indeed part of the $CD4^+$ T cell response primed by FI-RSV was specific for FCS by using D1 cells cultured in naïve mouse serum or FCS as antigen presenting cells in the lung T cell stimulation assay (**Figure 1D**). However, using RSV infected D1 cells to stimulate lung-derived T cells a significant response against viral antigens could also be visualized.

In early studies with cotton rats and BALB/c mice the role of contaminating tissue culture antigens present in RSV preparations, used to prepare the vaccine and challenge virus, has been described [24, 29, 30]. In fact, the immune response elicited to viral antigens and tissue culture contaminants was not essentially different ([10, 24, 28-30] and **Figure 1**). We additionally showed that the FCS component alone could be used during vaccination to elicit a Th2 response accompanied by lung eosinophilia and FCS specific serum IgE (**Figure 2**), without a necessary contribution of vaccine characteristic factors like formalin treatment of

FIGURE 2. Aluminum hydroxide and formalin inactivation of mock vaccine are not essential to induce Th2 skewed pulmonary inflammation. Three groups of mice were i.m. vaccinated with 1. formalin inactivated HEp-2 cell supernatant (FI-mock) and 2. HEp-2 cell supernatant (mock), both adjuvanted with aluminum hydroxide and 3. no vaccination. All groups were i.n. infected with RSV on day 28. At day 36 lung cells were analyzed for T cell responses by measuring intracellular cytokine production after *in vitro* stimulation with RSV infected or uninfected D1 cells. (**A**). The percentage of $CD8^+$ or $CD4^+$ T cells producing Th1 or Th2 cytokines. (**B**). Percentage of neutrophils, eosinophils, macrophages and lymphocytes in BAL. This result showed that formalin inactivation of the mock vaccine was not crucial to induce the Th2 response. (**C**). Five groups of mice were i.m. vaccinated with 1. formalin inactivated HEp-2 cell supernatant, 2. formalin inactivated IMDM + FCS, both adjuvanted with aluminum hydroxide, 3. FCS in IMDM, 4. IMDM + aluminum hydroxide, 5. no vaccination. All groups were i.n. infected with RSV on day 28 and lung T cell responses were measured at day 36 after restimulation *in vitro* with RSV infected or uninfected D1 cells. (**D**). Percentage of neutrophils, eosinophils, macrophages and lymphocytes in BAL. (**E**). Mice were either infected with RSV i.n., vaccinated with FI-RSV or FI-mock i.m. and challenged i.n. with RSV at day 28, 8 days after challenge serum was analyzed for FCS specific IgE. Data shown are OD_{450} values after subtraction of background OD_{450} obtained with pooled sera of naïve mice. Error bars represent the SEM. Results are means of 5 mice per group and are representative for two independent experiments. In A. and C. significance was calculated using a Students *t* test comparing the response to D1 cells and RSV infected D1 cells. For B. and D. significance was calculated with One-Way ANOVA for the eosinophil population. * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$.


A
% CD8+ IFN-γ+
% CD4+ IFN-γ+
% CD4+ IL-5+
D1
D1+RSV
mock - + +
formalin inactivation - + -
aluminum hydroxide - + +
C
IMDM - + + + +
FCS - + + + -
HEp-2 cell components - + - - -
formalin inactivation - + + - -
aluminum hydroxide - + + - +
B
% cells in BAL
eosinophils
neutrophils
macrophages
lymphocytes
mock - + +
formalin inactivation - + -
aluminum hydroxide - + +
D
IMDM - + + + +
FCS - + + + -
HEp-2 cell components - + - - -
formalin inactivation - + + - -
aluminum hydroxide - + + - +
E
αFCS
IgE
OD450
RSV / RSV
FI-RSV / RSV
FI-mock / RSV
1/2
1/10
1/33
1/100
1/300
1/900
1/2700
1/8100
1/24300
1/72900
plasma dilution

the mock vaccine (**Figure 2A and B**) or the alum adjuvant (**Figure 2C and D**). No FCS-specific Th2 response was observed in unvaccinated primary RSV-infected mice. These experiments suggest that FI-RSV and (FI-)mock vaccination induced similar allergic immune responses.

A viral trigger is not necessary during challenge to induce the FCS-specific allergic immune response

The allergic inflammatory response primed by FCS was elicited upon challenge infection with *in vitro* cultured RSV. To examine if the FCS-induced Th2 response is explained by a challenge with FCS (present in the viral preparation) and whether or not the viral trigger is necessary during the secondary response, we vaccinated mice with FI-mock, followed at day 28 after vaccination with an i.n. challenge with either FCS alone or RSV cultured in IMDM with FCS. At day 36, i.e. 8 days after challenge, T cell responses in the lungs of these mice were analyzed and the BAL cell composition was determined. We observed similar secondary IL-5 responses (**Figure 3A**) and BAL eosinophilia (**Figure 3B**) upon challenge with FCS alone and RSV cultured in IMDM supplemented with FCS. IL-4 and IL-13 producing $CD4^+$ T cell numbers showed the same pattern (data not shown). Furthermore, a primary RSV-specific $CD8^+$/IFN-γ T cell response was induced in FI-mock-vaccinated mice challenged with RSV, that was absent in the FI-mock-primed and FCS-challenged mice. This IFN-γ response of virus specific $CD8^+$ T cells was substantially higher at day 8 than at day 6 after challenge in FI-mock-vaccinated mice as expected because the peak of the RSV-specific T cell response after primary RSV infection is at day 8 (compare **Figure 1A**). IFN-γ producing $CD4^+$ T cells in the lung were non-virus-specific in both FI-mock-primed and RSV-challenged mice, as well as FI-mock-primed and FCS-challenged mice. In conclusion, these data showed that a viral trigger is not necessary during local antigenic challenge to elicit Th2 cytokine-producing secondary $CD4^+$ T cell responses in the lungs of FI-mock-vaccinated mice. Moreover, the RSV challenge and the virus-induced primary $CD8^+$ T cell response did not prevent the onset of the Th2 response and lung eosinophilia.

Primary RSV infection induces an inflammatory response in the lungs resulting in DC activation and migration to mediastinal lymph nodes, the location where subsequently a primary virus-specific T and B cell response is efficiently initiated ([42] and **Figure 1**). Yet it appeared that the inflammation induced by RSV infection in FI-RSV or FI-mock-primed mice did not affect the Th2-primed allergic response when the virus was present during challenge. The inflammatory cell influx during antigen exposure is influenced by the local cytokine and chemokine environment that results from innate responses induced by the pathogen, recruited innate immune cells and adaptive immune cells primed during earlier antigen exposure. CCL2/MCP1 has been shown to be a signature chemokine associated with Th2-biased immune pathology in a G protein vaccination model [31]. We performed a kinetic experiment to track the recruitment of $CD4^+$ T cells, $CD8^+$ T cells and eosinophils to the lungs

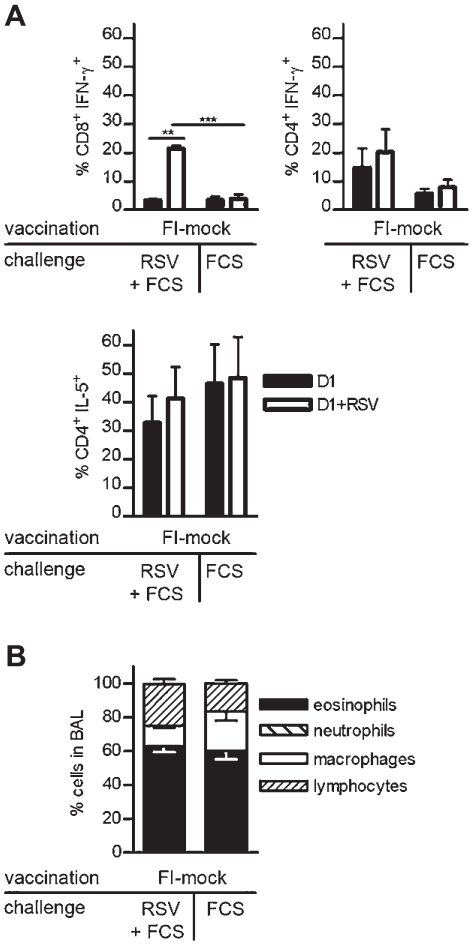


FIGURE 3. A viral trigger is not required to induce Th2/eosinophilia in FI-mock primed FCS challenged mice. Two groups of mice were i.m. vaccinated with FI-mock and at day 28 i.n. challenged with FCS or RSV cultured in IMDM + FCS. Eight days after challenge, lung cells were analyzed for T cell responses and BAL was analyzed for cellular influx. (**A**). The percentage of $CD8^+$ or $CD4^+$ T cells producing Th1 or Th2 cytokines. (**B**). Percentage of neutrophils, eosinophils, macrophages and lymphocytes in BAL. Error bars represent the SEM of 4 mice per group. Significance was calculated using a Two-Way ANOVA in A. and students *t* test in B. for the eosinophil population. This experiment has been performed three times with similar results. ** $p<0.01$ and *** $p< 0.001$.

as well as dendritic cell influxes, since allergic responses are associated with enhanced influx of lung dendritic cells [43] that depends on CCR2 expression, the receptor for CCL2/MCP1 on DC precursors [44].

The kinetics of $CD4^+$ T cells, virus-specific $CD8^+$ T cells and dendritic cell responses in lung tissue during primary (i.n. mock/RSV challenge), secondary (i.n. RSV/RSV) and i.m. FI-RSV-vaccinated/i.n. RSV-challenged mice are shown in **Figure 4**. In contrast to i.m. mock vaccination, i.n. exposure to mock did not prime a T cell response. 96 hours after challenge

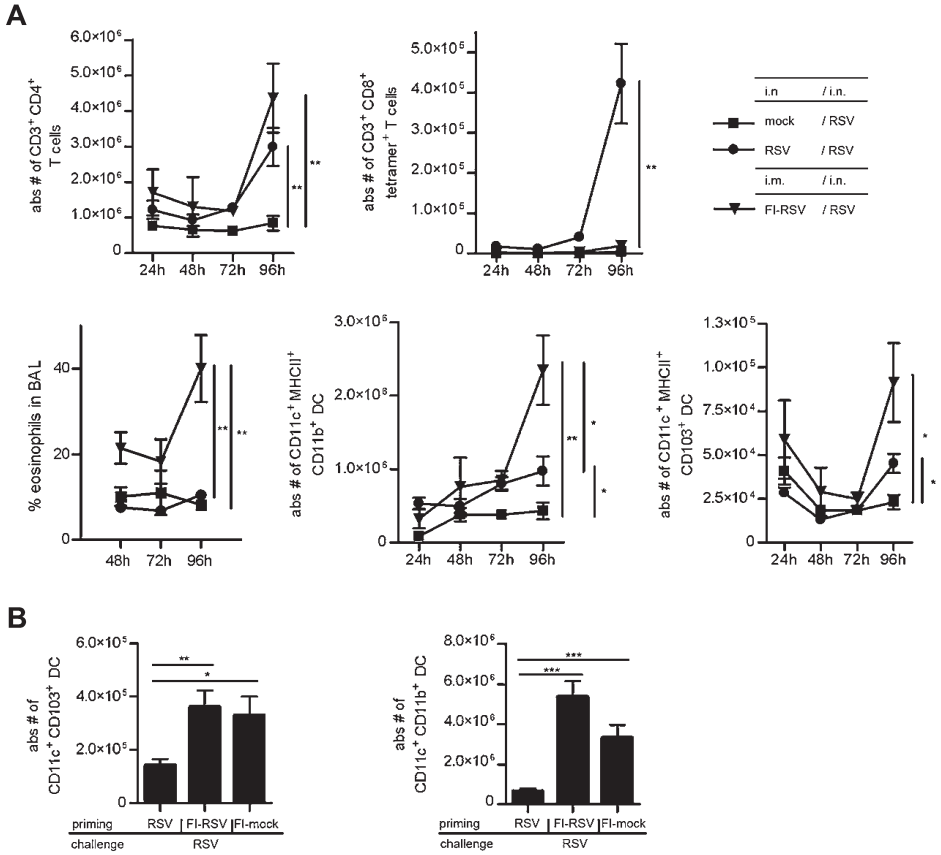


FIGURE 4. Kinetics of the cellular influx into the lungs during allergic versus non-allergic airway inflammation. (A). Three groups of mice were i.n. infected with RSV, i.n. primed with mock (HEp-2 cell culture supernatant) or i.m. vaccinated with FI-RSV. At day 28, mice were i.n. challenged with RSV. 24h, 48h, 72h and 96h after challenge lung cells were analyzed for absolute numbers of $CD3^+$ $CD4^+$ T cells, $CD3^+$ $CD8^+$ H-$2D^b$ $M_{187-195}$ $tetramer^+$ T cells, $CD11c^+$ $MHCII^{high}$ $CD11b^{high}$ DC and $CD11c^+$ $MHCII^{high}$ $CD103^+$ DC. 48h, 72h and 96h after challenge BAL was analyzed for the percentage of eosinophils. (**B**). Two groups of mice were vaccinated with either FI-RSV or FI-mock and a third group was i.n. infected with RSV. All groups were i.n. challenged at day 36 with RSV. Six days after challenge lung cells were analyzed for absolute numbers of $CD11c^+$ $MHCII^{high}$ $CD103^+$ DC and $CD11c^+$ $MHCII^{high}$ $CD11b^{high}$ DC. Error bars represent the SEM of 5 mice per group. In A. the significance was calculated using a Two-Way ANOVA and significance is shown comparing the three groups of mice at 96h after challenge. In B. significance is calculated using a One-Way ANOVA. * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$.

$CD4^+$ T cell responses became evident in the lungs of FI-RSV-vaccinated and secondary RSV-infected mice (**Figure 4A**). Virus-specific $CD8^+$ T cells arrived at the same time in the group exposed to secondary RSV infection. At this early time point no virus-specific $CD8^+$ T cells were found in FI-RSV or i.n. mock-exposed and RSV-challenged mice (**Figure 4A**). 96h after challenge eosinophil influx was observed in FI-RSV-vaccinated, RSV-challenged mice, but a slight increase in the percentage of eosinophils was already observed 48h after challenge

when compared to a secondary and primary RSV infection (**Figure 4A**). There was a strong recruitment of CD11c^{high}/MHC class II^{high} dendritic cells into the lung tissue in intramuscular FI-RSV and FI-mock-vaccinated mice compared to mice with secondary RSV infection (**Figure 4A and B**) or i.n. mock primed mice (Figure 4A). Both CD103^{+} CD11b^{low} MHC class II^{high} and CD103^{-} CD11b^{high} MHC class II^{high} DC contributed to the enhanced influx (**Figure 4A and B**;

DC gating strategy shown in Supplemented data **Figure S1**). These kinetic experiments show that the onset of the virus-specific (primary) CD8^{+} T cell response in FI-RSV-vaccinated or i.n. mock-exposed mice is not measurable at 96h post challenge in the lungs, while at this time Th2 responses/eosinophilia and DC influxes, parameters associated with allergic responses, occur with similar kinetics in FI-RSV and FI-mock-vaccinated animals.

Influenza virus infection during FCS challenge ameliorates FI-mock-primed Th2/eosinophilia and DC influx

So far our work showed that the FI-RSV/FI-mock vaccination model is similar to standard (OVA, house dust mite) mouse allergy models. These are presumably Th2-mediated diseases, where IL-4 has been shown to contribute to Th2 responses and IgE production, IL-5 to airway eosinophilia, and IL-13 to goblet cell metaplasia and mucin production and induction of airway hyper-responsiveness [45]. Moreover, the presence of neither inactivated RSV during priming nor the presence of live virus during challenge had a major impact on the Th2/eosinophilic component of the response. To establish whether the lack of an effect of the RSV infection during allergen (FCS) challenge was RSV-specific we tested whether infection with influenza virus during FCS challenge affected the balance of the Th1/Th2 response differently. For this purpose mice were vaccinated i.m. with FI-mock and i.n. challenged with FCS, or FCS and a co-administered live virus: RSV/FCS and influenza virus/FCS. To study the early innate effects of the viral infections, before substantial virus specific T cell responses were elicited, we measured RSV (RSV infected D1 cells, cultured in IMDM + FCS) and FCS- (D1 cells, cultured in IMDM + FCS) specific T cell responses in the lungs on day 5 after challenge (**Figure 5**). We found that the total cellular influx into the lungs did not differ upon infection with influenza virus and RSV (data not shown). Furthermore, no differences were observed in the percentage of allergen (FCS) specific IFN-γ producing CD4^{+} T cells in the three groups. RSV-specific-IFN-γ producing CD4^{+} and CD8^{+} T cells were absent as expected this early after primary exposure to the virus (**Figure 5A**). FCS-specific CD4^{+} Th2 responses were substantial. The fraction and absolute numbers (not shown) of FCS-specific CD4^{+} IL-4^{+} and CD4^{+} IL-13^{+} T cells was significantly lower in the influenza virus-infected mice compared to RSV-infected mice. A decrease was observed for CD4^{+} IL-5^{+} T cells upon influenza virus infection although this was not significantly different compared to RSV infected mice (**Figure 5A**). Eosinophil influx into the airways was also lower in the influenza virus-challenged mice compared to the FCS/RSV (not significant) and FCS (significant) challenged groups. In

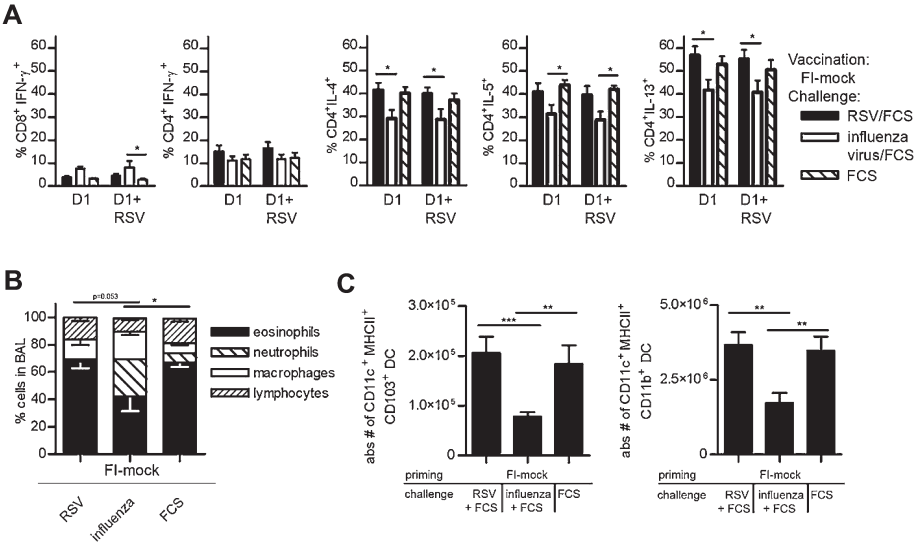


FIGURE 5. Influenza virus infection during FCS challenge decreases FI-mock primed allergic airway inflammation. Three groups of C57BL/6 mice were vaccinated with FI-mock. At day 28 mice were i.n. exposed to 1. RSV cultured in IMDM + FCS, 2. influenza virus plus FCS or 3. FCS alone. Five days after challenge, lung cells were analyzed for T cell responses and DC influx and BAL was analyzed for cellular influx. (**A**). The percentage of $CD8^+$ or $CD4^+$ T cells producing Th1 or Th2 cytokines. (**B**). Cellular composition of BAL. (**C**). Lung cells were analyzed for absolute numbers of $CD11c^+$ $MHCII^{high}$ $CD103^+$ DC and $CD11c^+$ $MHCII^{high}$ $CD11b^{high}$ DC. Error bars represent the SEM of 5 mice per group. In A. significance was calculated using a Two-Way ANOVA. In B. and C. significance was calculated using a One-Way ANOVA (in B. for the eosinophil populations). This experiment was performed three times with similar results. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

contrast to RSV-challenged and FCS-challenged mice, in influenza virus-challenged mice the decreased percentage of eosinophils was accompanied by an increase in the percentage of neutrophils (**Figure 5B**). The FCS-specific IgG1 and IgG2a/c levels in serum was similar in all three groups (data not shown) However, influenza virus infection resulted in a significant decrease in $CD11c^+$ $CD103^+$ DC and $CD11c^+$ $CD11b^{high}$ DC, compared to the RSV/FCS and FCS only challenged mice (**Figure 5C**). These findings suggest that the observed decreased Th2/eosinophilia upon influenza virus infection might be a local innate effect of this virus during antigenic challenge.

The route of priming and early recruited Th1 and $CD8^+$ T cells alter the intensity of allergic airway inflammation.

It has been shown that $CD8^+$ T cells can inhibit FI-RSV-mediated enhanced disease [20, 21]. We studied the impact of early $CD8^+$ T cell recruitment on the lung inflammatory cell composition and serum antibody response. **Figure 6A** shows that mice intravenously injected with lipopolysaccharide (LPS)-matured BM-DC loaded with the dominant RSV epitope

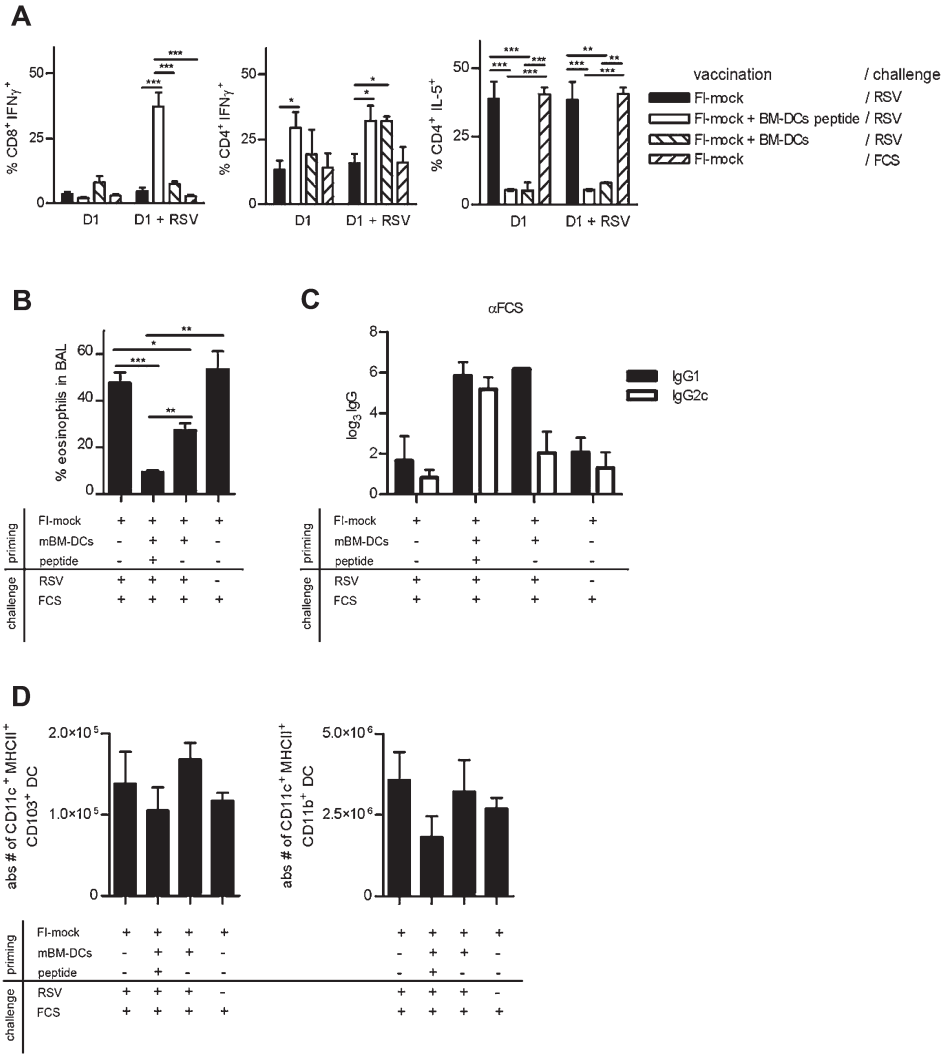


FIGURE 6. CD4⁺/8⁺ T cell priming by LPS matured BM-DC results in suppression of Th2 responses and lung eosinophilia. Three groups of mice were i.m. vaccinated with FI-mock. In addition to FI-mock vaccination, one group received mature bone marrow derived dendritic cells (BM-DC) i.v. and a second group received mature BM-DC loaded with the dominant RSV epitope $M_{187\text{-}195}$ i.v. At day 28 all mice were i.n. challenged with RSV. A fourth group was FI-mock vaccinated and i.n. challenged with FCS. (**A**). The percentage of $CD8^+$ or $CD4^+$ T cells producing Th1 or Th2 cytokines in the lungs at day 5 after challenge. (**B**). Percentage of eosinophils in BAL. (**C**). FCS specific IgG1 and IgG2a/c in sera at day 5 after challenge. (**D**). Lung cells were analyzed for absolute numbers of $CD11c^+$ $MHCII^{high}$ $CD103^+$ DC and $CD11c^+$ $MHCII^{high}$ $CD11b^{high}$ DC. Error bars represent the standard error of the mean (SEM). Results are shown for four mice per group. In A. significance was calculated using a Two-Way ANOVA. In B., C. and D. significance was calculated using One-Way ANOVA. * $p<0.05$, ** $p<0.01$ and *** $p< 0.001$.

$M_{187-195}$ at the day of FI-mock vaccination (i.m.), mounted an RSV-specific IFN-γ^+/$CD8^+$ T cell response upon i.n. challenge with RSV. Peptide loaded and unloaded BM-DC infused at the day of FI-mock vaccination led to an increased $CD4^+$ T-cell-mediated IFN-γ response. Both BM-DC and BM-DC that had been loaded with $M_{187-195}$ peptide administered at the time of FI-mock vaccination down-regulated the CD4/Th2 response to a similar extent. However, the additional RSV-specific $CD8^+$ T cell response more potently suppressed lung eosinophilia (**Figure 6B**). FCS-specific IgG1 levels increased when LPS matured BM-DC were administered intravenously while the presence of the $CD8^+$ epitope additionally enhanced the IgG2a/c response (**Figure 6C**). In mice primed with FI-mock and BM-DC no significant alterations in lung DC population were found compared to mice that did not have BM-DC-primed $CD4^+$ and $CD8^+$ T cells (**Figure 6D**). All groups of mice were analyzed for lung plasmacytoid DC (pDC) populations, however, no differences were observed in absolute numbers of pDC between the groups (data not shown). In comparison to the influenza virus-mediated local effect, BM-DC priming induced a systemic Th1/$CD8^+$ T cell response that during local RSV infection resulted in a shift of the Th2 to Th1 response against the co-administered allergen FCS.

DISCUSSION

In the present work we identified two mechanisms influencing Th2-mediated inflammatory responses in the FI-RSV vaccination model. The nature of a local virus infection during allergen (FCS or viral proteins in our study) challenge and the setting and route of vaccination could both influence the balance of Th2/Th1 responses and eosinophil influx in the lung. As previously reported early local recall of a primed $CD8^+$ T cell response during allergen challenge strongly suppressed lung eosinophilia [20, 21], but also $CD4^+$ T-cell priming by mature DC loaded with antigen (FCS) and administered intravenously, strongly suppressed lung Th2 responses and eosinophilia upon FCS challenge (**Figure 6**).

We further showed that FI-RSV vaccine-specific components that have been suggested earlier to contribute to the induction of FI-RSV induced enhanced respiratory disease, such as the formalin inactivation [17], the alum adjuvant [46-48] or particular viral proteins [49], are not essential for the induction of Th2 skewed inflammatory responses in the lung (**Figure 2 and 3**). Rather intramuscular priming with protein (either derived from inactivated virus or from contaminating FCS) was sufficient to elicit a systemic $CD4^+$ T cell response that upon recall sets the stage for the allergic secondary response. In contrast to intramuscular priming, intranasal exposure to HEp-2 cell supernatant (containing FCS) did not prime a $CD4^+$ T cell response underscoring the difference in efficacy or the nature of the immune response elicited via different vaccination routes. This difference might reflect the different access of the administered antigen to antigen presenting cell types, or the efficacy of migrating dendritic cell populations in the two different locations in transporting antigen to the lymph node, or priming naïve T cell responses.

It has been shown that a vaccinia virus recombinant expressing the G protein of RSV, administered by skin scarification causes Th2-biased RSV-specific T cell responses in the lung upon viral challenge [31, 49] despite a supposedly strong Th1-type immune priming by this virus [50]. These observations suggest that during local RSV/antigen challenge the final phenotypic switch of T cells occurs in the lungs. A similar final differentiation step in the lung has been described for $CD8^+$ T cells during influenza virus infections [51]. In line with these studies is the observation that in several models of RSV-enhanced (Th2-biased) disease, splenic T cells are not Th2 skewed while lung T cells are [52]. Thus primed T cells leaving the draining lymph nodes of the infection site seed secondary lymph nodes and spleen and migrate to the site of infection, where in the context of the local inflammatory milieu final differentiation occurs into effector cells. This scenario is supported by our observation that different virus infections might induce different innate responses locally (i.n. influenza virus versus RSV infection, **Figure 5**) that influence final T cell maturation.

Furthermore, we observed that MHC class II presented antigen at the surface of *in vitro* cultured (in medium + FCS), LPS matured conventional DC (cDC) administered intravenously primed a Th1-type response protecting against eosinophilia. This effect was enhanced when a MHC class I viral epitope was also presented during priming and local challenge (**Figure 6**). Whether this decreased Th2/eosinophilic-response was caused by the priming of naïve T cells by the administered DC themselves or by a different DC population by cross priming or whether the priming location (spleen) played a role remains to be established. Thus, besides a clear local effect on Th cell differentiation we observed that the route and setting of T cell priming might also imprint their final differentiation status. Interestingly, for vaccinia recombinant vaccines expressing RSV G protein, the intra-peritoneal priming route did not result in Th2-mediated enhanced disease during i.n. challenge with RSV [53]. This observation suggests that in the spleen T cell priming might be different from intramuscular or intradermal priming routes in predisposing $CD4^+$ T cells to become Th1 cells already during the induction phase of the response. Together these findings show that the circumstances during T cell priming as well as circumstances during challenge can both influence the ultimate nature of the $CD4^+$ T cell response during challenge infection in the lung.

Different viral infections differ in the quality and longevity of the innate immune responses they induce due to the pathogen specific interaction with innate immune receptors. This was illustrated in **Figure 5** where it was shown that influenza virus infection during i.n. FCS exposure enhanced the Th1/Th2 balance in FI-mock-primed mice at day 5 after challenge, while RSV did not alter the allergic response. A recent study compared early innate effects of influenza virus and RSV in the mouse lung and found that RSV-induced type I IFN production was low and transient (24h) whereas influenza virus mediated type I IFN production was more robust and increased over time (5 days) [54]. Because STAT 1-mediated signaling, a downstream consequence of type I IFN stimulation, could play a role in the balance of Th1/Th2 $CD4^+$ T cell responses these differences in type I IFN production might be a possible

explanation for the observed differences in the effect of both viruses during antigenic challenge [55].

Different level, quality or timing of innate immune responses caused significantly different recruitment of cDC types to the lung tissue when influenza virus infection or RSV infection coincided with FCS challenge in FI-mock-primed mice (**Figure 5**). Decreased cDC responses in comparison to FI-RSV or FI-mock-vaccinated mice were also observed in secondary or primary RSV-infected mice (**Figure 4**), conditions where Th2 responses and eosinophilia were also absent. Whether enhanced $CD11c^+$ $CD103^+$ or $CD11c^+$ $CD11b^{high}$ DC recruitment played part in the Th2-skewing of the responses or reflected a similar responsiveness of these DC types and Th2-primed T cells to local inflammatory factors (cytokines and chemokines) remains to be established. However, a role of both cDC subtypes in enhancing allergic lung inflammation has been described [43, 56]. Depletion of $CD11c^+$ DC in an OVA allergic model resulted in a reduced Th2 response [57]. The ratio of pDC with myeloid DC (mDC) (both $CD11c^+$ $CD11b^{high}$ and $CD11c^+$ $CD103^+$) present in lung tissue determined the initiation of allergic responses, whereby pDC suppressed and mDC enhanced allergic inflammation [58, 59]. In addition to DC, the lung epithelium has been shown to play an important role in inducing allergic airway inflammation. Studies with house dust mite (HDM) showed that binding of Derp2 to TLR4 specifically on epithelial cells, not on subepithelial DC, resulted in a strong Th2 response by secretion of GM-CSF, IL-33, TSLP and IL-25 [60]. Hence, different interactions of viruses with respiratory epithelium might also contribute to the eventual differentiation of T cells responding to infections in the lungs.

We and others have shown that the route of T cell priming and early recall of $CD8^+$ T cell responses at the time of antigen challenge in the lung can potently suppress lung Th2/eosinophilia responses (**Figure 6**, ref. [20-22]). Interestingly, under these conditions no significant differences were found in cDC populations of mice that did or did not develop eosinophilia (**Figure 6D**). Therefore, early innate responses depending on the type of virus infection or early adaptive immune responses could both affect the outcome of the ultimate immune response upon antigen challenge in the lung, whereby the Th2/Th1 balance might be regulated by different mechanisms.

In earlier work we have shown that primary RSV infections induce DC maturation and migration to the mediastinal lymph nodes [42]. Both sub-epithelial $CD103^+$ DC and parenchymal $CD11b^{high}$ DC migrated in roughly equal amounts and both DC types expressed MHC class I and II bound viral antigens and are therefore supposedly competent to prime naïve T cell responses, provided they reach the proper location in the lymph nodes to interact with naïve $CD4^+$ and $CD8^+$ T cells. These studies showed that RSV infections in the mouse induce sufficient inflammation to trigger a full blown adaptive T cell response. However, RSV infection in murine cells is less productive than RSV infections in human epithelium and RSV replication efficiency differs between mouse strains. Therefore, the differences in innate effects of RSV and influenza virus infections in our mouse model can not directly be

extrapolated to other mouse strains and to the human situation. Nevertheless, also in man different innate effects of viruses locally might have a different impact on the outcome of the secondary immune response in vaccinated individuals. In fact, a formalin-inactivated parainfluenza type I virus vaccine did not result in enhanced disease in vaccinated children [6]. Clearly, these factors should be addressed critically during the development of vaccines.

Viral damage, exuberant inflammation or immunopathology caused by T cells might all contribute to RSV-mediated disease. Highly neutralizing antibodies contribute to protection most likely by lowering viral load with as a consequence less viral damage to the lung tissue and lower virus induced inflammatory responses. Via lowering antigenic load, they may also affect the level of secondary T cell responses and hence protect against T cell mediated damage [15]. It has recently been shown that poorly neutralizing antibodies contribute to enhanced disease in the mouse FI-RSV-vaccination model. The major reason for the ineffective antibody response was a lack of antibody affinity maturation due to poor Toll-like receptor stimulation by inactivated RSV vaccines. Interestingly, TLR stimulation on B cells and not $CD11c^+$ cells determined the extent of antibody affinity maturation [23]. In addition, structural alterations in the fusion protein resulting from formalin treatment could also contribute to poor antibody recognition of viral epitopes in the wild type virus and impaired virus neutralization [11, 14, 17, 23]. Inclusion of a TLR ligand in the inactivated RSV vaccine protected against enhanced disease presumably by increasing antibody affinity and lowering viral load resulting in lower T cell activation and inflammatory cell influx. In our experiments we showed that similar allergic immune responses could be induced in FI-RSV-vaccinated mice that were either exposed to a live virus challenge or challenge with FCS, an innocuous antigenic contaminant present in *in vitro* cultured virus preparations. In this model we show that with the same i.n. antigenic challenge dose (FCS; whereby antibody neutralization does not play a role) Th2/eosinophilia can be effectively prevented by the mode of T cell priming. TLR-(LPS)-stimulated DC induced protective T cell immunity that counteracted Th2/eosinophilia. Therefore, TLR adjuvants might work both for the proper induction of RSV-neutralizing antibody responses and protective T cell immunity.

Extrapolation from the mouse model suggests that protective immunity to RSV requires strongly neutralizing antibodies and in addition a balanced $CD4^+/CD8^+$T cell response, whereby the quality of the local innate response during vaccination might be crucial for both the T cell and B cell arms of the immune response.

MATERIALS AND METHODS

Mice, viruses and infections

Pathogen-free 6-8 week old C57BL/6cjo mice were purchased from Charles River (Maastricht, The Netherlands). The mouse study protocols were approved by the Animal Ethics

Committee of the University Medical Centre (UMC) Utrecht. RSV A2 strain was grown in HEp-2 cells, purified by polyethylene glycol precipitation, resuspended in PBS supplemented with 10% sucrose, and stored in liquid nitrogen. Mice were lightly anesthetized with isoflurane and intranasally infected with 2-3 x 10^6 plaque-forming units RSV in a volume of 50 µl. Influenza virus strain A HK/2/68 was kindly provided by Dr. T. Rygiel (Wilhelmina Children's Hospital, Utrecht, The Netherlands). The virus was grown in fertilized hen's eggs. Tissue culture infective dose $(TCID)_{50}$ was determined after titration in MDCK cells. Mice were anesthetized with isoflurane and intranasally infected with 50 µl of an influenza virus preparation (350 $TCID_{50}$ in PBS containing 1% FCS).

Vaccine formulations

FI-RSV was prepared by the method used for the original vaccine tested in the 1960's [8]. RSV A2 strain was grown for 48 hrs in HEp-2 cells. Culture medium was cleared from cell debris by low speed centrifugation (1000xg, 10 min, 4°C). Formalin was added to 3 x 10^6 plaque-forming units RSV/ml containing supernatant at a final dilution of 1:4000 and incubated at 37°C for 3 days with stirring. After ultra-centrifugation (50,000xg_{av}, 1 h, 4°C) of the formalin-inactivated culture medium, resulting pellets were resuspended in 1/25th of the original volume in IMDM without supplements. The vaccine was adsorbed to 4 mg/ml aluminium hydroxide overnight at room temperature while stirring. Finally, the vaccine was pelleted by centrifugation (1000xg, 30 min) and resuspended in 1/4th volume in PBS. This procedure resulted in a final vaccine that was concentrated 100-fold and contained 16 mg/ml aluminum hydroxide. FI-mock was prepared with culture medium of uninfected HEp-2 cells.

Cell cultures

HEp-2 cells were cultured in IMDM (Gibco, 21980-065), supplemented with 5% FCS and 1% penicillin/streptomycin. RSV viral cultures and titration assays on HEp-2 cells were performed in IMDM containing 1% FCS and 1% penicillin/streptomycin. D1, a mouse dendritic cell line derived from C57BL/6 mice [37] used for restimulation of lung T cells was maintained in IMDM, 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin and 50 µM β-mercapto-ethanol and supplemented with 30% conditioned medium from GM-CSF producing R1 cells (mouse fibroblast NIH3T3, transfected with GM-CSF gene [37]). When indicated in the figure legends D1 cells were cultured in heat-inactivated mouse serum (of naïve C57BL/6 mice) supplemented with conditioned medium from GM-CSF producing R1 cells, that had also been cultured in mouse serum, to avoid the presentation of FCS derived antigens by MHC molecules on the surface of D1 cells. Bone marrow derived dendritic cells (BM-DC) were obtained by culturing BM derived from C57BL/6 femurs for 7 days in

RPMI1640 (Gibco, 61870-044) supplemented with 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin and 50 μM β-mercapto-ethanol as described before [38], using 30% culture supernatant from R1 cells. The percentage of $CD11c^+$ cells, determined by staining with anti-CD11c (BD, clone HL3), was (routinely > 70%) determined by analysis with a Facs Canto Flowcytometer. To obtain mature antigen loaded antigen presenting cells, BM-DC were incubated with 1 μg/ml LPS (Sigma, L4516-1MG) for 24h followed by a 1h incubation with the $H\text{-}2D^b$ restricted peptide from the RSV M protein ($M_{187-195}$, NAITNAKII) [39]. A total number of 0.8 x 10^6 $CD11c^+$ mature and peptide loaded BM-DC were intravenously injected into FI-mock-vaccinated mice. As a control, mice were injected with mature LPS-BM-DC not exposed to $M_{187-195}$.

Vaccination and challenge of C57BL/6 mice

Mice were i.m.-vaccinated with 50 μl FI-RSV, FI-mock or subunit vaccine preparations at day 0. After 28 or 36 days (as described in the figure legends) mice were challenged with RSV and at various days after challenge (see figure legends) T cell responses in lung tissue of vaccinated mice were compared to responses during a secondary RSV infection. When the effect of vaccination was compared to a primary RSV infection, mice were challenged with RSV at day 28 after vaccination and sacrificed at day 8 after challenge i.e. the peak of the primary T cell response. In experiments studying innate virus effects (influenza virus and RSV) during antigen (FCS)-challenge on bronchoalveolar lavage (BAL) and lung inflammatory cell recruitment in FI-mock-vaccinated mice, animals were sacrificed 5 days after challenge before substantial T cell responses were elicited in the lung. Kinetic studies were performed to optimize the dose response for antigen specific T cell responses in lung tissue upon primary and secondary infection.

Tissue sampling

Mice were sacrificed by i.p. injection of 300 μl pentobarbital. Cells from the airways were obtained by BAL with 3 x 1 ml of 0.15 M NaCl. Prior to removal, the lungs were perfused with PBS containing 100 U/ml heparin. Lungs were cut to 1 x 1 mm pieces and incubated with collagenase (2.4 mg/ml, Roche Applied Science) and DNase (1 mg/ml, Roche Applied Science) for 20 min. at 37°C. Single cell suspensions were prepared by processing the tissue trough 70 μm cell strainers (BD Falcon).

Flow cytometry

To identify DC populations in lung tissue, lung single cell suspensions were preincubated with anti-CD16/CD32 (BD, clone 2.4G2) to reduce non-specific binding. Cells were washed

with PBS containing 2% FCS, 2 mM EDTA and 0.02% NaN_3 (FACS buffer) and incubated with anti-CD11c (BD, clone HL3), anti-MHC-II (I-A^b/I-E^b) (BD, clone AF6-120.1), anti-CD103 (BD, clone M290) and anti-CD11b (BD, clone M1/70). Stained samples were acquired on a FACS-Canto flowcytometer (BD) and data was analyzed using FacsDiva software (BD).

For intracellular cytokine staining, single cell suspension of lung cells (10^6) were stimulated for 6 h at 37°C, 5% CO_2, with 0.2 x 10^6 RSV infected D1 cells or uninfected D1 cells in 200 µl IMDM, 5% FCS, penicillin/streptomycin, 50 µM β-mercapto-ethanol and 50 U/ml recombinant human IL-2 (Roche, 11147528001). Brefeldin A (10 µg/ml; Sigma, B7651) was added for the duration of the stimulation to facilitate intracellular accumulation of cytokines. D1 cells were infected for 48 hours with RSV (multiplicity of infection, moi 2) before addition to the lung cell suspension. Cytokine production by $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. Cells were washed with FACS buffer and stained for surface markers with anti-CD8 (BD, clone 53-6.7) and anti-CD4 (BD, clone RM4-5). Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (BD, 554722) solution and Perm/Wash buffer (BD, 554723). Intracellular cytokines were detected with anti-IFN-γ (BD, clone XMG1.2), anti-IL-5 (BD, clone TRFK5), anti-IL-4 (BD, clone 11B11) and anti-IL-13 (eBioscience, clone eBio13a). Stained samples were acquired on a FACSCanto flowcytometer (BD) and data were analyzed using FacsDiva software (BD).

May-Grünwald/Giemsa staining

BAL cell morphology was determined by May-Grünwald/Giemsa staining. Cells were fixed with methanol for 5 min. and stained with a 1:1 dilution of May-Grünwald (Mallinckrodt Baker, 3855) with Na_2HPO_4 and KH_2PO_4 buffered water pH 6.8 for 5 minutes. After washing with buffered water pH 6.8, cells were stained with a 1:8 dilution of Giemsa (Merck, 1.09204.500) with buffered water pH 6.8 for 15 min. and finally washed with water. One hundred cells were counted to determine the percentages of eosinophils, neutrophils, macrophages and lymphocytes.

ELISA

ELISA plates (NUNC) were coated with denatured RSV lysate from RSV-infected HEp-2 cells or with FCS in PBS for 18 h at 4°C. After removal of unbound RSV-lysate/FCS, plates were blocked in 200 µl 1% bovine serum albumin (BSA) in 0.05% $Tween_{20}$/ PBS for 1h at 37°C. Serum of FI-RSV, FI-mock-vaccinated, RSV-infected mice or pooled serum samples of naïve mice, diluted in 0.1% BSA/ 0.05% $Tween_{20}$/PBS, was added (25 µl/well). After 2h incubation (RT) and washing (0.05% $Tween_{20}$ in PBS) plates were incubated with secondary HRP-labelled antibodies, anti-IgG1 (Invitrogen, 04-6120), anti-Ig2a/c or anti-IgE (Invitrogen, 04-7000) diluted in PBS supplemented with 0.1% BSA/0.05% $Tween_{20}$ for 2h at RT. C57BL/6

mice express the Igh1-b allele encoding for antibodies of the IgG2c isotype and lack the allele of the IgG2a isotype [40]. Therefore, we used in addition to an IgG2a (Invitrogen, 04-6220) specific secondary antibody also an IgG2c (immunology consultant laboratory, GG2c-90P) specific antibody. With both secondary antibodies we obtained similar results. The data for the IgG2c-specific antibodies were shown in the figures. After removal of the secondary antibody, the substrate 3,3',5,5'-Tetramethylbenzidine in NaAc (pH 5.5) and H_2O_2 were added to the wells for 15 min. The enzymatic activity was stopped by adding 9.8% H_2SO_4 and measured at an OD_{450}. Elisa titers were expressed as the reciprocal of the last dilution with an OD_{450}>0.2 after subtraction of background OD_{450} measured with control mouse serum.

Statistical analysis

Data were analyzed for statistical significance using Students *t*, One-Way ANOVA or Two-Way ANOVA tests as indicated in the figure legends. Data are expressed as the mean +/- standard error of the mean (SEM). A *P* value <0.05 was taken as the level of significance.

ACKNOWLEDGEMENTS

BAL cell morphology determination was assisted by W.E. van Dijk-Bosch and G. den Engelsman.

Abbreviations used in this paper: RSV, respiratory syncytial virus; FI-RSV, formalin inactivated RSV; FI-mock, formalin inactivated mock; DC, dendritic cell; BM-DC, bone marrow derived DC; IMDM, Iscove's Modified Dulbecco's Medium; BAL, bronchoalveolar lavage; moi, multiplicity of infection; SEM, standard error of the mean.

REFERENCES

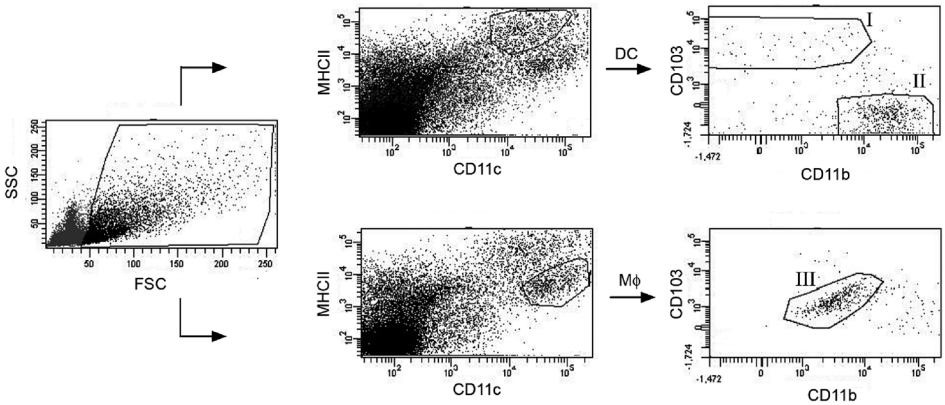
1. P. Glezen and F.W. Denny, Epidemiology of acute lower respiratory disease in children, N. Engl. J. Med. 288 (1973) 498-505.
2. A.R. Falsey, P.A. Hennessey, M.A. Formica, C. Cox, E.E. Walsh, Respiratory syncytial virus infection in elderly and high-risk adults, N. Engl. J. Med. 352 (2005) 1749-1759.
3. R.A. Karron, R.J. Singleton, L. Bulkow, A. Parkinson, D. Kruse, I. DeSmet et al, Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group, J. Infect. Dis. 180 (1999) 41-49.
4. A.Z. Kapikian, R.H. Mitchell, R.M. Chanock, R.A. Shvedoff, C.E. Stewart, An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine, Am. J. Epidemiol. 89 (1969) 405-421.
5. V.A. Fulginiti, J.J. Eller, O.F. Sieber, J.W. Joyner, M. Minamitani, G. Meiklejohn, Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine, Am. J. Epidemiol. 89 (1969) 435-448.
6. H.W. Kim, J.G. Canchola, C.D. Brandt, G. Pyles, R.M. Chanock, K. Jensen et al, Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine, Am. J. Epidemiol. 89 (1969) 422-434.
7. J. Chin, R.L. Magoffin, L.A. Shearer, J.H. Schieble, E.H. Lennette, Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population, Am. J. Epidemiol. 89 (1969) 449-463.
8. G.A. Prince, S.J. Curtis, K.C. Yim, D.D. Porter, Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine, J. Gen. Virol. 82 (2001) 2881-2888.
9. H.W. Kim, S.L. Leikin, J. Arrobio, C.D. Brandt, R.M. Chanock, R.H. Parrott, Cell-mediated immunity to respiratory syncytial virus induced by inactivated vaccine or by infection, Pediatr. Res. 10 (1976) 75-78.
10. P.A. Piedra, H.S. Faden, G. Camussi, D.T. Wong, P.L. Ogra, Mechanism of lung injury in cotton rats immunized with formalin-inactivated respiratory syncytial virus, Vaccine 7 (1989) 34-38.
11. G.A. Prince, A.B. Jenson, V.G. Hemming, B.R. Murphy, E.E. Walsh, R.L. Horswood et al, Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus, J. Virol. 57 (1986) 721-728.
12. M.E. Waris, C. Tsou, D.D. Erdman, S.R. Zaki, L.J. Anderson, Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern, J. Virol. 70 (1996) 2852-2860.
13. R.L. de Swart, T. Kuiken, H.H. Timmerman, G. van Amerongen, B.G. Van Den Hoogen, H.W. Vos et al, Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection, J. Virol. 76 (2002) 11561-11569.
14. B.R. Murphy and E.E. Walsh, Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity, J. Clin. Microbiol. 26 (1988) 1595-1597.
15. P.J. Openshaw, F.J. Culley, W. Olszewska, Immunopathogenesis of vaccine-enhanced RSV disease, Vaccine 20 Suppl 1 (2001) S27-S31.
16. M. Connors, A.B. Kulkarni, C.Y. Firestone, K.L. Holmes, H.C. Morse, III, A.V. Sotnikov et al, Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge

of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells, J. Virol. 66 (1992) 7444-7451.
17. A. Moghaddam, W. Olszewska, B. Wang, J.S. Tregoning, R. Helson, Q.J. Sattentau et al, A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines, Nat. Med. 12 (2006) 905-907.
18. F.P. Polack, M.N. Teng, P.L. Collins, G.A. Prince, M. Exner, H. Regele et al, A role for immune complexes in enhanced respiratory syncytial virus disease, J. Exp. Med. 196 (2002) 859-865.
19. T. Hussell, C.J. Baldwin, A. O'Garra, P.J. Openshaw, CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection, Eur. J. Immunol. 27 (1997) 3341-3349.
20. M.R. Olson and S.M. Varga, CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease, J. Immunol. 179 (2007) 5415-5424.
21. M.R. Olson, S.M. Hartwig, S.M. Varga, The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia, J. Immunol. 181 (2008) 7958-7968.
22. W.W. Stevens, J. Sun, J.P. Castillo, T.J. Braciale, Pulmonary eosinophilia is attenuated by early responding CD8(+) memory T cells in a murine model of RSV vaccine-enhanced disease, Viral Immunol. 22 (2009) 243-251.
23. M.F. Delgado, S. Coviello, A.C. Monsalvo, G.A. Melendi, J.Z. Hernandez, J.P. Batalle et al, Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease, Nat. Med. 15 (2009) 34-41.
24. A. Boelen, A. Andeweg, J. Kwakkel, W. Lokhorst, T. Bestebroer, J. Dormans et al, Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice, Vaccine 19 (2000) 982-991.
25. A.C. Rimaniol, G. Gras, F. Verdier, F. Capel, V.B. Grigoriev, F. Porcheray et al, Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type, Vaccine 22 (2004) 3127-3135.
26. H. Sun, K.G. Pollock, J.M. Brewer, Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation in vitro, Vaccine 21 (2003) 849-855.
27. M. Ulanova, A. Tarkowski, M. Hahn-Zoric, L.A. Hanson, The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism, Infect. Immun. 69 (2001) 1151-1159.
28. P.A. Piedra, G. Camussi, P.L. Ogra, Immune response to experimentally induced infection with respiratory syncytial virus: possible role in the development of pulmonary disease, J. Gen. Virol. 70 (Pt 2) (1989) 325-333.
29. P.A. Piedra, P.R. Wyde, W.L. Castleman, M.W. Ambrose, A.M. Jewell, D.J. Speelman et al, Enhanced pulmonary pathology associated with the use of formalin-inactivated respiratory syncytial virus vaccine in cotton rats is not a unique viral phenomenon, Vaccine 11 (1993) 1415-1423.
30. F. Vaux-Peretz, J.M. Chapsal, B. Meignier, Comparison of the ability of formalin-inactivated respiratory syncytial virus, immunopurified F, G and N proteins and cell lysate to enhance pulmonary changes in Balb/c mice, Vaccine 10 (1992) 113-118.
31. F.J. Culley, A.M. Pennycook, J.S. Tregoning, T. Hussell, P.J. Openshaw, Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology, J. Virol. 80 (2006) 4521-4527.
32. C.M. Percopo, Z. Qiu, S. Phipps, P.S. Foster, J.B. Domachowske, H.F. Rosenberg, Pulmonary eosinophils and their role in immunopathologic responses to formalin-inactivated pneumonia virus of mice, J. Immunol. 183 (2009) 604-612.

33. A.F. Antonis, R.S. Schrijver, F. Daus, P.J. Steverink, N. Stockhofe, E.J. Hensen et al, Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: exploring the parameters of pathogenesis, J. Virol. 77 (2003) 12067-12073.
34. W.V. Kalina, A.R. Woolums, R.D. Berghaus, L.J. Gershwin, Formalin-inactivated bovine RSV vaccine enhances a Th2 mediated immune response in infected cattle, Vaccine 22 (2004) 1465-1474.
35. R.L. de Swart, B.G. Van Den Hoogen, T. Kuiken, S. Herfst, G. van Amerongen, S. Yuksel et al, Immunization of macaques with formalin-inactivated human metapneumovirus induces hypersensitivity to hMPV infection, Vaccine 25 (2007) 8518-8528.
36. F.P. Polack, P.G. Auwaerter, S.H. Lee, H.C. Nousari, A. Valsamakis, K.M. Leiferman et al, Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody, Nat. Med. 5 (1999) 629-634.
37. M. Foti, F. Granucci, D. Aggujaro, E. Liboi, W. Luini, S. Minardi et al, Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site, Int. Immunol. 11 (1999) 979-986.
38. K. Inaba, M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara et al, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor, J. Exp. Med. 176 (1992) 1693-1702.
39. M.V. Lukens, E.A. Claassen, P.M. de Graaff, M.E. van Dijk, P. Hoogerhout, M. Toebes et al, Characterization of the CD8+ T cell responses directed against respiratory syncytial virus during primary and secondary infection in C57BL/6 mice, Virology 352 (2006) 157-168.
40. R.M. Martin, J.L. Brady, A.M. Lew, The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice, J. Immunol. Methods 212 (1998) 187-192.
41. P.J. Openshaw, Immunity and immunopathology to respiratory syncytial virus. The mouse model, Am. J. Respir. Crit Care Med. 152 (1995) S59-S62.
42. M.V. Lukens, D. Kruijsen, F.E. Coenjaerts, J.L. Kimpen, G.M. van Bleek, Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node, J. Virol. 83 (2009) 7235-7243.
43. H. Hammad, M. Plantinga, K. Deswarte, P. Pouliot, M.A. Willart, M. Kool et al, Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen, J. Exp. Med. 207 (2010) 2097-2111.
44. L.J. Robays, T. Maes, S. Lebecque, S.A. Lira, W.A. Kuziel, G.G. Brusselle et al, Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation, J. Immunol. 178 (2007) 5305-5311.
45. S.T. Holgate and R. Polosa, Treatment strategies for allergy and asthma, Nat. Rev. Immunol. 8 (2008) 218-230.
46. M. Kool, T. Soullie, M. van Nimwegen, M.A. Willart, F. Muskens, S. Jung et al, Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells, J. Exp. Med. 205 (2008) 869-882.
47. A. Sokolovska, S.L. Hem, H. HogenEsch, Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants, Vaccine 25 (2007) 4575-4585.
48. K.M. Neuzil, J.E. Johnson, Y.W. Tang, J.P. Prieels, M. Slaoui, N. Gar et al, Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine, Vaccine 15 (1997) 525-532.
49. P.J. Openshaw, S.L. Clarke, F.M. Record, Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized

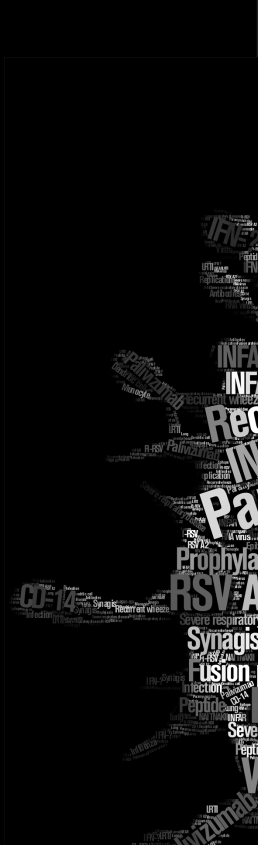
to the major surface glycoprotein G, Int. Immunol. 4 (1992) 493-500.
50. T. Tian, L. Liu, E.J. Freyschmidt, G.F. Murphy, T.S. Kupper, R.C. Fuhlbrigge, Overexpression of IL-1alpha in skin differentially modulates the immune response to scarification with vaccinia virus, J. Invest Dermatol. 129 (2009) 70-78.
51. C.W. Lawrence, R.M. Ream, T.J. Braciale, Frequency, specificity, and sites of expansion of CD8+ T cells during primary pulmonary influenza virus infection, J. Immunol. 174 (2005) 5332-5340.
52. A. Srikiatkhachorn and T.J. Braciale, Virus-specific memory and effector T lymphocytes exhibit different cytokine responses to antigens during experimental murine respiratory syncytial virus infection, J. Virol. 71 (1997) 678-685.
53. G.P. Bembridge, R. Garcia-Beato, J.A. Lopez, J.A. Melero, G. Taylor, Subcellular site of expression and route of vaccination influence pulmonary eosinophilia following respiratory syncytial virus challenge in BALB/c mice sensitized to the attachment G protein, J. Immunol. 161 (1998) 2473-2480.
54. N.A. Jewell, N. Vaghefi, S.E. Mertz, P. Akter, R.S. Peebles, Jr., L.O. Bakaletz et al, Differential type I interferon induction by respiratory syncytial virus and influenza a virus in vivo, J. Virol. 81 (2007) 9790-9800.
55. Z. Mikhak, C.M. Fleming, B.D. Medoff, S.Y. Thomas, A.M. Tager, G.S. Campanella et al, STAT1 in peripheral tissue differentially regulates homing of antigen-specific Th1 and Th2 cells, J. Immunol. 176 (2006) 4959-4967.
56. S.R. Beaty, C.E. Rose, Jr., S.S. Sung, Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation, J. Immunol. 178 (2007) 1882-1895.
57. L.S. van Rijt, S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez et al, In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma, J. Exp. Med. 201 (2005) 981-991.
58. B.N. Lambrecht, M. De Veerman, A.J. Coyle, J.C. Gutierrez-Ramos, K. Thielemans, R.A. Pauwels, Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation, J. Clin. Invest 106 (2000) 551-559.
59. H.J. de Heer, H. Hammad, T. Soullie, D. Hijdra, N. Vos, M.A. Willart et al, Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen, J. Exp. Med. 200 (2004) 89-98.
60. H. Hammad, M. Chieppa, F. Perros, M.A. Willart, R.N. Germain, B.N. Lambrecht, House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells, Nat. Med. 15 (2009) 410-416.

SUPPORTING INFORMATION



Supplementary figure 1. Gating strategy for lung DC populations. Dendritic cells were identified in the life cell gate (based on forward scatter, FSC and side scatter, SSC) CD11c^{pos}, and high expression of MHC class II. Two DC subsets were further defined on the bases of the expression of CD103 and CD11b respectively: MHC class II^{high} CD103^{+} CD11b^{low} (I) and MHC class II^{high} CD103^{-} CD11b^{high} (II). Pulmonary macrophages (MΦ) were identified as CD11c^{pos}, MHC class II^{intermediate} cells (III).

Chapter 3



Respiratory Syncytial Virus induced type I IFN production by pDC is regulated by RSV-infected airway epithelial cells, RSV-exposed monocytes and virus specific antibodies.

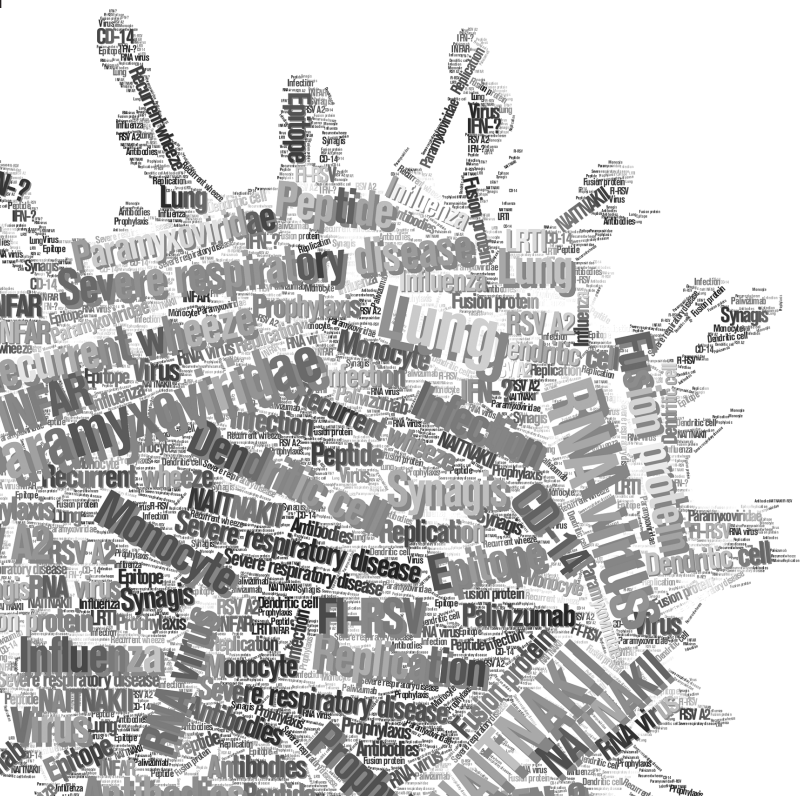
Marcel A. Schijf [1,2], Michael V. Lukens[1], Debby Kruijsen[1], Nathalie O.P. van Uden[1], Johan Garssen[2,4], Frank E.J. Coenjaerts[3], Belinda van't Land[1,2] and Grada M. van Bleek[1#]

[1]Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands.

[2]Department of Immunology, Danone Research - Centre for Specialised Nutrition, Wageningen, The Netherlands.

[3]Department of Medical Microbiology, University Medical Center, Utrecht, The Netherlands.

[4]Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands



PloS One (2013): In press

ABSTRACT

Innate immune responses elicited upon virus exposure are crucial for the effective eradication of viruses, the onset of adaptive immune responses and for establishing proper immune memory. Respiratory syncytial virus (RSV) is responsible for a high disease burden in neonates and immune compromised individuals, causing severe lower respiratory tract infections. During primary infections exuberant innate immune responses may contribute to disease severity. Furthermore, immune memory is often insufficient to protect during RSV re-exposure, which results in frequent symptomatic reinfections. Therefore, identifying the cell types and pattern recognition receptors (PRRs) involved in RSV-specific innate immune responses is necessary to understand incomplete immunity against RSV. We investigated the innate cellular response triggered upon infection of epithelial cells and peripheral blood mononuclear cells. We show that $CD14^+$ myeloid cells and epithelial cells are the major source of IL-8 and inflammatory cytokines, IL-6 and TNF-α, when exposed to live RSV. Three routes of RSV-induced IFN-α production can be distinguished that depend on the cross-talk of different cell types and the presence or absence of virus specific antibodies, whereby pDC are the ultimate source of IFN−α. RSV-specific antibodies facilitate direct TLR7 access into endosomal compartments, while in the absence of antibodies, infection of monocytes or epithelial cells is necessary to provide an early source of type I interferons, required to engage the IFNAR-mediated pathway of IFN−α production by pDC. However, at high pDC density infection with RSV causes IFN−α production without the need for a second party cell. Our study shows that cellular context and immune status are factors affecting innate immune responses to RSV. These issues should therefore be addressed during the process of vaccine development and other interventions for RSV disease.

INTRODUCTION

The innate immune system is triggered upon recognition of pathogen associated molecular patterns (PAMPS) and sets the stage for the subsequent initiation of an appropriate immune response against an invading pathogen [1]. Toll like receptors (TLRs), cytoplasmic sensors (RIG-I like receptors RLRs, RIG-I, MDA5, LGP2) and nucleotide-binding oligomerization domain receptors (NOD-like receptors, NLRs) have unique specificities for pathogen-specific molecular structures [2]. In general pathogens contain several PAMPS, and in addition evasion mechanisms to suppress innate or adaptive immune responses. Combined with a specific entry locale in the body and the specific mode of interaction with host cell types, each pathogen induces unique tailored immune responses. RSV is a negative stranded RNA virus causing respiratory tract infections with sometimes a severe disease course especially in infants, immunocompromised and elderly individuals [3–5]. Due to high infection rates, RSV causes a high disease burden during yearly epidemics [6]. Important issues that need to be solved for RSV are the exact sequence of events and correlates of disease upon RSV infection during primary exposure and the reason for inadequate immune protection against reinfections that are frequent for this virus.

Viral infections are characteristically accompanied by type I interferon responses resulting from interaction of viral RNA with TLR7 and TLR3, for respectively single- stranded RNA or double-stranded RNA getting access to endosomal compartments [7]. In addition, cytoplasmic RNA helicase-like sensors such as RIG-I and MDA detect viral RNA upon infection when viral RNA replication intermediates are present in the cytoplasm [8–11]. Type I interferon induction is a crucial step to initiate the cellular antiviral response, but in addition affects the nature and efficacy of the induction of adaptive immune responses [12]. For RSV it has additionally been reported that the membrane Fusion (F) and attachment (G) glycoproteins interact with TLR2 (F) and TLR4 (both F and G) [13,14]. The importance of proper TLR interactions during the initiation of RSV specific adaptive immune responses have been revealed by a human vaccination trial and in animal models using a formalin-inactivated RSV vaccine. The lack of proper TLR signals provided by this and other inactivated RSV vaccines precluded high affinity antibody production [15]. Ineffective virus neutralization upon subsequent natural RSV exposure and strong Th2-biased T cell responses caused dramatic disease enhancement in vaccinated children and animals [15,16]. Current knowledge of innate immune responses induced by RSV comes from murine *in vivo* models [17–21], *in vitro* studies on the interaction of the virus with human cell lines [22–24], purified cells [25–29] or *in vitro* cultured dendritic cells [30–32]. In the present work, we studied the interaction of RSV with a mixture of peripheral blood mononuclear cells (PBMC) that represent different cell types, each with a specific set of pattern recognition receptors. We determined the innate response of individual cells in the mixture, the reciprocal effects of different innate immune responses by different cell subsets in the mixture and the role of virus specific antibodies in these responses.

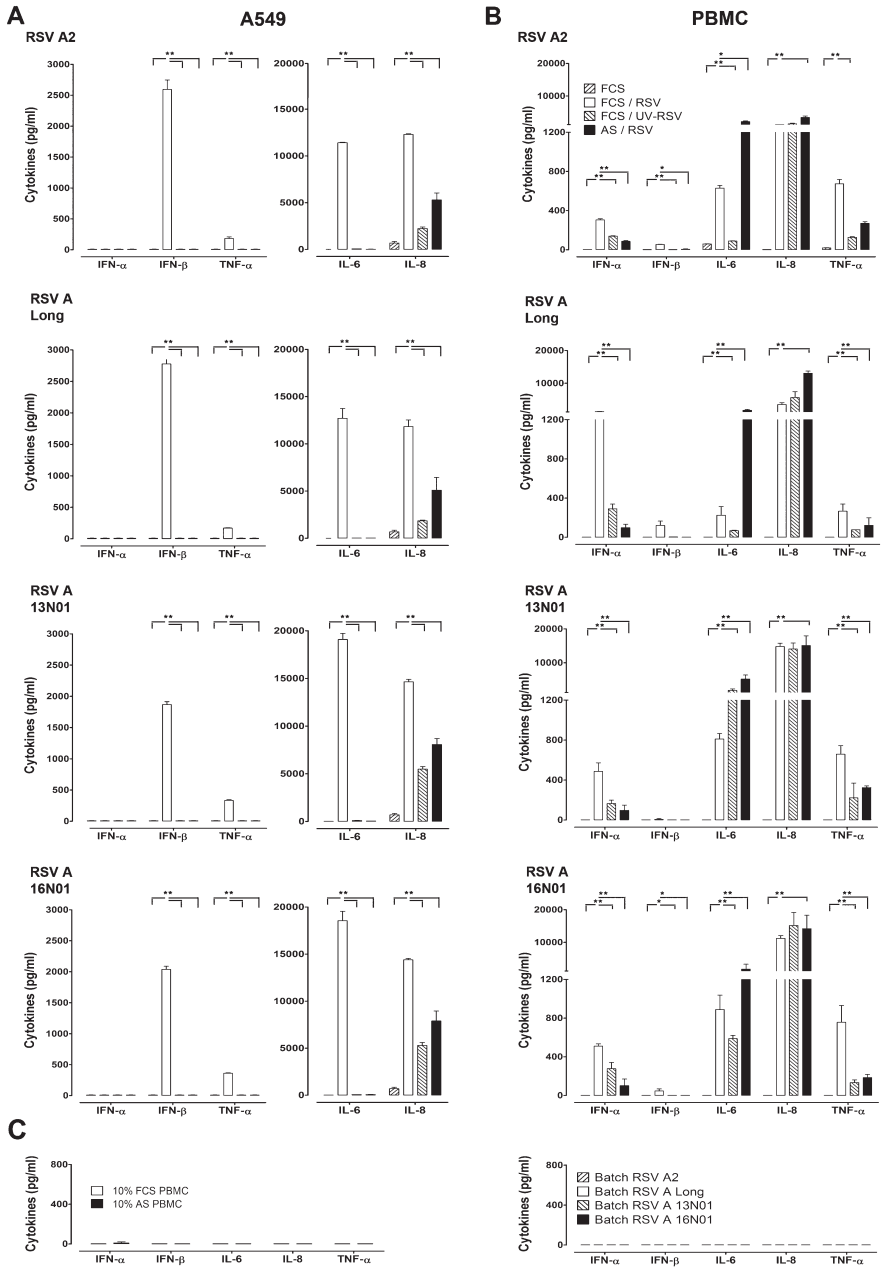
RESULTS

Cell specific interaction and innate immune response to RSV.

Host innate immune responses activated following RSV infection are suspected to contribute to RSV disease [33]. We studied the innate immune response induced by RSV in PBMC and an established cell line of human type II alveolar epithelial cells, A549. Each of the cells within PBMC and epithelial cells contain a cell specific characteristic set of innate immune receptors and potentially different attachment receptors. After exposure of PBMC and A549 cells to live, non-infectious UV-inactivated RSV or Ab-neutralized RSV for 20 hrs. at 37ºC, we measured a set of cytokines in the supernatants of these cultures. 10% autologous serum of all donors, used in all experiments described in this study, completely prevented infection of A549 with all strains of RSV at a multiplicity of infection (MOI) of 5 (confirmed by the absence of surface staining by RSV specific monoclonal Ab to the RSV F protein on A549 cells exposed to RSV for 24 hours, data not shown). The complete inhibition of infection in the presence of 10% autologous serum or UV-irradiation was further confirmed by the complete absence of IFN-β and IL-6 production by RSV exposed A549 cells, which depends for epithelial cell lines on the access of viral RNA to intra-cellular RLR (**Figure 1A**) [34,35]. The characteristic pattern of cytokines produced upon RSV exposure is shown for the A549 cell line and PBMC in **Figure 1**. The antiviral type I IFN response and inflammatory cytokine response elicited by RSV A2 and RSV Long strain (a laboratory strain that lost the ability of natural strains to efficiently suppress type I interferon induction) and two recently isolated strains, 13N01 and 16N01, were compared at MOI 5. All four strains failed to induce IFN-α protein synthesis by A549 cells (**Figure 1A**). The release of IFN–β protein by A549 cells depended on infection and was similar for all strains. In PBMC, all four strains induced significant amounts of IFN-α upon RSV infection and lower amounts when virus was inactivated by UV-irradiation or neutralized in autologous serum (**Figure 1B**). RSV Long strain induced the highest amount of IFN-α presumably due to ineffective suppression of type I IFN production by NS proteins [35]. A549 epithelial cells mainly produced the inflammatory cytokines IL-6 and TNF-α in an infection dependent process, while IL-8 was induced by live RSV and still in significant amounts by non-infectious virus. However, while the IL-8 induction by infectious RSV was always present, induction of IL-8 by inactivated virus was variable between experiments and may depend on cell culture conditions [36]. The pattern and level of inflammatory cytokines

---

FIGURE 1. Cell specific innate immune response to RSV. Cytokine responses upon RSV exposure by lung epithelial cell line A549 (**A**) and human PBMC (**B**). Cells were co-cultured with the virus strains: RSV A2, RSV Long strain or 2 recent subtype A isolates, 13NO1 and 16NO1 at MOI 5. In addition to live RSV, the response to UV-inactivated and neutralized RSV in autologous serum (AS) was measured. After 20 hrs. incubation, cytokines in supernatant were measured by multiplex immunoassay and ELISA. In **figure 1A** the data represent mean values from duplicate experiments using human serum from four different donors. Figure **1B** shows the



cytokine responses in human PBMC exposed to RSV A2, representing the mean values measured in 6 different donors. For the other virus strains, means for four different donors are depicted whereby similar results were obtained in duplicate experiments. (**C**) Control measurements of cytokines produced in PBMC cultured in the presence of medium containing 10% FCS or autologous serum (AS, left) and cytokine measurements in the virus batches used in panels A and B (right). Data represent the mean ± SEM and were analyzed using two way ANOVA followed by a Bonferroni post-test, *P< 0.05, **P <0.01.

produced in A549 cells were similar for all virus strains. The purified virus batches and supernatant of all PBMC cultures in autologous serum without virus were negative for the cytokines tested (**Figure 1C**).

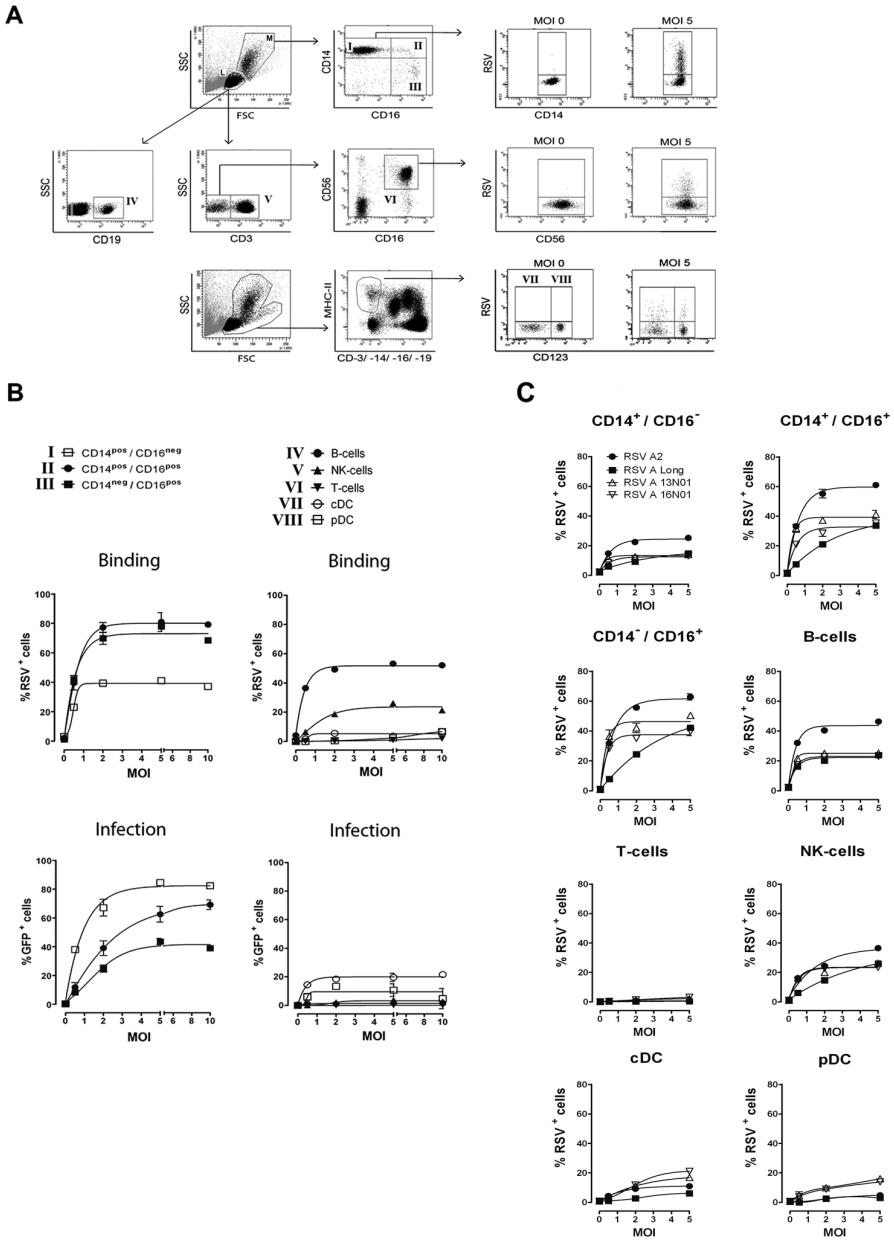
RSV infection of PBMC induced the production of significant amounts of IL-6, IL-8 and TNF-α in addition to IFN-α, while IFN-β was barely detectible. Similar to A549 cells the amount of TNF-α produced by PBMC was suppressed when the virus was inactivated by UV irradiation or neutralised by autologous serum. In contrast IL-8 production was not affected by virus inactivation. The production of IL-6 was enhanced in the presence of autologous serum. At a lower viral dose (MOI 1), IL-6 production was only observed when PBMC were exposed to RSV in the presence of autologous serum (**Figure S1**). In summary, these results show that in A549 epithelial cells cytokine responses against RSV are most efficiently induced upon infection. PBMC respond to infectious virus with the production of IFN-α and TNF-α, while IL-6 is produced at higher amounts in the presence of human serum at a concentration whereby RSV is neutralized. These results therefore suggest different mechanisms of virus-cell interaction that contribute to the production of these inflammatory mediators.

Binding efficiency of RSV is cell type specific and does not correlate with the susceptibility of the cells to infection.

The different cellular cytokine profiles evoked upon interaction with live, dead and Ab-bound RSV suggests that the innate immune response depends on the interaction of the virus with pattern recognition receptors expressed on both the cell surface and in the cytoplasm. Thus, the capacity of the virus to bind to and/or penetrate the host cell determines the immune response initiated. To further investigate the cell specific response against RSV, we studied the interaction of RSV with the individual cell types in PBMC. We performed binding and infection studies with different multiplicities of infection (MOI) of recombinant green fluorescent protein (GFP)-expressing RSV A2 (rgRSV224) [37]. For RSV binding experiments we co-cultured PBMC with rgRSV224 for 1 hour at 4ºC. **Figure 2A** shows the gating strategy used to detect cell type specific binding and infection. Cell subpopulations in the monocyte gate (based on forward/sideward scatter) were classified as classical $CD14^+/CD16^-$ monocytes, $CD14^+/CD16^{dim}$ monocytes and $CD14^-/CD16^+$ cells. We further identified $CD123^+$ pDC and $CD123^-$ MDC within the lineage-negative, HLA class II positive cell population in the

---

FIGURE 2. Binding efficiency of RSV is cell type specific and does not correlate with the susceptibility of the cells to infection. Binding and infection of rgRSV224 to different cell types in human PBMC was investigated. (**A**) FACS gating strategy for $CD14^+/CD16^-$, $CD14^+/CD16^+$ and $CD14^-/CD16^+$ monocytes (in M: monocyte gate), $CD19^+$ B-cells, $CD3^+$ T-cells and $CD3^-CD16^+CD56^+$ NK cells (in L: Lymphocyte gate). Dendritic cell populations were identified by CD3,CD14,CD16,$CD19^{negative}$ (Lin^-), MHC-II^{high}, and either $CD123^-$ (cDC) or $CD123^+$ (pDC) (in the total live cell gate, M+L). (**B**) Binding of RSV to specific cell types was measured after 1 hour incubation at 4ºC with polyclonal antibodies against RSV (**upper figures**). RSV



infection was determined after 24 hrs. incubation at 37°C by the percentage of GFP expressing cells within a specific cell population (**lower figures**). (**C**) Cell type specific binding characteristics of 4 RSV A strains. Binding of RSV A2, RSV A Long, and clinical RSV A isolates 13N01 and 16N01 to specific cell types in PBMC was visualized after 1 hour incubation at 4°C. Binding was detected using polyclonal antibodies against RSV. Data represent the percentage of RSV positive cells within a population and are expressed as mean ± SEM of triplicate measurements within 1 donor. Experiments were performed in 5 different donors with similar results.

total live cell gate. In the lymphogate we identified CD3^{-ve}CD56^{+} (both CD16^{+} and CD16^{-}) NK cells and CD3^{+} T cells. CD3^{+} cell subsets were not further divided into γ,δ T cells, CD4^{+} T cells, CD8^{+} T cells or NKT subsets because binding and infection characteristics were similar and extremely low in all subsets (data not shown). **Figure 2B** (upper figures) shows representative graphs of the binding characteristics of rgRSV224 to the different cell subsets and identifies B cells in the lymphocyte gate and CD16^{+} cells in the monocyte gate as the cells that most efficiently bind RSV (respectively 52% and 79 %). CD3^{+} T cells do not efficiently bind to RSV (1.9 %) and they are neither susceptible to infection. Classical CD14^{+}/CD16^{-} monocytes and NK cells show intermediate binding to RSV (39% and 26% positive cells within the population respectively). Despite a quite significant binding of RSV to B and NK cells this results in insignificant infection within 24 hrs (**Figure 2B**, lower right figure). In contrast binding efficiency to DC subtypes was low varying from 2-6 % in different donors and with different virus preparations. However, infection was noted in 15-21% of these populations. Optimal binding to all cell populations was found at MOI 5 and optimal infection rates were reached at MOI 5 and up. However, at MOI 10 and 20 increased cell death was observed. The experiment depicted in **Figure 2B** shows the data for binding studies with rgRSV224, the recombinant GFP-RSV strain. Similar results were obtained with RSV A2, RSV Long strain and the two natural isolates in binding experiments with PBMC from a different donor (**Figure 2C**). Using different virus strains and virus batches the percentage of binding and also the kinetics of binding varied somewhat, but in all experiments we found the same hierarchy in the cell types that were binding the virus most efficiently, as well as a similar hierarchy in the

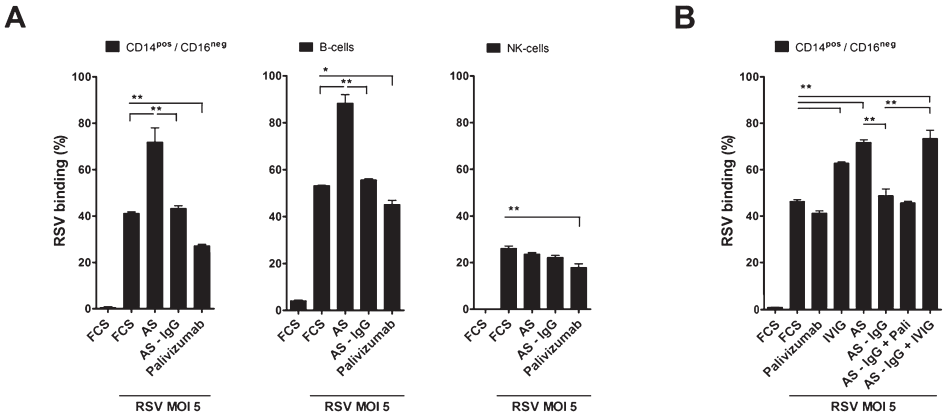


FIGURE 3. Virus specific polyclonal antibodies in human serum increase RSV binding to monocytes and B cells. (A) The effect of autologous serum, IgG-depleted serum and palivizumab in FCS on binding of RSV to monocytes, B cells and NK cells was measured after 1 hour incubation at 4ºC. **(B)** Polyclonal antibodies (IVIG) increase binding of RSV to CD14$^{+/}$CD16^{neg} cells. Data shown represent the mean ± SEM of triplicate measurements in two different donors (for A and B) and were analyzed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison analysis, *P< 0.05, **P <0.01. Experiments were performed in 3 additional donors with similar results.

susceptibility to infection. After 24 hours, about 84% of the $CD14^+$/ $CD16^-$ monocyte subpopulation, 62% of the $CD14^+$/ $CD16^{dim}$ and 43% $CD14^-$/ $CD16^+$ monocytes were infected with rgRSV224 (**Figure 2B**, lower left figure). These infection efficiency numbers show opposite hierarchy compared to the binding characteristics of these monocyte subpopulations. To determine the role of antibodies against RSV present in human serum on RSV binding capacity, we investigated the 3 cell populations (monocytes, B cells and NK cells) capable of binding RSV with high efficiency. **Figure 3A** shows that autologous serum (AS) significantly enhanced binding of RSV. This was limited to monocytes and B cells. Binding of RSV to NK cells in AS remained unaffected. Depletion of the IgG fraction from autologous serum with protein G Sepharose beads, and reconstitution with polyclonal IgGs (IVIG®) showed that IgGs present in AS enhanced binding of RSV to monocytes (**Figure 3B**). Only polyclonal antibodies and not the RSV-F specific monoclonal Ab palivizumab increased virus binding. In summary, RSV binds to various cell subtypes within the PBMC pool, but the level of binding does not correlate with the level of infection. DC and monocytes are the major cell types susceptible to RSV infection.

$CD14^+$ monocytes are necessary for the inflammatory cytokine response against RSV in PBMC cultures.

We next investigated the contribution of individual cell types in PBMC mixtures to the cytokine response upon RSV exposure, by removing single cell subsets. Removal of monocytes ($CD14^+$ cells) or pDC (BDCA-4^+ cells) was accomplished via magnetic cell separation. Specificity of depletion and composition of the remaining cell populations was checked by flow cytometric analysis and resulted in >98% depletion of $CD14^+$ and BDCA-4^+ cells, leaving the remaining cell populations intact (**Figure 4A**). Cell specific depletion was also confirmed by the complete lack of IL-6, IL-10 and TNF-α production upon stimulation of $CD14^+$ cell-depleted PBMC with LPS a specific ligand for the CD14-TLR4 complex (**Figure 4B**). Lack of IFN-α production upon stimulation with TLR9 ligand ODN 2216 confirmed pDC depletion. LPS and ODN 2216 stimulation in the presence of autologous serum resulted in increased cytokine production in PBMC.

PBMC exposed to live and UV-inactivated RSV A2 produced cytokines IL-6 and TNF-α. This response depended entirely on the presence of $CD14^+$ cells in the mixture while depletion of pDC did not affect these responses (**Figure 4C**). Autologous serum increased IL-6 production by PBMC exposed to RSV, but suppressed virus induced TNF-α (**Figure 4C**). The IFN-α response observed in PBMC cultures exposed to live RSV decreased after UV irradiation and depletion of either $CD14^+$ cells or pDC. Moreover, the amount of IFN-α and TNF-α produced in response to live RSV varied between experiments and between virus batches (**Figure 1B and 4C**). IFN-α production was facilitated in $CD14^+$ cell depleted PBMC in the presence of autologous serum (**Figure 4C**). These results show that, although RSV binds to various

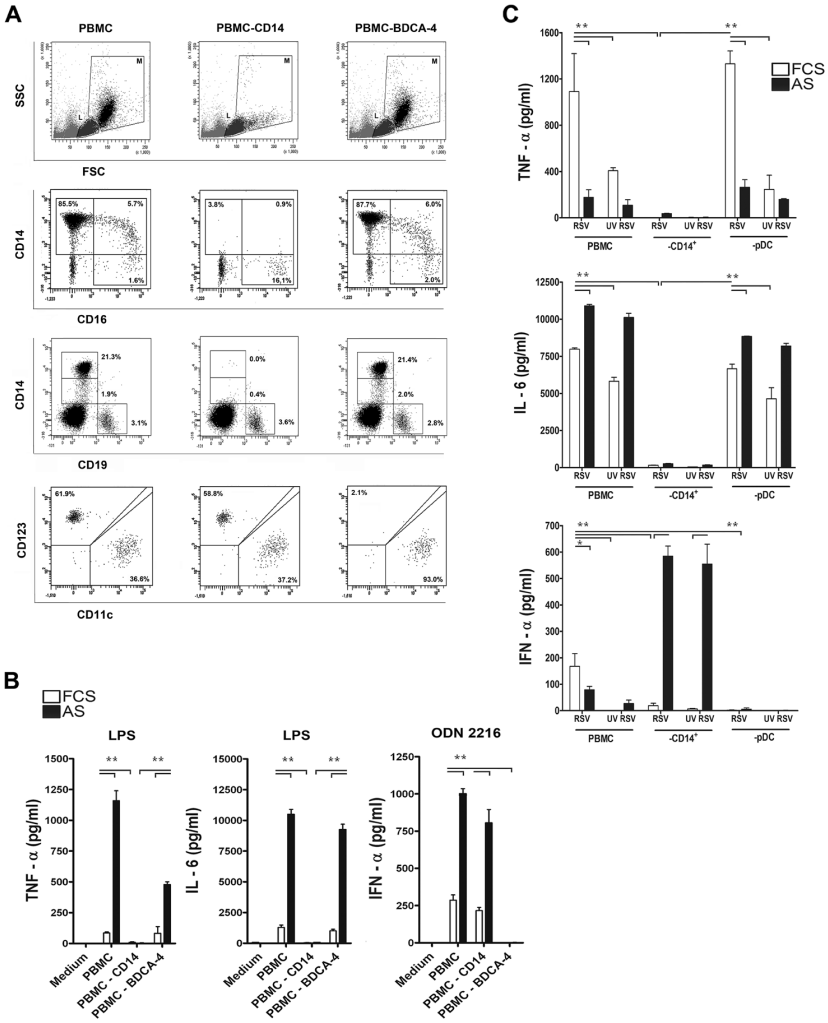


FIGURE 4. CD14⁺ cells are needed for the inflammatory cytokine response against RSV in PBMC cultures. (**A**) Confirmation of specific depletion of CD14⁺ and BDCA-4⁺ cells by FACS. M: monocyte gate, L: lymphocyte gate. Depletion of CD14⁺ cells leaves B cells (in M+L, CD19⁺ third row) and CD16⁺ monocyte/DC (in M, CD14⁻, CD16⁺, 2nd row), cDC (in M+L, lineage⁻, MHCII⁺, CD11c⁺, 4rd row) and pDC (in M+L, lineage⁻, MHCII⁺, BDCA-4⁺, 4rd row) compartments intact. Depletion of BDCA-4⁺ cells removes pDC but does not affect CD14⁺CD16⁻, CD14⁺CD16⁺, CD14⁻CD16⁺ cells in the M gate (2nd row), nor B cells (3rd row) and cDC (4rd row). The contribution of single cell types to anti-RSV cytokine responses in PBMC cultures was evaluated by depletion of specific cell populations. Cytokines in supernatant from the remaining cell populations were measured after 20 hrs. exposure to RSV or UV-RSV in the presence or absence of autologous serum. (**B**) Depletion of CD14⁺ cells and BDCA-4⁺ cells was confirmed by stimulation of depleted cell populations by TLR ligands, ultra-pure LPS to confirm the absence of TLR4⁺ (monocytes) and ODN 2216 to confirm the absence of TLR9⁺ (pDC). (**C**) PBMC, CD14 monocyte-depleted PBMC, or pDC-depleted PBMC were cultured with live and UV inactivated RSV (A2, MOI 5) in the presence or absence of 10% autologous serum. Data represent the mean ± SEM of 3 measurements in 1 donor and were analyzed using one way ANOVA followed by a Bonferroni post-test, *P< 0.05, **P <0.01. Experiments were performed in 3 different donors with similar results.

cell subtypes within the PBMC pool (**Figure 2**), CD14$^+$ monocytes play a central role in the inflammatory cytokine response against RSV, but pDC are required for the production of IFN-α. pDC appear to be the exclusive source of IFN-α when RSV is presented in the presence of autologous serum to CD14$^+$ cell depleted PBMC. However, this pathway of IFN-α production in the presence of serum is inhibited by CD14$^+$ monocytes.

RSV-specific antibodies inhibit IFN-α production in RSV-infected PBMC but enhance IFN-α production in CD14$^+$ cell-depleted PBMC.

We next addressed the question which component in human serum altered the cytokine responses induced by RSV in PBMC in comparison to the responses observed in medium supplemented with FCS. Everyone has been exposed to RSV by three years of age and is re-exposed frequently after. Therefore, adult sera contain RSV-specific neutralizing and non-neutralizing antibodies. In every donor we tested, 10% serum or 5 μg/ml of the RSV-F specific monoclonal Ab palivizumab completely blocked RSV infection in pDC **(Figure 5A**), CD14$^+$ monocytes and A549 cells (data not shown). Therefore, we evaluated whether RSV-specific antibodies in autologous serum affected IFN-α production in RSV-exposed PBMC and PBMC-CD14$^+$ cells. In IgG depletion-reconstitution studies (reconstitution with polyclonal IgG (IVIG), (**Figure 5B**) or virus specific monoclonal antibody palivizumab, (**Figure 5B, C**), we confirmed the role for RSV-specific antibodies in the AS dependent IFN-α responses. Interestingly, RSV-specific antibodies inhibited IFN-α production in RSV-exposed PBMC, but enhanced IFN-α production in CD14$^+$ cell depleted PBMC (**Figure 5C**).

CD14$^+$ monocytes inhibit IFN-α production via the TLR7 route triggered by Ab-RSV complexes in pDC.

We next performed experiments to determine the pathway of RSV induced IFN-α production and the dual role of virus specific antibodies in PBMC and CD14$^+$ cell depleted PBMC cultures. Single stranded viruses stimulate the production of IFN-α via different mechanisms i.e.; TLR7 recognizes viral RNA delivered to endosomal compartments in pDC and viral infection can elicit IFN-α responses via cytoplasmic sensors. Endosomal presence of double stranded RNA can also trigger type I IFN response via TLR3. Therfeore, the mechanism by which a virus interacts with a cell and the route of cell entry will affect the outcome of the innate response induced. From the experiments described in **Figure 1B**, **4C and 5C** it was concluded that in PBMC, live RSV in the absence of virus specific antibodies induced IFN-α, suggesting that an infection mediated process might be involved. In contrast IFN-α production in CD14$^+$ cell-depleted PBMC cultures in the presence of serum did not depend on infection because UV-RSV and live RSV induced equal amounts of IFN-α, (**Figure 4C**). Because pDC depletion aborted the latter IFN-α response (**Figure 4C**) we hypothesized that antibody-mediated

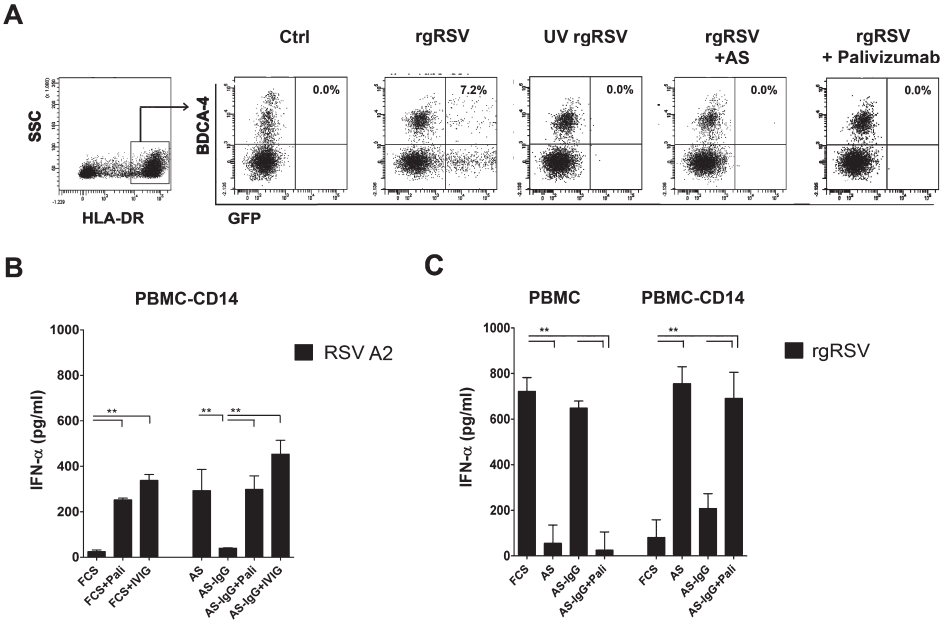


FIGURE 5. RSV-specific antibodies inhibit RSV-induced IFN-α production in PBMC, but enhance IFN-α production in CD14⁺ cell depleted PBMC. (**A**) Inhibition of RSV infection in pDC. Lineage^{-ve}, MHC-II^{high}, BDCA-4^{+} pDC are partially infected with rgRSV224 after a period of 20 hours. Infection is blocked after UV inactivation, after neutralization in 10% fresh human serum or in 5µg/ml Palivizumab. Similar results were obtained with sera from all donors used during our studies. IFN-α in supernatant of RSV-A2 exposed PBMC-CD14^{+} cell cultures (**B**) and rgRSV224 exposed PBMC-CD14^{+} cells or PBMC, (**C**) was measured after 20 hrs. The role of virus specific antibodies on the cytokine response was tested by removing IgGs from AS with protein G Sepharose® beads and reconstitution with 2 mg/ml IVIG (**B**) or 5 µg/ml palivizumab (**B, C**). Experiments represent the mean ± SEM of experiments performed in 4 different donors and were analyzed using one way ANOVA followed by a Bonferroni post-test, **P <0.01.

internalisation and endosomal TLR7 triggering might be the mechanism by which pDC produced IFN-α in PBMC-CD14^{+} cell cultures. To test this hypothesis we blocked endosomal acidification with bafilomycin A_1, a procedure that abrogates endosomal TLR7 and TLR9 mediated IFN-α production [38]. Indeed when endosomal acidification was abrogated by bafilomycin A_1, the Ab-mediated anti-viral IFN-α response was blocked in PBMC-CD14^{+} cells (**Figure 6A**). In addition the specific TLR7 inhibitor, IRS661 blocked the IFN-α response induced by RSV immune complexes, while a scrambled control nucleotide did not (**Figure 6B**). Furthermore, intracellular staining for IFN-α production identified lineage (CD3,-19,-14,-16,-56)$^{-ve}$, MHC class II^{+}, BDCA-4^{+} pDC as the source of IFN-α when palivizumab-RSV complexes were incubated with CD14^{+} cell depleted PBMC (**Figure 6C**). A lower amount of IFN-α production induced by live RSV not neutralized by palivizumab was also observed with similar kinetics (**Figure 6C**). Both the Ab-mediated and the infectious route of RSV-induced IFN-α occurred with slower kinetics than the production of IFN-α after TLR9 ligation by ODN 2216 (**Figure 6C**). The production of IFN-α in response to RSV infection and the

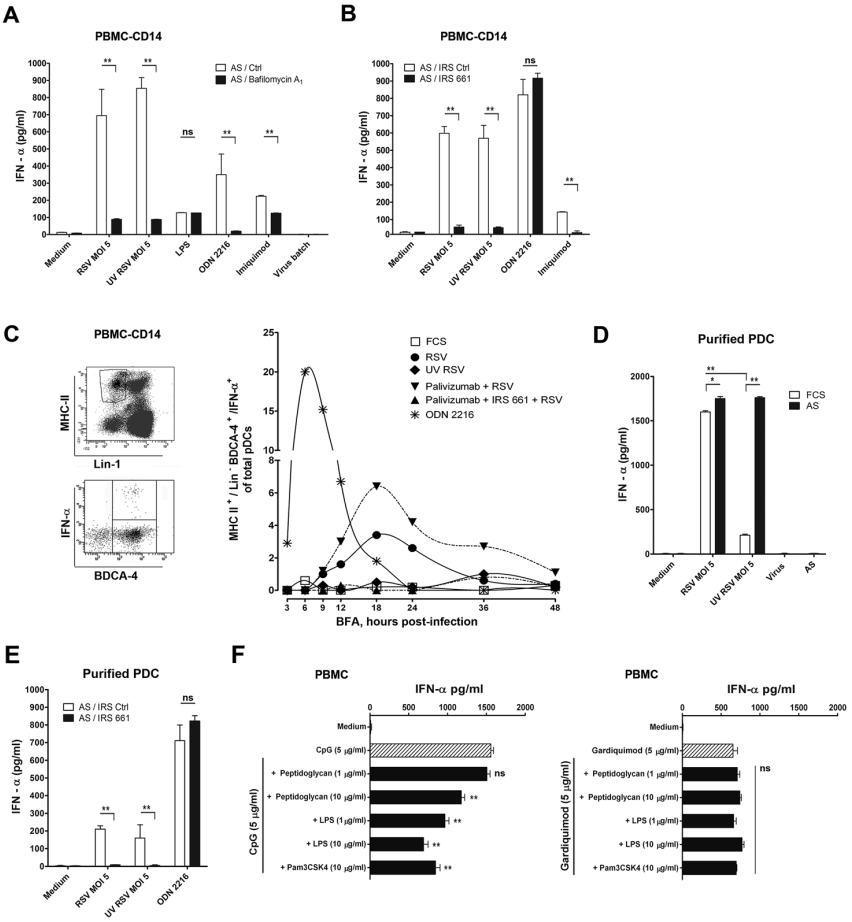


FIGURE 6. CD14⁺ monocytes inhibit IFN-α production triggered by Ab-RSV via TLR7 in pDC. (**A**) IFN-α production in CD14⁺ cell depleted PBMCs induced by Ab-RSV complexes, TLR9 ligand ODN 2216 and TLR7 ligand imiquimod is decreased by blocking endosomal acidification with 50nM Bafilomycin A_1. (**B**) Ab-RSV-induced IFN-α production in CD14⁺ cell depleted PBMCs was abrogated in the presence of immune regulatory sequence (IRS) 661 (1.4 µM) a specific blocking agent for endosomal TLR7 and not by a scrambled control nucleotide. (**C**) pDC are the source of Ab-RSV induced, TLR7 mediated production of IFN-α, as shown by intracellular staining for IFN-α in Lineage ($CD3^{-ve}$, $CD14^{-ve}$, $CD19^{-ve}$, $CD16^{-ve}$, $CD56^{-ve}$, Lin-1), MHC-II^{high}, BDCA-4⁺ cells. Inhibitor of cytokine secretion brefeldin A (BFA) was added at different time points post infection (shown on the X-axis). Cytokines were allowed to accumulate for 10 hrs. after addition of BFA. (**D**) Purified pDC (obtained by negative selection removing CD3⁺, CD19⁺ and CD16⁺ cells from fresh PBMC, followed by FACS purification of the BDCA-4⁺ cell population, which resulted in > 95% pure pDC) produce IFN-α upon infection with RSV. This response is abrogated after UV inactivation of RSV. In AS, both live RSV and UV-inactivated RSV induced IFN-α production to a similar extent. One representative experiment out of two performed with pDC isotated from two different donors is shown. (**E**) IFN-α production by Ab-RSV in purified pDC is blocked by IRS661 (1.4µM). (**F**) TLR1,-2 (PAM3CSK4, Peptidoglycan) and TLR4 (LPS) ligands suppress TLR9-triggered (ODN 2216) IFN-α production, but do not affect TLR7 (Gardiquimod) induced IFN-α production. All data represent mean ± SEM of triplicate measurements within 1 donor and analyzed using one way ANOVA followed by a Bonferroni post-test. ns not significant, *P< 0.05, **P <0.01. Experiments were performed in 3 different donors with similar results.

non-infectious Ab-mediated route via TLR7 were confirmed in experiments wherein we used purified pDC (**Figure 6D**). Also in purified pDC we found that live RSV infection elicited IFN-α production that was abrogated by UV irradiation of RSV. In contrast, in autologous serum that contains RSV specific antibodies, both live and UV-RSV elicited equal amounts of IFN-α, a response that could be specifically abrogated by TLR7 blocking oligonucleotide IRS 661 (**Figure 6E**).

It is not directly clear why this antibody-mediated route targeting RSV to endosomal TLR7 in pDC is less efficient in PBMC (**Figure 4C, 5C**). One explanation could be a mechanism similar to one that has been described for bacteria, where TLR2 and TLR4 ligands in *E. coli* lysates induced Prostaglandin (PG) E and IL-10 production in monocytes with a synergistic suppressive effect on IFN-α production by bacterial double stranded DNA in PDC [39]. Thus substantial amounts of IFN-α are produced by purified pDC exposed to *E. coli* lysates, but not in PBMC exposed to the same lysate. Because RSV has been reported to trigger a TLR4 response via RSV-F [14,40], we performed several experiments to test whether such a mechanism via TLR4 (and or TLR2) triggers could also decrease the TLR7 induced IFN-α response by RSV. However, we were unable to recover TLR7 mediated antiviral IFN-α response in PBMC, induced by RSV immune complexes, by inhibiting prostaglandin production (via cyclooxygenase inhibitor indomethacin), IL-10 signalling (via Ab-mediated IL-10R blocking) nor by combination of both treatments (data not shown). Also attempts to mimic the viral TLR4, TLR7 dual stimulation with synthetic ligands did not reveal a suppressive effect of TLR1/2 or TLR4 ligands on TLR7 induced IFN-α response, while the TLR9 mediated IFN-α response was significantly supressed by these ligands (**Figure 6F**). The suppressive mechanism of $CD14^+$ monocytes on IFN–α production by Ab-RSV complexes in pDC remains unresolved.

IFN-α production induced by live RSV in PBMC depends on IFNAR signalling.

After showing that Ab-neutralized RSV is targeted to endo-lysosomal compartments in PDC, where it activates TLR7 mediated IFN-α production, we next addressed the route of IFN-α production by *live* non-neutralized RSV in PBMC. We first attempted to define the cell type involved in IFN-α induction. IFN-α production in PBMC is abrogated when RSV is UV-inactivated, in the presence of neutralizing antibodies, or by depletion of either $CD14^+$ monocytes or pDC (**Figure 4C**). To explain the role of $CD14^+$ cells in the production of the type I interferon by pDC, we reasoned that RSV infection of monocytes may be required for the production of IFN-β, leading to IFN-α production through IFN-α,β receptor (IFNAR) signalling, JAK-STAT activation and an interferon regulatory factor 7 (IRF7) mediated pathway [41,42]. Such a role for monocytes would be in accord with the fact that they are the cell type within PBMC that are most easily infected by RSV (**Figure 2**). To test this hypothesis we performed experiments in which the secondary IFN-α pathway was blocked by an IFNAR

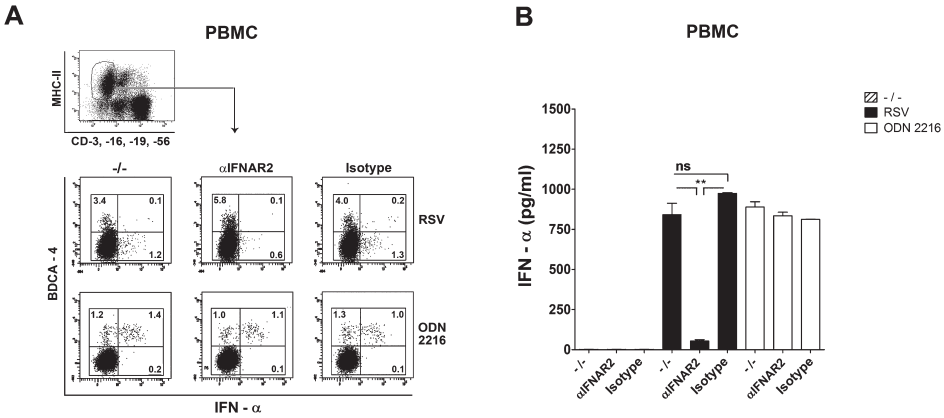


FIGURE 7. IFN-α production induced by live RSV in PBMC depends on IFNAR signalling.

PBMC were exposed to live RSV in the presence of interferon-α/β receptor (IFNAR) blocking antibody (5µg/ml), isotype control Ab or no Ab. Levels of IFN-α were determined via intracellular staining (**A**) or in 20hrs. supernatant by ELISA (**B**). IFN-α was trapped intracellular by BFA treatment initiated 6 hrs. after RSV infection, or at t=0 for the ODN 2216 control stimulus, because of different kinetics of anti-viral and ODN elicited IFN−α response. For both stimuli, intracellular staining for IFN-α was performed in $CD3^{-ve}$, $CD16^{-ve}$, $CD19^{-ve}$, $CD56^{-ve}$, $MHCII^{+}$, BDCA-$4^{+/-ve}$ cells after 10 hrs. BFA treatment. Experiments were performed in 3 different donors with similar results. Data represent the mean ± SEM of triplicate measurements within 1 donor and were analyzed using one way ANOVA followed by a Bonferroni post-test. ns not significant, **P <0.01.

blocking antibody. **Figures 7A and B** both show that indeed RSV-induced IFN-α production is decreased in the presence of IFNAR blocking Ab, while IFN-α production induced by ODN 2216 (IFN-α production via endo-lysosomal TLR9 activation is independent of the indirect IFNAR mediated route) was not affected by blocking the autocrine or paracrine type I IFN amplification loop. Furthermore, ODN 2216 clearly induced IFN-α production in BDCA-4^{+} pDC, whereas in RSV infected PBMC cultures, low numbers of BDCA-4^{+} cells as well as BDCA-4^{-ve} cells contributed to the IFN-α response. However, in the experiments shown in **Figure 7A**, the intracellular cytokine staining for IFN-α was performed within the first 6 hrs. of the cultures of PBMC exposed to ODN 2216, whereas RSV-induced IFN-α was measured by starting the Brefeldin A treatment to accumulate cytokines from 12 hrs. post infection. This was done because the kinetics of the response differs for these treatments (**Figure 6C**). When Brefeldin-A treatment was postponed in the ODN 2216 treated PBMC cultures, low numbers of IFN-α positive cells were found that were all BDCA-4 negative. These results indicate that BDCA-4 might be down regulated in time during culture and might not be a stable marker to identify pDC after prolonged culture in the context of PBMC with TLR ligands or ligands for other PRRs. For this reason we were unable to exclude pDC nor confirm pDC as the responding population. Also CD14 is down-regulated on monocytes in PBMC cultures and strongest in RSV infected samples. Therefore, CD14 staining could also not be used to determine the role of monocytes as a possible source of IFN-α. In conclusion, RSV infection

elicits IFN-α production in pDC via an IFNAR dependent mechanism in the context of PBMC. The involvement of monocytes in this process is suggested by the fact that $CD14^+$ cell depletion abrogates the IFN-α production (**Figure 4C and 5C**). Because UV-inactivation abrogates the IFN-α response in PBMC, combined with the fact that monocytes are the cells that are most efficiently infected (**Figure 2**), this suggests the involvement of an infection mediated route resulting in an early source of type I interferon production by monocytes that triggers IFN-α production by IFNAR binding on pDC.

CD14 plays a crucial role in the IFN-α production induced by RSV in PBMC.

The observation that UV-irradiation and virus neutralization by palivizumab prevented IFN-α production by pDC exposed to RSV in PBMC (**Figures 5C and 6C**), could suggest that infection with RSV might be required for IFN-α production. However, there may be another possible explanation for the abrogated IFN-α production when RSV is inactivated by UV irradiation or neutralized by antibodies. It has been reported that RSV-F interacts with the TLR4 complex, which could result in IFN-β production and has been shown to induce TNF-α and IL-6 production in human monocytes [14,40]. It is possible that F specific antibodies or UV treatment (by affecting the structure of the labile F molecule) could block TLR4 signalling. Therefore, Ab-mediated neutralization or UV-inactivation did not unequivocally distinguish the surface TLR-mediated or infection related innate immune pathways. To determine whether RSV-F/TLR4 interaction contributed to cytokine production in our experiments, we measured cytokine responses induced by RSV in PBMC cultures in the presence or absence of CD14-, or TLR4-blocking Abs and a LPS antagonist, LPS-RS, from *Rhodobacter sphaeroides*, that inhibits LPS and RSV-F interaction with TLR complex cofactor MD2 [40,43]. Both antibodies and LPS-RS blocked the LPS induced TLR4-mediated IL-6 response in PBMC (**Figure 8A, B and C**), while only MY4, the CD14 blocking antibody, decreased IL-6 and IFN-α production in PBMC exposed to RSV (**Figure 8C**). CD14-neutralizing antibody slightly decreased the level of infection of monocytes by rgRSV224 (data not shown). However, it seems unlikely that the marginal effect on the RSV infection of the monocyte population explained the significant inhibition of IFN-α production by PBMC. Therefore, our data are consistent with a cell surface TLR-complex mediated role during early IFN-type 1 production by RSV, although direct viral interaction with TLR4 or MD2 might not occur. Because A549 cells, that do not respond to inactivated RSV via TLR4, did produce substantial amounts of IFN-β upon RSV infection (**Figure 1**), we tested whether RSV-infected A549 cells could restore IFN-α production in monocyte depleted PBMC. In **Figure 8D** we show that indeed the IFN-α responses can be restored by co-culture of $CD14^+$ cell depleted PBMC with A549 exposed to live RSV and that pDC are the source of this IFN-α.

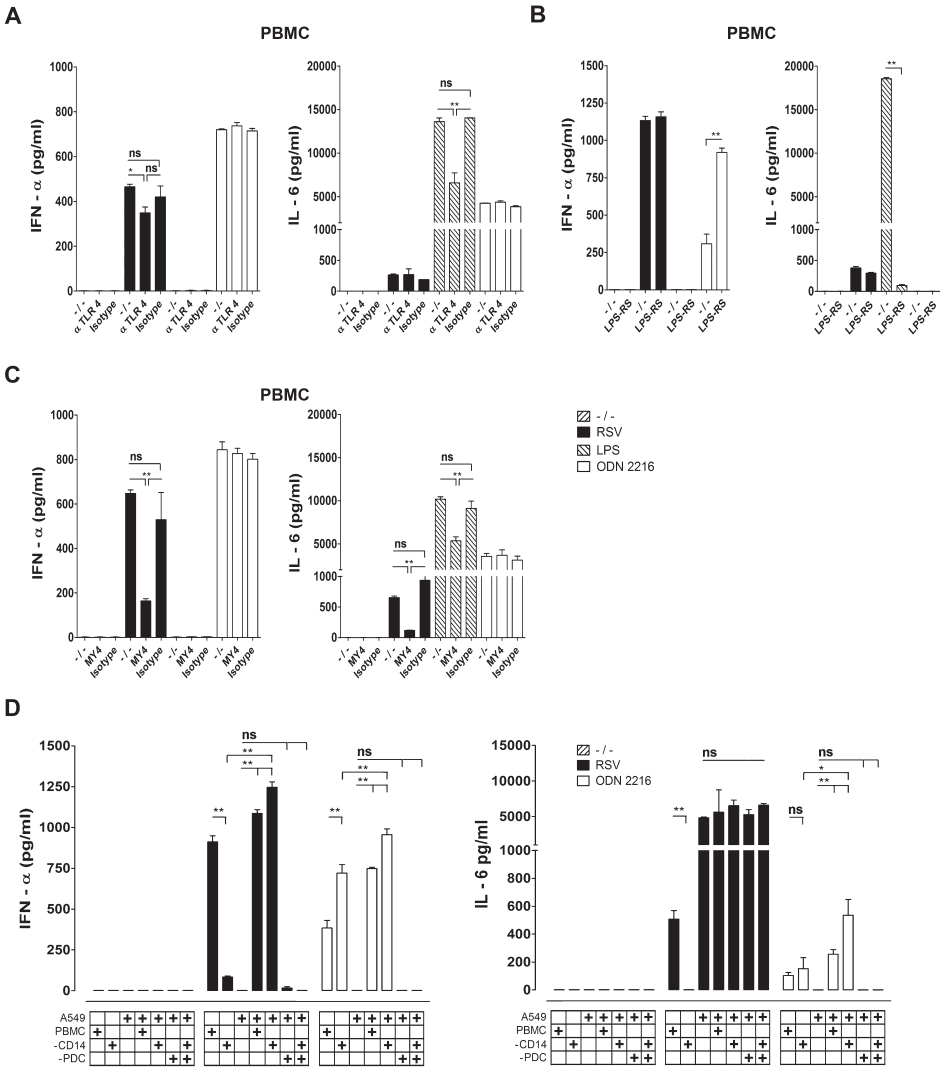


FIGURE 8. IFN-α production induced by live RSV in pDC depends on IFNAR and CD14.

The role of the TLR4/CD14/MD2 complex in the IFN-α and IL-6 response by PBMC after RSV infection (strain A2, MOI 5) was investigated (**A**) with a blocking monoclonal antibody specific for human TLR4 (20µg/ml), (**B**) the MD2 antagonist lipopolysaccharide from the bacterium *Rhodobacter sphaeroides* (1µg/ml) or (**C**) via neutralization of CD14 with monoclonal antibody MY4 (10µg/ml). (**A-C**) Cytokine responses were measured by ELISA. Control stimuli; LPS: 10ng/ml, ODN: 5µg/ml. (**D**) A549 epithelial cells were co-cultured with PBMC, $CD14^+$ cell depleted PBMC, pDC depleted PBMC, or $CD14^+$ and BDCA-4^+ double depleted PBMC, in the presence of RSV at MOI 5, ODN 2216 or no stimulus, for a period of 20 hrs. IL-6 and IFN-α production were measured by ELISA. Experiments were performed in 3 different donors with similar results. Data represent the mean ± SEM of triplicate measurements within 1 donor and were analyzed using one way ANOVA followed by a Bonferroni post-test. ns not significant, *P, 0.05, **P, 0.01.

DISCUSSION

In the present work we showed that RSV-specific (neutralizing) antibodies play a crucial role in dictating the nature of innate immune reactions to RSV. Moreover, the innate immune reaction elicited by RSV depends on the cellular context and the mechanism by which the virus interacts with specific cells in complex mixtures, and most likely in tissues. This was demonstrated by the role of CD14$^+$ cells in suppressing IFN-α production by pDC upon Ab-RSV exposure (**Figure 9A, D**). Furthermore, in the absence of antibodies, an indirect mechanism initiated by RSV-infected epithelial cells or RSV-exposed CD14$^+$ cells activated IFN-α release via IFNAR-signalling in pDC (**Figure 9C**). During natural exposure to RSV, airway epithelial cells, alveolar macrophages in the airway lumen and dendritic cells underneath the epithelium are the primary cells initially exposed to RSV rather than PBMC. As shown in mouse models, the early innate response causes the influx of DC types into the lung

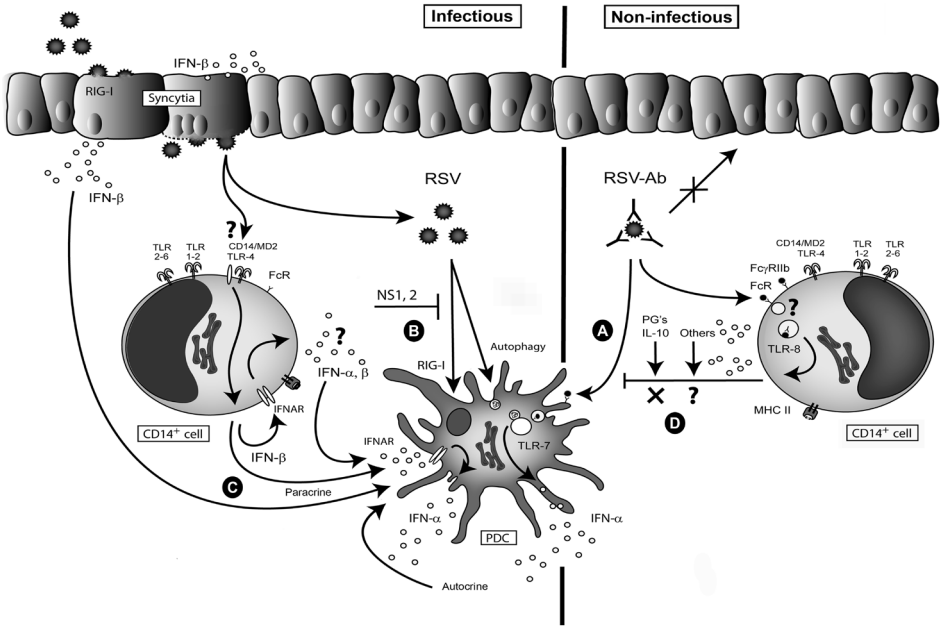


FIGURE 9. Cellular cross talk and the role of antibodies during IFN-α production.

pDC produce IFN-α via multiple pathways. i: TLR7 activation after Ab-mediated uptake of RSV (**A**), ii: via infection with RSV in purified pDC (**B**) or iii: indirect via IFNAR-mediated signalling triggered by IFN-β produced by RSV-infected A549 (as a model for airway epithelium) or RSV-exposed CD14$^+$ cells (**C**). In the context of PBMC the presence of CD14$^+$ monocytes suppresses the production of IFN-α by pDC upon exposure to Ab-RSV complexes. (**D**). The mechanism of the suppression by monocytes of this TLR7-mediated response elicited by Ab-RSV complexes in pDC remains unresolved, but appears not to be mediated via PG's and IL-10, mediators that suppress TLR9 induced IFN-α production when multiple PAMPS in bacterial lysates simultaneously trigger TLR9 and TLR2/4 responses in PBMC.

tissue and migration of DC to the draining lymphnodes, followed by T cell activation in the lymphnodes and trafficking to the infected tissue [44]. Clearly, the structural restraints, cell density and trafficking, the sequence of subsequent cellular exposure to RSV and cellular cross-talk in different environments (airway, lung-parenchyma and lymphnode) will affect the outcome of the innate immune response *in vivo*. Indeed *in vivo* mouse models for RSV implicated the contribution of different cell types and PRR systems, whereby IPS-1 signalling and presumably infection related routes of innate immune triggering, in both immune and non-immune cells, were important in viral clearance. On the otherhand, TLRs (3 and 7) were less important in virus clearance but affected disease parameters like mucus production, presumably via skewing of Th responses towards Th2 or Th17 respectively [17,18,31,45,46].

The efficacy of RSV infection is species specific and human TLRs have different target specificities and cellular distribution than mice. However, during the course of RSV infection in humans a similar contribution of different cell types will occur and the cellular cross-talk will be completely different from the short exposure of a static PBMC cell mixture *in vitro*. Nevertheless, our experiments demonstrate that the outcome of the inflammatory response in a complex environment, in which different cells are activated in their own specific manner, cannot be predicted from sum of the responses of each individual cell type. Furthermore, the level of viral neutralization by RSV-specific antibodies and, as shown in the present study, the altered access of RSV as Ab-RSV complexes to FcR-bearing cells will most likely affect the nature and amplitude of the inflammation. For all of these reasons *in vitro* analysis using single cell types to predict the potency and safety of attenuated RSV vaccine candidates is a poor approach to test such vaccine candidates.

We described cell specific interactions with RSV leading to different innate immune pathways. As reported earlier, epithelial A549 cells produced mainly inflammatory cytokines (IL-6, TNF-α, IFN-β) in a process that depended on RSV infection and is presumably mediated via RIG-I [47,48]. pDC produced type I interferon (IFN-α) i. via TLR7 activation after Ab-mediated uptake of RSV (**Figure 9A**), ii. via infection with RSV in purified pDC (**Figure 9B**), or iii. indirect via IFNAR-mediated signalling triggered by type I interferon produced by RSV-infected A549 (as a model for airway epithelium) or RSV-exposed $CD14^+$ cells (**Figure 9C**). $CD14^+$ cells were the main producers of inflammatory cytokines in PBMC.

The mechanism by which $CD14^+$ cells are triggered to produce cytokines is still controversial. Kurt-Jones *et al* first reported the role of RSV-F as a ligand for the TLR4/CD14 complex and the same group later reported that RSV could also trigger TLR2 and TLR6 mediated responses [13,14]. Recently Rallabhandi *et al*. demonstrated that purified RSV-F interacted with MD2 [40]). However, other groups were unable to confirm TLR4-mediated RSV interactions [49], or were unable to confirm a role of the viral F protein in the process [50]. Importantly, Rallabhandi and colleagues did not show MD2 dependent TLR4 activation with intact RSV. Also in studies *in vivo* using TLR4-deficient mice conflicting results for a role of TLR4 in RSV specific immunity were reported [19,51,52]. It is difficult to reconcile why the

results in different laboratories are so different. However, many variables in experimental setup may contribute to different results; i.e. virus culture in HEp versus Vero cells, the use of human monocytes, murine macrophages or reporter cell lines, differences in purification protocols for RSV or RSV-F. TLRs in different species although quite homologous still differ substantially in amino acid residues, changes that lead to differences in leucine-rich repeat organisation and also ligand specificity [53,54]. Moreover, in TLR-mutant or knockout mice it is conceivable that altered lung development [55,56] or differences in microbiota [57] could affect *in vivo* disease development after RSV infection. Therefore, altered immune responses elicited upon viral infection in knockout mice could occur via different mechanisms and do not unequivocally prove (the lack of) interaction of the virus with the TLR studied, as the reason for the altered immune response. Similar caution is warranted in the interpretation of human TLR polymorphisms and their involvement in severity of RSV disease.

In this study we found that live RSV elicited IL-6, IL-10, IL-8 and TNF-α production in PBMC and not in PBMC-CD14$^+$ cells. In contrast to responses in A549 cells UV-RSV still stimulated production of these cytokines although in most experiments in slightly lower amounts. TLR4 blocking antibody in a dose that diminished LPS induced IL-6 production by > 50% did not significantly inhibit IL-6 and IFN-α production by RSV in PBMC, neither did LPS-RS affect RSV-induced IL-6 and IFN-α production, while it did inhibit IL-6 production triggered by LPS completely (**Figure 8**). The latter observation suggests that in the context of RSV particles, MD2-RSV-F interaction might not occur in a similar fashion as was reported for purified F protein [40]. We did reproduce the reported effect of blocking CD14 on RSV induced cytokine responses in CD14$^+$ cells [14] and show that in addition to IL-6 production also the primary burst of type I interferon in PBMC cultures depends on CD14. CD14 is an important cofactor for the activation of endosomal TLRs. In addition to be required for the TRIF/TRAM pathway downstream of TLR4 [58], CD14 has been reported to associate with TLR7, TLR8 and TLR9 and to act as a co-receptor for these TLRs in endosomes [59]. Because CD14 is dispensable for cytoplasmic sensing of viral infection, our data suggest that IFN-α production by pDC in the context of PBMC depends on a TLR mediated route. It remains to be established which of the TLR pathways is involved.

pDC and type I interferons exert a crucial role in regulating adaptive immunity [60–63] and have been implicated in virus clearance and protection against immune mediated pathology caused by RSV infection [64]. It is still unclear why IFN-α induction is ineffective in PBMC-CD14$^+$ cells a cell mixture wherein pDC are present. We confirmed earlier studies that have shown that purified pDC could produce IFN-α when exposed to infectious RSV (**Figure 6D**), although the amount of IFN-α might be suppressed once viral non-structural proteins are expressed in infected pDC [26,27,35,65]. In contrast to influenza virus [66] and vesicular stomatitis virus [67], RSV infection and propagation does not follow an endo-lysosomal route [68]. The absence of endosomal uptake as the natural route of cell entry might explain the absence of TLR7-mediated IFN-α production by UV-inactivated RSV. Ab-mediated uptake

presumably enhances access of UV-RSV and live RSV to endosomal TLR7 in pDC explaining the increased IFN-α production when virus specific antibodies are present (**Figure 9A**). Moreover, the neutralizing effect of RSV-specific monoclonal Ab palivizumab or polyclonal serum antibodies prevents infection and expression of viral non-structural proteins that interfere with type I interferon production pathways [26,35]. However, we observed that live RSV can elicit IFN−α production in purified pDC in an IRS661 sensitive fashion (**Figure 6E**). Most likely this IFN-α production might be the result of autophagy [69] that occurs after infection of low numbers of pDC which is then followed by a second burst of IFN-α via IFNAR signalling in non/infected bystanders (**Figure 9B**). This process has been described before and has been shown to critically depend on the density of *in vitro* cultured pDC [70]. It is possible that in the context of PBMC-CD14 the pDC are too low in numbers for this process to occur.

By means of early expressed nonstructural proteins, RSV evades the type I interferon pathway and antiviral response in infected cells by suppression of the activation and nuclear translocation of interferon regulatory factor 3 [35,71]. However IRF3 activation occurs in the first 10 hrs. of infection before substantial amounts of NS proteins are expressed. This window of IRF3 activation suffices to contribute together with NF-κB activation to the production of IFN-β in A549 and presumably $CD14^+$ cells which in turn potentially stimulates secondary IFN-α responses via IFNAR signaling in pDC (**Figure 9C**). In addition NS2 suppresses Stat2 levels interfering with IFN-α/β signaling via IFNAR, the secondary route, leading to expression of interferon stimulated genes and a secondary burst of type I interferons [72]. Therefore, uninfected bystander pDC are an important source of type I interferon production.

In summary, our findings show that the interaction of RSV with a mixture of primary human cells (PBMC) leads to a cytokine response pattern that depends on cellular cross-talk and the presence or absence of RSV specific antibodies. We show a key role for CD14 monocytes in the control of antiviral type I IFN responses to RSV via a direct antibody mediated and an indirect, infection mediated, mechanism. Because RSV specific antibodies are transmitted from mother to child during pregnancy and via breast milk the role of these antibodies should be carefully evaluated with respect to their effect on innate immune responses when new vaccine candidates are tested.

MATERIALS AND METHODS

Ethics Statement

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood donated by healthy adult volunteers. All studies using primary cells were performed after written approval from the ethics Committee of the Medical Faculty of the Utrecht University and after obtaining written informed consent from the donors.

Human hematopoietic cell isolation and single cell depletion

In vitro experiments were performed using PBMC isolated from EDTA-anticoagulated whole blood isolated by Ficoll-Hypaque density gradient centrifugation (Biochrom, Germany). Specific cell type depletion was achieved by positive selection using antibody labeled magnetic micro beads (from Miltenyi Biotec unless otherwise stated) against CD14 (IgG2a, 130-050-201) and BDCA-4 (IgG1, 130-092-402) following standard procedures. PBMCs were incubated with bead labeled antibody at 4°C for 30 min in deoxygenated PBS with 0.5% BSA and 2 mM EDTA (MACS buffer) and passed through a magnetic separation column once (LD column, 130-042-901; Miltenyi Biotec). Depletion of the cell type of interest and specificity of depletion was confirmed using flowcytometry. Efficiency of depletion was at least 98%. pDC purification by positive selection with BDCA-4 coupled magnetic beads suppressed IFN-α production in our experiments. Therefore, we purified pDC by negative selection (by magnetic beads), removing $CD3^{+-}$, $CD19^{+}$- and $CD16^{+}$ cells from freshly isolated PBMC, followed by FACS purification of the BDCA-4^{+} cell population (FACSAria; Becton Dickinson). Purity of isolated pDC was >95%. Cell viability was assessed by trypan blue exclusion. $5x10^{4}$-10^{5} cells were used per stimulus in duplicate.

Cell lines and virus

HEp-2 cells were cultured in IMDM (21980-065, Life Technologies, Rockville, MD) and the A549 cell line was cultured in DMEM (31965-080, Life Technologies, Rockville, MD) both supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 5% FCS, and 1% penicillin/streptomycin. RSV strain A2 (VR-1540, ATCC), RSV Long strain (VR-26, ATCC), RSV patient strain (A, 13N01), RSV patient strain (A, 16N01) and recombinant GFP expressing RSV A2 (rgRSV224, GFP gene inserted into a naturally occurring BstXI site before the gene coding for non-structural protein 1 (NS1), kindly provided by Dr. M.E. Peeples and manufactured as previously described by Hallak *et al*) [37], were grown on HEp-2 cells (CCL-23, ATCC) in 1% FCS, purified by polyethylene glycol 6000 precipitation, resolved in phosphate-buffered saline (PBS) in the presence of 10% sucrose and stored in liquid nitrogen. The 50% tissue culture–infective dose (TCID-50) was determined post-titration in HEp-2 cells.

RSV binding and infection

For cell culture experiments, freshly isolated, specific cell depleted or whole PBMC suspensions (4×10^{5}) were cultured in round-bottom 96-wells plates (Costar) in a final volume of 200ul. For RSV binding experiments, cells were incubated for a period of 1hr. at 4ºC with rgRSV224 in IMDM supplemented with, 5% FCS, 2 mM L-glutamine, 25 mM Hepes buffer and 1% penicillin/streptomycin. RSV infection studies were performed with live or UV-inactivated

RSV (2300 μW/cm^2 of 254 nm UVA light for 10 min) and measured after an overnight (20-24h) incubation period at 37ºC in 5% CO_2, or as indicated in the Figure legends. Supernatants were collected and stored at −20°C until further analysis.

Cell identification and cytokine production

To identify different cell populations, cultured single cell suspensions were washed with PBS containing 2% FCS, 2mM EDTA and 0.02%NaN3 (FACS buffer), blocked with 10% human AB serum for 20 min. at 4ºC to reduce non-specific binding and stained for 4- or 5-color flow cytometry with the following antibodies (from BD Biosciences unless otherwise stated) anti-CD3 (clone SK7), anti-CD4 (clone SK3), anti-CD8 (clone SK1), anti-CD11b (clone D12,), anti-CD11c (clone B-ly6), anti-CD14 (clone MϕP9), anti-CD16 (clone 3G8), anti-CD19 (clone 4G7), anti-CD56 (clone NCAM16.2), anti-CD123 (clone 9F5), anti-BDCA-1 (clone AD5-8E7, Miltenyi Biotec), anti-BDCA-4 (clone AD5-17F6, Miltenyi Biotec) or anti-MHC-II (clone G46-6). To identify cell specific cytokine production, cells were first surface stained with specific antibodies to phenotype cell populations before fixation and permeabilisation with CytoFix/CytoPerm (554722, BD) solution and Perm/Wash buffer (554723, BD). Intracellular cytokines were detected with anti-IFN-α (clone 7N4-1) antibody. Stained samples were measured on a FACS Canto II flow cytometer (BD Biosciences, San Diego, CA) and analysed using FACS Diva software (BD, San Diego, CA).

For detection of cytokines in supernatant, culture supernatants were collected after a 20hrs. incubation period, or as indicated in Figure legends. Enzyme-linked immunosorbent assay (ELISA) was used to quantify human IFN-α (IFN-α, -α2a, 2b and 2c, BMS216MST, Bender MedSystems), IFN-β (*Verikine*™, PBL, 41410-1A), IL-6 (M9316, Sanquin Pelipair), IL-8 (M9318, Sanquin Pelipair) and TNF-α (M9323, Sanquin Pelipair) production in the supernatants. For some experiments a multiplex immunoassay (MIA) of IL-1β, -6, -8, -10, -12, IP-10 and TNF-α in culture supernatant was performed using the Bio-Plex System in combination with Bio-Plex Manager software V.4.1 using five parametric curve fitting (Bio-Rad laboratories, Hercules, California, USA) [73].

Cell stimulation

In experiments in which the effect of autologous serum or RSV specific AB was tested, RSV or control ligands were preincubated for 15 min. at 37ºC with 10% autologous serum, 2mg/ml IVIG® or 5ug/ml Palivizumab before co-culture with single cell suspensions. When indicated, IgGs were depleted from serum with Protein G Sepharose beads (P3296, Sigma-Aldrich). The following control ligands (from Invivogen unless otherwise stated) were used; TLR1,-2 (PAM3CSK4, tlrl-pms), TLR2 (Peptidoglycan, tlrl-pgn), TLR3 (Poly I:C, tlrl-pic), TLR4 (ultra-pure LPS, *E. coli* K12), TLR7 (Imiquimod, tlrl-imqs or Gardiquimod, tlrl-gdqs), TLR8 (CL-075,

tlrl-c75) and TLR9 (ODN 2216, tlrl-2216). PBMC or A549 cells were co-cultured with RSV for 20 hrs. or different duration as stated in the figure legends.

Inhibition assays

To determine the involvement of specific innate immune pathways we employed inhibitors to block endosomal acidification (Bafilomycin A_1, 50nM, Calbiochem) and protein transport (Brefeldin A, 40nM, Sigma-Aldrich). For investigation of TLR4 signalling, a blocking monoclonal antibody specific for human TLR4 (20μg/ml, anti-hTLR4-IgG1, Invivogen), the MD2 antagonist lipopolysaccharide from the bacterium *Rhodobacter sphaeroides* (1μg/ml LPS-RS, Invivogen) or CD14 blocking monoclonal antibody MY4 (10μg/ml, A83482, Beckman Coulter) were used. The human interferon-α/β receptor (IFNAR) was neutralized with a mouse monoclonal antibody (5μg/ml, clone MMHAR-2, PBL Interferon Source). All antibody based neutralizing experiments were performed with matching isotype controls. Endosomal Toll like receptor 7 signalling was blocked with specific immune-regulatory DNA sequences (IRS) to TLR7 (1.4 μM, IRS661: 5′-TGCTTGCAAGCTTGCAAGCA-3′, Eurofins MWG Operon). As a control, a non-TLR-specific oligodeoxyribonucleotide sequence was used in the same concentration as the inhibitor. Cell populations were preincubated with specific (TLR) inhibitors for a period of at least 30 min. at 37°C before adding virus or control TLR ligands.

Statistical analysis

For all experiments, data are expressed as the mean +/- standard error of the mean (SEM). Measurements were compared using a one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc analysis. Non parametric analysis was performed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. A P-value of <0.05 was considered to be statistically significant.

ACKNOWLEDGEMENTS

We thank Dr. M.E. Peeples (Nationwide Children's Hospital, Columbus, Ohio, USA) for providing us with recombinant GFP expressing RSV (rgRSV224).

REFERENCES

1. Janeway CA (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology 54 Pt 1: 1–13.
2. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11: 373–384.
3. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, et al. (2009) The burden of respiratory syncytial virus infection in young children. The New England journal of medicine 360: 588–598.
4. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE (2005) Respiratory syncytial virus infection in elderly and high-risk adults. The New England journal of medicine 352: 1749–1759.
5. Van Drunen Littel-van den Hurk S, Watkiss ER (2012) Pathogenesis of respiratory syncytial virus. Current opinion in virology 2: 300–305.
6. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, et al. (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380: 2095–2128.
7. Blasius AL, Beutler B (2010) Intracellular toll-like receptors. Immunity 32: 305–315.
8. Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, et al. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science (New York, NY) 314: 997–1001.
9. Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, et al. (2006) 5'-Triphosphate RNA is the ligand for RIG-I. Science (New York, NY) 314: 994–997.
10. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, et al. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441: 101–105.
11. Barral PM, Sarkar D, Su Z, Barber GN, DeSalle R, et al. (2009) Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity. Pharmacology & therapeutics 124: 219–234.
12. Le Bon A, Tough DF (2002) Links between innate and adaptive immunity via type I interferon. Current opinion in immunology 14: 432–436.
13. Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, et al. (2009) Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. Journal of virology 83: 1492–1500.
14. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, et al. (2000) Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nature immunology 1: 398–401.
15. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, et al. (2009) Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nature medicine 15: 34–41.
16. Boukhvalova MS, Prince GA, Soroush L, Harrigan DC, Vogel SN, et al. (2006) The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 24: 5027–5035.
17. Rudd BD, Smit JJ, Flavell RA, Alexopoulou L, Schaller MA, et al. (2006) Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. Journal of immunology (Baltimore, Md: 1950) 176: 1937–1942.
18. Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, et al. (2010) Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. Journal of immunology (Baltimore, Md: 1950) 185: 2231–2239.

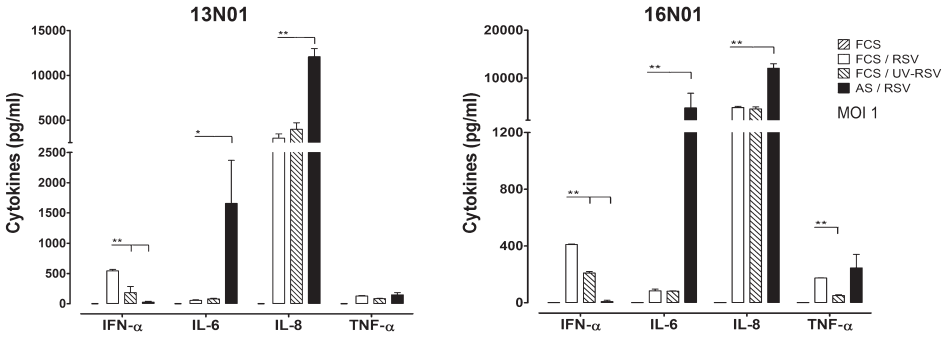
19. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich H-J, et al. (2002) Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. The Journal of infectious diseases 186: 1199–1206.
20. Klein Klouwenberg P, Tan L, Werkman W, Van Bleek GM, Coenjaerts F (2009) The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. Critical reviews in immunology 29: 531–550.
21. Lukacs NW, Smit JJ, Schaller MA, Lindell DM (2008) Regulation of immunity to respiratory syncytial virus by dendritic cells, toll-like receptors, and notch. Viral immunology 21: 115–122.
22. Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, et al. (2006) Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. Journal of immunology (Baltimore, Md: 1950) 176: 1733–1740.
23. Van Diepen A, Brand HK, Sama I, Lambooy LHJ, Van den Heuvel LP, et al. (2010) Quantitative proteome profiling of respiratory virus-infected lung epithelial cells. Journal of proteomics 73: 1680–1693.
24. Swedan S, Andrews J, Majumdar T, Musiyenko A, Barik S (2011) Multiple functional domains and complexes of the two nonstructural proteins of human respiratory syncytial virus contribute to interferon suppression and cellular location. Journal of virology 85: 10090–10100.
25. Villenave R, Thavagnanam S, Sarlang S, Parker J, Douglas I, et al. (2012) In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. Proceedings of the National Academy of Sciences of the United States of America 109: 5040–5045.
26. Schlender J, Hornung V, Finke S, Günthner-Biller M, Marozin S, et al. (2005) Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. Journal of virology 79: 5507–5515.
27. Hornung V, Schlender J, Guenthner-Biller M, Rothenfusser S, Endres S, et al. (2004) Replication-dependent potent IFN-alpha induction in human plasmacytoid dendritic cells by a single-stranded RNA virus. Journal of immunology (Baltimore, Md: 1950) 173: 5935–5943.
28. Johnson TR, Johnson CN, Corbett KS, Edwards GC, Graham BS (2011) Primary human mDC1, mDC2, and pDC dendritic cells are differentially infected and activated by respiratory syncytial virus. PloS one 6: e16458.
29. Johnson TR, McLellan JS, Graham BS (2012) Respiratory syncytial virus glycoprotein G interacts with DC-SIGN and L-SIGN to activate ERK1 and ERK2. Journal of virology 86: 1339–1347.
30. Jie Z, Dinwiddie DL, Senft AP, Harrod KS (2011) Regulation of STAT signaling in mouse bone marrow derived dendritic cells by respiratory syncytial virus. Virus research 156: 127–133.
31. Rudd BD, Schaller MA, Smit JJ, Kunkel SL, Neupane R, et al. (2007) MyD88-mediated instructive signals in dendritic cells regulate pulmonary immune responses during respiratory virus infection. Journal of immunology (Baltimore, Md: 1950) 178: 5820–5827.
32. Wang H, Peters N, Schwarze J (2006) Plasmacytoid dendritic cells limit viral replication, pulmonary inflammation, and airway hyperresponsiveness in respiratory syncytial virus infection. Journal of immunology (Baltimore, Md: 1950) 177: 6263–6270.
33. Ubol S, Halstead SB (2010) How innate immune mechanisms contribute to antibody-enhanced viral infections. Clinical and vaccine immunology: CVI 17: 1829–1835.
34. Jiang Z, Kunimoto M, Patel JA (1998) Autocrine regulation and experimental

modulation of interleukin-6 expression by human pulmonary epithelial cells infected with respiratory syncytial virus. Journal of virology 72: 2496–2499.
35. Spann KM, Tran K-C, Chi B, Rabin RL, Collins PL (2004) Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. Journal of virology 78: 4363–4369.
36. Mastronarde JG, Monick MM, Mukaida N, Matsushima K, Hunninghake GW (1998) Activator protein-1 is the preferred transcription factor for cooperative interaction with nuclear factor-kappaB in respiratory syncytial virus-induced interleukin-8 gene expression in airway epithelium. The Journal of infectious diseases 177: 1275–1281.
37. Hallak LK, Spillmann D, Collins PL, Peeples ME (2000) Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. Journal of virology 74: 10508–10513.
38. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A (2003) Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. The Journal of experimental medicine 198: 513–520.
39. Poth JM, Coch C, Busch N, Boehm O, Schlee M, et al. (2010) Monocyte-mediated inhibition of TLR9-dependent IFN-α induction in plasmacytoid dendritic cells questions bacterial DNA as the active ingredient of bacterial lysates. Journal of immunology (Baltimore, Md: 1950) 185: 7367–7373.
40. Rallabhandi P, Phillips RL, Boukhvalova MS, Pletneva LM, Shirey KA, et al. (2012) Respiratory syncytial virus fusion protein-induced toll-like receptor 4 (TLR4) signaling is inhibited by the TLR4 antagonists Rhodobacter sphaeroides lipopolysaccharide and eritoran (E5564) and requires direct interaction with MD-2. mBio 3.
41. Prakash A (2005) Tissue-specific Positive Feedback Requirements for Production of Type I Interferon following Virus Infection. Journal of Biological Chemistry 280: 18651–18657.
42. Ng CS, Kato H, Fujita T (2012) Recognition of viruses in the cytoplasm by RLRs and other helicases--how conformational changes, mitochondrial dynamics and ubiquitination control innate immune responses. International immunology 24: 739–749.
43. Stevens CW, Aravind S, Das S, Davis RL (2013) Pharmacological characterization of LPS and opioid interactions at the toll-like receptor 4. British journal of pharmacology 168: 1421–1429.
44. Lukens M V, Kruijsen D, Coenjaerts FEJ, Kimpen JLL, Van Bleek GM (2009) Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. Journal of virology 83: 7235–7243.
45. Demoor T, Petersen BC, Morris S, Mukherjee S, Ptaschinski C, et al. (2012) IPS-1 signaling has a nonredundant role in mediating antiviral responses and the clearance of respiratory syncytial virus. Journal of immunology (Baltimore, Md: 1950) 189: 5942–5953.
46. Bhoj VG, Sun Q, Bhoj EJ, Somers C, Chen X, et al. (2008) MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus. Proceedings of the National Academy of Sciences of the United States of America 105: 14046–14051.
47. Jamaluddin M, Wang S, Garofalo RP, Elliott T, Casola A, et al. (2001) IFN-beta mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. American journal of physiology Lung cellular and molecular physiology 280: L248–57.
48. Okabayashi T, Kojima T, Masaki T, Yokota S-I, Imaizumi T, et al. (2011) Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. Virus research 160: 360–366.

49. Marr N, Turvey SE (2012) Role of human TLR4 in respiratory syncytial virus-induced NF-κB activation, viral entry and replication. Innate immunity 18: 856–865. Available: http://www.ncbi.nlm.nih.gov/pubmed/22535679. Accessed 18 March 2013.
50. Lizundia R, Sauter K-S, Taylor G, Werling D (2008) Host species-specific usage of the TLR4-LPS receptor complex. Innate immunity 14: 223–231.
51. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, et al. (2001) Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. Journal of virology 75: 10730–10737.
52. Ehl S, Bischoff R, Ostler T, Vallbracht S, Schulte-Mönting J, et al. (2004) The role of Toll-like receptor 4 versus interleukin-12 in immunity to respiratory syncytial virus. European journal of immunology 34: 1146–1153.
53. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nature immunology 3: 354–359. Available: http://www.ncbi.nlm.nih.gov/pubmed/11912497. Accessed 5 March 2013.
54. Marr N, Hajjar AM, Shah NR, Novikov A, Yam CS, et al. (2010) Substitution of the Bordetella pertussis lipid A phosphate groups with glucosamine is required for robust NF-kappaB activation and release of proinflammatory cytokines in cells expressing human but not murine Toll-like receptor 4-MD-2-CD14. Infection and immunity 78: 2060–2069.
55. Benjamin JT, Smith RJ, Halloran BA, Day TJ, Kelly DR, et al. (2007) FGF-10 is decreased in bronchopulmonary dysplasia and suppressed by Toll-like receptor activation. American journal of physiology Lung cellular and molecular physiology 292: L550–8.
56. Benjamin JT, Carver BJ, Plosa EJ, Yamamoto Y, Miller JD, et al. (2010) NF-kappaB activation limits airway branching through inhibition of Sp1-mediated fibroblast growth factor-10 expression. Journal of immunology (Baltimore, Md: 1950) 185: 4896–4903.
57. Schijf MA, Kruijsen D, Bastiaans J, Coenjaerts FEJ, Garssen J, et al. (2012) Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. Journal of virology 86: 11472–11482.
58. Jiang Z, Georgel P, Du X, Shamel L, Sovath S, et al. (2005) CD14 is required for MyD88-independent LPS signaling. Nature immunology 6: 565–570.
59. Baumann CL, Aspalter IM, Sharif O, Pichlmair A, Blüml S, et al. (2010) CD14 is a coreceptor of Toll-like receptors 7 and 9. The Journal of experimental medicine 207: 2689–2701.
60. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, et al. (2002) Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. Science (New York, NY) 297: 2063–2066.
61. Kadowaki N, Antonenko S, Lau JY, Liu YJ (2000) Natural interferon alpha/beta-producing cells link innate and adaptive immunity. The Journal of experimental medicine 192: 219–226.
62. Kim D, Niewiesk S (2013) Synergistic Induction of Interferon α through TLR-3 and TLR-9 Agonists Identifies CD21 as Interferon α Receptor for the B Cell Response. PLoS pathogens 9: e1003233.
63. Szabo G, Dolganiuc A (2008) The role of plasmacytoid dendritic cell-derived IFN alpha in antiviral immunity. Critical reviews in immunology 28: 61–94.
64. Smit JJ, Rudd BD, Lukacs NW (2006) Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. The Journal of experimental medicine 203: 1153–1159.
65. Ling Z, Tran KC, Teng MN (2009) Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting

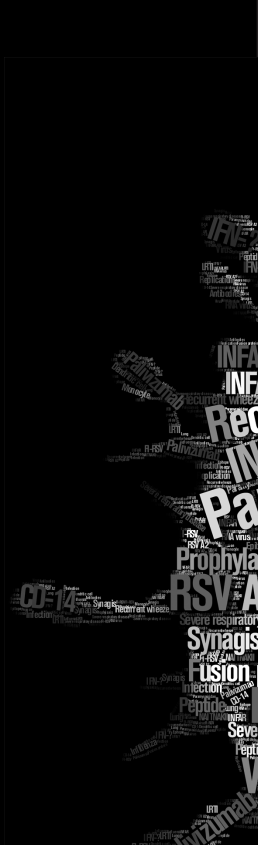
with RIG-I. Journal of virology 83: 3734–3742.
66. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science (New York, NY) 303: 1529–1531.
67. Sun X, Yau VK, Briggs BJ, Whittaker GR (2005) Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology 338: 53–60.
68. Srinivasakumar N, Ogra PL, Flanagan TD (1991) Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. Journal of virology 65: 4063–4069.
69. Morris S, Swanson MS, Lieberman A, Reed M, Yue Z, et al. (2011) Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses. Journal of immunology (Baltimore, Md: 1950) 187: 3953–3961.
70. Liao AP, Salajegheh M, Morehouse C, Nazareno R, Jubin RG, et al. (2010) Human plasmacytoid dendritic cell accumulation amplifies their type 1 interferon production. Clinical immunology (Orlando, Fla) 136: 130–138.
71. Spann KM, Tran KC, Collins PL (2005) Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. Journal of virology 79: 5353–5362.
72. Lo MS, Brazas RM, Holtzman MJ (2005) Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. Journal of virology 79: 9315–9319.
73. De Jager W, Prakken B, Rijkers GT (2009) Cytokine multiplex immunoassay: methodology and (clinical) applications. Methods in molecular biology (Clifton, NJ) 514: 119–133.

SUPPORTING INFORMATION



Supplementary Figure S1. Cytokine responses in PBMC exposed to RSV A 13NO1 and 16NO1 at MOI 1. PBMC cultured with the RSV strains 13N01 and 16N01 at MOI 1, UV inactivated RSV, or RSV neutralized in autologous serum. After 20 hrs. incubation, cytokines were measured in supernatant by ELISA. Experiments were performed in 3 different donors with similar results. Data shown represent the mean ± SEM of 3 measurements within 1 representative donor and were analyzed using one way ANOVA followed by a Bonferroni post-test, *P< 0.05, **P <0.01.

Chapter 4


Prophyla
RSVA
Synagis
Fusion
Peptide
Severe respirator
CD=14

Intranasal Administration of Antibody-Bound Respiratory Syncytial Virus Particles Efficiently Primes Virus-Specific Immune Responses in Mice

Debby Kruijsen,[a] Marcel A. Schijf,[a,c] Helga K. Einarsdottir,[b] Frank E. Coenjaerts,[d] Ellen C. van der Schoot,[b] Gestur Vidarsson,[b] Grada M. van Bleek[a]

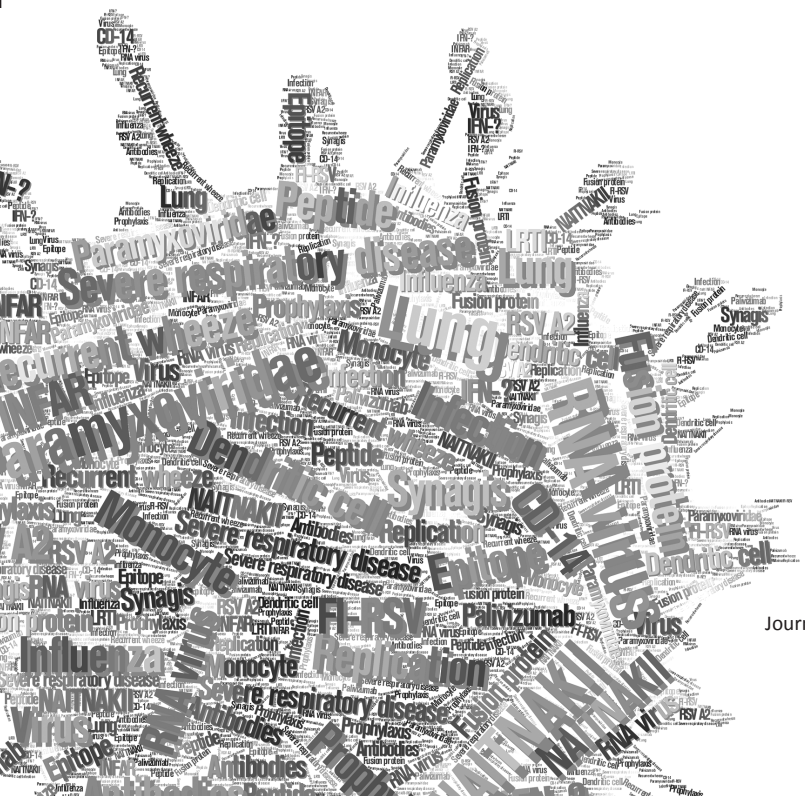
[a]Department of Pediatrics, the Wilhelmina Children's Hospital, University Medical Center (UMC), Utrecht, The Netherlands.

[b]Department of Experimental Immunohematology, Sanquin Research, Amsterdam and Landsteiner Laboratory, AMC University of Amsterdam, Amsterdam, The Netherlands.

[c]Department of Immunology, Danone Research-Centre for Specialised Nutrition, Wageningen, The Netherlands.

[d]Department of Medical Microbiology, UMC, Utrecht, The Netherlands.

M.A.S. and H.K.E. contributed equally to this article.



Journal of Virology (2013):13:7550-7557.

ABSTRACT

Infants are protected from a severe respiratory syncytial virus (RSV) infection in the first months of life by maternal antibodies or by prophylactically administered neutralizing antibodies. Efforts are under way to produce RSV-specific antibodies with increased neutralizing capacity compared to the currently licensed palivizumab. While clearly beneficial during primary infections, preexisting antibodies might affect the onset of adaptive immune responses and the ability to resist subsequent RSV infections. Therefore, we addressed the question of how virus neutralizing antibodies influence the priming of subsequent adaptive immune responses. To test a possible role of the neonatal Fc receptor (FcRn) in this process, we compared the responses in C57BL/6 wild-type (WT) and $FcRn^{-/-}$ mice. We observed substantial virus-specific T-cell priming and B-cell responses in mice primed with RSV IgG immune complexes resulting in predominantly Th1-type $CD4^+$ T-cell and IgG2c antibody responses upon live-virus challenge. RSV-specific $CD8^+$ T cells were primed as well. Activation of these adaptive immune responses was independent of FcRn. Thus, neutralizing antibodies that localize to the airways and prevent infection-related routes of antigen processing can still facilitate antigen presentation of neutralized virus particles and initiate adaptive immune responses against RSV.

INTRODUCTION

Antibodies are an important correlate of protection for many viral infections. Neutralizing antibodies reduce viral load and virus-induced pathogenesis. Virus infection may be directly cytopathogenic or cause indirect tissue damage by host immune responses that follow viral exposure. In addition to lowering viral load, virus-specific antibodies might reduce or alter innate immune responses, affect antigen presentation and thereby the level of T-cell activation (1–3), and potentially enhance disease (4–7).

Children experiencing a primary respiratory syncytial virus (RSV) infection are protected against lower respiratory tract infections (LRTIs) by maternal antibodies. However, maternal antibodies decline rapidly within a few months after birth (8), and high levels of serum antibodies are required to provide efficient local protection in the airways. Adults with acquired immunity to RSV, including RSV neutralizing serum antibodies and memory T cells, still experience recurrent reinfections (9). Reduced titers of serum antibodies correlate with increased RSV-associated hospitalization in patients of all ages (9–11). Based on these observations current vaccine development is focused on a vaccine preparation that induces the production of highly neutralizing antibodies. Alternatively, palivizumab, a neutralizing antibody to the fusion protein of RSV, can be administered prophylactically to protect high-risk infants against LRTIs (12).

Because better neutralizing antibodies are being developed to protect against severe RSV disease (13), it is essential to understand the consequences of the presence of antibodies *in vivo* on the outcome of the immune response upon infection. Previously, we showed that the neutralizing capacity of RSV-specific antibodies influenced the level of virus-specific $CD8^+$ and $CD4^+$ T-cell responses in immune mice (14). Highly neutralizing serum antibodies decreased the $CD8^+$ T-cell response, while both neutralizing and non-neutralizing antibodies increased $CD4^+$ T-cell responses. The increased T-cell responses in the presence of antibodies were mediated by increased antigen presentation of RSV immune complexes (IC-RSV) to $CD4^+$ T cells in an FcγR-dependent process (14).

Recently, yet another IgG Fc receptor, the neonatal Fc receptor (FcRn), was also shown to participate in phagocytosis processes of neutrophils, and it facilitates antigen presentation by dendritic cells (DCs) of soluble antigens opsonized by IgG (15, 16). FcRn was first described as a transporter of IgG across epithelial barriers, including transmission of IgG across the placenta from mother to infant. In addition, FcRn binds albumin and IgG Fc within endosomes at low pH and increases the serum half-life of these ligands by recycling them back to the cell surface, where the ligands are released from FcRn at higher pH (17, 18). FcRn is also responsible for antigen sampling from the airways and gut, by transporting IgG complexes across epithelium for uptake into macrophages and dendritic cells residing underneath the epithelial layer (19).

In the present work, we studied the effect of neutralizing antibodies to RSV on the induction of RSV-specific adaptive immune responses initiated after intranasal (i.n.) administration of antibody bound RSV in mice. The role of FcRn in the local antigen presentation of RSV-palivizumab complexes was investigated by comparing the immune responses of C57BL/6 wild-type (WT) and FcRn$^{-/-}$ mice.

RESULTS

H-2A^{b}-mediated antigen presentation of IC-RSV by DCs is facilitated by activating FcγRs and not FcRn.

In previous work, we showed that activating FcγRs are involved in antigen presentation of RSV-derived antigens to CD4^{+} T cells during *in vitro* antigen presentation with dendritic cells and during *in vivo* RSV infection in mice (14). To determine whether FcRn also played a role during presentation of RSV-derived antigenic peptides, we first performed *in vitro* antigen presentation assays in the presence or absence of virus-specific antibodies, using BM-DCs as antigen presenting cells obtained from WT, FcRn$^{-/-}$, or FcR common-γ- chain^{-}/$^{-}$ (γ^{-}/$^{-}$) mice. Expression of FcRn in WT BM-DCs and D1 cells and its absence in FcRn$^{-/-}$ BM-DCs were confirmed by RT-qPCR (**Figure 1A**). As a source of polyclonal RSV-specific T cells, lung cells were harvested at day 8 after a primary i.n. RSV infection. T-cell activation was measured by intracellular staining for IFN-$^{+}$. A similar increased percentage of IFN-γ-producing CD4^{+} T cells was observed when WT BM-DCs or BM-DCs from FcRn$^{-/-}$ mice were pulsed with UV-inactivated RSV in the presence of serum derived from mice after secondary RSV infection (immune serum) compared to the response of pre immune serum (**Figure 1B**). However, as described in our previous work, the IgG mediated enhanced antigen presentation of IC-RSV to CD4^{+} T cells completely depended on functional expression of FcγRs on BM-DCs (14) (**Figure 1C**).

Individual DC cultures originating from bone marrow harvested from different mice might vary in the percentage of immature DCs. To exclude this experimental variation, we performed experiments with a dendritic cell line (D1) expressing functional FcRn (Figure 1A) and used mutant antibodies unable to bind to FcRn in the *in vitro* antigen presentation assay. We used either WT anti-TNP or recombinant monoclonal antibodies with the same antigen specificity but mutated at a single amino acid residue (H435A) or three residues (IHH: I253A, H310A, and H345A) in the Fc domain. These positions are involved in FcRn binding but do not interfere with binding to FcγRs (15, 29). TNP-labeled RSV alone or opsonized with these recombinant WT or mutant (H435A and IHH) IgG1 monoclonal antibodies, was incubated with D1 cells, and antigen presentation was monitored using RSV specific lung T cells. The anti-TNP WT and mutant antibodies all increased the percentage of IFN-γ-producing CD4^{+} T cells responding to D1 cells exposed to RSV-TNP to a similar extent compared to the

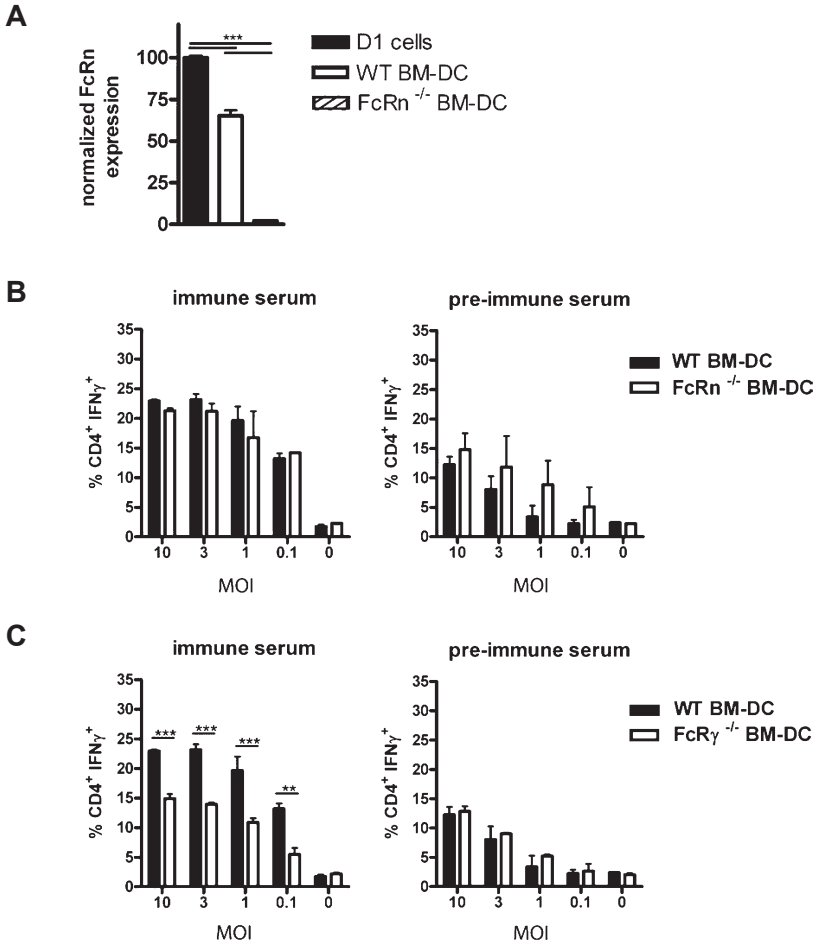


FIGURE 1. Activating FcγR, but not FcRn, expressed by BM-DCs are involved in antigen presentation of IC-RSV to RSV-specific CD4⁺ T cells. (**A**) WT BM-DC, D1 cells but not BM-DCs from FcRn$^{-/-}$ mice express FcRn. FcRn expression levels were analyzed with RTq-PCR and normalized for RPL27 expression. Result of one representative culture out of three is shown. Error bars represent the SEM of a duplicate within one experiment. Significance was calculated using a One-way ANOVA. ***p<0.001. (**B**) BM-DCs obtained from WT, FcRn$^{-/-}$ or (**C**) WT and γ$^{-/-}$ mice were incubated with UV-RSV (MOI 10, 3, 1, 0.1 and 0) in the presence of 2% serum of naïve mice or from RSV immune mice for 24h. Lung cells harvested 8 days after a primary RSV infection (a source of RSV-specific T cells) were added to the BM-DC and responding CD4⁺ T cells were visualized by intracellular staining for IFN-γ. Significance was calculated using a Two-way ANOVA. Error bars represent the SEM on data of one out of three individual experiments with similar results. **p<0.01 and ***p<0.001.

samples in which non-opsonized virus was incubated with D1 cells (**Figure 2**). These results confirmed that FcRn was not involved in the *in vitro* presentation by DCs of IC-RSV-derived antigenic peptides to virus-specific CD4⁺ T cells.

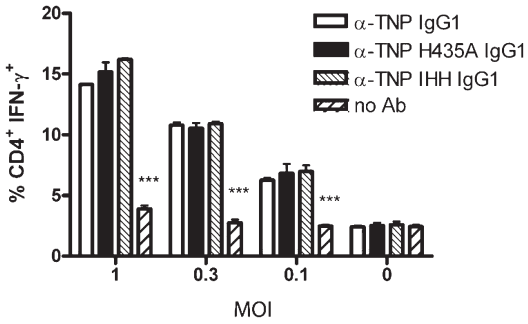


FIGURE 2. Abrogated binding of IC-RSV to FcRn does not affect antigen presentation to RSV-specific CD4⁺ T cells. C57BL/6-derived dendritic D1 cells were incubated with haptenized RSV (RSV-TNP) at MOI 1, 0.3, 0.1 and 0 in the presence of 0.1μg/ml of either anti-TNP (α-TNP) IgG1, or anti-TNP H435A mutant IgG1, or anti-TNP IHH or without antibodies for 24h. The percentage of responding CD4⁺ T cells (in lung cells harvested at eight days post infection) was measured by intracellular staining for IFN-γ. One representative experiment (out of three) is shown. Significance was calculated using a Two-way ANOVA. Error bars represent SEM of a duplicate within one experiment. ***p<0.001 comparing: no antibody, to all three antibody-mediated responses.

Noninfectious IC-RSV efficiently primes virus-specific T-cell responses after i.n. inoculation.

We next examined the impact of antibody neutralization of RSV in the airways on the initiation of the T- and B-cell responses by measuring recall responses during a virus challenge 5 weeks after immune priming. Abortive replication has been described as occurring in RSV-immune animals (34). This process of abortive replication is defined as viral penetration followed by replication of viral genetic material in the cytoplasm, without the production of infectious virus particles. Abortive replication *in vivo* can be monitored by comparing lung viral loads using the PFU assay and by the PCR detection technique and has been observed in animals treated prophylactically by virus neutralizing antibodies instilled systemically. Abortive replication still induces inflammatory immune responses via cytoplasmic recognition of genomic viral RNA and replication intermediates (35). Also, viral antigen expression might still occur. To focus only on the contribution of neutralized virus particles in priming of the immune response, we administered *in vitro*-preformed RSV immune complexes intranasally. Moreover, this approach also minimized the effect of increased antibody degradation in $FcRn^{-/-}$ mice that could lead to differences in virus neutralization in the lung, when antibody was administered parenterally. We used a monoclonal humanized IgG1 antibody, palivizumab (12), specific for the fusion protein of RSV in these experiments. Binding of palivizumab to RSV completely abolished virus infection of A549 epithelial cells *in vitro* (**Figure 3A**). RSV-palivizumab complexes could still be detected by RT-qPCR at a level of one-third of the input virus in the washed A549 samples incubated for 24 h with the different virus preparations. However, the amount of viral material still detected represented noninfectious virus bound to A549 cells because expression of viral pathogen-associated molecular patterns in the

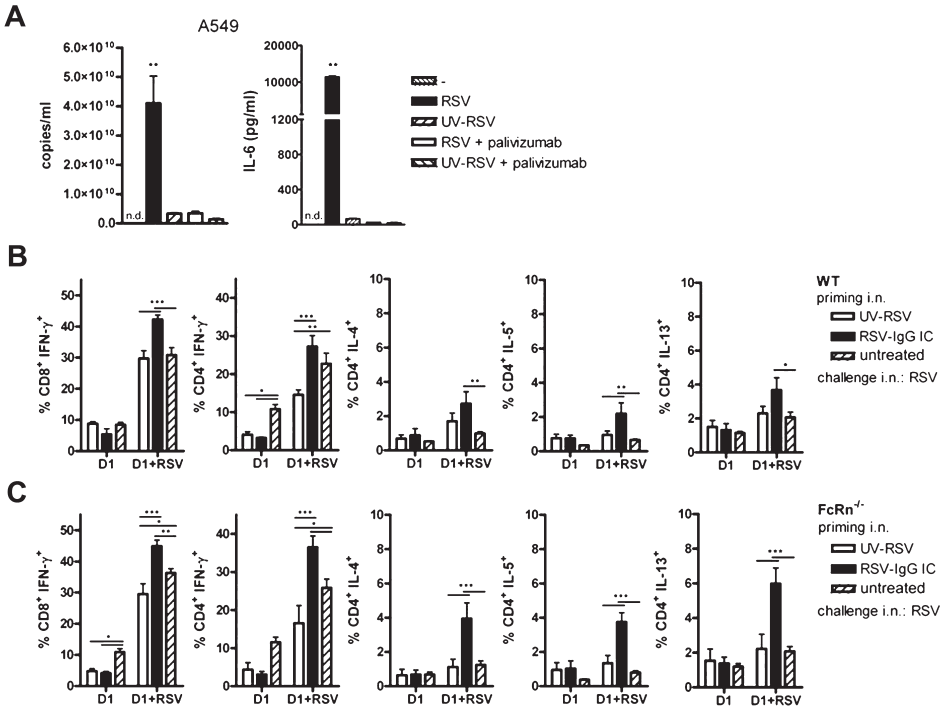


FIGURE 3. Intranasal inoculation of IC-RSV efficiently primes RSV-specific CD4⁺ and CD8⁺ T cell responses in both WT and FcRn-deficient mice. (**A**) Palivizumab neutralizes RSV. RSV (4.7×10^7 PFU) was preincubated for 15 min with 50 µg/ml palivizumab. UV-RSV was used as a negative control for replication. RSV, RSV plus palivizumab, UV-RSV, or UV-RSV plus palivizumab was added to epithelial A549 cells (equivalent of an MOI of 2), and the cells were incubated for 24 h. The results of the RT-qPCR performed on the RSV N gene are shown for one representative experiment of two. Error bars represent the SEM of a duplicate within one experiment. IL-6 production was measured in the 24-h supernatant of A549 cells treated with similar live- and inactivated-RSV preparations. One representative experiment out of 6 is shown. Significance was calculated using a one-way ANOVA. **, *P*<0.01. n.d., non- detectable. (**B**) WT mice and (**C**) $FcRn^{-/-}$ mice were primed with UV-RSV or IC-RSV at day 0. A third group was untreated. At day 35, all groups were challenged with RSV, and 6 days later, lungs were analyzed for T-cell responses in the presence of uninfected (D1) or RSV- infected D1 cells (D1+RSV). The percentage of cytokine producing $CD8^+$ and $CD4^+$ T cells is shown. Error bars represent the SEM of 5 individual mice per group. Results are shown for five mice per group of one of two representative experiments. Significance was calculated using a Two-way ANOVA. *p<0.05, **p<0.01 and ***p<0.001.

cytoplasm, which leads to inflammatory cytokine production in RSV-infected A549 cells, was completely abolished. This was shown by the complete absence of IL-6 production by A549 cells exposed to RSV-palivizumab (**Figure 3A**). We used these antibody-inactivated RSV immune complexes and UV-inactivated RSV to prime immune responses *in vivo*. Five weeks after intranasal inoculation of mice with RSV immune complexes (equivalent of 2.10^6 PFU and 50 µg/ml palivizumab in a total volume of 50 µl) or control UV inactivated RSV (equivalent of 2.10^6 PFU in 50 µl), T- and B-cell responses were measured in the lungs at day 6 after an i.n. challenge with live RSV. A third group only received a primary RSV infection. Despite

the failure of FcRn to enhance antigen presentation of IgG-complexed RSV *in vitro*, the possibility remained that FcRn might mediate translocation of ICs across the respiratory epithelial layer and increase antigen presentation in the lungs of mice (36). Therefore, we determined whether FcRn was involved in the *in vivo* priming of RSV-specific immune responses upon i.n. exposure to IC-RSV by comparing the priming of immune responses in C57BL/6WTand FcRn$^{-/-}$ mice. Palivizumab contains the complementarity-determining regions of a mouse neutralizing anti-RSV-F antibody grafted into the framework of a human IgG1 constant region (37). Human IgG1 (and thus palivizumab) binds mouse FcRn via its constant region with high affinity at pH 6.0 (34). In addition, mouse FcRn has been shown to extend the half-life of human IgG1 to a similar extent to mouse IgG (17, 38, 39). Importantly, mouse FcRn has also been demonstrated to enhance antigen presentation of immune complexes through human IgG1 (15). The activation of T cells in the lungs after viral challenge was measured by *in vitro* restimulation with untreated or RSV-infected D1 cells and subsequent intracellular cytokine staining. T cells from mice inoculated with IC-RSV showed an increased percentage of RSV-specific CD8$^+$ IFN-γ$^+$ T cells compared to T cells from UV-RSV-inoculated mice and non-immunized mice when re-stimulated with RSV-infected D1 cells. An increase in IFN-γ- and IL-5-producing CD4$^+$ T cells was also observed for IC-RSV-immunized mice in comparison to UV-inoculated mice. For IL-4$^+$ and IL-13$^+$ CD4$^+$ T cells, the increases were only significant in comparison to untreated mice but not in comparison to UV-inoculated mice (**Figure 3B**). The slight increase in the percentage of Th2 cytokine-producing cells in IC-RSV-inoculated mice was not accompanied by an increase in the percentage of eosinophils in BAL specimens (data not shown). In both WT and FcRn$^{-/-}$ mice, UV-RSV inoculation did not prime Th1 or Th2 responses against RSV. A slight decrease in IFN-γ production was noticed in UV-RSV-immunized mice after challenge compared to primary RSV-infected mice. Similar increased Th1 and Th2 responses after immune complex priming were observed in FcRn$^{-/-}$ mice (Figure 3C) and WT mice (**Figure 3B**), suggesting again that FcRn-mediated processes were not involved in the IgG mediated enhancement of the immune responses to RSV. In addition to increasing virus-specific T-cell responses, binding of palivizumab to RSV strongly potentiated RSV-specific IgG2c antibody responses (Figure 4). In FcRn$^{-/-}$ mice, the antibody responses were slightly lower than those in wild-type mice at day 28 after priming, probably due to the shorter antibody half-life in the knockout mice (**Figure 4A**) (18). However, at day 41 (i.e., 6 days after challenge), the virus-specific antibody response was robust and the responses were of equal magnitude in WT and FcRn$^{-/-}$ mice inoculated with immune complexes (**Figure 4B**). Due to the shorter half-life of IgG2c in FcRn$^{-/-}$ mice, this may indicate that the relative net production of anti-RSV IgG in these mice may be increased.

In conclusion, these experiments showed strong immune priming with opsonized RSV that was negligible when non-opsonized, non-infectious RSV was used. The enhanced immune priming was independent of FcRn.

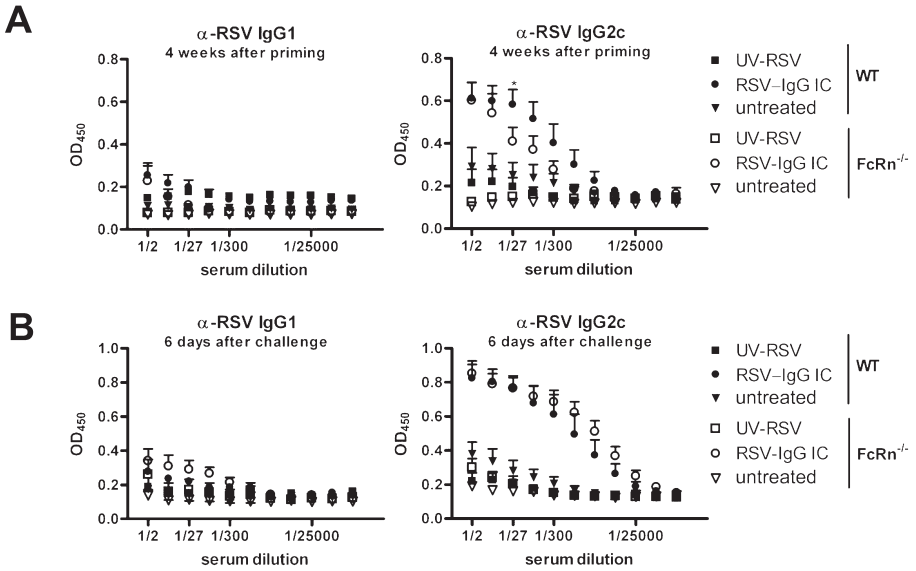


FIGURE 4. Intranasal administration of IC-RSV efficiently primes RSV-specific IgG2c responses in both WT and FcRn-deficient mice. WT mice and FcRn$^{-/-}$ mice were inoculated with UV-RSV or IC-RSV (palivizumab) at day 0. A third group was left untreated. At day 35, all groups were challenged with RSV. Blood was collected to analyze serum antibodies 7 days prior to RSV challenge (4 weeks after priming in panel A and 6 days after challenge in panel B). (**A**) RSV-specific IgG2c levels were significantly increased in both WT and FcRn$^{-/-}$ mice (P<0.001) after inoculation with IC-RSV. However, the responses were lower in FcRn$^{-/-}$ mice compared to those in WT mice (*, P<0.05 at 1/27 serum dilution). (**B**) At day 6 after challenge in both WT and FcRn$^{-/-}$ mice, virus-specific IgG2c levels differed significantly in IC-RSV-treated mice from non-primed and UV-RSV-inoculated mice (P<0.001), but no significant differences were found between the WT and FcRn$^{-/-}$ mice who received IC-RSV priming. These experiments were performed twice with similar results. Error bars represent the SEM of 5 individual mice per group. Significance was calculated using a two-way ANOVA.

DISCUSSION

In the present work, we showed that intranasal administration of noninfectious IC-RSV primed both virus-specific T- and B-cell responses in mice, while administration of non-opsonized UV inactivated RSV did not. The increased RSV-specific immune response observed after RSV challenge in IC-RSV-primed mice was independent of FcRn. Therefore, FcRn-mediated transcytosis of these immune complexes across epithelium was not the route by which priming by IC-RSV was facilitated, and also a role in antigen processing of FcRn during *in vivo* priming can be ruled out. These observations were further substantiated in *in vitro* experiments using IC-RSV-pulsed DCs as antigen-presenting cells to stimulate RSV-specific T cells. Comparing WT BM-DCs with FcRn$^{-/-}$ BM-DCs (**Figure 1**) and WT IgG with mutant IgG1 unable to bind FcRn (**Figure 2**), we did not find a contribution of FcRn in antigen processing.

Previously, we found that neutralizing antibodies present in immune mice affected RSV-specific T-cell responses by increasing CD4^{+} and decreasing CD8^{+} T-cell responses *in vivo* after

an i.n. live-RSV challenge (14). These findings suggested that virus-specific $CD8^+$ T cells are most efficiently primed upon live-virus exposure, presumably via the classical cytoplasmic route of antigen presentation and in the context of an innate immune response triggered by the virus. Depending on the titer and affinity of serum neutralizing antibodies, the efficacies of virus neutralization in the airways may differ. In previous work, we studied the secondary T and B-cell responses in mice pre-exposed to RSV (14). In those studies, both B-cell and T-cell responses were primed. The situation is different in infants before primary RSV exposure when only antiviral antibodies are present without primed T cells (i.e., maternal IgG obtained via the placenta and/or prophylactically administered antibody in children with a high risk of developing severe RSV disease). It has been shown in both laboratory animals and humans, that systemic antibodies do not completely prevent early viral replication steps and innate immune responses (34, 35, 40). This may be explained by inadequate serum titers and/or insufficient access of antibodies to the airway lumen. Systemic antibodies need to access the airways from the serum through a process in which basolateral-to-apical transport across airway epithelium facilitated by FcRn might play a role (19). Furthermore, the half-life of IgG antibodies in serum is affected by FcRn. Therefore, several mechanisms might contribute to altered immune priming when RSV neutralizing antibody is administered parenterally in $FcRn^{-/-}$ compared to WT mice. To circumvent the factors potentially affecting the efficacy of RSV neutralization and to exclude residual RSV (abortive) infection in the lung by systemically administered palivizumab the way in which earlier studies have been performed (41), we opted for the intranasal application of *in vitro* neutralized virus.

In the present study, we show that in comparison to an i.n. challenge with non-opsonized UV-inactivated virus particles, both $CD4^+$ and $CD8^+$ virus-specific T-cell responses were enhanced if the virus was opsonized with palivizumab. Thus, while live-virus infection might be the most effective way of priming $CD8^+$ T-cell responses in this mouse model due to viral replication, we demonstrate that preexisting neutralizing antibodies do not block initiation of virus-specific $CD8^+$ T responses completely. It can be envisioned that the inflammatory environment during T-cell priming is different when live RSV or immune complexes are present in the airways. This difference in innate immune response might affect the quality of the memory response and the functionality of effector cells recalled during subsequent infections. Low numbers of $CD4^+$ T cells producing Th2 cytokines were detected in RSV-palivizumab-primed mice after a challenge with live virus. The lack of infection of RSV-palivizumab complexes might alter early innate immune responses that affect the nature of the local adaptive immune response during challenge. However, these numbers of Th2 $CD4^+$ T cells are 1 to 4% higher than we usually find after secondary RSV infections in C57BL/6 mouse lungs and much lower than after intramuscular vaccination with inactivated-RSV vaccines (30 to 40%) (14, 42).

Similar T-cell responses were observed after i.n. RSV challenge with or without prior intranasal priming with UV-RSV. Thus, intranasal exposure with noninfectious virus appears

to be an ineffective route of T-cell priming and even may slightly decrease Th1 priming (**Figure 3**). We used UV-inactivated virus in these i.n. challenge experiments, because we wanted to evaluate the role of antibodies in the *in vivo* antigen presentation of noninfectious virus (i.e., mimicking the situation when the virus is bound to serum-derived neutralizing antibodies). It could be argued that UV inactivation of RSV might damage the virus proteins or the viral RNA, decreasing the efficiency of antigen presentation or interfering with virus-induced innate immune responses. However, in *in vitro* antigen presentation experiments, we found that antigen presentation to virus-specific T cells was equally efficient when antibody-bound live RSV or antibody-bound UV-RSV was incubated with antigen-presenting cells (14). Furthermore, the pattern and intensity of cytokines produced by antigen-presenting cells (monocytes, myeloid DCs [mDCs], and plasmacytoid DCs [pDCs]) were similar when these cells were exposed to live or UV-inactivated opsonized virus particles (data not shown). FcRn-mediated transport across the airway epithelium has been reported by others to be an effective way to introduce Ig antigen fusion proteins into the systemic circulation (36) and a route of immune priming (43). Herpes simplex virus 2 (HSV-2)- specific T- and B-cell responses were primed upon i.n. administration of an IgG Fc-gD fusion protein in an FcRn-dependent way, resulting in protection against a lethal dose of an intra-vaginal challenge with HSV-2 (43). These observations might be explained by FcRn-mediated transcytosis of gD-Fc across respiratory epithelium. The findings with respect to the role of FcRn in immune priming with gD-Fc fusion proteins and reports showing a role of FcRn in antigen processing in DC of ovalbumin immune complex (OVA-IC) contrast with our observations (15, 44, 45). The observed difference between our findings and those published with OVA and Fc-gD might be explained by the soluble nature of the antigens; an Fc fusion protein or ovalbumin versus RSV viral particles in our study, or perhaps due to inherent yet unknown properties of RSV itself. However, a different explanation might be that there was a difference in the antigen-presenting cell populations involved in the processes described in the different studies. A recent publication showed that FcRn exclusively facilitated processing of multimeric Ig-complexed OVA in macrophages and not in DCs (46). Apparently, the phagosomes in DCs did not reach the low pH required for IgG binding to FcRn, while in macrophages the pH rapidly dropped to 6.5, allowing FcRn binding. Monovalent internalization of IgG-OVA was, however, FcRn dependent in both DCs and macrophages. Thus, FcRn independent IC-RSV antigen presentation might be simply due to different mechanisms of uptake and presentation (phagocytosis versus endocytosis), or as described by Liu et al. (46) due to the neutral pH in the phagosomes of some types of DCs. In our studies, it might be possible that lung DCs efficiently captured IC-RSV via FcγR but did not (efficiently) process them and only transported captured viral material to the draining lymph nodes. Here antigen presentation could occur via cross-priming, possibly mediated by lymph node resident DCs (47). Dendritic cells located underneath the epithelial barrier in lung tissue contribute to the initiation of adaptive immune responses specific for RSV. DCs internalize antigen via a specific phagocytosis

or through FcγRs when the antigen is opsonized by IgGs. Here we confirm previous work, in a secondary RSV infection model in mice, that antigen presentation and subsequent RSV-specific T-cell activation were mediated via FcγRs in the presence of acquired antibodies (14). Therefore, it might be hypothesized that IC-RSV is sampled through FcγRs that are present on $CD103^+$ DCs in the airways. Moreover, antigen presentation in lymph nodes might depend on DCs that can more readily migrate to the lymph nodes compared to macrophages.

In conclusion, our study suggests that prophylactic RSV antibodies that suppress viral infection and the classical major histocompatibility complex (MHC) class I route of antigen presentation in the lung can boost the induction of adaptive $CD4^+$ T-cell responses through FcγR, while a significant virus specific $CD8^+$ T-cell response is elicited, presumably via cross-priming.

MATERIALS AND METHODS

Mice

Pathogen-free 6- to 8-week-old C57BL/6cjo wild-type mice were purchased from Charles River (Maastricht, The Netherlands). FcR common-γ-chain ($\gamma^{-/-}$) mice and $FcRn^{-/-}$ mice in a C57BL/6 background (20) were bred and maintained at the central animal facility at Utrecht University. Study protocols were approved by the UMC Utrecht Animal Ethics Committee.

Virus and cell lines

The RSV-A2 strain was grown in HEp-2 cells (ATCC CCL-23), purified by polyethylene glycol precipitation, and stored in liquid nitrogen in 10% sucrose in phosphate-buffered saline (PBS). The 50% tissue culture infective dose (TCID50) was determined after titration in HEp-2 cells. Detailed methods have been described previously (14). D1, a mouse dendritic cell line derived from C57BL/6 mice (21) used in antigen presentation assays, was maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen) containing 5% HyClone fetal calf serum (FCS) (Perbio, Logan, UT), 1% penicillin–streptomycin and 50 uM 2-mercaptoethanol (Bio-Rad, Hercules, CA) supplemented with 30% conditioned medium from granulocyte-macrophage colony stimulating factor (GM-CSF)-producing R1 cells (NIH 3T3 mouse fibroblasts transfected with the GM-CSF gene) (21). Bone marrow-derived dendritic cells (BM-DCs) were prepared as described before (14, 22). The percentage of $CD11c^+$ cells (routinely >70%) was determined by staining with anti-CD11c (clone HL3; BD Pharmingen, San Diego, CA).

TNP labeling of RSV

Trinitrophenol (TNP) labeling was performed as described by Hale et al. (23). In short, 200 µg/ml UV-inactivated RSV (UV-RSV) was incubated with 10mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Sigma-Aldrich, Steinheim, Germany) in 1 ml Hanks' buffer (Invitrogen) for 15 min at 37°C. Unbound TNBS was removed via dialysis against PBS overnight at 4°C while stirring. Precipitated RSV-TNP was removed by centrifugation at 1,500 rpm for 1 min. The concentration of stably linked TNP groups was measured by the optical density at 350 nm (OD350).

Generation of human anti-2, 4, 6-TNP antibodies

The variable regions of the heavy (VH) and light (VL) chains were cloned from an anti-2,4,6-trinitrophenol (anti-TNP) hybridoma (24) and expressed as chimeric human IgG as previously described (25). RNA was amplified using the SMART Race cDNA amplification kit with CH- and CL-specific primers (Clontech, Inc., CA). Variable (V) genes were identified after sequencing of clones. Codon-optimized V genes, including 5'-HindIII and 3'-EcoRI restriction sites, Kozak sequence, and HAVT20 leader sequence (26), were then designed and ordered from Geneart (Life Technologies), along with codon-optimized human $^{+}$ or $^{+}$1 constant regions for the variable light and heavy chains, respectively. The IHH IgG1 HC variant was created by inserting three mutations that negate binding to FcRn in the CH3 region (where IHH represents I253A, H435A, and H436A) (27). The HindIIIEcoRI fragment for the codon-optimized light chain was ligated into pEE14.4 (Lonza), and the HindIII-EcoRI fragment for the heavy chain was ligated into pEE6.4 (Lonza). A single-gene vector encoding eitherWT IgG1 or IHH-IgG1 was subsequently generated by ligation of the BamHINotI fragment from pEE6.4 [including a cytomegalovirus (CMV) promoter, IgG1 heavy chain, and poly(A)] into the light-chain-encoding pEE14.4 vector. The plasmids were then transfected in the FreeStyle 293 expression system (Life Technologies). Antibodies were purified on a protein A (WT IgG1) or protein G (IHH) HiTrap HP column (GE Life Sciences) and dialyzed against PBS overnight.

In vivo RSV infection, IC-RSV priming, and tissue sampling

Mice were lightly anesthetized with isoflurane and i.n. inoculated with $2x10^6$ PFU RSV in a volume of 50 µl. Lung cells were obtained from C57BL/6 mice 8 days after primary RSV infection at the peak of the T-cell response (28) when used as a readout in the *in vitro* antigen presentation assays. In the *in vivo* experiments testing the effect of immune priming induced with RSV immune complexes (IC-RSV), mice were i.n. inoculated with IC-RSV or UV-inactivated RSV (UV-RSV) in a volume of 50 µl at day 0. A third group of mice was left untreated. IC-RSV

was prepared by pre-incubation of $4.7x10^7$ PFU with 50 µg/ml palivizumab [humanized IgG1 monoclonal antibody; Synagis, Med Immune, Gaithersburg, MD) (12) for 15 min in a volume of 200 µl. At day 35, all groups were i.n. challenged with RSV. Six days after challenge, T-cell responses in the lung were analyzed. To obtain lung cell suspensions, mice were sacrificed by intra peritoneal (i.p.) injection of 300 µl pentobarbital. After bronchoalveolar lavage (BAL), lungs were perfused with 10 ml ice-cold PBS containing 100 U/ml heparin via the right ventricle. The lungs were removed, cut into pieces and incubated with collagenase (2.4 mg/ml; Roche Applied Science, Basel, Switzerland) and DNase (1 mg/ml; Roche) for 20 min at 37°C. Single-cell suspensions were prepared by processing the tissue through 70-um-pore cell strainers (BD Falcon, Franklin Lakes, NJ).

In vitro antigen presentation assay

In vitro mouse antigen presentation assays were performed with D1 cells or, in experiments to study the role of FcγRs and FcRn, bone marrow-derived DCs (BM-DCs) obtained from $FcγR^{-/-}$, $FcRn^{-/-}$, or C57BL/6 WT mice. To study antigen presentation of RSV immune complexes by BM-DCs or D1 cells, UV-inactivated RSV (UV-RSV) at multiplicities of infection (MOI) of 10, 3, 1, 0.1, and 0 was preincubated with either plasma derived from secondary RSV-infected mice or naive mice (pre immune serum) for 15 min at 37°C. UV-RSV was used to completely rule out infection-related effects. In parallel, RSV-TNP at MOI of 1, 0.3, 0.1, and 0 was preincubated with 0.1 µg/ml anti-TNP IgG1, anti-TNP-H435A mutant IgG1, or anti- TNP-IHH mutant IgG1 (IHH I253A, H310A, and H435A) (mutations that abolish FcRn binding capacity [29, 30]) or without antibodies as a control. RSV immune complexes were incubated with 5^{+} 104 BM-DCs or D1 cells for 24 h. Thereafter, antigen-presenting cells (APCs) were incubated with $5x10^5$ total lung cells in the presence of 25 U/ml recombinant human interleukin-2 (hIL-2) (Roche) and 10 µg/ml brefeldin A (Sigma, St. Louis, MO) to facilitate intracellular accumulation of cytokines, for 5 h at 37°C in 5% CO2. Lung cells of mice 8 days after a primary RSV infection were used as a source of RSV-specific T cells. Antigen presentation of RSV was analyzed by measuring gamma interferon (IFN-γ) production in lung $CD4^+$ T cells by intracellular fluorescence-activated cell sorter (FACS) staining.

Measurements of lung T-cell responses in IC-RSV priming experiments

Single-cell suspensions of lung cells (106) from RSV-infected wild type and $FcRn^{-/-}$ mice were stimulated for 5 h at 37°C in 5% CO2 with $2x10^5$ RSV-infected D1 cells or uninfected D1 cells in 200 µl IMDM supplemented with 5% FCS, 1% penicillin–streptomycin, 50 uM 2-mercaptoethanol, 25 U/ml recombinant hIL-2, and 10 µg/ml brefeldin A. D1 cells were infected for 48 h with RSV at an MOI of 2 before addition to the lung cell suspension. Cytokine production by $CD4^+$ and $CD8^+$T cells was analyzed by intracellular cytokine staining.

Intracellular cytokine staining

Cytokine production by $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. Cells were stained for surface markers with anti-CD8 (clone 53-6.7; BD) and anti-CD4 (clone RM4-5; BD). Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (BD) solution and Perm/Wash buffer (BD). Intracellular cytokines were detected with anti-IFN-γ (clone XMG1.2; BD), anti-IL-5 (clone TRFK5; BD), anti-IL-4 (clone 11B11; BD), and anti-IL-13 (clone eBio13a; eBioscience). Stained samples were acquired on a FACSCanto flow cytometer (BD), and data were analyzed using FacsDiva software (BD).

RT-PCR. (i) RSV-specific real-time PCR

A total of $2x10^5$ A549 cells were incubated with RSV or UV-RSV (MOI of 2) in the presence or absence of palivizumab. RSV ($4.7x10^7$ PFU) was preincubated with 50 µg/ml palivizumab for 15 min before addition to the cells. After 24 h, total RNA was extracted from these cells using MagnaPure LC (Roche) equipment. cDNA was synthesized and viral loads were determined by realtime quantitative PCR (RT-qPCR) as previously described (31). In short, extracted RNA was reverse transcribed using a MultiScribe reverse transcriptase kit (Applied Biosystems, Foster City, CA) and random hexamers. Reverse transcriptase was inactivated followed by RT-qPCR performed on 20 µl cDNA using primers and probes specific for the N gene (31). Amplification and detection were performed with an ABI 7900HT fast RT qPCR system for 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Sample threshold cycle (*CT*) values were compared with a standard curve of RSV-A2.

(ii) FcRn-specific RT-qPCR

RNA was isolated from cultured BMDCs of WT and $FcRn^{-/-}$ mice with the Qiagen RNA extraction kit, and cDNA was generated with random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. FcRn-specific mRNA was quantified by subsequent TaqMan RT-qPCR analysis with forward (5'-GTGGAAGGAGCCGCCGTCTATG-3') and reverse (5'-TGACCTCCAGCAATGACC ATGCG-3') primers and the 5'-ATCGTCATCGGTGTCTTGCTACTCACG G-3' probe. FcRn expression was normalized for the expression of the housekeeping gene *RPL27* (32).

ELISA. (i) RSV-specific IgG

RSV-specific IgG was determined in serum samples obtained from C57BL/6WTand $FcRn^{-/-}$ mice 28 days after i.n. inoculation with IC-RSV, UV-RSV, or control mice and from all groups at 6 days after i.n. challenge with live RSV, using an RSV enzyme linked immunosorbent assay

(ELISA) as described before (14). Denatured RSV lysate from RSV-infected HEp-2 cells in PBS was used as a coating. RSV-specific IgG was detected with secondary horseradish peroxidase (HRP)-labeled antibodies, anti-IgG1 (Invitrogen) or anti-IgG2c (Immunology Consultant Laboratory), and developed with the substrate 3,3',5,5'-tetramethylbenzidine in NaAc (pH 5.5) and H_2O_2. The enzymatic activity was stopped by adding 9.8% H_2SO_4 and measured at OD_{450} (14). C57BL/6 mice express the Igh1-b allele encoding antibodies of the IgG2c isotype and lack the allele of the IgG2a isotype (33). Therefore, we used an IgG2c-specific secondary antibody.

(ii) IL-6 ELISA

In the supernatant of A549 cells incubated for 24 h with RSV or UV-RSV (MOI of 2) in the presence or absence of palivizumab, IL-6 production was measured by ELISA (M9316; Sanquin Pelipair).

Statistical analysis

Data were analyzed for statistical significance using a one-way or two-way analysis of variance (ANOVA), as indicated in the figure legends. Data are expressed as means ± standard errors of the means (SEM). A *P* value of 0.05 was taken as the level of significance.

ACKNOWLEDGEMENTS

D. Kruijsen was financially supported by grant WK016 from the Wilhelmina Research Fund, M. Schijf by the Dutch Top Institute Pharma Project (D1-101-0) and H. Einarsdottir by grant 0721 from the Landsteiner Foundation for Blood Transfusion Research. We thank Derry Roopenian for the kind contribution of the $FcRn^{-/-}$ mice. The authors of this article declare they have no financial or commercial conflicts of interest.

REFERENCES

1. Hamano Y, Arase H, Saisho H, Saito T. 2000. Immune complex and Fc receptor-mediated augmentation of antigen presentation for in vivo Th cell responses. J. Immunol. 164: 6113– 6119.
2. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Thery C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S. 1999. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J. Exp. Med. 189:371– 380.
3. Schuurhuis DH, Ioan-Facsinay A, Nagelkerken B, van Schip JJ, Sedlik C, Melief CJ, Verbeek JS, Ossendorp F. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses in vivo. J. Immunol. 168: 2240 –2246.
4. Ponnuraj EM, Springer J, Hayward AR, Wilson H, Simoes EA. 2003. Antibody-dependent enhancement, a possible mechanism in augmented pulmonary disease of respiratory syncytial virus in the Bonnet monkey model. J. Infect. Dis. 187:1257–1263.
5. Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, Lirman DD, Rabold R, Hoffman SJ, Karp CL, Kleeberger SR, Wills-Karp M, Karron RA. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. J. Exp. Med. 196:859–865.
6. Gimenez HB, Chisholm S, Dornan J, Cash P. 1996. Neutralizing and enhancing activities of human respiratory syncytial virus-specific antibodies. Clin. Diagn. Lab. Immunol. 3:280 –286.
7. Ubol S, Halstead SB. 2010. How innate immune mechanisms contribute to antibody-enhanced viral infections. Clin. Vaccine Immunol. 17:1829–1835.
8. Brandenburg AH, Groen J, van Steensel-Moll HA, Claas EC, Rothbarth PH, Neijens HJ, Osterhaus AD. 1997. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. J. Med. Virol. 52: 97–104.
9. Hall CB, Walsh EE, Long CE, Schnabel KC. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163:693– 698.
10. Glezen WP, Taber LH, Frank AL, Kasel JA. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child. 140:543–546.
11. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. 2003. Correlates of immunity to respiratory syncytial virus (RSV) associated hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. Vaccine 21:3479 –3482.
12. IMpact Study Group-RSV. 1998. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 102: 531– 537.
13. Carbonell-Estrany X, Simões EA, Dagan R, Hall CB, Harris B, Hultquist M, Connor EM, Losonsky GA, Motavizumab Study Group, 2010. Motavizumab for prophylaxis of respiratory syncytial virus in highrisk children: a non-inferiority trial. Pediatrics 125:e35– e51.
14. Kruijsen D, Bakkers MJ, van Uden NO, Viveen MC, van der Sluis TC, Kimpen JL, Leusen JH, Coenjaerts FE, van Bleek GM. 2010. Serum antibodies critically affect virus-specific $CD4^+/CD8^+$ T cell balance during respiratory syncytial virus infections. J. Immunol. 185: 6489–6498.
15. Qiao SW, Kobayashi K, Johansen FE, Sollid LM, Andersen JT, Milford E, Roopenian DC, Lencer WI, Blumberg RS. 2008. Dependence of antibody-mediated presentation of antigen on FcRn. Proc. Natl. Acad. Sci. U. S. A. 105:9337–9342.

16. Vidarsson G, Stemerding AM, Stapleton NM, Spliethoff SE, Janssen H, Rebers FE, de Haas M, van de Winkel JG. 2006. FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis. Blood 108:3573–3579.
17. Stapleton NM, Andersen JT, Stemerding AM, Bjarnarson SP, Verheul RC, Gerritsen J, Zhao Y, Kleijer M, Sandlie I, de Haas M, Jonsdottir I, van der Schoot E, Vidarsson G. 2011. Competition for FcRn-mediated transport gives rise to short half-life of human IgG3 and offers therapeutic potential. Nat. Commun. 2:599. doi:10.1038/ncomms1608.
18. Chaudhury C, Mehnaz S, Robinson JM, Hayton WL, Pearl DK, Roopenian DC, Anderson CL. 2003. The major histocompatibility complex related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J. Exp. Med. 197:315–322.
19. Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A Roopenian DC, Lencer WI, Blumberg RS. 2004. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 20:769 –783.
20. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. 1994. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 76: 519 –529.
21. Foti M, Granucci F, Aggujaro D, Liboi E, Luini W, Minardi S, Mantovani A, Sozzani S, Ricciardi-Castagnoli P. 1999. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. Int. Immunol. 11:979 –986.
22. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176:1693–1702.
23. Hale AH. 1980. Molecular requirements for trinitrophenyl recognition by antihapten cytotoxic T lymphocytes. Cell. Immunol. 49: 408–414.
24. Ioan-Facsinay A, de Kimpe SJ, Hellwig SM, van Lent PL, Hofhuis FM, van Ojik HH, Sedlik C, da Silveira SA, Gerber J, de Jong YF, Roozendaal R, Aarden LA, van den Berg WB, Saito T, Mosser D, Amigorena S, Izui S, van Ommen GJ, van Vugt M, van de Winkel JG, Verbeek SJ. 2002. FcgammaRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. Immunity 16:391–402.
25. Vidarsson G, van de Winkel JG, van Dijk MA. 2001. Multiplex screening for functionally rearranged immunoglobulin variable regions reveals expression of hybridoma-specific aberrant V-genes. J. Immunol. Methods 249:245–252.
26. Kimura N, Toyonaga B, Yoshikai Y, Du RP, Mak TW. 1987. Sequences and repertoire of the human T cell receptor alpha and beta chain variable region genes in thymocytes. Eur. J. Immunol. 17:375–383.
27. Bitonti AJ, Dumont JA, Low SC, Peters RT, Kropp KE, Palombella VJ, Stattel JM, Lu Y, Tan CA, Song JJ, Garcia AM, Simister NE, Spiekermann GM, Lencer WI, Blumberg RS. 2004. Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway. Proc. Natl. Acad. Sci. U. S. A. 101: 9763–9768.
28. Lukens MV, Claassen EA, de Graaff PM, van Dijk ME, Hoogerhout P, Toebes M, Schumacher TN, van der Most RG, Kimpen JL, van Bleek GM. 2006. Characterization of the $CD8^+$ T cell responses directed against respiratory syncytial virus during primary and secondary infection in C57BL/6 mice. Virology 352:157–168.
29. Kim JK, Firan M, Radu CG, Kim CH, Ghetie V, Ward ES. 1999. Mapping the site on human IgG for binding of the MHC class I-related

receptor, FcRn. Eur. J. Immunol. 29:2819 –2825.
30. Medesan C, Matesoi D, Radu C, Ghetie V, Ward ES. 1997. Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1. J. Immunol. 158: 2211–2217.
31. Houben ML, Coenjaerts FE, Rossen JW, Belderbos ME, Hofland RW, Kimpen JL, Bont L. 2010. Disease severity and viral load are correlated in infants with primary respiratory syncytial virus infection in the community. J. Med. Virol. 82:1266 –1271.
32. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS One 2:e898. doi: 10.1371/journal.pone.0000898.
33. Martin RM, Brady JL, Lew AM. 1998. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. J. Immunol. Methods 212:187–192.
34. Boukhvalova MS, Prince GA, Blanco JC. 2007. Respiratory syncytial virus infects and abortively replicates in the lungs in spite of pre-existing immunity. J. Virol. 81: 9443–9450.
35. Pletneva LM, Haller O, Porter DD, Prince GA, Blanco JC. 2008. Induction of type I interferons and interferon-inducible Mx genes during respiratory syncytial virus infection and reinfection in cotton rats. J. Gen. Virol. 89:261–270.
36. Spiekermann GM, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, Lencer WI. 2002. Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. J. Exp. Med. 196:303–310.
37. Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M, O'Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E, Hall WC, Young JF. 1997. Development of a humanized monoclonal antibody (MED-493) with potent in vitro and in vivo activity against respiratory syncytial virus. J. Infect. Dis. 176:1215–1224.
38. Ober RJ, Radu CG, Ghetie V, Ward ES. 2001. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. Int. Immunol. 13: 1551–1559.
39. Dall'Acqua WF, Woods RM, Ward ES, Palaszynski SR, Patel NK, Brewah YA, Wu H, Kiener PA, Langermann S. 2002. Increasing the affinity of a human IgG1 for the neonatal Fc receptor: biological consequences. J. Immunol. 169:5171–5180.
40. Falsey AR, Formica MA, Walsh EE. 2002. Diagnosis of respiratory syncytial virus infection: comparison of reverse transcription-PCR to viral culture and serology in adults with respiratory illness. J. Clin. Microbiol. 40:817– 820.
41. Siber GR, Leombruno D, Leszczynski J, McIver J, Bodkin D, Gonin R, Thompson CM, Walsh EE, Piedra PA, Hemming VG, Prince GA. 1994. Comparison of antibody concentrations and protective activity of respiratory syncytial virus immune globulin and conventional immune globulin. J. Infect. Dis. 169:1368 –1373.
42. Kruijsen D, Schijf MA, Lukens MV, van Uden NO, Kimpen JL, Coenjaerts FE, van Bleek GM. 2011. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. Vaccine 29:2730 –2741.
43. Ye L, Zeng R, Bai Y, Roopenian DC, Zhu X. 2011. Efficient mucosal vaccination mediated by the neonatal Fc receptor. Nat. Biotechnol. 29:158–163.
44. Baker K, Qiao SW, Kuo TT, Aveson VG, Platzer B, Andersen JT, Sandlie I, Chen Z, de Haar C, Lencer WI, Fiebiger E, Blumber RS. 2011. Neonatal Fc receptor for IgG (FcRn) regulates cross-presentation of IgG immune complexes by CD8-CD11b^{+} dendritic cells. Proc. Natl. Acad. Sci. U. S. A. 108:9927–9932.
45. Mi W, Wanjie S, Lo ST, Gan Z, Pickl-Herk B, Ober RJ, Ward ES. 2008. Targeting the

neonatal Fc receptor for antigen delivery using engineered Fc fragments. J. Immunol. 181:7550 –7561.

46. Liu X, Lu L, Yang Z, Palaniyandi S, Zeng R, Gao LY, Mosser DM, Roopenian DC, Zhu X. 2011. The neonatal FcR-mediated presentation of immune-complexed antigen is associated with endosomal and phagosomal pH and antigen stability in macrophages and dendritic cells. J. Immunol. 186:4674–4686.

47. Lukens MV, Kruijsen D, Coenjaerts FE, Kimpen JL, van Bleek GM. 2009. Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung draini

Chapter 5


Prophyla
RSVA
Synagis
Fusion
Peptide
Severe respirator
CD=14

Influencing mucosal homeostasis and immune responsiveness; the impact of nutrition and pharmaceuticals

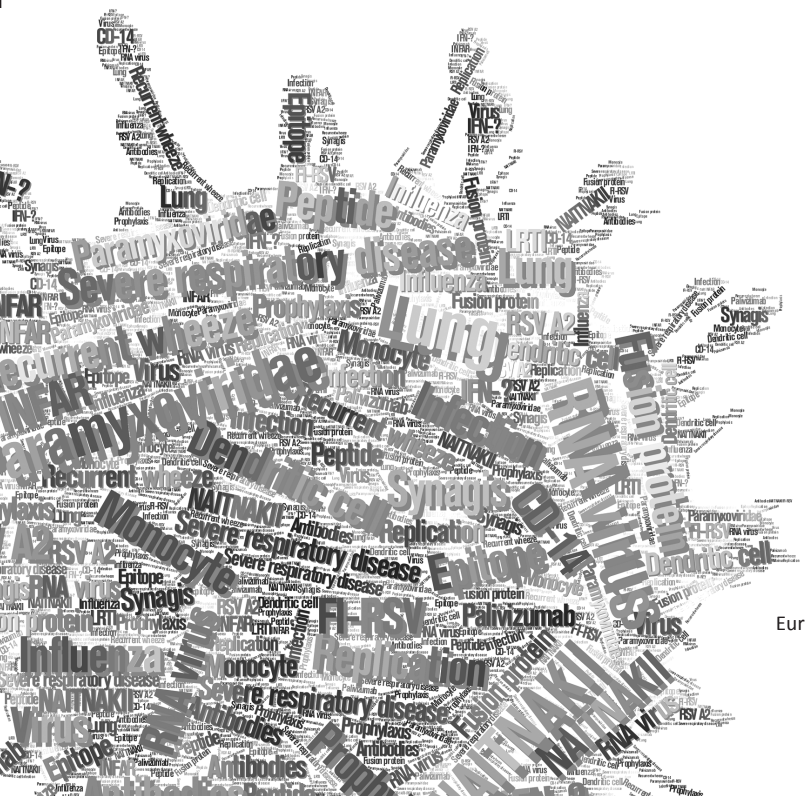
Belinda van't Land[1,2], Marcel A. Schijf[1,2], Rocio Martin[3], Johan Garssen[2,4] & Grada M. van Bleek[1]

[1]Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center, Utrecht, the Netherlands.

[2]Department of Immunology,

[3]Department of Microbiology, Danone Research – Centre for Specialised Nutrition, Wageningen, the Netherlands.

[4]Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, the Netherlands



Eur J Pharmacol (2011):(668):s101-s107

ABSTRACT

Both nutrition and orally ingested drugs pass the gastrointestinal mucosa and may affect the balance between the mucosal immune system and microbial community herein, i.e. affecting composition of the microbial community as well as the status of local immune system that controls microbial composition and maintains mucosal integrity. Numerous ways are known by which the microbial community stimulates mammalian host's immune system and vice versa. The communication between microbiota and immune system is principally mediated by interaction of bacterial components with pattern recognition receptors expressed by intestinal epithelium and various local antigen-presenting cells, resulting in activation or modulation of both innate and adaptive immune responses. Current review describes some of the factors influencing development and maintenance of a proper mucosal / immune balance, with special attention to Toll like receptor signaling and regulatory T cell development. It further describes examples (antibiotic use, HIV and asthma will be discussed) showing that disruption of the balance can be linked to immune function failure. The therapeutic potential of nutritional pharmacology herein is the main focus of discussion.

1. THE MUCOSAL / IMMUNE BALANCE

Interaction between microbial community and human host is known to play a crucial role in the mucosal homeostasis and health status of the host. The mucosa-associated lymphoid system tightly regulates this interaction and, under normal conditions, prevents damaging inflammatory reactions by maintaining a tolerogenic state. A proper mucosal barrier function therefore includes prevention of explicit pathogenic threats, while in mean time there is the need to suppress or moderate strong anti-microbial responses, and excessive inflammation.

1.1 The development of the balance starts at birth

The development of the immune system is enormously challenged to induce "specific" immune responses to dangerous intruders (pathogens) and tolerance to non dangerous substances such as food components and environmental factors. As far as it can be determined, the fetus is mostly sterile before birth. Although there is some evidence that maternal exposure to microbes occurs during pregnancy that may affect fetal immune development. Infections with parasites and viruses during gestation have been shown to affect the infant's immune response (Hughes et al., 1999; Santos et al., 2010). Infants born to mothers living in farm environments with high maternal microbial exposure during gestation appear to be protected from the development of asthma (Douwes et al., 2008; Ege et al., 2006). These infants have increased expression of innate immune system receptors at birth. Besides microbial exposure during gestation the infant is exposed to a highly diverse microbial community at birth and thereafter which requires a tremendous maturation of the immune system during the first months of life, a period when the infants is highly susceptible to infections. During the early period of life the infant is protected to some extend by maternal antibodies acquired via the placenta and breast milk. Different immune modulating substances are acquired via breast milk as well. The mechanisms involved in such protection are far from being elucidated, but the interaction with maternal delivered Toll-like receptor ligands may contribute to immune development (Conrad et al., 2009).

Directly upon delivery the neonate is for the first time exposed to a wide variety of microbes. The mode of delivery shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns (Dominguez-Bello et al., 2011). Although only a small proportion of the bacteria to which the baby is initially exposed colonizes, the delivery mode is to some extent related to disease predisposition, which shows that the earliest exposure has a crucial impact on the immune system of a child. For example: babies delivered by caesarian section are more susceptible to allergies and asthma compared to children who are vaginally delivered (Bager et al., 2008; Negele et al., 2004). To handle this first bacterial challenge without fatal inflammation, the babies are born with an immune system biased to induce immunological tolerance characterized by a preferential induction of Tregs (Mold

et al., 2008). Tregs are particularly abundant and functional at birth and inhibit excessive immune responses whereby ultimately the maintenance of peripheral T-cell tolerance and pathogen clearance are key (MarchantGoldman, 2005; Schonland et al., 2003).

1.2 First intervention establishing a lifelong balance

One important factor modulating both the infant's immune responsiveness and the gastrointestinal microbial community is nutrition, preferably breast milk. The composition of the microbial community can differ between infants that are fed with either breast milk or formula (Klaassens et al., 2009). The contents of breast milk have evolved over millions of years providing nutrition, supporting the development of proper microbiota and immune balance characterized by protection to pathogenic agents and tolerance towards environmental factors. Several components of breast milk seem to enhance mucosal barrier function and shape immune development both directly and indirectly. Breast milk contains several immune modulating components with specific modulating potential, that have a clear role in immune mediated disease resistance later in life (Van't Land et al., 2010a). Moreover, breastfeeding contributes to enrichment of lactic acid-producing bacteria in the baby's intestine (Martin et al., 2007).

Breastfeeding has been associated with protection against allergic disease development, neonatal diarrhea (Ruiz-Palacios et al., 1990), necrotizing colitis (McGuireAnthony, 2003), development of obesity (as reviewed in (Owen et al., 2005) and Type II diabetes later in life (Owen et al., 2006). However after weaning there is a level of microbial community maturation which seems to decrease the gross differences between the two feeding regimens in terms of fecal microbiota composition (Magne et al., 2005). The fact that early nutritional status (impacting the microbial community development) is associated with the subsequent development of disorders later in life, suggests an important role for the microbial community in the development and modulation of chronic diseases. This modulation might involve different processes. The composition of the intestinal microbial community influences energy balance (Backhed, 2009; Nicholson et al., 2005), pathogen colonization resistance (Boullier et al., 2003), and the maturation of the intestine (Are et al., 2008) and mucosal immune system (Mazmanian et al., 2005). These important features of modulation by the gut microbial community are likely similar to those of the non-gut body habitats although much less is known of those interactions. In the last few years we see increasing progress of understanding in the field of microbial interactions with the mucosal and systemic immune system.

2. CROSSTALK BETWEEN MICROBIOTA AND IMMUNE SYSTEM

2.1 Role of microbial recognition and response from the host

Pathogenic microbial eradication is characterized by an immediate recognition followed by a strong innate / inflammatory and adaptive immune response. Although effective in pathogen control the immune response often induces significant tissue damage. The mucosa is therefore permanently challenged to discriminate between commensal bacteria and potential pathogens and responds accordingly. Recently exhaustive metagenomic analysis displayed the enormous genomic diversity within the human gut microbial community (Qin et al., 2010), exposing the complexity of the interaction the immune system is challenged with. In addition to the pattern recognition receptor induced signaling by pathogens, they induce a particular level of stress which functions as additional danger signal, driving the immune response towards inflammation and ultimately eradication of the pathogen. These receptors can be found intracellular as well as on the cell surface and consist of different families like the Toll like receptors (TLR), C-type lectin receptors (including dectin-1 and DC-SIGN) and nucleotide-binding oligomerization domain (NOD) like receptors (NLRs, such as NOD2 the peptidoglycan receptor). Upon triggering a downstream intracellular signaling results ultimately in the induction of for instance cytokine responses. Pathogenic bacteria as well as commensals are both recognized by TLRs as they detect various conserved microbial structures including lipoteichoic acid (TLR-2), lipopolysaccharide (TLR-4), flagellin (TLR-5) and CpG DNA (TLR-9) recently reviewed in (Abreu, 2010). This interaction of the microbial community with TLRs is essential in maintenance of the intestinal integrity and immune homeostasis (Rakoff-Nahoum et al., 2004).

The microbial community induces immune modulating effects in different ways. For instance. the polysaccharide-A of *B. fragilis* was found to induce systemic Th1 responses, thereby reversing the Th1/ Th2 balance (RoundMazmanian, 2010) and, Peptidoglycan differences on commensal bacteria may affect neutrophil function through Nod1 (Clarke et al., 2010). Secretion of metabolites like short chain fatty acids can elicit responses, as is the ATP produced by some commensals which may enhance Th17 cell differentiation by the activation of Lamina propria located DCs (Atarashi et al., 2010b). These examples illustrate that we are only beginning to understand the pharmacological potential of microbial community modulation.

2.2 The role of microbiota in mucosal Treg development

The mucosa represents a unique environment that requires multiple levels of control. Intestinal contents are sampled by M-cells in the intestinal epithelial lining, after which the underlying dendritic cells situated mostly in the Peyer's patch present these antigens to

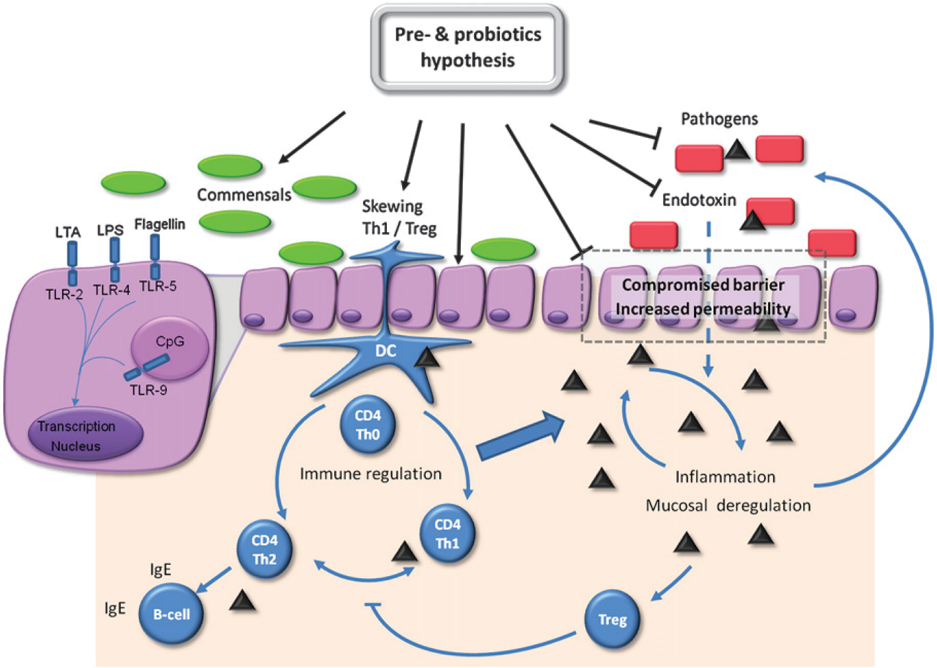


FIGURE 1. The microbial community in contact with the mucosal surface induces a state of non-responsiveness/tolerance through interaction with the mucosal immune system. There is cell specific limited recognition of bacterial products like lipopolysaccharide (LPS), lipoteichoic acid (LTA) and DNA fragments (CpG). Upon signaling through Toll like receptors (TLR) and NOD-like receptors the intestinal defense systems are activated. In addition, Dendritic cells (DCs) are activated and upon secretion of cytokines like IL-10 and TGF-β subsequent induction of the regulatory T-cell (Treg) tolerance induction is initiated. Mucosal barrier injury induced by external factors like infection, chemotherapy or other stressors disrupts homeostasis, which may create an autocrine loop inducing inflammation. More specifically, microbiota composition imbalance has been associated with an imbalance between populations of inflammation-mediating T-helper cells (Th1, Th2 and Th17) and anti-inflammatory Treg cells. Prolonged overproduction of Th1- and Th17-associated cytokines has been linked with inflammatory bowel diseases, while a Th2 skew is linked with allergic disorders, asthma, and ulcerative colitis. Such chronic inflammatory responses set up a 'vicious circle', disrupting the gastrointestinal microbiota. Therefore, an increased understanding of the complex microbial communities and subsequent cross-talk with the immune system will advance the development of efficacious prevention and treatment protocols of a diversity of chronic diseases.

naïve T cells. Upon antigen presentation, different cell lineages like Th1, Th2, Th17 cells or Tregs, are generated and enter the blood stream or home to the intestinal mucosa. Mucosal DCs specifically the $CD103^+$ DCs have a unique ability to generate specific Tregs (Sun et al., 2007). LPS in the diet for instance, provides sufficient TLR2 stimulation to expand Tregs in germ-free mice (Hrncir et al., 2008; Sutmuller et al., 2006). Moreover, TLR expression profiling studies revealed that multiple TLRs are expressed on $CD4^+$ T-cells as well as Tregs. Tregs express even higher levels of TLR4, TLR5, TLR7 and TLR8 in comparison to regular T-cells (LiuZhao, 2007). In addition, TLR2 seems to be important in regulating murine

Treg-mediated suppression as well as expansion (Sutmuller et al., 2006). In addition, Tregs are important for the induction of mucosal tolerance.

Several subsets of inducible Tregs have been defined, including Foxp3$^+$ Tregs, IL-10 producing type 1 regulatory T-cells (Tr1), cells that mediate suppression through IL-10 secretion (Roncarolo et al., 2006) and TGF-beta producing Tregs (Th3) that mediate regulation through TGF-beta production. These subsets however may not be mutually exclusive and act in synergy to achieve suppression. For instance orally induced Foxp3$^+$ Tregs are essential in the suppression of IL-4 production inducing tolerance in allergic airway inflammation. However, IL-5 production and eosinophilia seem to be controlled by an IFN-γ dependent, Foxp3-independent mechanism (Curotto de Lafaille et al., 2008). This may suggest that Foxp3-independent regulation may contribute to the conversion of Foxp3$^+$ Tregs in order to establish a tolerogenic microenvironment. This is only one detailed example of how eventually the development of Tregs can be influenced by microbial community present in the gut.

Inducible Tregs are generated from un-polarized CD4$^+$ T-cells in the intestine after contact with the lamina propria resident CD103$^+$ DC's. The CD103$^+$ DCs seem to be the major DC subset involved in the antigen trafficking from intestine to the lymph nodes and are potent activators of T cells. These DCs can convert vitamin A to retinoic acid, which triggers the expression of gut homing receptors during T cell priming and thereby convey signals from the microbial community to direct T cell and Treg differentiation (Coombes et al., 2007). Although, in the absence of microbiota there are still Tregs present, the microbial community is clearly capable of modulating the Treg phenotype. For example, the specific bacterial derived DNA suppresses Treg differentiation in a TLR-9 dependent way (Hall et al., 2008). The DNA was found to promote Th17 and Th1 cell responses by the restriction of Treg differentiation. However, commensal bacteria (specifically *B. fragilis*) have been found to increase the number of Tregs in the colon. More specifically, the spore forming *Clostridium* species belonging to clusters IV and XIVa induce Foxp3$^+$ and IL-10$^+$ Treg differentiation in the colonic lamina propria. The Treg differentiation was at least partially mediated by an epithelial derived TGF-beta induction. The increase in numbers of *Clostridium* made neonatal mice more resistant to colitis, and seemed to suppress systemic IgE responses (Atarashi et al., 2010a). So over time, the gut microbiota, oral pathogens, nutrition, but also orally taken pharmacological drugs may have an important role in shaping the repertoire of Foxp3$^+$ regulatory T cells. The relative contribution of these regulatory T cells to peripheral tolerance and the outcome of infections let alone the development of the immune system as a whole remains to be addressed with care. Immune modulating commensal species control to some extent the immune balance, in which species such as the Segmented Filamentous Bacteria favor the balance to Th17 whereas species belonging to *Clostridium* clusters IV and XIVa can be characterized as Treg inducing species. This may in part be linked to the intestinal localization of the different species, and their interaction to the mucosa, which is nicely reviewed recently. (IvanovLittman, 2011).

2.3 Gastrointestinal modulation and the respiratory immune response

A clear example of the influence of microbial community on the failure to develop a proper immune response comes from the associations found in the allergy / asthma field. The development of an adult–like microbiota composition occurs during the first years of life. Environmental exposure plays an important role in microbiota development (Palmer et al., 2007). The "Hygiene hypothesis" states that the encounter of infections in early life prevents later onset of allergic diseases, which is a well established working hypothesis in relation to the development of allergy (Yazdanbakhsh et al., 2002) and supported by several studies. For instance it has been shown that in a population with higher bacterial exposure early in life, the prevalence of atopy and asthma is lower (Vartiainen et al., 2002). Furthermore, a lower prevalence of asthma and atopy is found among children raised on a farm, which also correlates with the exposure to an increased bacterial diversity (Von MutiusRadon, 2008). Moreover, many studies using microbial products, like endotoxin and muramic acid as simple markers of microbial exposure have corroborated these observations (Sordillo et al., 2010). This illustrates the importance of a well balanced interaction between the mucosal immune system and the external microbial community.

The microbiota composition at 1 month of age is associated with susceptibility to develop atopic diseases in childhood (Penders et al., 2007b). In addition, the existence of an altered microbiota composition in children developing allergy up to 5 years of age suggested an early imprinting of a long lasting cross-talk (Sjogren et al., 2009). The importance of the development of a stable microbial community for the protection against the development of allergic disorders like asthma is further illustrated by studies showing detrimental effects of the use of antibiotics. Antibiotic use in early life, besides disrupting the gut microbiota composition, it appears to contribute to increased risk for atopy in certain subgroups of children. Moreover, neonatal antibiotic treatment is suggested to be a risk factor for early wheezing (Alm et al., 2008). It is clearly established that the microbial community has a role in the prevalence of allergic diseases. However, intestinal microbial composition is just one of the contributing factors in this process. It is very well known that there are also other factors playing an important role such as genetic background, early (non-intestinal) pathogenic exposures, environmental factors, etc. It is known that a disturbed microbial immunity might have detrimental effect on the development of the immune system. However, it is not known whether this effect is also vice versa, i.e. that immune disruption can affect the composition of the microbial community.

In diseases like Obesity, inflammatory bowel diseases and Crohn's disease more and more correlations are found between the microbial community composition and the disease, although it is difficult to determine if a certain microbial composition reflects a cause or an effect of the disease. It could be speculated that it is the microbial community which induces intestinal inflammation and thereby plays a role in disease etiology, or vice versa, the onset

of a disease is due to a genetic or immune based failure which is at the same time responsible for the disturbed microbial community, with neither of both hypothesis being completely wrong. There are however examples where an immunologic failure is clearly responsible for the onset of a chronic disease, like for HIV, where HIV patients develop a disturbed gut microbiota composition(Gori et al., 2008).

3. BALANCE DISTURBANCES

3.1 Immune disruption and the negative effect on microbial composition and disease progression (HIV)

HIV infection disrupts intestinal homeostasis as it directly affects the host immune system by killing $CD4^+$ T cells. Decreased $CD4^+$ T cell numbers indirectly affect the intestinal microbiota composition (Brenchley et al., 2006; Gori et al., 2008). The loss of intestinal $CD4^+$ T-cells occurs early in disease. Epithelial function and immune regulation, in combination with an increased pathogen-enriched microbiota composition leads to an increase in intestinal permeability, bacterial translocation and an increased inflammatory state of the mucosal immune system (Hummelen et al., 2010). The increased activation of $CD8^+$ as well as $CD4^+$ T-cells occurs systemically, but is most likely also present within the mucosa. Increased T cell activation is highly associated with HIV disease progression. In addition, the success of $CD4^+$ T-cell repopulation during anti-retroviral therapy depends on the level of $CD4^+$ T-cell activation and death prior to the start of therapy. Therefore, a strategy aimed to reduce immune activation (by for instance improvement of intestinal barrier integrity / immune regulation support) was envisaged to influence HIV disease progression as a whole (Gori et al., 2011). In a double-blind placebo controlled clinical trial, worldwide 340 HIV-1 adults pre-treatment received a nutritional formula containing specific prebiotic oligosaccharides or an isocaloric/isonitrogenous control for 1 year. This study showed a positive effect diminishing the $CD4^+$ T-cell decline in HIV-1 infection due to the specific nutritional intervention (Lange et al., 2009). Thus modulation on the mucosal side may support the immune system positively. In addition this is a clear example in which it is shown that cross-talk is effective in both directions, as HIV is known to deregulate the immune system directly, with microbial community changes as a consequence. Moreover, the nutritional intervention at the mucosal site shows clear microbial composition changes that impact on disease progression, as measured by the change in $CD4^+$ T cell decline.

TABLE 1: The intestinal microbiota and epithelial barrier interruptions are major factors in disease progression

Disease	*Evidence*	*Reference*
HIV	Aberrant intestinal microbiota composition in HIV patients	(Gori et al., 2008; McKenna et al., 2008)
	Increased bacterial translocation among HIV progressors and those non responding to anti-retroviral therapy	(Brenchley et al., 2006; Marchetti et al., 2008)
	Enhanced epithelial inflammation and scarring of gastrointestinal lymphoid system among HIV progressors	(Brenchley et al., 2006; Marchetti et al., 2008; Sankaran et al., 2005)
Asthma/Atopy	The microbiota composition at 1 month of age is associated with subsequent development of atopic diseases in childhood	(Penders et al., 2007a)
	Antibiotic use disturbing the microbiota of the gastrointestinal tract in early life appears to contribute to increased risk for atopy in certain subgroups of children.	(Johnson et al., 2005)
	Neonatal antibiotic treatment is a risk factor for early wheezing	(Alm et al., 2008)
IBD/Crohn	Microbial imbalances in inflammatory bowel diseases and Crohn's	(Frank et al., 2007; Kassinen et al., 2007)
	Host –mediated inflammation disrupts the intestinal microbial homeostasis and promotes the overgrowth of specific bacteria.	(Lupp et al., 2007; Willing et al., 2009)
Obesity/Diabetes	Altered microbiota composition in cesarean section delivered infants might be linked to the development of diabetes later in life	(Cardwell et al., 2008)
	The microbiota may affect lipid metabolism and function as an environmental factor that influences the development of obesity and related diseases by activating the innate immune system	(Caesar et al., 2010)

Disease progression / severity is the major focus of this table and only some examples of many more to be found in literature are displayed within this table.

3.2 Sensitive measurement of mucosal barrier disruption is lacking which complicates detailed investigation of the working mechanism of immune modulation

Knowing that there is a relation between immune dysfunction and intestinal barrier function, it raises the need for a sensitive method to analyze intestinal permeability in order to be able to investigate the effect of interventions closely. There are some relative non-invasive methods like the Lactulose / Rhamnose ratio measurements in urine to measure intestinal barrier function (Blijlevens et al., 2004). However, due to high variation, and difficulty to obtain standardized samples, detailed knowledge of intestinal barrier function in different settings is lacking. In addition, the possible role of intestinal disruption is not recognized as underlying immune deficiency cause. Even in therapies known to induce mucositis there is not a clear biomarker available to monitor intestinal disruption or barrier failure other than the culture of translocating bacteria, which only gives an indication, but not a detailed analysis of the intestinal barrier function. Moreover, the information necessary to correlate faltering intestinal barrier integrity in relation to immune responsiveness is still limited. Epithelial NF-κB activation plays an important role in the preservation of the integrity of

the mucosal epithelial barrier. In addition, it coordinates the antimicrobial actions of both innate as well as adaptive immune systems (Ben-NeriahSchmidt-Supprian, 2007). Therefore, a hyper-activation or deficiency in NF-κB regulated gene transcription results in chronic inflammation and disruption of homeostasis. This implicates that NF-κB can play a central role in immune functioning and mucosal barrier homeostasis and might be an interesting target for modulation (Van't Land et al., 2004). Pharmaceutical as well as nutritionally driven inhibition can be beneficial in the reduction of over-active immune responsiveness. However, as it affects intestinal barrier function, inhibition of NF-κB may reduce barrier integrity. This causes increased bacterial translocation, indirectly inducing inflammation which on itself may counteract the intended immune suppression. Therefore, there is a need for a sensitive method to analyze intestinal permeability in order to be able to investigate the effect of an intervention more closely.

3.3 Pharmaceutical interventions may unintentionally affect immune responsiveness

The main port of entry for respiratory and enteric viral pathogens is the mucosal epithelial surface, which is also involved in the modulation of the immune response at the innate/adaptive interface under the influence of the entire microbial community. Although respiratory and enteric viral infections cause significant morbidity and mortality world-wide, the majority of primary and secondary viral exposures induces only minor symptoms or occurs completely asymptomatic. It has recently been shown that the administration of some but not all antibiotics affects the capacity to respond to a respiratory pathogen (Ichinohe et al., 2011). Antibiotics are a frequently used and very effective pharmacological intervention during bacterial disease. However, although designed to counteract specific pathogens, antibiotic treatments might also disturb the microbial community in the human gut and therewith its homeostasis with the host. In a recent murine study it was shown that that the disturbance of commensal microbiota composition by antibiotic treatment critically influenced the generation of virus-specific immune responses following respiratory influenza virus infection (Ichinohe et al., 2011). Moreover, the administration of some TLR ligands could rescue the immune impairment in antibiotic-treated mice. Following intranasal influenza virus infection, the activation of the inflammasome leads to DC migration from the lung to the draining lymph node where antigen specific T-cell priming occurs and the antiviral immune response is elucidated. The antibiotic induced microbial disturbance and subsequent reduced response towards respiratory pathogens, subscribes the important role of the microbial community in the regulation of respiratory mucosal immunity, which leaves the question to be answered if this can be applied to all respiratory pathogens. In addition to the general use of antibiotics, it is suggested that also the use of pain killers like paracetamol and aspirin, regarded as safe and therefore regularly used even in early life may also influence the immune system development. The use of paracetamol has recently been associated with the development

of asthma later in life (Bakkeheim et al., 2010). This finding is in line with earlier reports stating that childhood asthma and recurrent wheezing and eczema are linked to the intake of paracetamol in the first year of life (Beasley et al., 2008). Paracetamol does not inhibit COX-2 in contrast to aspirin (acetylsicylic acid). COX-2 is involved in the PGE_2 production during the resolution of common respiratory infections. Recently it was shown that PGE_2 directly regulates the expression of retinal dehydrogenase enzymes in human DCs, known to be involved in the induction of gut homing receptors of primed T cells (Stock et al., 2011). PGE_2 is thereby linked to the Th1 / Th2 balance as it promotes Th2 and inhibits Th1 type of cytokine production. It might be that the use of paracetamol facilitates the relative Th1/Th2 imbalance in genetically predisposed children (Varner et al., 1998).

4. IMMUNE INTERVENTION

4.1 The possibilities through nutrition

As clearly illustrated by now, the commensal microbial community is essential in shaping the immune response in health and disease. Therefore, interventions affecting microbial composition gain increasing interest. This can be accomplished by oral administration of bacterial strains or prebiotics, i.e. special oligosaccharides that promote the growth of particular bacterial strains in the gut. It is however of key importance to unravel the mechanism of recognition and modulation induced by the different commensal bacterial species (Plantinga et al., 2011). The use of prebiotics is based on the observation that oligosaccharides in human breast milk appeared to influence the microbiota composition in the gut which is very different in infants receiving breast milk versus formula feeding. A clear bifidogenic effect on the gut microbiota has been ascribed to the non-digestible oligosaccharides present in human breast milk, and a positive effect on the incidence of infections and allergy related symptoms at short term and possibly also at long term was associated with the altered bacterial composition (Arslanoglu et al., 2008; Gibson et al., 1995). It has been suggested that the prebiotic oligosaccharides might act indirectly through microbiota-dependent mechanisms (i.e. rebalancing microbiota composition in the gut) and/or have a direct effect via activation or inhibition of cellular receptors on immune competent cells (Vos et al., 2007). More specifically, it was found recently that Tregs play a fundamental role in the immune modulation induced by the supplementation of these specific oligosaccharides (Schouten et al., 2010; Van't Land et al., 2010b).

4.2 In summary

Nutritional components, commensal bacteria and immunological processes are linked and play a role in diseases of different etiology. The relationship among these determinants is by

far not fully understood. Microbial interventions can be induced by addition of probiotics, prebiotics, or other nutrients, and by the administration of antibiotic therapies. All these interventions affect different bacterial species. It is of key importance to unravel the mechanism of recognition and modulation induced by the different commensal bacterial species. As the understanding of the cross talk between the mucosal immune system and the microbial community is developing, it is likely that interventions will become more and more specific, and we foresee a personalized approach in the future. Which implicates that microbiome adjustment to the core level with the gaps or excesses to be filled in or depleted respectively might affect disease status.

ACKNOWLEDGEMENTS

This review was initiated based on results obtained from TI pharma projects D1-101-0 and T1-214-1.

REFERENCES

1. Abreu, M.T., 2010, Toll-like receptor signaling in the intestinal epithelium: How bacterial recognition shapes intestinal function, Nat Rev Immunol 10, 131-144.
2. Alm, B., Erdes, L., Mollborg, P., Pettersson, R., Norvenius, S.G., Aberg, N.Wennergren, G., 2008, Neonatal antibiotic treatment is a risk factor for early wheezing, Pediatrics 121, 697-702.
3. Are, A., Aronsson, L., Wang, S., Greicius, G., Lee, Y.K., Gustafsson, J.A., Pettersson, S.Arulampalam, V., 2008, Enterococcus faecalis from newborn babies regulate endogenous ppargamma activity and il-10 levels in colonic epithelial cells, Proc Natl Acad Sci U S A 105, 1943-1948.
4. Arslanoglu, S., Moro, G.E., Schmitt, J., Tandoi, L., Rizzardi, S.Boehm, G., 2008, Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life, J Nutr 138, 1091-1095.
5. Atarashi, K., Tanoue, T.Honda, K., 2010a, Induction of lamina propria th17 cells by intestinal commensal bacteria, Vaccine 28, 8036-8038.
6. Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, Ii, Umesaki, Y., Itoh, K.Honda, K., 2010b, Induction of colonic regulatory t cells by indigenous clostridium species, Science 331, 337-341.
7. Backhed, F., 2009, Changes in intestinal microflora in obesity: Cause or consequence?, J Pediatr Gastroenterol Nutr 48 Suppl 2, S56-57.
8. Bager, P., Wohlfahrt, J.Westergaard, T., 2008, Caesarean delivery and risk of atopy and allergic disease: Meta-analyses, Clin Exp Allergy 38, 634-642.
9. Bakkeheim, E., Mowinckel, P., Carlsen, K.H., Haland, G.Lodrup Carlsen, K.C., 2010, Paracetamol in early infancy; the risk of childhood allergy and asthma, Acta Paediatr.
10. Beasley, R., Clayton, T., Crane, J., Von Mutius, E., Lai, C.K., Montefort, S.Stewart, A., 2008, Association between paracetamol use in infancy and childhood, and risk of asthma, rhinoconjunctivitis, and eczema in children aged 6-7 years: Analysis from phase three of the isaac programme, Lancet 372, 1039-1048.
11. Ben-Neriah, Y.Schmidt-Supprian, M., 2007, Epithelial nf-kappab maintains host gut microflora homeostasis, Nat Immunol 8, 479-481.
12. Blijlevens, N.M., Van't Land, B., Donnelly, J.P., M'rabet, L.De Pauw, B.E., 2004, Measuring mucosal damage induced by cytotoxic therapy, Support Care Cancer 12, 227-233.
13. Boullier, S., Nougayrede, J.P., Marches, O., Tasca, C., Boury, M., Oswald, E., De Rycke, J.Milon, A., 2003, Genetically engineered enteropathogenic escherichia coli strain elicits a specific immune response and protects against a virulent challenge, Microbes Infect 5, 857-867.
14. Brenchley, J.M., Price, D.A., Schacker, T.W., Asher, T.E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O., Altmann, D., Blazar, B.R., Rodriguez, B., Teixeira-Johnson, L.,
15. Landay, A., Martin, J.N., Hecht, F.M., Picker, L.J., Lederman, M.M., Deeks, S.G.Douek, D.C., 2006, Microbial translocation is a cause of systemic immune activation in chronic hiv infection, Nat Med 12, 1365-1371.
16. Caesar, R., Fak, F.Backhed, F., 2010, Effects of gut microbiota on obesity and atherosclerosis via modulation of inflammation and lipid metabolism, J Intern Med 268, 320-328.
17. Cardwell, C.R., Stene, L.C., Joner, G., Cinek, O., Svensson, J., Goldacre, M.J., Parslow, R.C., Pozzilli, P., Brigis, G., Stoyanov, D., Urbonaite, B., Sipetic, S., Schober, E., Ionescu-Tirgoviste, C., Devoti, G., De Beaufort, C.E., Buschard,

K.Patterson, C.C., 2008, Caesarean section is associated with an increased risk of childhood-onset type 1 diabetes mellitus: A meta-analysis of observational studies, Diabetologia 51, 726-735.
18. Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y.Weiser, J.N., 2010, Recognition of peptidoglycan from the microbiota by nod1 enhances systemic innate immunity, Nat Med 16, 228-231.
19. Conrad, M.L., Ferstl, R., Teich, R., Brand, S., Blumer, N., Yildirim, A.O., Patrascan, C.C., Hanuszkiewicz, A., Akira, S., Wagner, H., Holst, O., Von Mutius, E., Pfefferle, P.I., Kirschning, C.J., Garn, H.Renz, H., 2009, Maternal tlr signaling is required for prenatal asthma protection by the nonpathogenic microbe acinetobacter lwoffii f78, J Exp Med 206, 2869-2877.
20. Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y.Powrie, F., 2007, A functionally specialized population of mucosal cd103+ dcs induces foxp3+ regulatory t cells via a tgf-beta and retinoic acid-dependent mechanism, J Exp Med 204, 1757-1764.
21. Curotto De Lafaille, M.A., Kutchukhidze, N., Shen, S., Ding, Y., Yee, H.Lafaille, J.J., 2008, Adaptive foxp3+ regulatory t cell-dependent and -independent control of allergic inflammation, Immunity 29, 114-126.
22. Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N.Knight, R., 2011, Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns, Proc Natl Acad Sci U S A 107, 11971-11975.
23. Frank, D.N., St Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N.Pace, N.R., 2007, Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases, Proc Natl Acad Sci U S A 104, 13780-13785.
24. Gibson, G.R., Beatty, E.R., Wang, X.Cummings, J.H., 1995, Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin, Gastroenterology 108, 975-982.
25. Gori, A., Rizzardini, G., Van't Land, B., Ben Amor, K., Van Schaik, J., Torti, C., Quirino, T., Tincati, C., Bandera, A., Knol, J., Benhassan-Chahour, K., Trabattoni, D., Bray, D., Vriesema, A., Welling, G., Garssen, J.Clerici, M., 2011, Specific prebiotics modulate gut microbiota and immune activation in haart-naive hiv-infected adults: Results of the " copa " pilot randomized trial, Mucosal Immunol in press.
26. Gori, A., Tincati, C., Rizzardini, G., Torti, C., Quirino, T., Haarman, M., Ben Amor, K., Van Schaik, J., Vriesema, A., Knol, J., Marchetti, G., Welling, G.Clerici, M., 2008, Early impairment of gut function and gut flora supporting a role for alteration of gastrointestinal mucosa in human immunodeficiency virus pathogenesis, J Clin Microbiol 46, 757-758.
27. Hall, J.A., Bouladoux, N., Sun, C.M., Wohlfert, E.A., Blank, R.B., Zhu, Q., Grigg, M.E., Berzofsky, J.A.Belkaid, Y., 2008, Commensal DNA limits regulatory t cell conversion and is a natural adjuvant of intestinal immune responses, Immunity 29, 637-649.
28. Hrncir, T., Stepankova, R., Kozakova, H., Hudcovic, T.Tlaskalova-Hogenova, H., 2008, Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory t cells: Studies in germ-free mice, BMC Immunol 9, 65.
29. Hughes, C.H., Jones, R.C., Wright, D.E.Dobbs, F.F., 1999, A retrospective study of the relationship between childhood asthma and respiratory infection during gestation, Clin Exp Allergy 29, 1378-1381.
30. Hummelen, R., Vos, A.P., Van't Land, B., Van Norren, K.Reid, G., 2010, Altered host-microbe interaction in hiv: A target for intervention with pro- and prebiotics, Int Rev Immunol 29, 485-513.
31. Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S.Iwasaki, A., 2011, Microbiota regulates immune defense

against respiratory tract influenza a virus infection, Proc Natl Acad Sci U S A 108, 5354-5359.

32. Ivanov, IiLittman, D.R., 2011, Modulation of immune homeostasis by commensal bacteria, Curr Opin Microbiol 14, 106-114.
33. Johnson, C.C., Ownby, D.R., Alford, S.H., Havstad, S.L., Williams, L.K., Zoratti, E.M., Peterson, E.L.Joseph, C.L., 2005, Antibiotic exposure in early infancy and risk for childhood atopy, J Allergy Clin Immunol 115, 1218-1224.
34. Kassinen, A., Krogius-Kurikka, L., Makivuokko, H., Rinttila, T., Paulin, L., Corander, J., Malinen, E., Apajalahti, J.Palva, A., 2007, The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects, Gastroenterology 133, 24-33.
35. Klaassens, E.S., Boesten, R.J., Haarman, M., Knol, J., Schuren, F.H., Vaughan, E.E.De Vos, W.M., 2009, Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants, Appl Environ Microbiol 75, 2668-2676.
36. Lange, J., Gazzard, B., Diaz, R., Gori, A., Van't Land, B., Raijmakers, J., Vriesema, A., Kotler, D., Clerici, M., Garssen, J., Cahn, P.Group, B.S., 2009, Reduced cd4+ t cell decline and immune activation by nr100157, a specific multi-targeted nutritional intervention, in hiv-1 positive adults not on antiretroviral therapy (bite). ICAAC (Late-Breaker Abstract).
37. Liu, G.Zhao, Y., 2007, Toll-like receptors and immune regulation: Their direct and indirect modulation on regulatory cd4+ cd25+ t cells, Immunology 122, 149-156.
38. Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C.Finlay, B.B., 2007, Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of enterobacteriaceae, Cell Host Microbe 2, 204.
39. Magne, F., Suau, A., Pochart, P.Desjeux, J.F., 2005, Fecal microbial community in preterm infants, J Pediatr Gastroenterol Nutr 41, 386-392.
40. Marchant, A.Goldman, M., 2005, T cell-mediated immune responses in human newborns: Ready to learn?, Clin Exp Immunol 141, 10-18.
41. Marchetti, G., Bellistri, G.M., Borghi, E., Tincati, C., Ferramosca, S., La Francesca, M., Morace, G., Gori, A.Monforte, A.D., 2008, Microbial translocation is associated with sustained failure in cd4+ t-cell reconstitution in hiv-infected patients on long-term highly active antiretroviral therapy, Aids 22, 2035-2038.
42. Martin, R., Heilig, G.H., Zoetendal, E.G., Smidt, H.Rodriguez, J.M., 2007, Diversity of the lactobacillus group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut, J Appl Microbiol 103, 2638-2644.
43. Mazmanian, S.K., Liu, C.H., Tzianabos, A.O.Kasper, D.L., 2005, An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system, Cell 122, 107-118.
44. Mcguire, W.Anthony, M.Y., 2003, Donor human milk versus formula for preventing necrotising enterocolitis in preterm infants: Systematic review, Arch Dis Child Fetal Neonatal Ed 88, F11-14.
45. Mckenna, P., Hoffmann, C., Minkah, N., Aye, P.P., Lackner, A., Liu, Z., Lozupone, C.A., Hamady, M., Knight, R.Bushman, F.D., 2008, The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis, PLoS Pathog 4, e20.
46. Mold, J.E., Michaelsson, J., Burt, T.D., Muench, M.O., Beckerman, K.P., Busch, M.P., Lee, T.H., Nixon, D.F.Mccune, J.M., 2008, Maternal alloantigens promote the development of tolerogenic fetal regulatory t cells in utero, Science 322, 1562-1565.
47. Negele, K., Heinrich, J., Borte, M., Von Berg, A., Schaaf, B., Lehmann, I., Wichmann, H.E.Bolte,

G., 2004, Mode of delivery and development of atopic disease during the first 2 years of life, Pediatr Allergy Immunol 15, 48-54.
48. Nicholson, J.K., Holmes, E.Wilson, I.D., 2005, Gut microorganisms, mammalian metabolism and personalized health care, Nat Rev Microbiol 3, 431-438.
49. Owen, C.G., Martin, R.M., Whincup, P.H., Smith, G.D.Cook, D.G., 2005, Effect of infant feeding on the risk of obesity across the life course: A quantitative review of published evidence, Pediatrics 115, 1367-1377.
50. Owen, C.G., Martin, R.M., Whincup, P.H., Smith, G.D.Cook, D.G., 2006, Does breastfeeding influence risk of type 2 diabetes in later life? A quantitative analysis of published evidence, Am J Clin Nutr 84, 1043-1054.
51. Palmer, C., Bik, E.M., Digiulio, D.B., Relman, D.A.Brown, P.O., 2007, Development of the human infant intestinal microbiota, PLoS Biol 5, e177.
52. Penders, J., Stobberingh, E.E., Van Den Brandt, P.A.Thijs, C., 2007a, The role of the intestinal microbiota in the development of atopic disorders, Allergy 62, 1223-1236.
53. Penders, J., Thijs, C., Van Den Brandt, P.A., Kummeling, I., Snijders, B., Stelma, F., Adams, H., Van Ree, R.Stobberingh, E.E., 2007b, Gut microbiota composition and development of atopic manifestations in infancy: The koala birth cohort study, Gut 56, 661-667.
54. Plantinga, T.S., Van Maren, W.W., Van Bergenhenegouwen, J., Hameetman, M., Nierkens, S., Jacobs, C., De Jong, D.J., Joosten, L.A., Van't Land, B., Garssen, J., Adema, G.J.Netea, M.G., 2011, Differential tlr recognition and induction of cytokine profile by bifidobacterium breve and lactobacillus strains of probiotics, Clin Vaccine Immunol.
55. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Jian, M., Zhou, Y., Li, Y., Zhang, X., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P.Ehrlich, S.D., 2010, A human gut microbial gene catalogue established by metagenomic sequencing, Nature 464, 59-65.
56. Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S.Medzhitov, R., 2004, Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis, Cell 118, 229-241.
57. Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K.Levings, M.K., 2006, Interleukin-10-secreting type 1 regulatory t cells in rodents and humans, Immunol Rev 212, 28-50.
58. Round, J.L.Mazmanian, S.K., 2010, Inducible foxp3+ regulatory t-cell development by a commensal bacterium of the intestinal microbiota, Proc Natl Acad Sci U S A 107, 12204-12209.
59. Ruiz-Palacios, G.M., Calva, J.J., Pickering, L.K., Lopez-Vidal, Y., Volkow, P., Pezzarossi, H.West, M.S., 1990, Protection of breast-fed infants against campylobacter diarrhea by antibodies in human milk, J Pediatr 116, 707-713.
60. Sankaran, S., Guadalupe, M., Reay, E., George, M.D., Flamm, J., Prindiville, T.Dandekar, S., 2005, Gut mucosal t cell responses and gene expression correlate with protection against disease in long-term hiv-1-infected nonprogressors, Proc Natl Acad Sci U S A 102, 9860-9865.
61. Santos, P.A., Sales, I.R., Schirato, G.V., Costa, V.M., Albuquerque, M.C., Souza, V.M.Malagueno, E., 2010, Influence of maternal schistosomiasis on the immunity of adult offspring mice, Parasitol Res 107, 95-102.
62. Schonland, S.O., Zimmer, J.K., Lopez-Benitez, C.M., Widmann, T., Ramin, K.D., Goronzy, J.J.Weyand, C.M., 2003, Homeostatic control of t-cell generation in neonates, Blood 102, 1428-1434.

63. Schouten, B., Van Esch, B.C., Hofman, G.A., Boon, L., Knippels, L.M., Willemsen, L.E.Garssen, J., 2010, Oligosaccharide-induced whey-specific cd25(+) regulatory t-cells are involved in the suppression of cow milk allergy in mice, J Nutr 140, 835-841.
64. Sjogren, Y.M., Jenmalm, M.C., Bottcher, M.F., Bjorksten, B.Sverremark-Ekstrom, E., 2009, Altered early infant gut microbiota in children developing allergy up to 5 years of age, Clin Exp Allergy 39, 518-526.
65. Sordillo, J.E., Hoffman, E.B., Celedon, J.C., Litonjua, A.A., Milton, D.K.Gold, D.R., 2010, Multiple microbial exposures in the home may protect against asthma or allergy in childhood, Clin Exp Allergy 40, 902-910.
66. Stock, A., Booth, S.Cerundolo, V., 2011, Prostaglandin e2 suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans, J Exp Med.
67. Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R.Belkaid, Y., 2007, Small intestine lamina propria dendritic cells promote de novo generation of foxp3 t reg cells via retinoic acid, J Exp Med 204, 1775-1785.
68. Sutmuller, R.P., Den Brok, M.H., Kramer, M., Bennink, E.J., Toonen, L.W., Kullberg, B.J., Joosten, L.A., Akira, S., Netea, M.G.Adema, G.J., 2006, Toll-like receptor 2 controls expansion and function of regulatory t cells, J Clin Invest 116, 485-494.
69. Van't Land, B., Blijlevens, N.M., Marteijn, J., Timal, S., Donnelly, J.P., De Witte, T.J.M'rabet, L., 2004, Role of curcumin and the inhibition of nf-kappab in the onset of chemotherapy-induced mucosal barrier injury, Leukemia 18, 276-284.
70. Van't Land, B., Boehm, G.Garssen, J., 2010a, Breast milk: Components with immune modulating potential and their possible role in immune mediated disease resistance.
71. Van't Land, B., Schijf, M., Van Esch, B.C., Van Bergenhenegouwen, J., Bastiaans, J., Schouten, B., Boon, L.Garssen, J., 2010b, Regulatory t-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides, Vaccine 28, 5711-5717.
72. Varner, A.E., Busse, W.W.Lemanske, R.F., Jr., 1998, Hypothesis: Decreased use of pediatric aspirin has contributed to the increasing prevalence of childhood asthma, Ann Allergy Asthma Immunol 81, 347-351.
73. Vartiainen, E., Petays, T., Haahtela, T., Jousilahti, P.Pekkanen, J., 2002, Allergic diseases, skin prick test responses, and ige levels in north karelia, finland, and the republic of karelia, russia, J Allergy Clin Immunol 109, 643-648.
74. Von Mutius, E.Radon, K., 2008, Living on a farm: Impact on asthma induction and clinical course, Immunol Allergy Clin North Am 28, 631-647, ix-x.
75. Vos, A., M'rabet, L., Stahl, B., Boehm, G.Garssen, J., 2007, Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates, Crit Rev Immunol 27, 97-140.
76. Willing, B., Halfvarson, J., Dicksved, J., Rosenquist, M., Jarnerot, G., Engstrand, L., Tysk, C.Jansson, J.K., 2009, Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal crohn's disease, Inflamm Bowel Dis 15, 653-660.
77. Yazdanbakhsh, M., Kremsner, P.G.Van Ree, R., 2002, Allergy, parasites, and the hygiene hypothesis, Science 296, 490-494.

Chapter 6


Prophyla
RSVA
Synagis
Fusion
Severe respirator
Treatment with
Palivizumab
Peptide
Injection
CD=14

Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides.

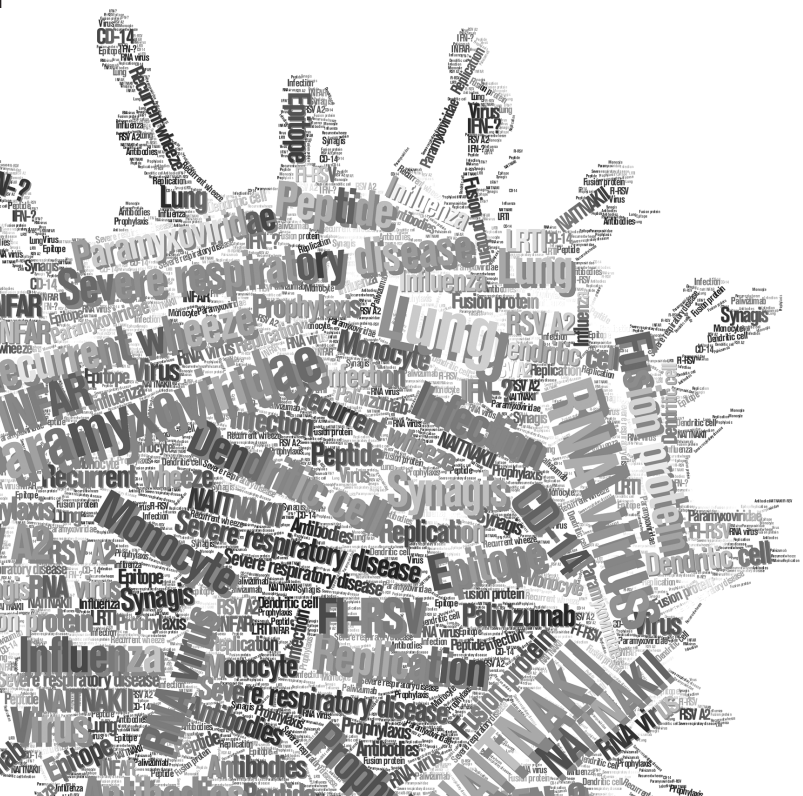
Belinda van't Land[1,2], Marcel Schijf[2,3], Betty C.A.M. van Esch[1,2], Jeroen van Bergenhenegouwen[1,2], Jacqueline Bastiaans[2], Bastiaan Schouten[1], Louis Boon[4], Johan Garssen[1,2]

[1]Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands

[2]Nutricia Advanced Medical Nutrition, Danone Research – Centre for Specialised Nutrition, Wageningen, The Netherlands

[3]Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center, Utrecht.

[4]Bioceros, Utrecht, The Netherlands.



Vaccine (2010): (35): 5711-5717

ABSTRACT

Regulatory T-cells are increasingly important in vaccine strategies. In a Flu-vaccination model the role of $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) and the immune modulation by orally supplied prebiotic oligosaccharides consisting of scGOS/lcFOS/pAOS, was assessed using anti-CD25 (PC61) mediated depletion studies. As expected, in C57BL/6J mice the Flu-vaccination resulted in significantly ($p<0.001$) increased DTH responses when receiving scGOS/lcFOS/pAOS. In addition, increased T-bet expression of activated $CD4^+$ T-cells was detected compared to placebo. *In vivo* depletion of $CD25^+$ Tregs significantly ($p<0.05$) increased basal DTH responses, indicating the suppressive function of these $CD25^+$ Tregs normally present. Surprisingly, *in vivo* Tregs depletion diminished scGOS/lcFOS/pAOS induced immune modulation completely to control levels ($p<0.05$). Although no difference in number, percentage or activation of Tregs could be determined after scGOS/lcFOS/pAOS supplementation, changes in Treg function still remains to be investigated. In conclusion, $CD25^+$ Tregs have an important role in modulated Flu-vaccine responses induced by scGOS/lcFOS/pAOS.

INTRODUCTION

Although influenza vaccination is widely recommended for immune suppressed individuals (i.e infants, elderly, etc), the same immune dysfunction that increases the risk and consequences of influenza infection also compromises vaccine responses and effectiveness *[1]*. Immune suppression can be the result from many different biological mechanisms, ranging from the developing immune system, rare congenital immune deficiencies to more common causes such as immunosuppressive drugs, aging or HIV infection. On the other hand, to maintain a proper immunological balance in the host, specialized T-cell subsets like $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) and CD1d-restriced NKT-cells are crucial. Tregs are also increasingly important in vaccine strategies as they serve to limit activation, trafficking, and/or effector functions of both CD4+ and CD8+ T-cells *[2,3].* During a primary immune response, the expansion and attraction of conventional and regulatory T-cell populations occur in synchrony *[4]*. More importantly, the relative accumulation of Tregs at peak response significantly exceeds that of conventional T-cells, reflecting an extensive relationship between Tregs and conventional T-cells, which control the magnitude of the response *[5]*. Little is known about the intracellular factors and external cues for the differentiation and function of distinct Treg populations. Although, recently it has been shown that both Fox-p3 as well as T-bet (a transcription factor both necessary and sufficient for Th1 differentiation) act jointly within Tregs to produce unique functional outcomes in Th1 type of inflammation *[6]*, suggesting the possibility of specific immune response related Tregs development.

Investigation of the role of Tregs in model systems is facilitated by their *in vivo* depletion using anti-CD25 monoclonal antibodies. One of the first studies using anti-CD25 mAb in combination with therapeutic vaccination resulted in increased eradication of tumors and the development of a strong tumor-directed T-cell response. Furthermore C57BL6 mice depleted for $CD25^+$ Tregs, showed a more potent Th1 response *[7]*, resulting in the resolution of established infections *[5]*. Thus, it seems that breaking immunological tolerance through depletion of Tregs can evoke strong immune responses in otherwise non-responding animals *[8]*. Although there has been considerable debate about the effectiveness of the anti-CD25 mAb treatment *in vivo* to deplete Tregs, numerous articles report significant clinical effects following anti-CD25 pre-treatment in a variety of animal models *[9-12].* This indicates that PC61 administration affects functionality of $CD25^+Foxp3^+$ Tregs *[13].* This approach is therefore used to study the role of Tregs in immune modulations induced by non-digestible oligosaccharides.

Specific non-digestible oligosaccharides can improve the immune balance in infants, resulting in lower incidence of infections early in life *[14].* In addition, both mucosal and systemic immune modulatory effects induced by orally applied prebiotic oligosaccharides have been reported *[14]*. Recent data indicate that a mixture of scGOS/lcFOS and pAOS can have an impact on allergy related symptoms *[14]*. Prebiotic oligosaccharides can possibly

act indirectly through microbiota-dependent mechanisms (i.e. rebalancing microbiota composition in the gut) and/or have a direct effect via activation or inhibition of cellular receptors on immune competent cells *[15]*. The pre- and probiotic concept is based on the fact that indigenous microbiota are considered to contribute to induction and maintenance of immune homeostasis possibly via of $CD25^+$ Tregs *[16]*. The exact underlying mechanism whereby prebiotic oligosaccharides induce immune modulatory effects however remains to be elucidated and is subject of current investigation. Given the importance of Tregs in maintenance of immune homeostasis and vaccine efficiency in the host, the possible role of Tregs in the immune-modulating effects of dietary supplementation with a unique mixture of prebiotic oligosaccharides scGOS/lcFOS/pAOS has been investigated.

RESULTS

Dietary scGOS/lcFOS/pAOS enhances Th1 type of specific immunity towards the Flu vaccination.

Immune modulation effects of the dietary interventions were analyzed by measuring antigen specific DTH responses, as an *in vivo* parameter for Th1 type of cellular immunity. In mice receiving scGOS/lcFOS/pAOS, influenza vaccination resulted in significantly ($p<0.001$) increased influenza-specific DTH responses as compared to mice receiving placebo diets (**Figure 1A**). These responses are comparable with earlier described immune modulation capacities of orally supplied oligosaccharides *[21]*. In addition, the expression of T-bet and Gata-3 in the activated ($CD69^+$) $CD4^+$ T-cell population was assessed (**Figure 1B and C**). A significant increase in T-bet expression ($p<0.05$) could be detected in splenocytes of mice receiving scGOS/lcFOS/pAOS as compared to mice receiving placebo diet (5515 +/− 295 placebo vs 6720 +/− 236 scGOS/lcFOS/pAOS MFI (mean +/− SE)). In contrast, no differences were found in Gata-3 expression levels. The increased DTH as well as increased T-bet expression level, through supplementation of oligosaccharides are indicative for improved Th1 type of immune responsiveness in C57BL/6J mice towards a fixed antigen dose.

$CD25^+$ T-cells are involved in scGOS/lcFOS/pAOS induced increased DTH response.

To investigate the role of $CD25^+$ T-cells in immune modulation induced by oligosaccharides, antibody mediated depletion studies were performed. *In vivo* depletion of $CD25^+$ T-cells with PC61 significantly ($p<0.05$) increased basal DTH responses (from 68.44 +/− 2.9 to 89.38+/− 9.4 mean +/− SEM), which indicates the suppressive function of these cells normally present (**Figure 2A**). As expected, Flu specific DTH responses were significantly ($p<0.001$) increased (117.3 ± 8.8 μm mean ± SEM) in mice receiving scGOS/lcFOS/pAOS in the diets compared to placebo (68.44 ± 2.9 μm mean ± SEM) as shown in earlier studies. On the contrary DTH

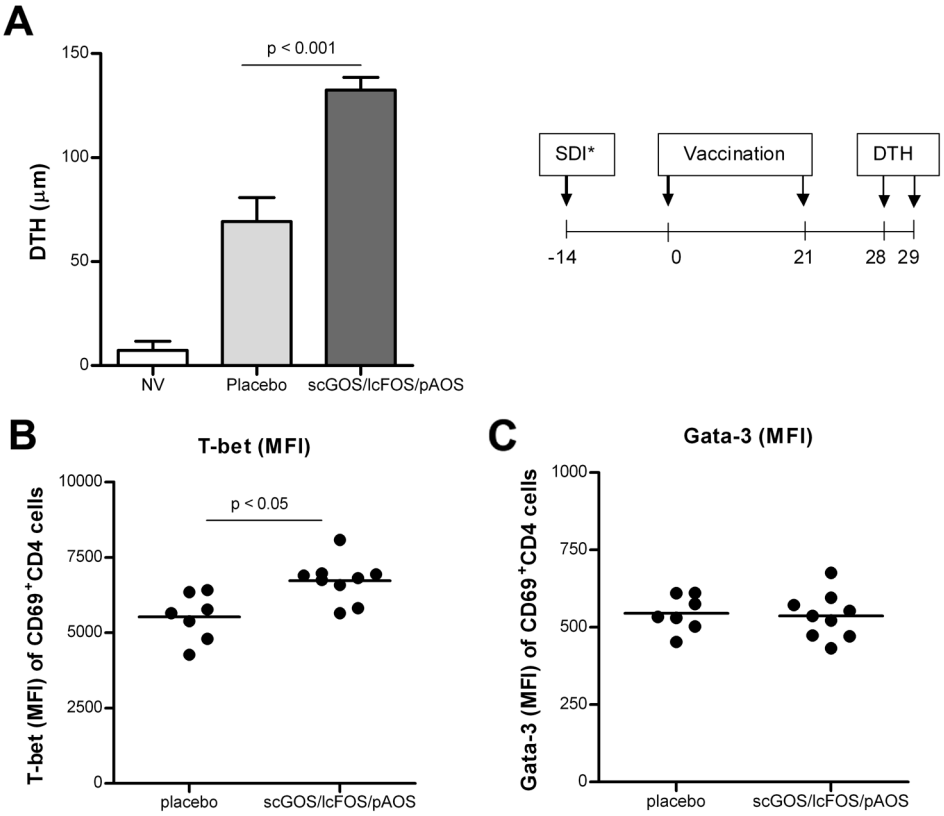


FIGURE 1. Dietary intervention with scGOS/lcFOS/pAOS significantly increases Flu specific Th1 response. Two groups of mice (n = 10 per group) received a sub-maximal vaccination and dietary intervention of placebo or scGOS/lcFOS/pAOS during the entire vaccination procedure as indicated in the experimental scheme (SDI = Start Dietary Intervention). A non-vaccinated group (n=3) was used as control (NV). Antigen specific DTH responses (**A**) were measured by ear swelling (24 hours post antigen injection) and corrected for background (PBS) swelling. Bars represent mean +/- SEM of the different groups. In addition, spleen cells isolated from mice (n = 10 per group) receiving either placebo or scGOS/lcFOS/pAOS were labeled with CD4/CD69/T-bet/Gata-3 and analyzed using flowcytometry. Lines represent mean MFI of T-bet (**B**) or Gata-3 (**C**) on $CD4^+CD69^+$ T-cells in individual spleen cell populations (as indicated through separate dots). Statistically significant differences between the groups receiving vaccinations are indicated in the graph.

responses of mice receiving scGOS/lcFOS/pAOS were completely ($p<0.05$) reduced to control levels (89.4 ± 9.4 and 91.1 ± 8.9 µm mean ± SEM of placebo and scGOS/lcFOS/pAOS respectively) upon depletion of CD25 positive cells. This indicates that depleting of $CD25^+$ T-cells with normally a suppressive function are involved in the immune modulation induced by scGOS/lcFOS/pAOS.

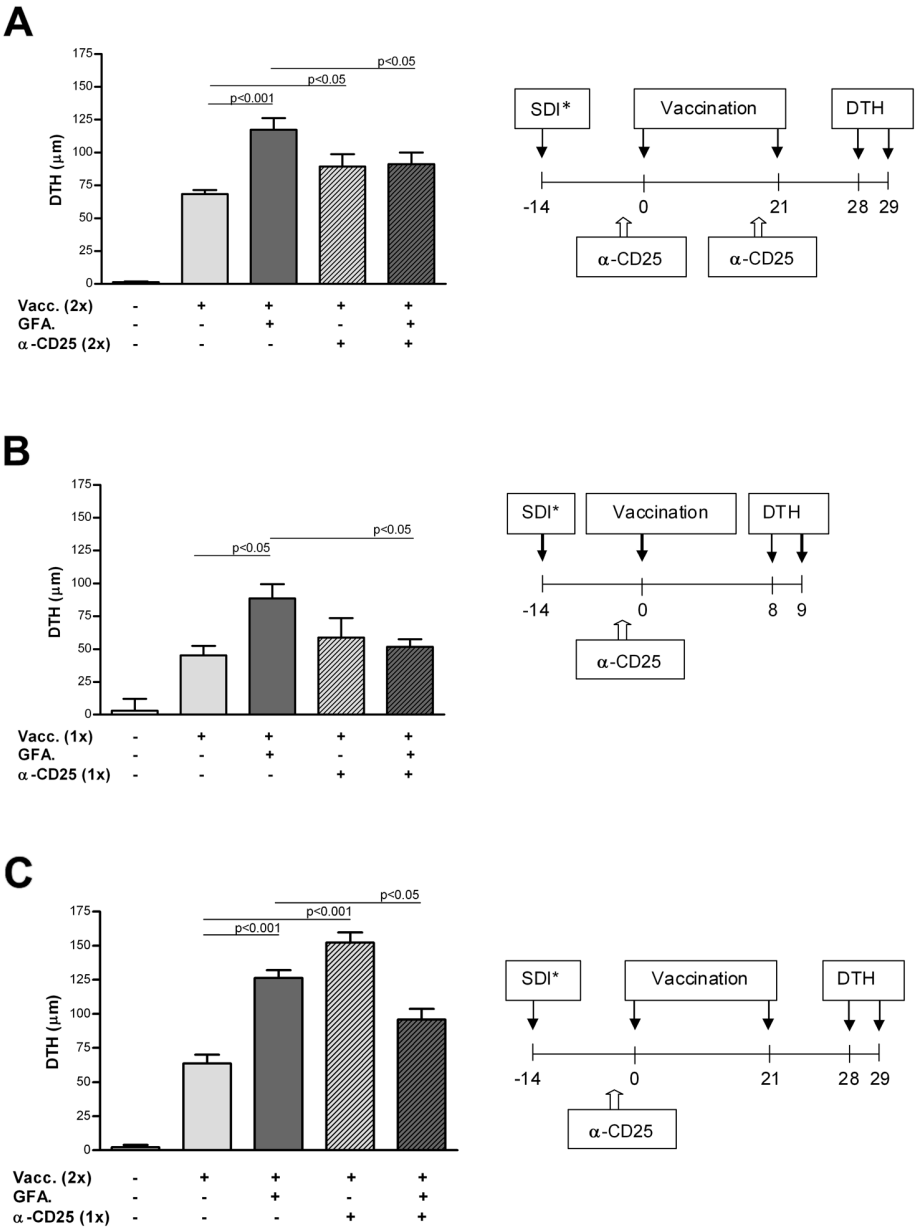


FIGURE 2. CD25 depletion reduces scGOS/lcFOS/pAOS induced Flu specific DTH responses. Four groups of mice (n=10 per group) received a sub-maximal vaccination and dietary intervention of placebo (gray bars) or scGOS/lcFOS/pAOS (dark gray bars) during the entire vaccination procedure as indicated in the experimental schemes (SDI = Start Dietary Intervention). In addition two groups of mice received anti-CD25 mAb (200 µg per inj. i.p. PC61 Biosceros) two days prior to vaccinations as indicated (dashed bars). Antigen specific DTH responses were measured by ear swelling (24 hours post antigen injection) and corrected for background (PBS) swelling. Bars represent mean +/- SEM of the different groups. All intervention groups are statistically different from placebo ($p<0.01$) and additional statistical significant differences are indicated in the graphs.

The involvement of CD25$^+$ T-cells in scGOS/lcFOS/pAOS induced DTH responses is already present during the primary Ag encounter.

As recently activated T-cells are expressing CD25, a treatment with PC61 prior the booster vaccination, may influence these CD25$^+$ cells as well as Tregs. In a non-vaccinated mice normally a low level of recently activated T-cells are present, therefore the accuracy and role of CD25$^+$ T-cell depletion was investigated during the primary vaccination. Flu specific DTH responses which were significantly ($p<0.05$) increased (85.6 ± 10.7 μm mean ± SEM) in mice receiving scGOS/lcFOS/pAOS in the diets compared to placebo (45.2 ± 7.3 μm mean ± SEM) (**Figure 2B**). These DTH responses were lower as seen in the model using two vaccinations as expected. DTH responses in mice receiving scGOS/lcFOS/pAOS were significantly ($p<0.05$) reduced to control levels (51.7 ± 5.8 μm mean ± SEM) upon treatment with anti-CD25 mAb. This indicates that CD25$^+$ Tregs have a role in the scGOS/lcFOS/pAOS induced effects.

Tregs are clearly involved in scGOS/lcFOS/pAOS induced DTH increment.

Treatment with PC61 is effective for at least 10 days post administration. This means that at the time DTH were set (11 days post PC61 administration), the mAb may affect DTH responses. Therefore it was tested whether anti-CD25 treatment prior to primary vaccination only could influence the vaccination response as the total vaccination procedure was applied. Anti-CD25 treatment resulted in a significantly increased DTH response of mice receiving placebo (**Figure 2C**), which clearly postulates a selective reduction in regulatory function during priming of the Flu specific immune response. In addition, it was found that the immune modulatory effect induced by scGOS/lcFOS/pAOS was almost completely diminished to control levels due to the *in vivo* depletion of CD25$^+$ Tregs prior to the primary vaccination. The DTH increase in mice receiving scGOS/lcFOS/pAOS was statistically significant reduced from 126.3 ± 5.8 to 95.7 ± 5.8 μm mean ± SEM ($p<0.05$) due to anti-CD25 treatment 2 days prior to primary vaccination (**Figure 2C**), which indicates that Tregs are involved in the immune modulatory effect of scGOS/lcFOS/pAOS.

Presence and activation of Tregs is not altered in spleen and MLN due to scGOS/lcFOS/pAOS supplementation.

Level of total immune activation as detected by measuring membrane expression of CD25, CD38 and CD69 were equal in mice regardless of diet, both in the MLN and spleen *(data not shown).* In both spleen and MLN's the percentage of Tregs were not altered significantly in the mice receiving scGOS/lcFOS/pAOS compared to mice receiving placebo in this vaccination model. Upon *in vivo* depletion of CD25$^+$ cells using anti-CD25 antibody treatment a significant reduction in the percentage of CD25$^+$Foxp3$^+$ expressing cells ($p<0.01$) in the

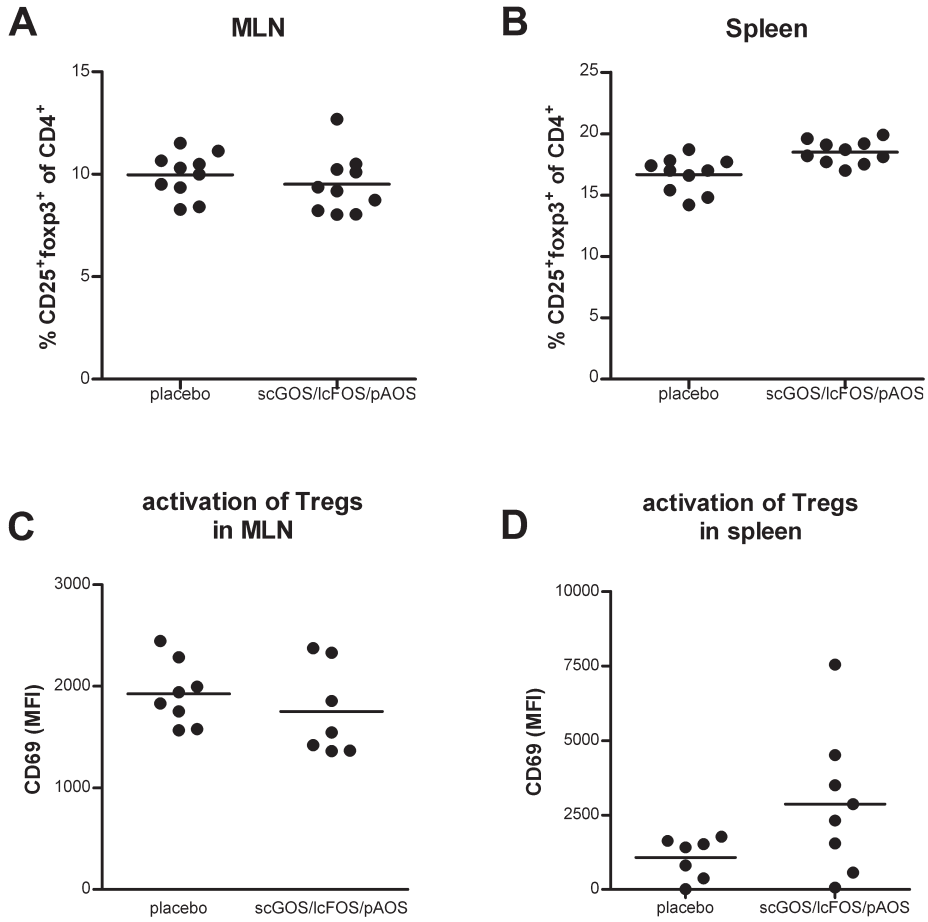


FIGURE 3. Activation of Tregs after first Flu vaccination. Spleen and MLN cells isolated from mice (n=10 per group) receiving either placebo or scGOS/lcFOS/pAOS were labeled with CD4/CD25/Foxp3/CD69 and analyzed using flowcytometry. Percentage of Tregs in MLN (**A**) and spleen (**B**) are depicted as individual dots, with the line representing mean percentage Foxp3$^+$CD25$^+$ T-cells. In addition the activation status of the Tregs as measured with CD69 MFI are shown for MLN (**C**) or spleen (**D**) on CD4$^+$CD25$^+$Foxp3$^+$ Tregs in individual cell populations (as indicated through separate dots, with the line representing the mean MFI of CD69).

MLNs could be detected at the end of the study *(data not shown)*. No effects could be detected in the spleen or the MLN's on the percentage of CD25$^+$Foxp3$^+$ T-cells or on the CD25$^+$ T-cells or on Foxp3$^+$ T-cells alone (*Figure III*). In addition, the activation status of Tregs was analyzed using CD69 activation surface marker. Although the levels of CD69 on CD25$^+$Foxp3$^+$CD4$^+$ cells increased from 1074 ± 258.1 to 2862 ± 848.1 MFI (mean ± SEM) in mice receiving placebo as compared to scGOS/lcFOS/pAOS respectively, these alterations did not reach statistical significance (**Figure 3**). This suggests that differences found in DTH responses due to scGOS/lcFOS/pAOS supplementation were not due to a change in percentage or clear activation of Tregs, but may be due to an altered function of the CD25$^+$ Treg population.

DISCUSSION

The objective of this study was to examine the possible role of CD25$^+$ Tregs in oligosaccharide induced immune modulation which results in increased vaccine responses. Dietary scGOS/lcFOS/pAOS clearly induces a more pronounced Th1 responsiveness, as shown by increased Flu-specific DTH responses and T-bet expression levels. The mechanism whereby the improved Th1 responsiveness develops however remains elusive. Data herein clearly shows that CD25$^+$ Tregs play a prominent role in the early stages of the development of enhanced Th1 responsiveness induced by the prebiotic oligosaccharides scGOS/lcFOS/pAOS.

Antibody mediated depletion of Tregs *in vivo* is not complete, neither with PC61 *[17,18]*, nor with 7D4 *[9]*, nor the combination of both treatments completely depletes the Treg population from circulation. Regulatory function however is clearly suppressed by i.p. administration of PC61 as shown in our experiments, by the increased vaccination responses in mice receiving placebo diets upon PC61 treatment. This indicates that the CD25$^+$ T-cells depleted are not the recently activated T-cells but rather the Treg. In addition a possible suppressive effect of the PC61 administration on DTH responses can be excluded, since also in the vaccination regimen, whereby only prior to the first vaccination and not the booster vaccination the anti-CD25 was given, a clear effect was detected on the scGOS/lcFOS/pAOS induced increased DTH responses. CD25$^+$ Treg depletion using PC61 treatment diminished the immune stimulation by non-digestible prebiotic oligosaccharides up to levels observed in placebo treated mice. These experiments indicate a prominent role for CD25$^+$ Tregs in this immune modulation by the prebiotic oligosaccharides. Although it is not suggested that Tregs are the sole cell type effected by scGOS/lcFOS/pAOS supplementation. As shown in *Figure II*, the administration of PC61 in mice receiving scGOS/lcFOS/pAOS completely reverses the DTH responsiveness, even below the levels detected in mice receiving placebo diets and PC61. This observation suggests that more cell types can be involved in the immune modulation induced through scGOS/lcFOS/pAOS supplementation.

There are several means for Tregs to influence the magnitude of the vaccination response as reviewed by *[22]*. Short-range mechanisms such as cytokine deprivation *[23]* and regulatory molecule secretion like IL-10, adenosine, IL-35, galectins and carbon monoxide will suppress the local effector cells during adaptive immune responses. In the longer run, Tregs create a regulatory environment by producing a.o. TGF-β and/or change DC functions to stimulate additional Treg production. In our experiments the prebiotic treatment with scGOS/lcFOS/pAOS does not seem to influence the homeostasis of Tregs, since the total amount, percentages nor did activation state of Tregs in oligosaccharide fed mice differ from placebo fed mice neither in the spleen, nor in MLNs. A change in Treg function can influence the magnitude of the response. It has been hypothesized *[24]* that each type of immune response either Th1 type of immune response or Th2 develops its own regulatory population. Recently published studies *[25,26]* establish that Tregs itself are subject to differentiation and functional

specialization. As illustrated in **Figure 4**, a balanced immune response can therefore be controlled by distinct Treg populations. This indicates that increased Treg mediated suppression of the Th2 response would favor the development of a more pronounced Th1 type of response. This indicates that there may be a more subtle difference present in the Treg

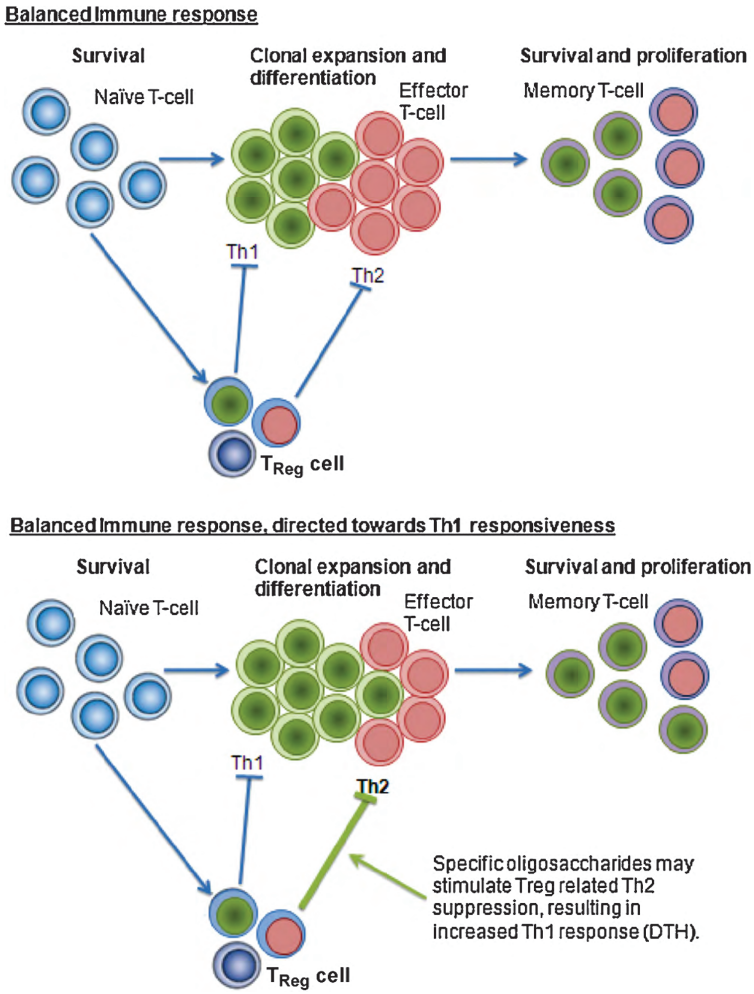


FIGURE 4. Hypothetical mode of scGOS/lcFOS/pAOS action. The balanced immune response develops after antigen exposure into a for the challenge appropriate balance between immune response types (Th1 and Th2 are illustrated, as many more are known to develop). The development of the immune response is regulated by among others the Treg population. As for each immune response a specific Treg types is hypothesized to develop, both may have an impact on total balance. In presence of prebiotic oligosaccharides like scGOS/lcFOS/pAOS the immune response in currently used vaccination model is shifted towards a more pronounced Th2 type of responsiveness. As Tregs play a prominent role herein, it can be hypothesized that the specific oligosaccharides like scGOS/lcFOS/pAOS strengthen the Th2 suppression, favoring the development of Th1 immune response.

population developed in mice receiving scGOS/lcFOS/pAOS which need to be investigated further explaining fully the discussed results.

In conclusion: This study clearly shows that $CD25^+$ Tregs play a prominent role in the development of prebiotic scGOS/lcFOS/pAOS induced immune modulation towards enhanced Th1 vaccine responsiveness. Knowing more about the mechanism whereby vaccine induced immune responses can be increased using alternative strategies such as described within this article, may lead to the development of improved vaccine strategies. On the other hand, immune modulations as induced by this specific prebiotic mixture may benefit individuals with suppressed immune system/responses in general.

MATERIALS AND METHODS

Animals and diets

Eight-week-old old male specific pathogen-free inbred C57BL/6J mice were obtained from Charles River (Someren, the Netherlands) and housed under standard housing conditions with a 12 h dark and light cycle. All animals had free access to tap water and the semi-purified AIN-93G diet (Research Diet Services, Wijk bij Duurstede, the Netherlands), with or without oligosaccharide mixture consisting of three different prebiotic oligosaccharide materials, i.e. short-chain galactooligosaccharides (scGOS: Borculo Domo, Zwolle, 45% scGOS), long-chain fructooligosaccharides (lcFOS: Orafti, Wijchen, 100% lcFOS) and pectin hydrolysate derived acidic-oligosaccharides (pAOS: Sudzucker, 85% galacturonic acid). The prebiotics were mixed in a ratio of 9:1:10 based on carbohydrate purity. Although other combinations of these specific prebiotic oligosaccharides are known to be effective as well, it was this specific ratio which within currently used mouse vaccination model gave the largest immune modulation as detected by an DTH increase at the time and was therefore used for these mechanistically studies. A small negative control group of animals (n=3) was included only to show specificity of the vaccination procedure. Therefore, this group was not used for any statistical comparisons to supplemented groups (n=10 per group). Fourteen days prior to the first vaccination dietary supplementation started which was maintained during the entire experimental protocol. The study protocol was reviewed and approved by the Animal Experimental Committee of the Utrecht University.

Vaccination protocol

All C57BL/6J mice except the small negative control group received primary vaccination (day 1) and if indicated a booster vaccination (day 21) with a human influenza subunit vaccine consisting of haemagglutinin proteins of 3 different influenza strains (Influvac, Solvay Pharmaceuticals, Weesp, the Netherlands). Vaccinations were performed by subcutaneous

injection of a 1:1 mix of vaccine (0.30 µg/ml per subunit) and adjuvant (Stimune, previously known as Specol: Cedi-diagnostics, Lelystad, the Netherlands) in a total volume of 100 µL. The negative control group received concurrent injections with a 1:1 mix of PBS and adjuvant in a total volume of 100 µL. As previous studies have shown that anti-CD25 mAb PC61 was capable of inactivation and/or depletion of $CD25^+$ Tregs *in vivo* 200 µg PC61 was administered i.p. to C57BL/6 mice two days prior to both primary vaccination as well as booster vaccination at day 21 *[17,18]*. To deplete $CD25^+$ Tregs mice received an i.p. administration of anti-CD25 (200 µg) two days prior to the vaccinations as indicated. Control mice received rat IgG. Dose and procedure were similar to manuscripts published earlier *[19,20]*, in which a reduction of 85% percent of $CD25^+$ Tregs was detected in the mesenteric lymph nodes (MLNs) two days post antibody treatment. In our hands similar reductions were detected (data not shown).

Delayed type hypersensitivity (DTH)

Vaccine-specific DTH reactions were induced 9 days after the last vaccination, by subcutaneous injection of 25 µL Influvac (30 µg/mL per haemagglutinin subunit) into the ear pinnea of one ear. As control, the other ear was injected with 25 µL PBS. Ear thickness was measured in duplicate before challenge, and 24 hours thereafter, with a digital micrometer (Mitutoyo Digimatic 293561, Veenendaal, the Netherlands). The Influvac specificity of the DTH response was calculated by subtracting the basal ear thickness from the value at 24 hours after challenge and was corrected for the control swelling.

Flowcytometry analysis

Splenocytes and cells from the MLNs were isolated by gently pressing the organs through nylon mesh filters (Falcon cell strainer, Becton Dickinson, Alphen a/d Rijn, the Netherlands). After erythrocyte lysis (spleens only), cells were counted and diluted to appropriate concentrations. $5*10^5$ cells were once washed with 100 µl PBS containing 1% FBS and were stained with anti-CD3-FITC (1/200 BD cat 555274), anti-CD4-PE-CY5 (1/200 BD cat 553654) and either with anti-CD25-PE (1/100 Immunotech PNIM 2795), anti-CD38-PE (1/200 BD cat 553764) or anti-CD69-PE (1/200 BD cat 553237) for 30 minutes. Intracellular Foxp3 staining was executed following manufacturer's protocol, (Ebioscience, FITC anti-Foxp3 staining set, Bio connect, The Netherlands). Same procedure was used for intracellular anti-GATA-3-PE (1/50 BD cat 12-9966), as well as anti-T-bet-PerCP-Cy5 (1/100 BD cat 45-5825) staining. The whole staining procedure was performed on ice and stained cells were protected from light. In total a minimum of 10.000 lymphocytes were counted and analyses were performed using FACS Calibur and Cell Quest software (BD Biosciences).

Statistical analysis

All statistical calculations were performed using SPSS version 12.0.1 software. Statistical differences between test and control groups were analyzed by ANOVA followed by multi-group comparison analysis using the Bonferroni test. All values are presented as mean ± SEM. P-values <0.05 were considered significant.

ACKNOWLEDGEMENTS

The authors would like to thank Suzanne v Doorn for technical assistance. This research was partly funded by TIPharma.

ABBREVATIONS

(pAOS) Acidic oligosaccharides derived from hydrolyzed pectin, (DTH) Delayed-type hypersensitivity, (lcFOS) long chain fructo-oligosaccharides, (scGOS) short chain galacto-oligosaccharides, (SDI) start dietary intervention, (NV) non-vaccinated.

REFERENCES

1. Kunisaki, K.M. & Janoff, E.N. Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses. *Lancet Infect Dis* 2009, 9(8), 493-504.
2. Vignali, D.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nat Rev Immunol* 2008, 8(7), 523-532.
3. Shevach, E.M., DiPaolo, R.A., Andersson, J., Zhao, D.M., Stephens, G.L. & Thornton, A.M. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev* 2006, 212, 60-73.
4. Haribhai, D., Lin, W., Relland, L.M., Truong, N., Williams, C.B. & Chatila, T.A. Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* 2007, 178(5), 2961-2972.
5. Belkaid, Y. & Rouse, B.T. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005, 6(4), 353-360.
6. Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G. & Glimcher, L.H. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000, 100(6), 655-669.
7. Oldenhove, G., de Heusch, M., Urbain-Vansanten, G. *et al.* CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. *J Exp Med* 2003, 198(2), 259-266.
8. Sutmuller, R.P., van Duivenvoorde, L.M., van Elsas, A. *et al.* Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001, 194(6), 823-832.
9. Kohm, A.P., McMahon, J.S., Podojil, J.R. *et al.* Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol* 2006, 176(6), 3301-3305.
10. Shimizu, J., Yamazaki, S. & Sakaguchi, S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 1999, 163(10), 5211-5218.
11. Lewkowich, I.P., Herman, N.S., Schleifer, K.W. *et al.* CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 2005, 202(11), 1549-1561.
12. Fecci, P.E., Sweeney, A.E., Grossi, P.M. *et al.* Systemic anti-CD25 monoclonal antibody administration safely enhances immunity in murine glioma without eliminating regulatory T cells. *Clin Cancer Res* 2006, 12(14 Pt 1), 4294-4305.
13. McNeill, A., Spittle, E. & Backstrom, B.T. Partial depletion of CD69low-expressing natural regulatory T cells with the anti-CD25 monoclonal antibody PC61. *Scand J Immunol* 2007, 65(1), 63-69.
14. Arslanoglu, S., Moro, G.E., Schmitt, J., Tandoi, L., Rizzardi, S. & Boehm, G. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *J Nutr* 2008, 138(6), 1091-1095.
15. Vos, A., M'Rabet, L., Stahl, B., Boehm, G. & Garssen, J. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. *Crit Rev Immunol* 2007, 27(2), 97-140.
16. Ishikawa, H., Tanaka, K., Maeda, Y. *et al.* Effect of intestinal microbiota on the induction of regulatory CD25+ CD4+ T cells. *Clin Exp Immunol* 2008, 153(1), 127-135.
17. McHugh, R.S. & Shevach, E.M. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J Immunol* 2002, 168(12), 5979-5983.

18. Taguchi, O. & Takahashi, T. Administration of anti-interleukin-2 receptor alpha antibody in vivo induces localized autoimmune disease. *Eur J Immunol* 1996, 26(7), 1608-1612.
19. Billiard, F., Litvinova, E., Saadoun, D. *et al.* Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation. *J Immunol* 2006, 177(4), 2167-2174.
20. van Wijk, F., Nierkens, S., de Jong, W. *et al.* The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administered peanut proteins. *J Immunol* 2007, 178(11), 6894-6900.
21. Vos, A.P., Haarman, M., Buco, A. *et al.* A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. *Int Immunopharmacol* 2006, 6(8), 1277-1286.
22. Tang, Q. & Bluestone, J.A. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008, 9(3), 239-244.
23. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M.J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 2007, 8(12), 1353-1362.
24. Romagnani, S. Immunologic influences on allergy and the TH1/TH2 balance. *J Allergy Clin Immunol* 2004, 113(3), 395-400.
25. Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B. & Campbell, D.J. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 2009, 10(6), 595-602.
26. Zheng, Y., Chaudhry, A., Kas, A. *et al.* Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 2009, 458(7236), 351-356.

Chapter
7

Palivizumab
Recurrent wheeze
Prophyla
RSV
Synagis
Fusion
Peptide
CD-14
Infection
Severe respiratory
RSV A2
Monocyte

Specific dietary oligosaccharides increase Th1 responses in a mouse Respiratory Syncytial Virus infection model.

Marcel A. Schijf [a,b], Debby Kruijsen [a], Jacqueline Bastiaans [b], Frank E.J. Coenjaerts [c], Johan Garssen [b,d], Grada M. van Bleek [a] and Belinda van't Land [a,b,#]

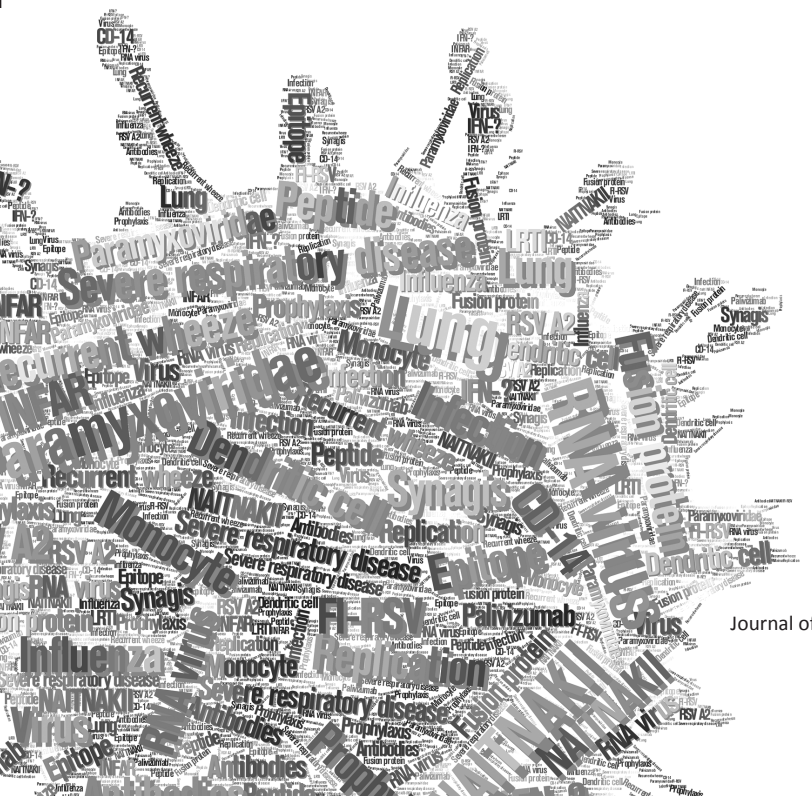
[a] Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

[b] Department of Immunology, Danone Research - Centre for Specialised Nutrition, Wageningen, The Netherlands

[c] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

[d] Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands

G.M. van Bleek and B. van't Land contributed equally to this work



Journal of Virology (2012):(21):11472-11482.

ABSTRACT

Breast feeding reduces the risk to develop severe RSV infections in infants. In addition to maternal antibodies other immune modulating factors in human milk contribute to this protection. Specific dietary prebiotic oligosaccharides, similar to oligosaccharides present in human milk, were evaluated in a C57BL/6 mouse RSV infection model. During primary RSV infection, increased numbers of RSV specific $CD4^+$ T cells producing IFN-γ were found in the lungs at day 8 to 10 post infection in mice receiving diet containing short chain Galacto-oligosaccharides, long chain Fructo-oligosaccharides and pectin derived Acidic-oligosaccharides (*scGOS/lcFOS/pAOS*). In a Th2-skewed formalin-inactivated (FI)-RSV vaccination model the prebiotic diet reduced RSV specific Th2 cytokine (IL-4, IL-5 and IL-13)-producing $CD4^+$ T cells in the lung and the magnitude of airway eosinophilia at day 4 and 6 after infection. This was accompanied by a decreased influx of inflammatory DCs ($CD11b^+/CD11c^+$) and increased numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells at day 8 after viral challenge. These findings suggest that specific dietary oligosaccharides can influence trafficking and/or effector functions of innate immune, $CD4^+$ and $CD8^+$ T cell subsets in the lungs of RSV infected mice. In our models, *scGOS/lcFOS/pAOS* had no effect on weight but increased viral clearance in FI-RSV vaccinated mice 8 days after infection. The increased systemic Th1 responses potentiated by *scGOS/lcFOS/pAOS* might contribute to an accelerated Th1/Th2 shift of the neonatal immune system, which might favour protective immunity against viral infections with a high attack rate in early infancy such as RSV.

INTRODUCTION

Respiratory syncytial virus (RSV), a pneumovirus in the family of Paramyxoviridae, infects nearly all children within the first 3 years of life (15). Primary RSV infections can cause severe bronchiolitis and pneumonia which are associated with significantly increased risk of developing wheeze during childhood that lasts until teenage years (31,45,46). Symptomatic re-infections occur in every age group but frequency and severity of symptoms are highest in children below 5 years of age. The mechanism behind the onset of severe RSV infections is still not completely clear. Severe RSV infections that require hospitalization are most frequent in infants 2-4 months of age (44). Therefore, it has been proposed that inadequate innate or adaptive responses of the immature immune system might contribute to disease severity, especially Th2-bias of the immature immune system has been suggested to be an important factor contributing to RSV disease (5,36).

Formation of the intestinal microbiota population, starting directly after birth, is shaped during infancy and is unique for each individual throughout life (23,37). The intestinal microbiota composition is important for establishment of gut homeostasis and affects local mucosal immunity (20). Although it has been documented that the host genetic background facilitates a core microbiome (52), factors like caesarian section, diet and reduced microbial pressure in western countries, shape host microbial populations (9,32). There is increasing evidence that environmental factors and diet correlate with host immune function and disease susceptibility. Best known examples are correlations found in allergy related diseases like atopy and asthma but also a relatively new disease like obesity appears to be linked to a specific composition of the intestinal microbiota (30,47). This suggests that the microbial community and the host immune system continuously cross-communicate and reorganize, leading to a delicate balance. It is however largely unknown how exactly the intestinal bacterial community interferes with systemic immune processes. Some insight has come from studies in conventional SPF animals, in which a microbiota depletion approach showed that the enhanced killing of *S. pneumonia* and *Staph. Aureus* by bone marrow derived neutrophils was regulated by bacterial peptidoglycans derived from the gut (8). This suggests that manipulation of microbiota can induce systemic priming of the innate immune system, by systemic shedding of bacterial components that act as ligands on pattern recognition receptors.

Breast feeding reduces the risk of severe RSV bronchiolitis (6). In addition to maternal antibodies, human milk contains immune modulating components including oligosaccharides. Some non-digestible oligosaccharides are called prebiotics, because they stimulate the growth of commensal bacteria known to be beneficial to the host (38). It has been shown that infant formula including specific non-digestible carbohydrates like short chain Galacto-oligosacharides (scGOS) and long chain Fructo-oligosaccharides (lcFOS) affects the incidence of upper respiratory tract infections, severity of asthma and lowers IgE antibody titers in atopic disease

(1,54,55). In addition, in infants receiving scGOS, lcFOS and pectin-derived oligosaccharides (pAOS) a preventive effect was found for development of atopic dermatitis (17).

In this study, the effect of a specific dietary intervention (*scGOS/lcFOS/pAOS*) with proven gut microbiota modulating capacities in humans and mice (1,16,33,56) is evaluated on virus specific lung T cell responses in a C57BL/6 mouse model of primary RSV infection. Because earlier work in an influenza vaccination model has shown that prebiotic treatment induced a Th2→Th1 shift we also tested the prebiotic diet in a formalin-inactivated (FI)-RSV vaccine model. This FI-RSV mouse model is an enhanced disease model whereby increased Th2 responses and lung eosinophilia are critical features of enhanced disease. Modulation of systemic immunity potentiated by these oligosaccharides might contribute to an accelerated Th1/Th2 shift of the neonatal immune system and thereby favours protective immunity against viral infections with a high attack rate in early infancy such as RSV.

RESULTS

Dietary intervention with prebiotic scGOS/lcFOS/pAOS increases RSV specific CD4$^+$ T cell mediated IFN-γ production during primary RSV infection.

The effect of prebiotic dietary intervention on primary immune responses initiated after respiratory virus infection was investigated. We used an intranasal RSV infection model in female C57BL/6 mice. In this model, development of the RSV specific CD4$^+$ and CD8$^+$ T cell responses in the lungs peak around day 8-10 after viral exposure (28). Dietary intervention was started in 3 week old mice and continued until the end of the experiment. Six weeks after the start of dietary intervention mice were i.n. infected with RSV. At 4 different time points after infection, mice were sacrificed and bronchoalveolar lavage (BAL) samples and lung single cell suspensions were analyzed for inflammatory cell influx and T cell responses (**Figure 1A**). During the course of infection a significantly ($p<0.05$) lower cellular influx in the BAL was observed in mice receiving *scGOS/lcFOS/pAOS* compared to control mice (3.2 ± 0.2 vs. 5.0 ± 0.8x10^5 cells) at day 8 post infection. The cellular composition of BAL samples at all time points was similar compared to control (**Figure 1B**).

The effect of *scGOS/lcFOS/pAOS* on developing CD4$^+$ T cell responses was measured by intracellular staining of IFN-γ (Th1) and IL-4, IL-5, IL-13 (Th2) cytokine production, after *in vitro* re-stimulation of lung cells with RSV infected or uninfected D1 cells. The RSV specific CD4$^+$ T cell response developed from day 4 post infection onward as has been shown before (28). Mainly IFN-γ was produced by the responding virus specific cells. A significantly increased percentage of virus specific IFN-γ producing CD4$^+$ T cells was detected in the mice receiving *scGOS/lcFOS/pAOS* as compared to control mice at day 8 after RSV infection (11.8 ± 1.6 vs. 7.8 ± 0.7 and at day ten 20.6 ± 1.2 vs. 15.3 ± 1.3 CD4$^+$ IFN-γ$^+$ of total CD4$^+$ T cells, **Figure 1C**). Expressed in absolute numbers/lung this resulted in increased numbers of CD4$^+$

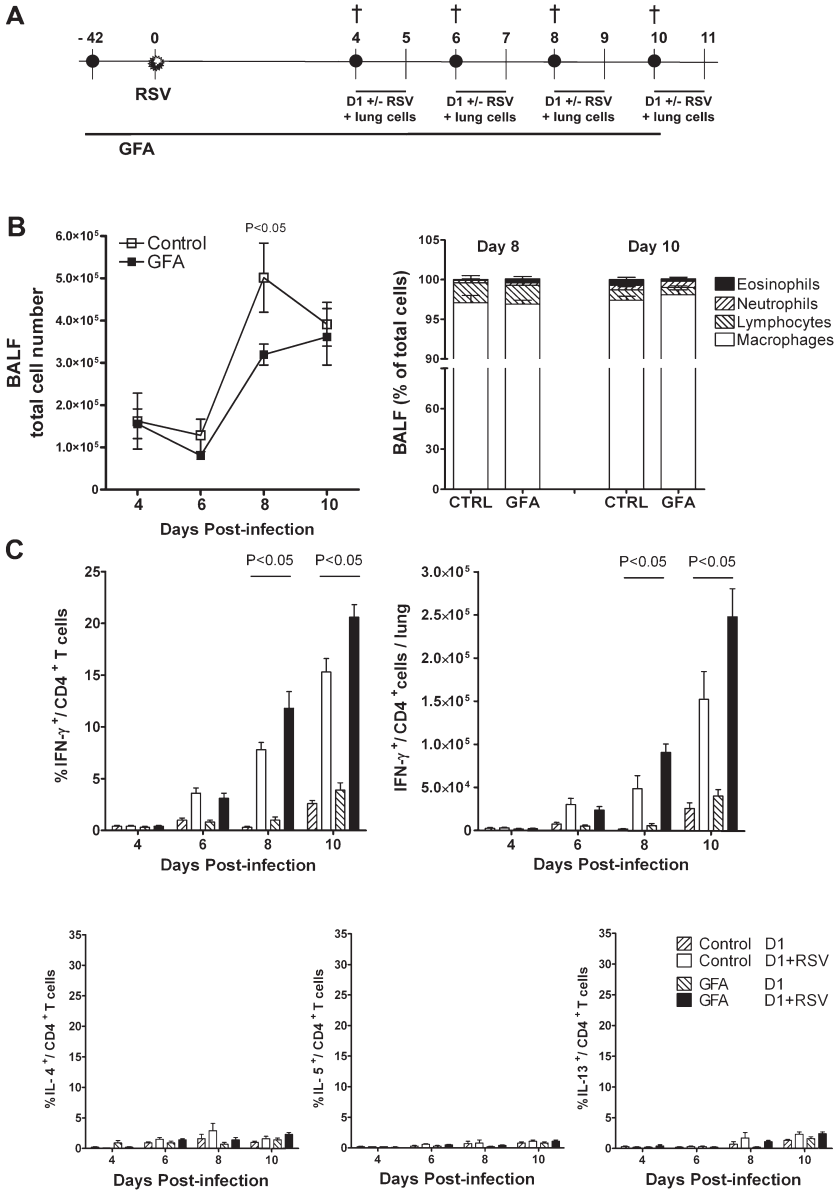


FIGURE 1. Dietary intervention with scGOS/lcFOS/pAOS (GFA) increases RSV specific CD4⁺ T cell-mediated IFN-γ production during primary RSV infection. (**A**) Mice received prebiotic or control diet, starting 6 weeks before intranasal infection with RSV and were sacrificed at indicated time points. (**B**) Cell composition of BAL fluid, left total cell number, right indicated cell type as a percentage of total BAL cells. (**C**) Percentage of cytokine producing CD4⁺ T cells (upper left IFN-γ producing CD4⁺ T cells, bottom figures IL-4⁺, IL-5⁺ and IL-13⁺ CD4⁺ T cells as a fraction of total CD4⁺ T cells) and absolute numbers of IFN-γ⁺ CD4⁺ T cell numbers (upper right) measured after *in vitro* restimulation of lung cell suspensions with unloaded (D1) or RSV loaded (D1+RSV) myeloid dendritic cells. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group.

TABLE I: Dietary intervention with scGOS/lcFOS/pAOS does not affect RSV-specific lung CD8$^+$ T cell responses during primary RSV infection.

	% NKG2A ^{POS}/ CD8 ^{POS}		*% IFN-γ ^{POS}/ CD8 ^{POS} (D1-RSV) §*		*%TET ^{POS}/ CD8 ^{POS}*		*% IFN-γ ^{POS}/ CD8 ^{POS} (NAITNAKII) §*	
	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA
Day 4	9.9±1.3	8.7±1.3	1.7±0.2	1.6±0.3	0.3±0.1	0.5±0.1	1.4±0.1	1.9±0.2
Day 6	19.9±2.0	20.4±1.6	8.7±0.7	8.6±1.0	7.9±1.0	10.0±1.3	10.3±1.8	10.8±1.3
Day 8	45.1±2.4	47.2±1.8	24.0±1.8	26.2±1.3	17.5±1.9	17.7±1.2	17.9±1.2	20.9±2.4
Day 10	59.5±1.9	59.8±2.9	26.4±2.1	29.2±2.1	25.5±1.7	28.8±2.1	19.6±1.1	20.8±1.1

C57BL/6 mice received scGOS/lcFOS/pAOS (GFA) or control diet, starting 6 weeks before i.n. infection with 2.0x10^6 p.f.u. RSV. At indicated time points after infection, lymphocytes were isolated from the lung parenchyma and stained for CD8 in combination with NKG2A or H-2D^b/M$_{187\text{-}195}$ tetramer. For *in vitro* T cell restimulation experiments, lung cells were stimulated with RSV infected D1 cells or the RSV epitope NAITNAKII and intracellularly stained for IFN-γ §. The values depicted represent the number of NKG2A$^+$/CD8$^+$, IFN-γ$^+$/CD8$^+$ or TET$^+$/CD8$^+$ double positive cells as a percentages of total CD8$^+$ T cells. The table shows the mean ± SEM of control or GFA treated mice from 2 individual experiments, n=8 / group.

IFN-γ$^+$ producing cells (9.1 ± 1.0 vs. 4.9 ± 1.5x10^4 cells) at day 8 and (2.5 ± 0.3 vs. 1.5 ± 0.3x10^5 cells/lung) at day 10 post infection. Th17 cells were barely detectable in the lungs of infected mice and no effect of the intervention with *scGOS/lcFOS/pAOS was* found on IL-17 producing CD4$^+$ T cells (data not shown).

The total number of recently activated CD8$^+$ T cells present in the lung was visualized using activation marker NKG2A. Although increased NKG2A$^+$ CD8$^+$ T cell numbers could be detected in the lungs after RSV infection, no difference was found between the two dietary intervention groups (**Table 1**). Virus specific CD8$^+$ T cell responses, visualized by staining with the H-2D^b/M$_{187–195}$ tetrameric complex (containing the dominant RSV epitope derived from the viral matrix protein M$_{187–195}$; NAITNAKII) increased with similar kinetics in both groups. The absolute number of tetramer positive cells that produced IFN-γ was slightly increased in the *scGOS/lcFOS/pAOS* treated group (9.1 ± 1.7 vs. 6.9 ± 0.9x10^5 cells/lung) at day 10 post infection but didn't reach statistical significance. These data show that dietary intervention with *scGOS/lcFOS/pAOS* selectively increased RSV specific CD4$^+$ T cell mediated IFN-γ production in the lungs of RSV infected mice.

Dietary intervention with scGOS/lcFOS/pAOS lowers the Th2 type immune response and lung eosinophilia in FI-RSV vaccinated mice.

Because the prebiotic intervention showed an increased Th1 response during primary RSV infection, it was further tested in a Th2 disease model to evaluate whether dietary intervention could alter the Th2/Th1 balance. We used a murine formalin-inactivated (FI)-RSV vaccination model based on the original vaccine as tested in the 1960's in infants (39). From this model it is known that Th2 cells play an important role in the development of enhanced

disease (7). Dietary intervention started 2 weeks prior to i.m. FI-RSV vaccination, and was administered until the end of the experiment. Thirty five days after vaccination mice were i.n. challenged with RSV. At 3 different time points after infection mice were sacrificed and bronchoalveolar lavage (BAL) samples and lung cell suspensions were analyzed for inflammatory cell influx and T cell responses (**Figure 2A**). Cellular infiltration in the lung airways peaked at day 6 post infection and was significantly ($p<0.05$) decreased in the mice receiving *scGOS/lcFOS/pAOS* (1.4 ± 0.2 vs. 4.3 ± 0.6x10^6 cells), compared to control mice. Furthermore, analysis of the BAL fluid cell composition showed that eosinophil influx, characteristic for this model, was significantly ($p<0.05$) decreased in mice receiving prebiotic diet compared to control diet at day 4 (30.8 ± 5.5 vs. 59.9 ± 3.1 %) and day 6 (38.1 ± 7.3 vs. 63.3 ± 1.7 %) after infection (**Figure 2B**).

In this model the specific T cell response against RSV, represented as the absolute numbers of IFN-γ, IL-4, IL-5, and IL-13 producing $CD4^+$ T cells, peaked at day 6 after infection. Dietary intervention with the specific prebiotic diet significantly ($p<0.05$) decreased the absolute numbers of IL-4 (2.8 ± 0.5 vs. 4.8 ± 1.1x10^6 cells), IL-5 (1.1 ± 0.2 vs. 2.2 ± 0.5x10^6 cells), and IL-13 (2.3 ± 0.4 vs. 4.4 ± 1.1x10^6 cells) producing $CD4^+$ T cells/lung at day 6 after challenge. Moreover, the percentages of $CD4^+$ T cells that produced these Th2 type cytokines were significantly lower in mice receiving *scGOS/lcFOS/pAOS* at both day 4 and day 6 post infection (Figure 2*D*). In this model the absolute numbers of $CD4^+$ T cells that produced RSV specific IFN-γ remained unaffected while the percentage followed a similar pattern compared to the primary infection i.e. they were significantly ($p<0.05$) increased in mice receiving the specific prebiotic diet compared to control (44.1 ± 4.4 vs. 32.6 ± 3.8 $CD4^+$ IFN-γ^+ cells) at day 8 post infection (**Figure 2C**).

The development in the total number of activated ($NKG2A^+$) $CD8^+$ T cells (**Table 2**) was similar between the two diet groups. However, virus specific $CD8^+$ T cell response, measured by $M_{187-195}$ tetramer staining, showed a significant ($p<0.05$) increase in virus specific $CD8^+$ T cell numbers in the mice receiving *scGOS/lcFOS/pAOS* compared to control (18.3 ± 2.6 vs. 12.8 ± 2.6 % $CD8^+$ IFN-γ^+ cells) 8 days post infection. *In vitro* restimulation of lung cells with $M_{187-195}$ peptide showed that these $CD8^+$ T cells were functional, since similar fractions of IFN-γ producing $CD8^+$ T cells (13.8 ± 2.5 for control versus 19.7 ± 2.8 % $CD8^+$ IFN-γ^+ cells in prebiotic receiving mice) were measured at this time point. However, expressed in absolute numbers of tetramer positive cells that produced IFN-γ, this increase (9.1 ± 1.7 vs. 6.9 ± 0.9x10^5 cells/lung) in the *scGOS/lcFOS/pAOS* receiving group did not reach statistical significance. Reduced airway eosinophila at day 4 and 6 after challenge (**Figure 2B**) correlated with significantly decreased numbers of GATA-3 expressing $CD4^+$ T cells (data not shown) and percentages of RSV specific IL-4, -5, -13 producing $CD4^+$ T cells (**Figure 2D**) in the lungs of mice receiving the prebiotics. These data show that dietary intervention with *scGOS/lcFOS/pAOS* modulates FI-RSV induced RSV specific $CD4^+$ and $CD8^+$ T cell responses to a more Th1 type of response in the lungs of RSV infected mice.

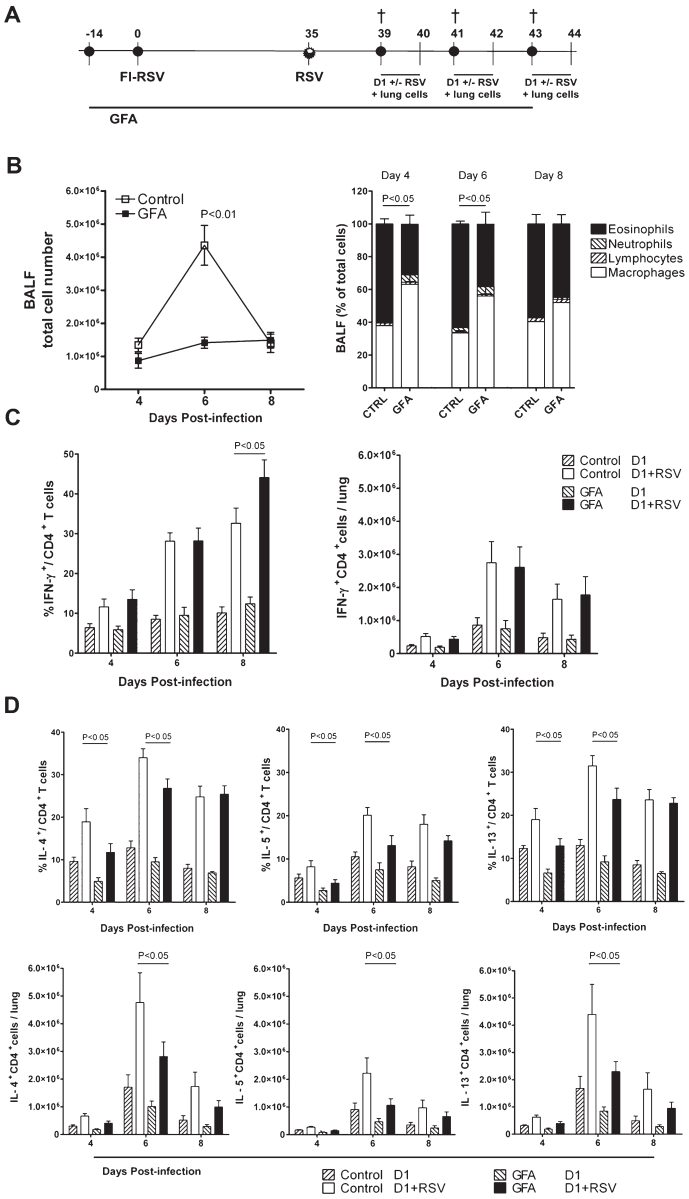


FIGURE 2. Dietary intervention with scGOS/lcFOS/pAOS (GFA) decreases the Th2 type immune response and lung eosinophilia upon challenge in FI-RSV vaccinated mice. (**A**) Mice received prebiotic diet or control diet starting 2 weeks before i.m. vaccination with 50µl of FI-RSV. After RSV challenge at day 35 after vaccination mice were sacrificed at indicated time points. (**B**) Cell composition of BAL fluid, left total cell number, right indicated cell type as a percentage of total BAL cells. Percentages of eosinophils were significantly decreased in mice treated with the GFA diet (P<0.05). (**C**, **D**) Percentages of IFN-γ, IL-4, -5, -13 producing cells of total $CD4^+$ T cells and total IFN-γ, IL-4, -5, -13 producing $CD4^+$ T cell numbers after *in vitro* restimulation of lung $CD4^+$ T cells with unloaded (D1) or RSV loaded (D1+RSV) myeloid dendritic cells. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group.

TABLE II. Dietary intervention with scGOS/lcFOS/pAOS increases the RSV-specific lung CD8$^+$ T cell response in FI-RSV vaccinated mice.

	% NKG2A ^{POS}/ CD8 ^{POS}		*% IFN-γ ^{POS}/ CD8 ^{POS} (D1-RSV) §*		*%TET ^{POS}/ CD8 ^{POS}*		*% IFN-γ ^{POS}/ CD8 ^{POS} (NAITNAKII) §*	
	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA
Day 4	19.1±1.5	17.4±0.8	6.2±0.6	5.2±0.2	1.5±0.3	1.3±0.2	4.7±0.5	3.4±0.2
Day 6	22.7±2.1	25.0±1.9	13.0±1.2	14.1±1.3	5.1±0.5	6.8±0.9	6.3±0.8	8.3±1.4
Day 8	47.6±4.7	52.4±4.7	26.1±2.0	29.6±3.0	12.8±2.6	18.3±2.6 *	13.8±2.5	19.7±2.8 *

C57BL/6 mice received scGOS/lcFOS/pAOS (GFA) or control diet, starting 2 weeks before i.m. vaccination with 50µl of FI-RSV. After 35 days mice were i.n. challenged with 2.0x10^6 p.f.u. RSV. At indicated time points, lymphocytes were isolated from the lung parenchyma and stained for CD8 in combination with NKG2A or H-2D^b/M$_{187-195}$ tetramer. For *in vitro* T cell restimulation experiments, lung cells were stimulated with RSV infected D1 cells or the RSV epitope NAITNAKII and intracellularly stained for IFN-γ §. The values depicted represent the number of NKG2A$^+$/CD8$^+$, IFN-γ$^+$/CD8$^+$ or TET$^+$/CD8$^+$ double positive cells as a percentages of total CD8$^+$ T cells. The table shows the mean ± SEM of control or GFA treated mice from 2 individual experiments, n=8/group. Values denoted with * represent a significant (p<0.05) increase in NAITNAKII-specific (IFN-γ producing) CD8$^+$ T cells.

Dietary intervention with scGOS/lcFOS/pAOS lowers absolute numbers of CD11c$^+$ CD11b$^+$ DCs in lung tissue of FI-RSV vaccinated mice 6 days after RSV infection.

In the mouse lung two major subsets of DC have been described, CD11c^{low}/mPDCA-1$^+$ plasmacytoid DC (pDC) and myeloid or "conventional" CD11c$^+$ DC (cDC). These CD11c$^+$ cDCs can be further divided into CD11c$^+$MHCclass-II$^+$CD103$^-$CD11b^{high} (CD11b$^+$ DC), a subset located in the lung parenchyma that is important in leukocyte recruitment, and CD11c$^+$MHCclass-II$^+$CD103$^+$CD11b^{low} (CD103$^+$ DC) located underneath the epithelium, that can sample antigens from airways (3,50). Different DC populations might locally be involved in polarization of Th cell subsets (29,48). In this study the kinetics of lung DC populations present during the acute RSV response as well as the FI-RSV induced disease was investigated (Figure 3). During the acute RSV infection, no differences between the two dietary interventions on DC populations in the lungs were detected (data not shown). Within the FI-RSV vaccination model, the pDC population, known for its function in anti-viral immunity and rapid production of IFN-α, decreased from day 4 to 8 after intranasal challenge with RSV-A2, but no differences were observed between the mice receiving prebiotic or control diet. No differences were found in CD103$^+$ DC between the two intervention groups. CD11b$^+$ DC were the most abundant DC population on day 4-8 after viral challenge. This population was significantly larger (p<0.05) at day 6 in animals receiving control diet compared to mice receiving specific prebiotic diet (**Figure 3B**). However, at day 8 CD11b$^+$ DC numbers in the lungs decreased in both groups and reached similar levels. These data indicate that dietary intervention with *scGOS/lcFOS/pAOS* affected the lung CD11b$^+$ DC population, a subset known to be important in leukocyte recruitment, at day 6 after RSV challenge in the FI-RSV vaccination model.

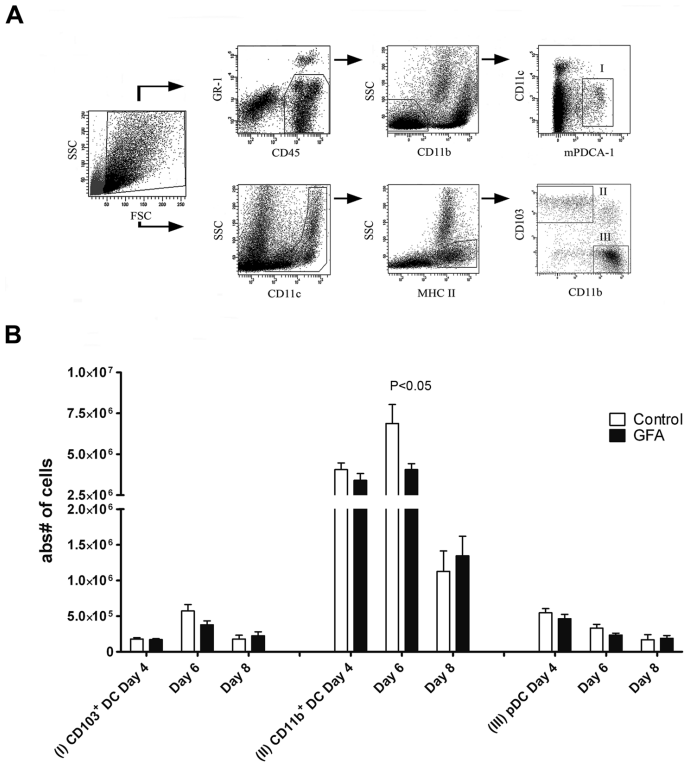


FIGURE 3. Dietary intervention with scGOS/lcFOS/pAOS (GFA) decreases CD11c$^+$ CD11b$^+$ DC influx in lung tissue of FI-RSV vaccinated mice at day 6 after RSV infection. (**A**) Lungs of naïve mice were used for gating strategies of lung DC populations, I (pDC), II (CD103$^+$ DC), III (CD11b$^+$ DC). (**B**) Day 4, 6 and 8 after intra-nasal challenge with RSV, absolute cell numbers of pDC, CD103$^+$ DC and CD11b$^+$ DC were determined. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group.

Dietary intervention with scGOS/lcFOS/pAOS in the C57BL/6 FI-RSV vaccination model does not affect weight loss, but improves viral clearance.

Weight loss can be used as a correlate of illness severity in mice models of respiratory infection. Therefore, we performed daily weight measurements in individual mice to monitor the effect of dietary intervention. During the 5 week period prior to infection the diet with *scGOS/lcFOS/pAOS* did not change the growth pattern of the mice compared to control diet (data not shown). In comparison to the BALB/c mouse model, C57BL/6 mice are relatively resistant to viral growth and weight loss (22,40,49). In accordance with these earlier studies, we did not observe significant weight loss during primary RSV infection and no differences were observed between the diet groups (data not shown). In addition, no significant weight loss was observed in the FI-RSV infection model, and again weight changes between the two diet groups were not significantly different (**Figure 4A**).

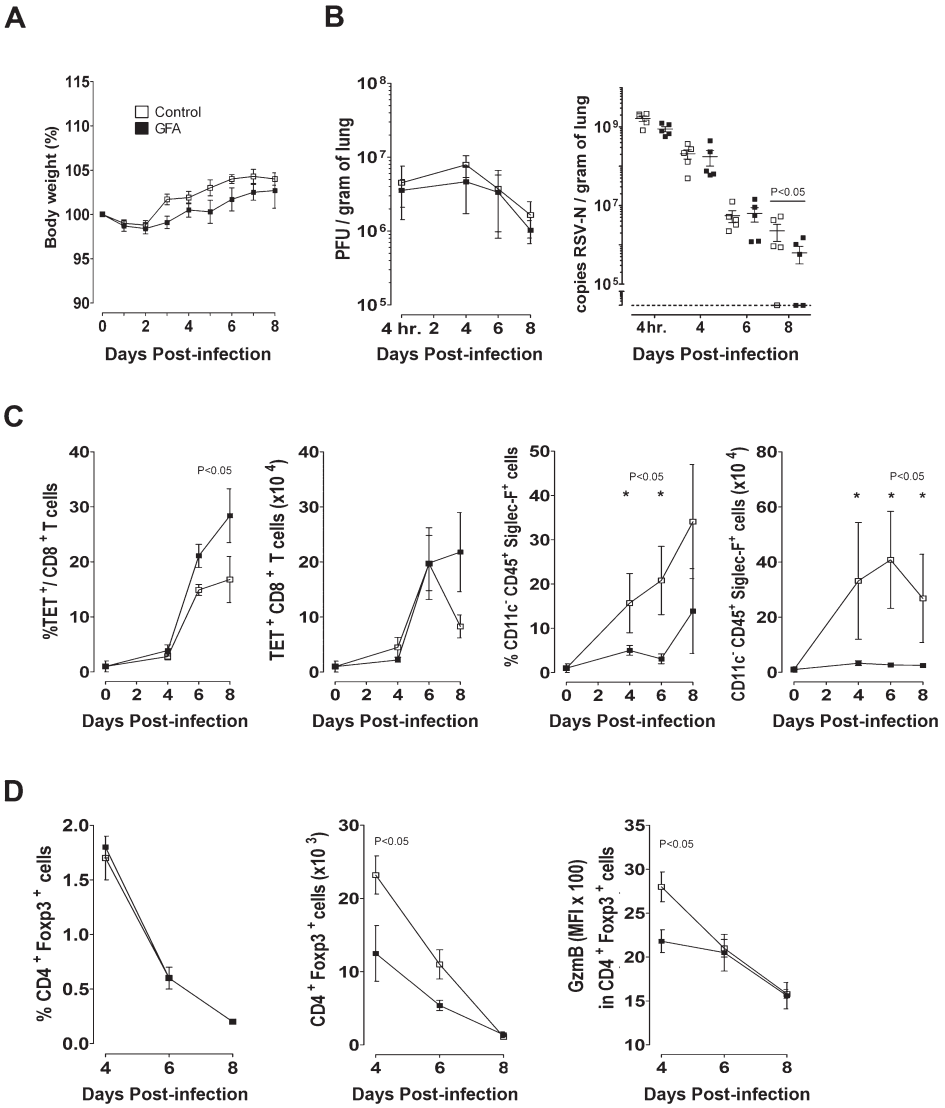


FIGURE 4. Dietary intervention with scGOS/lcFOS/pAOS (GFA) does not affect weight loss but has a significant effect on viral clearance in FI-RSV vaccinated mice. (**A**) Daily weight measurements were performed for 8 days after RSV infection. The percentage of original body weight is shown. (**B**) Viral replication in lungs of mice was measured by plaque assay and shown as PFU /gram of lung. Amounts of RSV A2 present in lung of FI-RSV vaccinated mice was measured by RT-PCR. Concentrations of RSV-N RNA were determined by comparing sample threshold values with a standard curve of RSV A2 and shown as copies RSV-N/gram of lung. (**C**) Development of the RSV-specific $CD8^+$ T cell response measured as H-$2D^b/M_{187-195}$ tetramer positive cells as a percentage of total $CD8^+$ T cells and absolute numbers in BAL. Kinetics of eosinophil ($CD45^+CD11c^-$Siglec-F^+) influx in BAL as a percentage of total BAL cells and depicted as absolute numbers. (**D**) Kinetics of $CD4^+Foxp3^+$ regulatory T cells in BAL of FI-RSV vaccinated mice at day 4, 6 and 8 after infection and GrzmB expression in $CD4^+Foxp3^+$ cells at these time points. Data shown represent the mean ± SEM of n=5 group. Significant differences were depicted as (*p<0.05)

Next, we addressed the question whether changes observed in immune responses caused by the dietary intervention affected the lung viral load and virus elimination kinetics. Lung viral load was measured in total lung tissue both in the primary and FI-RSV infection models after bronchoalveolar lavage. Virus titration was performed on HEp2 cells and total viral particle counts based on PCR were determined. Eight days after i.n infection with RSV significantly ($p<0.05$) lower viral copy counts were recovered from lungs of mice receiving *scGOS/lcFOS/pAOS* diet as compared to mice receiving control diet in the FI-RSV vaccinated mice (**Figure 4B**). During this experimental setup immunological parameters were monitored in the BAL. As observed before in the FI-RSV infection model (**Figure 2**), a substantial influx of eosinophils was observed in the BAL in the control diet group which was strongly inhibited in mice receiving the *scGOS/lcFOS/pAOS* diet. In addition, as observed in lung tissue (**Table 2**) we found a significantly ($p<0.05$) increased percentage of virus specific $CD8^+$ T cells in the BAL at day 8 after RSV challenge in FI-RSV vaccinated mice (**Figure 4C**).

Previously, changes in $CD25^+$ regulatory T cells (Tregs) were observed in an influenza vaccination model when the scGOS/*lcFOS/pAOS* diet was administered. In the experiments described in **figure 2** we observed a systemic decrease in Treg number in lung, spleen and mesenteric lymph nodes at day 4 after infection in FI-RSV vaccinated mice receiving the *scGOS/lcFOS/pAOS* diet (data not shown). Tregs have previously been found to regulate RSV specific primary immune responses after *in vivo* Treg depletion with antibodies (14,26,41) or after diphtheria toxin induced depletion in mice expressing diphtheria toxin receptor under the control of the *foxp3* gene locus (27). In the latter study the expression of granzyme B (GrzmB) in Treg locally in the lung was shown to be involved in the immune regulatory function of Tregs in the primary RSV infection model. Although the percentage of Tregs did not differ, a significantly decreased absolute influx of Tregs was detected in the BAL at day 4 after challenge in FI-RSV vaccinated mice receiving *scGOS/lcFOS/pAOS* diet. At this time point GrzmB expression was also significantly lower in Tregs of mice receiving the *specific prebiotic* diet (**Figure 4D**). This indicates that the dietary intervention with *scGOS/lcFOS/pAOS* has an effect on the function of regulatory immune cells which correlates with altered RSV specific immune responses.

DISCUSSION

In recent years, research in the field of immunology and microbiology has revealed that commensal bacteria in the mammalian host play a role that is not limited to digestive help alone. The composition and products of gut microbiota can influence host immune and inflammatory responses (32). Its effect on systemic immunity however, is still largely unknown and is a growing field of interest. In this study we demonstrate that a specific mixture of orally applied non-digestible oligosaccharides *scGOS/lcFOS/pAOS*, with known prebiotic properties (16,56) can regulate $CD4^+$ and $CD8^+$ T cell mediated immune responses in the lungs of RSV infected mice.

During primary infection, dietary intervention with *scGOS/lcFOS/pAOS* resulted in a lower cellular infiltrate in BAL and an increased virus specific $CD4^+$ IFN-γ response in the lungs of RSV infected mice. In a formalin-inactivated RSV (FI-RSV) vaccination model typical asthmatic parameters like airway hypersensitivity, airway eosinophilia, RSV specific IgE and a Th2 skewed cytokine profile are present (4,10,25). In this FI-RSV model dietary intervention with *scGOS/lcFOS/pAOS* reduced lung cell infiltration and airway eosinophila at day 4 and 6 after challenge and correlated with significant decreased numbers of GATA-3 expressing (data not shown) and numbers of RSV specific IL-4, -5, -13 producing $CD4^+$ T cells in the lungs of *scGOS/lcFOS/pAOS* receiving mice. Although a slightly different ratio of oligosaccharides was used in previous studies (9:1:2), these findings extend earlier observations that a specific prebiotic mixture decreases lower airway hyperreactivity, IgE serum levels and lung inflammatory cell influx in an ovalbumin induced murine asthma model (57). Furthermore, it has been reported that administration of *scGOS/lcFOS* in combination with Bifidobacterium breve M-16V for a period of 4 weeks significantly reduced systemic production of Th2 cytokines after allergen challenge in patients with asthma and house dust mite allergy (54). These studies all underscore the immune modulating and more specifically a Th1 supporting effect of these specific oligosaccharide mixtures.

In the FI-RSV induced Th2-dominated model we observed that decreased Th2 responsiveness at days 4 and 6 was accompanied by the development of an increased IFN-γ response in $CD4^+$ as well as $CD8^+$ T cells at day 8 after infection in the mice receiving scGOS/lcFOS/pAOS. Interestingly, the increase in IFN-γ production at day 8 post infection was also seen during primary RSV infection when Th2 responses do not play a significant role. The impact detected on viral load and physical condition of the mice was marginal in this model. How the Th1 and Th2 arms of the virus specific immune response reciprocally interact in these models is unclear. Suppression of Th2-induced disease in the FI-RSV model can be accomplished when $CD8^+$ T cell responses are boosted early during challenge i.e in mice pre-immunized with the dominant $CD8^+$ T cell epitope (34,35). However, the virus specific $CD8^+$ T cell response and $CD4^+$ T cell mediated IFN-γ responses were not different early after viral challenge. Furthermore, dietary induced differences in the magnitude of the Th2 response (day 4 and 6 in the FI-RSV model) preceded the difference in Th1 response by $CD4^+$ T cells and virus specific $CD8^+$ T cells (day 8/10) as well as viral clearance difference between the diets. Recently, a regulatory function of $CD4^+Foxp3^+$ T cells has been described during primary RSV infection. Several approaches; *in vivo* depletion with CD25-specific antibody, conditional depletion with diphtheria toxin during primary RSV infection in DEREG mice that express the diphtheria toxin receptor under control of the Foxp3 locus, or *in vivo* Treg activation by IL-2- immune complexes have shown that Tregs can regulate the early inflammatory response and the magnitude and quality of adaptive immune responses (14,26,27). In the present study we found a correlation between diminished $CD4^+Foxp3^+$ T cells in the lungs and increased virus specific $CD8^+$ T cell numbers at day 8 post challenge in the FI-RSV vaccination model. In addition, we

also found decreased GzmB expression in the $CD4^+Foxp3^+$ cells in the BAL of *scGOS/lcFOS/pAOS* receiving mice, another indication that regulatory T cell function in these mice might be influenced. In the FI-RSV experiment mice received the *scGOS/lcFOS/pAOS* diet from two weeks before vaccination through the entire experiment until they were sacrificed 8 days after viral challenge. Therefore, it is possible that dietary effects on Treg (or other immune cells) affected immune responses during priming as well as during the recall response. It has been shown in a murine model of experimental allergic airway inflammation performed with DEREG mice that depletion of Treg during the priming phase of the response led to an exacerbation of allergic airway inflammation affecting serum IgE levels and lung eosinophilia (2). Because of the similarities between OVA induced allergy models and the FI-RSV model (25), a direct effect of altered Treg function on the Th2 component in the immune response might be envisioned in the FI-RSV model without a role of a Th1-mediated suppression of Th2 responses and eosinophilia. More in-depth studies looking into the role of Tregs during dietary intervention are needed to address their specific role and function in time.

The exact mechanism(s) involved in systemic immune modulation by *scGOS/lcFOS/pAOS* is currently unknown. Because of the prebiotic capacities of the oligosaccharides tested in this study, it is tempting to speculate that microbial products or composition influences gut and systemic immunity. Studies with germ free, restricted flora or antibiotic treated mice have shown that commensal microbiota can influence systemic immunity by targeting specific cell types like plasmacytoid dendritic cells, invariant NKT cells, virus-specific $CD8^+$ memory cells and marginal zone B cells (13,51,58,59). Products from gut bacteria provide signals for pattern recognition receptors like NOD or Toll like receptors. Systemic immune response alterations appear to be caused by interaction of gut microbial components and such innate immune receptors. Ichinohe *et al.* showed that immune responses against respiratory tract influenza A virus infections could be influenced by gut commensal bacteria. Administration of broad spectrum antibiotics in mice resulted in incompetent virus-specific $CD4^+$ and $CD8^+$ T cell responses, a defect that could be completely restored by intra-rectal injection of the TLR4 ligand lipopolysaccharide (LPS) (21). Another recent study in mice showed that gut microbiota derived peptidoglycan is translocated from the gut into the systemic circulation. Systemic availability of peptidoglycan is sensed by Nod1 receptors and results in enhanced neutrophil mediated innate immunity (8). In addition to effects of bacterial composition or bacterial components, the observed immune modulation might be a result of direct interactions between oligosaccharides provided in the diet and host immune cells. Eiwegger *et al.* showed that small amounts of scGOS and lcFOS can cross the gut epithelial barrier, therefore these components may become systemically available (11). Lectins are known to bind carbohydrate structures and modulate immune responses. Galectins have been shown to control immune homeostasis and inflammation. Galectin-9/Tim-3 interactions regulate virus specific primary and memory $CD8^+$ T cell responses in Herpes Simplex virus infections and promote the induction of regulatory T cells (Tregs) (42,43).

In summary, in our study we show that dietary intervention with a specific prebiotic oligosaccharide mixture can influence host innate and T cell responses during a respiratory virus infection by modulation of the Th1/Th2 responses in the lungs. Therefore, prophylactic dietary supplementation of *scGOS/lcFOS/pAOS* in infant formula could be beneficial by accelerating post natal maturation of the infant immune system and potentiating protective immunity against respiratory virus infections with a high attack rate in early infancy such as RSV.

MATERIALS AND METHODS

Mice

Specific pathogen-free 3-4 week-old female C57BL/6 mice (Charles River Nederland Maastricht, The Netherlands) were housed under standard housing conditions. All animals had *ad libitum* access to tap water and diet. All study protocols were approved by the Animal Ethics Committee of the Medical Faculty of the Utrecht University.

Diet

AIN-93G-based diets were mixed with a specific oligosaccharide mixture (Research Diet Services, Wijk Bij Duurstede, The Netherlands) containing short chain Galacto-oligosacharide (scGOS: Borculo, Domo, Zwolle, The Netherlands, 45% scGOS), long-chain Fructo-oligosaccharide (lcFOS: Orafti, Wijchen, The Netherlands, 100% lcFOS) and Pectin derived Acidic oligosaccharides (pAOS: Sudsucker, Mannheim, Germany, 5% galacturonic acid) in a 9:1:10 ratio based on carbohydrate purity. This specific ratio is based on earlier described immune modulating capacities in human and mice (16,53). The specific oligosaccharides were exchanged against 2% (wt:wt) of total carbohydrates present in the control diet.

Virus and infection

RSV strain A2 (VR-1302, ATCC) was grown on HEp-2 cells (CCL-23, ATCC) purified by polyethylene glycol 6000 precipitation and stored in phosphate-buffered saline (PBS) with 10% sucrose in liquid nitrogen until further use. Mice were anesthetized with isoflurane and intranasally infected with $4x10^6$ plaque forming units (pfu) RSV in a volume of 50 µl diluted in PBS.

FI-RSV vaccine and vaccination

Formalin-inactivated (FI)-RSV was prepared by the method used for the original vaccine as tested in the 1960's in infants (39). RSV A2 strain was grown for 48 hrs in HEp-2 cells. Culture

medium was cleared from cell debris by low speed centrifugation (1000xg, 10 min, 4°C). Formalin (F8775, Sigma-Aldrich) was added to $3x10^6$ pfu RSV/ml containing supernatant at a final dilution of 1:4000 and incubated at 37°C for 3 days with stirring. After ultra-centrifugation (50,000 g_{av}, 1h, 4°C) of the FI-RSV preparation, resulting pellets were resuspended to $1/25^{th}$ of the original volume in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Invitrogen) without supplements. FI-RSV was adsorbed to 4 mg/ml aluminium hydroxide (A1577, Sigma-Aldrich) overnight at room temperature while stirring. Finally, FI-RSV was pelleted by centrifugation (1000xg, 30 min) and resuspended to $1/4^{th}$ volume in PBS. This procedure resulted in a final dosage that was concentrated 100-fold and contained 16 mg/ml aluminium hydroxide. At day 0, mice were intramuscularly (i.m.) injected with 50 µl FI-RSV vaccine preparation.

Tissue isolation and preparation

Mice were sacrificed at different time points, as indicated in the figure legends, by i.p. injection of 300 µl pentobarbital. Cells from the airways were obtained by broncho-alveolar lavage (BAL) with 3 x 1 ml of 0.15 M NaCl. Prior to removal, the lungs were perfused with PBS containing 100 U/ml heparin. Lungs were cut to 1 x 1 mm pieces and incubated with collagenase (2.4 mg/ml; 10103586001, Roche Applied Science) and DNase (1 mg/ml; 10104159001, Roche Applied Science) for 20 min. at 37°C. Single cell suspensions were prepared by processing the tissue trough 70 µm cell strainers (BD Falcon, BD Biosciences).

RSV-specific real-time PCR

For preparation of lung tissue homogenates, isolated snap-frozen whole lungs were crushed on dry ice, dissolved in PBS and stored at -80 ^{0}C until further use. Viral replication in lung homogenates was determined, as earlier described, via plaque assay (24). To measure total virus copy numbers by PCR, total RNA from lung homogenates was extracted using MagnaPure LC equipment, cDNA was synthesized, and viral loads were determined by real-time PCR as recently described (19). In short, viral genomic RNA was isolated using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). The isolated viral RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA), according to the manufacturer's guidelines. Primers and probes designed on the basis of highly conserved genomic regions of the N gene for both RSV subgroup A (RSV-A) and B (RSV-B) were used for subtyping of the RSV patient strains. The following primers and probes were used:

RSA-1: 5'-AGATCAACTTCTGTCATCCAGCAA-3'
RSA-2: 5'-TTCTGCACATCATAATTAGGAGTATCAAT-3'
RSB-1: 5'-AAGATGCAAATCATAAATTCACAGGA-3'

RSB-2: 5'-TGATATCCAGCATCTTTAAGTATCTTTATAGTG-3'
RSA probe: 5'-CACCATCCAACGGAGCACAGGAGAT-3'
RSB probe: 5'-TTCCCTTCCTAACCTGGACATAGCATATAACATACCT-3'

Murine encephalomyocarditis virus (RNA) was used as an internal control. Samples were assayed in a 25 µl reaction mixture containing 10 µl of cDNA, TaqMan universal PCR master mix (Applied Biosystems, ABI), primers (900 nM RSV-A primers and 300 nM RSV-B primers), and fluorogenic probes (58.3 nM RSV-A probe and 66.7 nM RSV-B probe) labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an ABI 7900 HT system for 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 sec at 95°C and 1 min at 60°C. Samples were controlled for the presence of possible inhibitors of the amplification reaction by the indicated internal control, signals of which had to range within a clear-cut interval. Sample cycle threshold values (C^t) were compared with a standard curve of RSV A2.

In vitro re-stimulation

Isolated lung cells were re-stimulated with a synthetic peptide representing a dominant H-2 restricted RSV epitope or a dendritic cell line infected with RSV. For peptide stimulation, single cell suspensions of lung cells ($1x10^6$ cells) were incubated with the H-$2D^b$ restricted peptide (1 µg/ml) from the RSV M protein ($M_{187-195}$, NAITNAKII) (28). Restimulation with RSV was accomplished by co-culturing lung cells ($1x10^6$ cells) with RSV infected D1 cells ($2x10^5$ cells). D1 is a non-transformed, growth factor-dependent, myeloid dendritic cell line derived from C57BL/6 mice (60). D1 cells were maintained in IMDM, 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin and 50µM β-mercapto-ethanol and supplemented with 30% conditioned medium from GM-CSF producing R1 cells (mouse fibroblast NIH3T3, transfected with the GM-CSF gene (12). D1 cells were infected for a period of 48 hours with RSV (multiplicity of infection, m.o.i 2) before addition to the lung cell suspension. Cell suspensions were stimulated for 6 h at 37°C, 5% CO_2 in 200 µl IMDM supplemented with 2 mM L-glutamine, 25 mM Hepes buffer, 5% FCS, penicillin/streptomycin, 50 µM β-mercapto-ethanol and 50 U/ml recombinant human IL-2 (11147528001, Roche). Brefeldin A 10 µg/ml (B7651, Sigma-Aldrich) was added for the duration of the stimulation to facilitate intracellular accumulation of cytokines.

Cell surface and intracellular cytokine staining

Lung single cell suspensions were first pre-incubated with 2.4.G2 an Fc receptor specific Ab (anti-CD16/32) to reduce non-specific binding and then stained for 4- or 5-color flow cytometry with the following antibodies (from BD Biosciences unless otherwise stated). To identify mDC populations cells were stained with: anti-CD11c (clone HL3), anti-MHC-II (I-A^b/I-E^b,

clone AF6-120.1), anti-CD103 (clone M290) and anti-CD11b (clone M1/70). For pDC populations cells were incubated with anti-Ly6C (clone RB6-8C5), anti-CD45R (clone RA3-6B2), anti-CD11b (clone M1/70), anti-CD11c (clone HL3) and anti-mPDCA-1(Miltenyi Biotec, clone JF05-1C2.4.1). BAL eosinophils were identified with anti-CD45R (clone RA3-6B2), anti CD11c (clone HL3) and anti-Siglec-F (clone E50-2440). RSV specific $CD8^+$ T cells were visualized with MHC class I - $M_{187-195}$ tetramer, manufactured as previously described (18). Cytokine production by $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. Cells were washed with FACS buffer and stained for surface markers with anti-NKG2A (clone 20d5), anti-CD8 (clone 53-6.7) and anti-CD4 (clone RM4-5). For intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (BD, 554722) solution and Perm/Wash buffer (BD, 554723). Intracellular cytokines were detected with anti-IFN-γ (clone XMG1.2), anti-IL-5 (clone TRFK5), anti-IL-4 (clone 11B11), anti-IL-13 (eBioscience, clone eBio13a). Stained samples were measured on a FACSCanto II flowcytometer (BD, San Diego, CA) and analyzed using FacsDiva software (BD, San Diego, CA).

BAL fluid leukocyte composition

Lung cells were spun onto glass slides (Shandon cytospin, Pittsburgh, Pa) and fixed with 100% methanol for 5 min. Subsequently, cell nuclei were stained in a 1:1 dilution of May-Grünwald (Mallinckrodt Baker, 3855) with buffered water pH 6.8 for 5 min. After washing with buffered water pH 6.8, cells were stained with a 1:8 dilution of Giemsa (Merck, 1.09204.500) with buffered water pH 6.8 for 15 min. and finally washed with water. For determination of cell composition one hundred cells per sample were counted.

Statistical analysis

For all experiments, the difference between groups was calculated using a Two-Way ANOVA followed by the Bonferroni test (Graphpad Prism version 4: Graphpad, San Diego). Data are expressed as the mean $^+/-$ standard error of the mean (SEM). The p values deemed significant are indicated in the figures as follows: $p < 0.05$.

ACKNOWLEDGEMENTS

This study was financially supported and performed within the framework of the Dutch Top Institute Pharma project: D1-101-0. M.A. Schijf, J. Bastiaans, J. Garssen and B. van't Land are employees of Dept. of Immunology, Danone Research–Center for Specialised Nutrition, Wageningen, The Netherlands.

REFERENCES

1. Arslanoglu, S., G. E. Moro, J. Schmitt, L. Tandoi, S. Rizzardi, and G. Boehm. 2008. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. J. Nutr. 138:1091-1095.
2. Baru, A. M., A. Hartl, K. Lahl, J. K. Krishnaswamy, H. Fehrenbach, A. O. Yildirim, H. Garn, H. Renz, G. M. Behrens, and T. Sparwasser. 2010. Selective depletion of Foxp3+ Treg during sensitization phase aggravates experimental allergic airway inflammation. Eur. J. Immunol. 40:2259-2266.
3. Beaty, S. R., C. E. Rose, Jr., and S. S. Sung. 2007. Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. J. Immunol. 178:1882-1895.
4. Becker, Y. 2006. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. Virus Genes 33:235-252.
5. Belderbos, M. E., G. M. van Bleek, O. Levy, M. O. Blanken, M. L. Houben, L. Schuijff, J. L. Kimpen, and L. Bont. 2009. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin. Immunol. 133:228-237.
6. Bryan, D. L., P. H. Hart, K. D. Forsyth, and R. A. Gibson. 2007. Immunomodulatory constituents of human milk change in response to infant bronchiolitis. Pediatr. Allergy Immunol. 18:495-502.
7. Castilow, E. M., M. R. Olson, and S. M. Varga. 2007. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol. Res. 39:225-239.
8. Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 16:228-231.
9. de Filippo, C., D. Cavalieri, P. M. Di, M. Ramazzotti, J. B. Poullet, S. Massart, S. Collini, G. Pieraccini, and P. Lionetti. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc. Natl. Acad. Sci. U. S. A 107:14691-14696.
10. de Swart, R. L., T. Kuiken, H. H. Timmerman, A. G. van, B. G. van den Hoogen, H. W. Vos, H. J. Neijens, A. C. Andeweg, and A. D. Osterhaus. 2002. Immunization of macaques with formalin-inactivated respiratory syncytial virus (RSV) induces interleukin-13-associated hypersensitivity to subsequent RSV infection. J. Virol. 76:11561-11569.
11. Eiwegger, T., B. Stahl, P. Haidl, J. Schmitt, G. Boehm, E. Dehlink, R. Urbanek, and Z. Szepfalusi. 2010. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr. Allergy Immunol. 21:1179-1188.
12. Foti, M., F. Granucci, D. Aggujaro, E. Liboi, W. Luini, S. Minardi, A. Mantovani, S. Sozzani, and P. Ricciardi-Castagnoli. 1999. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. Int. Immunol. 11:979-986.
13. Fujiwara, D., B. Wei, L. L. Presley, S. Brewer, M. McPherson, M. A. Lewinski, J. Borneman, and J. Braun. 2008. Systemic control of plasmacytoid dendritic cells by CD8+ T cells and commensal microbiota. J. Immunol. 180: 5843-5852.
14. Fulton, R. B., D. K. Meyerholz, and S. M. Varga. 2010. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. J. Immunol. 185:2382-2392.

15. Glezen, W. P., L. H. Taber, A. L. Frank, and J. A. Kasel. 1986. Risk of primary infection and reinfection with respiratory syncytial virus. Am. J. Dis. Child 140:543-546.
16. Gori, A., G. Rizzardini, B. van't Land, K. Ben Amor, J. van Schaik, C. Torti, T. Quirino, C. Tincati, A. Bandera, J. Knol, K. lhassan-Chahour, D. Trabattoni, D. Bray, A. Vriesema, G. Welling, J. Garssen, and M. Clerici. 2011. Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the "COPA" pilot randomized trial. Mucosal. Immunol. 5: 544-563.
17. Gruber, C., S. M. van, F. Mosca, G. Moro, G. Chirico, C. P. Braegger, J. Riedler, G. Boehm, and U. Wahn. 2010. Reduced occurrence of early atopic dermatitis because of immunoactive prebiotics among low-atopy-risk infants. J. Allergy Clin. Immunol. 126: 791-797.
18. Haanen, J. B., M. Toebes, T. A. Cordaro, M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher. 1999. Systemic T cell expansion during localized viral infection. Eur. J. Immunol. 29:1168-1174.
19. Houben, M. L., F. E. Coenjaerts, J. W. Rossen, M. E. Belderbos, R. W. Hofland, J. L. Kimpen, and L. Bont. 2010. Disease severity and viral load are correlated in infants with primary respiratory syncytial virus infection in the community. J. Med. Virol. 82:1266-1271.
20. Hummelen, R., A. P. Vos, B. van't Land, K. van Norren, and G. Reid. 2010. Altered host-microbe interaction in HIV: a target for intervention with pro- and prebiotics. Int. Rev. Immunol. 29:485-513.
21. Ichinohe, T., I. K. Pang, Y. Kumamoto, D. R. Peaper, J. H. Ho, T. S. Murray, and A. Iwasaki. 2011. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. U. S. A 108: 5354-5359.
22. Jessen, B., S. Faller, C. D. Krempl, and S. Ehl. 2011. Major histocompatibility complex-dependent cytotoxic T lymphocyte repertoire and functional avidity contribute to strain-specific disease susceptibility after murine respiratory syncytial virus infection. J. Virol. 85:10135-10143.
23. Koenig, J. E., A. Spor, N. Scalfone, A. D. Fricker, J. Stombaugh, R. Knight, L. T. Angenent, and R. E. Ley. 2011. Succession of microbial consortia in the developing infant gut microbiome. Proc. Natl. Acad. Sci. U. S. A 108 Suppl 1:4578-4585.
24. Kruijsen, D., M. J. Bakkers, N. O. van Uden, M. C. Viveen, T. C. van der Sluis, J. L. Kimpen, J. H. Leusen, F. E. Coenjaerts, and G. M. van Bleek. 2010. Serum antibodies critically affect virus-specific CD4+/CD8+ T cell balance during respiratory syncytial virus infections. J. Immunol. 185:6489-6498.
25. Kruijsen, D., M. A. Schijf, M. V. Lukens, N. O. van Uden, J. L. Kimpen, F. E. Coenjaerts, and G. M. van Bleek. 2011. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. Vaccine 29:2730-2741.
26. Lee, D. C., J. A. Harker, J. S. Tregoning, S. F. Atabani, C. Johansson, J. Schwarze, and P. J. Openshaw. 2010. CD25+ natural regulatory T cells are critical in limiting innate and adaptive immunity and resolving disease following respiratory syncytial virus infection. J. Virol. 84:8790-8798.
27. Loebbermann, J., H. Thornton, L. Durant, T. Sparwasser, K. E. Webster, J. Sprent, F. J. Culley, C. Johansson, and P. J. Openshaw. 2012. Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. Mucosal. Immunol. 5:161-172.
28. Lukens, M. V., E. A. Claassen, P. M. de Graaff, M. E. van Dijk, P. Hoogerhout, M. Toebes, T. N. Schumacher, R. G. van der Most, J. L. Kimpen, and G. M. van Bleek. 2006. Characterization of the CD8+ T cell responses directed against respiratory syncytial virus during primary and secondary infection in C57BL/6 mice. Virology 352:157-168.

29. Lukens, M. V., D. Kruijsen, F. E. Coenjaerts, J. L. Kimpen, and G. M. van Bleek. 2009. Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. J. Virol. 83: 7235-7243.
30. Ly, N. P., A. Litonjua, D. R. Gold, and J. C. Celedon. 2011. Gut microbiota, probiotics, and vitamin D: Interrelated exposures influencing allergy, asthma, and obesity? J. Allergy Clin. Immunol.
31. Martinez, F. D. 2003. Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. Pediatr. Infect. Dis. J. 22: S76-S82.
32. Maslowski, K. M. and C. R. Mackay. 2011. Diet, gut microbiota and immune responses. Nat. Immunol. 12:5-9.
33. Moro, G., I. Minoli, M. Mosca, S. Fanaro, J. Jelinek, B. Stahl, and G. Boehm. 2002. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. J. Pediatr. Gastroenterol. Nutr. 34:291-295.
34. Olson, M. R., S. M. Hartwig, and S. M. Varga. 2008. The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia. J. Immunol. 181:7958-7968.
35. Olson, M. R. and S. M. Varga. 2007. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. J. Immunol. 179:5415-5424.
36. Olson, M. R. and S. M. Varga. 2008. Pulmonary immunity and immunopathology: lessons from respiratory syncytial virus. Expert. Rev. Vaccines. 7:1239-1255.
37. Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown. 2007. Development of the human infant intestinal microbiota. PLoS. Biol. 5:e177.
38. Pineiro, M., N. G. Asp, G. Reid, S. Macfarlane, L. Morelli, O. Brunser, and K. Tuohy. 2008. FAO Technical meeting on prebiotics. J. Clin. Gastroenterol. 42 Suppl 3 Pt 2:S156-S159.
39. Prince, G. A., S. J. Curtis, K. C. Yim, and D. D. Porter. 2001. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. J. Gen. Virol. 82: 2881-2888.
40. Prince, G. A., R. L. Horswood, J. Berndt, S. C. Suffin, and R. M. Chanock. 1979. Respiratory syncytial virus infection in inbred mice. Infect. Immun. 26:764-766.
41. Ruckwardt, T. J., K. L. Bonaparte, M. C. Nason, and B. S. Graham. 2009. Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. J. Virol. 83:3019-3028.
42. Sehrawat, S., P. B. Reddy, N. Rajasagi, A. Suryawanshi, M. Hirashima, and B. T. Rouse. 2010. Galectin-9/TIM-3 interaction regulates virus-specific primary and memory CD8 T cell response. PLoS. Pathog. 6:e1000882.
43. Sehrawat, S., A. Suryawanshi, M. Hirashima, and B. T. Rouse. 2009. Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. J. Immunol. 182:3191-3201.
44. Shay, D. K., R. C. Holman, R. D. Newman, L. L. Liu, J. W. Stout, and L. J. Anderson. 1999. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 282: 1440-1446.
45. Sigurs, N., R. Bjarnason, F. Sigurbergsson, and B. Kjellman. 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am. J. Respir. Crit Care Med. 161:1501-1507.
46. Simoes, E. A., X. Carbonell-Estrany, C. H. Rieger, I. Mitchell, L. Fredrick, and J. R. Groothuis. 2010. The effect of respiratory syncytial virus on subsequent recurrent wheezing in atopic and nonatopic children. J. Allergy Clin. Immunol. 126:256-262.

47. Sin, D. D. and E. R. Sutherland. 2008. Obesity and the lung: 4. Obesity and asthma. Thorax 63:1018-1023.
48. Smit, J. J., D. M. Lindell, L. Boon, M. Kool, B. N. Lambrecht, and N. W. Lukacs. 2008. The balance between plasmacytoid DC versus conventional DC determines pulmonary immunity to virus infections. PLoS. One. 3: e1720.
49. Stark, J. M., S. A. McDowell, V. Koenigsknecht, D. R. Prows, J. E. Leikauf, A. M. Le Vine, and G. D. Leikauf. 2002. Genetic susceptibility to respiratory syncytial virus infection in inbred mice. J. Med. Virol. 67:92-100.
50. Sung, S. S., S. M. Fu, C. E. Rose, Jr., F. Gaskin, S. T. Ju, and S. R. Beaty. 2006. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J. Immunol. 176:2161-2172.
51. Tanaka, K., S. Sawamura, T. Satoh, K. Kobayashi, and S. Noda. 2007. Role of the indigenous microbiota in maintaining the virus-specific CD8 memory T cells in the lung of mice infected with murine cytomegalovirus. J. Immunol. 178:5209-5216.
52. Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan, R. E. Ley, M. L. Sogin, W. J. Jones, B. A. Roe, J. P. Affourtit, M. Egholm, B. Henrissat, A. C. Heath, R. Knight, and J. I. Gordon. 2009. A core gut microbiome in obese and lean twins. Nature 457: 480-484.
53. van't Land, B., M. Schijf, B. C. van Esch, J. Bastiaans, B. Schouten, L. Boon, and J. Garssen. 2010. Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. Vaccine 28:5711-5717.
54. van de Pol, M. A., R. Lutter, B. S. Smids, E. J. Weersink, and J. S. van der Zee. 2011. Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics. Allergy 66:39-47.
55. van Hoffen, E., B. Ruiter, J. Faber, L. M'rabet, E. F. Knol, B. Stahl, S. Arslanoglu, G. Moro, G. Boehm, and J. Garssen. 2009. A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. Allergy 64: 484-487.
56. Vos, A. P., J. Knol, B. Stahl, L. M'rabet, and J. Garssen. 2010. Specific prebiotic oligosaccharides modulate the early phase of a murine vaccination response. Int. Immunopharmacol. 10:619-625.
57. Vos, A. P., B. C. van Esch, B. Stahl, L. M'rabet, G. Folkerts, F. P. Nijkamp, and J. Garssen. 2007. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. Int. Immunopharmacol. 7:1582-1587.
58. Wei, B., T. T. Su, H. Dalwadi, R. P. Stephan, D. Fujiwara, T. T. Huang, S. Brewer, L. Chen, M. Arditi, J. Borneman, D. J. Rawlings, and J. Braun. 2008. Resident enteric microbiota and CD8+ T cells shape the abundance of marginal zone B cells. Eur. J. Immunol. 38: 3411-3425.
59. Wei, B., G. Wingender, D. Fujiwara, D. Y. Chen, M. McPherson, S. Brewer, J. Borneman, M. Kronenberg, and J. Braun. 2010. Commensal microbiota and CD8+ T cells shape the formation of invariant NKT cells. J. Immunol. 184:1218-1226.
60. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J. Exp. Med. 185:317-328.

Chapter 8

Broad spectrum antibiotics increase Th2 responses and lung eosinophilia in a mouse Respiratory Syncytial Virus vaccination model.

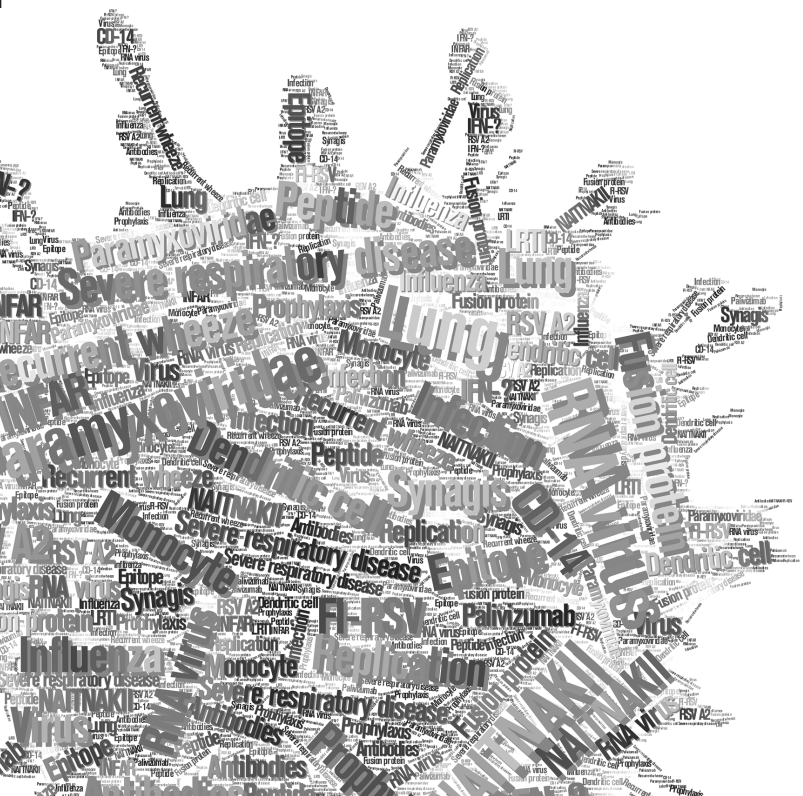
Schijf M.A.*, Wopereis H.†, Coenjearts F.E.# , Garssen J†‡, van Bleek G.M.*, van't Land B*†

*Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands.

#Department of Medical Microbiology, University Medical Center, Utrecht, The Netherlands.

†Department of Immunology and Microbiology, Nutricia Research, Utrecht, The Netherlands.

‡Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands



In preparation

ABSTRACT

The host-microbiome interplay is one of the new areas in human health research, including airway diseases. Bacterial colonization of mucosal surfaces is an important factor for maturation of neonatal immunity in the first year of life shifting immune responses from Th2 biased towards a more Th1 type of response. Factors like, caesarean section, antibiotics and diet affect microbial populations and could potentially influence immune maturation. Neonatal Th2 biased immunity has been suggested to contribute to increased risk of developing respiratory syncytial virus (RSV) induced severe disease. RSV is estimated to be responsible for up to 22% of severe lower respiratory tract infections (LRTI) in children under 5 years of age. We investigated the impact of microbiome disruption, induced by broad spectrum antibiotics, on immune responses against RSV, in a murine formalin-inactivated (FI-) RSV vaccination model for enhanced disease. Orally administered antibiotics applied during vaccination had no effect on body weight and viral clearance but affected both cellular and humoral arms of the adaptive immune response against RSV. Antibiotic treatment reduced microbial diversity and increased RSV specific Th2 cytokine (IL-4, IL-5 and IL-13)-producing $CD4^+$ T cell numbers and airway eosinophilia in the lung at day 6 after challenge infection. This was accompanied by a significant decrease in IFN-γ and TNF-α producing $CD4^+$ T cells in the lung and lower RSV specific IgG2c antibody titers in serum. Our model suggests that microbiome disruption via broad spectrum antibiotics affects host immune responses against RSV, causing a Th2 biased response. Because the infant microbiome is important to develop the capacity to mount Th1-type immune responses, our study indicates that the effect of antibiotics administered to new-borns should be evaluated carefully especially for their potential to inhibit immune maturation. Extrapolation from our mouse model suggests that antibiotics used in the neonatal period might increase the risk of developing severe RSV bronchiolitis and susceptibility to develop allergic disease later in life.

INTRODUCTION

Respiratory syncytial virus (RSV) infection remains a serious threat to neonates, immune compromised individuals and elderly. Primary RSV infections occur within the first 3 years of life and can cause severe bronchiolitis and pneumonia, particularly in infants < 6 months of age [1,2]. In children diagnosed with RSV-induced severe lower respiratory tract infections (LRTI), the use of antibiotics is only recommended when a secondary bacterial co-infection is suspected. However, numerous pathogens may cause similar symptoms of respiratory illness and clinical characteristics are rarely distinctive to accurately diagnose RSV induced disease [3,4] and therefore antibiotics are frequently prescribed to children hospitalised with RSV-induced bronchiolitis [5–7]. The benefits of antibiotic treatment in children diagnosed with RSV-induced severe LRTI are minor [8] and the use of antibiotics to prevent persistent respiratory symptoms in the RSV induced post-acute bronchiolitis phase has shown to be ineffective [9]. The outcome of early antibiotic treatment on the onset of adaptive immune responses or immunological memory is not known.

Neonatal exposure to antibiotics and severe episodes of RSV bronchiolitis are both factors associated with the susceptibility to develop asthma-like symptoms later in life. In early life antibiotic usage, time of prescription and frequency are both factors that determine the risk of subsequent development of atopic asthma and allergic disease [10–16]. A relation between RSV bronchiolitis and the susceptibility to develop asthma/recurrent wheeze (RW) has been suggested [17]. Several studies have identified LRTIs occurring during the first few years of life as independent risk factors for subsequent development of asthma [18–20] and others showed a strong correlation between severe RSV bronchiolitis in infancy and recurrent wheeze [21,22]. The reason why children diagnosed with severe RSV bronchiolitis are at a risk of developing recurrent wheeze and atopic disease is not known, but there is evidence that prevention of RSV infection by passive antibody therapy reduces the risk of long-term respiratory illness, indicating that severe RSV infection at a critical period of postnatal development may lead to persistent problems in later life [23]. Neonatal immunity is immature, Th2 biased and less effective compared to the stronger Th1 biased responses in older children and adults. To provide complete protection against infections, the neonatal immune system relies on innate immune responses and maternal antibodies transferred in utero and through breast milk. Microbial stimuli play a key role in the maturation process of neonatal immunity [24,25]. Since the first description of the "hygiene hypothesis" in 1989 [26] it is now well recognized that environmental factors, either directly or indirectly, have a decisive role in microbiome formation and the maturation of mucosal immunity [27]. In addition to the role of the microbiome as a factor in the development of uncontrolled inflammatory responses in asthmatic disease [28,29] correlations between the microbiome present in lung and gut and viral airway infections are also emerging [30]. A recent mouse respiratory viral infection study reported that commensal microbiota composition in the gut regulated the

generation of virus-specific immune responses following influenza virus infection [31] via a inflammasome mediated mechanism. Another study showed that pharyngeal microflora disruption by antibiotics promoted airway hyperresponsiveness after primary respiratory syncytial virus infection [32]. This suggests a clear correlation between the influence of the microbiome in the lung and gut ameliorating viral airway infections.

For RSV it is well known that inactivated RSV vaccine cause disease increase upon later natural virus exposure. This was first shown in a vaccine trial with formalin- inactivated RSV formulated with alum as the adjuvant [33]. Many vaccinated children needed hospitalization during the RSV season following vaccination, with fatal outcome for two children. Similar enhanced disease symptoms have been reproduced in animal models and appears to result from a strong Th2 type RSV specific CD4 T cell response and eosinophilia accompanied by low affinity antibody responses and low virus specific CD8 T cell numbers in mouse models [34]. The presumed mechanism is a lack of TLR activation during the priming phase of the adaptive immune response [35] and possibly there is an additional role of formalin treatment, disrupting antigenic structures recognized by antibodies.

In this study, the effect of oral administration of broad spectrum antibiotics during the vaccination phase is evaluated on RSV specific lung T cell responses in the murine formalin-inactivated (FI)-RSV vaccination model and preliminary correlates between microbiome composition and host immunity were evaluated.

RESULTS

Antibiotics do not affect viral clearance and disease severity, but affect the cellular response against RSV six days post infection.

To address the question whether microbal disruption through antibiotic (Abt) treatment, could affect local lung immune responses against RSV, C57BL/6 mice were fed a combination of broad spectrum antibiotics consisting of Ampicillin, Vancomycin, Neomycin sulfate and Metronidazole in drinking water for a period of 4 weeks. In a bio-contained environment (Isocage®), antibiotic treatment started 2 weeks prior to intra muscular (i.m.) FI-RSV vaccination (d14) until 2 weeks thereafter. Thirty five days after vaccination, mice were intra nasally (i.n.) challenged with RSV. Six days post infection mice were sacrificed, lung cell composition analyzed and lung T cell responses determined after *in vitro* re-stimulation of T cells with RSV infected dendritic cells (**Figure 1A**). Weight loss can be used as a correlate for disease severity in mouse models of respiratory infection. Orally applied antibiotics resulted in significantly ($p<0.05$) lower body weights compared to control mice (98.3 ±3.1% vs. 108.9 ±1.0%) in the first week of treatment, presumably due to the foul taste of Metronidazole [36] (**Figure 1B**). At the time of vaccination and in the subsequent 5 weeks before i.n. challenge, antibiotic treated mice recovered in weight and during this phase weights in both

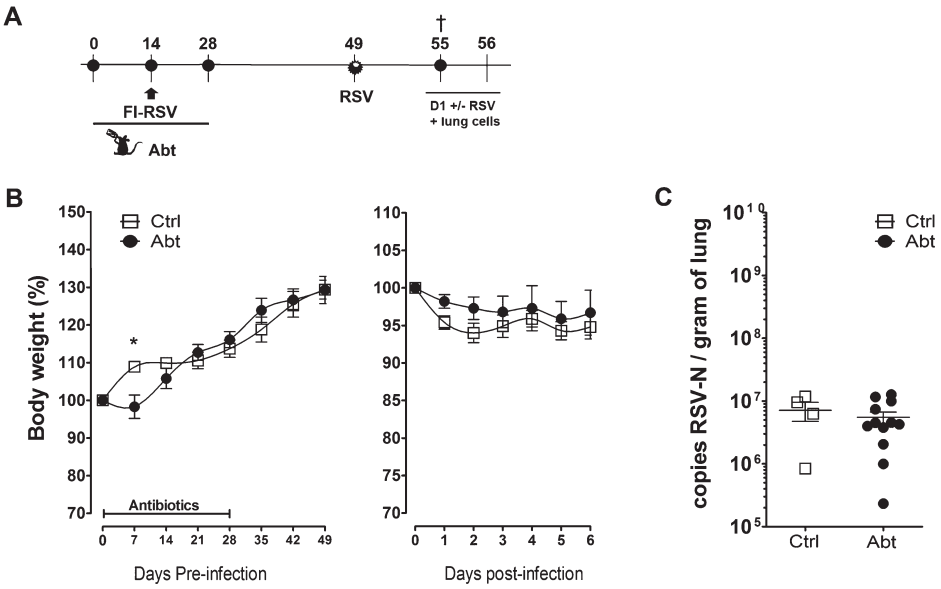


FIGURE 1. Broad spectrum antibiotics have no effect on viral clearance and RSV specific disease severity **(A)** Mice housed under bio-contained sterile conditions using HEPA® filtered isocages® received plain or antibiotics supplemented water 2 weeks before and 2 weeks after i.m. vaccination with FI-RSV. 5 weeks after vaccination, mice were intranasally infected with $4x10^6$ plaque forming units (pfu) RSV and sacrificed 6 days post infection. **(B)** Weight measurements, as a parameter for disease severity, were performed weekly pre-infection and daily post-infection. Data are shown as a percentage of the original body weight measured. **(C)** Amounts of RSV A2 present in lung of FI-RSV vaccinated mice day 6 post infection was measured by RT-PCR. Concentrations of RSV-N RNA were determined by comparing sample threshold values with a standard curve of RSV A2 and shown as copies RSV-N/gram of lung. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group, except for control group figure B (n=4). Significant differences were depicted as (*p<0.05).

groups were similar. Although C57BL/6 mice are relatively resistant to viral growth and weight loss upon RSV infection [37,38], a decrease in body weight (2-5%) was observed in the first 3 days post i.n. infection. Antibiotic treated and control animals showed no significant differences in bodyweight post infection (**Figure 1B**). The amount of virus present on day 6 post infection was measured by PCR in lung tissue after bronchoalveolar lavage. No difference was detected between antibiotic treated and control animals (**Figure 1C**). The possible effect of antibiotic treatment on the influx of lung leucocyte subpopulations was investigated via phenotyping of parenchymal $CD45^+$ $CD11c^-$ Siglec-F^+ eosinophils, $NK1.1^+$ $CD3^-$ NK cells, $CD3^-$ $CD19^+$ $B220^+$ B cells and $CD3^+$ $CD4^+$ or $CD8^+$ T cells using flowcytometric analysis (**Figure 2A**). Antibiotic treated mice showed significantly lower infiltration of absolute lung $CD4^+$ T cell numbers compared to control mice (1.1 ± 0.1 vs. 1.7 ± 0.1 $x10^7$ cells/lung), but antibiotic treatment had no effect on absolute numbers of other cell populations investigated. (**Figure 2B**). Analysis of the cellular composition of the BAL fluid showed that eosinophil influx, characteristic for this model, was significantly ($p<0.05$) increased in mice

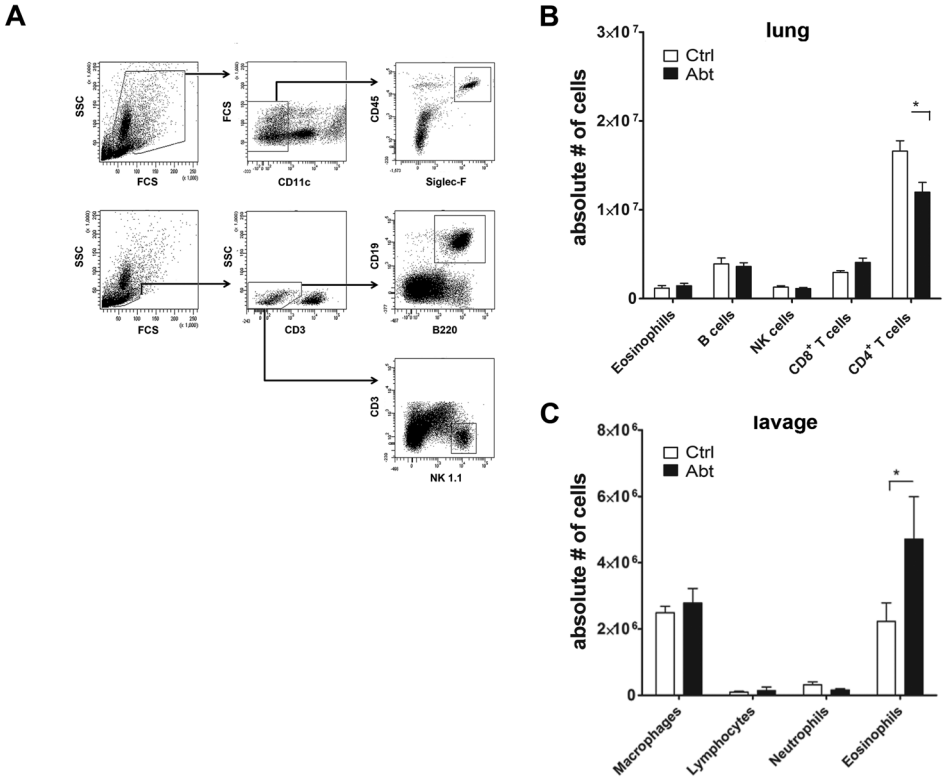


FIGURE 2. Antibiotics affect the RSV specific cellular response 6 days post infection. (A) Phenotyping of leukocyte subpopulations in murine lung by flow cytometric analysis. Populations were identified as: Eosinophils ($CD45^+$ $CD11c^-$ Siglec-F^+), NK cells ($NK1.1^+$ $CD3^-$), B cells ($CD3^-$ $CD19^+$ $B220^+$), T cells ($CD4^+$ or $CD8^+$). **(B)** Cellular composition of specific cell types in the lung, depicted in absolute cell numbers. **(C)** BAL fluid, depicted in absolute numbers. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*$p<0.05$)

treated with antibiotics compared to controls (4.7 ± 1.3 vs. 2.2 ± 0.5 $x10^6$ cells/lung) at day 6 after infection (**Figure 2C**).

Reduced numbers of lung $CD103^+$ DC and increased numbers of $CD11b^+$ DC are present at day six post infection in antibiotic treated mice.

Dendritic cells play an important role in the initiation of both innate and adaptive immune responses to pathogens. In the mouse lung two major subsets of DC have been described, $CD11c^{low}$/mPDCA-1^+ plasmacytoid DC (PDC) and myeloid or "conventional" $CD11c^+$ DC (cDCs). These $CD11c^+$ cDCs can be further divided into $CD11c^+$MHC class-$II^+CD103^-CD11b^{high}$ ($CD11b^+$ DC), a subset located in the lung parenchyma that is important in leukocyte recruitment, and $CD11c^+$MHC class-$II^+CD103^+CD11b^{low}$ ($CD103^+$ DC) located underneath the

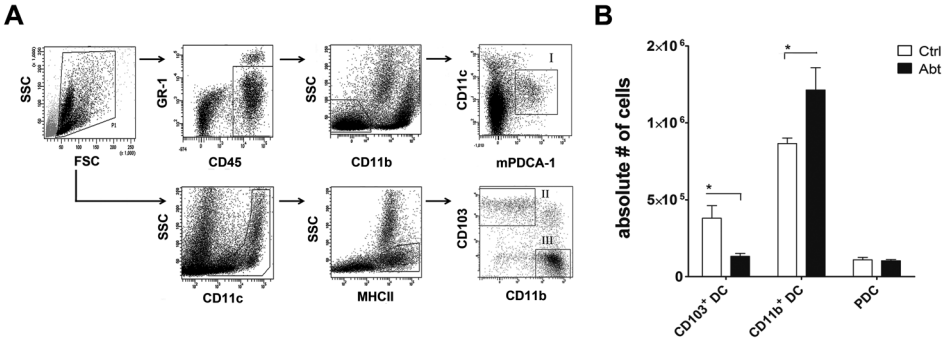


FIGURE 3. Reduced numbers of lung CD103⁺ DC and increased numbers of CD11b⁺ DC are present at day 6 post infection in antibiotic treated mice. (A) Lungs of naïve mice were used for gating strategies of lung DC populations, I (PDC), II (CD103⁺ DC), III (CD11b⁺ DC). **(B)** Day 6 after intra-nasal challenge with RSV, absolute cell numbers of PDC, CD103⁺ DC and CD11b⁺ DC were determined. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*p<0.05).

epithelium, that sample antigens from the airways [39,40], (**Figure 3A**). We investigated the impact of antibiotic treatment on dendritic cell populations. Antibiotic treatment during vaccination significantly decreased the number of CD103⁺ DCs (1.3 ± 0.2 vs. 3.8 ± 0.8 x10^5 cells/lung) 6 days post infection compared to control mice. Furthermore, antibiotic treatment resulted in a significant increase in the number of CD11b⁺ DC (12.1 ± 1.9 vs. 8.6 ± 0.3x10^5 cells/lung) compared to control mice while pDC numbers remained unaffected (**Figure 3B**). This pattern of increased CD11b⁺ DC correlated with the Th2/eosinophillic immune response in this model [41,42].

Oral administration of antibiotics during FI-RSV vaccination increases regulatory T cell numbers and Th2 type effector cell numbers in lungs of RSV challenged mice.

We investigated RSV-specific CD4⁺ and CD8⁺ T cell responses at day 6 post infection. Lung lymphocytes were stained with an H-$2D^b$/$M_{187-195}$ tetrameric complex (containing the dominant RSV epitope derived from the viral matrix protein $M_{187-195}$; NAITNAKII), and antibodies specific for the migration markers CD11a and CD62L and activation marker NKG2a. At day 6 post infection the percentage of activated lung CD8⁺ T cell population was significantly decreased in the antibiotic treated mice compared to control mice (37.1 ± 2.6 vs. 54.5 ± 3.7% based on NKG2a⁺ staining) and (50.2 ± 3.4 vs. 63.6 ± 3.6% based on $CD11a^{hi}$/$CD62L^{low}$ staining). Identification of RSV specific CD8⁺ T cells with the H-$2D^b$/$M_{187-195}$ tetrameric complex showed that approximately 34% of recently activated NKG2a⁺ CD8⁺ T cells were specific for the major RSV epitope NAITNAKII. Furthermore, antibiotic significantly reduced the percentage of recently activated NKG2a⁺ NAITNAKII specific CD8⁺ T cells (11.0 ± 1.3 vs. 20.8 ± 2.4%) compared to control animals (**Figure 4A**). The effect of antibiotic treatment on RSV

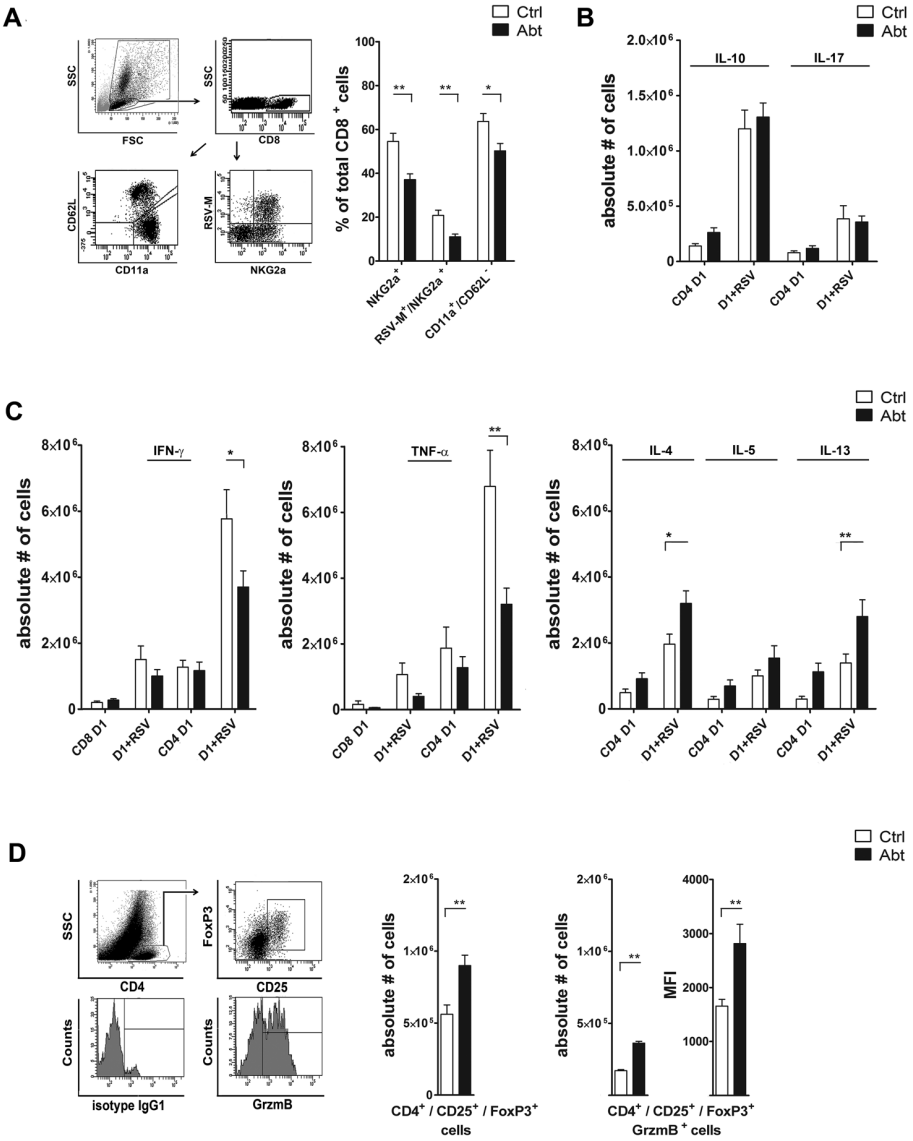


FIGURE 4. Oral administration of antibiotics during FI-RSV vaccination increases regulatory T cell numbers and Th2 type effector cell numbers in lungs of RSV challenged mice. (A) At day 6 post infection, leucocytes were isolated from the lung parenchyma and stained for CD4 or CD8 in combination with CD11a/CD62L or NKG2A/H-2D^b/M$_{187-195}$ tetramer to investigate T cell activation. Figure represents the number double positive cells as a percentage of total CD8$^+$ T cells. **(B)** Cytokine production capacity was investigated by *in vitro* T cell re-stimulation. Lung single cell suspensions were co-cultured with unloaded (D1) or RSV loaded (D1+RSV) myeloid dendritic cells and intracellularly stained for IL-10 and -17 or **(C)** IL-4, -5, -13, IFN-γ and TNF-α**. (D)** Regulatory T cells were stained for CD4, CD25 and FoxP3 in combination with Granzyme B or isotype control and depicted as the absolute number of Granzyme B producing regulatory T cells or granzyme B expression per cell (MFI) present in the lung at day 6 post infection. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*p<0.05, **p<0.01).

specific lung $CD4^+$ T effector cell responses was measured via intracellular staining of TNF-α (inflammatory) , IFN-γ (Th1), IL-4, IL-5, IL -13 (Th2) and IL-10, IL-17 (regulatory) cytokine production, after *in vitro* re-stimulation of lung cells with RSV infected or uninfected myeloid dendritic cells (D1 cells). RSV specific IL-10 and IL-17 producing $CD4^+$ T cell numbers measured at day 6 post infection were not affected by antibiotic treatment (**Figure 4B**). However, a significant increase in absolute numbers of lung $CD4^+$ T cells producing Th2-type cytokines IL-4 (3.2 ± 0.4 vs. 1.9 ± 0.3 $x10^6$ cells/lung) and IL-13 (2.8 ± 0.5 vs. 1.4 ± 0.3 $x10^6$ cells/lung) was found in antibiotic treated animals compared to control animals (**Figure 4C**). The significantly increased numbers of virus specific IL-4 and IL-13 producing $CD4^+$ T cells was accompanied by significantly lower number of virus specific TNF-α^+ (3.2 ± 0.5 vs. 6.8 ± 2.0 $x10^6$) cells/lung and IFN-γ^+ (3.7 ± 0.5 vs. 5.8 ± 1.2 $x10^6$) $CD4^+$ T cells/lung in the antibiotic treated group compared to control mice (**Figure 4C**). The inflammatory response to lung infections must be tightly regulated. Host immune responses should efficiently eliminate pathogen while maintaining crucial gas exchange. Regulatory T cells control local immunity to suppress excessive inflammation and damage. In our model, antibiotic treatment resulted in significant increased number of local $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (9.0 ± 0.7 vs. 5.6 ± 0.6 $x10^5$ cells/lung) compared to control. The perforin/granzyme pathway is a central component of anti-tumor and anti-viral cytotoxic responses mediated by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Recent data show that granzymes are also involved in immune regulation. Granzyme B producing regulatory T cells, exclusively present in lung tissue, are able to control RSV induced disease severity by the suppression of virus specific $CD8^+$ T cell responses, cellular infiltration and local cyto-/chemo-kine release in the lungs of RSV infected mice [43,44]. We investigated possible effects of antibiotic treatment on granzyme B expression in regulatory T cells in the lungs in the FI-RSV induced severe RSV model. The number of granzyme B positive regulatory T cells (3.6 ± 1.3 vs. 1.7 ± 0.7 $x10^5$ cells/lung) and granzyme B expression per cell (2980 ± 447 vs. 1737 ± 498 MFI) were significantly increased in antibiotic treated compared to control mice (**Figure 4D**).

Antibiotic treatment lowers serum IgG2c levels six days post challenge in FI-RSV vaccinated mice.

Next to the impact microbial distortion has on cellular immunity against RSV we also investigated the effect of antibiotic treatment on the RSV specific humoral response. The impact of antibiotic treatment during FI-RSV vaccination on the virus specific antibody response against RSV was measured by ELISA of serum IgG1 and IgG2c levels present on day 6 post challenge. C57BL/6 mice express the Igh1-b allele encoding for antibodies of the IgG2c isotype and lack the allele of the IgG2a isotype [45], therefore we used an IgG2c specific secondary antibody. In antibiotic treated mice the RSV specific IgG1 antibody response remained unaffected while the virus specific IgG2c levels in serum were significantly ($p<0.05$) lower

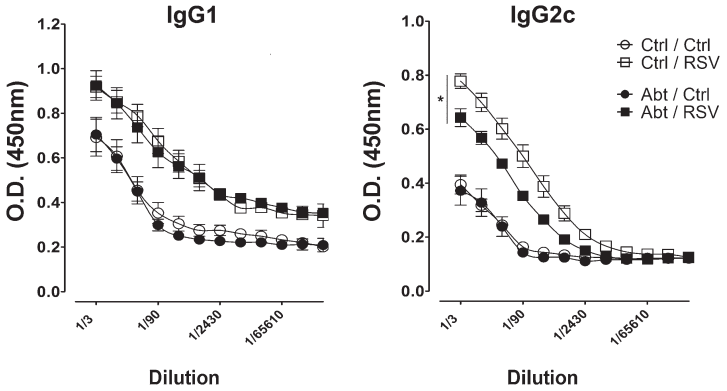


FIGURE 5. Antibiotic treatment lowers serum IgG2c levels 6 days post challenge in FI-RSV vaccinated mice. Serum from mice sampled at day 6 post RSV infection was analyzed for RSV specific IgG1 and IgG2c levels. Symbols represent the $O.D._{450}$ value measured after subtraction of background. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*p<0.05).

in the antibiotic treated animals compared to controls (**Figure 5**). These data correlate with the DC and cytokine profiles depicted in **figure 3** and **4**. Interestingly, similar DC and T cell responses were measured by Roux *et al* in neonatal mice [46]. This study shows that both the cellular and humeral response RSV can be modulated via orally applied antibiotics.

Intra-rectally administered LPS during FI-RSV vaccination reduces eosinophilia and increases RSV specific IFN-γ producing T cell numbers in the lungs of RSV infected mice.

We next tested whether local innate immune responses in the gut could alter adaptive immune responses in the lung. As a model for increased microbial pressure in the intestine, we rectally inoculated mice with a single dose of ultra-pure LPS (the major bacterial cell wall component of gram-negative bacteria) prior FI-RSV vaccination (d14), to mimic the effect of a high load of gram-negative bacteria present in the colon. Thirty five days after vaccination, mice were intra nasally (i.n.) challenged with live RSV. At day 6 post infection mice were sacrificed, bronchoalveolar lavage (BAL) samples were collected and lung cell suspensions phenotyped and analyzed for RSV specific T cell responses (**Figure 6A**). A single rectal dose of ultrapure LPS from *Escherichia coli* (5μg) prior FI-RSV vaccination had no effect on the body weight compared to mice receiving control PBS (**Figure S1**). However, a significantly reduced eosinophillic cell influx (2.1 ± 0.4 vs. 4.0 ± 0.7x10^6 cells) into the bronchoalveolar space was observed in LPS treated animals vs. control mice at day 6 post RSV infection. (**Figure 6B**). *In vitro* re-stimulation of RSV specific lung T cells with RSV infected or uninfected D1 cells resulted in significant increased percentage of $CD8^+$ (36.9 ± 4.7 vs. 24.1 ± 2.5%, not shown) T cells, non-significant increased number of IFN-γ producing $CD8^+$ T cells (2.5 ± 0.3

vs. 1.8 ± 0.3 x10^6 cells/lung) and significant increased numbers of IFN-γ producing $CD4^+$ T cells (12.0 ± 1.9 vs. 5.6 ± 1.0 x10^6 cells/lung) in LPS treated animals compared to control treated mice (**Figure 6C**). LPS treatment had no effect of the number of T cells producing Th2 associated cytokines IL-4, IL-5 and IL-13 (**Figure 6D**). These data show that a rectally applied single dose of LPS during an intra-muscular vaccination skews the RSV specific T cell response in the lung towards a stronger Th1 type of response 41 days later at the peak of the response i.e. 6 days after the live virus challenge.

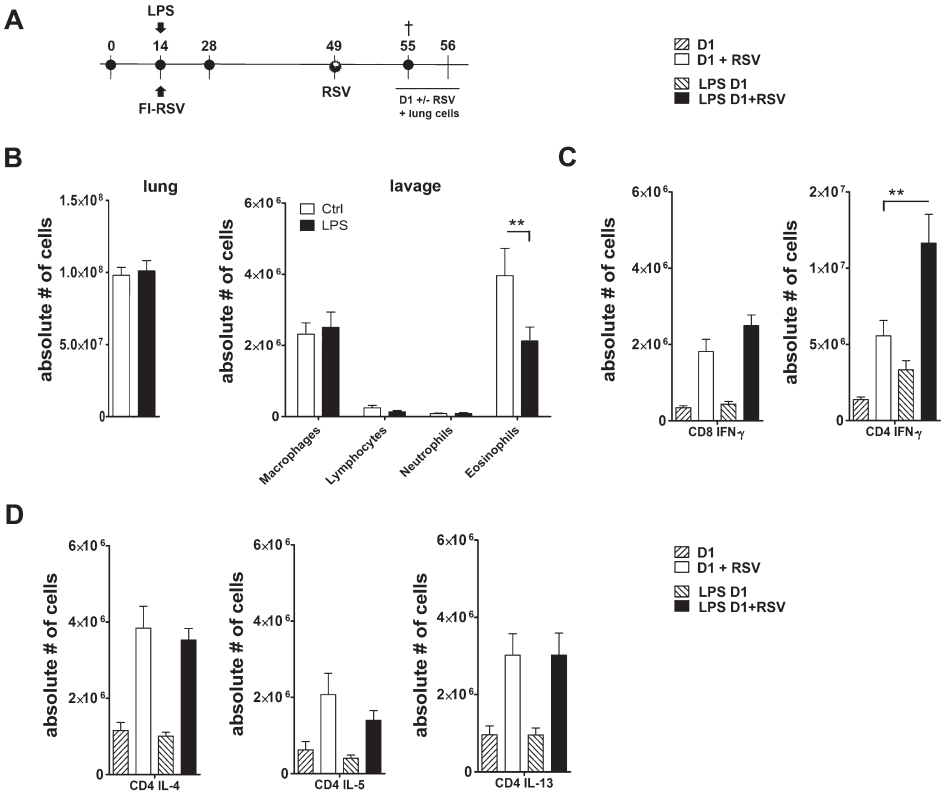


FIGURE 6. Intra-rectally administered LPS during FI-RSV vaccination reduces eosinophilia and increases RSV specific IFN-γ producing $CD4^+$ T cell numbers in the lungs of RSV infected mice (A) SPF mice housed under bio-contained sterile conditions using HEPA® filtered isocages, received intra rectally a single dose of 50µL PBS or 50µL PBS containing ultrapure LPS *from Escherichia coli* (5µg), before i.m. vaccination with FI-RSV. 5 weeks after vaccination, mice were intranasally infected with 4x10^6 plaque forming units (pfu) RSV and sacrificed 6 days post infection. **(B)** Lung single cell suspensions **(left)** and cellular composition of BAL fluid **(right)**, depicted in absolute numbers. **(C)** For *in vitro* T cell re-stimulation experiments, lung cell suspensions were co-cultured with unloaded (D1) or RSV loaded (D1+RSV) myeloid dendritic cells and intracellularly stained for IFN-γ and **(D)** IL-4, -5, 13. The values depicted represent the absolute numbers of cytokine producing T effector cells 6 days post infection. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=10 group. Significant differences were depicted as (*$p<0.05$, **$p<0.01$).

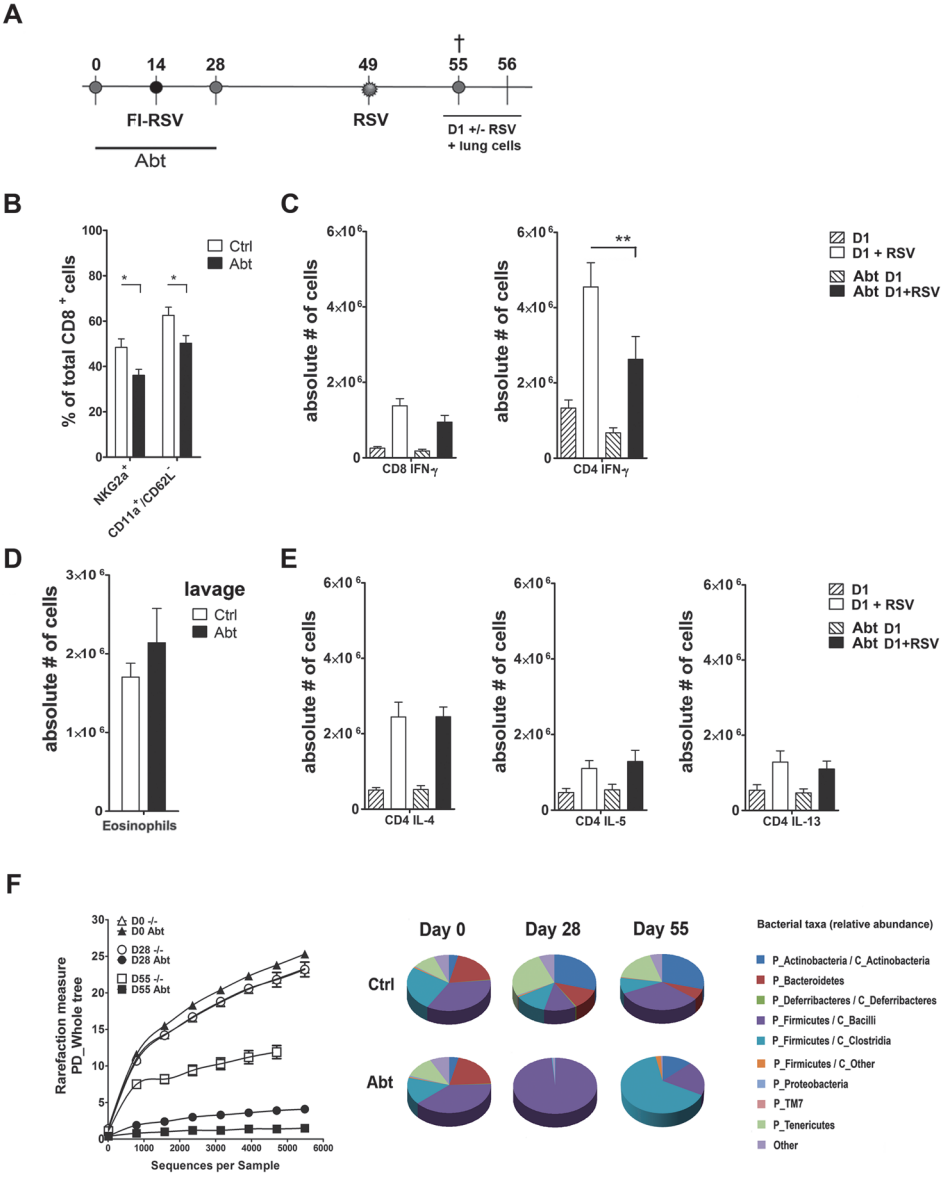
Oral administration of broad spectrum antibiotics during FI-RSV vaccination reduces fecal bacterial taxonomic composition and the absolute numbers of RSV-specific IFN-γ producing T cells in the lungs of RSV infected mice.

Our experimental models for altered microbial pressure showed that it was possible to modulate lung RSV specific T cell responses. We repeated the antibiotic treatment in the FI-RSV vaccination model and collected isolated fecal samples before, 4 weeks after antibiotic treatment and 6 days post infection (**Figure 7A**). In this experiment we reproduced the antibiotic treatment effect on IFN-γ in this model i.e. at day 6 post infection the number of activated cells of the total lung $CD8^+$ T cell population were significantly decreased in the antibiotic treated mice compared to controls (37.5 ± 2.1 vs. 46.8 ± 2.7% based on $NKG2a^+$) and (50.1 ± 1.7 vs. 61.5 ± 1.6% based on $CD11a^{hi}/CD62L^{low}$ staining, **Figure 7B**). Furthermore, *in vitro* restimulation of lung cells with RSV loaded D1 showed that antibiotic treatment significantly reduced absolute number of lung $CD4^+$ T cells producing IFN-γ (2.6 ± 0.6 vs. 4.6 ± 0.6 $x10^6$ cells/lung) compared to control animals (**Figure 7C**). Antibiotic treatment showed a non-significant increase of eosinophils in the BAL fluid compared to control animals (**Figure 7D**) and had no effect of the absolute number of IL-4, IL-5 and IL-13 producing T cells (**Figure 7E**). Analysis of fecal microbial taxonomic composition and alpha/beta diversity via 16S gene amplicon sequencing [47] and QIIME data mining, showed that all animals had a divers microbial taxon, expressed as a rarefaction and operational taxonomic unit plot (OTU), at the start of the experiment (**Figure 7F**). Administration of sterile food and drinking water for a period of 55 days in a bio-contained environment resulted in a significant decrease in variety of microbial taxonomic composition in control animals, showing that HEPA® filtered isocages effectively reduce exposure to external pathogens. Antibiotic treatment for a period of 4 weeks dramatically reduced variation in fecal bacterial species, resulting in a dominant composition of mostly gram-positive bacteria from the phylum

---

FIGURE 7. Oral administration of broad spectrum antibiotics during FI-RSV vaccination reduces fecal bacterial taxonomic composition and the absolute numbers of RSV-specific IFN-γ producing CD4+ T cells in the lungs of RSV infected mice. (A) Mice received plain or antibiotics supplemented water 2 weeks before until 2 weeks after i.m. vaccination with FI-RSV. 5 weeks after vaccination, mice were intranasally infected with $4x10^6$ plaque forming units (pfu) RSV and sacrificed 6 days post infection. **(B)** At day 6 post infection, leucocytes were isolated from the lung parenchyma and analyzed for activation status by staining with CD8 in combination with CD11a/CD62L or NKG2A. Figure represents the number double positive cells as a percentage of total $CD8^+$ T cells. **(C)** Virus specific $CD4^+$ and $CD8^+$ T cell responses, visualized after *in vitro* re-stimulation of lung cells with unloaded (D1) or RSV loaded (D1+RSV) myeloid dendritic cells and intracellularly stained for IFN-γ **(E)** IL-4, IL-5 and IL-13 **(D)** Eosinophillic influx measured in BAL fluid 6 days after infection is depicted in absolute numbers/lung. **(F)** Roche-454 16S rRNA gene pyrosequencing was performed to longitudinally characterize fecal microbiome composition before antibiotic treatment (D0), after antibiotic treatment (D28) and 6 days post infection (D55). Figures represent fecal taxa depicted as a rarefaction plot **(left)** or taxon/species pie chart **(right).** The values depicted represent the absolute numbers of cytokine producing T effector cells 6 days post infection. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*$p<0.05$, **$p<0.01$).

Firmicutes. Twenty-seven days after ending antibiotic treatment, diversity in Phyla was still significantly reduced. Moreover, the relative abundance of Bacilli present after 28 days of antibiotic treatment was almost completely substituted by Clostridia, as measured 6 days post infection (**Figure 7F**). Furthermore, macroscopical examination of the intestinal tract showed that antibiotic treatment results in an enlarged cecum **Figure S2**). These data show that in a bio-contained environment orally applied broad spectrum antibiotics irreversibly



reduce fecal microbial diversity. Whether reduced microbial colonization due to antibiotic treatment affects immune priming upon vaccination or the onset of the adaptive immune response triggered upon RSV infection remains elusive.

DISCUSSION

In the present work we demonstrate that orally applied broad spectrum antibiotics had drastic and long term effects on intestinal microbiota composition and skewed adaptive immune responses specific for RSV in the FI-RSV vaccination model towards stronger Th2-biased responses accompanied with increased lung eosinophilia and decreased RSV specific serum IgG2c titers. Opposite effects were observed when TLR4 ligand LPS was administered rectally prior to i.m. vaccination with FI-RSV. Antibiotics treatment did not significantly alter weight or viral loads at day six in the lungs. Recently we reported a prebiotic diet intervention study in the same FI-RSV vaccination model and in a primary RSV infection model [41]. In both models we observed an increased Th1/Th2 ratio of RSV specific $CD4^+$ T cell responses. Together, our studies suggest that alterations in commensal microbiota composition have an impact on airway immune responses to RSV.

In a recent study Ichinohe *et al*. reported that the same mixture of antibiotics that we used in our studies decreased both humoral (IgG) and cellular ($CD4^+$ and $CD8^+$ T cell responses) in a primary influenza virus infection model [31]. However, this antibiotic treatment did not affect the immune response triggered by ovalbumin in complete Freund's adjuvant administered in the footpad, nor the $CD4^+$ and $CD8^+$T cell response initiated after intranasal administered herpes simplex type 2 virus. This difference was explained by the authors as a difference in the requirement of inflammasome activation in immune priming to influenza virus, but may also be a location specific aspect. A different antibiotic intervention; i.e. oral administration of cefoperazone promoted airway hyperresponsiveness after primary RSV infection, which was not observed in untreated mice [32]. The authors of this study suggested that the antibiotic effect might result from the eradication of pharyngeal commensal bacteria, but the mechanism was not further elucidated. Clearly, antibiotic treatment will affect microbial content in different locations. Therefore, local effects on innate immune responses or systemic effects on immune parameters might have contributed to the observed immune modulation. Moreover, we showed in the present study that in the isocage® bio-contained model, antibiotic treatment for a period of 4 weeks irreversibly reduced gut microbiome diversity until at least 4 weeks after ending antibiotic treatment. Therefore, a direct immunosuppressive effect of the antibiotic treatment in the RSV infection can be excluded. However, it is not possible to determine whether the observed immune manipulation via antibiotics (or LPS) treatment occur during the priming phase of the immune response or during the effector phase, i.e. the recall response upon the RSV challenge. Nevertheless, our study shows that in addition to the role of microbial stimuli during the perinatal maturation of the immune system, also in

mature mice altered microbial composition has an impact on immune responses directed to a virus or a vaccine when the immune systems has already matured towards the exposure of a diverse microbiome. Therefore, it is important for clinicians to be aware of the possible side effects on RSV specific adaptive immune responses when antibiotics are administered during RSV infections or for other causes during the neonatal period.

MATERIALS AND METHODS

Mice

Specific pathogen-free 3-4 week-old female C57BL/6 mice (Charles River Nederland Maastricht, The Netherlands) were housed under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Italy). All animals had *ad libitum* access to sterile water and diet. All study protocols were approved by the Animal Ethics Committee of the Medical Faculty of the Utrecht University.

Antibiotic treatment

For a period of 4 weeks animals had *ad libitum* access to sterile water supplemented with a broad spectrum antibiotic cocktail containing: Ampicillin (2g/L, A1593, Sigma-Aldrich), Vancomycin (500mg/L, V2002, Sigma-Aldrich), Neomycin sulfate (1g/L, N1876, Sigma-Aldrich) and Metronidazole (1g/L, M3761, Sigma-Aldrich).

Virus and infection

RSV strain A2 (VR-1302, ATCC) was grown on HEp-2 cells (CCL-23, ATCC) purified by polyethylene glycol 6000 precipitation and stored in phosphate-buffered saline (PBS) with 10% sucrose in liquid nitrogen until further use. Mice were anesthetized with isoflurane and intranasally infected with $4x10^6$ plaque forming units (pfu) RSV in a volume of 50 µl diluted in PBS.

FI-RSV vaccine and vaccination

Formalin-inactivated (FI)-RSV was prepared by the method used for the original vaccine as tested in the 1960's in infants [48]. RSV A2 strain was grown for 48 hrs in HEp-2 cells. Culture medium was cleared from cell debris by low speed centrifugation (1000xg, 10 min, 4°C). Formalin (F8775, Sigma-Aldrich) was added to $3x10^6$ pfu RSV/ml containing supernatant at a final dilution of 1:4000 and incubated at 37°C for 3 days with stirring. After ultra-centrifugation (50,000 g_{av}, 1h, 4°C) of the FI-RSV preparation, resulting pellets were resuspended

to 1/25th of the original volume in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Invitrogen) without supplements. FI-RSV was adsorbed to 4 mg/ml aluminium hydroxide (A1577, Sigma-Aldrich) overnight at room temperature while stirring. Finally, FI-RSV was pelleted by centrifugation (1000xg, 30 min) and resuspended to 1/4th volume in PBS. This procedure resulted in a final dosage that was concentrated 100-fold and contained 16 mg/ml aluminium hydroxide. At day 0, mice were i.m. injected with 50 µl FI-RSV vaccine preparation.

Tissue isolation and preparation

Mice were sacrificed at different time points, as indicated in the figure legends, by i.p. injection of 300 µl pentobarbital. Cells from the airways were obtained by broncho-alveolar lavage (BAL) with 3 x 1 ml of 0.15 M NaCl. Prior to removal, the lungs were perfused with PBS containing 100 U/ml heparin. Lungs were cut to 1 x 1 mm pieces and incubated with collagenase (2.4 mg/ml; 10103586001, Roche Applied Science) and DNase (1 mg/ml; 10104159001, Roche Applied Science) for 20 min. at 37°C. Single cell suspensions were prepared by processing the tissue trough 70 µm cell strainers (BD Falcon, BD Biosciences).

RSV-specific real-time PCR

For preparation of lung tissue homogenates, isolated snap-frozen whole lungs were crushed on dry ice, dissolved in PBS and stored at -80 ^{0}C until further use. To measure total virus copy numbers by PCR, total RNA from lung homogenates was extracted using MagnaPure LC equipment, cDNA was synthesized, and viral loads were determined by real-time PCR as recently described [49]. In short, viral genomic RNA was isolated using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). The isolated viral RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA), according to the manufacturer's guidelines. Primers and probes designed on the basis of highly conserved genomic regions of the N gene for both RSV subgroup A (RSV-A) and B (RSV-B) were used for subtyping of the RSV patient strains. The following primers and probes were used:

RSA-1: 5'-AGATCAACTTCTGTCATCCAGCAA-3'
RSA-2: 5'-TTCTGCACATCATAATTAGGAGTATCAAT-3'
RSB-1: 5'-AAGATGCAAATCATAAATTCACAGGA-3'
RSB-2: 5'-TGATATCCAGCATCTTTAAGTATCTTTATAGTG-3'
RSA probe: 5'-CACCATCCAACGGAGCACAGGAGAT-3'
RSB probe: 5'-TTCCCTTCCTAACCTGGACATAGCATATAACATACCT-3'

Murine encephalomyocarditis virus (RNA) was used as an internal control. Samples were assayed in a 25 µl reaction mixture containing 10 µl of cDNA, TaqMan universal PCR master mix (Applied Biosystems, ABI), primers (900 nM RSV-A primers and 300 nM RSV-B primers),

and fluorogenic probes (58.3 nM RSV-A probe and 66.7 nM RSV-B probe) labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an ABI 7900 HT system for 2 min at 50ºC, 10 min at 95ºC, and 45 cycles of 15 sec at 95ºC and 1 min at 60ºC. Samples were controlled for the presence of possible inhibitors of the amplification reaction by the indicated internal control, signals of which had to range within a clear-cut interval. Sample cycle threshold values (C^t) were compared with a standard curve of RSV A2.

Microbiome analysis

For fecal microbiome analysis, DNA extracts were prepared from snap frozen freshly collected fecal samples. Identification of microbial taxonomic composition and alpha/beta diversity was performed via 16S gene amplicon sequencing [47] with Roche 454 technology and QIIME datamining.

In vitro re-stimulation

Isolated lung cells were re-stimulated with a synthetic peptide representing a dominant H-2 restricted RSV epitope or a dendritic cell line infected with RSV. For peptide stimulation, single cell suspensions of lung cells ($1x10^6$ cells) were incubated with the H-$2D^b$ restricted peptide (1 µg/ml) from the RSV M protein ($M_{187-195}$, NAITNAKII) (28). Restimulation with RSV was accomplished by co-culturing lung cells ($1x10^6$ cells) with RSV infected D1 cells ($2x10^5$ cells). D1 is a non-transformed, growth factor-dependent, myeloid dendritic cell line derived from C57BL/6 mice [50]. D1 cells were maintained in IMDM, 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin and 50µM β-mercapto-ethanol and supplemented with 30% conditioned medium from GM-CSF producing R1 cells (mouse fibroblast NIH3T3, transfected with the GM-CSF gene [51]. D1 cells were infected for a period of 48 hours with RSV (multiplicity of infection, m.o.i 2) before addition to the lung cell suspension. Cell suspensions were stimulated for 6 h at 37°C, 5% CO_2 in 200 µl IMDM supplemented with 2 mM L-glutamine, 25 mM Hepes buffer, 5% FCS, penicillin/streptomycin, 50 µM β-mercapto-ethanol and 50 U/ml recombinant human IL-2 (11147528001, Roche). Brefeldin A 10 µg/ml (B7651, Sigma-Aldrich) was added for the duration of the stimulation to facilitate intracellular accumulation of cytokines.

Cell surface and intracellular cytokine staining

Lung single cell suspensions were first pre-incubated with 2.4.G2 an Fc receptor specific Ab (anti-CD16/32) to reduce non-specific binding and then stained for 4- or 5-color flow cytometry with the following antibodies (from BD Biosciences unless otherwise stated):

Anti-CD3 (clone 145-2C11), anti CD4 (clone RM4-5), anti CD8 (clone 53-6.7), anti CD11a (clone 2D7), anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti CD19 (clone 1D3), anti CD25 (clone PC61), anti-CD45 (clone RA3-6B2), anti CD62L (clone MEL-14), anti-CD103 (clone M290) anti B220 (clone RA3-6B2), anti-MHC-II (I-A^b/I-E^b, clone AF6-120.1), anti-Ly6C (clone RB6-8C5) anti-mPDCA-1 (Miltenyi Biotec, clone JF05-1C2.4.1) anti-Siglec-F (clone E50-2440) and anti-NKG2A (clone 20d5). RSV specific $CD8^+$ T cells were visualized with MHC class I - $M_{187-195}$ tetramer, manufactured as previously described [52]. Granzyme B and cytokine production by $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry. Cells were washed with FACS buffer and stained for surface markers with anti-CD8 (clone 53-6.7) and anti-CD4 (clone RM4-5). For intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (BD, 554722) solution and Perm/Wash buffer (BD, 554723). Intracellular cytokines were detected with anti-IFN-γ (clone XMG1.2), anti TNF-α (clone MP6-XT22), anti-IL-4 (clone 11B11), anti-IL-5 (clone TRFK5), anti-IL-10 (clone JES5-16E3), anti-IL-13 (eBioscience, clone eBio13a), anti-IL-17 (clone TC11-18H10). T_{reg} detection using anti FoxP3 (clone MF23). Granzyme B expression was determined via anti Granzyme B (clone GB11) or matching IgG1 isotype control. Stained samples were measured on a FACSCanto II flowcytometer (BD, San Diego, CA) and analysed using FacsDiva software (BD, San Diego, CA).

BAL fluid leukocyte composition

Lung cells were spun onto glass slides (Shandon cytospin, Pittsburgh, Pa) and fixed with 100% methanol for 5 min. Subsequently, cell nuclei were stained in a 1:1 dilution of May-Grünwald (Mallinckrodt Baker, 3855) with buffered water pH 6.8 for 5 min. After washing with buffered water pH 6.8, cells were stained with a 1:8 dilution of Giemsa (Merck, 1.09204.500) with buffered water pH 6.8 for 15 min. and finally washed with water. For determination of cell composition one hundred cells per sample were counted.

ELISA

To determine RSV specific IgG's, NUNC MaxiSorp® plates were coated with denatured lysate from RSV infected or uninfected Hep-2 cells in PBS and incubated overnight at 4^0C. After removal of unbound lysate, plates were blocked with a bovine serum albumin (BSA), 0.05% Tween 20 in PBS solution for 1 hr. at 37^0C. Serum dilutions of animals infected with RSV were incubated for 2 hr. at 37^0C and after wash incubated for 2 hr. with the secondary HRP-labelled antibodies anti-IgG1 (04-6120, Invitrogen) or anti-IgG2c (GG2C-90P, Immunology Consultant Laboratory). The substrate 3,3',5,5'-Tetramethylbenzidine was used for colour development. Enzymatic activity was stopped by adding 9.8% H_2SO_4 and measured at OD_{450}.

Statistical analysis

For all experiments, the difference between groups was calculated using a Two-Way ANOVA followed by the Bonferroni test (Graphpad Prism version 4: Graphpad, San Diego).

Data are expressed as the mean $^{+}$/- standard error of the mean (SEM). The p values deemed significant are indicated in the figures as follows: * $p < 0.05$, ** $p < 0.01$.

ACKNOWLEDGEMENTS

This study was financially supported and performed within the framework of the Dutch Top Institute Pharma project: D1-101-0. M.A. Schijf, H. Wopereis, J. Garssen and B. van't Land are employees of Dept. of Immunology and Microbiology, Nutricia Research Utrecht, The Netherlands.

REFERENCES

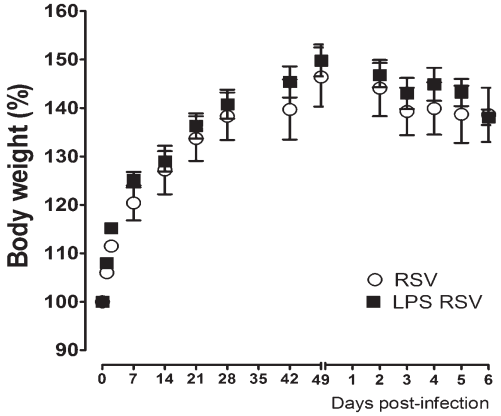
1. Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. Journal of Virology 2008;82: 2040–55.
2. Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-Associated Hospitalizations Among US Children, 1980-1996. JAMA: The Journal of the American Medical Association 1999; 282:1440–6.
3. Peltola V, Reunanen T, Ziegler T, Silvennoinen H, Heikkinen T. Accuracy of clinical diagnosis of influenza in outpatient children. Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America 2005;41:1198–200.
4. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clinical Microbiology Reviews 2010;23:74–98.
5. Haque F, Husain MM, Ameen KMH, Rahima R, Hossain MJ, Alamgir ASM, et al. Bronchiolitis outbreak caused by respiratory syncytial virus in southwest Bangladesh, 2010. International Journal of Infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases 2012;16: e866–71.
6. Popow-Kraupp T, Aberle JH. Diagnosis of respiratory syncytial virus infection. The Open Microbiology Journal 2011;5:128–34.
7. Byington CL, Castillo H, Gerber K, Daly JA, Brimley LA, Adams S, et al. The effect of rapid respiratory viral diagnostic testing on antibiotic use in a children's hospital. Archives of Pediatrics & Adolescent Medicine 2002;156: 1230–4.
8. Spurling GK, Doust J, Del Mar CB, Eriksson L. Antibiotics for bronchiolitis in children. Cochrane Database of Systematic Reviews (Online) 2011:CD005189.
9. McCallum GB, Morris PS, Chang AB. Antibiotics for persistent cough or wheeze following acute bronchiolitis in children. Cochrane Database of Systematic Reviews (Online) 2012;12:CD009834.
10. Henderson J, Hilliard TN, Sherriff A, Stalker D, Al Shammari N, Thomas HM. Hospitalization for RSV bronchiolitis before 12 months of age and subsequent asthma, atopy and wheeze: a longitudinal birth cohort study. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2005;16: 386–92.
11. Johnson CC, Ownby DR, Alford SH, Havstad SL, Williams LK, Zoratti EM, et al. Antibiotic exposure in early infancy and risk for childhood atopy. The Journal of Allergy and Clinical Immunology 2005;115:1218–24.
12. Thomas M, Custovic A, Woodcock A, Morris J, Simpson A, Murray CS. Atopic wheezing and early life antibiotic exposure: a nested case-control study. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2006;17:184–8.
13. Marra F, Lynd L, Coombes M, Richardson K, Legal M, FitzGerald JM, et al. Does antibiotic exposure during infancy lead to development of asthma? A systematic review and metaanalysis. 2006. Chest 2009;136:e30.
14. Wickens K, Pearce N, Crane J, Beasley R. Antibiotic use in early childhood and the development of asthma. Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology 1999; 29:766–71.
15. McKeever TM, Lewis SA, Smith C, Collins J, Heatlie H, Frischer M, et al. Early exposure to infections and antibiotics and the incidence of allergic disease: a birth cohort study with the West Midlands General Practice Research Database. The Journal of Allergy and Clinical Immunology 2002;109:43–50.
16. Kozyrskyj AL, Ernst P, Becker AB. Increased risk of childhood asthma from antibiotic use in early life. Chest 2007;131:1753–9.

17. Blanken MO, Rovers MM, Molenaar JM, Winkler-Seinstra PL, Meijer A, Kimpen JLL, et al. Respiratory syncytial virus and recurrent wheeze in healthy preterm infants. The New England Journal of Medicine 2013;368: 1791–9.
18. Stein RT. Long-term airway morbidity following viral LRTI in early infancy: recurrent wheezing or asthma? Paediatric Respiratory Reviews 2009;10 Suppl 1:29–31.
19. Hollams EM, Deverell M, Serralha M, Suriyaarachchi D, Parsons F, Zhang G, et al. Elucidation of asthma phenotypes in atopic teenagers through parallel immunophenotypic and clinical profiling. The Journal of Allergy and Clinical Immunology 2009;124: 463–70, 470.e1–16.
20. Kusel MMH, De Klerk NH, Holt PG, Kebadze T, Johnston SL, Sly PD. Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life: a birth cohort study. The Pediatric Infectious Disease Journal 2006;25:680–6.
21. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax 2010;65:1045–52.
22. Szabo SM, Levy AR, Gooch KL, Bradt P, Wijaya H, Mitchell I. Elevated risk of asthma after hospitalization for respiratory syncytial virus infection in infancy. Paediatric Respiratory Reviews 2013;13 Suppl 2:S9–15.
23. Habibi MS, Openshaw PJM. Benefit and harm from immunity to respiratory syncytial virus: implications for treatment. Current Opinion in Infectious Diseases 2012;25:687–94.
24. Stockinger S, Hornef MW, Chassin C. Establishment of intestinal homeostasis during the neonatal period. Cellular and Molecular Life Sciences: CMLS 2011;68:3699–712.
25. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229–41.
26. Strachan DP. Hay fever, hygiene, and household size. BMJ (Clinical Research Ed) 1989; 299:1259–60.
27. Celedón JC, Weiss ST. Use of antibacterials in infancy: clinical implications for childhood asthma and allergies. Treatments in Respiratory Medicine 2004;3:291–4.
28. Lau S. What is new in the prevention of atopy and asthma? Current Opinion in Allergy and Clinical Immunology 2013;13:181–6.
29. Noverr MC, Huffnagle GB. The "microflora hypothesis" of allergic diseases. Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology 2005;35:1511–20.
30. Hansel TT, Johnston SL, Openshaw PJ. Microbes and mucosal immune responses in asthma. Lancet 2013;381:861–73.
31. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proceedings of the National Academy of Sciences of the United States of America 2011;108:5354–9.
32. Ni K, Li S, Xia Q, Zang N, Deng Y, Xie X, et al. Pharyngeal microflora disruption by antibiotics promotes airway hyperresponsiveness after respiratory syncytial virus infection. PloS One 2012;7:e41104.
33. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. American Journal of Epidemiology 1969;89:422–34.
34. Graham BS, Johnson TR, Peebles RS. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology 2000;48:237–47.
35. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nature Medicine 2009;15:34–41.

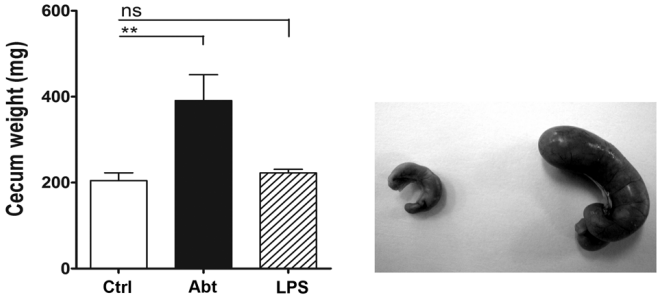
36. Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, et al. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. PloS One 2011;6:e17996.
37. Jessen B, Faller S, Krempl CD, Ehl S. Major histocompatibility complex-dependent cytotoxic T lymphocyte repertoire and functional avidity contribute to strain-specific disease susceptibility after murine respiratory syncytial virus infection. Journal of Virology 2011;85:10135–43.
38. Prince GA, Horswood RL, Berndt J, Suffin SC, Chanock RM. Respiratory syncytial virus infection in inbred mice. Infection and Immunity 1979;26:764–6.
39. Beaty SR, Rose CE, Sung S-SJ. Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. Journal of Immunology (Baltimore, Md: 1950) 2007; 178:1882–95.
40. Sung S-SJ, Fu SM, Rose CE, Gaskin F, Ju S-T, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. Journal of Immunology (Baltimore, Md: 1950) 2006;176:2161–72.
41. Schijf MA, Kruijsen D, Bastiaans J, Coenjaerts FEJ, Garssen J, Van Bleek GM, et al. Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. Journal of Virology 2012;86:11472–82.
42. Kruijsen D, Schijf MA, Lukens M V, Van Uden NO, Kimpen JL, Coenjaerts FE, et al. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. Vaccine 2011;29:2730–41.
43. Loebbermann J, Thornton H, Durant L, Sparwasser T, Webster KE, Sprent J, et al. Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. Mucosal Immunology 2012;5:161–72.
44. Salti SM, Hammelev EM, Grewal JL, Reddy ST, Zemple SJ, Grossman WJ, et al. Granzyme B regulates antiviral CD8+ T cell responses. Journal of Immunology (Baltimore, Md: 1950) 2011;187:6301–9.
45. Martin RM, Brady JL, Lew AM. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. Journal of Immunological Methods 1998; 212:187–92.
46. Roux X, Remot A, Petit-Camurdan A, Nahori M-A, Kiefer-Biasizzo H, Marchal G, et al. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells. European Journal of Immunology 2011;41:2852–61.
47. Wu GD, Lewis JD, Hoffmann C, Chen Y-Y, Knight R, Bittinger K, et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiology 2010;10:206.
48. Prince GA, Denamur F, Deschamps M, Garçon N, Prieels JP, Slaoui M, et al. Monophosphoryl lipid A adjuvant reverses a principal histologic parameter of formalin-inactivated respiratory syncytial virus vaccine-induced disease. Vaccine 2001;19:2048–54.
49. Houben ML, Coenjaerts FEJ, Rossen JWA, Belderbos ME, Hofland RW, Kimpen JLL, et al. Disease severity and viral load are correlated in infants with primary respiratory syncytial virus infection in the community. Journal of Medical Virology 2010;82: 1266–71.
50. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. The Journal of Experimental Medicine 1997;185: 317–28.
51. Foti M, Granucci F, Aggujaro D, Liboi E, Luini W, Minardi S, et al. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated

for recruitment and maintenance of DC at the inflammatory site. International Immunology 1999;11:979–86.
52. Haanen JB, Wolkers MC, Kruisbeek AM, Schumacher TN. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. The Journal of Experimental Medicine 1999;190:1319–28.

SUPPORTING INFORMATION



Supplementary figure S1. Single intra-rectal dose of LPS during vaccination had has no effect on bodyweight development. After intra rectal LPS administration and FI-RSV vaccination, weekly weight measurements pre-infection and daily weight measurements post-infection were performed. Data shown are the mean ± SEM percentage of original body weight measured and represent data of 2 individual experiments performed with similar results, n=8 group.



Supplementary figure S2. Oral administration of antibiotics during FI-RSV vaccination increases cecum weight in RSV infected mice. In mice receiving sham, oral antibiotic or i.r. ultra-pure LPS during FI-RSV vaccination, cecum and colon were isolated and weighted 6 days post RSV infection. Data shown represent the mean ± SEM of 2 individual experiments performed with similar results, n=8 group. Significant differences were depicted as (*p<0.05, **p<0.01).

Chapter 9

Alterations in regulatory T cells induced by specific oligosaccharides improve vaccine responsiveness in mice.

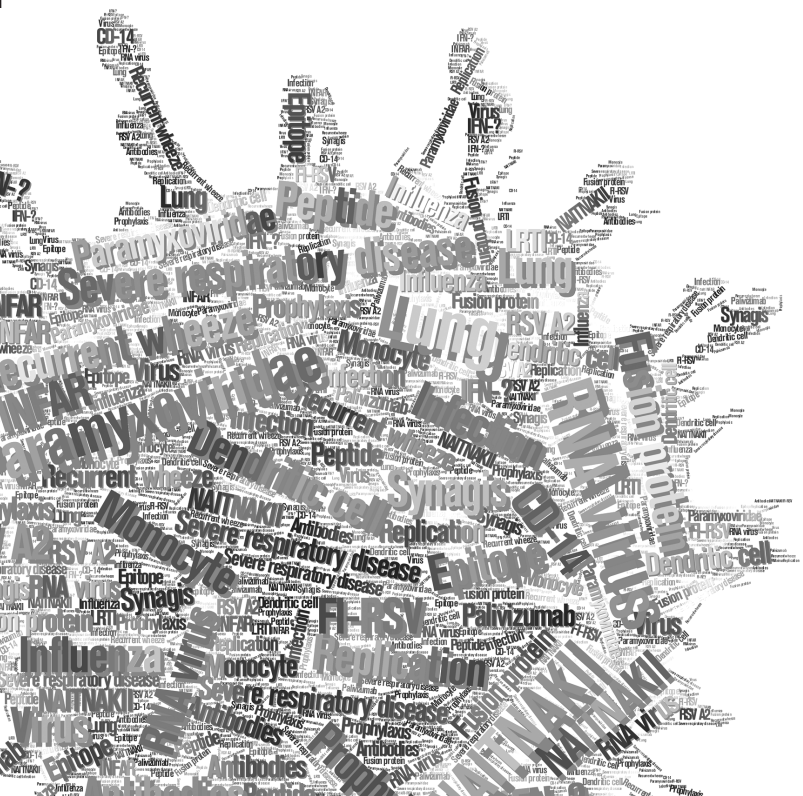
Marcel A. Schijf[1,2], JoAnn Kerperien[3], Jacqueline Bastiaans[2,3], Kirsten Szklany[2], Jenny Meerding[1], Gerard Hofman[3], Louis Boon[4], Femke van Wijk[1], Johan Garssen[2,3], Belinda van't Land[1,2*]

[1]Department of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, the Netherlands

[2]Department of Immunology, Nutricia Research, Utrecht, the Netherlands

[3]Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, the Netherlands.

[4]Bioceros, Utrecht, the Netherlands.



PLoS One (2013): (9): e75148

ABSTRACT

Prophylactic vaccinations are generally performed to protect naïve individuals with or without suppressed immune responsiveness. In a mouse model for Influenza vaccinations the specific alterations of $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) in the immune modulation induced by orally supplied oligosaccharides containing scGOS/lcFOS/pAOS was assessed. This dietary intervention increased vaccine specific DTH responses. In addition, a significant increased percentage of T-bet$^+$ (Th1) activated $CD69^+CD4^+$ T cells ($p<0.001$) and reduced percentage of Gata-3$^+$ (Th2) activated $CD69^+CD4^+$T cells ($p<0.001$) was detected in the mesenteric lymph nodes (MLN) of mice receiving scGOS/lcFOS/pAOS compared to control mice. Although no difference in the number or percentage of Tregs ($CD4^+Foxp3^+$) could be determined after scGOS/lcFOS/pAOS intervention, the percentage of CXCR3$^+$/T-bet$^+$ (Th1-Tregs) was significantly reduced ($p<0.05$) in mice receiving scGOS/lcFOS/pAOS as compared to mice receiving placebo diets. Moreover, although no absolute difference in suppressive capacity could be detected, an alteration in cytokine profile suggests a regulatory T cell shift towards a reducing Th1 suppression profile, supporting an improved vaccination response.

In conclusion: These data are indicative for improved vaccine responsiveness due to reduced Th1 suppressive capacity in the Treg population of mice fed the oligosaccharide specific diet, showing compartmentalization within the Treg population. The modulation of Tregs to control immune responses provides an additional arm of intervention using alternative strategies possibly leading to the development of improved vaccines.

INTRODUCTION

The induction of proper immune responsiveness to vaccinations shortly after birth or in immune compromised individuals is challenging. The highly protective environment and need to avoid immunological interactions of the fetus against the mother seem to be the main reason for this "physiological" immaturity of the immune system in newborn infants. Regulatory T cells (Tregs) are particularly abundant and potent during pregnancy and at birth and inhibit excessive immune responses whereby ultimately the maintenance of peripheral T-cell tolerance and pathogen clearance are key [1,2,3,4]. Moreover the signaling within the intestine through pathogen recognition receptors like the toll like receptor (TLR) family for instance is crucial for the generation of effective immunity. Illustrative for this is that TLR2 influences the function of Tregs [5] and establishes a direct link between the intestinal microbiota and the control of immune responses through Tregs [6]. Tregs are important cells involved in immune regulation and play an increasing role in many immune related disorders of which many are found to be related to disturbed Treg function. For instance in HIV disease progression, the up regulation of fork head box p3 (Foxp3) expression in $CD4^+$ T cells seems to be a marker of disease severity [7] and also in HAART-therapy treatment the Treg activity is associated with persistently reduced $CD4^+$ T-cell counts during antiretroviral therapy [8]. Even, in chronic diseases like asthma, allergy, cancer an altered Treg number or function has been described. This is exemplified by the increased number of Tregs in children with eosinophilic esophagitis and explicit role of Tregs in tumor immunity [9]. In addition, related towards acute infections and innate immunity, an important role for Tregs is the suppression of innate immune pathology during influenza A virus infection [10]. $Foxp3^+CD4^+$ Tregs limit pulmonary immune pathology by modulating the $CD8^+$ T cell responses during respiratory syncytial virus infection [11,12]. These findings contribute to the notification, that next to immune response induction, a regulated suppression is essential for maintaining proper immune balance.

The diversity in immune responses evoked upon pathogen recognition may require several subsets of $CD4^+Foxp3^+$ Tregs to maintain proper immune homeostasis. There have been important functions reported for T-bet (Th1-specific T box transcription factor) and IRF-4 (Interferon regulatory factor 4) in Tregs demonstrating that the suppression ability requires the expression of transcription factors typically associated with the effector T cell function at place [13]. IRF4 is a decisive factor during Th17 development by influencing the balance of Foxp3, retinoid-related orphan receptor (RORα), and RORγt [14]. In response to IFN-γ, the Tregs are found to up regulate T-bet which promotes the expression chemokine receptor CXCR3, and T-bet^+ Tregs accumulate at sites of Th1 cell–mediated inflammation. T-bet expression is required for the homeostasis and function of Treg cells during type 1 inflammation [15]. This subscribes the hypothesis that specific subsets of $Foxp3^+$ Tregs develop for the suppression of Th1 responses *in vivo* [13]. Moreover, besides strong stimulation of effector

T cells a modulation of Tregs to control immune responses provide an additional arm of intervention in the development of improved vaccines.

Early in life establishment of immune responsiveness is influenced by several factors, including nutrition. Breast milk contains several interesting immune modulating components with specific modulating potentials, which are known to have a clear role in immune mediated disease resistance later in life [16]. Specific oligosaccharides are known to modulate immune responses, as they can improve the immune balance in infants, resulting in lower incidence of infections and simultaneously can have an impact on allergy related symptoms [17]. Prebiotic oligosaccharides can have a direct effect via activation or inhibition of cellular receptors on immune competent cells [18] and may act indirectly through microbiota-dependent mechanisms (i.e. rebalancing microbiota composition in the gut) [19] The pre- and probiotic concept is based on the fact that our microbiota are considered to contribute to induction and maintenance of immune homeostasis possibly via $CD25^+$ Tregs [20]. More specifically, it was found recently that Tregs play a fundamental role in the immune modulation induced by the supplementation of these specific oligosaccharides [21]. The exact underlying mechanism by which prebiotic oligosaccharides induce immune modulation effects however remains to be elucidated and is subject of current investigation. Given the importance of Tregs in maintenance of immune homeostasis and vaccine efficiency in the host, the specific role of Tregs in the immune-modulating effects of dietary supplementation with a unique mixture of prebiotic oligosaccharides scGOS/lcFOS/pAOS in a vaccination model has been investigated.

RESULTS

Oligosaccharide induced T-bet / Gata-3 differentiation increase the Th1 responsiveness.

Immune modulation effects of the dietary intervention were analyzed by measuring antigen specific DTH responses, representing an *in vivo* parameter for Th1 type of cellular immunity. In mice receiving scGOS/lcFOS/pAOS, influenza vaccination results significantly ($p<0.001$) increased influenza-specific DTH responses compared to mice receiving placebo diets (**Figure 1**) which are comparable with earlier described immune modulation capacities of orally supplied oligosaccharides [22]. In addition, the expression of T-bet and Gata-3 in the activated ($CD69^+$) $CD4^+$ T-cell population was assessed both in spleen (data not shown) and MLN cells (**Figure 2**). "Neither in the total $CD4^+$ T cell, nor in the percentage of activated $CD69^+CD4^+$ T cell populations (**Figure 2B**) is a significant change seen between the dietary interventions Interestingly a statistical significant increase in percentage of T-bet positive $CD4^+CD69^+$ T cells (*p<0.001*) could be detected in MLNs of mice receiving scGOS/lcFOS/pAOS compared to mice receiving placebo diet (25.41 ± 1.85 placebo vs. 41.19 ± 1.92 scGOS/lcFOS/pAOS % of $CD4^+CD69^+$ T cells (mean +/- SE)) as depicted in **Figure 2C**. In addition, a

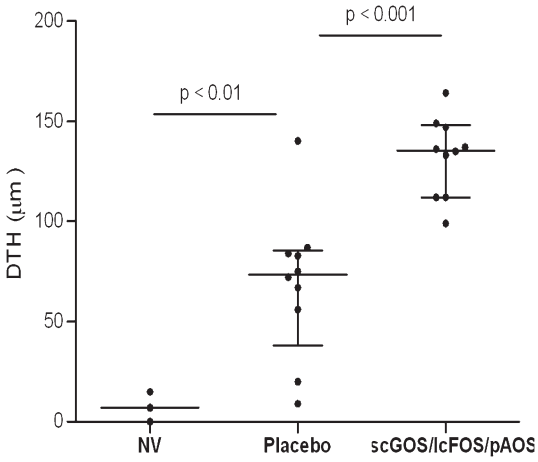


FIGURE 1. Dietary intervention alters Flu-specific DTH responses. C57Bl/6J mice (n = 10 per group) received a sub-maximal vaccination and dietary intervention with or without scGOS/lcFOS/pAOS during the entire vaccination procedure. Antigen specific DTH responses were measured by ear swelling (24 hr. post-antigen injection) and corrected for background (PBS) swelling. A non-vaccinated group (n = 3) was used as control (NV). Lines represent median with interquartile range of the DTH responses from individual mice per group (as indicated through separate dots). The statistical differences are indicated in the graph.

statistical significant reduction ($p<0.001$) was found in the percentage of Gata-3 expressing activated $CD69^+CD4^+$ T cells (20.30 ± 1.28 placebo vs. 10.66 ± 1.13 scGOS/lcFOS/pAOS % (mean +/- SE)) as depicted in **Figure 2C**. Moreover, a significant ($p<0.001$) positive linear correlation ($r^2 = 0,54$) was detected between the percentage of activated T-bet$^+$ T cells and *in vivo* DTH response as well as a significant ($p<0.01$) negative correlation ($r^2 = 0,47$) between the percentage of activated Gata-3$^+$ T cells and DTH response (**Figure 3**). These data combined are indicative for improved Th1 type of immune responsiveness in C57BL/6J mice towards a fixed antigen dose due to specific dietary oligosaccharides.

Changes within Treg population are in line with increased Th1 responsiveness.

If the development of Tregs are in line with the developed immune response as postulated by Barnes *et al* [13], than alterations in immune response should be accompanied with changes in Treg population accordingly. In order to test this hypothesis for the scGOS/lcFOS/pAOS induced Th1 responsiveness, the Treg population was analyzed using flowcytometry with surface staining of CXCR3 as additional marker besides T-bet expression (Th1 polarization) (**Figure 4A**). In mice receiving scGOS/lcFOS/pAOS the percentage of $CD4^+Foxp3^+$ T cells did not change in the MLNs (**Figure 4B**) or in the spleen (data not shown). Strikingly however, the percentage of CXCR3+ and T-bet+ Tregs were significantly lower ($p<0.05$) in the MLNs of mice receiving scGOS/lcFOS/pAOS compared to mice receiving placebo diets (2.99 ± 0.53 placebo vs 1.06 ± 0.11 scGOS/lcFOS/pAOS % of Tregs (mean +/- SE)) (**Figure 4C**). Tregs

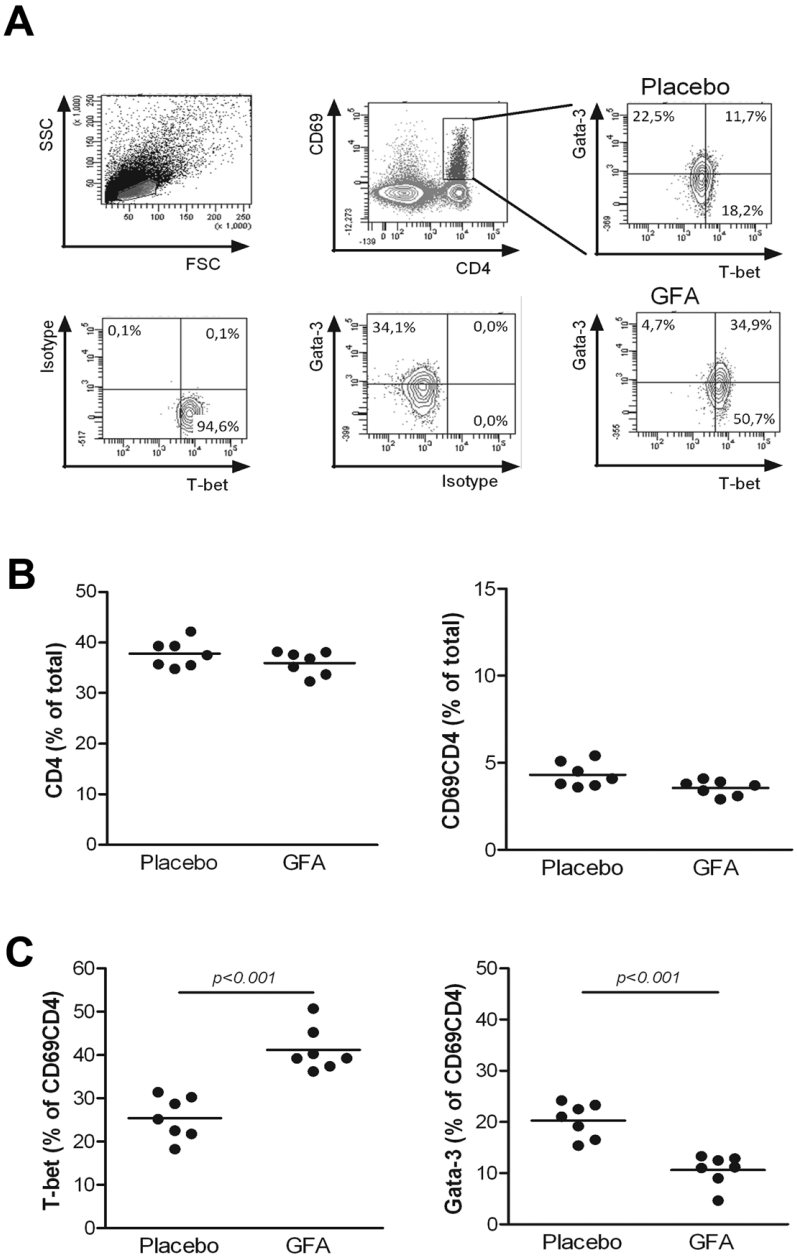


FIGURE 2. Activated CD4⁺ T-cells are modulated towards Th1 type of immune responsiveness. MLN cells were isolated from mice (n = 7 per group) receiving either placebo or scGOS/lcFOS/pAOS and were labeled with CD4/CD69/T-bet/Gata-3 flowcytometric analysis. The characterization of different cell populations is indicated in the gating strategy (**A**). Lines represent mean % of CD4⁺ T cells, activated CD69⁺CD4⁺ T cells (**B**), T-bet⁺ activated T-cells and Gata-3⁺ activated T cells (**C**). In addition, individual measurements are indicated through separate dots. Data presented is representative for 3 individual experiments. Statistically significant differences between the groups are indicated in the graphs.

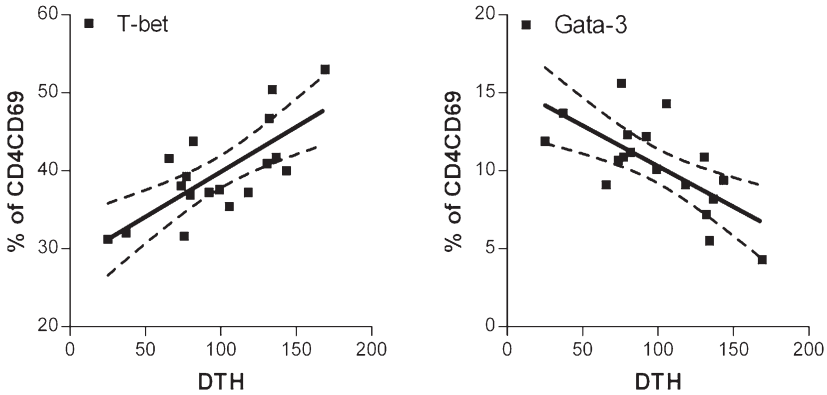


FIGURE 3. Correlation between DTH response and percentage of T-bet$^+$, Gata-3$^+$ of activated CD4 T cells in MLN. From individual mice the DTH values were correlated to the percentage of T-bet$^+$ or percentage Gata-3$^+$ of the activated CD4$^+$ T cells irrespective of dietary intervention, using Pearson and Spearman correlation tests. Mean correlation (line) and 95% CI (dashed) are indicated in the graphs next to the individual data points. With r2 = 0.5438 and r2 = 0.4653 for T-bet and Gata-3 respectively these percentages of activated T cells correlate significantly p<0.001 and p<0.01 respectively.

(Foxp3$^+$CD4$^+$) cells which are positive for both CXCR3 and T-bet are hypothesized to down regulate an increased Th1 response. Therefore these data are indicative for a reduced Th1 suppressive capacity in the Treg population of mice fed the scGOS/lcFOS/pAOS diet. Although the changed percentages are small some strongly significant immune modulatory changes in Treg population are detected due to specific oligosaccharides in the diet, showing their functional capacity as evidenced by increased DTH responses.

Finally the suppressive capacity of isolated Tregs from spleen as well as MLNs was analyzed. From C57BL/6-Foxp3tm1Flv/J mice the regulatory T cells (characterized as CD4$^+$CD25$^+$mRFP$^+$) were cell sorted and cultured together with PBSE labeled effector T cells for 96 hours. Cell division was observed after CD3 stimulation for 96h. Although Tregs isolated from both the spleen as well as the MLN suppressed the CD3 induced T cell proliferation, in none of the ratios tested a difference could be observed between the Tregs isolated from the mice on the different diets (data not shown). In addition to an overall reduction in cytokines produced with increasing percentage of Tregs, a small change in some cytokines was detected between the diets. As shown in **Figure 5** no difference in IL-2 (proliferation) could be detected, but a significant ($p<0.05$) increased IFN-γ (Th-1) as well as significant ($p<0.05$) reduced IL-17 (Th-17) response was detected in spleen cell populations suppressed by Tregs from mice receiving the specific oligosaccharides. The production of IL-13 (Th2), IL-10 as well as IL-6 (inflammatory), were suppressed with increasing amounts of Tregs, but not different between the diets. These changes are in line with improved Th1 type of immune responsiveness in C57BL/6J mice towards a fixed antigen dose due to specific dietary oligosaccharides.

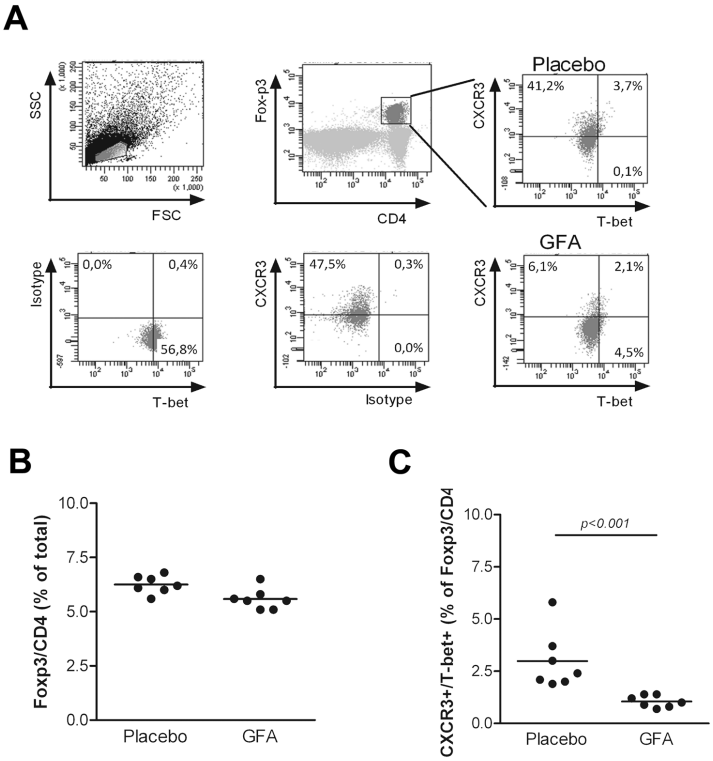


FIGURE 4. Treg population in MLN is influenced by the dietary intervention. Cells from MLNs were isolated from mice (n = 7 per group) receiving either placebo or scGOS/lcFOS/pAOS and were labeled with CD4/Foxp3 in combination with CXCR3, T-bet, for flowcytometric analysis (**A**). Lines represent mean percentages of Tregs in total ($CD4^+Foxp3^+$ T cells) (**B**), and sub-populations of Tregs including % of $CXCR3^+/T\text{-}bet^+$ Tregs (**C**) In addition, individual measurements are indicated through separate dots. Data presented is representative for 3 individual experiments. Statistically significant differences between the groups are indicated in the graphs.

DISCUSSION

A better understanding of the mechanism by which vaccine induced immune responses can be increased using alternative strategies will improve vaccine development. In our current study we show that specific modulation of the immune response by specific oligosaccharide containing diet results in alteration in Treg population. More specifically, a reduced percentage of $T\text{-}bet^+$ Tregs were induced in mice fed scGOS/lcFOS/pAOS diet during vaccination, resulting in increased vaccine responsiveness. Although the involvement of Tregs in dietary immune modulations has been indicated before [21,23], this is the first study showing that an alteration of activated $CD4^+$ T cells (increased T-bet (Th1) and reduced Gata-3 (Th2)) is accompanied with a reduced population Tregs expressing T-bet. Moreover, although suppressive capacity does not seem to be altered, the Tregs seem to suppress through a different and to hereunto unknown mechanism.

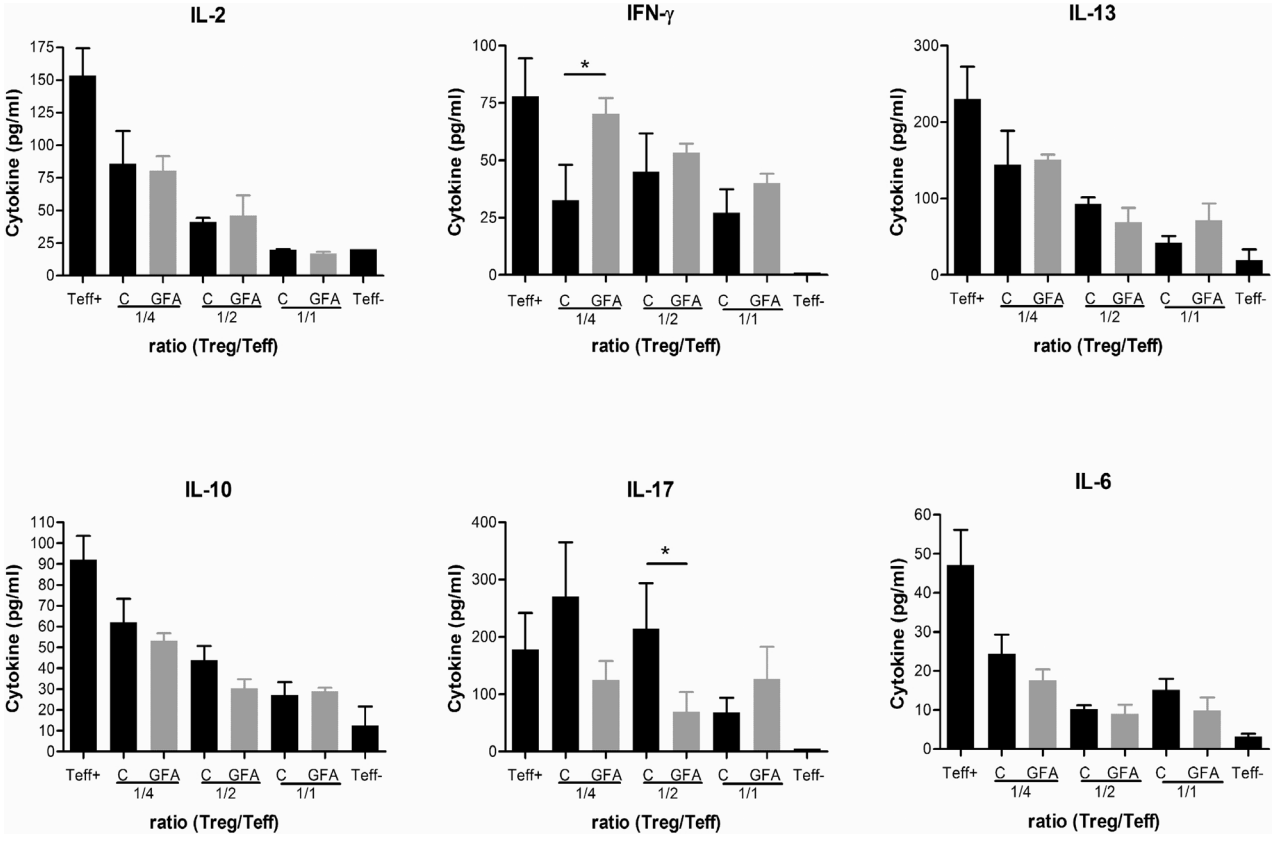


FIGURE 5. Suppression method but not capacity of Tregs is influenced by dietary intervention. $CD4^{+}CD25^{+}mRFP^{+}$ regulatory T cells were isolated from MLN of the C57BL/6-Foxp3tm1Flv/J mice (n=4) using a FACS Aria cell sorter and cultured with Teff cells in ratio of 1/1, 1/2, 1/4 (Treg /Teff) for 96hr. after stimulation with α-CD3. Cell culture supernatant contained different cytokines including (IL-2, IFN-γ, IL-13, IL-10 and IL-17A). Only the significant differences between the diets were indicated using * for $p<0.05$.

Recent observations have challenged the notification of one stable Treg sub lineage. Some reports suggest that there are multiple, functional Treg subsets. One of these subsets expresses T-bet, and has shown to be specifically adapted for the suppression of Th1 responses [15]. In addition another Treg subset expressing IRF-4 was identified, which is essential for Th2 differentiation. The absence of IRF-4$^+$ Tregs even resulted in spontaneous Th2 mediated inflammation, suggesting the necessity of the IRF-4 positive Tregs to control Th2 type of responses [14]. Furthermore a third Treg subtype expressing STAT-3 seemed required suppressing Th17 responses [24]. Therefore as the plasticity of T cell population seems to require plasticity of Tregs to control excessive immune responses, this provides an additional arm of intervention. Within our studies we clearly show increased vaccination responsiveness, by dietary intervention. Both the increased DTH response as well as increased percentage of T-bet expressing T-cells is indicative for improved Th1 responsiveness, which is accompanied by alterations within the Treg population.

Previously, we observed a systemic decrease in Treg numbers in lung, spleen, and mesenteric lymph nodes at day 4 after infection in FI-RSV-vaccinated mice receiving the scGOS/lcFOS/pAOS diet. Tregs have previously been found to regulate RSV-specific primary immune responses. Within this study the expression of granzyme B (GrzmB) in Treg locally in the lung was shown to be involved in the immune-regulatory function of Tregs in the primary RSV infection model [25]. Although the percentage of Tregs did not differ, a significantly decreased absolute number of Tregs was detected in the BAL fluid at day 4 after challenge in FI-RSV-vaccinated mice receiving the scGOS/lcFOS/pAOS diet. This indicates that the dietary intervention with scGOS/lcFOS/pAOS has an effect on the function of regulatory immune cells which correlates with altered immune responses.

The changes detected in the Treg population are multiple, they include a reduced percentage of CXCR3$^+$/T-bet$^+$ Tregs, GITR$^+$CXCR3$^+$ Tregs (data not shown) in the MLNs of mice receiving scGOS/lcFOS/pAOS diet compared to mice fed control diet. T-bet controls migration to inflammatory sites through CXCR3 up regulation as CXCR-3 is a direct transcript of T-bet [26]. Thus, T-bet may therefore play an important role in the direction and regulation of regulatory T cells. This indicates that it is not the percentage of Tregs in total, but merely the functionality which changes due to dietary intervention. Tregs also express receptors for inflammatory chemokines (CCR4, CCR9, and CXCR3), integrin's, and tissue-homing receptors like CD103 [27]. Indeed, it was demonstrated that CD103$^+$ Tregs are attracted and retained in inflamed tissues where they may exert their suppressive function [28]. Functional compartmentalization of Tregs is linked to the expression of different phenotypes, with Tregs found in tissues expressing CD103, IL-10, IL-2R, and CCR5.

In conclusion; this study shows that with dietary intervention using specific oligosaccharides improved vaccine responsiveness can be induced due to reduced Th1 suppressive capacity in the Treg population of mice. The better understanding of the mechanism by

which vaccine induced immune responses can be amplified using alternative strategies will improve vaccine development.

MATERIALS AND METHODS

Animals and diets

Eight-week-old old male specific pathogen-free inbred C57BL/6J mice and C57BL/6-Foxp3tm1Flv/J were obtained from Charles River (Someren, the Netherlands) and housed under standard housing conditions with a 12 hr. dark and light cycle. All animals had free access to tap water and the semi-purified AIN-93G diet (Research Diet Services, Wijk bij Duurstede, the Netherlands), with or without oligosaccharide mixture consisting of three different prebiotic oligosaccharide materials, i.e. short-chain galacto-oligosaccharides (scGOS: Borculo Domo, Zwolle, 45% scGOS), long-chain fructo-oligosaccharides (lcFOS: Orafti, Wijchen, 100% lcFOS) and pectin hydrolysate derived acidic-oligosaccharides (pAOS: Sudzucker, Mannheim, 85% galacturonic acid). The oligosaccharides were mixed in a ratio of 9:1:10 based on carbohydrate purity. Although other combinations of these specific prebiotic oligosaccharides are known to be effective as well, it was this specific ratio within currently used mouse vaccination model which gives the largest immune modulation, detected by a DTH increase at the time and was therefore used for these mechanistically studies. A small negative control group of animals (n=3) was included only to show specificity of the vaccination procedure. Therefore, this group was not used for any statistical comparison to supplemented groups (n=10 per group). Fourteen days prior to the first vaccination dietary supplementation started which was maintained during the entire experimental procedure. The study protocol was reviewed and approved by the Animal Experimental Committee of the Utrecht University (permit number 2011.II.06.102).

Vaccination protocol

All mice except the small negative control group received primary vaccination (day 1) and a booster vaccination (day 21) with a human influenza subunit vaccine consisting of haemagglutinin proteins of 3 different influenza strains (Influvac® , Solvay Pharmaceuticals, Weesp, the Netherlands). Vaccinations were performed by subcutaneous injection of vaccine (30 µg/ml per subunit) in a total volume of 100 µL. The negative control group received concurrent injections with PBS in a total volume of 100 µL. Vaccine-specific DTH reactions were induced 7 days after the last vaccination, by subcutaneous injection of 25 µL Influvac (30 µg/mL per haemagglutinin subunit) into the ear pinnea of one ear. For control, the other ear was injected with 25 µL PBS. Ear thickness was measured in duplicate before challenge, and 24 hours thereafter, with a digital micrometer (Mitutoyo Digimatic 293561, Veenendaal,

the Netherlands). The influvac specificity of the DTH response was calculated by subtracting the basal ear thickness from the value at 24 hours after challenge and was corrected for the control swelling.

Flowcytometric analysis

Splenocytes and cells from the mesenteric lymph nodes (MLNs) were isolated by gently pressing the organs through nylon mesh filters (Falcon cell strainer, Becton Dickinson, Alphen a/d Rijn, the Netherlands). After erythrocyte lysis (spleens only), a total of $1x10^6$ cells were washed with PBS containing 1% FCS and incubated for 15 min with an anti-mouse CD16/CD32 antibody (BD Pharmingen cat# 553142) blocking the Fc receptors. The cells were then stained with different combinations of anti-CD4-FITC (BD Pharmingen cat# 553046), anti-CD4-PE (BD Pharmingen cat# 553049), CD4-PeCy5 (BD Pharmingen cat# 553654), anti-CD25-PE (Beckman Coulter 732091), anti-CD69-APC (eBioscience cat# 17-0691), anti-CXCR3-APC (eBioscience cat# 17-1831), anti-CD103-PE (eBioscience cat# 12-1031), anti GITR-PE (eBioscience cat# 12-5874), or with anti CTLA-4-PE (eBioscience cat#12-1522) for 30 minutes. Intracellular staining was performed according manufacturer's protocol, (EBioscience, Foxp3 staining set, Bio connect, The Netherlands). For intracellular staining the antibodies anti-Foxp3-FITC (eBioscience cat# 11-5773), anti-Gata-3-PE (eBioscience cat# 12-9966), anti-T-bet-PerCP-Cy5 (eBiosience cat# 45-5825) were used in combination with above mentioned surface markers. Matching Isotype controls were used for all staining to minimize the influence of nonspecific binding, and proper gate setting. All staining procedures were performed on ice and protected from light. In total a minimum of 50.000 cells were counted and analyses were performed using FACSCanto II and FACSDiva software (BD Biosciences).

Suppression assay

After labeling of MLN and spleen single cell suspensions obtained from C57BL/6-Foxp3tm1Flv/J mice (Charles river) with CD4-FITC and CD25-APC the $CD4^+CD25^+mRFP^+$ Tregs were isolated by flow cytometry using a FACS Aria cell sorter. Sorted Tregs were cultured together with 2 μM pacific blue succinimidyl ester (PBSE Invitrogen) labeled total spleen cells (20.000 per well ((Teff)) in ratio's as indicated in the graphs. Cells were stimulated with 1ug/ml anti-CD3 (BD Pharmingen clone 145-2c11) for 96hr. at 37°C. Cell culture supernatants were collected and cytokines were measured according to manufacturer's protocol using Bio-Plex Pro Mouse Cytokine Grp I panel 23-Plex (BioRad). For cell division analysis using PBSE positivity, the cells were stained with CD4-FITC (BD Pharmingen cat# 553046), CD8-APC (BD Pharmingen cat# 553932) and cell division was measured using flowcytometry.

Statistical analysis

All statistical calculations were performed using SPSS version 12.0.1 software and Graph Path prism 4.03 software. Statistical differences between test and control groups were analyzed by one way ANOVA followed by multi-group comparison analysis using the Bonferroni test; otherwise an un-paired two sided t-test was used. Correlations were identified using Spearman and Pearson tests. All values are presented as mean ± SEM. P-values <0.05 were considered significant.

Funding Statement

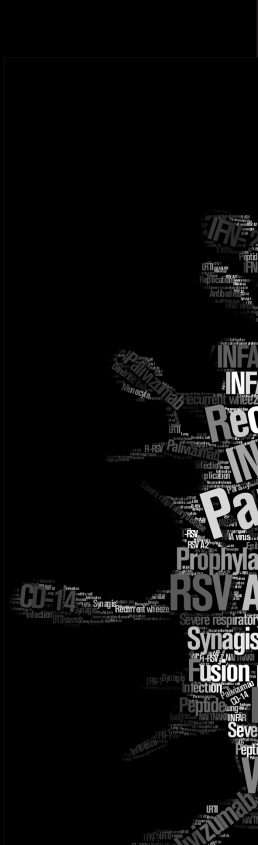
This study was financially supported and performed within the framework of the Dutch Top Institute Pharma project: D1-101-0 (http://www.tipharma.com). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

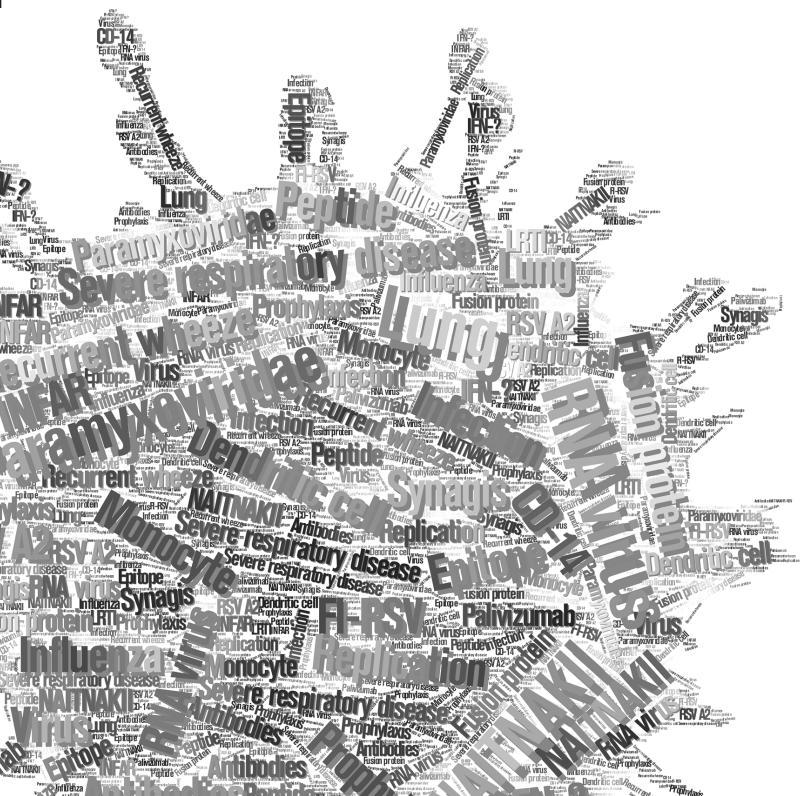
1. Belkaid Y, Rouse BT (2005) Natural regulatory T cells in infectious disease. Nat Immunol 6: 353-360.
2. Marchant A, Goldman M (2005) T cell-mediated immune responses in human newborns: ready to learn? Clin. Exp Immunol 141: 10-18.
3. Schonland SO, Zimmer JK, Lopez-Benitez CM, Widmann T, Ramin KD, et al. (2003) Homeostatic control of T-cell generation in neonates. Blood 102: 1428-1434.
4. Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, et al. (2008) Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J Immunol 180: 5737-5745.
5. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, et al. (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 116: 485-494.
6. Van't Land B, Schijf MA, Martin R, Garssen J, van Bleek GM (2011) Influencing mucosal homeostasis and immune responsiveness: The impact of nutrition and pharmaceuticals. Eur J Pharmacol 668 Suppl 1:S101-7
7. Suchard MS, Mayne E, Green VA, Shalekoff S, Donninger SL, et al. (2010) FOXP3 expression is upregulated in CD4+ T cells in progressive HIV-1 infection and is a marker of disease severity. PLoS One 5: e11762.
8. Piconi S, Trabattoni D, Gori A, Parisotto S, Magni C, et al. (2010) Immune activation, apoptosis, and Treg activity are associated with persistently reduced CD4+ T-cell counts during antiretroviral therapy.AIDS 24:1991-2000.
9. Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. Int J Cancer 127: 759-767.
10. Antunes I, Kassiotis G (2010) Suppression of Innate Immune Pathology by Regulatory T Cells During Influenza A Virus Infection of Immunodeficient Mice. J Virol 84:12564-12575.
11. Brandenburg AH, Kleinjan A, van't Land B, Moll HA, Timmerman HH, et al. (2000) Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. J Med Virol 62: 267-277.
12. Fulton RB, Meyerholz DK, Varga SM (2010) Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. J Immunol 185: 2382-2392.
13. Barnes MJ, Powrie F (2009) Hybrid Treg cells: steel frames and plastic exteriors. Nat Immunol 10: 563-564.
14. Zheng Y, Chaudhry A, Kas A, de Roos P, Kim JM, et al. (2009) Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature 458: 351-356.
15. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, et al. (2009) The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol 10: 595-602.
16. Van't Land B, Boehm G, Garssen J (2011) Breast milk: components with immune modulating potential and their possible role in immune mediated disease resistance. Dietary Components and Immune Function, ISBN 978-1-60761-060-1.
17. Arslanoglu S, Moro GE, Schmitt J, Tandoi L, Rizzardi S, et al. (2008) Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. J Nutr 138: 1091-1095.
18. Vos A, M'Rabet L, Stahl B, Boehm G, Garssen J (2007) Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Crit Rev Immunol 27: 97-140.

19. Gori A, Rizzardini G, Van't Land B, Amor KB, van Schaik J, et al. (2011) Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the "COPA" pilot randomized trial. Mucosal Immunol.4:544-63.
20. Ishikawa H, Tanaka K, Maeda Y, Aiba Y, Hata A, et al. (2008) Effect of intestinal microbiota on the induction of regulatory CD25+ CD4+ T cells. Clin Exp Immunol 153: 127-135.
21. Van't Land B, Schijf M, van Esch BC, van Bergenhenegouwen J, Bastiaans J, et al. (2010) Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. Vaccine 28: 5711-5717.
22. Vos AP, Haarman M, van Ginkel JW, Knol J, Garssen J, et al. (2007) Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatr Allergy Immunol 18: 304-312.
23. Schouten B, van Esch BC, Hofman GA, de Kivit S, Boon L, et al. (2011) A potential role for CD25+ regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. Br J Nutr: 1-10.
24. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, et al. (2009) CD4+ regulatory T cells control Th17 responses in a Stat3-dependent manner. Science 326: 986-991.
25. Schijf MA, Kruijsen D, Bastiaans J, Coenjaerts FE, Garssen J, et al. (2012) Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. J Virol 86: 11472-11482.
26. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, et al. (2005) T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood 106: 3432-3439.
27. Sather BD, Treuting P, Perdue N, Miazgowicz M, Fontenot JD, et al. (2007) Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. J Exp Med 204: 1335-1347.
28. Suffia I, Reckling SK, Salay G, Belkaid Y (2005) A role for CD103 in the retention of CD4+CD25+ Treg and control of *Leishmania* major infection. J Immunol 174: 5444-5455.

Chapter 10



Summary and Discussion



Modern vaccination strategies are based on the induction of highly neutralizing antibodies. In the first part of this thesis we addressed several immunological questions concerning the role of antibodies in recurrent infections and innate and adaptive immunity against RSV.

In the second part of this thesis we discussed the impact of microbial colonization of mucosal surfaces on the development of immunity against RSV. We demonstrate the impact of dietary antibiotic or prebiotic use on microbiome composition and the adaptive immune response evoked in lungs of RSV infected mice.

GENERAL DISCUSSION

The exact cause of severe disease in children during primary RSV infections is not completely clear. There is a link with viral load, but differences in virus strains do not seem to be the major reason why in some children the disease manifests as a mild cold while others suffer from a severe lower respiratory tract disease. The prevalence of severe lower respiratory tract infection due to RSV is high in the neonatal period. The immune response in this age group is characterized by suppressed innate immune responses and a Th2 bias, reasons for poorly effective immune responses to infection and vaccination. During the neonatal period maternally derived antibodies are an important factor to protect the newborn against pathogens upon the first encounter. For RSV high levels of serum neutralizing antibodies are required to neutralize the virus in the airways [1–3]. This is also underscored by the fact that antibody presence is no guarantee that re-infections are prevented. Re-infections with genetically similar RSV strains still occur even within the same RSV season. These reinfections, despite the presence of virus specific $CD4^+$ and $CD8^+$ T cell responses and antibodies suggest insufficient efficacy of acquired immunity. Specific virus characteristics, i.e. high replication rate and specific immune evasion mechanisms might contribute to the potency of RSV to re-infect the host. The ability of RSV to suppress the initiation of an effective T cell response has been shown by us (**Figure 1**) and described by several research groups.

RSV infected dendritic cells suppress the proliferative response and the maturation of effector function in $CD4^+$ and $CD8^+$ T cells. Two mechanisms have been described that may contribute to this inhibitory process, i.e. a soluble factor produced by RSV infected dendritic cells [4] and a contact-mediated inhibitory mechanism that prevents effective APC-T cell synapse formation [5]. Furthermore, Kruijsen *et al* showed that preexisting antibodies might prevent efficient induction of functional RSV specific $CD8^+$ T cell responses [6]. This process might also contribute to decreased efficiency of virus eradication. Moreover, RSV specific antibodies might eventually lead to less effective $CD8^+$ T cell memory. In healthy adults RSV specific $CD4^+$ T memory cells can be detected with high frequencies while $CD8^+$ T cell numbers appear to be low [7]. This situation is in contrast to the situation for influenza virus where virus specific CD8/CD4 T cell ratios in peripheral blood are usually much higher. From our murine studies we can conclude that virus neutralizing antibodies prevent the classical

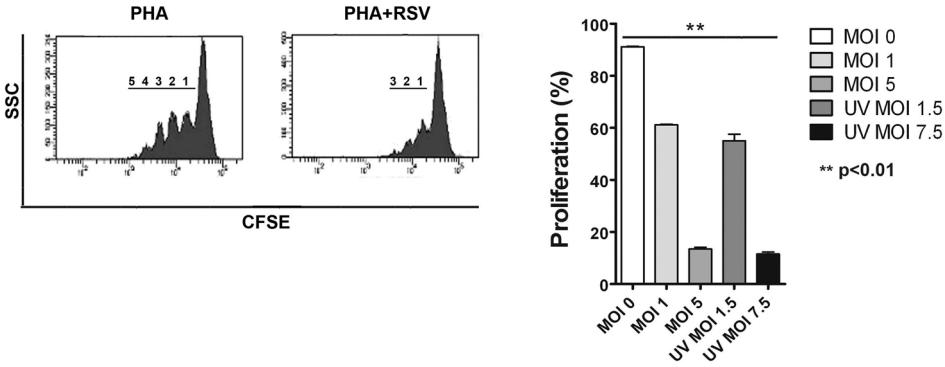


FIGURE 1. Proliferation of PHA activated T cells is suppressed by RSV via a non-infectious mechanism. Carboxyfluorescein succinimidyl ester (CFSE) labeled human PBMC were stimulated with 10ug/ml Phytohaemagglutinin (PHA) and simultaneously infected with different multiplicities of infection (MOI) of (UV inactivated) RSV. Data represent the percentage of divided $CD3^+$ cells compared to non-stimulated $CD3^+$ cells 5 days post infection.

proteasomal degradation route after infection of APC and thereby prevent effective class I presentation to $CD8^+$ T cells [6]. While Ab-RSV complexes present in the airways can still induce $CD8^+$ T cell responses (**chapter 4**), this process is less effective than the induction of $CD8^+$ T cell responses by RSV in the absence of virus neutralizing antibodies. Some caution is warranted though when extrapolating this mechanism to humans, because virus replication is much more effective in humans compared to mice. It is possible that neutralizing antibodies are therefore less effective in the inhibition of the infection mediated route of antigen presentation. In addition to the effect of virus specific (neutralizing) antibodies on the amount of viral antigenic materials displayed on antigen presenting cells to virus specific T cells, we showed in **chapter 3** that antibodies also strongly affect the nature of the innate immune response to RSV, depending on the particular context wherein the virus and antibodies are present. Therefore, cytokines and chemokines induced locally in the lung, in the presence or absence of RSV specific antibodies, might affect the cellular repertoire attracted to the infected lung and the adaptive immune responses primed and reactivated in the lung draining lymphnodes or lung tissue respectively. These processes might also be extremely important to consider when attenuated viral vaccines are tested for safety and efficacy, since in our opinion attenuated replication and cytokine profiles in cultured epithelial cell lines does not suffice as safety indications [8,9]. Moreover, the effects of neutralizing antibodies on innate immunity and the initiation and maturation of RSV specific memory and effector cells should also be considered during the development of (stronger) neutralizing RSV specific antibodies used for prophylaxis. Antibody/FcR mediated reduced capacity to induce protective antiviral T cell responses might add to the inability to produce protective Th1 like cytokines and may increase the risk of excessive viral replication and disease severity. However the most successful current strategy in the protection against severe RSV infections

is the prophylactic use of the neutralizing antibody Palivizumab [10,11]. Reduced titers of serum antibodies correlate with increased RSV-associated hospitalization in patients of all ages and the additional use of neutralizing antibodies prevents infection and limit cell recruitment into the airways. Vaccination strategies are therefore aimed to induce high levels of neutralizing antibodies. The fact that the more potent monoclonal Ab Motavizumab [12] does not result in reduced incidence of severe RSV disease in high risk groups compared to Palivizumab suggests that inducing highly neutralizing Ab is not the only solution to a successful vaccine.

The difficulty to develop a protective vaccine candidate against RSV was demonstrated in the 1960s when children intra muscularly vaccinated with formalin inactivated (FI-) RSV developed severe lower respiratory tract disease upon infection with community acquired RSV [13–15]. Many animal models have been used to understand RSV induced severe disease and the FI-RSV vaccination trial (described in **chapter 1**). Vaccination with FI-RSV in BALB/c mice results in airway hyperresponsiveness, mucus production, increased numbers of neutrophils and eosinophils in lung lavages, poor neutralizing antibodies and a T cell response dominated by Th2 cells. In these mice, several groups have investigated the contribution of individual components to the harmful immune response evoked upon infection. Factors described to contribute to FI-RSV induced immune pathology are; viral epitope disruption by formalin [16], RSVs G protein [17,18] and the use of aluminum hydroxide as an adjuvant [19–21]. We performed FI-RSV vaccination experiments in C57BL/6 mice with similar results as in the BALB/c mouse model with respect to pulmonary influx of neutrophils/eosinophils and a Th2 biased $CD4^+$ T cell response in **chapter 2**. Investigation of the individual components demonstrated that neither formalin treatment nor the adjuvant aluminum hydroxide where essential components in the induction of eosinophilia and the Th2 response in this C57BL/6 mouse model. Furthermore i.m. priming with a control vaccine, FI-RSV and FCS all induced eosinophilia upon infection with RSV cultured in FCS containing medium, demonstrating that not only the specific characteristics of the individual proteins of RSV but any protein presented via i.m. priming can contribute to enhanced disease in this murine model. Interestingly i.m. FCS primed mice i.n. infected with FCS+Influenza showed reduced Th2 T cell responses compared to mice challenged with FCS+RSV. This finding suggests that the outcome of the local innate immune response triggered upon viral challenge depends on the nature of the infecting virus and this might explain the difference in success of vaccination strategies with inactivated viral vaccines. The infectious mechanism of influenza virus depends on receptor mediated entry and routing to endo/lysosomal compartments where fusion occurs in a pH dependent mechanism. This process targets viral particles to endosomal TLRs. RSV does not require endosomal entry and during natural infection endosomes are bypassed. These different entry mechanisms could be a reason for the differences in the efficacy to trigger innate immunity, and the difference in the potency of these viruses to locally shift the effector response towards a Th1 type response.

The question whether RSV infection creates an innate response in the lung that favors the attraction of Th2 type cells or fails to suppress the Th2 setting, initiated during priming remains elusive. We also showed that during vaccination, systemic availability of RSV matured dendritic cells trough i.v. injection induced a robust $CD8^+$ response and reduced eosinophilia 5 days after an intranasal RSV challenge (**chapter 2**). This confirms earlier observations that one of the important factors to prevent the Th2 shifted response associated with severe disease, is the priming of a $CD8^+$ T cell response during the initial exposure to virus particles [22–24]. This was shown before in BALB/c mice experiments, where disease was ameliorated when in addition to a strong G-specific $CD4^+$ T cell response also $CD8^+$ T cells were primed against the M_{2-2} –H-K^d epitope.

From the sequence of events observed in children hospitalized with severe RSV infections, it has been suggested that the innate immune response might be an important factor contributing to disease, while the acquired T cell response against the virus is protective [25]. During primary infections the maternally derived antibodies limit viral replication and innate immune responses might contribute to this process as well, but at the cost of collateral damage caused by inflammatory cytokines, chemokines and attracted innate immune cells, altogether resulting in cloughing of the small bronchioles by dead cell debris and increased mucus production. Since the exact nature of the innate immune response caused by RSV has been studied in murine models [26–30], cultured human cell lines [31–33] or *in vitro* cultured dendritic cells [34–36], detailed knowledge in primary human cell types and the impact of viral infection in mixtures of different cell types, as it occurs *in vivo* in human tissues is lacking. In **chapter 3** we investigated the innate response triggered upon RSV infection in a complex mixture of primary human cells (PBMC) and the human epithelial cell line A549. Although our setting of a short exposure of a static human PBMC cell mixture to RSV infection in *vitro* will not resemble the *in vivo* situation, our experiments demonstrate that the outcome of the inflammatory response in a complex environment, in which different cells are activated in their own specific manner, cannot be predicted from sum of the responses of each individual cell type. We showed that within PBMC, binding of RSV is cell type specific. Moreover, the amount of viral particles that binds to specific cell types does not necessarily correlate with the cell types that become infected. These data show that both infection related routes of innate immune triggering and receptor mediated triggering of surface PRR can contribute to outcome of the innate response. RSV binds and infects primarily monocyte subtypes, DC and epithelial cells (**chapter 3**). During natural exposure to RSV, airway epithelial cells, alveolar macrophages in the airway lumen and dendritic cells underneath the epithelium are the primary cells initially exposed to RSV. These cell types contribute to innate immunity by controlling viral replication via the production of type I interferons and the induction of a local inflammatory cytokine and chemokine environment [37]. We showed that the broadly used RSV A2, the RSV A long strain and 2 recently isolated clinical strains all evoke a similar inflammatory response in epithelial A549 cells and human PBMC. The inflammatory

response in epithelial A549 cells was mediated via a process that depended on RSV infection and is presumably mediated via RIG-I [38,39], whereas CD14$^+$ cells were the main producers of inflammatory cytokines within the PBMC mixture and showed a specific cytokine profile specific for infectious, non-infectious or antibody opsonized virus. With depletion of specific cell types within PBMC we demonstrated that pDC were the solitary producers of IFN-α via different pathways; I: TLR7 activation after Ab-mediated uptake of RSV, II: Infectious pathway of RSV in purified pDC and III: Indirectly via IFNAR-mediated signaling triggered by type I interferon produced by RSV-infected A549 or RSV-exposed CD14$^+$ cells.

Although we confirmed earlier studies where it was shown that purified pDC could produce IFN-α when exposed to infectious RSV [40], we also showed that in the absence of epithelial cells or CD14$^+$ monocytes, pDC are poor producers of IFN-α upon infection with RSV compared to the exposure to viral components via endosomal TLR7 or via IFNAR mediated pathways described above. Also in contrast to the laboratory Long strain virus used by Hornung *et al* (36) A2 strain and natural isolates are poor inducers of IFN-α by pDC and these strains reflect more the natural situation than the pDC infection with LONG strain virus, that was selected for it high potential to induce type I interferon. Possible explanations could be that the amount of IFN-α might be suppressed once viral non-structural proteins are expressed in RSV-infected pDC [8,41,42]. And secondly, RSV infection and propagation does not follow an endo-lysosomal route [40], avoiding the production of TLR7 mediated IFN-α production, that is readily observed for influenza virus, where even inactivated viral particles efficiently enter endosomes causing IFN-α production [43]. The inability to enter endosomal compartments can be overcome by presenting RSV as immune complexes to pDC (**chapter 3**, **Figure 2**).

Antibody opsonized viral particles might be beneficial to the production of IFN-α in two ways; by the prevention of infection and thereby preventing the expression of viral non-structural proteins that interfere with type I interferon production pathways and secondly, facilitating FcR mediated viral uptake and activation of the TLR7/MyD88/ IFN-α pathway. The efficiency of this process *in vivo* remains to be established. In **chapter 3** we showed a key role for CD14$^+$ monocytes in inhibiting or inducing pDC antiviral type I IFN responses to RSV via respectively a direct antibody mediated and an indirect, infection mediated, mechanism. Therefore, the amount of type I interferon induced in lung tissue or lymphnodes will depend on the cellular context and the efficiency by which IgG-RSV complexes are produced *in vivo*. Some groups have shown that IFN-α production induced by bacterial CpG in pDC could be inhibited by Prostaglandin (PG) E and IL-10 released from monocytes that were stimulated with TLR2 and 4 ligands [44]. Our data suggest that the inhibition of TLR7 activation, after Ab-mediated uptake of RSV and subsequent IFN-α release, by CD14 monocytes in our model is not mediated via these factors. However, RSV interacting with TLR4 complex or TLR2 complex might decrease type I interferon by RSV exposed pDC via different mediators. Moreover, local commensal bacteria or co-infecting virus could also play a role in this process.

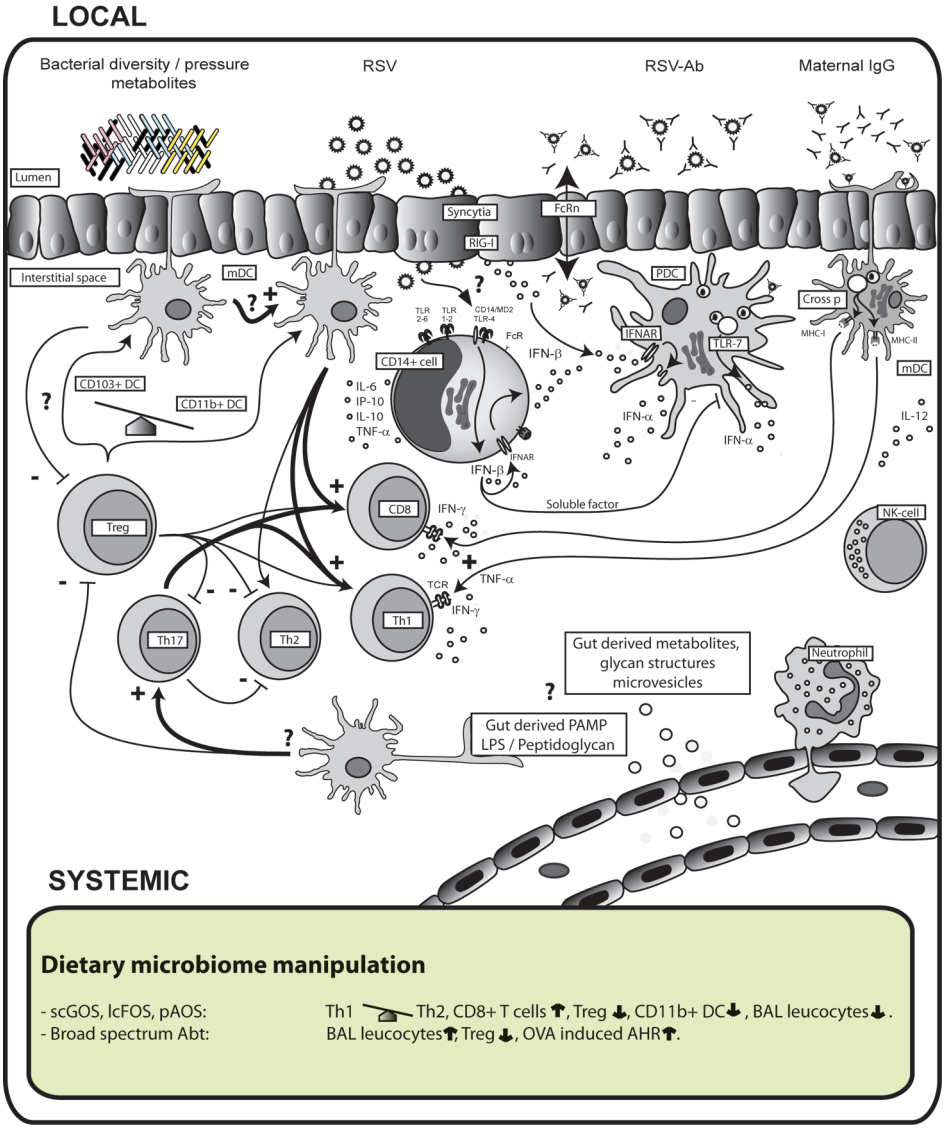


FIGURE 2. Aspects of local cellular immunity upon primary RSV infection described in this thesis and possible mechanisms involved in specific dietary pre- and antibiotic influenced RSV specific T cell responses. In human primary RSV infection, pDC contribute to the antiviral innate type I IFN response by producing IFN-α via 3 different pathways; I: TLR7 activation after Ab-mediated uptake of RSV, II: Infectious pathway of RSV probably via RIG-I and III: Indirectly via IFNAR-mediated signaling triggered by type I interferon produced by RSV-infected epithelial cells or RSV-exposed CD14$^+$ cells (**chapter 3**). In mice, the cellular immune response against primary infection is dominated by RSV specific IFN-γ producing CD4$^+$ and CD8$^+$ T cells 4-10 days post infection (**chapter 7**). Prophylactic anti- and pre-biotics both alter microbiome composition and may influence mucosal and systemic immunity, resulting in an altered cellular immune response against RSV **[98]**. Lungs of mice treated with dietary scGOS/lcFOS/pAOS contained reduced numbers of BAL leucocytes, Treg and inflammatory CD11b$^+$ DC and increased numbers of IFN-γ producing CD4$^+$ T cells 6-10 days post RSV infection (**chapter 7**).

Because RSV specific antibodies are transmitted from mother to child during pregnancy and via breast milk, the role of these antibodies should be carefully evaluated with respect to their effect on innate immune responses when new vaccine candidates are tested. In addition to FcR mediated viral uptake and signaling on the cell membrane, the neonatal Fc receptor (FcRn) is an important receptor for antigen sampling at multiple mucosal sites [45]. FcRn is known to regulate serum albumin and IgG concentrations, provide passive immunity trough transport of IgG across the placenta from mother to infant and the bidirectional transfer of (antigen bound) IgG across the epithelial layer via transcytotic pathway [46,47]. The transfer of antigen bound IgG from the lumen into the lamina propria have been demonstrated in the intestine and a similar process occurs in the upper airways in humanized mice [45]. The immune complexes transported into the lamina propria, are internalized by antigen presenting cells and presented to T cells at different lymphatic sites. The importance for FcRn has been shown in specific antigen presenting cells for MHC II presentation to $CD4^+$ cells as well as cross-presentation to $CD8^+$ T cells in both *in vitro* and *in vivo* studies[45,48–50]. In **chapter 4** we investigated how pre-existing RSV specific antibodies could influence the priming of subsequent adaptive immunity against RSV and a possible role for FcRn in this process. Kruijsen *et al. previously* showed in mice that neutralizing antibodies systemically present could affect RSV-specific T-cell responses by shifting the CD4/CD8 T cell balance, resulting in decreased $CD8^+$ T cells present in the lung [6]. Antibody opsonized virus may prevent viral infection and thereby reducing the ability for class I presentation and $CD8^+$ T cell priming. We showed that i.n. administration of UV-inactivated virus particles resulted in poor T cell priming, while both $CD4^+$ and $CD8^+$ virus-specific T-cell responses were induced after viral challenge when virus was opsonized with palivizumab during priming (**chapter 4**, **Figure 3**). These experiments showed that presenting non-infectious virus particles in immune complexes facilitates $CD8^+$ T cell priming, possibly via more efficient targeting to class I routes of (cross-) presentation in APC. Also the different cytokine and presumably chemokines produced upon interaction of cells with RSV-IC might contribute to $CD8^+$ T cell priming. Both processes might be facilitated by FcγR that were shown to be crucial for enhanced $CD8^+$ T cell priming (**chapter 4**, [51]).

In summary, prophylactic administration of RSV neutralizing antibodies clearly has beneficial effects in high risk children presumably by lowering viral load and decreasing over exuberant innate immune responses [10,52,53]. However, the presentation of antibody opsonized virus to various cell types important in the initiation of innate and adaptive immune responses may alter innate pathogen recognition pathways, affect the onset of subsequent immune response evoked by these cells and the ability to resist subsequent RSV infections. When testing new vaccine candidates or prophylactic Ab treatment, awareness of the mechanism of cellular cross talk and the role of antibodies in T cell priming and innate immunity to RSV might contribute to the evaluation of vaccine efficacy and safety.

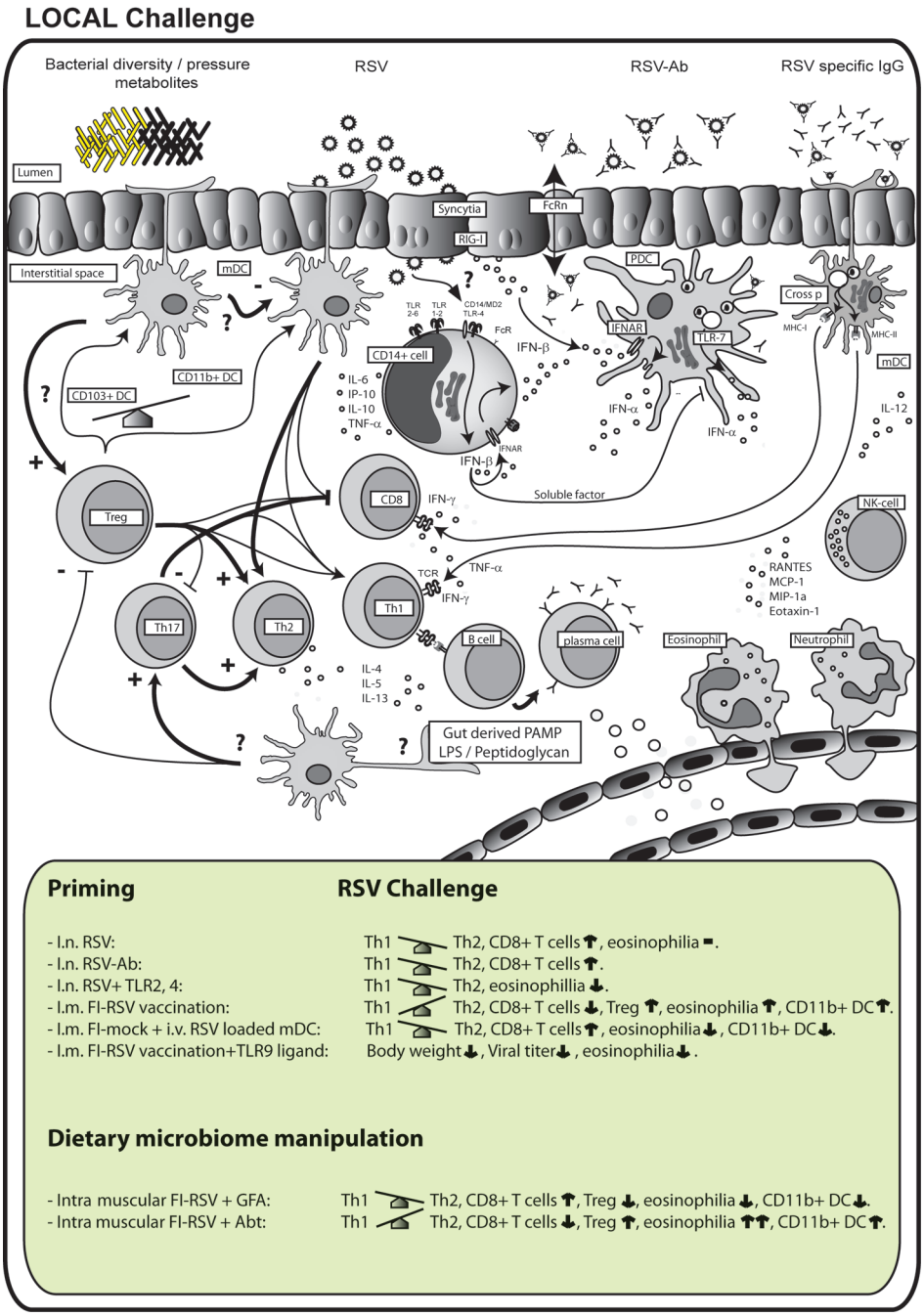


FIGURE 3. Aspects of local immunity upon RSV infection in RSV or FI-RSV vaccinated mice. In mice, intra muscular vaccination with formalin inactivated RSV (FI-RSV) results upon challenge with live virus in lung eosinophilia, RSV specific antibodies and Th2 biased T cell responses. Interestingly, i.m. vaccination with FI-RSV +TLR9 ligand **[107]**, i.n. RSV priming or FI mock vaccination in combination with i.v. RSV loaded mDC does not lead

During the neonatal period exposure to the microbial environment contributes to maturation of the immune system. Interestingly, colonization of mucosal sites including skin, lung and intestinal tract with substantial amounts of microorganisms, does not result in extensive pro-inflammatory responses. Several studies have shown that, during the first month of life, *ex vivo* re-stimulation of isolated neonatal monocytes with Toll ligands showed an inflammatory cytokine response similar to adults [54,55]. This suggests that specialized tolerogenic mechanisms regulate immune adaption of the immune system to microorganisms in the first months of life. Immune programming trough bacterial colonization in the first months of life might be a window of opportunity for the development of a balanced immune responsiveness. Indeed several mouse studies [56] and human clinical trials [57] have demonstrated that the most promising strategy for immune modulatory effects in atopic disease is trough administration of specific bacterial strains to pregnant woman, colonizing the infant's mucosal surfaces at birth. In this period altered colonization due to antibiotic treatment, environmental challenges or pharmaceutical components might affect the development of neonatal immunity. Correlations between bacterial colonization of different mucosal surfaces, allergic/infectious disease and the influence of pharmaceutical and nutritional components on particularly gastrointestinal microbial colonization are discussed in **chapter 5**. The link between microbial composition and its influence on the immune system and immune mediated diseases originate from 1989 where Strachan *et al* first described the "hygiene hypothesis" suggesting that the presence of infectious agents in early life might protect us from Th2 related immune responses and atopic disease in later life [58]. However after 24 years, epidemic studies have shown the increase of atopic diseases in children with Th1-promoting disease [59] and the protection of Th2 promoting parasitic infections against atopic disease [60]. Furthermore, although several clinical studies have shown some influence of specific bacteria on the prevention of allergic diseases [61], major improvements to prevent or treat allergic disease have not been accomplished. These findings demonstrate not only the complexity of allergic disease, but also that we are only beginning to understand how microbial communities can influence mucosal immunity and mucosal derived immunological disorders. Metagenomic studies like the human microbiome project suggest the presence of an intestinal core microbiome in human individuals. For example, it seems that deviation from this core microbiome

---

to severe disease upon viral challenge, demonstrating the importance of either location of vaccination and/or aspects of pathogen recognition in the priming phase of the response (**chapter 2**). Infectious and antibody opsonized virus can both prime RSV specific T and B cell immunity via a FcRn independent mechanism resulting in dominant IFN-γ producing $CD4^+$ and $CD8^+$ T cells upon challenge (**chapter 4**). Prophylactic anti- and pre-biotics both alter microbiome composition and may influence mucosal and systemic immunity (**chapter 5**), resulting in an altered cellular immune response against RSV (**chapters 7 and 8**) and influenza (**chapters 6 and 9**). Lungs of FI-RSV vaccinated mice treated with dietary scGOS/lcFOS/pAOS contained reduced numbers of BAL leucocytes, eosinophilia, Treg and inflammatory $CD11b^+$ DC and increased numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells 6-10 days post RSV infection (**chapter 7**). Orally applied broad spectrum antibiotics during FI-RSV priming resulted in an increased RSV specific inflammatory response upon viral challenge (**chapter 8**).

can be associated with diseases like obesity [62]. Once formed, microbiome composition is relatively stable throughout adulthood [63]. Microbiome manipulation through the use of a single bacterial strain generates the intrinsic challenge of changing the composition of an established microbiome in balance with a certain setting of the immunological responses. To manipulate bacterial composition and immune parameters is a big challenge also because of the differences in stability and colonization capacity between bacterial strains, which will result in altered life/dead ratios between strains and altered length of presence on the mucosal surfaces. Moreover, we are just at the beginning to understand some of the mechanisms by which mucosal or systemic immunity can be regulated by commensal bacterial products. In **chapters 6, 7 and 9** we used dietary non-digestible oligosaccharides with known prebiotic characteristics stimulating the growth of different commensal bacteria already present within the microbiome. Several groups have described microbiome modulating properties of short-chain Galactooligosacharides (scGOS), long-chain Fructooligosaccharides (lcFOS) and pectin-derived Acidic oligosaccharides (pAOS) that mimic microbiome modulating properties of oligosaccharide structures present in breast milk [64–67]. We show that a diet based on these carbohydrates could systemically alter immune responses in mouse models. In **chapter 6** we demonstrated that dietary intervention with a combination of scGOS/lcFOS/pAOS, starting 2 weeks before vaccination, can enhance the influenza specific Type IV hypersensitivity vaccination response in C57BL/6 mice induced by a human influenza subunit vaccine. Furthermore, i.p. injection of monoclonal antibodies against CD25, a well-accepted method to deplete $CD4^+CD25^+Foxp3^+$ regulatory T cells [68,69], reduced the GFA mediated increase in influenza specific DTH response. Furthermore, injection of monoclonal antibody in vaccinated mice treated with control diet showed no inhibition of the DTH response demonstrating that depletion in this model is mostly like limited to regulatory T cell (Treg) populations. Our data suggest that $CD25^+$ regulatory T cells control the early stages of the development of enhanced Th1 responsiveness. Vos *et al* have shown in a similar influenza specific Type IV hyper-sensitivity vaccination model that timing of GFA supplementation was crucial for enhanced responsiveness[70]. Supplementation of GFA before the primary vaccination was necessary to increase delayed-type hypersensitivity responses (DTH) significantly at day 30, indicating that immune modulation occurred during the priming phase [70,71].

Breastfeeding reduces the risk of hospitalization for lower respiratory tract infections in the first year of life [72]. Moreover; several clinical studies have demonstrated the protective effects of breast milk against RSV bronchiolitis [73]. This effect is usually ascribed to virus specific neutralizing antibodies derived from the mother via the placenta and in milk. However, several other components present in breast milk might also have direct or indirectly, via microbiome formation or microbial metabolites, immune stimulatory potential. In **chapter 7** we showed that GFA oligosaccharides also affect adaptive immunity in a primary RSV infection model and in the FI-RSV vaccination model for sever disease. In both models

the balance of the immune response shifted to stronger Th1 type cytokine milieu and is in accord with the enhanced the Th1 driven influenza specific Type IV hyper-sensitivity in an influenza vaccination model (**chapter 6**) and [70]. Further research is needed to unravel the mechanism behind the effect of the GFA diet. Studies of the intestinal and pharyngeal bacterial composition before and after implementing the GFA diet might give indications of the involvement of specific bacterial strains in the process.

The bias of neonatal immunity towards mucosal immune tolerance avoids extensive inflammation towards microbial colonization. However, this period might increase the susceptibility to viral infections in newborns or even later in life. Studies in mice have shown that neonatal infection with RSV causes more severe bronchial inflammation upon adult re-infection compared to re-infections in adult mice [74]. In the neonatal period regulatory T cells are easily induced. Their function might not only be the induction of mucosal tolerance but may also play an important role in this neonatal period in controlling viral infections and the prevention of allergic disease in later life [75]. We investigated Treg populations, and especially granzyme B producing Treg, a population uniquely present in the lung [76], in our murine RSV models. Our data showed that dietary intervention with GFA resulted in decreased numbers of regulatory T cells present in the lung on day 4 and 6 post infection at the peak of the effector phase of the RSV specific T cell response. Although we did not measured the functional (suppressive) capacity of these cells our data suggest that combined with decreased eosinophilia and Th2 cytokine responses, lower Treg numbers are a result of the suppressed inflammatory response induced by dietary GFA. Future research should study this role of Treg in more detail, with special focus also during the initiation phase of the immune response against RSV, when Treg play a crucial role in the regulation of potentially pathogenic $CD8^+$ T cell responses [76–79].

The question how oligosaccharides influence the infection and vaccination models as described above cannot be answered conclusively on the basis of the studies presented in this thesis. Since 1% of the HMOs are absorbed and reach the systemic circulation [80–83], potential direct receptor mediated mechanisms of HMO on intestinal epithelial cells or systemic available immune cells like PBMC are feasible. Indeed, in *in vitro* models several groups have described direct effects of HMO on leucocyte recruitment [84] and leucocyte cytokine production in stimulated cord blood cells [85] and PBMC [86,87]. Direct effects of specific oligosaccharides have been described on cytokine production by PBMC [88], epithelial cell lines responses [89] and PBMC/epithelial cell line co-cultures [90]. Since GFA is a mixture of specific carbohydrates, lectin receptors on cells including DCs and epithelial cells, which come in direct contact with GFA, are most likely stimulated through these receptors. Naarding *et al.* recently showed that that HMOS are able to bind specifically to the lectin receptor DC-SIGN [91]. Furthermore, de Kivit *et al*. did not show interaction of specific oligosaccharides with a specific receptor, but showed that *in vitro* stimulation of IEC lines with GOS/FOS could enhance the release of (bacterial) DNA induced Galectin-9 by these cells [89], affecting local

intestinal mucosal immunity. However, galectin-9 knockout (G9KO) mice infected with influenza A virus mounted a more robust acute phase virus-specific $CD8^+$ T-cell response as well as increased viral clearance compared to WT mice [92]. Moreover, we and others could not detect any dose dependent immune stimulatory effects of GFA on human cytokine responses with or without co-stimulation with RSV or Concanavalin A in PBMC (Vos AP, thesis).

Most studies published with HMO and specific oligosaccharides ascribe the immune modulatory effects of oligosaccharides to the changes in intestinal microbiome composition trough growth of specific bacterial species that are considered beneficial to humans [66,93–95]. To address the question whether a change in microbiome composition rather that GFA stimulation, could be responsible for the effects seen on GFA induced changes in RSV specific T cell responses in the lung of infected mice (**chapter 7**), we disrupted the balance in microbiome composition in mice by orally applied broad spectrum antibiotics (**chapter 8**). In this study we showed that in the isocage® contained model, antibiotic treatment for a period of 4 weeks irreversibly reduced gut microbiome diversity until at least 4 weeks after ending antibiotic treatment. Oral treatment with antibiotics during FI-RSV vaccination had significant effects on the development of the local immune response measured 6 days post infection. We show that reduced $CD8^+$ T cell activation after antibiotic treatment and reduced numbers of IFN-γ and TNF-α producing $CD4^+$ T cells (**Figure 3**). This was accompanied by an increase in numbers of IL-4, IL-5 and IL-13 producing $CD4^+$ T cells and ratio in numbers of "inflammatory" $CD11b^+$ DC vs. tolerogenic $CD103^+$ DC (CD103 expression on DC is associated with tolerance induction in lung and intestinal mucosal surfaces, however not necessary in the lung, [96]) in Abt treated mice. Furthermore reduced numbers of (GrzmB producing) regulatory T cells were present, suggesting that orally applied antibiotics during the priming phase resulted in an amplification of the Th2 biased immune response characteristic for this model (**chapter 8**). Interestingly, similar cDC and T cell response differences were measured by Roux *et al* in neonatal mice compared to adult BALB/c mice when i.n. vaccinated with Bacillus Calmette-Guérin (BCG) [97]. Because antibiotics were only applied around the time of vaccination, a direct immunosuppressive effect of the antibiotic treatment on RSV infection can be excluded. However, signs of a disturbed intestinal microbiome were still visible 5 weeks after ending antibiotic treatment, therefore it is not possible to determine whether the observed immune manipulation via antibiotic treatment affected the RSV specific immune response during the priming phase or during the effector phase, i.e. the recall response upon the RSV challenge. Furthermore, in all studies described above, the nature of the species and the settings used cannot exclude the possibility that the immune modulatory effects observed are induced at other mucosal surfaces e.g. the lung [98]. Clearly, dietary prebiotic and pharmaceutical antibiotic treatment will affect microbial content in different locations. Therefore, both local effects on innate immune responses as well as systemic effects on immune parameters might have contributed to the observed immune modulation. Based on our *in vivo* mouse studies with the known bacterial stimulating properties of oligosaccharides and

the effects of antibiotics on reducing bacterial populations it is tempting to speculate that regulatory T cell numbers and the bias to a more allergic phenotype correlates with bacterial diversity. Studies in mice [99] and human [100,101] have indeed shown that reduced intestinal microbiota diversity during childhood can be associated with allergic diseases in later life. Although mechanistic details need to be worked on our data clearly show that dietary factors and antibiotics significantly affect immune responses in different mouse models. These observations are important with respect to medical treatment during RSV infections or even before RSV infections occur in infants, because our data show that there might be a certain risk to alter RSV specific immune responses (**chapter 7, 8**).

In **chapter 6** we demonstrated that in influenza specific Type IV hyper-sensitivity vaccination response could be enhanced in C57BL/6 mice fed with a GFA containing diet probably via a regulatory T cell mediated mechanism. However, in this model we could not detect differences in Treg numbers present in the spleen or gut associated lymphoid structures like the mesenteric lymphnodes (MLN). We investigated the questions if dietary intervention with GFA could induce differences in Treg sub-populations or functionality in **chapter 9**. We confirmed the increased influenza specific DTH response by dietary intervention with GFA seen in **chapter 6**. Furthermore, we found a correlation with an increased percentage of T-bet expressing T-cells, an indication for enhanced Th1 responsiveness. Recent observations have challenged the notification of one stable Treg sub lineage. Some reports show the presence of T-bet expressing Treg suppressing Th1 responses [102], IRF-4 expressing Treg that suppress Th2 responsiveness [103] and a STAT-3 expressing Treg subtype suppressing Th17 responses [104] in mice. T-bet controls migration to inflammatory sites through CXCR3 up regulation as CXCR-3 is a direct transcript of T-bet [105]. We demonstrated that GFA induced improved Th1 responsiveness was accompanied with a reduced population of CXCR3 /T-bet expressing Tregs in the MLN (**chapter 9**). To investigate if dietary intervention with GFA could modify Treg function, we performed a suppression assay with isolated RFP expressing Treg from GFA treated C57BL/6- Foxp3tm1Flv/J mice. We show that MLN derived Tregs from GFA treated animals could enhance T effector IFN-γ release and the Treg/Th17 balance by suppressing IL-17 production. In **chapter 7** we observed a similar increase in $CD4^+$ IFN-γ producing cells and decrease numbers of regulatory T cells in the lungs of GFA fed animals. However the differences in immune responses between Influenza vs. RSV upon challenge (elegantly shown by Kruijsen *et al* [6]), mode of vaccination (**Figure 3**) and location of subsequent infection are all parameters shown in this thesis and by others to have an impact on the outcome of the subsequent immune response against the pathogen. Whether a similar Treg subtype is involved in the GFA induced local immune modulatory effects seen in the FI-RSV vaccination model for severe disease and the systemic influenza vaccination model needs therefore further investigation. The role of regulatory T cells (especially GrzmB producing Treg) in controlling immunity in primary RSV infections and the FI-RSV vaccination model was demonstrated by Loebbermann *et al.* They show that Treg suppress disease

enhancement and enhance viral clearance after primary RSV infection. However in FI-RSV-vaccinated mice depletion of Treg does not cause additional enhancement of disease upon live virus infection, illustrating the inability of local Treg to control disease upon infection in this model [76,77]. Therefore it is tempting to speculate that direct or indirect modulation of mucosal and systemic immunity (including Treg) through (GFA induced) microbiome composition might affect priming phase of the vaccination response leading to the altered immune response seen upon infection.

In the second part of this thesis we show that changes in bacterial populations caused by pharmaceutical antibiotics or specific oligosaccharides in the diet could affect local immunity in the lungs of primary infected or FI-RSV vaccinated mice. Follow up studies should carefully address at which site these bacterial changes affect the immune responses in the different models, because bacterial changes induced by diet and antibiotics will impact bacterial composition in the intestine but also in the nasopharynx. It can be envisioned that direct viral-bacterial interactions or competitive interaction with epithelia in the nasopharynx also affect the efficacy of infection and as a result the nature and extent to which innate and adaptive immune responses are induced. Furthermore, our studies demonstrated that specific oligosaccharides altered Treg and DC populations. To address the immune modulating effects of GFA to (each of) these cell types, future experiments on the effects of GFA on these cells before vaccination or viral infection are necessary. These issues need to be settled first before further studies addressing the question how intestinal mucosal immunity could affect local responses in the lung. Nevertheless, several groups show that communication between different mucosal sites exits and that they function together as a system-wide organ [106].

In conclusion, our studies show that the use of highly neutralising antibodies or antibiotics in new-borns should carefully be evaluated for their potential to alter innate immune responses and the development of adaptive immunity to RSV and certainly also other pathogens. Extrapolation from our mouse model suggests that antibiotics used in the neonatal period might increase the risk of developing severe RSV bronchiolitis and susceptibility to develop allergic disease in later life. A better understanding of the mechanism by which vaccine induced immune responses can be amplified or altered by microbiome manipulation, might in the future also lead to promising options whereby dietary manipulation might support vaccine efficacy.

REFERENCES

1. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. American Journal of Diseases of Children (1960) 1986; 140:543–6.
2. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. The Journal of Infectious Diseases 1991;163:693–8.
3. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. Vaccine 2003;21:3479–82.
4. De Graaff PMA, De Jong EC, Van Capel TM, Van Dijk MEA, Roholl PJM, Boes J, et al. Respiratory syncytial virus infection of monocyte-derived dendritic cells decreases their capacity to activate CD4 T cells. Journal of Immunology (Baltimore, Md: 1950) 2005; 175:5904–11.
5. González PA, Prado CE, Leiva ED, Carreño LJ, Bueno SM, Riedel CA, et al. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proceedings of the National Academy of Sciences of the United States of America 2008;105:14999–5004.
6. Kruijsen D, Bakkers MJ, Van Uden NO, Viveen MC, Van der Sluis TC, Kimpen JL, et al. Serum antibodies critically affect virus-specific CD4+/CD8+ T cell balance during respiratory syncytial virus infections. Journal of Immunology (Baltimore, Md: 1950) 2010; 185:6489–98.
7. De Bree GJ, Heidema J, Van Leeuwen EMM, Van Bleek GM, Jonkers RE, Jansen HM, et al. Respiratory syncytial virus-specific CD8+ memory T cell responses in elderly persons. The Journal of Infectious Diseases 2005; 191:1710–8.
8. Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. Journal of Virology 2005;79:5353–62.
9. Spann KM, Tran K-C, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. Journal of Virology 2004;78:4363–9.
10. Shadman KA, Wald ER. A review of palivizumab and emerging therapies for respiratory syncytial virus. Expert Opinion on Biological Therapy 2011;11:1455–67.
11. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics 1998; 102:531–7.
12. Carbonell-Estrany X, Simões EAF, Dagan R, Hall CB, Harris B, Hultquist M, et al. Motavizumab for prophylaxis of respiratory syncytial virus in high-risk children: a noninferiority trial. Pediatrics 2010;125: e35–51.
13. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. American Journal of Epidemiology 1969;89:422–34.
14. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. American Journal of Epidemiology 1969;89:435–48.
15. Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent

parainfluenza virus vaccine in a pediatric population. American Journal of Epidemiology 1969;89:449–63.

16. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nature Medicine 2006;12:905–7.
17. Openshaw PJ, Clarke SL, Record FM. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. International Immunology 1992;4:493–500.
18. Culley FJ, Pennycook AMJ, Tregoning JS, Hussell T, Openshaw PJM. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. Journal of Virology 2006; 80:4521–7.
19. Piedra PA, Faden HS, Camussi G, Wong DT, Ogra PL. Mechanism of lung injury in cotton rats immunized with formalin-inactivated respiratory syncytial virus. Vaccine 1989;7: 34–8.
20. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. Journal of Virology 1996; 70:2852–60.
21. Boelen A, Andeweg A, Kwakkel J, Lokhorst W, Bestebroer T, Dormans J, et al. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. Vaccine 2000;19: 982–91.
22. Olson MR, Varga SM. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. Journal of Immunology (Baltimore, Md: 1950) 2007;179:5415–24.
23. Olson MR, Hartwig SM, Varga SM. The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia. Journal of Immunology (Baltimore, Md: 1950) 2008;181:7958–68.
24. Hussell T, Baldwin CJ, O'Garra A, Openshaw PJ. CD8+ T cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. European Journal of Immunology 1997;27:3341–9.
25. Lukens M V, Van de Pol AC, Coenjaerts FEJ, Jansen NJG, Kamp VM, Kimpen JLL, et al. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. Journal of Virology 2010;84: 2374–83.
26. Rudd BD, Smit JJ, Flavell RA, Alexopoulou L, Schaller MA, Gruber A, et al. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. Journal of Immunology (Baltimore, Md: 1950) 2006; 176:1937–42.
27. Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, Lindell DM. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. Journal of Immunology (Baltimore, Md: 1950) 2010;185:2231–9.
28. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich H-J, Van Rooijen N, et al. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. The Journal of Infectious Diseases 2002;186:1199–206.
29. Klein Klouwenberg P, Tan L, Werkman W, Van Bleek GM, Coenjaerts F. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. Critical Reviews in Immunology 2009;29: 531–50.
30. Lukacs NW, Smit JJ, Schaller MA, Lindell DM. Regulation of immunity to respiratory syncytial virus by dendritic cells, toll-like

receptors, and notch. Viral Immunology 2008;21:115–22.
31. Groskreutz DJ, Monick MM, Powers LS, Yarovinsky TO, Look DC, Hunninghake GW. Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells. Journal of Immunology (Baltimore, Md: 1950) 2006; 176:1733–40.
32. Van Diepen A, Brand HK, Sama I, Lambooy LHJ, Van den Heuvel LP, Van der Well L, et al. Quantitative proteome profiling of respiratory virus-infected lung epithelial cells. Journal of Proteomics 2010;73:1680–93.
33. Swedan S, Andrews J, Majumdar T, Musiyenko A, Barik S. Multiple functional domains and complexes of the two nonstructural proteins of human respiratory syncytial virus contribute to interferon suppression and cellular location. Journal of Virology 2011;85:10090–100.
34. Jie Z, Dinwiddie DL, Senft AP, Harrod KS. Regulation of STAT signaling in mouse bone marrow derived dendritic cells by respiratory syncytial virus. Virus Research 2011; 156:127–33.
35. Rudd BD, Schaller MA, Smit JJ, Kunkel SL, Neupane R, Kelley L, et al. MyD88-mediated instructive signals in dendritic cells regulate pulmonary immune responses during respiratory virus infection. Journal of Immunology (Baltimore, Md: 1950) 2007;178:5820–7.
36. Wang H, Peters N, Schwarze J. Plasmacytoid dendritic cells limit viral replication, pulmonary inflammation, and airway hyperresponsiveness in respiratory syncytial virus infection. Journal of Immunology (Baltimore, Md: 1950) 2006;177:6263–70.
37. Lotz MT, Peebles RS. Mechanisms of respiratory syncytial virus modulation of airway immune responses. Current Allergy and Asthma Reports 2012;12:380–7.
38. Jamaluddin M, Wang S, Garofalo RP, Elliott T, Casola A, Baron S, et al. IFN-beta mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. American Journal of Physiology Lung Cellular and Molecular Physiology 2001; 280:L248–57.
39. Okabayashi T, Kojima T, Masaki T, Yokota S-I, Imaizumi T, Tsutsumi H, et al. Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells. Virus Research 2011; 160:360–6.
40. Hornung V, Schlender J, Guenthner-Biller M, Rothenfusser S, Endres S, Conzelmann K-K, et al. Replication-dependent potent IFN-alpha induction in human plasmacytoid dendritic cells by a single-stranded RNA virus. Journal of Immunology (Baltimore, Md: 1950) 2004;173:5935–43.
41. Ling Z, Tran KC, Teng MN. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. Journal of Virology 2009;83:3734–42.
42. Schlender J, Hornung V, Finke S, Günthner-Biller M, Marozin S, Brzózka K, et al. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. Journal of Virology 2005;79:5507–15.
43. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science (New York, NY) 2004;303:1529–31.
44. Poth JM, Coch C, Busch N, Boehm O, Schlee M, Janke M, et al. Monocyte-mediated inhibition of TLR9-dependent IFN-α induction in plasmacytoid dendritic cells questions bacterial DNA as the active ingredient of bacterial lysates. Journal of Immunology (Baltimore, Md: 1950) 2010;185:7367–73.
45. Rath T, Kuo TT, Baker K, Qiao S-W, Kobayashi K, Yoshida M, et al. The immunologic functions of the neonatal Fc receptor for IgG. Journal of Clinical Immunology 2013;33 Suppl 1:S9–17.

46. Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC, et al. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 2004;20:769–83.
47. Israel EJ, Patel VK, Taylor SF, Marshak-Rothstein A, Simister NE. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. Journal of Immunology (Baltimore, Md: 1950) 1995;154:6246–51.
48. Baker K, Rath T, Lencer WI, Fiebiger E, Blumberg RS. Cross-presentation of IgG-containing immune complexes. Cellular and Molecular Life Sciences: CMLS 2013;70: 1319–34.
49. Qiao S-W, Kobayashi K, Johansen F-E, Sollid LM, Andersen JT, Milford E, et al. Dependence of antibody-mediated presentation of antigen on FcRn. Proceedings of the National Academy of Sciences of the United States of America 2008;105:9337–42.
50. Liu X, Lu L, Yang Z, Palaniyandi S, Zeng R, Gao L-Y, et al. The neonatal FcR-mediated presentation of immune-complexed antigen is associated with endosomal and phagosomal pH and antigen stability in macrophages and dendritic cells. Journal of Immunology (Baltimore, Md: 1950) 2011;186:4674–86.
51. Kruijsen D, Einarsdottir HK, Schijf MA, Coenjaerts FE, Van der Schoot EC, Vidarsson G, et al. Intranasal administration of antibody-bound respiratory syncytial virus particles efficiently primes virus-specific immune responses in mice. Journal of Virology 2013; 87:7550–7.
52. Hu J, Robinson JL. Treatment of respiratory syncytial virus with palivizumab: a systematic review. World Journal of Pediatrics: WJP 2010;6:296–300.
53. Simões EAF, Carbonell-Estrany X, Rieger CHL, Mitchell I, Fredrick L, Groothuis JR. The effect of respiratory syncytial virus on subsequent recurrent wheezing in atopic and nonatopic children. The Journal of Allergy and Clinical Immunology 2010;126:256–62.
54. Belderbos ME, Van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, et al. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clinical Immunology (Orlando, Fla) 2009;133:228–37.
55. Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA, et al. Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo. Pediatric Research 2006;60:205–9.
56. Buddington RK, Williams CH, Kostek BM, Buddington KK, Kullen MJ. Maternal-to-infant transmission of probiotics: concept validation in mice, rats, and pigs. Neonatology 2010;97:250–6.
57. Ovid: Effect of Maternal Consumption of Lactobacillus GG on Transfer and Establishment of Fecal Bifidobacterial Microbiota in Neonates. n.d.
58. Strachan DP. Hay fever, hygiene, and household size. BMJ (Clinical Research Ed) 1989; 299:1259–60.
59. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013:n/a–n/a.
60. Smits HH, Hartgers FC, Yazdanbakhsh M. Helminth infections: protection from atopic disorders. Current Allergy and Asthma Reports 2005;5:42–50.
61. Isolauri E, Rautava S, Salminen S. Probiotics in the development and treatment of allergic disease. Gastroenterology Clinics of North America 2012;41:747–62.
62. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. The Journal of Physiology 2009;587:4153–8.
63. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. Nature Reviews Gastroenterology & Hepatology 2012;9:577–89.

64. Moro GE, Mosca F, Miniello V, Fanaro S, Jelinek J, Stahl B, et al. Effects of a new mixture of prebiotics on faecal flora and stools in term infants. Acta Paediatrica (Oslo, Norway: 1992) Supplement 2003;91:77–9.
65. Fanaro S, Jelinek J, Stahl B, Boehm G, Kock R, Vigi V. Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: effect on intestinal flora, stool characteristics, and pH. Journal of Pediatric Gastroenterology and Nutrition 2005;41: 186–90.
66. Jeurink P V, Van Esch BC, Rijnierse A, Garssen J, Knippels LM. Mechanisms underlying immune effects of dietary oligosaccharides. The American Journal of Clinical Nutrition 2013.
67. Nauta AJ, Garssen J. Evidence-based benefits of specific mixtures of non-digestible oligosaccharides on the immune system. Carbohydrate Polymers 2013;93:263–5.
68. Betts RJ, Ho AWS, Kemeny DM. Partial depletion of natural $CD4^{+}CD25^{+}$ regulatory T cells with anti-CD25 antibody does not alter the course of acute influenza A virus infection. PloS One 2011;6:e27849.
69. Ruckwardt TJ, Bonaparte KL, Nason MC, Graham BS. Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. Journal of Virology 2009;83:3019–28.
70. Vos AP, Knol J, Stahl B, M'rabet L, Garssen J. Specific prebiotic oligosaccharides modulate the early phase of a murine vaccination response. International Immunopharmacology 2010;10:619–25.
71. Vos AP, Haarman M, Van Ginkel J-WH, Knol J, Garssen J, Stahl B, et al. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2007;18:304–12.
72. Lanari M, Prinelli F, Adorni F, Di Santo S, Faldella G, Silvestri M, et al. Maternal milk protects infants against bronchiolitis during the first year of life. Results from an Italian cohort of newborns. Early Human Development 2013;89 Suppl 1:S51–7.
73. Bryan D-L, Hart PH, Forsyth KD, Gibson RA. Immunomodulatory constituents of human milk change in response to infant bronchiolitis. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2007; 18:495–502.
74. Culley FJ, Pollott J, Openshaw PJM. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. The Journal of Experimental Medicine 2002;196:1381–6.
75. Krishnamoorthy N, Khare A, Oriss TB, Raundhal M, Morse C, Yarlagadda M, et al. Early infection with respiratory syncytial virus impairs regulatory T cell function and increases susceptibility to allergic asthma. Nature Medicine 2012;18:1525–30.
76. Loebbermann J, Thornton H, Durant L, Sparwasser T, Webster KE, Sprent J, et al. Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. Mucosal Immunology 2012;5:161–72.
77. Loebbermann J, Durant L, Thornton H, Johansson C, Openshaw PJ. Defective immunoregulation in RSV vaccine-augmented viral lung disease restored by selective chemoattraction of regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America 2013; 110:2987–92.
78. Liu J, Ruckwardt TJ, Chen M, Nicewonger JD, Johnson TR, Graham BS. Epitope-specific regulatory CD4 T cells reduce virus-induced illness while preserving CD8 T-cell effector function at the site of infection. Journal of Virology 2010;84:10501–9.
79. Fulton RB, Meyerholz DK, Varga SM. Foxp3+ CD4 regulatory T cells limit pulmonary

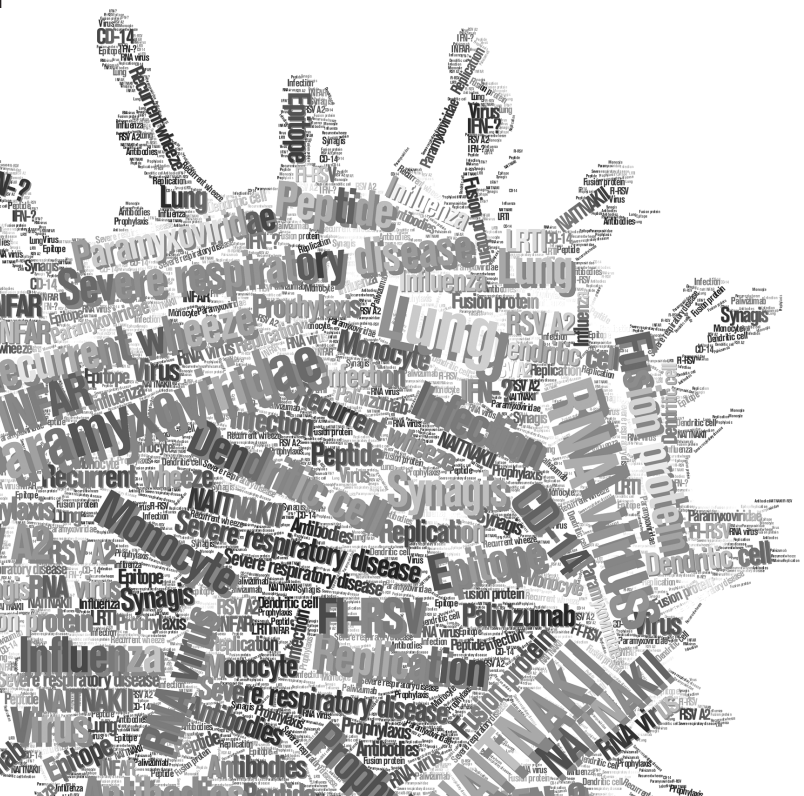
immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. Journal of Immunology (Baltimore, Md: 1950) 2010;185:2382–92.

80. Gnoth MJ, Rudloff S, Kunz C, Kinne RK. Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. The Journal of Biological Chemistry 2001;276:34363–70.
81. Kunz C, Rudloff S. [Structural and functional aspects of oligosaccharides in human milk]. Zeitschrift Für Ernährungswissenschaft 1996;35:22–31.
82. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annual Review of Nutrition 2000;20:699–722.
83. Rudloff S, Kunz C. Milk oligosaccharides and metabolism in infants. Advances in Nutrition (Bethesda, Md) 2012;3:398S–405S.
84. Bode L, Kunz C, Muhly-Reinholz M, Mayer K, Seeger W, Rudloff S. Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. Thrombosis and Haemostasis 2004;92:1402–10.
85. Eiwegger T, Stahl B, Schmitt J, Boehm G, Gerstmayr M, Pichler J, et al. Human milk--derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. Pediatric Research 2004;56:536–40.
86. Atochina O, Daly-Engel T, Piskorska D, McGuire E, Harn DA. A schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1(+) macrophages that suppress naive CD4(+) T cell proliferation via an IFN-gamma and nitric oxide-dependent mechanism. Journal of Immunology (Baltimore, Md: 1950) 2001; 167:4293–302.
87. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, et al. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology 2010;21:1179–88.
88. Vendrig JC, Coffeng LE, Fink-Gremmels J. In vitro evaluation of defined oligosaccharide fractions in an equine model of inflammation. BMC Veterinary Research 2013;9:147.
89. De Kivit S, Saeland E, Kraneveld AD, Van de Kant HJG, Schouten B, Van Esch BCAM, et al. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012;67: 343–52.
90. De Kivit S, Kraneveld AD, Knippels LMJ, Van Kooyk Y, Garssen J, Willemsen LEM. Intestinal Epithelium-Derived Galectin-9 Is Involved in the Immunomodulating Effects of Nondigestible Oligosaccharides. Journal of Innate Immunity 2013.
91. Naarding MA, Ludwig IS, Groot F, Berkhout B, Geijtenbeek TBH, Pollakis G, et al. Lewis X component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4+ T lymphocytes. The Journal of Clinical Investigation 2005;115:3256–64.
92. Sharma S, Sundararajan A, Suryawanshi A, Kumar N, Veiga-Parga T, Kuchroo VK, et al. T cell immunoglobulin and mucin protein-3 (Tim-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. Proceedings of the National Academy of Sciences of the United States of America 2011;108:19001–6.
93. Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients 2013;5: 1417–35.
94. Barile D, Rastall RA. Human milk and related oligosaccharides as prebiotics. Current Opinion in Biotechnology 2013;24:214–9.
95. Bertino E, Peila C, Giuliani F, Martano C, Cresi F, Di Nicola P, et al. Metabolism and biological functions of human milk oligosaccharides. Journal of Biological Regulators and Homeostatic Agents n.d.;26:35–8.

96. Guilliams M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, et al. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. Blood 2010;115:1958–68.
97. Roux X, Remot A, Petit-Camurdan A, Nahori M-A, Kiefer-Biasizzo H, Marchal G, et al. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells. European Journal of Immunology 2011;41:2852–61.
98. Ni K, Li S, Xia Q, Zang N, Deng Y, Xie X, et al. Pharyngeal microflora disruption by antibiotics promotes airway hyperresponsiveness after respiratory syncytial virus infection. PloS One 2012;7:e41104.
99. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Reports 2012;13:440–7.
100. Storrø O, Avershina E, Rudi K. Diversity of intestinal microbiota in infancy and the risk of allergic disease in childhood. Current Opinion in Allergy and Clinical Immunology 2013;13:257–62.
101. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. The Journal of Allergy and Clinical Immunology 2011;128: 646–52.e1–5.
102. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nature Immunology 2009;10: 595–602.
103. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu T-T, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature 2009;458:351–6.
104. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science (New York, NY) 2009;326:986–91.
105. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, et al. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood 2005;106:3432–9.
106. Gill N, Wlodarska M, Finlay BB. The future of mucosal immunology: studying an integrated system-wide organ. Nature Immunology 2010;11:558–60.
107. Johnson TR, Rao S, Seder RA, Chen M, Graham BS. TLR9 agonist, but not TLR7/8, functions as an adjuvant to diminish FI-RSV vaccine-enhanced disease, while either agonist used as therapy during primary RSV infection increases disease severity. Vaccine 2009;27:3045–52.


Prophyla
RSVA
Synagis
Fusion
Severe respirator
Palivizumab
CD=14

Nederlandse samenvatting (Dutch Summary)



Griep of verkoudheid zijn ziektes waar iedereen elk jaar weer last van heeft. Ze worden gekenmerkt door karakteristieke verschijnselen als geprikkelde slijmvliezen van neus en ogen, hoesten, benauwdheid, lusteloosheid en koorts. Deze ziektebeelden worden meestal veroorzaakt door virussen als gevolg van replicatie in luchtweg epitheel, de schade die dat teweegbrengt en de door de virale infectie veroorzaakte immuun respons. Dit scenario onderscheid zich van vergelijkbare ziektebeelden veroorzaakt door luchtweg over-gevoelligheids reacties geinitieerd door een bepaald micro-organisme of eiwit waarbij het lichaam heftig reageert tegen een onschuldig (niet infectieus) partikel (astma/allergie). Bacteriële luchtweg-ontstekingen worden meestal veroorzaakt door *Streptococcus pneumoniae* en *Haemophilus influenzae* en worden, als er genoeg aanwijzingen zijn dat het om een bacteriële infectie gaat, bestreden met antibiotica. Als de bron van een luchtweginfectie viraal is heeft het toedienen van antibiotica geen zin. Infecties door virussen worden daarom dan ook op een andere manier bestreden. De ziekte wordt meestal aangepakt door veel te drinken, te rusten en preventief op te treden. Preventie van virale luchtweg infecties kan worden bereikt door tegen bepaalde luchtweg virussen te vaccineren met een afgedood virus of een stukje virus. Een bekend voorbeeld hiervan is de vaccinatie tegen het *Influenza* (griep) virus, waar vaccinatie beschermt tegen een natuurlijke infectie. Helaas werkt vaccinatie niet tegen elk virus. In 1960 is dit ook geprobeerd voor het respiratoir syncytieel (RS) virus. Omdat dit virus vooral bij jonge kinderen ernstige infecties in de diepere luchtwegen kan veroorzaken is hier toen een vaccinatie programma met formaline geinactiveerd virus voor opgezet. Helaas waren de gevaccineerde kinderen niet beschermd tegen het virus. Na een natuurlijke luchtweg infectie werden deze gevaccineerde kinderen ziek, waarschijnlijk door een verhevigde immuun reactie tegen het virus. De reden waarom het lichaam op deze manier tegen het virus reageerde is tot op de dag van vandaag reden voor onderzoek en een belangrijke vraag die tot in detail beantwoord moet worden om in de toekomst dit soort vaccinatie problemen te voorkomen.

Het virus

Het RS virus dankt zijn naam aan de karakteristieke eigenschap dat het in de long de samensmelting van epitheel cellen (Syncytia) kan veroorzaken. De mens raakt in zijn leven regelmatig in de bovenste luchtwegen geinfecteerd met dit virus en blijft in de meeste gevallen symptoomloos of het leidt tot een (milde) verkoudheid of loopneus. Kinderen raken voor het eerst besmet met RSV in het 1e of 2e levens jaar en hebben in de meeste gevallen voor hun vijfde al meerdere besmettingen doorgemaakt. In deze groep, zeker in de pasgeborenen, kan een RSV infectie een ernstig ziektebeeld veroorzaken als het ook de onderste luchtwegen infecteert. In 1-3% van de kinderen geinfecteerd met RSV bereikt het replicerende virus ook de onderste luchtwegen (waar de gasuitwisseling plaats vindt) en veroorzaakt daar een ernstige onderste luchtweg ontsteking (broncheolitis). In 10% van deze gevallen leidt dat tot

een ziekenhuis opname. Dit betekent in Nederland alleen al een jaarlijkse ziekenhuisopname van 2000-3000 kinderen met RSV gerelateerde broncheolitis. Deze kinderen lijden aan symptomen als piepende borst, verhoogde mucus productie, zuurstof tekort en een gebrek aan eetlust. Helaas blijft het in sommige gevallen niet alleen bij luchtweg problemen vroeg na de geboorte. Onderzoek heeft uitgewezen dat bij kinderen die vroeg in het leven RSV geïnduceerde broncheolitis hebben doorgemaakt, er een grotere kans bestaat dat zij later in het leven astmatische verschijnselen en luchtweg overgevoeligheid onwikkelen.

Het afweer systeem

Om lichaams vreemde stoffen en potentieel gevaarlijke micro-organismen buiten te houden maakt het menselijk lichaam gebruik van verschillende strategieën. Ten eerste maakt het gebruik van een fysieke barriëre zoals de huid, luchtweg- en darm epitheel. Lichaams cellen die deze scheidingslijn vormen hebben behalve de taak om als fysieke barriëre op te treden, ook de functie het lichaam te laten weten wat voor een soort micro-organismen zich nestelen aan de oppervlakte en op te treden als deze barriere doorbroken wordt. Als deze barriere door schade of (virale) infectie niet meer toereikend is, treedt het immuun systeem (Latijn *immunis*, betekenis: vrijheid van belasting) van het lichaam in werking. Deze imuun reactie bestaat uit 2 gedeelten. In eerste instantie treedt het intrinsieke (innate) immuun systeem in werking. De eigenschap van deze immuun reactie is dat deze afweer aangeboren is, niet pathogeen speciek is en snel in werking treedt (minder dan 24 uur). Cellen die onderdeel uitmaken van deze reactie zijn voornamelijk epitheel cellen, cellen die lichaamsvreemd materiaal kunnen opnemen (fagocyten) en cellen die geinfecteerde cellen kunnen doden, de Natural Killer (NK) cellen. Deze cellen worden bij een virale infectie geactiveerd doordat ze bepaalde algemene structuren (zogenaamde antigenen) die voorkomen op micro-organismen kunnen herkennen. Cellen die in contact met het virus komen produceren hierdoor verschillende signaal stoffen (cytokines en chemokinen) en grote hoeveelheden interferonen (met name IFN-α en β), wat resulteert in de migratie van immuun cellen naar de plaats van infectie en remming van virale replicatie. Tijdens deze eerste immuun reactie heeft het lichaam tijd voor het initiëren van een meer specifieke manier van pathogeen eliminatie. Om specifieker tegen het pathogeen op te treden maakt het lichaam gebruik van de adaptieve (geheugen) immuun response. Deze reactie wordt ook wel de aangeleerde (verworven) immuun reactie genoemd en wordt pas later actief. De reactie wordt geinitieerd door antigeen presenterende cellen (APCs) en dan met name de dendritische cel (DC). Deze cellen worden geinfecteerd of nemen (een stukje) virus op, knippen dit in kleine stukjes eiwit (epitopen) en migreren naar lokale lymfeknopen waar zij deze epitopen aan hun oppervlakte via zogenaamde MHC moleculen presenteren aan B en T cellen. Via epitopen op MHC-I moleculen worden dan zogenaamde $CD8^+$ cytotoxische T cellen geactiveerd. Deze cytotoxische antigeen specifieke T cellen zullen zich vermenigvuldigen, naar de plek van infectie migreren en daar geinfecteerde cellen

doden. Via MHC-II moleculen op DC worden, afhankelijk van het type antigeen en het type cytokine co-stimulatie aanwezig in het lokale milieu, verschillende $CD4^+$ T helper cel subtypen geactiveerd die effectief een anti-virale of anti-bacteriële immuun reactie op gang kunnen zetten. Op dit moment zijn er 4 typen T helper cells bekend namelijk: Th1, Th2, Th17 en de regulatoire T cel (Treg). Deze antigeen specifieke geactiveerde T cellen zullen zich gaan vermenigvuldigen en op de plek van infectie mediatoren (cytokines) uitscheiden die de immuun reactie kunnen sturen. Een belangrijk aspect van de adaptieve immuun reactie is de geheugen functie. Een aantal van de $CD4^+$ en $CD8^+$ T cellen in de lymfknopen zullen na antigeen presentatie door DC differentieren in geheugen (memory) T cellen. Verder zullen lichaamsvreemde structuren, DCs die antigeen bevatten en geactiveerde T cellen in de lymfknoop tezamen B cellen activeren. Deze geactiveerde B cellen zullen zich gaan vermenigvuldigen, veranderen in plasmacellen en antigeen specifieke antilichamen gaan produceren (humorale immuun reactie). De aanwezigheid van antigeen specifieke antilichamen en geheugen T en B cellen na afloop van de infectie zorgt ervoor dat bij de eerst volgende infectie met virus (antigeen) er een snellere en betere immuun reactie kan optreden o.a. doordat in het bloed circulerende antilichamen het virus kunnen neutraliseren. Dit mechanisme wordt gebruikt bij vaccinaties.

In dit proefschrift

De precieze oorzaak van ernstige ziekte bij kinderen tijdens primaire RSV-infecties is niet helemaal duidelijk. Waarom krijgt 1-3% van de geinfecteerde kinderen ernstige lagere luchtweg infecties? Een interessant gegeven is dat kinderen die daadwerkelijk lagere luchtweg infectie door RSV krijgen, in de meeste gevallen niet eens tot de traditionele risico groepen behoren zoals: te vroeg geboren kinderen, kinderen met een chronische long ziekte, hartproblemen of een immuun deficiëntie. Er zijn verbanden aangetoond tussen de hoeveelheid virus en de ernst van RSV infecties. Maar dit verklaart niet alles. Tijdens de neonatale periode zijn maternale (van de moeder) antilichamen een belangrijke factor om de pasgeborenen te beschermen tegen pathogenen bij de eerste blootstelling. Onderzoek heeft uitgewezen dat er een correlatie bestaat tussen de hoeveelheid RSV specifieke antilichamen aanwezig in het bloed en RSV-geassocieerde ziekenhuisopnames [1,2]. Verder zijn kinderen die in hun neonatale periode preventief behandeld worden met Palivizumab, een antilichaam gericht tegen het fusie eiwit van RSV, beter beschermd tegen ernstige luchtweg infecties wat resulteert in 50% minder ziekenhuis opnames [3]. Maar ondanks de blijkbaar preventieve rol van antilichamen is dit maar een gedeelte van de oplossing. De ontwikkeling van een antilichaam tegen RSV F eiwit (Motavizumab) dat sterker neutraliseert geeft geen betere resultaten met betrekking tot infectie preventie en ziekenhuis opnames [4]. Verder kunnen gezonde volwassenen binnen 4 maanden na een RSV infectie weer opnieuw geinfecteerd raken met een identieke virus stam. Omdat in volwassenen kort na zo'n recente blootstelling zeker antilichamen tegen het RSV aanwezig zijn, geeft dit ook aan dat de aanwezigheid van

antilichamen geen garantie is dat re-infecties worden voorkomen. De niet volledige bescherming door de adaptieve immuun reactie tegen her-infectie, ondanks de aanwezigheid van virus specifieke $CD4^+$ en $CD8^+$ T cellen en antilichamen, kan een gevolg zijn van specifieke (immuun ontwijkende) kenmerken van het RS virus. RSV geïnfecteerde dendritische cellen onderdrukken de proliferatieve respons en de rijping van $CD4^+$ en $CD8^+$ T-effector cellen. Twee mechanismen die kunnen bijdragen aan de onvolledige adaptieve immuunreactie. Een derde mechanisme zou virale herkenning kunnen zijn. Het initieren van een robuuste virus specifieke $CD8^+$ T cell reactie vereist de presentatie van virale epitopen op MHC-I moleculen van DC. Door infectie van DCs worden stukjes viral eiwit via de klassieke proteasomale afbraak route op hun MHC klasse I molecuul aan T cellen gepresenteerd. De aanwezigheid van antilichaam verkregen via de moeder of door eerdere infecties, kan het virus neutraliseren waardoor infectie niet meer mogelijk is. Wel kan de DC het virus/antilichaam complex opnemen door het te omsluiten (endosoom), in een intern compartiment (endo-lysosoom) af te breken en via MHC-II moleculen te presenteren aan $CD4^+$ T cellen of te kruis-presenteren aan $CD8^+$ cellen. Onze groep (Kruijsen *et al* [5]) heeft laten zien dat antilichamen tegen RSV de verhouding geactiveerde $CD4^+/CD8^+$ T cellen kan verschuiven naar meer $CD4^+$ T cellen en dus een andere adaptieve immuun reactie opwekt dan bij infectie in afwezigheid van virus neutraliserende antilichamen. Dit zou dus mogelijk kunnen leiden tot een minder effectief $CD8^+$ T-cel geheugen en een niet volledig adaptieve immuun reactie. In kinderen opgenomen met een ernstige luchtweg infectie veroorzaakt door primaire RSV infectie zijn veel RSV specifieke $CD8^+$ T cellen en weinig $CD4^+$ T cellen in het bloed aanwezig. In volwassen, die meerdere malen geinfecteerd zijn met RSV, is dit precies andersom. Onze groep heeft ook laten zien (in de muis) dat de hierboven beschreven verschuiving in de verhouding $CD4^+/CD8^+$ T cellen veroorzaakt door antilichamen een gevolg is van herhaalde infecties. Aangezien een robuuste RSV specieke $CD8^+$ T cel response aanwezig is in bloed van kinderen die opgenomen zijn met een ernstige luchtweg infectie veroorzaakt door primaire RSV infectie, zouden $CD8^+$ T cellen, door de schade die zij aanrichten als zij geïnfecteerde cellen dood maken en door cytokines die zij produceren, de bron van de symptomen kunnen zijn. In muizen modellen is inderdaad aangetoond dat het cytokine TNF-α, door $CD8^+$ T cellen geproduceerd, verantwoordelijk kan zijn voor de ziekteverschijnselen. Echter wij denken dat dit mechanisme in mensen onwaarschijnlijk is, omdat we hebben laten zien dat de piek van de $CD8^+$ T cel response van deze kinderen ligt op 14-16 dagen na verschijning van de eerste RSV geïnduceerde ziekteverschijnselen, op een tijdstip dat de meeste kinderen het ziekenhuis alweer verlaten. Deze studies suggeren dus eerder dat $CD8^+$ T cellen mogelijk betrokken zijn bij het herstel proces en dat de eerder optredende intrinsieke immuun reactie en schade geïnduceerd door de virale infectie eerder in aanmerking komen als de veroorzakers van de ziekte verschijnselen.

Om zo min mogelijk schade te ondervinden van een RSV infectie moet een effectieve immuun response bestaan uit het aanwezig zijn van voldoende antilichamen die het virus

neutraliseren voordat het schade aan weefsel toebrengt. Verder helpen een intrinsieke immuun reactie die niet te hevig is, maar wel bijdraagt aan het in toom houden van de infectie, o.a door de productie van interferon en een adequate $CD8^+$ T cell reactie om geinfecteerde cellen te doden. Een vaccinatie programma om hoge RSV specifieke serum titers te realiseren lijkt dus een goede strategie. Helaas is gebleken dat de ontwikkeling van zo'n vaccin de nodige uitdagingen met zich mee brengt. Ten eerste geven natuurlijke her-infecties al geen efficiënte adaptieve immuun reacties. Ten tweede; ondanks het feit dat her-infecties in alle leeftijds catagoriën voorkomen ligt de risico groep in de neonatale periode, een periode waarin het immuun systeem een immuun suppressief karakter heeft en het dus moeilijk is om immuniteit op te wekken. Een derde uitdaging voor de ontwikkeling van een beschermend kandidaat-vaccin, is de vaccinatie trial uit de jaren 1960. De moeilijkheid om een vaccin te ontwikkelen tegen RSV werd aangetoond in dit programma toen kinderen die intra musculair gevaccineerd werden met formaline geïnactiveerd (FI-)RSV ernstige lagere luchtweg aandoeningen onwikkelden na natuurlijke infectie. In de periode van 50 jaar na dit dramatische vaccinatie programma zijn vele (dierlijke) RSV modellen gebruikt om ernstige lagere luchtweg ziekte veroorzaakt door RSV en de FI-RSV vaccinatie beter te begrijpen.

In BALB/C muizen resulteert intra musculaire (i.m.) vaccinatie met FI-RSV in luchtweg hyperreactiviteit, verhoogde aantallen neutrofielen, eosinofielen in de long lavage, slecht neutraliserende antilichamen en een adaptieve T-cel response gedomineerd door Th2 cellen. Sommige groepen hebben in het BALB/C muis model de afzonderlijke componenten van het FI-RSV vaccin onderzocht om de bijdrage van elk afzonderlijk component aan de FI-RSV geïnduceerde immuunpathologie na infectie te onderzoeken. Wijzelf laten in **hoofdstuk 2** in een C57BL/6 model zien dat FI-RSV vaccinatie ook hier tot pulmonaire influx van neutrofielen / eosinofielen en Th2 biased $CD4^+$ T cel responsen leidt. Verder laten wij zien dat noch formaline behandeling (ooit gesuggereerd als oorzaak omdat het virale structuren zou veranderen en daardoor minder effectieve antilichamen zou induceren), noch het adjuvans aluminiumhydroxide verantwoordelijk zijn voor de inductie van eosinofilie en Th2-gedomineerde immuun response in dit muizenmodel. Uit onze experimenten blijkt verder dat de eerder gesuggereerde specifieke eigenschappen van het RSV G eiwit ook niet de belangrijkste oorzaak zijn van het Th2 gerelateerde ziektebeeld, maar dat elk willekeurig eiwit gepresenteerd via intra musculaire vaccinatie (priming) kan bijdragen tot de inflammatoire reactie in dit muizenmodel. Van belang is daarbij de locale intrinsieke immuun reactie in de long tijdens de challenge met virus. Bij systemische vaccinatie met een dood virus is RSV, in tegenstelling tot influenza virus, niet instaat om locaal een inflammatoir milieu de kant van een Th1 response op te sturen. Deze bevinding suggereert dat elke benadering van een RSV vaccine gebaseerd op een gedood vaccine of een subunit vaccin een mogelijk risico inhoudt gezien deze characteristieke eigenschappen van een natuurlijke RSV infectie. Kortom, deze bevinding suggereert dat de uitkomst van de lokale adaptieve immuun reactie die na infectie geactiveerd wordt, mede afhankelijk is van de aard van het infecterende virus. Een mogelijke

verklaring voor deze verschillen tussen RSV en influenza virus zou het verschil kunnen zijn in de manier waarop deze virussen een cel binnen dringen. Influenza virus maakt gebruik van een receptor gemedieerde endo/lysosomale route naar compartimenten waar niet alleen fusie optreedt, maar ook herkenning door endosomale Toll receptoren (TLR). Receptoren die een belangrijke rol spelen bij het initiëren van de innate immuun response. RSV maakt geen gebruik van deze route. Daarom worden virus structuren na infectie voornamelijk herkend door cytoplasmatische pathogeen receptoren van het innate immuun systeem. Dit verschil in herkenning zou dus een oorzaak kunnen zijn voor het uiteindelijke verschil in de werkzaamheid van de aangeboren immuniteit na infectie in gevaccineerde muizen. In **hoofdstuk 2** laten we ook nog zien dat long eosinophilie en Th2 reacties onderdrukt kunnen worden door immuun responsen tegen RSV te induceren met geinfecteerde dendritische cellen. De $CD8^+$ T cel response die via deze weg wordt geinduceerd heeft een beschermend effect in de challenge fase omdat ze de balans verschuift naar een verhoogde Th1/Th2 ratio.

Uit de reeks van gebeurtenissen waargenomen bij kinderen die opgenomen zijn met ernstige RSV-infecties is gesuggereerd dat de intrinsieke (innate) immuun reactie een belangrijke rol speelt in de pathogenesis van RSV, terwijl de adaptieve T cel reactie juist beschermend is. In het verleden is de intrinsieke immuun reactie veroorzaakt door RSV voornamelijk bestudeerd in muismodellen, gekweekte humane cellijnen of *in vitro* gekweekte dendritische cellen. Omdat gedetailleerde kennis van RSV infecties in primaire humane celtypes en het effect van RSV infectie op co-culturen van verschillende humane celtypes ontbreekt, bestuderen wij in **hoofdstuk 3** de intrinsieke immuun reactie na RSV-infectie in een complex mengsel van primaire humane cel typen (perifere bloed mononucleaire cellen; PBMC) in combinatie met de humane long epitheel cellijn A549. Wij laten zien dat men er niet zomaar vanuit kan gaan dat immuun reacties tegen RSV voorspeld kunnen worden door infectie geïnduceerde immuun reacties van individuele celtypes bij elkaar op te tellen. Cellen in complexe samenstelling hebben invloed op elkaar. Zo laten wij zien dat monocyten die blootgesteld zijn aan RSV, de type I interferon response van plasmacytoide dendritische cellen reguleren. In PBMC produceren deze pDC in aanwezigheid van andere cel typen bijna geen IFN-α na blootstelling aan RSV In **hoofdstuk 3** laten wij via 3 mechanismen zien dat het wel degelijk mogelijk is dat deze cellen bij een RSV infectie IFN-α produceren, namelijk: I: Via activatie van TLR7 na antilichaam-gemedieerde opname (in plaats van infectie) van RSV II: Via een infectieuze route van RSV in gezuiverde pDC als andere cellen niet ook in de kweek aanwezig zijn en III: Indirect via IFNAR (de type I interferon receptor die geactiveerd wordt door binding van IFN-α of IFN-β) gemedieerde signalering. Receptor activatie geschied bijvoorbeeld door type I interferonen die geproduceerd zijn door RSV geïnfecteerde A549 cellen of RSV-blootgestelde $CD14^+$ cellen (CD14 is een cell marker die voornamelijk veel op monocyten voor komt). Interessant is dat deze $CD14^+$ cellen de activatie van TLR7 door antilichaam gebonden virus juist remmen. Het mechanisme of oplosbare factor die dit laatste proces remt is tot nu toe nog onbekend. Verder laten wij zien dat in PBMC binding van

RSV celtype specifiek is en dat het aantal virale deeltjes dat bindt aan een specifiek celtype niet noodzakelijkerwijs correleert met de mate van infectie in dat cel type (**hoofdstuk 3**).

Bij een natuurlijke blootstelling aan RSV zijn epitheelcellen, alveolaire macrofagen in het lumen van de luchtwegen en dendritische cellen onder het epitheel de eerste cellen die in contact komen met RSV. Ondanks dat dit een andere setting is dan ons *in vitro* PBMC mengsel, spelen soortgelijke kruisreacties ook daar naar alle waarschijnlijkheid een rol. De relevantie van onze bevindingen is daarom dat men bij het beoordelen van verzwakte virale vaccins op hun veiligheid in beschouwing moet nemen wat zo'n virus doet in de complexe omgeving van de long. Het is dus niet voldoende om een dergelijk verzwakt viraal vaccin te testen in een enkel *in vitro* gekweekt cel type.

RSV specifieke antilichamen worden overgedragen van moeder op kind tijdens de zwangerschap en na de geboorte via moedermelk. De aanwezigheid van antilichamen kan invloed hebben op de intrinsieke immuunreactie en is daarom een factor die bestudeerd moet worden bij de ontwikkeling van nieuwe vaccins. Naast de opname van antilichaam gebonden virale deeltjes via Fcγ receptor (de receptor die de IgG klasse van antilichamen bindt) op de membraan van verschillende cellen, speelt de neonate Fc receptor (FcRn) ook een belangrijke rol in antigeen bemonstering op verschillende mucosale locaties. Van FcRn is bekend dat het serum albumine en IgG-concentraties kan reguleren, kan zorgen voor passieve immuniteit via transport van IgG via de placenta van moeder op kind en de bidirectionele overdracht van IgG gebonden antigeen over de epitheellaag via trancytose (waar bij het complex een cel laag wordt getransporteerd). In zowel *in vitro* als *in vivo* studies is aangetoond dat FcRn op antigen presenterende cellen (DC) een rol speelt in MHC II presentatie aan $CD4^+$ T cellen, alsmede cross-presentatie aan $CD8^+$ T-cellen. In **hoofdstuk 4** onderzochten wij (als model voor passief verkregen immuniteit) hoe reeds aanwezige RSV specifieke antilichamen de priming van de immuun reactie en de adaptieve immuun reactie bij her-infectie beïnvloeden. Daarnaast onderzochten wij of FcRn hier een rol bij speelt. Onze groep toonde al eerder in muizen aan dat systemische aanwezigheid van neutraliserende antilichamen RSV-specifieke T-cel responsen in de long kunnen beïnvloeden, door het verschuiven van de $CD4^+/CD8^+$ T cel balans. Het is mogelijk dat antilichaam gebonden aan RSV de infectie kan verhinderen, met als gevolg gereduceerde MHC klasse I presentatie en opeenvolgende $CD8^+$ T-cel priming. Wij laten zien dat i.n. toediening van UV-geïnactiveerd RSV (dat niet meer instaat is een cel te infecteren) alleen resulteert in slechte T-cel priming, terwijl antilichaam (palivizumab) gebonden UV-RSV zowel $CD4^+$ als $CD8^+$ RSV specifieke T-cel responsen kan induceren (**hoofdstuk 4**). Deze experimenten tonen aan dat de presentatie van niet-infectieuze virusdeeltjes via immuuncomplexen $CD8^+$ T-cel priming kan faciliteren, waarschijnlijk door het activeren van MHC klasse I routes via cross-presentatie in APC. In dit hoofdstuk is een rol voor FcRn in RSV specifieke $CD4^+$ en $CD8^+$ adaptieve T cel reacties hierin uitgesloten.

Samengevat kunnen we concluderen dat profylactische toediening van RSV neutraliserende antilichamen duidelijk gunstige effecten heeft bij hoog risico kinderen. Deze

antilichamen remmen virale replicatie en verlagen daardoor de hoeveelheid virus aanwezig in de long. Verlaging van de hoeveelheid virus aanwezig in de long onderdrukt ook waarschijnlijk de uitbundige innate immuun reactie die kenmerkend is voor kinderen die opgenomen worden met ernstige lagere luchtweg infecties. In deze eerste hoofdstukken laten wij echter ook zien dat ondanks de bewezen beschermende rol in primaire infecties, virus/antilichaam complexen ook intrinsieke en adaptieve immuun reacties kunnen beïnvloeden doordat het virus via een alternatieve (niet infectieuze) weg herkend wordt. Dit kan gevolgen hebben voor latere immuun reacties en het vermogen om weerstand te bieden gedurende RSV her-infecties.

De rol van het microbioom in RSV infecties

Gedurende de neonatale periode draagt de blootstelling van lichaams oppervlakken zoals de huid, long en darmen aan micro-organismen bij aan de rijping van het immuun systeem. In deze periode reguleren gespecialiseerde tolerogene mechanismen van het immuun systeem de balans tussen excessieve immuun reacties op micro-organismen waaraan de neonaat wordt blootgesteld en een noodzakelijke immuun rijping die de neonaat moet beschermen tegen toekomstige infecties die ziekte veroorzaken. In de eerste maanden na de geboorte is er theoretisch gezien dus een mogelijkheid om via microbiome manipulatie de ontwikkeling van evenwichtige immuun reacties te sturen. In muis modellen en in klinische studies heeft men al eerder een modulerend effect aangetoond van specifieke bacteriestammen op de ontwikkeling van atopische ziekte, door deze toe te dienen aan zwangere vrouwen of via kolonisatie met specifieke bacteriestammen tijdens de geboorte. Omgekeerd zou dit ook kunnen betekenen dat in deze periode veranderingen in bacteriële kolonisatie door toediening van bijvoorbeeld antibiotica van invloed zouden kunnen zijn op de ontwikkeling van neonatale immuniteit. In **hoofdstuk 5** bediscussiëren wij correlaties tussen bacteriële kolonisatie, astmatische /allergische ziekten en luchtweg infecties. Verder beschrijven wij in dit hoofdstuk wat er bekend is over de effecten van farmaceutische en voedingscomponenten op gastro-intestinale microbiële kolonisatie en immuun (dys-)functie. Het idee dat de blootstelling aan micro-organismen vroeg in het leven ons zou kunnen beschermen tegen Th2 gerelateerde astmatische immuunreacties en atopische ziekten op latere leeftijd bestaat al enige jaren. Maar jaren van onderzoek hebben aangetoond dat deze denkwijze te simpel is en dat wij nu slechts aan het begin staan van de wetenschap hoe microbiële gemeenschappen mucosale immuniteit en immunologische aandoeningen kunnen beïnvloeden.

Het is aangetoond dat borstvoeding in het eerste levensjaar het risico van ziekenhuisopname voor lagere luchtweg infecties vermindert. Bovendien hebben een aantal klinische studies de beschermende effecten van moedermelk tegen RSV bronchiolitis aangetoond. Naast de aanwezigheid van passief via de moeder verkregen virus neutraliserende antilichamen via de placenta en melk, is het echter ook mogelijk dat andere in moedermelk aanwezige

componenten immuun stimulerende potentie hebben. Humane melk oligoschariden (HMOS) zijn complexe suiker structuren waarvan is aangetoond dat deze immuun modulerende eigenschappen hebben. In de **hoofdstukken 6, 7 en 9** maken wij gebruik van diëten die onverteerbare oligosacchariden bevatten die lijken op HMOS. Deze specifieke oligosacchariden (GFA, korte-keten Galacto oligosacharides (scGOS), lange-keten Fructooligosacchariden (lcFOS) en pectine-afgeleide zure oligosachariden (pAOS)) hebben aangetoonde "prebiotische" eigenschappen wat wil zeggen dat ze de groei van speciefieke commensale bacteriën kunnen stimuleren. Wij tonen aan dat een dieet op basis van deze oligosacchariden systemische immuniteit kan wijzigen in verschillende infectie en vaccinatie gerelateerde muismodellen. In **hoofdstuk 6** laten we zien dat een dieet met een combinatie van scGOS/lcFOS/pAOS tijdens een influenza subunit vaccinatie, de influenza specifieke type IV overgevoeligheids reactie (DTH) in C57BL/6 muizen kan versterken. In dit hoofdstuk laten we zien dat dit mogelijk komt via effecten op $CD25^+$ regulatoire T cellen, omdat verwijdering van dit cel type door middel van intra peritoneale i.p.injectie van monoklonale antilichamen tegen CD25 (een marker aanwezig op geactiveerde T cellen en regulatoire T cellen) deze versterkte effecten van GFA op de influenza specifieke DTH reactie teniet doet. In **hoofdstuk 9** gaan we hier dieper op in door te onderzoeken of voedingsinterventie met GFA verschillen in regulatoire T cel subpopulaties of hun functionaliteit kan induceren. In dit hoofdstuk tonen we aan dat in GFA behandelde dieren een correlatie bestaat tussen de verhoogde DTH reactie en een verhoogd percentage van T-bet expresserende T-cellen, een aanwijzing voor een sterkere Th1 reactie. Verder vonden we in lymfeknopen die in contact met de darm staan een verlaagd aantal CXCR3/T-bet expresserende regulatoire T cellen, een regulatoir T cel subtype dat de Th1 reactie onderdrukt. Hoewel wij verschillen vonden in aantallen Treg subtypes konden we geen effecten van GFA op Treg functionaliteit detecteren. Vervolg onderzoek is nodig om te onderzoeken hoe deze regulatoire T cellen de adaptieve immuun reactie beïnvloeden. In **hoofdstuk 7** laten wij zien dat het GFA oligosacchariden dieet ook adaptieve immuniteit in zowel een primaire RSV-infectie model als in een FI-RSV vaccinatie model in muizen kan beïnvloeden. In beide modellen verschuift het dieet de balans van de immuunrespons naar meer RSV specifieke $CD8^+$ T cellen en een Th1 gedreven (IFN-γ producerende cellen) cytokine milieu. In het FI-RSV model resulteert dit in verlaagde long cell influx en long eosinophilia, parameters die kenmerkend zijn voor dit model.

De meeste studies naar HMOS en specifieke oligosacchariden schrijven de immuun modulerende effecten van oligosacchariden toe aan hun capaciteit om veranderingen in intestinale microbiome samenstelling te bewerkstelligen door de groei van, voor de mens gunstige, specifieke bacteriën. Om inzicht te krijgen op de vraag of een wijziging in de samenstelling van microbiome compositie door GFA verantwoordelijk is voor de veranderingen in de RSV-specifieke T cel responsen beschreven in **hoofdstuk 7**, hebben we in **hoofdstuk 8** het microbiome van C57BL/6 muizen gemanipuleerd met behulp van breed spectrum antibiotica. In deze studie laten wij zien dat een orale antibiotica behandeling voor een periode

van 4 weken, de diversiteit van darm bacteriën tot tenminste 4 weken na het beëindigen van de behandeling onomkeerbaar beinvloedt. Wij tonen in het FI-RSV vaccinatie model aan dat oraal toegediende antibiotica de adaptieve immuun respons, geinduceerd na RSV infectie verandert, resulterende in een verlaagde $CD8^+$ T cel activatie en lagere aantallen TNF-α/IFN-γ producerende RSV-specifieke T cellen. Dit ging gepaard met een versterkte inflammatoire type Th2 immuune reactie in de long gekenmerkt door een toegenomen aantal "ontstekings" $CD11b^+$ DC versus tolerogene $CD103^+$ DC, toegenomen aantallen eosinofielen in de long lavage en een verlaagd aantal regulatoire T cellen. Dit hoofdstuk toont aan dat orale antibiotica toegedoend gedurende een korte periode langdurige negatieve effecten heeft op de RSV specifieke T cell reacties in de long van volwassen muizen. Een effect dat waarschijlijk toe te schrijven valt aan immuun dysfunctie veroorzaakt door een sterk gereduceerde microbiële diversiteit. De vraag of veranderingen in bacterie populaties beschreven in de **hoofdstukken 6 t/m 9** systemische of lokale immuniteit beinvloed blijft open. Ondanks dat wij grote verschillen zagen in microbiële populaties in de darm, is het ook mogelijk dat directe interacties tussen virus en bacteriën in de nasofarynx de infectiegraad beïnvloeden en daarmee ook de aard en de mate waarin de intrinsieke en adaptieve immuun reacties geïnduceerd worden.

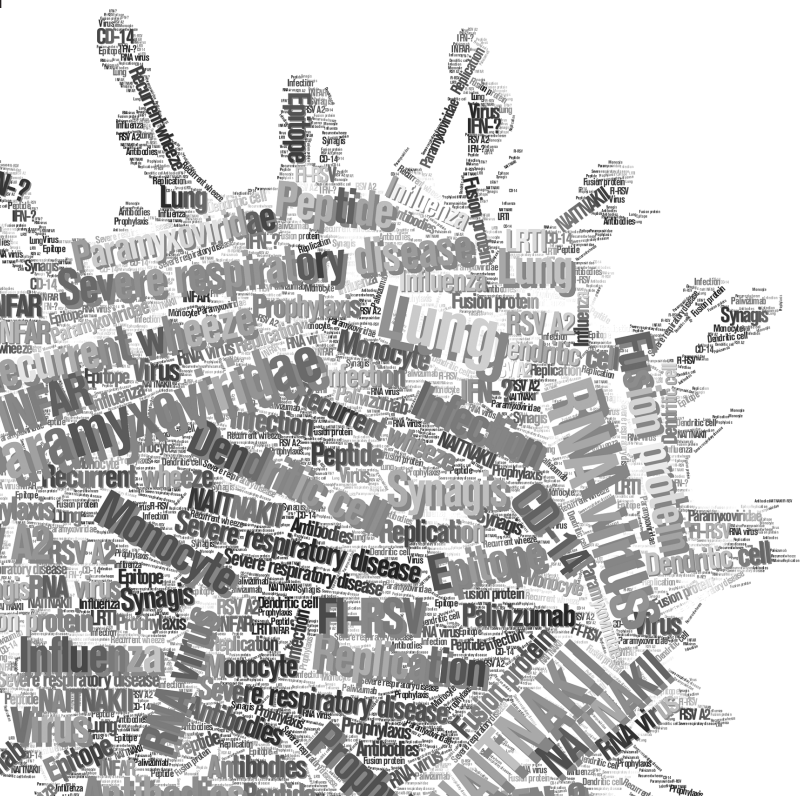
In dit proefschrift demonstreren wij dat voorzichtigheid geboden is bij het gebruik van hoog neutraliserende antilichamen of antibiotica in pasgeborenen. Wij laten zien dat beide invloed kunnen hebben op de intrinsieke immuun reactie en de ontwikkeling van adaptieve immuniteit tegen pathogenen zoals RSV. Bij onderzoek naar nieuwe vaccin kandidaten of profylactische antilichamen is het belangrijk dat er gekeken wordt naar de invloed van complexe cel interacties en de rol van antistoffen zowel in T-cel priming als aangeboren (innate) immuniteit na (her-)infecties. Verder zou vanuit ons muis model opgemaakt kunnen worden dat antibiotica gebruik in de neonatale periode de kans op ernstige RSV bronchiolitis en de ontwikkeling van allergische aandoeningen op latere leeftijd zou kunnen verhogen. Aangezien wij de mogelijkheid van modulatie van RSV specifieke immuun reacties via dieet aantonen, is meer onderzoek naar het mechanisme hoe vaccin geïnduceerde immuun reacties kunnen worden veranderd door microbiome manipulatie nodig. Dit zou in de toekomst kunnen bijdragen aan strategieën waarbij microbioom manipulatie via dieet leidt tot ondersteuning van of meer efficientere vaccinatie strategieën.

REFERENTIES

1. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. Vaccine, 2003.
2. Prince GA, Horswood RL, Chanock RM. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. Journal of Virology, 1985.
3. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics, 1998.
4. Carbonell-Estrany X, Simões EAF, Dagan R, Hall CB, Harris B, Hultquist M, et al. Motavizumab for prophylaxis of respiratory syncytial virus in high-risk children: a noninferiority trial. Pediatrics, 2010.
5. Kruijsen D, Bakkers MJ, Van Uden NO, Viveen MC, Van der Sluis TC, Kimpen JL, et al. Serum antibodies critically affect virus-specific CD4+/CD8+ T cell balance during respiratory syncytial virus infections. Journal of Immunology (Baltimore, Md: 1950) 2010.


Prophyla
RSVA
Synagis
Fusion
Severe respirator
Palivizumab
CD=14

About the author



CURRICULUM VITAE

Marcel Schijf was born on June 11th, 1974 in De Meern, Utrecht. In 1993 he graduated from secondary school (HAVO) at the St. Gregorius College in Utrecht. Thereafter he started the study Biology and Medical Laboratory Research at the Hogeschool van Utrecht (HvU). In his final year an internship was done at the department of Pharmacology and Pathophysiology of the Utrecht University under supervision of Dr. Sjef de Kimpe, where he studied the influence of endogenous IL-4 and IFN-γ on the regulation of blood pressure in hypertensive NZWF1 hybrid mice. In 1998 he received his Bachelor's degree with specializations in Histology and Zoology. In the same year he started working as a technician in the department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University. Under supervision of Dr. Nanne Bloksma and Dr. Paul Henricks he studied the involvement of alveolar macrophages in occupational asthma and assisted in the development of a mouse model for Chronic Obstructive Pulmonary Disease (COPD). In 2004 he started working as a technician at the department of Toxicology & Applied Pharmacology of TNO, Nutrition and Food research, Zeist. Under supervision of Dr. Ir. Josje Arts, Dr. Hans Muijser and Dr. Frieke Kuper he worked in the inhalation toxicology group on the development of tools to identify contact and respiratory allergens. In 2006 he joined the experimental immunology group of the department of Toxicology & Applied Pharmacology, where he conducted immunogenicity and (immuno) toxicity studies on pharmaceutical compounds and food allergens, under supervision of Dr. Andre Penninks and Dr. Jolanda van Bilsen. In August 2008 he started, as a Ph.D. student funded by TIPharma, his research on Respiratory Syncytial virus (RSV) at the division of Pediatrics of the Wilhelmina's Children's Hospital, UMC Utrecht. Under supervision of Dr. Ir. G.M. van Bleek (UMC Utrecht), Dr. B. van't Land (Danone research) and Prof. Dr. J. Garssen (Danone Research and Faculty of Pharmaceutical Sciences, Utrecht University) he investigated innate immune responses upon viral recognition and the effect this has on immune priming against RSV and subsequent re-infections. In addition he studied the influence of microbiome composition on the susceptibility and outcome of RSV induced respiratory disease. The results are described in this thesis.

LIST OF PUBLICATIONS

1) **Schijf MA**, Kruijsen D, Lukens M, Coenjaerts FE, Garssen J, van't Land B, van Bleek GM. Respiratory Syncytial Virus induced type I IFN production by pDC is regulated by RSV-infected airway epithelial cells, RSV-exposed monocytes and virus specific antibodies. PLoS One (2013): In press.
2) **Schijf MA**, Kerperien J, Bastiaans J, Szklany K, Meerding J, Hofman G, Boon L, van Wijk F, Garssen J, van't Land B. Alterations in regulatory T cells induced by specific oligosaccharides improve vaccine responsiveness in mice. PLoS One (2013): 9: e75148.
3) Kruijsen D, **Schijf MA**, Einarsdottir HK, Coenjaerts FE, van der Schoot EC, Vidarsson G, van Bleek GM. Intranasal administration of antibody-bound respiratory syncytial virus particles efficiently primes virus specific immune responses in mice. Journal of Virology (2013): 13: 7550-7557.
4) **Schijf MA**, Kruijsen D, Bastiaans J, Coenjaerts FE, Garssen J, van Bleek GM, van't Land B. Specific dietary oligosaccharides increase Th1 responses in a mouse respiratory syncytial virus infection model. Journal of Virology (2012): 21: 11472-11482.
5) van't Land B, **Schijf MA**, Martin R, Garssen J, van Bleek GM. Influencing mucosal homeostasis and immune responsiveness: the impact of nutrition and pharmaceuticals. Eur J Pharmacol (2011): 668: s101-s107.
6) Kruijsen D, **Schijf MA**, Lukens MV, van Uden NO, Kimpen JL, Coenjaerts FE, van Bleek GM. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. Vaccine (2011): 29: 2730-2741.
7) van't Land B, **Schijf MA**, van Esch BC, van Bergenhenegouwen J, Bastiaans J, Schouten B, Boon L, Garssen J. Regulatory T-cells have a prominent role in the immune modulated vaccine response by specific oligosaccharides. Vaccine (2010): 35: 5711-5717.
8) De Jong WH, Arts JH, De Klerk A, **Schijf MA**, Ezendam J, Kuper CF, Van Loveren H. Contact and respiratory sensitizers can be identified by cytokine profiles following inhalation exposure. Toxicology (2009): 3:103-111.
9) Arts JH, de Jong WH, van Triel JJ, **Schijf MA**, de Klerk A, van Loveren H, Kuper CF. The respiratory local lymph node assay as a tool to study respiratory sensitizers. Toxicol. Sci. (2008): 2: 423-434.
10) Arts JH, **Schijf MA**, Kuper CF. Preexposure to amorphous silica particles attenuates but also enhances allergic reactions in trimellitic anhydride-sensitized brown Norway rats. Inhal Toxicol. (2008): 10: 935-948.
11) Kuper CF, Stierum RH, Boorsma A, **Schijf MA**, Prinsen M, Bruijntjes JP, Bloksma N, Arts JH. The contact allergen dinitrochlorobenzene (DNCB) and respiratory allergy in the Th2-prone Brown Norway rat. Toxicology (2008): 2-3: 213-221.

12) Valstar DL, **Schijf MA**, Stelekati E, Nijkamp FP, Bloksma N, Henricks PA. Trimellitic anhydride-conjugated serum albumin activates rat alveolar macrophages in vitro. J. Occup. Med. Toxicol. (2006): 23: 1-13.
13) Valstar DL, **Schijf MA**, Arts JH, Kuper CF, Nijkamp FP, Storm G, Bloksma N, Henricks PA. Alveolar macrophages suppress non-specific inflammation caused by inhalation challenge with trimellitic anhydride conjugated to albumin. Arch. Toxicol. (2006): 9: 561-71.
14) Valstar DL, **Schijf MA**, Nijkamp FP, Storm G, Arts JH, Kuper CF, Bloksma N, Henricks PA. Alveolar macrophages have a dual role in a rat model for trimellitic anhydride-induced occupational asthma. Toxicol. Appl. Pharmacol. (2006): 1: 20-29.
15) Valstar DL, **Schijf MA**, Nijkamp FP, Bloksma N, Henricks PA. Glutathione-conjugated toluene diisocyanate causes airway inflammation in sensitized mice. Arch. Toxicol. (2004): 9: 533-539.
16) Smit JJ, Van Loveren H, Hoekstra MO, **Schijf MA**, Folkerts G, Nijkamp FP. Mycobacterium vaccae administration during allergen sensitization or challenge suppresses asthmatic features. Clin. Exp. Allergy. (2003): 8: 1083-1089.

ACKNOWLEDGEMENTS (DANKWOORD)

Niets leuker dan op een zondagmiddag onder het genot van een kopje thee terug te kijken naar de afgelopen jaren van dit promotie onderzoek. De keuze om te gaan promoveren heeft geleid tot veel (wetenschappelijke) uitdagingen, nieuwe omgevingen en het ontmoeten van veel kleurrijke personen. Natuurlijk waren er, zoals bij elke AIO, genoeg momenten waarop je jezelf afvraagt waarom je hier ook alweer mee begonnen was, maar achteraf gezien was het een leuke en vooral leerzame periode, waar ik met plezier aan terug denk. Het feit dat je aangenomen wordt op een TI-Pharma promotie traject, in dienst treedt bij Danone research, geplaatst wordt bij de RSV groep van de divisie Kinderen en op het lab van NIDOD in het Wilhelmina kinderziekenhuis komt te werken, heeft ervoor gezorgd dat ik de afgelopen jaren een hoop leuke mensen heb mogen ontmoeten. Graag wil ik in dit laatste hoofdstuk aandacht besteden aan een aantal personen die het tot stand komen van dit proefschrift mogelijk hebben gemaakt.

Allereerst de promotor en copromotoren die mij de kans hebben gegeven om dit promotie traject te starten en mijzelf als wetenschappelijk onderzoeker te ontwikkelen. Johan Garssen, mijn promotor, ondanks dat jij het onderzoek van een afstand volgde, heeft jouw interesse en enthousiasme voor het onderzoek mij altijd het gevoel van steun en vertrouwen gegeven, bedankt hiervoor. Beste Bea, bedankt voor de kans die je mij gegeven hebt om te promoveren. Dankzij jouw heb ik de wereld van Danone Research en het WKZ leren kennen. Naast de drempelloze begeleiding waren jouw enthousiasme en aanstekelijke lach voor mij een bron van motivatie. Bedankt! Dankzij mijn 2^{e} copromotor heb ik kennis gemaakt met de wetenschappelijke wereld van het RS virus. Beste Grada, bedankt voor jouw tijd en geduld om mij de kunst van het doen van goed en gefundeerd wetenschappelijk onderzoek bij te brengen. Naast het regelmatig lanceren van eerlijke, maar kritische opmerkingen over het onderzoek (je mist een controle…) ben je ook warm en betrokken persoon waar ik zeker in de laatste maanden van het traject, zowel voor privé als werk gerelateerde onderwerpen, veel steun aan heb gehad. Bedankt hiervoor.

De familie. De voordelen van een kleine groep zijn toch wel de hechte banden en samenwerkingen. Michael bedankt voor je bijdrage om mij wegwijs te maken op het lab en het “opweg” helpen in de eerste maanden van het RSV onderzoek. Debby, dankzij jou heb ik niet alleen veel praktische handelingen op het lab geleerd maar door de wetenschappelijke discussies ook veel over het virus en de rol van Fc receptoren in RSV pathogenese. Ondertussen mama geworden, ik wens jou en je gezin veel geluk toe en succes met je verdere carrière. Nathalie, jammer dat je al zo snel weg ging bij de groep, het was met jou nooit saai op het lab, bedankt voor de hulp bij de luminex experimenten. De studenten Tetje en Mark. Ook al waren jullie studenten van Debby, bedankt voor de hulp bij de *in vivo* studies. Student Anniek, het blokkeren van pathogen recognition receptors is niet altijd even simpel, dat weet jij nu ook.

Ondanks de tegenslagen is er toch een mooie publicatie uit voortgekomen, bedankt hiervoor. Paranimfen Joost en Patrick. Beide een beetje verbonden met het RSV onderzoek. De ene als postdoc bij RSV autoriteit Nick Lukacs, de ander als iemand die er achter gekomen is dat promoveren op RSV onderzoek toch niet helemaal zijn ding is. Hoe dan ook, 2x het bewijs dat (ex) collega zijn en vriendschap heel goed samen gaan.

De adoptie familie: Naast de RSV groep heb ik kennis mogen maken met psycho neuro immunologie, HI en pijn. Naast het gebruik van lab faciliteiten heeft de NIDOD groep van Prof. dr. Cobi Heijnen en Prof. dr. Annemiek Kavelaars mij de broodnodige wintersport, sinterklaas en koffietafel gezelligheid gegeven, zeker toen het wat rustiger werd binnen de RSV groep. De afgelopen jaren zijn in deze dynamische groep nogal wat kleurrijke personen (lees Mexicaan, Arts-assistenten en suikerjunkies) voorbij gekomen die ik graag wil bedanken. Naast pijngoeroe Niels, altijd in voor een standpunt, wil ik Cora, Cindy en Hanneke graag bedanken voor hun gezelligheid en interesse. Naast het brengen van mooie wetenschappelijke output zijn julie, wat zeker net zo belangrijk is, mede verantwoordelijk voor de goede sfeer binnen de groep. Buurvrouw Hilde, na Anibal is het er zeker niet saaier op geworden. Behalve koffie over mij heen gooien, heb je ook een hoop gezelligheid gebracht. Bedankt hiervoor. Veel succes met de afronding van je proefschrift en natuurlijk de nieuwe fase in je leven als mama! Naast de overheersende vrouwelijke kant van het lab, was NIDOD 's avonds een mannen bolwerk. Adobe Illustrator Emiel, bedankt voor je bijdrage aan de figuren en het laten kennismaken met placenta onderzoek. Het wordt vast een interessant proefschrift met ongetwijfeld mooie plaatjes. De (ex) collega's van de AIO kamer: Jessica, Lotte en Mirjam (niet helemaal…). Bedankt voor de gezellige wintersport, promotie feestjes, koffie pauzes en lunches. Ik wens jullie veel succes in je verdere carrière. Vanessa en Elke, succes met het afronden van je proefschrift. Fundamenteel onderzoek is gewoon hard werken met vaak weinig resultaat. Elke ik bewonder je doorzettingsvermogen, je komt er wel!

De buitenland delegatie Anibal, Luca, Huijing. Anibal, although hard to understand, it's nice to have a real man with a good sense of humor in the AIO room. Huijng, another example of a hardworking, motivated Chinese, I loved your candies. Luca, it seems to me that you're having a great time in the Netherlands; I hope that the exosomes will deliver some interesting answers and add some nice papers to your PhD.

De steunpilaren van elk (AIO) onderzoek zijn zonder twijfel de analisten. Jitske, Mirjam, Karima, Marijke en Sabine (goed van je dat je de keuze hebt gemaakt), bedankt voor het helpen met de humane PBMC studies. Jullie "bijdrage" aan de 51 *in vitro* studies (als jullie de exacte bijdrage in ml wil weten kan je altijd even langs komen…;-) heeft toch maar tot een mooie publicatie geleid. Koos, bedankt voor het beantwoorden van vragen over flow cytometrie en plan B als ik weer eens mis greep in onze eigen antilichamen voorraad. Iemand

die altijd in is voor een praatje, de koffiehoek versiert met kerst en taarten bakken een hele nieuwe dimensie heeft gegeven, zou eigen nooit weg mogen gaan. Pirkko, bedankt voor je gezelligheid, het AZU mag best blij zijn met zo'n aanwinst.

Het doen van RSV infectie studies betekent dat je ook heel wat uurtjes in de GDL infectie unit doorbrengt. Gelukkig kon ik met mijn vragen en problemen terecht bij Wout en Gerard (geniet lekker van je pensioen). Het meedenken bij de complexe studies en de bereidheid om te helpen hebben een grote bijdrage geleverd aan de studies, bedankt hiervoor.

Beste Danone collega's. Je kan nooit op 2 plekken tegelijk zijn. In dit geval had dit tot gevolg dat het zwaartepunt van het promotietraject bij het WKZ lag. Ondanks dat het contact met de meeste personen van het Danone immunologieplatform beperkt is gebleven tot half jaarlijkse werkbesprekingen en luxe bedrijfsuitjes heb ik, mede omdat een groot gedeelte uit ex-Farmacologie collega's bestaat, het gevoel gehad dat jullie betrokken waren bij het onderzoek, bedankt hiervoor. Speciale aandacht gaat uit naar een paar mensen. Leon Knippels, het doorsturen van mijn CV naar Bea heeft het balletje doen rollen…, bedankt. Hulp op het lab kwam van Jaqueline. Helaas is wetenschappelijk onderzoek geen 9-5 werk en betekent dit meestal dat op de experimentele dagen extra handjes van harte welkom zijn. Jouw hulp, meestal in de vroege uurtjes, heeft een grote bijdrage geleverd aan de dieet studies. Bedankt hiervoor. De interesse voor veranderingen in bacteriële populaties heeft geleid tot kennismaking met Phyla, rarefaction plots en Harm. Het is leuk om de werelden van de microbiologie en immunologie te combineren, iets waar jij met enthousiasme aan mee hebt gewerkt, bedankt hiervoor en succes met je (AIO?) carrière.

Geen succes zonder een stabiele basis. De broodnodige afleiding naast het werk is regelmatig geleverd door mensen die zich bezig houden met o.a.: Pfalzerwald, Barista- praat, racefietsen, winter BBQ, Bowmore, Noordwijk, Pandoraatjes en ff een downloadje kijken. Niets leuker dan mensen om je heen te hebben die excuusjes verzinnen waarom ze die berg niet meer op komen, zich druk maken over melk figuurtjes in de koffie en eindeloos slap praten over fiets onderdelen en whisky. Ik hoop dat wij hier nog lang mee door mogen gaan.

Beste (schoon)-familie, bedankt voor alle onvoorwaardelijke steun en vertrouwen. Ook al was het niet altijd te begrijpen waar het onderzoek over ging, jullie waren altijd heel erg geïnteresseerd en vol vertrouwen dat het zou gaan lukken. Bedankt hiervoor.

Leontien, een promotie traject kent pieken en dalen, dat weet jij nu ook. Bedankt voor de steun die je mij gegeven hebt. Het onderzoek is nu af en wordt het tijd voor een nieuwe fase. Naast het sfinxen van de buikschuiver hoop ik dat de toekomst ons mooie dingen gaat brengen.

Marcel

LIST OF ABBREVIATIONS

Ab:	Antibody
APC:	Antigen presenting cell
BALT:	Broncho alveolar lymphoid tissue
BFA:	Brefeldin A
CFSE:	Carboxyfluorescein succimidyl ester
DC:	Dendritic cell
DTH:	Delayed Type Hypersensitivity reaction
ELISA:	Enzyme linked Immunosorbent assay
FACS:	Fluorescence-activated cell sorting
FcRn:	Neonatal Fc receptor
GALT:	Gut associated lymphoid tissue
GFA:	GOS/FOS/AOS
Type I IFN:	Type I Interferon
i.n.:	Intra nasal
i.m.:	Intra muscular
i.p.:	Intra peritoniaal
i.r.:	Intra rectal
lcFOS:	Long chain Fructo oligosaccharides
Palivizumab:	Monoclonal antibody against RSV F (fusion) protein
PAMPs:	Pathogen associated molecular patterns
pAOS:	Pectin derived acetic oligosaccharides
PRR:	Pathogen recognition receptor
RSV:	Respiratory syncytial virus
RSV-IC:	Respiratory syncytial virus immune complex
scGOS:	short chain Galacto oligosaccharides
Th-1, -2, -17:	T helper cell subsets
TLR:	Toll like receptor
UV-RSV:	UV inactivated RSV