Mechanisms of Respiratory Syncytial Virus specific T cell activation

Debby Kruijsen

Author: Debby Kruijsen
Lay-out: Medialen B.V., Best
Print: proefschriftmaken.nl
ISBN: 978-90-393-5647-0

Mechanisms of Respiratory Syncytial Virus specific T cell activation

Mechanismes van Respiratoir Syncytieel Virus specifieke T cel activatie

(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
vrijdag 11 november 2011 des middags te 2.30 uur

door

Debby Kruijsen

geboren op 28 mei 1983 te Tilburg

Promotor: Prof.Dr. J.L.L. Kimpen

Co-promotor: Dr.Ir. G.M. van Bleek

Financial support by the Heart & Long Foundation Utrecht for the publication of this thesis is gratefully acknowledged.
Printing of this thesis was financially supported by AIMM Therapeutics, Luminex B.V., Danone Research - Centre for Specialised Nutrition, Novartis Pharma B.V., GlaxoSmithKline B.V., Abbott B.V., J.E. Juriaanse stichting, Stichting Astma Bestrijding and The Infection and Immunity Center Utrecht.

Contents

Chapter 1:	General introduction	8
Chapter 2:	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: 7235-7243*	36
Chapter 3:	Serum antibodies critically affect virus-specific $CD4^+$/$CD8^+$ T cell balance during Respiratory Syncytial Virus infections. *The Journal of Immunology (2010) 185: 6489-6498*	56
Chapter 4:	Intranasal administration of antibody-opsonized Respiratory Ryncytial Virus particles efficiently primes immune responses in mice.	84
.		
Chapter 5:	Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. *Vaccine (2011) 29: 2730-2741*	102
Chapter 6:	Specific dietary oligosaccharides increase Th1 responses in a mouse Respiratory Syncytial Virus infection model.	128
Chapter 7:	Summary and general discussion	146
	Nederlandse samenvatting	165
	Dankwoord	173
	Curriculum Vitae	177
	List of publications	179

chap

ter 1

General Introduction

Respiratory syncytial virus : The virus

Respiratory syncytial virus (RSV) was first isolated in 1957 from chimpanzees who experienced an upper respiratory tract illness with coryza, runny nose and malaise. The virus was first named chimpanzee coryza agent (1) and later RSV for its ability to induce syncytia of infected epithelial cells (2). RSV belongs to the family of Paramyxoviridae and has a single stranded, negative sense RNA genome that encodes 11 proteins on 10 genes. Infection of host cells with RSV is mediated via two viral transmembrane glycoproteins. The glycoprotein (G), expressed as a soluble and membrane attached isoform, mediates attachment of the virus to the host cell (3). The viral fusion protein (F) is responsible for fusion with the cell membrane which allows the nucleocapsid to enter the cellular cytoplasm (4). The function of a third minor membrane protein, the small hydrophobic (SH) protein is not exactly known. Some suggested that the protein has a negative effect during viral fusion (5), plays a role in inhibiting Tumor Necrosis Factor (TNF)-α mediated apoptotic signaling pathways (6) and due to its pentameric structure could possibly form cationic ion channels that might induce the formation of pores in infected cells (7). As a consequence of viral fusion with the host cell membrane, viral RNA accompanied by the viral nucleocapsid proteins RNA polymerase (L), nucleoprotein (N) and phosphoprotein (P) is injected into the cytosol of the host cell [reviewed in (8, 9)]. These nucleocapsid proteins are involved in RSV RNA transcription. Transcription of the genome of RSV is initiated at the 3' terminus and generates a full-length positive-sense RNA strand with a promoter site at the 5' terminus. Genes encoding non-structural (NS) protein 1 and 2 are located at the 3' terminus of the RSV genome and are expressed early after infection. NS proteins suppress the anti-viral interferon (IFN) response of the host cell (10, 11). In addition, NS proteins inhibit early apoptosis of lung epithelial cells *in vitro* suggesting that this might prevent early cell death upon infection and thereby promote viral replication (12). The matrix (M) protein is crucial for viral assembly and budding at the plasma membrane. The M2 protein is transcribed in two open reading frames, resulting in two proteins after translation. The M2-1 protein is a co-factor involved in efficient RSV RNA transcription. The M2-2 protein promotes RNA replication and inhibits transcription. Both M2 proteins are structural proteins. During transcription of the RSV RNA genome, the RNA polymerase crosses from one gene to the next gene by a gene start and gene end sequence. As a result of inefficient crossing during transcription of the RSV genome, the genes encoded at the 3' end are transcribed with higher efficiency compared to genes at the 5' terminus [reviewed in (8)].

Disease

RSV infections are most common in infants, elderly people and immunocompromised individuals (13-17). Most children under the age of 2 experience a primary RSV infection usually during the first winter season and 50% experience a second infection before 24 months of life. Nearly all children infected with RSV develop an upper respiratory tract infection with mild symptoms, cough and rhinorrhea (18). A small percentage of these children develop a lower respiratory tract infection where

RSV infects epithelial cells of bronchioles and alveoli. This results in bronchiolitis with severe coughing, wheezing and dyspnea and about 30% of these children require hospitalization. Children with severe lower respiratory tract infections may require mechanical ventilation. Severe RSV infections in hospitalized children are associated with an increased risk (6- to 8-fold in the first year of life) for developing asthmatic symptoms. Conversely, children with asthma experience a long term 3-fold increased susceptibility for severe RSV disease (19-21).
Re-infection with RSV occurs throughout life, indicating that natural infection does not induce immune memory that can completely prevent re-infections. The reason for this incomplete immune protection is not clear. T cells are required for effective clearance of the virus since T cell deficient children are not able to clear RSV efficiently (14, 16). RSV specific neutralizing antibodies are present in human serum and provide partial protection against severe RSV lower respiratory tract infections (22-24). At the moment there is still no licensed vaccine available to prevent RSV mediated serious lower respiratory tract infections.

The innate immune response to RSV

During natural exposure to RSV primarily respiratory epithelial cells from the small airways and alveoli, and antigen presenting cells (APC) like alveolar macrophages and dendritic cells (DC) become infected (25-28). DCs are present underneath the epithelial cell layer. Epithelial cells lining the respiratory tract, macrophages and DC express or up-regulate pattern recognition receptors (PRR) and recognize patterns on invading pathogens. In initial virus detection three classes of pattern recognition proteins (PRP) are important, nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), RIG-I (retinoic acid-inducible gene I)-like helicases (RLH) and toll like receptors (TLR) (29). Viral infection or binding of the virus to PRR results in PRR mediated signaling and cytokine plus chemokine release. This induces recruitment of inflammatory cells into the inflamed tissue. Different cell types express different sets of pattern recognition receptors and expression varies depending on environmental conditions.
Infection mediated replication of RSV in respiratory epithelial cells or APCs results in single stranded (ss) RNA replication with double stranded (ds) RNA as an intermediate product. In the cytoplasm of infected cells viral dsRNA can be recognized by RIG-I. This leads to a signaling cascade that stimulates transcription of type I interferons such as IFN-α and IFN-β via transcription factor Interferon Regulatory Factor (IRF)-3, that have anti-viral effects (30, 31). In addition, TLR3 located in the endosomes of APCs and in epithelial cells interacts with dsRNA. Upon infection of airway epithelial cells with RSV early RIG-I signaling results in IFN-β transcription. IFN-β stimulates TLR3 up-regulation that can be expressed at the cell surface on epithelial cells in addition to the endosomal TLR3 (32). TLR3 signals via the Toll/Interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) thereby activating IRF-3. This pathway activates type I interferon production, cytokine/chemokine production and DC maturation. C57BL/6 TLR3$^{-/-}$ mice studies showed that TLR3 is not required for RSV clearance but plays a role in the maintenance of the inflammatory milieu (33).

TLR7 and TLR9 mainly expressed by pDCs and TLR8 expressed by monocytes/DCs sense viral nucleic acid structures internalized via the endosomal route. However, a role of TLR7, 8 and 9 has not been described in RSV disease (34). TLR4 is expressed by monocytes and epithelial cells and is up-regulated on both cell types upon RSV infection (35, 36). Strong evidence exists that the F protein of human RSV interacts with the TLR4/CD14 complex on the cell surface of human blood derived monocytes (37, 38). Studies in TLR4$^{-/-}$ mice showed impaired viral clearance and innate immune responses in RSV infected TLR4$^{-/-}$ mice compared to wild type mice (37, 39). Subsequent studies in mice described a role for TLR4 in NF-κB activation upon RSV infection. Since UV-inactivated RSV did not induce NF-κB activation viral replication seemed to be required for *in vivo* TLR4 up-regulation in lung epithelial cells and TLR4 signaling (36, 40). TLR2 forms a heterodimeric complex with either TLR1 or TLR6 and is expressed at the cell surface of immune cells (34). Stimulation of the TLR2/-6 complex by RSV promotes innate signaling which results in TNF-α and IL-6 production in mouse models (41).

The viral NS proteins are transcribed upon replication of the virus in the host cell and are not present in the virus particle in significant amounts. These proteins prevent phosphorylation of IRF-3 and translocation to the nucleus which is necessary for the transcription of IFN genes. NS proteins synthesized in RSV infected cells additionally inhibit signal transducer and activator of transcription (STAT)-2 activation upon binding of IFN to interferon α/β receptor (IFNAR) and thereby prevent gene transcription that leads to the cellular anti-viral state (10, 42-44).

The innate signaling and regulation of transcription after viral infection sets the stage for an anti-viral response. This includes chemokine and cytokine production that stimulates immune cell recruitment to the infection site and activation of inflammatory cells.

Chemokine and cytokine production during RSV infection

The inflammatory milieu is created by chemokines and cytokines, produced by the infected cells and influences the type of immune response. In addition, the recruitment of different cell types mediated by the inflammatory milieu is an important component for further specifying the immune response.

Studies on respiratory secretions of RSV infected infants with bronchiolitis showed increased concentrations of IL-8, IL-6, TNF-α and CCL5 (RANTES; regulated upon activation, normal T-cell expressed and presumably secreted) compared to respiratory secretions of healthy, uninfected controls. These cytokines stimulate recruitment of neutrophils and T cells to the respiratory tract (45-49). *In vitro* studies with RSV infected respiratory epithelial cell lines, like A549 cells, showed increased RANTES, MCP-1, MIP-1α and 1β, IL-8 and fractalkine mRNA levels (50, 51). These products are chemotactic for monocytes, basophils, eosinophils and T cells. The G protein of RSV contains a conserved region that mimics the CX3C chemokine motif and structurally mimics fractalkine. The G protein is able to bind to the fractalkine receptor (CX3CR1). Fractalkine as well as the soluble RSV G protein recruit CX3CR1 expressing leukocytes in *in vitro* migration assays and presumably also *in vivo* in mice (52-54).

The adaptive immune response to RSV

The generation of an efficient immune response is controlled by various cellular and soluble components of the innate and acquired immune system which includes T cells and antibodies. Understanding the relative contribution of antibodies and T cells to efficient clearance of RSV is an important aspect for vaccine development. The involvement of T cells and antibodies differ during primary and secondary viral infections. During a primary infection of host cells infection related products activate innate signaling cascades to initiate the immune response. Consequently, inflammatory cells are recruited and antigen presentation to T cells and B cells is stimulated. Maternally derived antibodies acquired by the child via the placenta or via breast milk are present during a primary infection and might provide some protection. These maternal antibody levels wane rapidly in the first months of life (55). The acquired antibody response is elicited after the primary infection and is assumed to contribute to protection during subsequent infections. In the presence of neutralizing antibodies the immune response might be modulated. Neutralization of the virus results in reduction of the viral load which might affect the innate signaling cascade, alter the route of antigen presentation and the level of T cell activation.

Antigen presentation

DCs are professional APCs and play an important role during initiation of innate and acquired immunity (25). They can capture antigens, process the antigen and transport antigenic material to the infected tissue draining lymph nodes. In both humans and mice DCs are a heterogeneous population divided into two main subpopulations, conventional DCs (cDC) and plasmacytoid DCs (pDC) (56-58). Upon antigen processing PRR signaling stimulates up-regulation of co-stimulatory molecules like CD80 and CD86 that facilitate optimal antigen presentation upon arrival of the DC in the lymph nodes (59). After arrival in the lymph nodes, the APCs present antigens to $CD4^+$ and $CD8^+$ T-cells via MHC class II and MHC class I molecules respectively. Different DC subsets are involved in efficient antigen presentation (60). Two cDC subpopulations present in the mouse lung are the sub-epithelial $CD103^+$ DC and the parenchymal $CD11b^{high}$ DC. $CD11b^+$ DC are the main producers of chemokines resulting in cellular recruitment of inflammatory cells into the lungs (61). $CD103^+$ DCs are located directly underneath the epithelial cell layer and express αEβ7 which is the receptor for E-cadherin expressed by epithelial cells. These cells further express the tight junction proteins ZO-2 and claudin-7 which enable the $CD103^+$ DC to sample the airways (62). In addition to the lung derived APCs two cDC subpopulations are present in secondary lymphoid organs in mice, $CD8\alpha^+$ and $CD8\alpha^-$ DCs (63, 64). These lymph node resident DCs are derived from bone marrow precursors in contrast to the lung derived DCs that differentiate from blood monocytes (65). Recently a $CD141^+$ DC population in human peripheral blood and $DNGR\text{-}1^+$ $BDCA3^{high}$ DC population in human spleen have been described which resemble the $CD8\alpha^+$ DC population in mice (66-69). Different DC populations might have different abilities to present antigenic material to T cells. This might depend on

the type of antigenic material e.g. infectious or innocuous, the uptake mechanism and the ability of the DC to process and present antigenic material to T cells (27, 70, 71).

Antigen presentation to $CD4^+$ and $CD8^+$ T cells occurs via MHC class II and MHC class I molecules, respectively. MHC class II molecules are synthesized in the endoplasmic reticulum of APCs (monocytes, macrophages, B cells and DCs and can be up-regulated on epithelia). The newly synthesized MHC class II molecules are associated with an invariant chain of which a specific domain, the CLIP peptide, is present in the peptide binding groove. Association with the invariant chain is required for stable assembly and transport of MHC class II molecules to late endosomal compartments. After endosomal uptake of external antigens HLA-DM catalyzes the exchange of CLIP for an antigenic peptide (72, 73).

MHC class I is expressed by all nucleated cells. Loading of MHC class I is accomplished via different intracellular routes. After infection viral proteins and the viral genome enter the cytosol of the host cell. The viral genome is transcribed and the synthesized proteins enter the cytosol where they can be cleaved by the proteasome (74, 75). This process results in peptides that are actively transported to the endoplasmic reticulum via transporter associated with antigen processing (TAP). MHC class I is located in the endoplasmic reticulum and encounters TAP translocated peptides. In the endoplasmic reticulum the co-localization of MHC class I and TAP is catalyzed and the translocated peptide is deposited into the peptide binding groove of MHC class I molecules (76, 77). This peptide loaded complex is then translocated to the cell surface. In addition to peptides derived from cytoplasmic proteins, professional APCs have the ability to cross-present external antigens onto MHC class I, a process referred to as cross-presentation. Cross-presentation involves MHC class I loading of an antigen internalized via the endosomal route. Two distinct pathways for MHC I loading of exogenous antigens have been described. Via one route the antigen gains access to the cytosol and enters the conventional MHC class I processing pathway. The second route involves internalization of loaded MHC class I molecules from the cellular membrane into the endosomal route where the peptide is replaced by an antigenic peptide processed in the endosomes (78, 79). A different mechanism that mediates cross presentation is trogocytosis (80). This process involves transfer of plasma membrane fragments including the antigen loaded MHC molecules present on infected APCs to the lymph node resident DCs.

In the lymph nodes antigen loaded APCs present antigen to naïve or memory T cells. Tissue derived APCs can present or cross-present antigens to T cells via MHC class II or MHC class I directly. In mice it has been described that during an intranasal influenza virus infection or with innocuous ovalbumin, $CD11b^+$ DCs presented antigen mainly to $CD4^+$ T cells, while $CD103^+$ DCs were important for the induction of $CD8^+$ T cell responses (27, 70). Lymph node resident DCs might also participate in antigen presentation. Tissue derived DCs might deliver MHC class I or MHC class II loaded molecules to lymph node resident DCs via trogocytosis or $CD8\alpha^+$ DCs acquire antigens via uptake of death cell material in the lymph node (56, 81).

In addition to T cell activation in the lymph nodes, it has been described that T cells can be activated locally in the lung in tertiary lymphoid structures described as iBALT (inducible bronchus associated lymphoid tissue) in mice (82, 83).

T cell responses

T cell responses are a crucial component in RSV clearance since immunocompromised (T cell deficient) individuals are not able to clear RSV efficiently (14, 16). $CD8^+$ T cells might directly kill virus infected cells, and by this mechanism eliminate the virus. $CD4^+$ T cells produce cytokines and other molecules to provide $CD8^+$ T cell and B cell help by promoting effector activity (84). Both T cell subsets might contribute to an anti-viral state via IFN-γ production. However, while both $CD4^+$ and $CD8^+$ T cells are involved in viral clearance they may also be implicated in immune pathology via IFN-γ and TNF-α production (85-88). In RSV research it is still a debate what causes damage in lung tissue upon infection. It might be damage caused by viral infection, the innate immune response, anti-viral T cells or combinations. Moreover, the reason for the occurrence of re-infections despite the presence of RSV specific $CD4^+$ and $CD8^+$ T cells is still an important issue for vaccine development.

Lukens *et al.* studied the kinetics of RSV specific $CD8^+$ T cell responses and viral load in children admitted to the intensive care unit with a severe primary RSV infection. The peak of a strong primary $CD8^+$ T cell response measured in peripheral blood was detected at convalescence and not when the children were severely ill, i.e. at the peak or just after the peak of viral load. This indicated that cytotoxic T lymphocytes might not be responsible for the severe lower respiratory tract disease during severe primary infections (89). However, in this study the T cell response was measured in peripheral blood and might not reflect the kinetics of T cell influx in lung tissue. Welliver *et al.* performed lung tissue analysis in autopsy material from RSV infected children and observed a very low number of $CD8^+$ T cells in lung tissue (90). This might support the conclusion by Lukens *et al.* that $CD8^+$ T cells are indeed absent during the peak of disease and thus not responsible for immunopathology but important for recovery.

Re-infection with RSV occurs regularly. The reason for the high re-infection rate even in healthy adults is still not understood. Hall *et al.* showed that re-infection with RSV is correlated with low serum IgG titers at the moment of RSV challenge (91). An additional reason for repeated re-infections could be explained by a low number of RSV specific memory $CD8^+$ T cells in peripheral blood. In humans, RSV specific T cells are readily detectable by assays measuring IFN-γ production by peripheral blood cells of healthy adults, but are mainly $CD4^+$. We and others detected substantial numbers of $CD8^+$ T cells after a primary RSV infection in human peripheral blood (48, 89), however, the frequency of RSV specific $CD8^+$ T cells in the memory pool of healthy adult individuals who experienced multiple RSV infections is low and adequate detection requires T cell expansion (92-96).

Mouse models have been used to study immune responses to RSV in more detail. The role of $CD4^+$ and $CD8^+$ T cells in viral clearance and immune pathology in BALB/c mice was studied by Graham *et al.* (88). Depletion of both $CD4^+$ and $CD8^+$ T cells prior to a primary RSV infection resulted in prolonged viral replication and reduced illness. However, single depletion of $CD8^+$ or $CD4^+$ T cells showed a dominant role for $CD8^+$ T cells in reducing viral load and increasing illness. In addition, infection of naïve mice with RSV, after adoptive transfer of $CD8^+$ T cells from RSV primed mice,

resulted in viral clearance but enhanced lung pathology (86, 97).

An important role for regulatory T cells (Treg) has been described during primary RSV infections in mice. Treg are a subpopulation of $CD4^+$ T cells and are essential in maintaining peripheral tolerance, prevent autoimmune diseases and limit chronic inflammatory disease. In addition, Treg have a suppressive effect on the adaptive immune response (98). Depletion of Treg in mice prior to an RSV infection results in delayed viral clearance with increased weight loss, enhanced morbidity and airway restriction (99). In Treg-depleted mice early recruitment of $CD8^+$ T cells and NK cells was observed with elevated IFN-γ and especially TNF-α secretion by T cells, factors which might contribute to the observed enhanced pathology (87, 99, 100). A balance in effector $CD8^+$ and regulatory $CD4^+$ T cell activation during RSV infection is essential. Liu *et al.* showed that stimulating both effector $CD8^+$ T cells and regulatory $CD4^+$ T cells resulted in diminished RSV induced illness without affecting viral clearance compared to mice in which $CD8^+$ T cells were stimulated alone (101). This study suggested that viral load and tempo of viral clearance did not affect disease severity but indicated that regulatory T cells impacted on the kinetics of innate (NK) and adaptive ($CD8^+$ T cell) responses.

Modulation of the adaptive immune response by RSV

Many viruses have evolved mechanisms to evade an immune response initiated by the host (102, 103). RSV has developed several mechanisms to suppress or subvert the host innate and adaptive immune response. Directly after infection and RSV replication NS proteins are transcribed. NS proteins suppress the innate anti-viral IFN response as described above. In addition, several studies have shown that infection of antigen presenting cells like monocytes and dendritic cells with RSV has suppressive effects on T cell activation (104-108). De Graaff *et al.* showed that proliferation of naïve $CD4^+$ T cells stimulated by RSV infected monocyte derived DC was inhibited by a soluble factor secreted by the infected DC (105). Addition of supernatant from RSV infected DC to IL-1β plus TNF-α matured DCs (MF-DC) decreased the induction of T cell proliferation in these cultures while addition of MF-DC supernatant to RSV infected DCs reversed the suppressive effect on T cell proliferation induced by RSV infected DCs. The soluble factor produced by RSV infected DCs also suppressed the maturation of responding T cells into cytokine producing effector cells. No differences were observed in the maturation status of the DC used in the assay after stimulation with IL-1β plus TNF-α or with RSV. Soluble mediators like type I and type III interferons and IL-1Ra have been identified by some groups as RSV induced inhibitory factors suppressing T cell proliferation, but the role of these components is not supported by other studies. Chi *et al.* reported that blocking both IFNAR2 and IFN-λR1, the receptors for IFN-α and IFN-λ, reduced the RSV mediated suppression of $CD4^+$ T cell proliferation using monocyte derived DCs (104). Preston and colleagues showed that IFN-α itself produced by RSV infected monocytes suppressed T cell proliferation (109) while de Graaff *et al.* did not detect IFN-α in supernatant of RSV infected monocyte derived DCs and found no effects of IFN α/β receptor (IFNAR) blocking (105). Le Nouën *et al.* studied suppression of T cell proliferation using monocyte derived DCs and autologous T cells and did not find a suppressive

effect of RSV infected APCs on $CD4^+$ memory T cell proliferation and cytokine production (110). The reason for all these differences is unclear and warrants further investigation.
Despite all the *in vitro* inhibitory effects on T cell proliferation and differentiation, primary RSV infections induce strong $CD8^+$ T cell responses in infants (89). Therefore, it is unclear how the suppressive mechanisms observed in *in vitro* stimulation assays affect T cell responses *in vivo* and which populations (effector/ memory cells) are affected. It is interesting to speculate that inhibitory effects somehow contribute to low RSV specific $CD8^+$ memory T cell numbers in peripheral blood in healthy adults.
In addition to the possible suppressive soluble factor, it has been shown that the F protein of RSV might play a role in contact mediated inhibition of T cell proliferation (106, 111). A co-culture of a T7/5 cell line expressing the RSV-F protein with peripheral blood leukocytes resulted in the suppression of mitogen-induced proliferation. In contrast, co-cultures of T7/5 cells expressing the G protein did not influence proliferation.

Humoral immune response

For many viruses antibodies are the correlate of protection. For RSV it has been shown that antibodies derived from the mother or acquired via natural infection are protecting when present in high serum titers. However, re-infections occur quite frequently indicating that protection is incomplete.
Antibodies are produced by antigen specific B cells that are activated in secondary lymphoid tissues like the lymph nodes or the spleen. B cell activation can occur in a T cell dependent and independent fashion. During T cell dependent B cell activation, germinal centers are formed in the B cell follicles of secondary lymphoid tissue. In these germinal centers the B cells undergo clonal expansion, class switching and somatic hypermutation to produce high affinity antibodies. B cells that are activated outside the germinal centers produce low affinity antibodies with low magnitude of somatic hypermutation. Naïve mature B cells express cell surface IgM and IgD (B cell receptors, BCR). Upon T cell independent B cell activation IgM recognizes structures present on the antigen which results in antigen specific IgM production by the B cells. Upon T cell dependent B cell activation, the BCR internalizes the bound antigen and presents antigenic epitopes on MHC class II to activate T cells. These activated T cells stimulate B cell activation. Eventually in germinal centers this results in a class switch from IgM or IgD to IgG, IgA and IgE antibodies (112-115). In mice it has been shown that cytokines produced by inflammatory cells determine the class switch to, IgA or IgE, and IgG-isotypes. As a reflection of Th2 cell activity and IL-4 production γ1 and ε transcripts are produced that are translated to IgG1 and IgE, respectively. This type of class switch is stimulated with alum precipitated antigens. In the presence of IFN-γ, produced by NK cells and T cells, switching to γ2a occurs which is a transcript for IgG2a (113, 116-118).
The antibodies produced differ in structure and location in the body. IgA is a dimer and is mainly present at mucosal surfaces like the upper respiratory tract. IgM is a pentamer and is one of the first

antibodies produced after a primary infection. Allergic immune responses are associated with elevated levels of antigen specific IgE. IgG is the main isotype present in peripheral blood and is present as a monomeric structure. Ig's in complex with antigens can interact with different FcReceptors (FcR) at the cell surface of antigen presenting cells which results in activation of effector cell functions like phagocytosis, degranulation, transcription of cytokine genes and release of inflammatory mediators. One common FcR is the FcγReceptor (FcγR) which binds IgG Fc structures (119).
In mammalian species four FcγRs (FcγR I, II, III and IV) are described (120-122). The FcγR family exists of activating FcγRs and one inhibitory FcγR. Mammalian activating FcγRs exist of an alpha chain that associates in most cases with a γ-chain dimer. This γ-chain has a crucial function in the translocation of the receptor complex to the cell surface and it functions as the signaling component of the receptor complex (122, 123). In the human FcγR system there are two exceptions. The human activating FcγRIIA and IIC signal autonomously via a signaling motif present on the α-chain. The signaling motif present on activating FcγR is described as the intracellular immunoreceptor tyrosine-based activation motif (ITAM). Both human and mice have one inhibitory FcγRIIB which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) on the intracellular domain of the α-chain. The murine FcγR family is less complex then the human FcγR system; activating FcγRI, III and IV signal via the ITAM motif on the associated γ-chain (Tabel 1) [reviewed in (121)]. The net result of activating and inhibitory signals upon FcγR ligation determines the final activation status of the FcγR mediated immune response (119, 121-125). Many of the mechanistic studies on the role of FcγRs during immune responses are performed in mouse models and are extrapolated to the human FcγR system. Despite sequence similarity in the extracellular domains and genomic localization of the FcγR, the intracellular domain and cellular expression pattern differ between mice and man. Therefore, extrapolation of observations made in mouse models should be interpreted with caution and cannot directly be translated to the human situation.

Table 1 Human and mouse FcγReceptors.

	Mouse		**Human**	
Activating Fc Receptor	FcγRI	α-chain, γ-chain ITAM	FcγRI	α-chain, γ-chain ITAM
	FcγRIII	α-chain, γ-chain ITAM	FcγRIIA	α-chain, ITAM
			FcγRIIC	α-chain, ITAM
	FcγRIV	α-chain, γ-chain ITAM	FcγRIIIA	α-chain, γ-chain ITAM
			FcγRIIIB	α-GPI
Inhibitory Fc Receptor	FcγRIIB	α-chain, ITIM	FcγRIIB	α-chain, ITIM

FcR expression is different on different hematopoetic cell types. A summary of FcR expression on the different cell types is presented in table 2 (123). Regulation of FcγR expression on these cells is mediated by cytokines. IL-4, IL-10 and TGF-β up-regulate FcγRIIB and IFN-γ, TNF, lipopolysaccharide and complement protein C5a, stimulate up-regulation of the activating FcγR (123).
Different antibody isotypes differ in the binding affinity for FcRs. In humans FcγRI binds IgG1 and IgG3 with high affinity. In mice FcγRI binds IgG2a exclusively with high affinity. The other FcγR, both human and mice, bind antibodies of a broader IgG isotype specificity but with a 100-1000 fold lower affinity (Tabel 2) (123). As a result of the low binding affinity of FcγRs for IgG, monomeric IgG will not saturate these FcγR. Mouse FcγRI is the only FcγR that is able to bind monomeric IgG (120, 121).

Table 2 Murine and human FcγR expression pattern and IgG isotype preference.

Mouse FcγR		**Expression pattern**	**IgG isotype preference**	**affinity**
Activating FcγR	FcγR I	Monocytes, MΦ, DCs	IgG2a	High
	FcγR III	Monocytes, MΦ, DCs, neutrophils, mast cells, NK cells	IgG1, IgG2a, IgG2b	Low
	FcγR IV	Monocytes, MΦ, DCs, neutrophils	IgG2a, IgG2b	Intermediate
Inhibitory FcγR	FcγRIIB	B cells, Monocytes, MΦ, DCs, neutrophils, mast cells	IgG1, IgG2a, IgG2b	Low
Human FcγR		**Expression pattern**	**IgG isotype preference**	**affinity**
Activating FcγR	FcγRI	MΦ, DCs, neutrophils, eosinophils	IgG1/IgG3, IgG4, IgG2	High
	FcγRIIA	MΦ, DCs, neutrophils, eosinophils, mast cells, platelets	IgG1, IgG2/IgG3, IgG4	Low
	FcγRIIIA	MΦ, DCs, NK cells, mast cells, basophils	IgG1/IgG3	Low
	FcγRIIIB	neutrophils	IgG1/IgG3	Low
Inhibitory FcγR	FcγRIIB	MΦ, DCs, neutrophils, eosinophils, mast cells, FDC, B cells	IgG1, IgG2/IgG3, IgG4	Low

A different IgG binding molecule is the neonatal FcR (FcRn). FcRn has structural resemblance with MHC class I and it is endosomally expressed by APCs, endothelial and epithelial cells. The original function of FcRn was described as transporting IgG across epithelial barriers (126, 127) and recycling of internalized albumin and IgG to prevent lysosomal degradation (128, 129).

Qiao *et al.* described a role for FcRn in antigen presentation and stimulation of T cell proliferation when soluble antigens are opsonized by antibodies (130). FcRn directs multimeric IC consisting of large globular proteins opsonized by multiple IgGs to lysosomes for initiation of antigen presentation, while monomeric IgG-antigen complexes are not efficiently processed. Most likely ICs are internalized via FcγR present at the cell surface. Once internalized, the acidic pH in the endosomes induces antibody-FcγR release and stimulates binding of IgG to FcRn. The Fc binding site for FcRn is distinct from FcγR binding sites (131, 132).

During viral infections antibodies can have different effects on the immune response. Neutralizing antibodies lower the viral load and thereby reduce pathogenesis induced by viral infection. By lowering viral load antibodies might also reduce innate immune responses and inflammation or alter innate immune responses by targeting pathogens to intracellular compartments where different TLRs are present compared to the cell surface. Both neutralizing and non-neutralizing antibodies might play a role in the antigen presentation process and determine the level of $CD8^+$ and $CD4^+$ T cell activation. Internalization of IC into the endosomal route might facilitate MHC class II antigen presentation (133, 134). In addition, antigens in complex with antibodies are more efficiently cross-presented via MHC class I compared to soluble antigens (135, 136).
Internalization of IC can also be facilitated by the classical complement system and their receptors (137, 138). C1q is the first component of the classical complement pathway and interacts with multiple immunoglobulin molecules like the pentameric structure of IgM or IgG complexed with an antigen. Bound C1q might interact with the complement receptor, C1qR, present at the cell surface of antigen presenting cells. In addition, C1q can activate the alternative complement pathway by activation of C3 which will further activate the complement pathway (139, 140).

Role of antibodies during RSV infections

Children under the age of 2 months experiencing a primary RSV infection are protected by maternally derived RSV neutralizing antibodies. These antibodies are transferred via the placenta or via breast milk. Although RSV specific antibodies are detectable in infants, the maternal antibody levels decline rapidly after birth (55). In line with this rapid decline of maternal antibodies, the highest incidence of RSV associated bronchiolitis occurs in the first 4-6 months of life (55, 141-144). In adults RSV specific neutralizing antibodies, acquired via natural infection, are present in serum. The titer of neutralizing antibodies in serum is inversely correlated with the risk for RSV re-infection. In addition, about 0.5% to 1.3% of the antibody level in serum crosses the airway epithelium in steady state conditions and post RSV infection in infants, respectively (145). Thus, high titers of RSV specific neutralizing antibodies are required to inhibit a natural RSV infection (18, 91, 142, 144). For this reason studies were performed with palivizumab (Synagis), a monoclonal antibody directed to the fusion protein of RSV (146). Children who received palivizumab prior to a natural RSV infection showed a 55% reduction in RSV related hospitalization compared to children receiving a placebo.

At the moment high risk groups for RSV infection receive palivizumab prophylactically.
RSV infects mainly the upper respiratory tract and only a small percentage of RSV infected individuals will develop a lower respiratory tract infection. The upper respiratory tract is occupied by IgA whereas IgGs are located in the lower respiratory tract where they more easily cross the alveoli. Thus it might be considered that RSV specific IgGs present in serum might not effectively prevent RSV infection in the upper respiratory tract.
Thus, strong neutralizing antibodies contribute to prevention against a severe RSV infection, however serum pharmacokinetics and lung bio-availability of the antibodies need to be taken into account. For this reason higher affinity antibodies are now being developed like motavizumab (147, 148).
Once viral replication occurs antibodies might block further spread and, consequently, block additional production of possible soluble modulators of the innate and adaptive immune response. In addition, antibodies might block or mask the fusion protein present on the cell surface of infected cells. This might affect viral pathogenesis by inhibition of large syncytia formation of infected epithelial cells. In addition, antibodies might affect RSV induced evasion of the adaptive immune response by blocking the possible contact mediated suppression of RSV infected cells on T cell proliferation.
Not only for RSV but also for Murray Valley encephalitis virus (149), dengue virus (150-152), West Nile virus (153, 154), feline infectious peritonitis virus (155) and Ross River virus (156-158) it has been described that (non-)neutralizing antibodies might enhance infection or affect immune responses. Antibodies might enhance the binding of opsonized virus to the FcγR on APCs and thereby stimulate close proximity of the host cell and the virus particle. It might also stimulate enhanced internalization into the host cell via FcγR or complement receptor mediated internalization into intracellular compartments that might facilitate infection. Enhanced infection of opsonized viruses might also occur via decreased innate immunity caused by the neutralization of the virus (159).

RSV vaccine development

A licenced RSV vaccine is not available and the development of a vaccine has been set back due to the dramatic vaccine trial failure in the 1960s with a formalin-inactivated RSV preparation. When FI-RSV vaccinated children experienced a natural respiratory infection with life RSV increased lower respiratory tract infections were observed compared to children vaccinated with a control vaccine (142, 160-162). About 80% of the vaccinated children required hospitalization with two fatal cases among them. Post mortem examination of lung tissue of the diseased children showed strong T cell proliferation with neutrophil and eosinophil influx into the lungs and complement deposition (142, 160, 161, 163). Different animal models were used to study FI-RSV mediated enhanced disease (164-167). Mice vaccinated with FI-RSV developed airway hyper-reactivity, a Th2 type immune response with influx of neutrophils and eosinophils into the lungs and a poor neutralizing antibody response (167-169). $CD4^+$ T cells were shown to play a central role in FI-RSV mediated enhanced disease in which they

outnumbered the $CD8^+$ T cell response (170). Depletion of $CD4^+$ T cells in FI-RSV vaccinated mice prior to RSV challenge showed reduced pulmonary histopathology. This was not observed in RSV primed and challenged mice. Depletion of $CD8^+$ T cells did not drastically affect FI-RSV induced pulmonary histopathology. Upon a primary infection of unvaccinated mice virus specific $CD8^+$ T cells dominate the response in the lungs. Adoptive transfer of RSV primed $CD8^+$ T cells into naïve mice prior to FI-RSV vaccination ameliorated respiratory disease upon RSV challenge (171-173).

An additional important aspect of the FI-RSV vaccine failure is attributed to the fact that antibodies were non-neutralizing, since administration of highly neutralizing antibodies prior to a primary natural RSV infection protects infants and mice from re-infection (141, 146, 174). Arguments for the non-neutralizing character of FI-RSV induced antibodies might be formalin disruption of RSV epitopes which affect inefficient binding of antibodies to the antigen (166, 168, 175, 176) and lack of B cell maturation (177). The absence of TLR stimulation by the inactivated vaccine preparation resulted in lack of B cell maturation and consequently production of low affinity of antibodies. Addition of TLR signals like poly(I:C) (TLR3 agonist) and polyU (TLR8 agonist) during vaccination with FI-RSV resulted in higher affinity antibodies and reduced enhanced respiratory disease (177). Polack *et al.* showed in FI-RSV vaccinated mice that non-neutralizing antibodies caused IC deposition in the lungs upon RSV challenge which was partially responsible for enhanced respiratory illness (163). FI-RSV vaccination of both complement component $C3^{-/-}$ mice and B $cell^{-/-}$ mice showed comparable cellular infiltrate in the lungs compared to wild type mice. However, in both mice respiratory hyper-reactivity was absent while wild type mice suffered from bronchoconstriction. These results indicated that FI-RSV mediated hyper-reactivity was caused by IC deposition depending on both IgG and C3.

Many attempts have been made to design the optimal RSV vaccine that prevents serious lower respiratory tract disease without causing immune pathology. Until now most attempts were unsuccessful because subunit vaccines tended to cause immune pathology while attenuated life vaccine were over attenuated (178). Moreover, an important target group for RSV vaccination are infants <6 months of age. However, these children have a relatively immature immune system and might have maternal antibodies. Maternal antibodies can neutralize RSV which might result in an incomplete priming of the immune response (e.g. lack innate immune response via viral infection, lack of $CD8^+$ T cell activation and dampened B cell response). It is possible that neonates are not capable of somatic hypermutation upon B cell activation in the lymph nodes and therefore lack the development of high affinity RSV specific serum IgG and mucosal IgA antibodies. In children older than two years the difficulty of vaccination is the induction of an immune response in the presence of pre-existing immunity.

The presence of high neutralizing serum antibody levels might not always be favorable since the infant might not develop an anti-viral $CD8^+$ T cell response sufficient to prevent a secondary infection. However, it might be a priority to prevent infection related damage in neonates.

The fact that a natural infection can occur in the presence of neutralizing antibodies and RSV specific T cells is an important issue that needs clarification in the process of vaccine development.

Therefore, it is important to study and understand RSV infection, antigen presentation, T cell activation and the capacity of antibodies to modulate RSV specific immune responses.

Outline of this thesis

Since the discovery of RSV, and especially since the vaccine trial in the 1960s with FI-RSV, many studies have been performed to understand RSV specific immunity. However, it is still not completely understood how RSV modulates the host immune response and the reason for the host's incomplete immune protection is also not clear. Thus a lot of questions still remain in RSV research like: I. what exactly are the correlates of protection against RSV disease? How do different arms of the innate and adaptive immune response against RSV interact? II. How can factors that are responsible for the lack of natural protection similarly affect the efficacy of vaccines? What to learn from the FI-RSV vaccine trial?
In this thesis we answered some aspects of these issues in mouse models and using adult human peripheral blood. We studied antigen presentation of RSV and the cell types involved during RSV infection in the presence or absence of acquired immunity.

RSV re-infects individuals throughout life presumably because of insufficient acquired immunity and the ability of the virus to suppress the initiation and propagation of an immune response. DCs are essential in the initiation of acquired immunity by transporting antigenic material to the draining lymph nodes. However, DC are also know to be involved in directing the inflammatory response in lung tissue as was shown in murine asthma models wherein the lung was exposed to allergens like HDM (179). In **chapter 2** we describe the contribution of different lung DC subsets and lymph node resident DCs on $CD4^+$ and $CD8^+$ T cell activation during a primary RSV infection in C57BL/6 mice. In addition, we studied the kinetics of the DCs migrating out of the lungs to the lung draining lymph nodes upon RSV infection and the re-population of the lungs with DCs after induction of migration. The knowledge on the involvement of different DC subsets during RSV infections on T cell activation might be interesting for vaccine development by targeting antigens to specific DC subsets.

Most of the strategies for vaccine development to prevent RSV infection and the resulting lower respiratory tract infections in high risk groups are focussed on the induction of a good neutralizing antibody response in de vaccinated individuals. Upon RSV infection, high titres of neutralizing antibodies are required to prevent infection of airway epithelial cells and respiratory antigen presenting cells (18, 91, 144, 180). However, after a natural infection these antibodies are not completely protecting and are not preventing secondary immune responses. It is of great importance to understand the consequences of the presence of antibodies during RSV infections. For this reason we studied the role of antibodies and Fcγ receptors in antigen presentation to virus specific T cells during RSV exposure of human peripheral blood mononuclear cells (PBMC) *in vitro* and in mouse models (**chapter 3**).

In addition to the role of FcγRs in RSV immune complex antigen presentation to T cells we studied the role of the neonatal FcR. The function of FcRn was first described as the transporter of IgG across epithelial barriers like lung epithelial cells and the transfer of IgGs across the placenta from the mother to the infant (126, 127, 129). The transfer of IgGs across the airway epithelium towards the lumen where exposure to RSV occurs might be important for protection during secondary RSV infections. In addition, FcRn is known to be involved in antigen presentation of OVA:IgG immune complexes (130). In **chapter 4** we describe studies on the role of FcRn in antigen presentation and transcytosis across airway epithelium *in vitro* and *in vivo* in a mouse $FcRn^{-/-}$ model.

An RSV vaccine is not available and the dramatic failure of the formalin-inactivated RSV vaccine administered to children in the 1960's has set back the development of a safe and efficacious vaccine. The 1960 vaccinees developed severe lower respiratory disease upon natural infection with high T cell proliferation and cellular influx of mononuclear cells, eosinophils and neutrophils into the lungs. It is still not completely understood what the exact mechanism of the failure of protection was, however, an important role for formalin disruption of RSV epitopes (176), the alum adjuvant (181-183), the inefficient priming of an efficient B cell and T cell response by inactivated virus and location of priming have been described (177, 184, 185) in animal studies. In **chapter 5** we describe FI-RSV vaccination experiments in C57BL/6 mice and evaluated the role of different aspects of the vaccine in the induction of the allergic/Th2 type immune response.
High titres of RSV specific antibodies in serum protect infants during a primary infection. These antibodies are obtained via the mother and can be transmitted from the mother to the infant via the placenta or via breast milk. In addition to neutralizing antibodies, breast milk also provides immune modulating components like non-digestable oligosaccharides. Specific non-digestible oligosaccharide supplements are now being developed as immune modulating components in formula. Such oligosaccharide mixtures have been shown to play a role in the disease severity of asthma and reduce antibody levels during atopic diseases by modulation of intestinal and possibly nasopharyngeal microbial flora (186, 187). In **chapter 6** we describe the effects of non-digestible prebiotic oligosaccharides on the modulation of Th2/Th1 balance during primary and secondary (FI-RSV vaccination model) RSV infections in mice.

References

1. Blount, R. E., Jr., J. A. Morris, and R. E. Savage. 1956. Recovery of cytopathogenic agent from chimpanzees with coryza. Proc. Soc. Exp. Biol. Med. 92: 544-549.
2. Chanock, R., B. Roizman, and R. Myers. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. Am. J. Hyg. 66: 281-290.
3. Levine, S., R. Klaiber-Franco, and P. R. Paradiso. 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J. Gen. Virol. 68 (Pt 9): 2521-2524.
4. Collins, P. L., and G. Mottet. 1991. Post-translational processing and oligomerization of the fusion glycoprotein of human respiratory syncytial virus. J. Gen. Virol. 72 (Pt 12): 3095-3101.
5. Techaarpornkul, S., N. Barretto, and M. E. Peeples. 2001. Functional analysis of recombinant respiratory syncytial virus deletion mutants lacking the small hydrophobic and/or attachment glycoprotein gene. J. Virol. 75: 6825-6834.
6. Fuentes, S., K. C. Tran, P. Luthra, M. N. Teng, and B. He. 2007. Function of the respiratory syncytial virus small hydrophobic protein. J. Virol. 81: 8361-8366.
7. Gan, S. W., L. Ng, X. Lin, X. Gong, and J. Torres. 2008. Structure and ion channel activity of the human respiratory syncytial virus (hRSV) small hydrophobic protein transmembrane domain. Protein Sci. 17: 813-820.
8. Cowton, V. M., D. R. McGivern, and R. Fearns. 2006. Unraveling the complexities of respiratory syncytial virus RNA synthesis. J. Gen. Virol. 87: 1805-1821.
9. Barik, S. 1992. Transcription of human respiratory syncytial virus genome RNA *in vitro*: requirement of cellular factor(s). J. Virol. 66: 6813-6818.
10. Ramaswamy, M., L. Shi, S. M. Varga, S. Barik, M. A. Behlke, and D. C. Look. 2006. Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. Virology 344: 328-339.
11. Spann, K. M., K. C. Tran, and P. L. Collins. 2005. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J. Virol. 79: 5353-5362.
12. Bitko, V., O. Shulyayeva, B. Mazumder, A. Musiyenko, M. Ramaswamy, D. C. Look, and S. Barik. 2007. Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and facilitate virus growth. J. Virol. 81: 1786-1795.
13. Falsey, A. R., P. A. Hennessey, M. A. Formica, C. Cox, and E. E. Walsh. 2005. Respiratory syncytial virus infection in elderly and high-risk adults. N. Engl. J. Med. 352: 1749-1759.
14. Fishaut, M., D. Tubergen, and K. McIntosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96: 179-186.
15. Glezen, P., and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288: 498-505.
16. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315: 77-81.
17. 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.
18. 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.
19. Stensballe, L. G., J. B. Simonsen, S. F. Thomsen, A. M. Larsen, S. H. Lysdal, P. Aaby, K. O. Kyvik, A. Skytthe, V. Backer, and H. Bisgaard. 2009. The causal direction in the association between respiratory syncytial virus hospitalization and asthma. J. Allergy Clin. Immunol. 123: 131-137.
20. Kusel, M. M., N. H. de Klerk, T. Kebadze, V. Vohma, P. G. Holt, S. L. Johnston, and P. D. Sly. 2007. Early-life respiratory

viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J. Allergy Clin. Immunol. 119: 1105-1110.

21. Mohapatra, S. S., and S. Boyapalle. 2008. Epidemiologic, experimental, and clinical links between respiratory syncytial virus infection and asthma. Clin. Microbiol. Rev. 21: 495-504.
22. Bruhn, F. W., and A. S. Yeager. 1977. Respiratory syncytial virus in early infancy. Circulating antibody and the severity of infection. Am. J. Dis. Child 131: 145-148.
23. Lamprecht, C. L., H. E. Krause, and M. A. Mufson. 1976. Role of maternal antibody in pneumonia and bronchiolitis due to respiratory syncytial virus. J. Infect. Dis. 134: 211-217.
24. Ogilvie, M. M., A. S. Vathenen, M. Radford, J. Codd, and S. Key. 1981. Maternal antibody and respiratory syncytial virus infection in infancy. J. Med. Virol. 7: 263-271.
25. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392: 245-252.
26. Beyer, M., H. Bartz, K. Horner, S. Doths, C. Koerner-Rettberg, and J. Schwarze. 2004. Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. J. Allergy Clin. Immunol. 113: 127-133.
27. del Rio, M. L., J. I. Rodriguez-Barbosa, E. Kremmer, and R. Forster. 2007. $CD103^-$ and $CD103^+$ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to $CD4^+$ and $CD8^+$ T cells. J. Immunol. 178: 6861-6866.
28. von Garnier, C., L. Filgueira, M. Wikstrom, M. Smith, J. A. Thomas, D. H. Strickland, P. G. Holt, and P. A. Stumbles. 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. J. Immunol. 175: 1609-1618.
29. Takeuchi, O., and S. Akira. 2007. Recognition of viruses by innate immunity. Immunol. Rev. 220: 214-224.
30. Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5: 730-737.
31. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity. 23: 19-28.
32. Liu, P., M. Jamaluddin, K. Li, R. P. Garofalo, A. Casola, and A. R. Brasier. 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. J. Virol. 81: 1401-1411.
33. Rudd, B. D., J. J. Smit, R. A. Flavell, L. Alexopoulou, M. A. Schaller, A. Gruber, A. A. Berlin, and N. W. Lukacs. 2006. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. J. Immunol. 176: 1937-1942.
34. Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194: 863-869.
35. Gagro, A., M. Tominac, V. Krsulovic-Hresic, A. Bace, M. Matic, V. Drazenovic, G. Mlinaric-Galinovic, E. Kosor, K. Gotovac, I. Bolanca, S. Batinica, and S. Rabatic. 2004. Increased Toll-like receptor 4 expression in infants with respiratory syncytial virus bronchiolitis. Clin. Exp. Immunol. 135: 267-272.
36. Monick, M. M., T. O. Yarovinsky, L. S. Powers, N. S. Butler, A. B. Carter, G. Gudmundsson, and G. W. Hunninghake. 2003. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J. Biol. Chem. 278: 53035-53044.
37. Kurt-Jones, E. A., L. Popova, L. Kwinn, L. M. Haynes, L. P. Jones, R. A. Tripp, E. E. Walsh, M. W. Freeman, D. T. Golenbock, L. J. Anderson, and R. W. Finberg. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. 1: 398-401.
38. Lizundia, R., K. S. Sauter, G. Taylor, and D. Werling. 2008. Host species-specific usage of the TLR4-LPS receptor complex. Innate. Immun. 14: 223-231.
39. Haynes, L. M., D. D. Moore, E. A. Kurt-Jones, R. W. Finberg, L. J. Anderson, and R. A. Tripp. 2001. Involvement of toll-

like receptor 4 in innate immunity to respiratory syncytial virus. J. Virol. 75: 10730-10737.

40. Haeberle, H. A., R. Takizawa, A. Casola, A. R. Brasier, H. J. Dieterich, N. Van Rooijen, Z. Gatalica, and R. P. Garofalo. 2002. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. J. Infect. Dis. 186: 1199-1206.
41. Murawski, M. R., G. N. Bowen, A. M. Cerny, L. J. Anderson, L. M. Haynes, R. A. Tripp, E. A. Kurt-Jones, and R. W. Finberg. 2009. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J. Virol. 83: 1492-1500.
42. Lo, M. S., R. M. Brazas, and M. J. Holtzman. 2005. Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. J. Virol. 79: 9315-9319.
43. Swedan, S., A. Musiyenko, and S. Barik. 2009. Respiratory syncytial virus nonstructural proteins decrease levels of multiple members of the cellular interferon pathways. J. Virol. 83: 9682-9693.
44. Ling, Z., K. C. Tran, and M. N. Teng. 2009. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. J. Virol. 83: 3734-3742.
45. Gill, M. A., K. Long, T. Kwon, L. Muniz, A. Mejias, J. Connolly, L. Roy, J. Banchereau, and O. Ramilo. 2008. Differential recruitment of dendritic cells and monocytes to respiratory mucosal sites in children with influenza virus or respiratory syncytial virus infection. J. Infect. Dis. 198: 1667-1676.
46. bu-Harb, M., F. Bell, A. Finn, W. H. Rao, L. Nixon, D. Shale, and M. L. Everard. 1999. IL-8 and neutrophil elastase levels in the respiratory tract of infants with RSV bronchiolitis. Eur. Respir. J. 14: 139-143.
47. Everard, M. L., A. Swarbrick, M. Wrightham, J. McIntyre, C. Dunkley, P. D. James, H. F. Sewell, and A. D. Milner. 1994. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. Arch. Dis. Child 71: 428-432.
48. Heidema, J., M. V. Lukens, W. W. van Maren, M. E. van Dijk, H. G. Otten, A. J. van Vught, D. B. van der Werff, S. J. van Gestel, M. G. Semple, R. L. Smyth, J. L. Kimpen, and G. M. van Bleek. 2007. $CD8^+$ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. J. Immunol. 179: 8410-8417.
49. Sheeran, P., H. Jafri, C. Carubelli, J. Saavedra, C. Johnson, K. Krisher, P. J. Sanchez, and O. Ramilo. 1999. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr. Infect. Dis. J. 18: 115-122.
50. Zhang, Y., B. A. Luxon, A. Casola, R. P. Garofalo, M. Jamaluddin, and A. R. Brasier. 2001. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. J. Virol. 75: 9044-9058.
51. Olszewska-Pazdrak, B., A. Casola, T. Saito, R. Alam, S. E. Crowe, F. Mei, P. L. Ogra, and R. P. Garofalo. 1998. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. J. Virol. 72: 4756-4764.
52. Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson. 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat. Immunol. 2: 732-738.
53. Harcourt, J. L., R. A. Karron, and R. A. Tripp. 2004. Anti-G protein antibody responses to respiratory syncytial virus infection or vaccination are associated with inhibition of G protein CX3C-CX3CR1 binding and leukocyte chemotaxis. J. Infect. Dis. 190: 1936-1940.
54. Harcourt, J., R. Alvarez, L. P. Jones, C. Henderson, L. J. Anderson, and R. A. Tripp. 2006. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect $CX3CR1^+$ T cell responses. J. Immunol. 176: 1600-1608.
55. Brandenburg, A. H., J. Groen, H. A. van Steensel-Moll, E. C. Claas, P. H. Rothbarth, H. J. Neijens, and A. D. Osterhaus. 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.
56. Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular

antigens. Immunol. Rev. 199: 9-26.
57. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets *in vivo*. Nat. Rev. Immunol. 7: 543-555.
58. Merad, M., and M. G. Manz. 2009. Dendritic cell homeostasis. Blood 113: 3418-3427.
59. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. 14: 233-258.
60. Schuurhuis, D. H., N. Fu, F. Ossendorp, and C. J. Melief. 2006. Ins and outs of dendritic cells. Int. Arch. Allergy Immunol. 140: 53-72.
61. Beaty, S. R., C. E. Rose, Jr., and S. S. Sung. 2007. Diverse and potent chemokine production by lung $CD11b^{high}$ dendritic cells in homeostasis and in allergic lung inflammation. J. Immunol. 178: 1882-1895.
62. 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.
63. Vremec, D., M. Zorbas, R. Scollay, D. J. Saunders, C. F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J. Exp. Med. 176: 47-58.
64. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J. Immunol. 164: 2978-2986.
65. Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. Nat. Rev. Immunol. 7: 19-30.
66. Bachem, A., S. Guttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, A. Salama, K. Movassaghi, C. Opitz, H. W. Mages, V. Henn, P. M. Kloetzel, S. Gurka, and R. A. Kroczek. 2010. Superior antigen cross-presentation and XCR1 expression define human $CD11c^{+}CD141^{+}$ cells as homologues of mouse $CD8^{+}$ dendritic cells. J. Exp. Med. 207: 1273-1281.
67. Crozat, K., R. Guiton, V. Contreras, V. Feuillet, C. A. Dutertre, E. Ventre, T. P. Vu Manh, T. Baranek, A. K. Storset, J. Marvel, P. Boudinot, A. Hosmalin, I. Schwartz-Cornil, and M. Dalod. 2010. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse $CD8alpha^{+}$ dendritic cells. J. Exp. Med. 207: 1283-1292.
68. Jongbloed, S. L., A. J. Kassianos, K. J. McDonald, G. J. Clark, X. Ju, C. E. Angel, C. J. Chen, P. R. Dunbar, R. B. Wadley, V. Jeet, A. J. Vulink, D. N. Hart, and K. J. Radford. 2010. Human $CD141^{+}$ $(BDCA-3)^{+}$ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J. Exp. Med. 207: 1247-1260.
69. Poulin, L. F., M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J. L. Chen, A. M. Keller, O. Joffre, S. Zelenay, E. Nye, A. Le Moine, F. Faure, V. Donckier, D. Sancho, V. Cerundolo, D. Bonnet, and C. Reis e Sousa. 2010. Characterization of human $DNGR-1^{+}$ $BDCA3^{+}$ leukocytes as putative equivalents of mouse $CD8alpha^{+}$ dendritic cells. J. Exp. Med. 207: 1261-1271.
70. Kim, T. S., and T. J. Braciale. 2009. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic $CD8^{+}$ T cell responses. PLoS. One. 4: e4204.
71. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by $CD8^{+}$ dendritic cells. J. Immunol. 166: 5327-5330.
72. van Niel, G., R. Wubbolts, and W. Stoorvogel. 2008. Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. Curr. Opin. Cell Biol. 20: 437-444.
73. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nat. Immunol. 5: 685-692.
74. Kloetzel, P. M. 2004. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. Nat. Immunol. 5: 661-669.

75. Rock, K. L., I. A. York, and A. L. Goldberg. 2004. Post-proteasomal antigen processing for major histocompatibility complex class I presentation. Nat. Immunol. 5: 670-677.
76. Cresswell, P., A. L. Ackerman, A. Giodini, D. R. Peaper, and P. A. Wearsch. 2005. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol. Rev. 207: 145-157.
77. Elliott, T., and A. Williams. 2005. The optimization of peptide cargo bound to MHC class I molecules by the peptide-loading complex. Immunol. Rev. 207: 89-99.
78. Gromme, M., and J. Neefjes. 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol. Immunol. 39: 181-202.
79. Jensen, P. E. 2007. Recent advances in antigen processing and presentation. Nat. Immunol. 8: 1041-1048.
80. Joly, E., and D. Hudrisier. 2003. What is trogocytosis and what is its purpose? Nat. Immunol. 4: 815.
81. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells *in vivo*. J. Exp. Med. 192: 1685-1696.
82. Halle, S., H. C. Dujardin, N. Bakocevic, H. Fleige, H. Danzer, S. Willenzon, Y. Suezer, G. Hammerling, N. Garbi, G. Sutter, T. Worbs, and R. Forster. 2009. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. J. Exp. Med. 206: 2593-2601.
83. Sminia, T., van der Brugge-Gamelkoorn GJ, and S. H. Jeurissen. 1989. Structure and function of bronchus-associated lymphoid tissue (BALT). Crit Rev. Immunol. 9: 119-150.
84. Doherty, P. C., D. J. Topham, R. A. Tripp, R. D. Cardin, J. W. Brooks, and P. G. Stevenson. 1997. Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. Immunol. Rev. 159: 105-117.
85. Graham, B. S., T. R. Johnson, and R. S. Peebles. 2000. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology 48: 237-247.
86. Cannon, M. J., P. J. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168: 1163-1168.
87. 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.
88. Graham, B. S., L. A. Bunton, P. F. Wright, and D. T. Karzon. 1991. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J. Clin. Invest 88: 1026-1033.
89. Lukens, M. V., A. C. van de Pol, F. E. Coenjaerts, N. J. Jansen, V. M. Kamp, J. L. Kimpen, J. W. Rossen, L. H. Ulfman, C. E. Tacke, M. C. Viveen, L. Koenderman, T. F. Wolfs, and G. M. van Bleek. 2010. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. J. Virol. 84: 2374-2383.
90. Welliver, T. P., R. P. Garofalo, Y. Hosakote, K. H. Hintz, L. Avendano, K. Sanchez, L. Velozo, H. Jafri, S. Chavez-Bueno, P. L. Ogra, L. McKinney, J. L. Reed, and R. C. Welliver, Sr. 2007. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. J. Infect. Dis. 195: 1126-1136.
91. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163: 693-698.
92. Brandenburg, A. H., L. de Waal, H. H. Timmerman, P. Hoogerhout, R. L. de Swart, and A. D. Osterhaus. 2000. HLA class I-restricted cytotoxic T-cell epitopes of the respiratory syncytial virus fusion protein. J. Virol. 74: 10240-10244.
93. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66: 2102-2110.
94. de Waal, L., Y. Suzer, L. S. Wyatt, K. Sintnicolaas, G. Sutter, B. Moss, A. D. Osterhaus, and R. L. de Swart. 2006. T cell responses to respiratory syncytial virus fusion and attachment proteins in human peripheral blood mononuclear cells. Viral Immunol. 19: 669-678.

95. Isaacs, D., C. R. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 2: 769-771.
96. Mbawuike, I. N., J. Wells, R. Byrd, S. G. Cron, W. P. Glezen, and P. A. Piedra. 2001. HLA-restricted $CD8^+$ cytotoxic T lymphocyte, interferon-gamma, and interleukin-4 responses to respiratory syncytial virus infection in infants and children. J. Infect. Dis. 183: 687-696.
97. Cannon, M. J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology 62: 133-138.
98. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. Nat. Rev. Immunol. 8: 523-532.
99. 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.
100. 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.
101. Liu, J., T. J. Ruckwardt, M. Chen, J. D. Nicewonger, T. R. Johnson, and B. S. Graham. 2010. Epitope-specific regulatory CD4 T cells reduce virus-induced illness while preserving CD8 T-cell effector function at the site of infection. J. Virol. 84: 10501-10509.
102. Vossen, M. T., E. M. Westerhout, C. Soderberg-Naucler, and E. J. Wiertz. 2002. Viral immune evasion: a masterpiece of evolution. Immunogenetics 54: 527-542.
103. Katze, M. G., Y. He, and M. Gale, Jr. 2002. Viruses and interferon: a fight for supremacy. Nat. Rev. Immunol. 2: 675-687.
104. Chi, B., H. L. Dickensheets, K. M. Spann, M. A. Alston, C. Luongo, L. Dumoutier, J. Huang, J. C. Renauld, S. V. Kotenko, M. Roederer, J. A. Beeler, R. P. Donnelly, P. L. Collins, and R. L. Rabin. 2006. Alpha and lambda interferon together mediate suppression of CD4 T cells induced by respiratory syncytial virus. J. Virol. 80: 5032-5040.
105. de Graaff, P. M., E. C. de Jong, T. M. van Capel, M. E. van Dijk, P. J. Roholl, J. Boes, W. Luytjes, J. L. Kimpen, and G. M. van Bleek. 2005. Respiratory syncytial virus infection of monocyte-derived dendritic cells decreases their capacity to activate CD4 T cells. J. Immunol. 175: 5904-5911.
106. Gonzalez, P. A., C. E. Prado, E. D. Leiva, L. J. Carreno, S. M. Bueno, C. A. Riedel, and A. M. Kalergis. 2008. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proc. Natl. Acad. Sci. U. S. A 105: 14999-15004.
107. Rothoeft, T., K. Fischer, S. Zawatzki, V. Schulz, U. Schauer, and C. Korner Rettberg. 2007. Differential response of human naive and memory/effector T cells to dendritic cells infected by respiratory syncytial virus. Clin. Exp. Immunol. 150: 263-273.
108. Guerrero-Plata, A., A. Casola, G. Suarez, X. Yu, L. Spetch, M. E. Peeples, and R. P. Garofalo. 2006. Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. Am. J. Respir. Cell Mol. Biol. 34: 320-329.
109. Preston, F. M., P. L. Beier, and J. H. Pope. 1995. Identification of the respiratory syncytial virus-induced immunosuppressive factor produced by human peripheral blood mononuclear cells *in vitro* as interferon-alpha. J. Infect. Dis. 172: 919-926.
110. Le Nouen, C., P. Hillyer, S. Munir, C. C. Winter, T. McCarty, A. Bukreyev, P. L. Collins, R. L. Rabin, and U. J. Buchholz. 2010. Effects of Human Respiratory Syncytial Virus, Metapneumovirus, Parainfluenza Virus 3 and Influenza Virus on $CD4^+$ T Cell Activation by Dendritic Cells. PLoS. One. 5: e15017.
111. Schlender, J., G. Walliser, J. Fricke, and K. K. Conzelmann. 2002. Respiratory syncytial virus fusion protein mediates inhibition of mitogen-induced T-cell proliferation by contact. J. Virol. 76: 1163-1170.
112. MacLennan, I. C. 1994. Germinal centers. Annu. Rev. Immunol. 12: 117-139.
113. MacLennan, I. C., K. M. Toellner, A. F. Cunningham, K. Serre, D. M. Sze, E. Zuniga, M. C. Cook, and C. G. Vinuesa. 2003.

Extrafollicular antibody responses. Immunol. Rev. 194: 8-18.
114. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. Nature 381: 751-758.
115. Cyster, J. G. 2010. B cell follicles and antigen encounters of the third kind. Nat. Immunol. 11: 989-996.
116. Snapper, C. M., and W. E. Paul. 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236: 944-947.
117. Vitetta, E. S., J. Ohara, C. D. Myers, J. E. Layton, P. H. Krammer, and W. E. Paul. 1985. Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. J. Exp. Med. 162: 1726-1731.
118. Sideras, P., S. Bergstedt-Lindqvist, and E. Severinson. 1985. Partial biochemical characterization of IgG1-inducing factor. Eur. J. Immunol. 15: 593-598.
119. Ravetch, J. V., and S. Bolland. 2001. IgG Fc receptors. Annu. Rev. Immunol. 19: 275-290.
120. Nimmerjahn, F., P. Bruhns, K. Horiuchi, and J. V. Ravetch. 2005. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity. 23: 41-51.
121. Nimmerjahn, F., and J. V. Ravetch. 2006. Fcgamma receptors: old friends and new family members. Immunity. 24: 19-28.
122. Ravetch, J. V. 1994. Fc receptors: rubor redux. Cell 78: 553-560.
123. Ravetch, J. V. 2003. In Fundamental Immunology, 5th ed. W. E. Paul, eds. Lippincott-Raven, Philadelphia, p. 685-700.
124. Daeron, M. 1997. Fc receptor biology. Annu. Rev. Immunol. 15: 203-234.
125. Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. Nature 383: 263-266.
126. Yoshida, M., S. M. Claypool, J. S. Wagner, E. Mizoguchi, A. Mizoguchi, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2004. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity. 20: 769-783.
127. Israel, E. J., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. J. Immunol. 154: 6246-6251.
128. Chaudhury, C., S. Mehnaz, J. M. Robinson, W. L. Hayton, D. K. Pearl, D. C. Roopenian, and C. L. Anderson. 2003. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J. Exp. Med. 197: 315-322.
129. Junghans, R. P., and C. L. Anderson. 1996. The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor. Proc. Natl. Acad. Sci. U. S. A 93: 5512-5516.
130. Qiao, S. W., K. Kobayashi, F. E. Johansen, L. M. Sollid, J. T. Andersen, E. Milford, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2008. Dependence of antibody-mediated presentation of antigen on FcRn. Proc. Natl. Acad. Sci. U. S. A 105: 9337-9342.
131. Shields, R. L., A. K. Namenuk, K. Hong, Y. G. Meng, J. Rae, J. Briggs, D. Xie, J. Lai, A. Stadlen, B. Li, J. A. Fox, and L. G. Presta. 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J. Biol. Chem. 276: 6591-6604.
132. Wines, B. D., M. S. Powell, P. W. Parren, N. Barnes, and P. M. Hogarth. 2000. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. J. Immunol. 164: 5313-5318.
133. de Jong, J. M., D. H. Schuurhuis, A. Ioan-Facsinay, M. M. Welling, M. G. Camps, E. I. van der Voort, T. W. Huizinga, F. Ossendorp, J. S. Verbeek, and R. E. Toes. 2006. Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4 T cells upon immune-complex uptake *in vivo*. Immunology 119: 499-506.

134. Hamano, Y., H. Arase, H. Saisho, and T. Saito. 2000. Immune complex and Fc receptor-mediated augmentation of antigen presentation for *in vivo* Th cell responses. J. Immunol. 164: 6113-6119.
135. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 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.
136. Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J. Immunol. 168: 2240-2246.
137. Chen, Y., Y. B. Park, E. Patel, and G. J. Silverman. 2009. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. J. Immunol. 182: 6031-6043.
138. van Montfoort, N., J. M. de Jong, D. H. Schuurhuis, E. I. van der Voort, M. G. Camps, T. W. Huizinga, C. van Kooten, M. R. Daha, J. S. Verbeek, F. Ossendorp, and R. E. Toes. 2007. A novel role of complement factor C1q in augmenting the presentation of antigen captured in immune complexes to $CD8^+$ T lymphocytes. J. Immunol. 178: 7581-7586.
139. Lu, J. H., B. K. Teh, L. Wang, Y. N. Wang, Y. S. Tan, M. C. Lai, and K. B. Reid. 2008. The classical and regulatory functions of C1q in immunity and autoimmunity. Cell Mol. Immunol. 5: 9-21.
140. Reid, K. B. 1986. Activation and control of the complement system. Essays Biochem. 22: 27-68.
141. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J. Virol. 55: 517-520.
142. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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: 405-421.
143. Englund, J. A. 1994. Passive protection against respiratory syncytial virus disease in infants: the role of maternal antibody. Pediatr. Infect. Dis. J. 13: 449-453.
144. Piedra, P. A., A. M. Jewell, S. G. Cron, R. L. Atmar, and W. P. Glezen. 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.
145. Murphy, B. R., B. S. Graham, G. A. Prince, E. E. Walsh, R. M. Chanock, D. T. Karzon, and P. F. Wright. 1986. Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. J. Clin. Microbiol. 23: 1009-1014.
146. The IMpact-RSV Study Group. 1998. Palivizumab, a Humanized Respiratory Syncytial Virus Monoclonal Antibody, Reduces Hospitalization From Respiratory Syncytial Virus Infection in High-risk Infants. Pediatrics 102: 531-537.
147. Wu, H., D. S. Pfarr, S. Johnson, Y. A. Brewah, R. M. Woods, N. K. Patel, W. I. White, J. F. Young, and P. A. Kiener. 2007. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. J. Mol. Biol. 368: 652-665.
148. Kwakkenbos, M. J., S. A. Diehl, E. Yasuda, A. Q. Bakker, C. M. van Geelen, M. V. Lukens, G. M. van Bleek, M. N. Widjojoatmodjo, W. M. Bogers, H. Mei, A. Radbruch, F. A. Scheeren, H. Spits, and T. Beaumont. 2010. Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nat. Med. 16: 123-128.
149. Hawkes, R. A. 1964. Enhancement of the infectivity of arboviruses by specific antisera produced in domestic fowls. Aust. J. Exp. Biol. Med. Sci. 42: 465-482.
150. Halstead, S. B., and E. J. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146: 201-217.
151. Halstead, S. B., and E. J. O'Rourke. 1977. Antibody-enhanced dengue virus infection in primate leukocytes. Nature 265: 739-741.
152. Halstead, S. B., J. S. Chow, and N. J. Marchette. 1973. Immunological enhancement of dengue virus replication.

Nat. New Biol. 243: 24-26.
153. Gollins, S. W., and J. S. Porterfield. 1984. Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. J. Gen. Virol. 65 (Pt 8): 1261-1272.
154. Gollins, S. W., and J. S. Porterfield. 1985. Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. J. Gen. Virol. 66 (Pt 9): 1969-1982.
155. Olsen, C., and F. Scott. 1993. Evaluation of antibody-dependent enhancement of feline infectious peritonitis virus infectivity using in situ hybridization. Microb. Pathog. 14: 275-285.
156. Suhrbier, A., and M. La Linn. 2003. Suppression of antiviral responses by antibody-dependent enhancement of macrophage infection. Trends Immunol. 24: 165-168.
157. Linn, M. L., J. G. Aaskov, and A. Suhrbier. 1996. Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with arthritis. J. Gen. Virol. 77 (Pt 3): 407-411.
158. Lidbury, B. A., and S. Mahalingam. 2000. Specific ablation of antiviral gene expression in macrophages by antibody-dependent enhancement of Ross River virus infection. J. Virol. 74: 8376-8381.
159. Ubol, S., and S. B. Halstead. 2010. How innate immune mechanisms contribute to antibody-enhanced viral infections. Clin. Vaccine Immunol. 17: 1829-1835.
160. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89: 422-434.
161. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. 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: 435-448.
162. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89: 449-463.
163. Polack, F. P., M. N. Teng, P. L. Collins, G. A. Prince, M. Exner, H. Regele, D. D. Lirman, R. Rabold, S. J. Hoffman, C. L. Karp, S. R. Kleeberger, M. Wills-Karp, and R. A. Karron. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. J. Exp. Med. 196: 859-865.
164. de Swart, R. L., T. Kuiken, H. H. Timmerman, G. van Amerongen, 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.
165. Prince, G. A., A. B. Jenson, V. G. Hemming, B. R. Murphy, E. E. Walsh, R. L. Horswood, and R. M. Chanock. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. J. Virol. 57: 721-728.
166. Piedra, P. A., H. S. Faden, G. Camussi, D. T. Wong, and P. L. Ogra. 1989. Mechanism of lung injury in cotton rats immunized with formalin-inactivated respiratory syncytial virus. Vaccine 7: 34-38.
167. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. 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: 2852-2860.
168. Openshaw, P. J., F. J. Culley, and W. Olszewska. 2001. Immunopathogenesis of vaccine-enhanced RSV disease. Vaccine 20 Suppl 1: S27-S31.
169. Murphy, B. R., and E. E. Walsh. 1988. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. J. Clin. Microbiol. 26: 1595-1597.
170. Connors, M., A. B. Kulkarni, C. Y. Firestone, K. L. Holmes, H. C. Morse, III, A. V. Sotnikov, and B. R. Murphy. 1992. 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: 7444-7451.

171. Olson, M. R., and S. M. Varga. 2007. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. J. Immunol. 179: 5415-5424.
172. 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.
173. Stevens, W. W., J. Sun, J. P. Castillo, and T. J. Braciale. 2009. Pulmonary eosinophilia is attenuated by early responding CD8(+) memory T cells in a murine model of RSV vaccine-enhanced disease. Viral Immunol. 22: 243-251.
174. Groothuis, J. R., E. A. Simoes, M. J. Levin, C. B. Hall, C. E. Long, W. J. Rodriguez, J. Arrobio, H. C. Meissner, D. R. Fulton, R. C. Welliver, and *et al.* 1993. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. N. Engl. J. Med. 329: 1524-1530.
175. Boelen, A., A. Andeweg, J. Kwakkel, W. Lokhorst, T. Bestebroer, J. Dormans, and T. Kimman. 2000. 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: 982-991.
176. Moghaddam, A., W. Olszewska, B. Wang, J. S. Tregoning, R. Helson, Q. J. Sattentau, and P. J. Openshaw. 2006. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat. Med. 12: 905-907.
177. Delgado, M. F., S. Coviello, A. C. Monsalvo, G. A. Melendi, J. Z. Hernandez, J. P. Batalle, L. Diaz, A. Trento, H. Y. Chang, W. Mitzner, J. Ravetch, J. A. Melero, P. M. Irusta, and F. P. Polack. 2009. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat. Med. 15: 34-41.
178. Graham, B. S. 2011. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol. Rev. 239: 149-166.
179. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J. Exp. Med. 207: 2097-2111.
180. Midulla, F., Y. T. Huang, I. A. Gilbert, N. M. Cirino, E. R. McFadden, Jr., and J. R. Panuska. 1989. Respiratory syncytial virus infection of human cord and adult blood monocytes and alveolar macrophages. Am. Rev. Respir. Dis. 140: 771-777.
181. Kool, M., T. Soullie, M. van Nimwegen, M. A. Willart, F. Muskens, S. Jung, H. C. Hoogsteden, H. Hammad, and B. N. Lambrecht. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205: 869-882.
182. Neuzil, K. M., J. E. Johnson, Y. W. Tang, J. P. Prieels, M. Slaoui, N. Gar, and B. S. Graham. 1997. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. Vaccine 15: 525-532.
183. Sokolovska, A., S. L. Hem, and H. HogenEsch. 2007. Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants. Vaccine 25: 4575-4585.
184. Culley, F. J., A. M. Pennycook, J. S. Tregoning, T. Hussell, and P. J. Openshaw. 2006. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. J. Virol. 80: 4521-4527.
185. Openshaw, P. J., S. L. Clarke, and F. M. Record. 1992. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. Int. Immunol. 4: 493-500.
186. Macfarlane, G. T., H. Steed, and S. Macfarlane. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J. Appl. Microbiol. 104: 305-344.
187. 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.

chap

ter 2

Respiratory Syncytial Virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node.

Michaël V. Lukens[1], Debby Kruijsen[1], Frank E.J. Coenjaerts[2], Jan L.L. Kimpen[1] and Grada M. van Bleek[1]

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

Journal of Virology (2009) 83: 7235-7243

Abstract

In the respiratory tract, different dendritic cell (DC) populations guard a tight balance between tolerance and immunity to infectious or harmless materials to which the airways are continuously exposed. For infectious and noninfectious antigens administered via different routes, different subsets of DC might contribute during the induction of T cell tolerance and immunity. We studied the impact of primary Respiratory Syncytial Virus (RSV) infection on respiratory DC composition in C57BL/6 mice. We also tracked the migration of respiratory DC to the lymph nodes and studied antigen presentation by lung-derived and lymph node-resident DC to $CD4^+$ and $CD8^+$ T cells. We observed a massive influx of mainly $CD103^-CD11b^{high}$ $CD11c^+$ conventional DC (cDC) and plasmacytoid DC during the first 7 days of RSV infection, while $CD103^+$ $CD11b^{low}$ $CD11c^+$ cDC disappeared from the lung. The two major subsets of lung tissue DC, $CD103^+$ $CD11b^{low}$ $CD11c^+$ and $CD103^-$ $CD11b^{high}$ $CD11c^+$ cDC, both transported RSV RNA to the lung-draining lymph node. Furthermore, these lung-derived cDC subsets as well as resident LN DC, which did not contain viral RNA, displayed viral antigen by major histocompatibility complex class I and class II to $CD8^+$ and $CD4^+$ T cells. Taken together, our data indicate that during RSV infections, at least three DC subsets might be involved during the activation of lymph node-homing naïve and memory $CD4^+$ and $CD8^+$ T cells.

Introduction

Respiratory Syncytial Virus (RSV) constitutes a major health burden for infants, elderly people and immune compromised individuals (1, 2). The virus infects most children in their first year of life and is the main cause of severe lower respiratory tract infections (LRTI) in infants (1). Despite many decades of research, the immune response to RSV is still not completely understood. Infection with RSV leads to poor development of immunity, and recurrent infections are common (3). In mice, it was found that RSV induces virus-specific $CD8^+$ T-cell responses in the lung that are functionally impaired (4). It has been suggested that a functional inactivation of $CD8^+$ T cells by RSV could be a reason for the short-lived immune response. Furthermore, we and others have previously shown that human monocyte-derived dendritic cells (DC) can be infected with RSV, which results in strong inhibition of their ability to support proliferative responses and induction of effector function in naive T cells (5, 6). An early vaccine trial with formalin-inactivated RSV in alum administered intramuscularly elicited a memory immune response that caused a strong aberrant secondary immune response in vaccinees upon natural exposure with live virus. This resulted in a high rate of morbidity in the vaccinated children (7). These observations underscore the necessity to understand the components of the immune response that are protective during RSV infections and the need to understand the mechanism by which protective immunity can be elicited for the development of an effective and safe vaccine.

DC play an important role in the initiation of both the innate and adaptive immune responses to pathogens including RSV (8). They are a heterogeneous population of cells represented by two main subsets, the myeloid or "conventional" $CD11c^+$ DC (cDC) and the $CD11c^{low}$/mPDCA-1^+ plasmacytoid DC (pDC) (9, 10). cDC can be further divided based on the expression of surface markers and anatomic location. cDC in the tissue and DC in lymph nodes (LN) appear to be different subsets arising from different pools of progenitor cells and with specialized function (11-15). In the mouse lung two major cDC populations are derived from blood monocytes. $CD11c^+$ major histocompatibility class II (MHC-II^+) $CD103^-$ $CD11b^{high}$ cDC ($CD11b^{hi}$ cDC) are localized in the parenchyma. These cells are the main producers of chemokines and are important for the recruitment of leukocytes (16). A second cDC population, $CD11c^+$ MHC-II^+ $CD103^+$ $CD11b^{low}$ ($CD103^+$ cDC), is located directly underneath the airway epithelium. These $CD103^+$ cDC express the integrin αEβ7; therefore, they are found mainly at the basal lamina of the bronchial epithelia and arterioles, which express E-cadherin, the ligand for αEβ7. Furthermore, $CD103^+$ cDC express tight junction proteins ZO-2 and claudin-7, which enables them to sample the airways with their extensions (17). In the lung-draining LN in addition to pDC, at least two steady-state populations of cDC are present, which are characterized by the expression or absence of CD8α. In contrast to the lung tissue DC, these cells enter the LN from the blood, and they are directly derived from a bone marrow precursor (18-20). In addition, minor fractions of tissue derived cDC also access draining LN in the steady state (21). Several studies have addressed the roles of different DC subsets that are present in the tissue and LN draining the infection site. In spleen and skin-draining LN, the role of $CD8α^+$ cDC seems to be important for the

initiation of anti-ovalbumin and antiviral CD8$^+$ T-cell responses (22-24). In mice exposed to innocuous (ovalbumin) or infectious (influenza virus) antigen, functional specialization was described for CD103$^+$ and CD11b^{hi} lung cDC subsets. CD11b^{hi} cDC presented intranasally administered ovalbumin or influenza virus antigen mainly to naïve CD4$^+$ T cells, while CD103$^+$ cDC were important for the induction of CD8$^+$ T-cell responses (25, 26).

The ability of DC to present or cross-present antigens depends on the type of antigenic materials and the uptake mechanism used by antigen presenting cells (APC). Hence different pathogens and innocuous antigens might be differently presented by different DC subsets. We studied the kinetics of lung DC migration and repopulation during primary RSV infection in C57BL/6 mice. We found that upon RSV infection, CD103$^+$ cDC disappeared from the lung, while there was a net increase in numbers of CD11b^{hi} cDC, pDC, and macrophages. Within the first 48 h after virus exposure, both CD103$^+$ and CD11b^{hi} cDC rapidly migrated to the lung-draining mediastinal LN (MLN), while this accumulation was absent in the non-lung draining axillary LN. The migrating cDC showed the highest level of expression of the co-stimulatory molecules CD40, CD80 and CD86, which are necessary for T-cell stimulation, compared to the MLN-resident cDC. Furthermore, the migrating cDC transported viral RNA to the MLN and were capable of stimulating RSV-specific CD4$^+$ and CD8$^+$ T-cell responses. Resident cDC in the LN were uniformly negative for viral RNA. However, resident cDC in the LN did present viral antigen to CD8$^+$ and CD4$^+$ T cells via MHC-I and MCH-II, respectively.

Results

Rapid alterations in DC composition in the lung during RSV infection.

To study the role of RSV infection in lung DC populations, we infected C57BL/6 mice intranasally with RSV or uninfected supernatant of HEp-2 cells as a mock infection. At several time points after infection, we performed a BAL to remove most of the alveolar macrophages before analysis of lung DC numbers and phenotypes with flow cytometry. The CD103$^+$ and CD11b^{hi} cDC as well as lung macrophages could easily be detected (Fig. 1A). Within 24 h, we found a rapid decline in absolute numbers of CD103$^+$ cDC and lung macrophages in RSV infected compared to mock infected animals (Fig. 1B). Absolute numbers of CD103$^+$ cDC remained significantly smaller throughout the first 6 days of infection. This suggested that the CD103$^+$ cDC had migrated out of the lung or died because of the infection and were not replenished by precursors. In contrast, absolute numbers of CD11b^{hi} cDC and pDC remained constant during the first 48 h of RSV infection and significantly increased during the following 5 days compared to mock infected mice (Fig. 1B).

We also enumerated CD103$^+$ and CD11b^{hi} cDC populations in BAL fluid sampels to determine whether migration into the airway lumen occurred. We found that the majority of cells in the BAL fluid consisted of alveolar macrophages. There were no significant alterations in absolute numbers of CD103$^+$ cDC in the BAL fluid during an RSV infection. Similar to the lung tissue, we observed an increase in the numbers of CD11b^{hi} cDC in BAL fluid which was delayed by 24 hours compared to

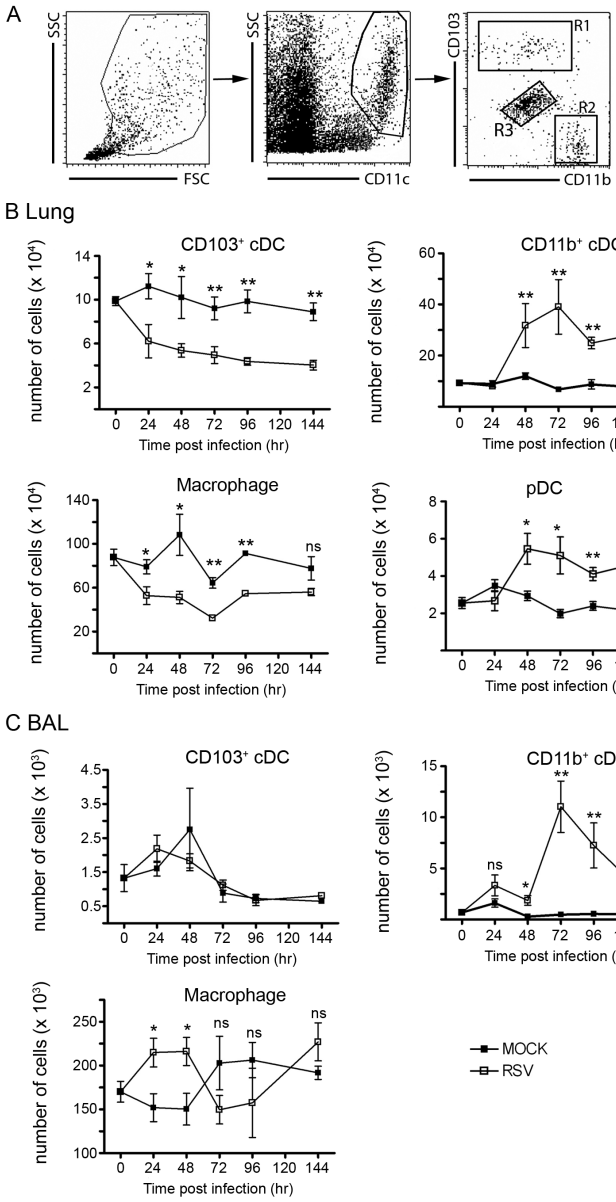


Figure 1. Migration characteristics of DC subsets in lung and BAL fluid during primary RSV infection.

A. Lungs of naïve mice were used for the gating strategies to identify the different cDC populations. R1, CD103^{+} cDC; R2, CD11b^{hi} cDC; R3, pulmonary macrophages; SSC, side scatter; FSC, forward scatter. **B.** At several time points after primary RSV and mock infections, the absolute numbers of CD103^{+}, CD11b^{hi} cDC, macrophages and pDC (CD11c^{low}, mPDCA-1^{+}, and CD45R/B220^{+}) were determined in the lungs. **C.** Similarly, absolute numbers of CD103^{+} and CD11b^{hi} cDC and alveolar macrophages in the BAL fluid were determined. The experiment was performed twice with five mice per time point. Average values of absolute DC number per mouse lung are depicted. Error bars represent the standard error of the mean (SEM). (*, $p < 0.05$; **, $p < 0.01$; ns, not significant).

that in the lung (Fig. 1C). In conclusion, RSV infection induced a rapid alteration of the DC composition in the lung. We observed a disappearance of $CD103^+$ cDC, while there was an accumulation of $CD11b^{hi}$ cDC and pDC in the lung during RSV infection.

Migration of lung DC to the lung draining LN.

Naïve T cell priming occurs in LN draining the site of infection. To study the kinetics of lung DC migration to the lung-draining MLN, we administered the fluorescent dye CFSE intranasally to mice to stably label all the cells in the lung 6 h before an intranasal RSV infection (29). This procedure enabled us to track DC migrating from the lung to the MLN during RSV infection and separate migrating from resident DC in the LN with flow cytometry. CFSE treatment alone did not induce significant DC migration, as shown by the minimal numbers of CFSE labeled cells in the MLN after mock infection (Fig. 2C). After intranasal infection with RSV, predominantly $CD11c^+$ cells were labeled with CFSE in the MLN (Fig. 2A). There was an 8-10 fold increase in absolute numbers of cDC during RSV infection in comparison to mice that received a mock infection (Fig. 2B). Absolute numbers of CFSE-labeled cDC in the MLN showed a steep rise in RSV-infected mice, which peaked at 36 h after RSV infection. After 36 h, there was a gradual decline in absolute numbers of CFSE-positive ($CFSE^+$) cDC, while there was still an accumulation of cDC in the MLN (Fig. 2B and C). This might be explained by the migration of CFSE-negative ($CFSE^-$) cDC that originated from cells that entered the lung later after CFSE labeling and hence were not efficiently labeled, or alternatively, these cells were not lung derived but entered the LN from the blood.
In the lungs, we found a net accumulation of $CD11b^{hi}$ cDC, while the $CD103^+$ cDC numbers declined in the lung during the RSV infection. To determine to what extent both populations migrated to the MLN during the RSV infection, we analyzed the fraction of $CFSE^+$ and $CFSE^-$ cells within these subpopulations in the MLN. We found equal numbers of total $CD103^+$ and $CD11b^{hi}$ cDC in the MLN during the first 36 to 48 h after RSV infection (Fig. 2D). Both $CD103^+$ and $CD11b^{hi}$ cDC contributed equally to the accumulation of cDC in the MLN during the first 36 to 48 h of RSV infection, as the same percentages of $CD103^+$ and $CD11b^{hi}$ cDC were labeled by CFSE (Fig. 2E). Later during the RSV infection, predominantly unlabeled $CD11b^{hi}$ cDC were responsible for the accumulation of cDC in the MLN (Fig. 2D and 2F). Thus, we observed a two-step accumulation of cDC in MLN after RSV infection. In the first 48 h, an influx of equal numbers of both subsets of lung cDC was followed in the next 48 h period by the influx of unlabeled cDC, which might not be lung derived.

Migrating cDC have a more mature phenotype than LN-resident cDC.

For efficient T-cell stimulation, DC have to be mature; i.e., they should express or up-regulate T-cell costimulatory molecules such as CD40, CD80, and CD86. Therefore, we measured the maturation status of the cDC in the MLN by staining these cells with CD40-, CD80-, and CD86-specific antibodies and compared the expression levels of these surface markers between resident and

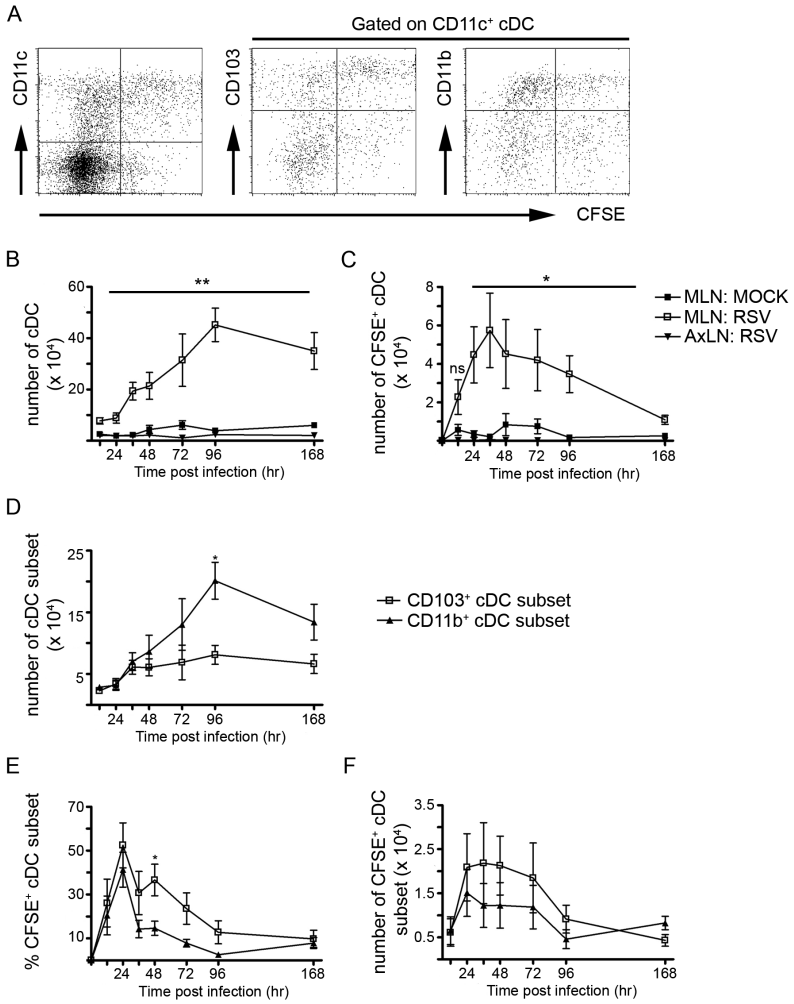


Figure 2. Migration of cDC to the lung-draining LN (MLN) during primary RSV infections.

A. Mice were given CFSE intranasally 6 h before RSV or mock infection. Using flow cytometry, $CFSE^+$ cDC subsets were identified in the MLN. **B.** At several time points after RSV or mock infection, absolute numbers of $CD11c^+$ cDC were determined in MLN and in RSV-infected mice in axillary LN (AxLN). **C.** Lung-derived $CFSE^+/CD11c^+$ cDC were quantified per lung-draining LN and in axillary LN after RSV exposure or mock infection. **D.** During primary RSV infection, absolute numbers of $CD103^+$ cDC and $CD11b^{hi}$ cDC per MLN were determined (E and F). Percentage (**E**) and absolute numbers (**F**) of lung-derived ($CFSE^+$) $CD103^+$ cDC and $CD11b^{hi}$ cDC per MLN during RSV infection were determined. The experiment was performed twice with five mice per time point. Average values of absolute cDC number per mouse MLN are depicted. Error bars represent the SEM. (*, $p< 0.05$; **, $p< 0.01$; ns, not significant).

migrant cDC. We found that at 36 h after RSV infection, the $CFSE^+$ migrating cDC had a higher level of expression of all three costimulatory molecules than did the CFSE- cDC (resident cDC), as judged by the mean fluorescence intensity (Fig. 3A). Over time, the level of expression of CD40 remained

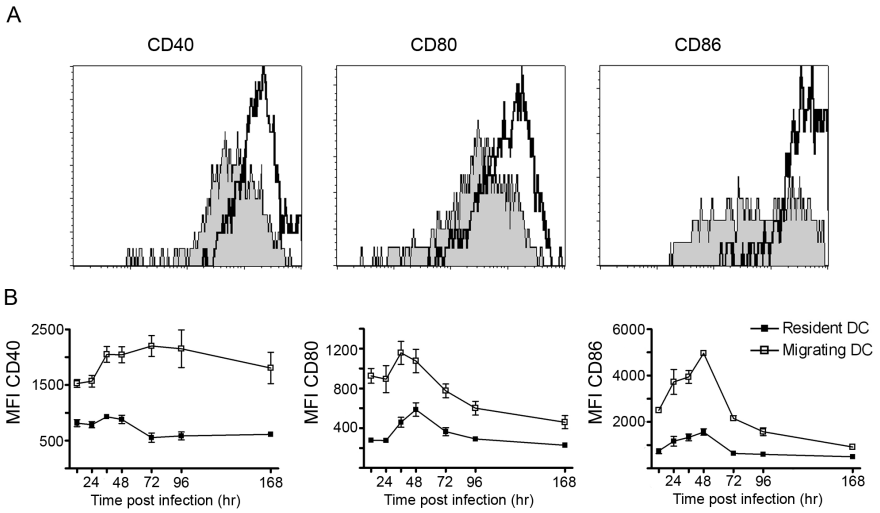


Figure 3. Migrating cDC have a more mature phenotype than do LN- resident cDC.

A. At 48 h after primary RSV infection, MLN cDC were stained with anti-CD40, -CD80, and -CD86. Based on CFSE the cDC were divided in LN-resident cDC (filled histogram) and migrating cDC (open histogram). **B.** At several time points after primary RSV infection, the levels of expression of CD40, CD80, and CD86 on LN-resident cDC and migrating cDC was determined. The experiment was performed twice with five mice per time point. Average mean fluorescence intensity (MFI) are depicted. Error bars represent the SEM.

significantly higher on migrating cDC than on resident cDC. The level of expression of CD80 and CD86 also remained significantly higher on migrant cDC than resident cDC, but in contrast to a constant level of CD40 expression, these molecules showed a clear peak between 36 and 48 hours after RSV infection (Fig. 3B).

Transport of viral RNA from the lung to the lung-draining LN by lung-derived cDC.

DC start a program of maturation after contact with inflammatory cytokines, pathogens, or tissue damage. The lung-derived cDC that arrived in the MLN had a mature phenotype required for efficient T-cell stimulation. To test whether these cells had acquired RSV either via direct infection or uptake of RSV-containing cell debris, we assayed MLN cDC for the presence of RSV RNA. The MLN were harvested 72 h after RSV infection, and the cDC were sorted based on the expression of CD11c and CD103 and labeling by CFSE (Fig. 4A). Since there was an inverse relationship between the expression of CD103 and CD11b on lung cDC subsets, we used the absence of expression of CD103 to identify $CD11b^{hi}$ cDC. RNA was isolated from 10^4 cells per population and assessed for the presence of RSV RNA with quantitative RT-PCR for the RSV N gene. We found most of the viral RNA in the cDC that had been labeled with CFSE and, thus, originated from the lung. There was no significant difference in the presence of RSV RNA in $CD103^+/CFSE^+$ or $CD103^-/CFSE^+$ cDC, suggesting that they had been infected or had internalized similar amounts of RSV. The $CD103^+$/CFSE- cDC contained very

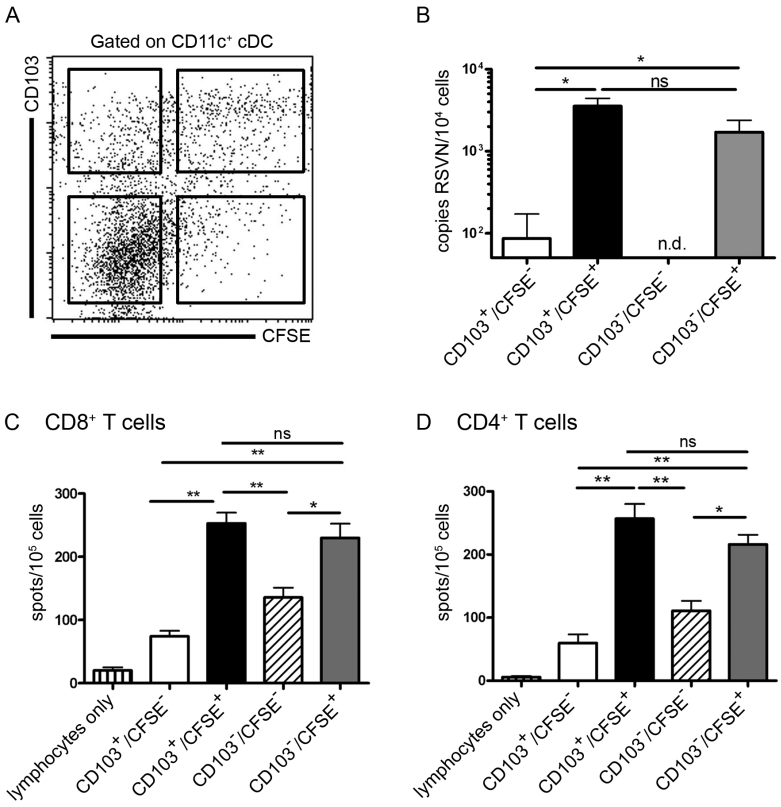


Figure 4. Migrating CFSE$^+$ cDC transport viral RNA to the lung-draining LN and present antigens by MHC-I and MHC-II molecules.

A. MLN cDC were harvested 72 h post-RSV infection and sorted into four populations based on CD103 expression and CFSE labeling. **B.** Sorted MLN cDC (10^4 cells/subset) were analyzed for the presence of the RSV N RNA by quantitative RT-PCR. **C.** and **D.** Antigen presentation was analyzed by culturing RSV-specific CD4$^+$ or CD8$^+$ T-cell populations for 24 h with sorted cDC subsets isolated from the MLN 72 h after RSV infection. To enrich for CD8$^+$ T cells, lung lymphocytes isolated on day 8 after primary RSV infection were depleted of CD4$^+$ T cells, B cells, and NK cells. Similarly, CD4$^+$ T cells were enriched by depletion of CD8$^+$ T cells, B cells, and NK cells. RSV-specific IFN-γ production was measured by ELISPOT assay. Data shown are the means of six individual experiments for the RT-PCR assay. For the ELISPOT assay, the mean values of five individual experiments using cDC purified from five individual mice for each experiment are shown. Error bars represent SEM. (*, $p< 0.05$; **, $p< 0.01$; ns, not significant; n.d., not detected).

low levels of viral material. The expression of CD103 identifies these cells as being lung derived. These cells might have been poorly labeled or most of these cells might have migrated before CFSE labelling. In unlabeled CD103$^-$ cells, the fraction composed of LN-resident DC and possibly inflammatory monocyte-derived DC originating from blood, no viral RNA could be detected (Fig. 4B).

MHC-I and MHC-II restricted antigen presentation by lung-derived DC.

The lung-derived cDC in the MLN contained most of the RSV RNA and were likely candidates to contribute to the induction of RSV-specific T-cell responses. However, several reports have shown that T-cell responses are induced primarily by the LN-resident DC (36) or by both LN-resident and airway-derived cDC (31). Furthermore, it is unclear how the different airway cDC types contribute to MHC-I and MHC-II antigen presentation during RSV infection. To investigate which cDC type was involved in antigen presentation to $CD4^+$ and $CD8^+$ T cells during RSV infection, we again sorted MLN cDC 72 h after RSV infection, based on the expression of CD11c and CD103 and CFSE labeling. These sorted MLN cDC were cocultured with lung lymphocytes isolated 8 days after primary RSV infection that were depleted of B and NK cells and either $CD4^+$ or $CD8^+$ T cells. The enriched $CD4^+$ or $CD8^+$ T-cell populations were cocultured with the different cDC subsets, and the level of IFN-γ production by the virus-specific effector $CD8^+$ or $CD4^+$ T cells was assessed with an IFN-γ ELISPOT assay. We found that the CFSE-labeled cDC subsets were most effective inducers of IFN-γ production by $CD8^+$ T cells. There was no significant difference in IFN-γ production by $CD8^+$ or $CD4^+$ T cells when these cells where stimulated with $CD103^+$ or $CD103^-$ lung-derived ($CFSE^+$) cDC in the T-cell DC ratios used (Fig. 4C and D). $CD103^+$/CFSE- cDC activated a smaller number of $CD4^+$ and $CD8^+$ T cells, which could be explained by the smaller amount of viral RNA detected in this population of lung-derived cells (Fig 4B to D). Interestingly, a clear response of $CD4^+$ and $CD8^+$ T cells was also observed against the $CD103^-$/CFSE- DC population of non-lung-derived DC that did not contain viral RNA was also observed (Fig. 4B to D). In summary, we found that virus-exposed lung cDC subsets migrating to the MLN as well as non-lung-derived cells present in MLN could present viral antigen to $CD4^+$ and $CD8^+$ effector T cells. Moreover, we found no indication that different cDC subsets preferably processed and presented viral materials for the presentation to either the $CD4^+$ or $CD8^+$ T-cell subset.

Discussion

In this study, we showed that both major lung cDC subsets, the sub-epithelial $CD103^+$ $CD11b^{low}$ and the parenchymal $CD103^-$ $CD11b^{high}$ DC, as well as a population of non-lung derived MLN cDC display viral antigen in the context of MHC-I and MHC-II molecules in MLN after intranasal RSV infection. Thus, migratory lung DC that might have been directly infected as judged from the observed presence of viral RNA, as well as MLN cDC that did not originate from the upstream infection site in which no viral RNA was detected, triggered T-cell activation *in vitro* (Fig. 4). In recent studies with influenza virus infection models and intranasal installation of ovalbumin, a functional specialization of cDC subsets in the lung has been described. $CD11b^{hi}$ and $CD103^+$ cDC were found to differ in their efficacy to present antigen to naïve $CD8^+$ and $CD4^+$ T cells (25, 26). It was shown that $CD103^+$ cDC had the unique capacity to cross-present exogenously acquired (noninfectious) antigen *in vitro* to naïve $CD8^+$ T cells (26). In contrast, $CD11b^{hi}$ lung DC appeared to be more effective in the induction of naïve $CD4^+$ T cell proliferation. However, the observed functional dichotomy might

be less pronounced when antigen is acquired via direct infection. Indeed, there was a clear difference in the abilities of CD11b^{hi} cDC to present live and noninfectious influenza virus particles (25). Upon intranasal infection with live influenza virus, CD11b^{hi} cDC could present antigen by MHC-I molecules to naïve CD8$^+$ T cells *in vitro* albeit at a somewhat lower efficiency than CD103$^+$ cDC. These differences might reflect the more effective uptake of influenza virus by the CD103$^+$ cDC subset that is located directly underneath the infected airway epithelium and/or the fact that these cells are more easily infected by influenza virus *in vitro* plus the characteristic of these cDC to also be able to capture and present influenza virus-derived antigen via a noninfectious route (25, 37). In our experiments with RSV, we found no substantial difference in the infection levels of both lung cDC subsets as measured by viral RNA content (Fig 4). Using effector T cells as a readout system to measure antigen display by MHC-I and MHC-II molecules we found that both migrating lung cDC subsets presented RSV-derived antigens to CD4$^+$ and CD8$^+$ T cells. The presence of viral RNA indicates that a proportion of these DC had been directly infected, but also, material transferred to DC from infected epithelial cells might have contributed to the antigen displayed to T cells. Migrating cDC had upregulated costimulatory molecules CD80, CD86 and CD40. The roles and relative efficacy of the two lung-derived cDC subsets and LN-resident cDC during the initiation of RSV-specific T-cell responses *in vivo* are unclear. Because T-cell receptor-transgenic mice are not available for RSV, it was not possible for us to study the *in vitro* stimulation of naïve CD4$^+$ and CD8$^+$ T-cell responses. However, such *in vitro* studies are inadequate to determine the exact *in vivo* role of DC in initiating T-cell responses. It was elegantly shown by Allenspach *et al.* that although migratory DC activated naïve CD4$^+$ T-cell proliferation *in vitro*, they were unable to initiate T-cell responses *in vivo*, possibly because they cannot anatomically interact with naïve T cells in the LN (38). Interestingly, the role of migratory cDC was not limited to antigen delivery into the node, as suggested by previous studies, because the full expansion of antigen-specific T cells required, in addition to primary contacts with LN-resident cDC, secondary contacts with migratory cDC (36, 38). A similar cooperation of migrating and resident cDC subsets might be needed for the CD8$^+$ T-cell response because studies *in vivo* in which CD8α$^+$ LN and splenic cDC were depleted showed the essential role of this DC subset in the initiation of antiviral, antitumor and ovalbumin-specific CD8$^+$ T-cell responses (22-24, 31). Because those studies did not look at the role of CD8α$^+$ cDC after the administration of antigen via the intranasal/intratracheal route, it is unclear whether the role of LN-resident cDC in the MLN is obligatory, as after antigen exposure via intravenous or intradermal administration (39). The CD103$^+$ lung cDC population has many similarities with the LN-resident CD8α$^+$ cDC subset. They share the ability to cross-present and to react to Toll-like receptor 3 stimulation, and in spleen, CD103 is coexpressed with CD8α. They may therefore function in a similar way in the MLN (17, 26, 40).

For effective *in vivo* activation of T cell responses, other cellular interactions might be required. In addition to multiple DC-T-cell contacts, licensing of cDC by CD4$^+$ T cells might contribute to the efficacy of DC to prime CD8$^+$ T-cell responses *in vivo*, and after primary triggers in the LN, an additional activation of T cells might occur when activated T cells migrate into the infected tissue (32, 41). Thus, the complete picture of how effective T-cell responses are elicited can be learned only

from experiments with intact hosts. Because it is currently not possible to delete or functionally inactivate single DC populations in the respiratory tract, the exact *in vivo* role during T-cell activation awaits tools or genetically engineered mice to perform these experiments.

We found a massive increase of total cDC in the MLN, with a peak influx 96 h after RSV infection. There were five- to eightfold-more cDC in the MLN during RSV infection than in mock infected mice. The peak influx of $CFSE^+$ cDC occurred 36 h after RSV infection. This is a slower accumulation of cDC in the MLN, as was reported previously for Influenza or Sendai virus infection, where the peak influx of respiratory cDC in the MLN is 12 to 24 h after infection (29, 32, 33). This can be explained by differences in virus dose or innate detection of the different viruses by pattern recognition receptors, resulting in a different pattern, and possibly, a different tempo of inflammatory response. The source of accumulating unlabeled $CD11b^{hi}$ cDC in MLN during RSV infection is not clear. These cells might not be lung derived but enter the LN via blood. Alternatively, they might be derived from cells that enter the lung after CFSE labeling or develop from DC precursors after extensive cell division, which results in dilution of the CFSE label. It was previously reported that DC precursors can divide extensively and develop into DC upon exposure to granulocyte-macrophage colony-stimulating factor exist in the lung (42). In contrast, immature DC and mature DC do not extensively divide in the course of 4 days, making these committed DC from the lung an unlikely source of CFSE- cells in the draining LN (43). It has been shown that respiratory cDC migrate only during a short period of time after infection and subsequently become refractory to activation signals resulting in the inability of subsequent infections to induce DC migration (29). Therefore, local development of DC from lung or monocyte precursors most likely contributes to the enhanced $CD11b^+$ DC numbers in the lung tissue (Fig. 1B), and the late-expanding unlabeled $CD11b^{hi}$ cells in MLN are most likely not lung derived.

We and others have shown that human DC infected with RSV are poor inducers of T-cell proliferation (5, 6, 44). Also, $CD4^+$ T cells that do expand upon exposure to RSV-infected DC do not develop effector function to an extent similar to that of T cells cultured with influenza-virus infected DC or DC matured with Toll-like receptor ligands or cytokines. The exact mechanism of the inhibition effect is still not completely clear. Cell contact mechanisms as well as unidentified soluble components in RSV-DC cultures might contribute to inefficient DC function (5, 6, 44-46). Despite the fact that RSV infection impairs DC function we have observed quite robust functional T-cell responses in peripheral blood of infants during primary RSV infections (47). These observations indicate that *in vivo* cross-presentation could be an important pathway of T-cell priming. Thus, a role for LN-resident DC that are not infected might be important during the activation of RSV-specific T-cell responses.

During respiratory infection with RSV and influenza virus an increase in DC populations in the lungs of humans and mice has been observed (48, 49) and (Fig.1B). These cells might play a role in the addition activation of LN-primed T cells and/or be important as local antigen-presenting cells that stimulate effector/memory T cells that are directly recruited to inflammatory sites (32, 50, 51). Furthermore, they could also play a role in the production of cytokines and chemokines that are in-

volved in the attraction of different inflammatory cell types (14). Previous studies in RSV-infected mice have reported the increase of cDC and pDC populations in the lung, similar to our observations (Fig. 1) (49, 52, 53). However, in those studies, the cDC subsets that accumulated were not further characterized. We show that upon RSV infection, both $CD103^{+}$ and $CD11b^{hi}$ cDC migrate in similar numbers to the draining LN but that only $CD11b^{hi}$ cDC are replenished and reach higher levels than originally present in the lung tissue. In contrast to LN-resident cDC that develop from precursor cells in the bone marrow, blood monocytes are the precursor cells that give rise to the two major lung tissue cDC subsets (13-15, 19). $Ly6C(Gr1)^{high}$ $CCR2^{high}$ $CX3CR1^{int}$ monocytes develop into $CD103^{+}$ cDC, whereas $Ly6C(Gr1)^{low}$ $CCR2^{low}$ $CX3CR1^{high}$ develop into $CD11b^{hi}$ cDC during homeostatic conditions (15). Landsman *et al.* previously showed that both monocyte subsets also give rise to cDC under inflammatory (lipopolysaccharide given intratracheal) conditions (13). However, the phenotype of the resulting cDC populations was not determined. It is unclear whether the accumulating $CD11b^{hi}$ cDC subset in the lung after RSV infection are derived from a single monocyte precursor and whether this is different during different viral infections. It is an intriguing idea that the type of inflammatory response induced by the infecting virus might impact on the pattern of DC repopulation. During influenza infection, $CD11b^{hi}$ cDC numbers were also increased, while it appears that $CD103^{+}$ cDC numbers stayed stable during the first 3 days after influenza virus infection, suggesting that these cells were replenished but to a lower extent than for $CD11b^{hi}$ cDC (25).

It was previously described that local ratios of cDC and pDC in the lung might affect the severity of RSV disease, whereby pDC might ameliorate disease by shifting the T-cell response to a Th1 type of response and potentiate more effective viral clearance (52, 53). The exact mechanism behind this more beneficial response by enhanced pDC/cDC ratios needs to be established. Possibly, enhanced IFN-γ producing $CD8^{+}$ T-cell numbers could ameliorate Th2 $CD4^{+}$ T-cell responses. However, it is currently unclear how pDC contribute to enhanced $CD8^{+}$ T-cell responses; clearly IFN-α is not crucial for this process (53).

We have shown that at least three subsets of cDC display RSV-derived antigen in the context of MHC-I and MHC-II molecules in the LN draining the infected lung. These subsets might thus be involved in activating RSV-specific T cells circulating through the LN. We further identified the cDC subset that enters the lung after RSV infection as being $CD11b^{hi}$ cells, while $CD103^{+}$ cells are not replenished in the first 8 days after RSV infection. Future work aimed at studying the contribution of DC subsets during secondary immune responses after natural infection or vaccination and in the presence of preexisting (maternal) antibodies will contribute to our understanding of the exact role that these cells play during protective or pathologic immune responses. These studies may therefore contribute to the design of safe vaccine approaches.

Materials and Methods

Mice

Pathogen-free 6- to 8-week-old female C57BL/6cjo mice were purchased from Charles River Nederland (Maastricht, The Netherlands). The mouse study protocol was approved by the Animal Ethics Committee of the University Medical Center Utrecht and Utrecht University.

Viruses and infections

RSV strain A2 strain was grown on HEp-2 cells and purified by polyethyleen glycol 6000 precipitation. Mice were lightly anesthetized with isofluorane and intranasally infected with 10^6 PFU RSV in a volume of 50 µl diluted in phosphate buffered saline (PBS) with 10 % sucrose.

Isolation of tissue DC

Mice were sacrificed by intraperitoneal injection of pentobarbital. After performing bronchial alveolar lavage (BAL), the lungs were perfused with 10 ml ice-cold PBS containing 100 U/ml heparin via the right ventricle. The lungs and mediastinal and axillary LN were removed and cut into pieces. The fragments were digested for 25 min at 37°C in 5 % CO_2 with 3.2 mg collagenase A and 1 mg DNase (Roche Applied Science, Basel, Switzerland). For the last 5 min, 1 mM EDTA was added. Single-cell suspensions were prepared from the pretreated LN by processing the tissue trough cell strainers (BD Falcon, Franklin Lakes, NJ).

Flow cytometry

The DC of the lung and LN were prepared as described above. All cell suspensions were preincubated with 5 µg/ml blocking antibody against CD16/CD32 (2.4G2), obtained from BD biosciences (San Diego, CA) before staining to reduce nonspecific binding. The cells were stained in PBS containing 2 % fetal calf serum, 2 mM EDTA and 0,02 % NaN_3 with the following monoclonal antibodies: anti-CD4 (L3T4), anti-CD8α (clone 53-6.7), anti-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD19 (clone 1D3), anti-CD40 (clone 3/23), anti-CD45R (B220,clone RA3-6B2), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-CD103 (clone M290) and anti-MHC-II ($I\text{-}A^b/I\text{-}E^b$) (clone M5/114.15.2), obtained from BD biosciences (San Diego, CA) and anti-mPDCA-1 (clone JF05-1C2.4.1) obtained from Miltenyi Biotec (Germany). Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences San Diego, CA). Data was analyzed using CellQuest software (BD Biosciences).

CFSE labeling of migrating DC

To detect migrating DC, mice were lightly anesthetized with isofluorane and 50 µl 8 mM carboxyfluorescein succinimidyl ester (CFSE) (Fluka, Buchs, Switzerland) diluted in PBS was intranasally

administered 6 h before intranasal infection with RSV. This method is based on labeling of intracellular proteins and is stable for at least 8 weeks in nondividing lymphocytes. Cells that undergo cell division can still be distinguished from unlabeled cells after at least five to six cell divisions (27, 28). This labeling procedure has frequently been used to stain cells in the lung after intranasal administration and allowed the tracking of respiratory DC migration. At least 95 to 100 % of all respiratory DC labeled *in vivo* remained CFSE positive up to 6 days after CFSE instillation (29-35 and our unpublished results). At various time points after RSV infection, DC were isolated from the MLN and axillary LN to study the kinetics of migration.

RSV-specific reverse transcriptase (RT)-PCR

LN were processed as described above. Subsequently, cDC subsets were sorted on a FACSAria apparatus (BD Biosciences San Diego, CA) based on the expression of CD11c and CD103 and the uptake of CFSE. RSV RNA was extracted from $1x10^4$ DC using the RNeasy minikit (Qiagen, Valencia, CA) according to the instructions provided by the manufacturer. cDNA was prepared with reverse transcriptase using 2.5 µM random hexamers for 5 min at 25 °C, 30 min at 48 °C, and 10 min at 95 °C. Real-time PCR for the RSV N gene was performed using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) using 10 µl of sample in a total volume of 25 µl master mix under the following run conditions: 1 cycle for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles for 15 s at 95 °C and 1 min at 60 °C. The following primers and 6-carboxy-fluorescein-labeled probe were used: RSV forward primer AGA TCA ACT TCT GTC ATC CAG CAA, RSV reverse primer TTC TGC ACA TCA TAA TTA GGA GTA TCA AT and RSV probe CAC CAT CCA ACG GAG CAC AGG AGA T . Known concentrations RSV A2 were used to derive a standard curve. Standards and negative controls were run together with each PCR mixture; the lower limit of detection of the assay was 12 viral copies/ml.

Detection of antigen presentation by LN DC

At several time points after RSV infection, the MLN cDC populations were isolated as described above and sorted on a FACSAria apparatus (BD Biosciences San Diego, CA). MLN cDC ($1x10^4$ cells) were cocultured with 10^5 lung lymphocytes harvested 8 days after primary infection and before coculture with cDC depleted of CD19-, NK1.1-, and CD4- or CD8- positive cells. An enzyme linked immunospot (ELISPOT) assay was performed to detect gamma interferon (IFN-γ) production. The mouse IFN-γ EISPOT pair (U-cytech, Utrecht, The Netherlands) and Multiscreen-IP filter plates (Millipore, Billerica, MA) were used according to the manufacturers' instructions. Cells were stimulated in 200 µl iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen) containing 10 % FCS, penicillin/streptomycin and 50 µM 2-mercapto-ethanol with 25 u/ml recombinant human IL-2 for 24 hrs at 37 °C, 5 % CO_2.

References

1. Glezen P, Denny FW. 1973. Epidemiology of acute lower respiratory disease in children. N Engl J Med 288:498-505.
2. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. 2005. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med 352:1749-1759.
3. 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.
4. Chang J, Braciale TJ. 2002. Respiratory syncytial virus infection suppresses lung $CD8^+$ T-cell effector activity and peripheral $CD8^+$ T-cell memory in the respiratory tract. Nat Med 8:54-60.
5. de Graaff PM, de Jong EC, van Capel TM, *et al.* 2005. Respiratory Syncytial Virus Infection of Monocyte-Derived Dendritic Cells Decreases Their Capacity to Activate CD4 T Cells. J Immunol 175:5904-5911.
6. Chi B, Dickensheets HL, Spann KM, *et al.* 2006. Alpha and lambda interferon together mediate suppression of CD4 T cells induced by respiratory syncytial virus. J Virol 80:5032-5040.
7. Kim HW, Canchola JG, Brandt CD, *et al.* 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 89:422-434.
8. Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.
9. von Garnier C, Filgueira L, Wikstrom M, *et al.* 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. J Immunol 175:1609-1618.
10. Wikstrom ME, Stumbles PA. 2007. Mouse respiratory tract dendritic cell subsets and the immunological fate of inhaled antigens. Immunol Cell Biol 85:182-188.
11. Varol C, Yona S, Jung S. 2009. Origins and tissue-context-dependent fates of blood monocytes. Immunol Cell Biol 87:30-38.
12. Geissmann F, Auffray C, Palframan R, *et al.* 2008. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol Cell Biol 86:398-408.
13. Landsman L, Varol C, Jung S. 2007. Distinct differentiation potential of blood monocyte subsets in the lung. J Immunol 178:2000-2007.
14. del Rio ML, Rodriguez-Barbosa JI, Bolter J, *et al.* 2008. $CX3CR1^+$c-kit^+ Bone Marrow Cells Give Rise to $CD103^+$ and C. J Immunol 181:6178-6188.
15. Jakubzick C, Tacke F, Ginhoux F, *et al.* 2008. Blood Monocyte Subsets Differentially Give Rise to $CD103^+$ and $CD103^-$ Pulmonary Dendritic Cell Populations. J Immunol 180:3019-3027.
16. Beaty SR, Rose CE, Jr., Sung SS. 2007. Diverse and potent chemokine production by lung $CD11b^{high}$ dendritic cells in homeostasis and in allergic lung inflammation. J Immunol 178:1882-1895.
17. Sung SS, Fu SM, Rose CE, Jr., Gaskin F, Ju ST, Beaty SR. 2006. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol 176:2161-2172.
18. Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. 2007. Identification of clonogenic common $Flt3^+$M-$CSFR^+$ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. Nat Immunol 8:1207-1216.
19. Naik SH, Sathe P, Park HY, *et al.* 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived *in vitro* and *in vivo*. Nat Immunol 8:1217-1226.
20. Shortman K, Naik SH. 2007. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 7:19-30.
21. Jakubzick C, Bogunovic M, Bonito AJ, Kuan EL, Merad M, Randolph GJ. 2008. Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. J Exp Med 205:2839-2850.
22. Lin ML, Zhan Y, Proietto AI, *et al.* 2008. Selective suicide of cross-presenting $CD8^+$ dendritic cells by cytochrome c

injection shows functional heterogeneity within this subset. Proc Natl Acad Sci U S A ;105:3029-3034.

23. Belz GT, Smith CM, Eichner D, *et al.* 2004. Cutting edge: conventional CD8 alpha$^+$ dendritic cells are generally involved in priming CTL immunity to viruses. J Immunol 172:1996-2000.
24. Hildner K, Edelson BT, Purtha WE, *et al.* 2008. Batf3 deficiency reveals a critical role for CD8alpha$^+$ dendritic cells in cytotoxic T cell immunity. Science 322:1097-1100.
25. Kim TS, Braciale TJ. 2009. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8$^+$ T cell responses. PLoS ONE 4:e4204.
26. del Rio ML, Rodriguez-Barbosa JI, Kremmer E, Forster R. 2007. CD103$^-$ and CD103$^+$ Bronchial Lymph Node Dendritic Cells Are Specialized in Presenting and Cross-Presenting Innocuous Antigen to CD4$^+$ and CD8$^+$ T cells. J Immunol 178:6861-6866.
27. Weston SA, Parish CR. 1990. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Methods 133:87-97.
28. Lyons AB, Parish CR. 1994. Determination of lymphocyte division by flow cytometry. J Immunol Methods 171:131-137.
29. Legge KL, Braciale TJ. 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. Immunity 18:265-277.
30. Hintzen G, Ohl L, del Rio ML, *et al.* 2006. Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. J Immunol 177:7346-7354.
31. Belz GT, Smith CM, Kleinert L, *et al.* 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. Proc Natl Acad Sci U S A 101:8670-8675.
32. McGill J, van RN, Legge KL. 2008. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. J Exp Med 205:1635-1646.
33. Grayson MH, Ramos MS, Rohlfing MM, *et al.* 2007. Controls for Lung Dendritic Cell Maturation and Migration during Respiratory Viral Infection. J Immunol 179:1438-1448.
34. Belz GT, Zhang L, Lay MD, Kupresanin F, Davenport MP. 2007. Killer T cells regulate antigen presentation for early expansion of memory, but not naive, CD8$^+$ T cell. Proc Natl Acad Sci U S A 104:6341-6346.
35. Jakubzick C, Helft J, Kaplan TJ, Randolph GJ. 2008. Optimization of methods to study pulmonary dendritic cell migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen. J Immunol Methods 337:121-131.
36. Allan RS, Waithman J, Bedoui S, *et al.* 2006. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity 25:153-162.
37. Hao X, Kim TS, Braciale TJ. 2008. Differential response of respiratory dendritic cell subsets to influenza virus infection. J Virol 82:4908-4919.
38. Allenspach EJ, Lemos MP, Porrett PM, Turka LA, Laufer TM. 2008. Migratory and lymphoid-resident dendritic cells cooperate to efficiently prime naive CD4 T cells. Immunity 29:795-806.
39. Belz GT, Shortman K, Bevan MJ, Heath WR. 2005. CD8alpha$^+$ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens *in vivo*. J Immunol 175:196-200.
40. Edwards AD, Chaussabel D, Tomlinson S, Schulz O, Sher A, Reis e Sousa. 2003. Relationships among murine CD11c(high) dendritic cell subsets as revealed by baseline gene expression patterns. J Immunol 171:47-60.
41. Smith CM, Wilson NS, Waithman J, *et al.* 2004. Cognate CD4($^+$) T cell licensing of dendritic cells in CD8($^+$) T cell immunity. Nat Immunol 5:1143-1148.
42. Wang H, Peters N, Laza-Stanca V, Nawroly N, Johnston SL, Schwarze J. 2006. Local CD11c$^+$ MHC Class II- Precursors Generate Lung Dendritic Cells during Respiratory Viral Infection, but Are Depleted in the Process. J Immunol 177:2536-2542.
43. Winzler C, Rovere P, Rescigno M, *et al.* 1997. Maturation stages of mouse dendritic cells in growth factor-depen-

dent long-term cultures. J Exp Med 185:317-328.

44. Guerrero-Plata A, Casola A, Suarez G, *et al.* 2006. Differential response of dendritic cells to human metapneumovirus and respiratory syncytial virus. Am J Respir Cell Mol Biol 34:320-329.
45. Gonzalez PA, Prado CE, Leiva ED, *et al.* 2008. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proc Natl Acad Sci U S A 105:14999-15004.
46. Schlender J, Walliser G, Fricke J, Conzelmann KK. 2002. Respiratory syncytial virus fusion protein mediates inhibition of mitogen-induced T-cell proliferation by contact. J Virol 76:1163-1170.
47. Heidema J, Lukens MV, van Maren WW, *et al.* 2007. $CD8^+$ T Cell Responses in Bronchoalveolar Lavage Fluid and Peripheral Blood Mononuclear Cells of Infants with Severe Primary Respiratory Syncytial Virus Infections. J Immunol 179:8410-8417.
48. Gill MA, Long K, Kwon T, *et al.* 2008. Differential Recruitment of Dendritic Cells and Monocytes to Respiratory Mucosal Sites in Children with Influenza Virus or Respiratory Syncytial Virus Infection. J Infect Dis 198:1667-1676.
49. Beyer M, Bartz H, Horner K, Doths S, Koerner-Rettberg C, Schwarze J. 2004. Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. J Allergy Clin Immunol 113:127-133.
50. Wakim LM, Waithman J, van RN, Heath WR, Carbone FR. 2008. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. Science 319:198-202.
51. Wakim LM, Gebhardt T, Heath WR, Carbone FR. 2008. Cutting edge: local recall responses by memory T cells newly recruited to peripheral nonlymphoid tissues. J Immunol 181:5837-5841.
52. Smit JJ, Lindell DM, Boon L, Kool M, Lambrecht BN, Lukacs NW. 2008. The Balance between Plasmacytoid DC versus Conventional DC Determines Pulmonary Immunity to Virus Infections. PLoS ONE 3:e1720.
53. Smit JJ, Rudd BD, Lukacs NW. 2006. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. J Exp Med 203:1153-1159.

chap

ter 3

Serum antibodies critically affect virus specific CD4+/CD8+ T cell balance during RSV infections.

Debby Kruijsen[1], Mark J. Bakkers[1], Nathalie O. van Uden[1], Marco C. Viveen[2], Tetje C. van der Sluis[1], Jan L. Kimpen[1], Jeanette H. Leusen[3,], Frank E. Coenjaerts[2,*] and Grada M. van Bleek[1]*

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

[*] J.H. Leusen and F.E. Coenjaerts contributed equally to this paper.

The Journal of Immunology (2010) 185: 6489-6498

Abstract

Following infection with respiratory syncytial virus (RSV), reinfection in healthy individuals is common and presumably due to ineffective memory T cell responses. In peripheral blood of healthy adults a higher $CD4^+/CD8^+$ memory T cell ratio was observed compared to the ratio of virus specific effector $CD4^+/CD8^+$ T cells that we had found in earlier work during primary RSV infections. In mice, we show that an enhanced ratio of RSV specific neutralizing to non-neutralizing Abs profoundly enhanced the $CD4^+$ T cell response during RSV infection. Moreover, Fcγ–receptors and complement factor C1q contributed to this Ab mediated enhancement. Therefore, the increase in $CD4^+$ memory T cell response likely occurs through enhanced endosomal Ag processing dependent on Fcγ–receptors. The resulting shift in memory T cell response was likely amplified by suppressed T cell proliferation caused by RSV infection of Ag presenting cells, a route important for Ag presentation via MHC class I molecules leading to $CD8^+$ T cell activation. Decreasing memory $CD8^+$ T cell numbers could explain the inadequate immunity during repeated RSV infections. Understanding this interplay of Ab mediated $CD4^+$ memory T cell response enhancement and infection mediated $CD8^+$ memory T cell suppression is likely critical for development of effective RSV vaccines.

Introduction

Respiratory Syncytial Virus (RSV) infections are a major health burden in infants and elderly people (1-3). Although severe RSV bronchiolitis and pneumonia usually occur in children during primary infections, symptomatic reinfections are frequent in healthy individuals (4). These reinfections can occur with the same RSV strain, indicating that acquired immunity is not effectively established or does not completely protect against reinfection. Precise correlates of protection for RSV-induced respiratory tract infections are not defined, and the reason for incomplete immune protection is not clear. Abs provide partial protection against severe RSV bronchiolitis and pneumonia (5-7). Prophylactic treatment with palivizumab (Synagis, MedImmune, Gaitersburg, MD), a neutralizing Ab specific for the F protein of RSV, is used to protect high-risk infants against severe lower respiratory tract disease (8). Furthermore, children with T cell deficiencies are unable to efficiently clear the virus, which shows that T cells play a role in virus eradication (9, 10). The susceptibility of elderly people to severe RSV pneumonia might be the consequence of T cell senescence and/or decreased RSV-specific memory T cell numbers (11-13). However, no detailed information is available on the immune status of individuals in different age groups with respect to RSV.

The role of RSV-specific Abs is likely diverse. Neutralizing Abs can contribute to the reduction of viral load and, as a result, decrease innate immune responses. Non-neutralizing Abs might enhance infection and might cause immune complex deposition, leading to enhanced respiratory disease (14). Abs play a role in the uptake of immune complexes by APCs via FcRs or complement receptors and influence $CD4^+$ and $CD8^+$ T cell activation (15-19). In addition, Abs might alter innate immune activation by targeting viral particles to endosomal compartments where TLRs get activated or by interacting with viral proteins or carbohydrates that bind to immune receptors [e.g. chemokine and pattern recognition receptors (20, 21)] that play regulatory roles during innate and adaptive immune responses.

In the present work, we studied the impact of RSV-specific Abs on initiation of adaptive immunity during Ag presentation. We observed that a higher ratio of neutralizing versus nonneutralizing Abs enhanced the balance of responding virus specific $CD4^+$/$CD8^+$ T cells *in vitro* as well as *in vivo*. A possible role for RSV-specific Abs in shaping the human virus-specific memory T cell pool is discussed.

Results

AS enhances *in vitro*-induced RSV-specific IFN-γ responses and suppresses influenza virus-specific IFN-γ production by PBMCs.

To determine the effect of virus-specific Abs on T cell activation, we performed *in vitro* T cell stimulation assays with PBMCs derived from healthy adult donors. Antigenic stimulus was achieved by treatment of PBMCs with RSV alone or RSV treated with AS. We performed IFN-γ ELISPOT assays and compared the response against RSV with the response against influenza virus. RSV-specific

IFN-γ responses increased in the presence of AS, whereas influenza virus-specific responses strongly decreased in the presence of AS (Fig. 1A). To confirm that Abs present in AS caused the altered IFN-γ responses, RSV and influenza virus were preincubated with IgG-depleted AS. Depletion of IgG significantly reduced the number of IFN-γ producing cells specific for RSV. In contrast, the IFN-γ response against influenza virus, which was strongly suppressed in the presence of AS, increased after IgG depletion (Fig. 1A).

To determine a possible role for complement during the Ag-presentation process, we first used heat-inactivated human serum to repeat IFN-γ ELISPOT assays. Fig. 1B (a representative experiment with one donor) and Fig. 1C (the mean of three individual donors) show that HI of human serum diminished the IFN-γ production induced by opsonized live and UV-inactivated RSV. Replenishing heat-inactivated serum with C1q, the initiator of the classical complement pathway, reconstituted the heat labile factor in serum. These experiments further showed that the increased T cell response in the presence of C1q depended on the presence of virus-specific serum Abs, because enhanced IFN-γ production was not observed when C1q was added to PBMCs stimulated with RSV in FCS (Fig. 1B) or in AS devoid of IgG (Fig. 1C). Fig. 1D shows that serum of all the adult donors contained RSV-specific (IgG) Abs, as measured by an RSV-specific ELISA using lysate of RSV-infected HEp-2 cells as a source of Ag. The polyclonal virus specific Abs found in adult donors potentially recognize neutralizing and nonneutralizing viral epitopes. We established the neutralizing capacity of the RSV-specific serum IgG Abs used in the ELISPOT experiments by testing the ability of the donor sera to block RSV infection of A549 lung epithelial cells and PBMCs. Using the same serum-to-virus ratio applied in the ELISPOT experiments, we observed a complete suppression of viral replication in A549 cells, as determined by real-time PCR (Fig. 1E). This neutralizing capacity was most likely a primary result of serum IgG. HI of AS had no effect on the RSV Ag recognition or neutralizing capacity of serum Abs (Fig. 1D, 1E). We further determined that replication was completely abrogated by UV treatment of the virus. In comparison to A549 cells, PBMCs were less permissive to viral infection (Fig. 1E), which could be explained in part by the fact that a smaller fraction of cells was infected. Specifically, confocal microscopy experiments (data not shown) revealed that only HLA class II-positive, mostly $CD14^+$ cells such as monocytes and possibly DCs (10-15% of PBMCs), were infected by RSV. In contrast to A549 cells, we detected low levels of RSV in PBMC samples exposed to serum-treated RSV, which might represent viral material internalized by APCs rather than viral infection. We found that IFN-γ responses were similar poststimulation with live and UV-inactivated RSV in ELISPOT assays performed in FCS and in AS (Supplemental Fig. 1 and data not shown).

AS enhances the IFN-γ response of RSV-specific $CD4^+$ T cells and decreases influenza virus and RSV-specific $CD8^+$ T cell activation.

Presentation of virus-derived antigenic peptides to $CD8^+$ T cells occurs after productive infection, whereby peptides derived from viral proteins synthesized in the cytoplasm are processed by proteasomes and translocated into the endoplasmic reticulum. Within the endoplasmic reticulum,

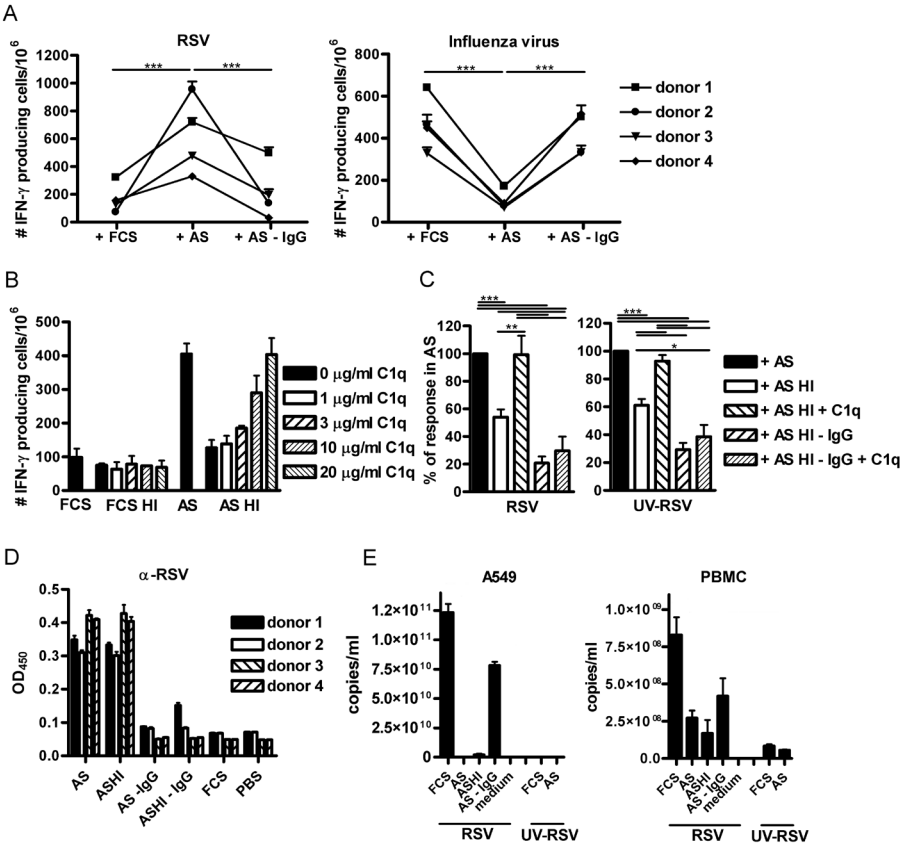


Figure 1. Virus-specific IgG enhances RSV-specific, and suppresses influenza virus-specific, T cell-mediated IFN-γ production.

A. Human PBMCs of four healthy donors were stimulated with RSV or influenza virus in the presence of FCS, AS, or IgG-depleted AS (AS – IgG) for 24 h. An IFN-γ ELISPOT was performed to quantify T cell activation. **B.** PBMCs were stimulated with RSV in the presence of FCS, FCS-HI, AS, or AS-HI for 24 h. Complement component C1q was added to FCS-HI and AS-HI in different concentrations: 0, 1, 3, 10 or 20 μg/ml. An IFN-γ ELISPOT was performed to quantify T cell activation; we depicted a representative experiment of one donor. **C.** RSV or UV-RSV was incubated with AS or AS-HI. AS-HI was either reconstituted with 75 μg/ml C1q, IgG-depleted, or IgG-depleted with the addition of 75 μg/ml C1q. These complexes were added to PBMCs in an IFN-γ ELISPOT for 24 h. The mean of 3 individual donors is shown. **D.** AS of the donors used in the ELISPOT experiments was analyzed for RSV-specific IgG levels with an ELISA on RSV-infected HEp-2 cell lysate. HI and IgG depletion (AS $^{+}$/- IgG and AS-HI $^{+}$/- IgG) were analyzed. **E.** RSV or UV-RSV was incubated with FCS, AS, AS-HI, or AS-IgG and added to either A549 cells or PBMCs for 24 h to analyze the neutralizing capacity of AS with real-time PCR performed on RSV nucleocapsid (N) gene. One representative experiment of one donor is depicted. Data from the ELISPOT experiments are presented as the number of IFN-γ producing cells/10^6 PBMCs with media control values subtracted. Significance was calculated with one-way ANOVA (A, C). Error bars represent the SEM of four individual donors (A) and three individual donors (C). In B, D, and E the error bars represent the SEM of a duplicate within one experiment. *p <0.05; **p < 0.01; ***p < 0.001.

viral peptides are loaded onto nascent HLA class I molecules (34). In contrast, the default route for HLA class II peptide presentation to $CD4^+$ T cells is internalization of antigenic material, followed by degradation in late endosomal compartments or lysosomes (35). A possible explanation for our contrasting observations with the RSV and influenza virus specific IFN-γ response in the presence of serum IgG could be the relative fraction of $CD4^+$ T cells and $CD8^+$ T cells in the antiviral memory pool. Thus, we hypothesized that neutralizing Abs in serum decreased influenza-specific $CD8^+$ T cell responses through inhibition of viral infection and HLA class I-mediated Ag presentation, whereas the RSV-specific $CD4^+$ T cell response was enhanced by Ab-mediated endosomal uptake and HLA class II Ag presentation.

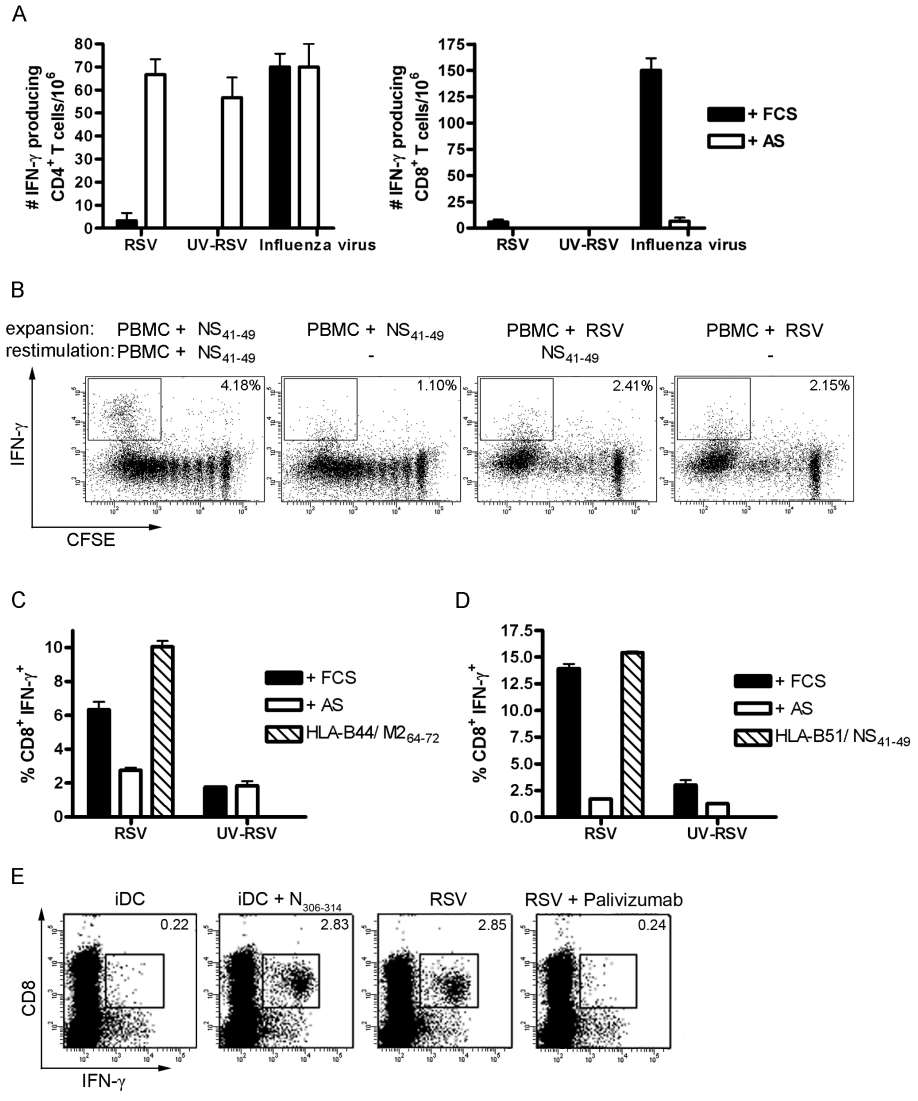
To test this hypothesis, we performed ELISPOT experiments using $CD4^+$ or $CD8^+$ T cells purified from PBMCs. The T cell subsets were stimulated with the monocyte/DC fraction of PBMCs from the same donor. The monocyte/DC fraction was exposed to live RSV, live influenza virus, or UV-inactivated RSV in the presence of AS or FCS. This experiment showed that the influenza virus-specific IFN-γ production by $CD8^+$ T cells was indeed strongly suppressed after exposure to serum treated and supposedly Ig-opsonized virus (Fig. 2A). $CD8^+$ T cell responses specific for RSV were absent or barely detectable by ELISPOT. For RSV, the IFN-γ response was dominated by $CD4^+$ T cells, and AS caused an increase in the RSV-specific IFN-γ production by these cells (Fig. 2A).

In earlier work, we and others have shown that RSV-specific memory $CD8^+$ T cells are present in

Figure 2. Human AS enhances Ag presentation to RSV-specific $CD4^+$ memory T cells and decreases specific $CD8^+$ T cell responses.

A. The enriched monocyte/DC fraction plus purified $CD4^+$ T cells, or purified $CD8^+$ T cells, from adult PBMCs were incubated with RSV, UV-RSV, or influenza virus in the presence of FCS or AS for 24 h. The number of IFN-γ-producing cells was analyzed with an ELISPOT assay. The experiment was repeated for two additional donors with similar results. One representative experiment of one donor was depicted. The data is presented as the number of IFN-γ-producing cells/10^6 purified $CD4^+$ or $CD8^+$ T cells with media control values subtracted. **B.** CFSE-labelled PBMCs (HLA-A2, -B7, -B51) were incubated with 1 μg/ml $NS_{41\text{-}49}$, a HLA-B51-restricted epitope from the non-structural protein NS1 or life RSV moi 2 for 7 d in the presence of rhIL-2. Expanded $CD8^+$ T cells were restimulated in the presence of Brefeldin A with 1 μg/ml $NS_{41\text{-}49}$ or left unstimulated. After 5 h, the percentage of IFN-γ-producing $CD8^+$ T cells was analyzed by intracellular staining and FACS analysis. Cells are gated on $CD8^+$ in the life gate. The experiment was repeated for two additional donors with similar results. One representative experiment of one donor is shown. PBMCs (HLA-A1, -A2, -B8, -B44, and D. HLA-A2, -A25, -B18, -B51) were incubated with 1 μg/ml $M2_{64\text{-}72}$, an HLA-B44-restricted epitope from the structural M2 protein of RSV **C.** or with 1 μg/ml $NS_{41\text{-}49}$ **D.** for 7 d at 37°C in the presence of rhIL-2. Expanded T cells were added to a 24-h culture of PBMCs of the same donor incubated with RSV or UV-RSV in the presence of FCS or AS. Restimulation with 1 μg/ml of the peptide used during expansion was applied as a positive control. After 24 h, the percentage of IFN-γ-producing $CD8^+$ T cells was analyzed by intracellular staining and FACS analysis. One representative experiment of one donor is shown. Similar results were obtained with two different donor/peptide combinations. **E.** PBMCs (HLA-A2, -B7, -B51) were incubated with 1 μg/ml $N_{306\text{-}314}$, an HLA-B7-restricted epitope from the structural Np protein of RSV, for 9 d at 37°C in the presence of rhIL-2. Immature monocyte-derived DCs were incubated with RSV or RSV opsonized by 5 μg/ml palivizumab for 48 h. Expanded $CD8^+$ T cells were added for 6 h in the presence of brefeldin A. Restimulation with 1 μg/ml of the peptide used during expansion was applied as a positive control. The percentage of IFN-γ-producing $CD8^+$ T cells was analyzed by intracellular staining and FACS analysis. Similar results were obtained with two different donor/peptide combinations. Error bars represent the SEM of a duplicate within one experiment.

adult PBMCs, although responses against single epitopes were low and, in most donors, not detectable without *in vitro* expansion (36-42). However, the virus-specific T cell response might be directed against a broad panel of HLA-presented peptides derived from the viral proteome (36, 39). In experiments presented in this study (Fig. 2A and data not shown), it also appeared that total RSV-specific $CD8^+$ T cells were low or undetectable. To exclude the possibility that technical reasons resulted in underestimated $CD8^+$ memory T cell numbers (i.e. that HLA class I presentation was somehow inefficient after RSV infection of the APC), we first expanded $CD8^+$ T cells specific for single viral epitopes. The ability of these T cell lines to respond to APCs loaded with the same synthetic peptide used for expansion or RSV-infected APCs was then evaluated. Figure 2B shows an



example of *in vitro* T cell expansion after stimulation of PBMCs with peptide $NS1_{41-49}$ derived from the nonstructural protein NS1, a peptide that is presented in the context of HLA-B51 (36). More than eight cell divisions were required to detect substantial numbers of IFN-γ-producing $CD8^+$ T cells in PBMCs. Similar observations were made for epitopes derived from structural proteins (M, Np, M2, data not shown). Expansion of $NS1_{41-49}$ specific $CD8^+$ T cells performed with live RSV was less efficient than expansion with the synthetic peptide. This finding illustrates the well documented inhibitory effect of RSV infection on T cell proliferation (28, 36, 43-46). Using expanded virus-specific $CD8^+$ T cell populations, we found similar IFN-γ responses induced by HLA class I molecules on both virus-infected APCs and peptide loaded APCs. Both the APC fraction in PBMCs (Fig. 2C, 2D) and monocyte-derived DCs (Fig. 2E) presented viral peptide to expanded $CD8^+$ T cells. This indicated that virus infection did not suppress HLA class I-mediated Ag presentation. Moreover, in contrast to the suppression of T cell proliferation, we found no indication for a suppressive effect of infectious virus on the production of IFN-γ by T cells.

Therefore, we concluded that results from IFN-γ ELISPOT assays showing low IFN-γ production from the RSV-specific $CD8^+$ T cell fraction could not be attributed to a lack of Ag presentation, but instead reflected a low frequency of RSV-specific $CD8^+$ memory T cells. Furthermore, RSV infection was required for effective HLA class I-mediated Ag presentation in the *in vitro* ELISPOT assay because the IFN-γ response of $CD8^+$ T cells was absent when APCs were stimulated with replication-defective UV-treated RSV or RSV in the presence of neutralizing serum Abs (Fig. 2C - 2E).

Activating FcγR are involved during Ag presentation to RSV-specific mouse $CD4^+$ T cells *in vitro* and *in vivo*.

To determine whether IgG plays a significant role during T cell activation *in vivo*, we initiated a series of experiments in mice. We first utilized an *in vitro* T cell stimulation assay to test preimmune serum and serum from mice obtained after secondary RSV infection. In these experiments, we used a mouse dendritic cell line, D1, as the APCs. As a source of polyclonal RSV-specific $CD4^+$ and $CD8^+$ T cells, lung cells were harvested at day 8 after a primary RSV infection administered by intranasal inoculation. T cell activation was measured by intracellular staining for IFN-γ. Similar to the results observed with human serum, we found that mouse immune serum, in comparison to preimmune serum, enhanced RSV-specific $CD4^+$ T cell responses (Fig. 3A). IgG depletion experiments confirmed the role of IgG.

To determine a possible role for FcγR in Ag uptake during virus specific Ag presentation, we proceeded with *in vitro* Ag presentation studies using BM-DCs from WT and $\gamma^{-/-}$ mice lacking expression of activating FcγRI, -III and -IV (Supplemental Fig. 2) (23). As shown in figure 3B, immune serum caused enhanced presentation of RSV Ag to virus-specific $CD4^+$ T cells when activating FcRs were expressed. In contrast, using DCs cultured from $\gamma^{-/-}$ bone marrow, $CD4^+$ T cell responses were unaltered when virus Ag was presented in the presence of immune serum as compared to preimmune serum. UV-inactivated RSV was used to completely rule out infection-related effects in these experi-

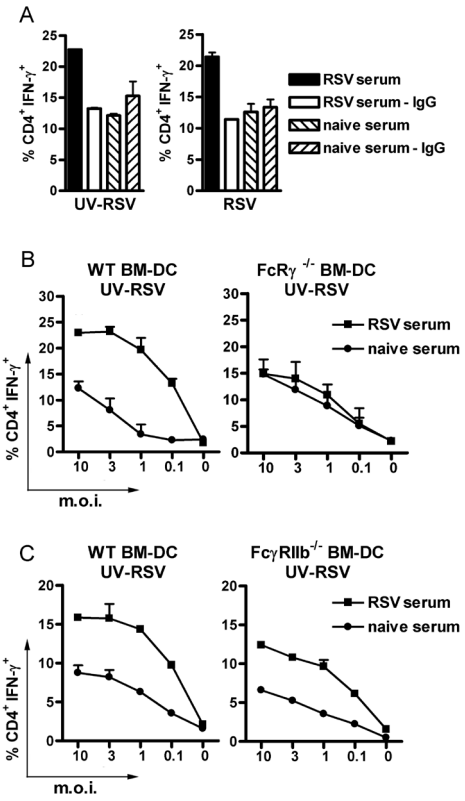


Figure 3. RSV-specific CD4+ T cell activation by mouse BM-DCs exposed to RSV immune complexes *in vitro* is enhanced by activating FcγRs.

A. Ag-presenting D1 cells were incubated with UV-RSV or RSV moi 1 in the presence of 2% serum of naïve mice or serum from RSV-infected mice or sera depleted for IgG. Lung cells of mice harvested 8 d after a primary RSV infection were used as a source of RSV-specific T cells. The percentage of responding CD4+ T cells was measured by intracellular staining for IFN-γ. BM-DCs from WT and $\gamma^{-/-}$ bone marrow **B.** and WT and FcγRIIb$^{-/-}$ **C.** bone marrow were incubated with UV-RSV (moi 0, 0.1, 1, 3 or 10) and serum from RSV-infected mice or serum from naïve mice for 24 h. Lung cells of mice 8 d after a primary RSV infection were harvested and added to the culture in the presence of brefeldin A. The percentage of IFN-γ-producing CD4+ T cells was analyzed by FACS. The experiments were performed twice (A) and three times (B, C) with similar results; one representative experiment is shown. Error bars represent the SEM of a duplicate within one experiment.

ments. Similar experiments performed with DCs derived from mice that lack expression of FcγRIIb, the only inhibitory FcγR in mice, did not show differences in Ag presentation in the presence of immune serum compared to WT BM-DCs (Fig. 3C). From these experiments, we concluded that activating FcγR played a role during Ag presentation when RSV specific Abs were present.

To test the relevance of these observations during *in vivo* T cell responses, we performed RSV infection studies in C57BL/6 WT, $\gamma^{-/-}$, and FcγRIIb$^{-/-}$ mice. T cell responses in the lungs of these mice were measured after ex vivo restimulation with RSV-infected and uninfected D1 cells. After secondary

RSV infections, similar RSV-specific Ab responses (IgG1 and IgG2c) were elicited in WT and $\gamma^{-/-}$ mice (Fig. 4A). RSV-specific $CD4^+$ T cell responses were significantly higher in WT compared to $\gamma^{-/-}$ mice (Fig. 4B), which was consistent with the enhanced Ag presentation observed during *in vitro* assays in the presence of immune serum. No differences were found in the level of the RSV-specific $CD4^+$ T cell response in $Fc\gamma RIIb^{-/-}$ mice compared to WT mice (Fig. 4B), while IgG2c Ab levels were slightly higher in the mice lacking the inhibiting FcγRIIb (Fig. 4A). During primary infection, no differences in immune responses were observed in WT, $\gamma^{-/-}$, or $Fc\gamma RIIb^{-/-}$ mice (data not shown).

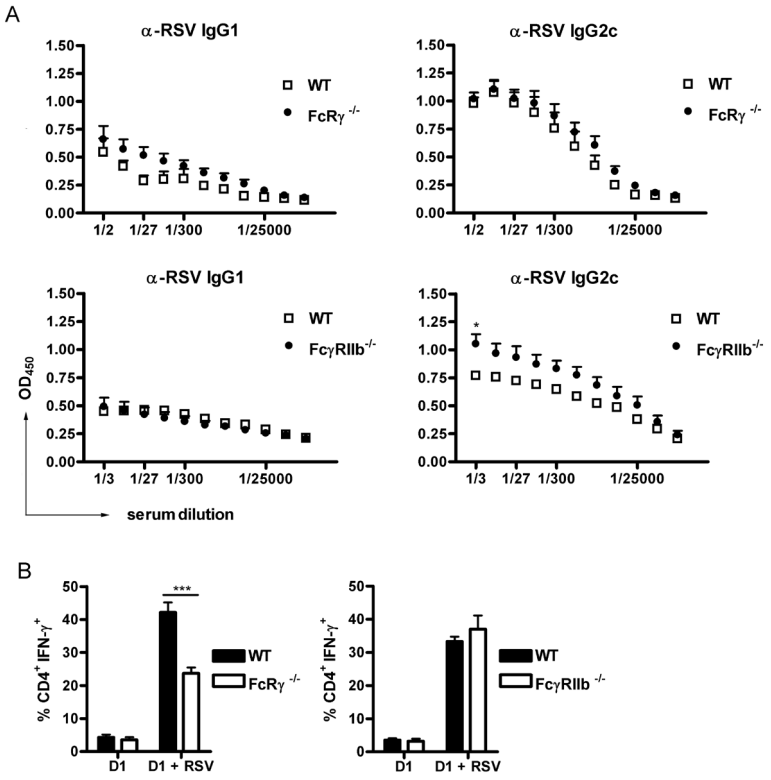


Figure 4. Activating FcγRs are involved during *in vivo* Ag presentation of RSV-derived Ag.

WT, $\gamma^{-/-}$, or $Fc\gamma RIIb^{-/-}$ mice were infected with RSV at day 0 and challenged with RSV at day 28. Six days postchallenge, blood was collected to analyze serum Abs, and lungs were analyzed for T cell responses. **A.** RSV specific IgG1 and IgG2c levels in serum (three-step serial dilution of serum starting at 3%). **B.** Percentage of IFN-γ–producing $CD4^+$ T cells in lung tissue responding to RSV-infected or uninfected D1 cells. Error bars represent the SEM of five individual mice per group. Results are shown for five mice per group of one representative experiment. In A, Significance was calculated using a two-way ANOVA. In B, significance was calculated using a Students t test. These experiments were performed three times with similar results. *$p < 0.05$; ***$p < 0.001$.

The ratio of neutralizing and nonneutralizing Abs in serum determines the activation of the CD8⁺ T cell response.

In different *in vivo* experiments, we observed a variable effect of virus-specific Abs on CD8⁺ T cell responses. These responses were sometimes equal and sometimes slightly lower in γ$^{-/-}$ mice compared to WT mice (two examples shown in supplemental Fig. 3). It is possible that RSV-specific Abs might affect RSV-specific CD8⁺ T cell responses *in vivo* via different mechanisms such as virus neutralization (resulting in lower antigenic load), altered innate immune responses, or Ab-mediated cross presentation of viral Ag. The resulting *in vivo* T cell response might therefore be determined by the sum of different effects of Abs that work synergistically or antagonistically. We tested different serum batches from immune mice in the *in vitro* Ag presentation assay and found that some sera inhibited *in vitro* CD8⁺ T cell responses while others did not. An example is shown in figure 5A, depicting immune sera A and B obtained from mice after secondary infection with RSV from different batches of virus. While both sera induced a similar IgG-mediated, enhanced RSV-specific CD4⁺

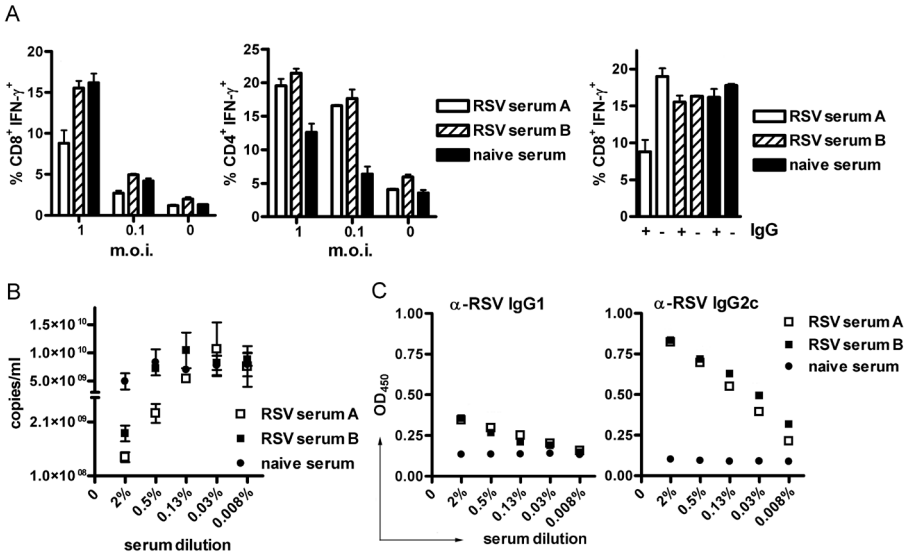


Figure 5. The ratio of neutralizing versus nonneutralizing Abs in serum determines the activation of CD8⁺ T cells.
A. D1 cells were incubated with RSV moi 1 and 0.1 in the presence of RSV mouse immune serum batch A or B or with serum from naïve mice +/- IgG depletion for 24 h. Lung cells from mice harvested 8 d after primary RSV infection were added to these APCs as a source of RSV-specific T cells. The percentage of IFN-γ-producing CD4⁺ and CD8⁺ T cells was analyzed by intracellular staining. RSV-immune serum batch A or B or serum from naïve mice were tested for virus-specific neutralizing capacity by PCR **B.** and virus-specific Ab levels by ELISA **C.** (four-step serial dilution of serum starting at 2%). To test neutralizing capacity of serum Abs, RSV was preincubated with a four-step serial dilution of the three serum batches starting at 2% before incubation with A549 cells. After 24 h, real-time PCR was performed on the RSV-N gene. Experiments were performed three times (A, B) and twice (C) with similar results, one representative experiment is shown. Error bars represent the SEM of a duplicate within one experiment.

T cell response, serum A decreased the CD8+ T cell response, whereas serum B did not change the CD8+ T cell response. The lower CD8+ T cell response found when RSV was opsonized with serum A was caused by the IgG fraction of serum (Fig. 5A). We reasoned that serum A might be a better neutralizing serum. Indeed, a virus neutralization assay confirmed that the neutralizing capacity of serum A was at least 4-fold stronger than the neutralizing capacity of serum B (Fig. 5B). Based on an RSV-specific ELISA, the virus-specific Ab levels were similar in both serum samples (Fig. 5C).

To confirm the different effects of neutralizing and nonneutralizing Abs on the activation of CD4+ and CD8+ T cells and to exclude a potential contribution of other serum components, we repeated *in vitro* T cell stimulation assays using two mAbs. We used nonneutralizing murine IgG1, specific for the G protein of RSV, and palivizumab, a strongly neutralizing humanized IgG1 Ab specific for the F protein (Fig. 6A). Despite their distinct origin, both Abs show similar binding affinities to FcRs. Again, both mAbs increased CD4+ T cell responses to a similar extent, whereas only opsonization with neutralizing palivizumab lowered the CD8+ T cell response (Fig. 6B). This result supports the conclusion that different ratios of neutralizing and nonneutralizing RSV-specific serum Abs might influence the relative ratio of RSV-specific CD4+ and CD8+ T cell responses.

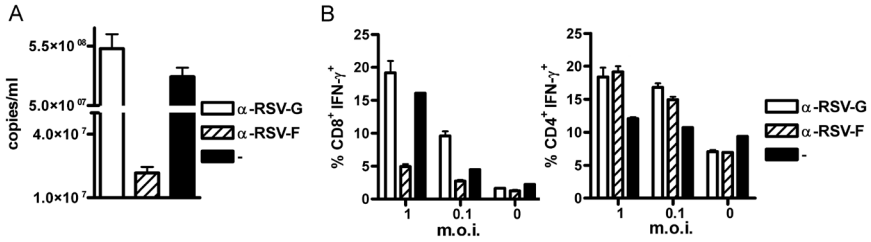


Figure 6. Neutralizing mAb, palivizumab, decreases RSV-specific CD8+ T cell responses and enhances CD4+ T cell activation.

A. RSV (moi 0.1) was preincubated for 15 min with 1 µg/ml anti-RSV-G or 1 µg/ml palivizumab before incubation with A549 cells and incubated for 24 h. Real-time PCR was performed on the RSV-N gene. **B.** D1 cells were incubated with RSV moi 1 or 0.1 in the presence of 1 µg/ml of anti-RSV-G or 1 µg/ml palivizumab for 24 h. Lung cells of mice were harvested 8 d after a primary RSV infection and added to the D1 cells in the presence of brefeldin A. The percentage of IFN-γ-producing CD4+ or CD8+ T cells was analyzed by FACS. These experiments were performed three times with similar results; one representative experiment shown. Error bars represent the SEM of a duplicate within one experiment.

Discussion

In the present work, we have shown that virus-specific Abs play a crucial role in the regulation of RSV-specific T cell responses. We base this conclusion on *in vitro* stimulation assays with human PBMCs and murine lung-derived effector T cells as well as experiments with an *in vivo* mouse model. Polyclonal virus-specific Abs generated *in vivo* are a complex mixture of neutralizing and nonneutralizing Abs. We showed that the ratio of neutralizing and nonneutralizing RSV-specific Abs in serum may affect the efficacy of Ag presentation to virus-specific CD4+ and CD8+ T cells.

Both neutralizing and nonneutralizing Abs enhanced Ag presentation to RSV-specific $CD4^+$ T cells, whereas neutralizing Abs lowered RSV- and influenza virus-specific $CD8^+$ T cell responses. Therefore, the presence of virus-specific maternal Abs in neonates before natural RSV infection, or induced by a RSV vaccination, might affect the activation of virus-specific T cells *in vivo*. In a vaccine trial with a formalin-inactivated RSV vaccine, poorly neutralizing RSV-specific Abs were induced (47-49), illustrating that during intervention with vaccines, Ab-mediated effects on T cell activation might differ from B and T cell responses during natural infection. Moreover, maternal Abs present during primary infections, or Abs delivered as a prophylactic, might affect the induction of the T cell response during natural infection.

From the present work, it appears that RSV-specific memory $CD8^+$ T cell numbers are low when compared to RSV-specific $CD4^+$ T cell numbers and influenza-specific memory $CD8^+$ T cell numbers. Extensive studies on the RSV-specific T cell immune status in healthy individuals are sparse. However, one elegant study by Lee *et al.* (12) compared $CD4^+$ and $CD8^+$ T cell responses specific for RSV and influenza virus in a group of healthy young adults and elderly people. Similar to our observations, this group found significantly higher ratios for IFN-γ-producing $CD4^+/CD8^+$ T cells specific for RSV than for influenza virus in both young and elderly groups. In contrast, during severe primary RSV infections, $CD8^+$ T cell responses dominate during the acute response (50). Because the immune status during the first year after primary infection and before a secondary RSV exposure has not been monitored, it is currently not clear whether a shift in the $CD4^+/CD8^+$ memory T cell ratio results from an ineffective differentiation or poor survival of RSV specific $CD8^+$ memory T cells. An alternative explanation could be that the switch in the $CD4^+/CD8^+$ memory T cell ratio occurs during multiple exposures to RSV in older children and adults. Our studies suggest that neutralizing RSV-specific Abs could potentially play a role in such a process. Because $CD8^+$ T cells are likely important for RSV clearance, diminished $CD8^+$ T memory cell numbers could be a reason why RSV-specific immune memory wanes and individuals become prone to reinfection.

In our studies, *in vitro* MHC class I-mediated Ag presentation presumably occurred via the classical proteasome-dependent pathway of Ag presentation that required viral infection of APCs (Fig. 2C, 2D). It is unclear how these *in vitro* observations might translate to the *in vivo* situation in humans. The route of viral entry and access of virus-derived antigenic material to different subsets of dendritic cells *in vivo* might differ from *in vitro* experiments performed with PBMC. Cross-presentation, the process by which acquired exogenous Ag gains access to the class I Ag-processing pathway of DC (34), did not play a significant role in the *in vitro* experiments (Fig. 2, 5). However, this process might contribute *in vivo* via uptake of infected epithelial cell debris or immune complexes by dendritic cell subsets specialized in cross presentation. In fact, our previous work in a murine intranasal RSV infection model showed that both RSV-infected lung-derived DCs and uninfected lymph node resident DCs presented Ag in the context of both MHC class I and class II molecules (51). Thus, uninfected lymph node resident DCs cross-presented virus Ag via MHC class I molecules and could therefore contribute *in vivo* to Ag presentation to $CD8^+$ T cells.

Dendritic cells specialized in class I cross presentation mainly reside in tissue draining lymph nodes

and spleen. Recently, human CD141$^+$ dendritic cells in peripheral blood, and closely related cells in lymph nodes, were found to be related to murine CD8$^+$ splenic DCs, a DC type specialized in cross presentation (52-55). However, the CD141$^+$ DC type in peripheral blood is present in extremely low numbers (0.03-0.05% of PBMCs) and might not play a significant role during the *in vitro* Ag presentation assays performed with human PBMCs.

The contribution of cross presentation might be important for effective activation and restimulation of RSV-specific CD8$^+$ T cell responses *in vivo*. We and others have shown that RSV infection of APCs results in impaired induction of T cell proliferation (28, 45, 46) (Fig. 2B). Therefore, it can be envisaged that for an effective *in vivo* expansion of RSV-specific CD4$^+$ and CD8$^+$ T cells, indirect routes of Ag presentation are most effective for efficient induction of T cell responses whereby DC acquire noninfectious material like opsonized virus particles or necrotic infected cells. Cross presentation is facilitated by stimulation of innate immune receptors expressed by DCs (56-60). The efficacy of the cross presentation route via MHC class I molecules, and the relative contribution of MHC class II presentation for *in vivo* RSV-specific T cell activation might depend on several factors. These include the local inflammatory milieu induced by RSV infection, the access route of viral material, and the innate immune process triggered by, for instance, Ab-opsonized virus or infected necrotic cells. Insight into these mechanisms is important in order to develop effective and safe intervention procedures for RSV disease.

Our experiments showed that FcγR and/or complement contributed to more effective RSV-specific CD4$^+$ T cell responses when virus was opsonized with Abs. These results indicated that the enhanced CD4$^+$ T cell response was caused by enhanced uptake and processing of viral antigenic material. Alternatively, C1q can induce cytokine production when added to APCs in the absence of immune complexes (61, 62). However, our experiments (Fig. 1B, 1C) showed that C1q did not contribute significantly to enhanced responses when IgGs were not present.

Most published work on CD8$^+$ T cell memory status in adults or effector CD8$^+$ T cell responses during primary disease in infants has been performed with expanded T cell populations, either using RSV-infected APCs or a-specific methods for T cell expansion (37-39, 41, 42). These methods confirm the presence of RSV CD8$^+$ T cells in individuals previously exposed to RSV, but are inadequate to measure the exact *in vivo* frequencies of these cells. IFN-γ ELISPOT assays performed on total PBMCs do not distinguish between the CD4$^+$ and CD8$^+$ T cells contributing to the response. Due to the low frequency of RSV-specific T cells in peripheral blood, intracellular cytokine staining methods are difficult to perform. Moreover, for effective expression of MHC class I-presented viral epitopes representing the total virus proteome, RSV infection of autologous APCs needs to be initiated at least 24 h before exposure to T cells. This makes such a procedure cumbersome for large studies.

Importantly, the present study underscores the crucial need to carefully develop *in vitro* T cell assays useful for monitoring RSV-specific T cell responses. Such assays could be employed for the evaluation of the efficacy of future RSV vaccine candidates. Furthermore, understanding the reason for frequent re-infections with RSV and the susceptibility of elderly people to severe morbidity caused by RSV infections requires careful analysis of the RSV-specific immune status of different

age groups. The dynamics of the antiviral adaptive immune responses during, and in between, RSV seasons should also be carefully considered. The observation that RSV-specific $CD8^+$ T cells might be present in low numbers in the peripheral memory pool and the role that virus-specific Abs could play during *in vivo* T cell activation are important issues that need to be understood in detail when considering the development of safe and effective vaccines and evaluating correlates of protection against severe RSV-induced respiratory infections.

Material and Methods

Mice

Pathogen-free 6-8-wk-old C57BL/6cjo wild-type (WT) mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). FcR common γ-chain-deficient ($\gamma^{-/-}$) mice and $Fc\gamma RIIb^{-/-}$ mice on a C57BL/6 background (22-24) were bred and maintained at the central animal facility at Utrecht University. The mouse study protocols were approved by the Animal Ethics Committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

Viruses and cell lines

RSV A2 strain was grown in HEp-2 cells, purified by polyethelene glycol precipitation, and stored in liquid nitrogen in 10% sucrose in PBS. The 50% tissue culture-infective dose was determined post-titration in HEp-2 cells. Influenza virus strain A Nanchang/933/95 (H3N2) was grown in fertilized chicken eggs. The 50% tissue culture-infective dose was determined by titration on Madin-Darby Canine Kidney cells (25).

HEp-2 and A549 cells were cultured in IMDM (21980-065, Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 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 (DC) line derived from C57BL/6 mice (26) used in Ag-presentation assays, was maintained in IMDM, 5% HyClone FCS (SH30080.03, Perbio, Lausanne, Switzerland), 1% penicillin/streptomycin, and 50 μM 2–ME supplemented with 30% conditioned medium from GM-CSF-producing R1 cells [mouse fibroblast NIH3T3, transfected with GM-CSF gene (26)].

IFN-γ ELISPOT

Human PBMC were isolated by Ficoll-Paque (17-1440-02, Pharmacia Biotech, Piscataway, NJ) gradient centrifugation. A total of 2×10^5 total PBMCs or 1×10^5 purified APCs plus 1×10^5 enriched $CD4^+$ or $CD8^+$ T cell populations were infected with RSV (multiplicity of infection [moi] 2), infected with influenza virus (moi 0.5) or stimulated with the same amount of virus that had been preincubated

15 min at 37°C with 10% autologous serum (AS) or FCS. To test the contribution of complement components, heat inactivation (HI) of AS was performed for 45 min at 57°C, and reconstitution experiments were performed by incubating preformed immune complexes for an additional 5 min at 37°C with C1q (C1740, Sigma-Aldrich, St. Louise, MO; amounts given in the figure legends). The amount of C1q necessary to reconstitute complement in heat-inactivated serum varied. We used 75 µg/ml routinely in experiments because this is an average physiological amount present in human serum. When indicated, IgGs were depleted using Protein G (P3296, Sigma-Aldrich).

An ELISPOT assay was performed as described before (27) to detect IFN-γ production by human PBMC or purified $CD4^+$ or $CD8^+$ T cells. Multiscreen-IP filter plates (MS1PN4510, Millipore, Bedfort, MA) were coated overnight with anti-IFN-γ coating Ab 1-D1K (100 µl, 15 µg/ml; 3002831, Mabtech, Nacka Strand, Sweden) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at 4°C. Before adding the cells, the plate was blocked for 1h at 37°C with RPMI 1640 (52400-041, Life Technologies) containing 10% FCS. After blocking, PBMC (2 x 10^5/well) were stimulated with virus preparations in 200 µl RPMI 1640 containing 5% FCS and penicillin/streptomycin for 24 h at 37°C 5% CO_2. Cells were removed, and 100 µl 1 µg/ml of detecting mAb 7-B6-1-biotin (3420-6-1000, Mabtech) in PBS-0.5% FCS was added for 2 h. After 2 h, 100 µl 1:1000 dilution of ExtraAvidine alkaline phosphatase conjugate (E-2636, Sigma-Aldrich) in PBS-0.5% FCS was added to the wells. Spots were visualized by adding 100 µl 5-bromo-4-chloro-3-indolylphosphate-NBT substrate (B5655, Sigma-Aldrich, one tablet dissolved in 10 ml H2O) per well.

MACS

To study $CD4^+$ and $CD8^+$ T cell activation in a human ELISPOT assay: 1) $CD4^+$: 2) $CD8^+$ T cells; and 3) APCs were negatively selected from total PBMCs using MACS. PBMCs were incubated with a mixture of FITC-labelled Abs at 4°C for 30 min in PBS with 0.5% BSA and 2 mM EDTA (MACS buffer): 1) anti-CD8 (clone SK1, BD Biosciences, San Jose, CA), anti-CD56 (clone NCAM16.2, BD Biosciences), anti-CD14 (clone MφP9, BD Biosciences), and anti-CD19 (clone 4G7, BD Biosciences); 2) anti-CD4 (clone SK3, BD Biosciences), anti-CD56 (clone NCAM16.2, BD Biosciences), anti-CD14 (clone MφP9, BD Biosciences), and anti-CD19 (BD, clone 4G7); and 3) anti-CD3 (SK7, BD Biosciences), anti-CD56 (clone NCAM16.2, BD Biosciences), and anti-CD19 (clone 4G7, BD Biosciences). After washing, the samples were incubated with 20 µl α-FITC beads/10^7 cells for 30 min at 4°C. The cells were washed and applied on a LD-column (130-042-901, Miltenyi Biotec, Auburn, CA) attached to a magnet. The flow-through were enriched $CD4^+$ T cells, $CD8^+$ T cells, or APCs (all populations were >90% pure). In some experiments, monocyte-derived DCs were used as APCs and were produced as described previously (28). In short, monocytes were isolated using CD14 beads (Miltenyi Biotec). Immature DCs were generated by culturing monocytes for 6 d in IMDM (21980-065, Life Technologies) supplemented with 10% FCS (HyClone, Breda, The Netherlands), 500 U/ml GM-CSF (a gift of Schering-Plough, Kenilworth, NJ), and 250 U/ml IL-4 (Strathmann, Biotec, Hamburg, Germany).

ELISA

ELISA plates (Nunc, Roskilde, Denmark) were coated with denatured RSV lysate from RSV-infected HEp-2 cells in PBS for 18 h at 4°C. Postremoval of unbound RSV lysate, plates were blocked with 200 µl 1% BSA in 0.05% $Tween_{20}$/PBS for 1 h at 37°C. A 10% serum of healthy donors, untreated, heat inactivated, and/or IgG depleted, diluted in 0.1% BSA/ 0.05% $Tween_{20}$/PBS, was added (25 µl/well). After 2 h incubation (room temperature [RT]) and washing (0.05% $Tween_{20}$ in PBS), plates were incubated with a secondary anti-human IgG (P040601, DakoCytomation, Carpinteria, CA) HRP-labelled Ab diluted in 0.1% BSA/0.05% $Tween_{20}$/PBS for 2 h at RT. Postremoval of the secondary Ab, the substrate 3,3',5,5'-tetramethylbenzidine (T3405, Sigma-Aldrich) in NaAc (pH 5.5) and H_2O_2 was added to the wells for 15 min. The enzymatic activity was stopped by 9.8% H_2SO_4 and measured at OD_{450}.
Mouse ELISAs were performed with serum of either naïve or RSV-infected mice diluted in 0.1% BSA/0.05% $Tween_{20}$/PBS. Serum batches from RSV-infected mice were prepared from blood derived 6 d after secondary RSV infection. Dilutions used in the assays were four-step serial dilutions starting at 2% or three-step serial dilutions starting at 3% as indicated in the figure legends. The secondary Abs were HRP-labelled Abs, anti-mouse IgG1 (04-6120, Invitrogen, Carlsbad, CA), or anti-mouse Ig2c (GG2c-90P, Immunology Consultant Laboratory, Newburg, OR) diluted in 0.1% BSA/0.05% $Tween_{20}$/PBS and incubated for 2 h at RT.

Human T cell expansion assay

RSV-specific $CD8^+$ T cells present in peripheral blood were expanded with or without prior CFSE labelling of PBMCs as described before (29). In short, PBMCs were washed twice with RPMI without FCS. 2.5 µM CFSE was added to 1 x 10^7 cells/ml for 10 min at 37°C. Cells were washed twice with cold RPMI 1640 (52400-041, Life Technologies) containing 5% FCS. A total of 1 x 10^6 CFSE-labelled PBMCs were stimulated with live RSV (moi 2) or with 1 µg/ml non-structural protein $(NS)_{41-49}$ in AIMV (Invitrogen) with 2% IgG-depleted heat-inactivated AS in the presence of 20 U/ml human (h)IL-2 (11147528001, Roche, Basel, Switzerland). After 24 h, cells stimulated with NS_{41-49} were restimulated with 1 µg/ml peptide/hIL-2. After 7 d, the expanded $CD8^+$ T cells were restimulated with 1 µg/ml peptide or not restimulated in the presence of 1 µg/ml anti-CD28 (340975, BD Biosciences), 1 µg/ml anti-CD49d (340976, , BD Biosciences), 20 U/ml hIL-2 (11147528001, Roche) and 10 µg/ml Brefeldin-A (B7651, Sigma-Aldrich). After 5 h at 37°C 5% CO_2, IFN-γ production by CFSE-labelled cells was analyzed by FACS staining as described below.
A total of 1 x 10^6 PBMCs were stimulated with 1 µg/ml $NS1_{41-49}$, 1 µg/ml $M2_{64-72}$ or 1 µg/ml $N_{306-314}$ in AIMV (Invitrogen) with 2% AB serum, 20 U/ml rhIL-2 (11147528001, Roche) and penicillin/streptomycin for 24 h at 37°C 5% CO_2. After 24 h, cells were restimulated with 1 µg/ml peptide/hIL-2. At day 8 ($NS1_{41-49}$ and $M2_{64-72}$) or at day 9 ($N_{306-314}$) poststimulation, expanded cells were harvested. $NS1_{41-49}$ and $M2_{64-72}$ expanded T cells were restimulated with freshly isolated PBMCs from the same donor incubated with RSV (moi 2) in the presence of FCS or AS for 24 h or 1 µg/ml of the corresponding pep-

tide ($NS1_{41-49}$ and $M2_{64-72}$) as a control for 1 h. $N_{306-314}$-expanded T cells were restimulated with monocyte-derived immature DCs incubated with RSV, RSV opsonized by 5 µg/ml palivizumab (Synagis, MedImmune) for 48 h, or with 1 µg/ml $N_{306-314}$ for 1 h. In both restimulation assays, expanded T cells were added in the presence of 1 µg/ml anti-CD49d (340976, BD Biosciences), 1 µg/ml anti-CD28 (340975, BD Biosciences), 20 U/ml hIL-2 (11147528001, Roche) and 10 µg/ml Brefeldin-A (B7651, Sigma-Aldrich). After an incubation period of 5 h at 37°C, intracellular cytokine staining was performed to analyze IFN-γ production by $CD8^+$ T cells. Cells were washed with PBS containing 2% FCS, 2 mM EDTA and 0.02% NaN_3 (FACS buffer) and stained for surface markers with anti-CD4 (clone SK3, BD Biosciences) and anti-CD8 (clone RPA-T8, BD Biosciences) Abs. Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (554722, BD Biosciences) solution and Perm/Wash buffer (554723, BD Biosciences). Intracellular cytokines were detected with anti-IFN-γ (clone 25723.11, BD Biosciences). Stained samples were acquired on a FACSCanto (BD Biosciences).

RSV-specific Real Time PCR

2 x 10^5 A549 cells or PBMCs were incubated with RSV or UV-inactivated RSV (UV-RSV; moi 2) in the presence of FCS or AS (fresh, heat inactivated, or IgG-depleted) as described above. The neutralizing capacity of mouse serum was analyzed by incubation of 5 x 10^4 A549 cells with RSV (moi 0.1) in the presence of four-step serial dilutions starting at 2% immune serum from RSV-infected mice, or preimmune serum from naïve mice. In parallel, RSV was preincubated with 1 µg/ml anti-F [humanized IgG1 mAb, Synagis, MedImmune (8)] or 1 µg/ml anti-G (mouse IgG1, MAB858-2-5, Chemicon International, Temecula, CA). After 24 h, total RNA was extracted from these cells using MagnaPure LC equipment, cDNA was synthesized, and viral loads were determined by real-time PCR as recently described (30). In short, RNA extraction was performed using a MagnaPure LC total nucleic acid kit (Roche Diagnostics Systems, Somerville, NJ). Extracted RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. RT inactivation was performed at 95°C for 5 min. Real-time PCR was performed with primers specific for the N gene of RSV: 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'; and RSB probe: 5'-TTCCCTTCCTAACCTGGACATAGCATATAACATACCT-3'. RT-PCR was performed with 20 µl cDNA, TaqMan universal PCR mastermix (Applied Biosystems), primers (RSA 900 nM each, RSB 300 nM each), and fluorogenic probes (58.3 and 66.7 nM for RSA and –B probes, respectively) labelled with the 5' reporter dye FAM and the 3' quencher dye TAMRA. Amplification and detection were performed with an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems) 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 cycle treshold values were compared with a standard curve of RSV A2.

Tissue sampling mice

Mice were sacrificed by i.p. injection of 300 µl pentobarbital. 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, Burgess Hill, U.K.) 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).

Mouse bone marrow-derived DC cultures

Bone marrow-derived DCs (BM-DCs) were prepared as described before (31). Bone marrow was depleted for erythrocytes using erythrocyte lysis mix (155 mM NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA [pH 7.4]). BM-DCs were enriched for 7 d in RPMI 1640 (61870-044, Life Technologies) supplemented with 2 mM L-Alanyl-Glutamine, 5% HyClone FCS (SH30080.03, Perbio), 1% penicillin/streptomycin and 50 µM 2-ME and as a source of GM-CSF, 30% culture supernatant from R1 cells [mouse fibroblast NIH3T3, transfected with GM-CSF gene (26)]. The percentage of $CD11c^+$ cells (routinely > 70%) and the expression of different FcRs was determined by staining with anti-CD11c (clone HL3, BD Biosciences), anti-FcγRI (clone X54-5/7.1, BD Biosciences), anti-FcγRII (clone Ly17.2), anti-FcγRII/III (clone 2.4G2, BD Biosciences), anti-FcγRIV [clone 9E9, (32)], PE isotype (mouse IgG1κ), Fitc isotype (rat IgG2b, BD Biosciences), and APC isotype (Armenian hamster IgG1) determined by analysis with a FACSCanto flow cytometer (BD Biosciences).

Mouse T cell activation assay

In vitro mouse Ag-presentation assays were performed with D1 cells and in experiments to study the role of FcγRs and FcγRIIb with BM-DCs cultured from bone marrow derived from knockout mice and compared with C57BL/6 WT BM-DCs.

To study Ag presentation of RSV immune complexes by BM-DC or D1 cells, RSV or UV-RSV at moi 10, 3, 1 or 0.1 were preincubated with either plasma derived from secondary RSV-infected mice, or naïve mice (preimmune serum) for 15 min at 37°C. When indicated, the IgG fraction was depleted from serum using protein G beads. In parallel, RSV or UV-RSV were preincubated with anti-F [humanized IgG1 monoclonal Ab, Synagis, MedImmune (8)] or anti-G (mouse IgG1, MAB858-2-5, Chemicon International). RSV immune complexes were incubated with 5 x 10^4 BM-DC/D1 cells per condition for 24 h. After 24 h, the APCs were incubated with 5 x 10^5 total lung cells in the presence of 25 U/ml recombinant hIL-2 (11147528001, Roche) and 10 µg/ml Brefeldin-A (B7651, Sigma) for 5 h at 37°C in 5% CO_2. Lung cells were obtained from C57BL/6 mice 8 d after primary RSV infection, at the peak of the T cell response (33). Ag presentation of RSV was analyzed by measuring IFN-γ production by lung $CD4^+$ and $CD8^+$ T cells by intracellular FACS staining. Cells were washed with FACS buffer and stained for surface markers with anti-CD8 (clone 53-6.7, BD Biosciences) and anti-

CD4 (clone RM4-5, BD Biosciences). Before intracellular staining, cells were fixed and permeabilized with CytoFix/CytoPerm (554722, BD Biosciences) solution and Perm/Wash buffer (554723, BD Biosciences). Intracellular cytokines were detected with anti-IFN-γ (clone XMG1.2, BD Biosciences). Stained samples were acquired on a FACSCanto flow cytometer (BD Biosciences), and the data was analyzed using FACSDiva software (BD Biosciences).

In vivo RSV infection experiments

Mice were lightly anesthetized with isoflurane and intranasally infected with 2 to 3 x 10^6 PFU RSV in a volume of 50 µl at day 0. At day 28, mice were challenged with RSV. Six days postchallenge, T cell responses in the lung were analyzed. Single-cell suspensions of lung cells (10^6) from RSV-infected WT, $\gamma^{-/-}$, and FcγRIIb$^{-/-}$ mice were stimulated for 5 h at 37°C, 5% CO_2, with 2 x 10^5 RSV-infected D1 cells or uninfected D1 cells in 200 µl IMDM (21980-065, Life Technologies) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 5% FCS, penicillin/streptomycin, 50 µM 2-ME, and 25 U/mL rhIL-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. D1 cells were infected for 48 h with RSV moi 2 before addition to the lung cell suspension. IFN-γ production by $CD4^+$ and $CD8^+$ T cells was analyzed by intracellular cytokine staining.

Statistical analysis

Data were analyzed for statistical significance using a Students t test or ANOVA, as indicated in the figure legends. Data are expressed as the mean $^+$/- SEM. A p value <0.05 was taken as the level of significance.

References

1. Falsey, A. R., P. A. Hennessey, M. A. Formica, C. Cox, and E. E. Walsh. 2005. Respiratory syncytial virus infection in elderly and high-risk adults. N. Engl. J. Med. 352: 1749-1759.
2. Glezen, P., and F. W. Denny. 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. 288: 498-505.
3. 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.
4. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163: 693-698.
5. Ogilvie, M. M., A. S. Vathenen, M. Radford, J. Codd, and S. Key. 1981. Maternal antibody and respiratory syncytial virus infection in infancy. J. Med. Virol. 7: 263-271.
6. Lamprecht, C. L., H. E. Krause, and M. A. Mufson. 1976. Role of maternal antibody in pneumonia and bronchiolitis due to respiratory syncytial virus. J. Infect. Dis. 134: 211-217.
7. Bruhn, F. W., and A. S. Yeager. 1977. Respiratory syncytial virus in early infancy. Circulating antibody and the severity of infection. Am. J. Dis. Child 131: 145-148.
8. The IMpact-RSV Study Group. 1998. Palivizumab, a Humanized Respiratory Syncytial Virus Monoclonal Antibody, Reduces Hospitalization From Respiratory Syncytial Virus Infection in High-risk Infants. Pediatrics 102: 531-537.
9. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315: 77-81.
10. Fishaut, M., D. Tubergen, and K. McIntosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96: 179-186.
11. de Bree, G. J., J. Heidema, E. M. van Leeuwen, G. M. van Bleek, R. E. Jonkers, H. M. Jansen, R. A. van Lier, and T. A. Out. 2005. Respiratory syncytial virus-specific $CD8^+$ memory T cell responses in elderly persons. J. Infect. Dis. 191: 1710-1718.
12. Lee, F. E., E. E. Walsh, A. R. Falsey, N. Liu, D. Liu, A. Divekar, J. E. Snyder-Cappione, and T. R. Mosmann. 2005. The balance between influenza- and RSV-specific CD4 T cells secreting IL-10 or IFNgamma in young and healthy-elderly subjects. Mech. Ageing Dev. 126: 1223-1229.
13. Looney, R. J., A. R. Falsey, E. Walsh, and D. Campbell. 2002. Effect of aging on cytokine production in response to respiratory syncytial virus infection. J. Infect. Dis. 185: 682-685.
14. Polack, F. P., M. N. Teng, P. L. Collins, G. A. Prince, M. Exner, H. Regele, D. D. Lirman, R. Rabold, S. J. Hoffman, C. L. Karp, S. R. Kleeberger, M. Wills-Karp, and R. A. Karron. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. J. Exp. Med. 196: 859-865.
15. de Jong, J. M., D. H. Schuurhuis, A. Ioan-Facsinay, M. M. Welling, M. G. Camps, E. I. van der Voort, T. W. Huizinga, F. Ossendorp, J. S. Verbeek, and R. E. Toes. 2006. Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4 T cells upon immune-complex uptake *in vivo*. Immunology 119: 499-506.
16. Hamano, Y., H. Arase, H. Saisho, and T. Saito. 2000. Immune complex and Fc receptor-mediated augmentation of antigen presentation for *in vivo* Th cell responses. J. Immunol. 164: 6113-6119.
17. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 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.
18. Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$

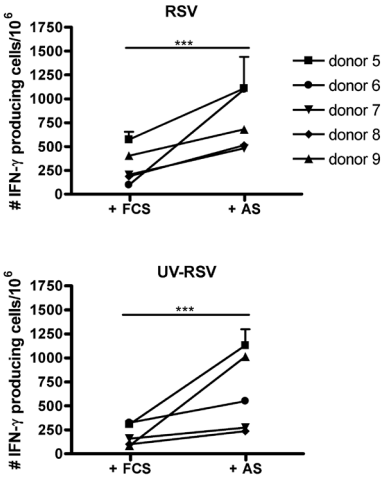
CTL responses *in vivo*. J. Immunol. 168: 2240-2246.
19. van Montfoort, N., J. M. de Jong, D. H. Schuurhuis, E. I. van der Voort, M. G. Camps, T. W. Huizinga, C. van Kooten, M. R. Daha, J. S. Verbeek, F. Ossendorp, and R. E. Toes. 2007. A novel role of complement factor C1q in augmenting the presentation of antigen captured in immune complexes to $CD8^+$ T lymphocytes. J. Immunol. 178: 7581-7586.
20. Klein Klouwenberg P., L. Tan, W. Werkman, G. M. van Bleek, and F. Coenjaerts. 2009. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. Crit Rev. Immunol. 29: 531-550.
21. Tripp, R. A., L. P. Jones, L. M. Haynes, H. Zheng, P. M. Murphy, and L. J. Anderson. 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat. Immunol. 2: 732-738.
22. Wernersson, S., M. C. Karlsson, J. Dahlstrom, R. Mattsson, J. S. Verbeek, and B. Heyman. 1999. IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. J. Immunol. 163: 618-622.
23. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 76: 519-529.
24. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. Nature 379: 346-349.
25. Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus. 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication *in vitro*. J. Virol. Methods 74: 57-66.
26. 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.
27. van Bleek, G. M., M. C. Poelen, R. van der Most, H. F. Brugghe, H. A. Timmermans, C. J. Boog, P. Hoogerhout, H. G. Otten, and C. A. van Els. 2003. Identification of immunodominant epitopes derived from the respiratory syncytial virus fusion protein that are recognized by human CD4 T cells. J. Virol. 77: 980-988.
28. de Graaff, P. M., E. C. de Jong, T. M. van Capel, M. E. van Dijk, P. J. Roholl, J. Boes, W. Luytjes, J. L. Kimpen, and G. M. van Bleek. 2005. Respiratory syncytial virus infection of monocyte-derived dendritic cells decreases their capacity to activate CD4 T cells. J. Immunol. 175: 5904-5911.
29. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods 171: 131-137.
30. 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.
31. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 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.
32. Nimmerjahn, F., P. Bruhns, K. Horiuchi, and J. V. Ravetch. 2005. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity. 23: 41-51.
33. 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.
34. Gromme, M., and J. Neefjes. 2002. Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. Mol. Immunol. 39: 181-202.
35. van Niel, G., R. Wubbolts, and W. Stoorvogel. 2008. Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. Curr. Opin. Cell Biol. 20: 437-444.
36. Heidema, J., G. J. de Bree, P. M. de Graaff, W. W. van Maren, P. Hoogerhout, T. A. Out, J. L. Kimpen, and G. M. van Bleek. 2004. Human $CD8(^+)$ T cell responses against five newly identified respiratory syncytial virus-derived

epitopes. J. Gen. Virol. 85: 2365-2374.

37. de Waal, L., Y. Suzer, L. S. Wyatt, K. Sintnicolaas, G. Sutter, B. Moss, A. D. Osterhaus, and R. L. de Swart. 2006. T cell responses to respiratory syncytial virus fusion and attachment proteins in human peripheral blood mononuclear cells. Viral Immunol. 19: 669-678.
38. Mbawuike, I. N., J. Wells, R. Byrd, S. G. Cron, W. P. Glezen, and P. A. Piedra. 2001. HLA-restricted $CD8^+$ cytotoxic T lymphocyte, interferon-gamma, and interleukin-4 responses to respiratory syncytial virus infection in infants and children. J. Infect. Dis. 183: 687-696.
39. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66: 2102-2110.
40. Goulder, P. J., F. Lechner, P. Klenerman, K. McIntosh, and B. D. Walker. 2000. Characterization of a novel respiratory syncytial virus-specific human cytotoxic T-lymphocyte epitope. J. Virol. 74: 7694-7697.
41. Brandenburg, A. H., L. de Waal, H. H. Timmerman, P. Hoogerhout, R. L. de Swart, and A. D. Osterhaus. 2000. HLA class I-restricted cytotoxic T-cell epitopes of the respiratory syncytial virus fusion protein. J. Virol. 74: 10240-10244.
42. Isaacs, D., C. R. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 2: 769-771.
43. Schlender, J., G. Walliser, J. Fricke, and K. K. Conzelmann. 2002. Respiratory syncytial virus fusion protein mediates inhibition of mitogen-induced T-cell proliferation by contact. J. Virol. 76: 1163-1170.
44. Salkind, A. R., D. O. McCarthy, J. E. Nichols, F. M. Domurat, E. E. Walsh, and N. J. Roberts, Jr. 1991. Interleukin-1-inhibitor activity induced by respiratory syncytial virus: abrogation of virus-specific and alternate human lymphocyte proliferative responses. J. Infect. Dis. 163: 71-77.
45. Gonzalez, P. A., C. E. Prado, E. D. Leiva, L. J. Carreno, S. M. Bueno, C. A. Riedel, and A. M. Kalergis. 2008. Respiratory syncytial virus impairs T cell activation by preventing synapse assembly with dendritic cells. Proc. Natl. Acad. Sci. U. S. A 105: 14999-15004.
46. Chi, B., H. L. Dickensheets, K. M. Spann, M. A. Alston, C. Luongo, L. Dumoutier, J. Huang, J. C. Renauld, S. V. Kotenko, M. Roederer, J. A. Beeler, R. P. Donnelly, P. L. Collins, and R. L. Rabin. 2006. Alpha and lambda interferon together mediate suppression of CD4 T cells induced by respiratory syncytial virus. J. Virol. 80: 5032-5040.
47. Murphy, B. R., and E. E. Walsh. 1988. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. J. Clin. Microbiol. 26: 1595-1597.
48. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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: 405-421.
49. Delgado, M. F., S. Coviello, A. C. Monsalvo, G. A. Melendi, J. Z. Hernandez, J. P. Batalle, L. Diaz, A. Trento, H. Y. Chang, W. Mitzner, J. Ravetch, J. A. Melero, P. M. Irusta, and F. P. Polack. 2009. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat. Med. 15: 34-41.
50. Lukens, M. V., A. C. van de Pol, F. E. Coenjaerts, N. J. Jansen, V. M. Kamp, J. L. Kimpen, J. W. Rossen, L. H. Ulfman, C. E. Tacke, M. C. Viveen, L. Koenderman, T. F. Wolfs, and G. M. van Bleek. 2010. A systemic neutrophil response precedes robust $CD8^{(+)}$ T-cell activation during natural respiratory syncytial virus infection in infants. J. Virol. 84: 2374-2383.
51. 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.
52. Bachem, A., S. Guttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, A. Salama, K. Movassaghi, C. Opitz, H. W. Mages, V. Henn, P. M. Kloetzel, S. Gurka, and R. A. Kroczek. 2010. Superior antigen cross-presentation and XCR1 expression define human $CD11c^+CD141^+$ cells as homologues of mouse $CD8^+$ dendritic cells. J. Exp. Med. 207: 1273-1281.

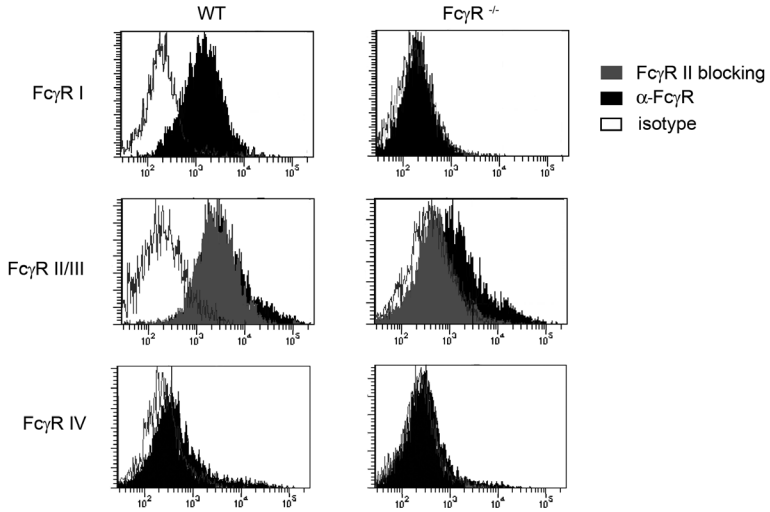
53. Crozat, K., R. Guiton, V. Contreras, V. Feuillet, C. A. Dutertre, E. Ventre, T. P. Vu Manh, T. Baranek, A. K. Storset, J. Marvel, P. Boudinot, A. Hosmalin, I. Schwartz-Cornil, and M. Dalod. 2010. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha$^+$ dendritic cells. J. Exp. Med. 207: 1283-1292.
54. Jongbloed, S. L., A. J. Kassianos, K. J. McDonald, G. J. Clark, X. Ju, C. E. Angel, C. J. Chen, P. R. Dunbar, R. B. Wadley, V. Jeet, A. J. Vulink, D. N. Hart, and K. J. Radford. 2010. Human CD141$^+$ (BDCA-3)$^+$ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J. Exp. Med. 207: 1247-1260.
55. Poulin, L. F., M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J. L. Chen, A. M. Keller, O. Joffre, S. Zelenay, E. Nye, A. Le Moine, F. Faure, V. Donckier, D. Sancho, V. Cerundolo, D. Bonnet, and C. Reis e Sousa. 2010. Characterization of human DNGR-1$^+$ BDCA3$^+$ leukocytes as putative equivalents of mouse CD8alpha$^+$ dendritic cells. J. Exp. Med. 207: 1261-1271.
56. Blander, J. M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature 440: 808-812.
57. Frleta, D., C. I. Yu, E. Klechevsky, A. L. Flamar, G. Zurawski, J. Banchereau, and A. K. Palucka. 2009. Influenza virus and poly(I:C) inhibit MHC class I-restricted presentation of cell-associated antigens derived from infected dead cells captured by human dendritic cells. J. Immunol. 182: 2766-2776.
58. Lopez-Albaitero, A., R. Mailliard, T. Hackman, P. A. Andrade Filho, X. Wang, W. Gooding, S. Ferrone, P. Kalinski, and R. L. Ferris. 2009. Maturation pathways of dendritic cells determine TAP1 and TAP2 levels and cross-presenting function. J. Immunother. 32: 465-473.
59. P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 433: 887-892.
60. van Montfoort, N., M. G. Camps, S. Khan, D. V. Filippov, J. J. Weterings, J. M. Griffith, H. J. Geuze, T. van Hall, J. S. Verbeek, C. J. Melief, and F. Ossendorp. 2009. Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc. Natl. Acad. Sci. U. S. A 106: 6730-6735.
61. Csomor, E., Z. Bajtay, N. Sandor, K. Kristof, G. J. Arlaud, S. Thiel, and A. Erdei. 2007. Complement protein C1q induces maturation of human dendritic cells. Mol. Immunol. 44: 3389-3397.
62. Baruah, P., I. E. Dumitriu, T. H. Malik, H. T. Cook, J. Dyson, D. Scott, E. Simpson, and M. Botto. 2009. C1q enhances IFN-gamma production by antigen-specific T cells via the CD40 costimulatory pathway on dendritic cells. Blood 113: 3485-3493.

Supplementary figures



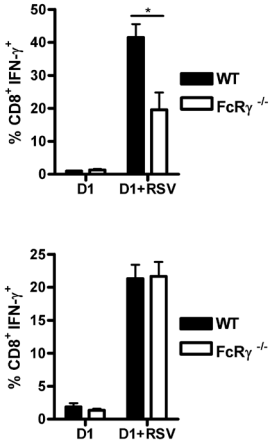
Supplementary figure 1. AS enhances IFN-γ production by PBMCs stimulated with both untreated RSV and UV-inactivated RSV to a similar extend.

Human PBMCs of five healthy donors (different from the donors represented in Fig. 1) were stimulated with RSV or UV-inactivated RSV in the presence of FCS or AS. An IFN-γ ELISPOT was performed to quantify T cell activation. The data is presented as the number of IFN-γ-producing cells/10^6 PBMCs with media control values subtracted. Significance was calculated using Students' t test. Error bars represent SEM of five individual donors.



Supplementary figure 2. WT BM-DCs express FcγRI, -II, -III and -IV while BM-DCs from γ$^{-/-}$ mice only express FcγRII.

The MFI of the anti-FcR Abs was compared to an isotype control by FACS analysis. One representative staining.



Supplementary figure 3. The involvement of activating FcγR during *in vivo* Ag presentation of RSV-derived Ag to CD8⁺ T cells.

WT or $\gamma^{-/-}$ mice were infected with RSV at day 0 and challenged with RSV at day 28. Six days postchallenge, lungs were analyzed for the percentage of RSV specific IFN-γ-producing $CD8^+$ T cells. Error bars represent the SEM. Results are shown for five mice per group. Significance was calculated using Students' t test.

chap

ter 4

Intranasal administration of antibody-opsonized Respiratory Syncytial Virus particles efficiently primes immune responses in mice.

Debby Kruijsen[1], Helga Einarsdottir[2,], Marcel A. Schijf[1,3,*], Frank E. Coenjaerts[4], Ellen C. van der Schoot[2], Gestur Vidarsson[2] and Grada M. van Bleek[1]*

[1]Department of Pediatrics, the Wilhelmina Children's Hospital, University Medical Center, Utrecht,
[2]Department of Experimental Immunohematology, Sanquin Research, Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam,
[3]Department of Immunology, Danone Research - Centre for Specialised Nutrition, Wageningen,
[4]Department of Medical Microbiology, University Medical Center, Utrecht, The Netherlands.

[*]Helga Einarsdottir and Marcel A. Schijf contributed equally to this work.

Submitted

Abstract

Infants are protected from a severe RSV infection in the first months of life by maternal antibodies or by prophylactic administered virus specific neutralizing antibodies. To evaluate the role of pre-existing antibodies on T cell priming, we studied the effect of a virus specific neutralizing antibody, palivizumab on RSV antigen presentation, when antibody opsonized particles were administered via the intranasal route in a C57BL/6 mouse model. We found substantial virus specific $CD4^+$ and $CD8^+$ T cell priming and B cell responses in intranasal RSV-IC primed mice compared to mice primed with non-opsonized RSV. Upon live virus challenge RSV-IC treated mice responded with predominantly Th1-type $CD4^+$ T cell responses (IFN-γ^+, accompanied by IgG2c antibody responses) with a minor component of Th2 cytokine (IL-4^+, IL-13^+, IL-5^+) producing $CD4^+$ T cells. Furthermore, administration of RSV-IC resulted in activation of a broad repertoire of RSV specific $CD8^+$ T cells. The activation of adaptive immune responses was independent of FcRn excluding a role of FcRn-mediated antigen processing or FcRn mediated translocation of viral materials across airway epithelium in this setting.

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 direct 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 (1-4) and potentially enhance infection (5-8).

Children experiencing a primary RSV infection are protected against lower respiratory tract infections (LRTI) by maternal antibodies. However, maternal antibodies decline rapidly within a few months after birth (9), 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 re-infections (10). Reduced titers of serum antibodies were shown to correlate with increased RSV associated hospitalization in patients of all ages (10-13). Thus neutralizing antibodies provide partial protection to RSV (re-)infections. Based on these observations current vaccine development is focussed on a vaccine preparation that induces the production of highly neutralizing antibodies. Moreover, palivizumab, a highly neutralizing antibody to the fusion protein of RSV, is administered prophylactically to protect high risk infants against LRTI (14).

The presence of RSV-specific antibodies although protective against (primary) RSV infections might interfere with the induction of an immune response to an administered vaccine preparation or a natural infection. It is therefore essential to understand the consequences of the presence of antibodies *in vivo* on the outcome of the immune response upon infection. We showed in previous work that the neutralizing capacity of RSV-specific antibodies influenced the level of virus specific $CD8^+$ and $CD4^+$ T cell responses in a murine *in vivo* infection model and *in vitro* in experiments performed with human PBMCs (15). Highly neutralizing antibodies decreased the $CD8^+$ T cell response while both neutralizing and non-neutralizing antibodies increased the $CD4^+$ T cell response. Antigen presentation of RSV immune complexes (IC) to $CD4^+$ T cells was mediated via activating Fcγ-Receptors (FcγR) expressed by antigen presenting cells.

Recently, the neonatal FcR (FcRn) an Ig/Fc binding protein predominantly located in endosomal compartments was found to participate in antigen presentation of antigens internalized as IC. The function of FcRn was first described as a transporter of IgG across epithelial barriers including transmission of IgG across the placenta from mother to infant (16) and epithelial layers in airways and gut (17, 18). In addition, FcRn mediates recycling of internalized albumin and IgG to prevent lysosomal degradation in a pH dependent manner (19) and thereby increases the serum half life of these molecules significantly. However, FcRn was also shown to be highly expressed in myeloid cells, enhancing phagocytosis of IC in neutrophils and monocytes, and playing a role during antigen presentation of soluble antigens opsonized by IgGs by dendritic cells (20, 21). FcRn preferably directed multimeric IC consisting of large globular proteins opsonized by multiple IgGs to lyso-

somes for initiation of antigen presentation, while monomeric IgG-antigen complexes were not efficiently processed.

In the present work we studied the role of FcRn in RSV antigen presentation *in vivo* mimicking the situation of pre-existing maternal antibodies or prophylactically administered neutralizing antibodies. We show that intranasal administration of RSV-IC primed both virus specific T and B cell response while priming with non-opsonized RSV did not occur. However, priming of the immune response via the intranasal route with RSV-IC was independent of FcRn, excluding a role of FcRn-mediated antigen processing or FcRn mediated translocation across airway epithelium in this setting.

Results

H-2A^b mediated antigen presentation of RSV-IC by DC is facilitated by activating FcγRs while FcRn is not involved.

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 (15). To determine whether also FcRn 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 as antigen presenting cells BM-DCs obtained from WT, FcRn$^{-/-}$ and as a control γ$^{-/-}$ mice. FcRn expression levels were determined by RT-PCR in WT and FcRn$^{-/-}$ BM-DC and D1 cells (supplementary figure 1). 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-γ. A similar increased percentage of IFN-γ producing CD4$^+$ T cells was observed when WT BM-DCs or BM-DC from FcRn$^{-/-}$ mice were pulsed with RSV in the presence of immune serum derived from mice after secondary RSV infection as compared to the response of pre-immune serum (Figure 1). However, unlike FcRn, the IgG-mediated enhanced antigen presentation of RSV-IC to CD4$^+$ T cells was completely dependent on functional expression of FcγRs on BM-DCs (Figure 1).

To confirm that FcRn is not involved during antigen presentation of RSV-IC we repeated antigen presentation assays using monoclonal antibodies mutated at a single amino acid residue (H435A) or three residues (I253A, H310A and H345A; IHH) in the Fc domain, positions involved in FcRn binding, but not interfering with binding to FcγRs (20, 29, 31). The antibody Fab domain was specific for TNP. TNP-labelled RSV opsonized with recombinant WT IgG1 or mutant IgG1 monoclonal antibodies was incubated with D1, a DC cell line expressing FcRn (supplementary figure 1), 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 (Figure 2). These results confirmed that in this experimental setup FcRn was not involved in RSV-IC antigen presentation by DC to virus specific CD4$^+$ T cells.

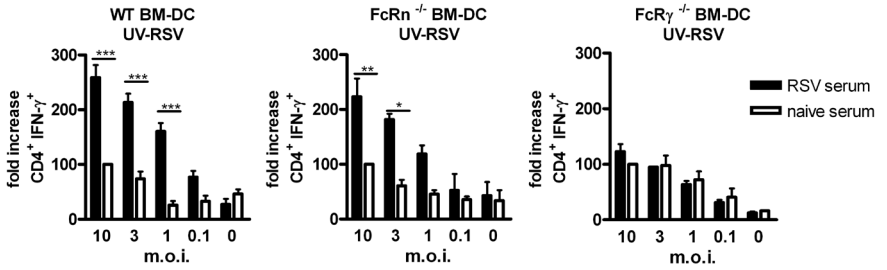


Figure 1. Activating FcγRs, but not FcRn, expressed by bone marrow derived DCs are involved in antigen presentation of RSV-IC to RSV-specific CD4+ T cells.

BM-DCs obtained from bone marrow of WT, FcRn$^{-/-}$ and γ$^{-/-}$ mice were incubated with UV-RSV (m.o.i. 10, 3, 1, 0.1 and 0) in the presence of 2% serum of naïve mice or serum from RSV immune mice for 24h. Lung cells of mice harvested 8 days after a primary RSV infection (as a source of RSV-specific T cells) were added to the culture in the presence of Brefeldin-A. Responding CD4+ T cells were visualized by intracellular staining for IFN-γ. Data are depicted as fold increase compared to the fraction of CD4+ T cells responding in naïve serum at m.o.i. 10. The experiments were performed three times with similar results. Significance was calculated using a Two-way ANOVA. Error bars represent the SEM on data out of two individual experiments. *p<0.05, **p<0.01 and ***p<0.001.

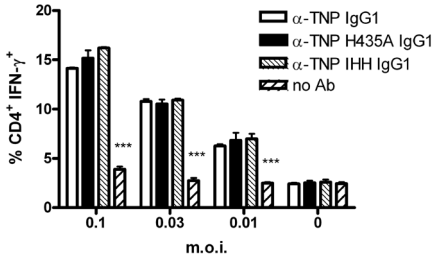


Figure 2. Abrogated binding of RSV-IC to FcRn does not affect antigen presentation to RSV-specific CD4+ T cells.

Antigen presenting D1 cells were incubated with RSV-TNP (m.o.i. 0.1, 0.03, 0.01 and 0) in the presence of 0.1 µg/ml anti-TNP IgG1, 0.1 µg/ml anti-TNP H435A mutant IgG1 or 0.1 µg/ml anti-TNP IHH mutant IgG1 (IHH: I253A, H310A and H435A) for 24h. Lung cells of mice harvested 8 days after a primary RSV infection (as a source of RSV-specific T cells) were added to the culture in the presence of Brefeldin-A. The percentage of responding CD4+ T cells was measured by intracellular staining for IFN-γ. The experiment was performed three times with similar results, one representative experiment is shown. Significance was calculated using a Two-way ANOVA. Error bars represent the SEM of a duplicate within one experiment. ***p<0.001 compared to all three antibody-mediated responses.

Non-infectious RSV-IC efficiently prime *in vivo* virus specific T cell responses after i.n. inoculation in a process independent of FcRn.

We hypothesized that FcRn might mediate translocation of ICs across the respiratory epithelial layer and increase antigen presentation in the lungs. Therefore, we determined whether FcRn was involved in the *in vivo* priming of RSV-specific immune responses upon i.n. exposure to RSV-IC. We used monoclonal humanized IgG1 antibody (palivizumab, 14) specific for the fusion protein of RSV

to opsonize virus particles in these experiments. Human IgG1 binds mouse FcRn with high affinity at pH 6.0 and is capable of extending its half-life (30, 33). Due to the neutralization by palivizumab, the opsonized virus preparation was non-infectious (supplementary figure 2), and control inoculation without antibodies was therefore performed with RSV inactivated by UV-irradiation. The palivizumab-RSV complexes (equivalent of $2x10^6$ PFU and 50 µg/ml palivizumab, in a total volume of 50µl) or control UV-inactivated RSV (equivalent of $2x10^6$ PFU, 50 µl) were used to inoculate WT and $FcRn^{-/-}$ mice i.n. A third group was left untreated. T cell priming was measured in all groups in the lungs 6 days after a live virus challenge administered five weeks after priming. Single cell suspensions from lungs were re-stimulated *in vitro* with RSV-infected dendritic (D1) cells or untreated D1 cells to measure T cell responses by intracellular cytokine staining. T cells from mice inoculated with RSV-IC showed an increased percentage of RSV-specific $CD8^+$ IFN-γ^+ and $CD4^+$ T cells compared to T cells from UV-RSV inoculated mice when re-stimulated with RSV infected D1 cells. An increase in IFN-γ^+, IL-4^+, IL-$5^+$and IL-13^+ cytokine producing $CD4^+$ T cells was observed with a dominant role for IFN-γ^+ cells (Figure 3A). No difference was observed between UV-RSV primed compared to primary RSV infected mice. Similar increased responses after immune complex priming were observed in $FcRn^{-/-}$ and WT mice suggesting that FcRn mediated processes were not involved (Figure 3B).

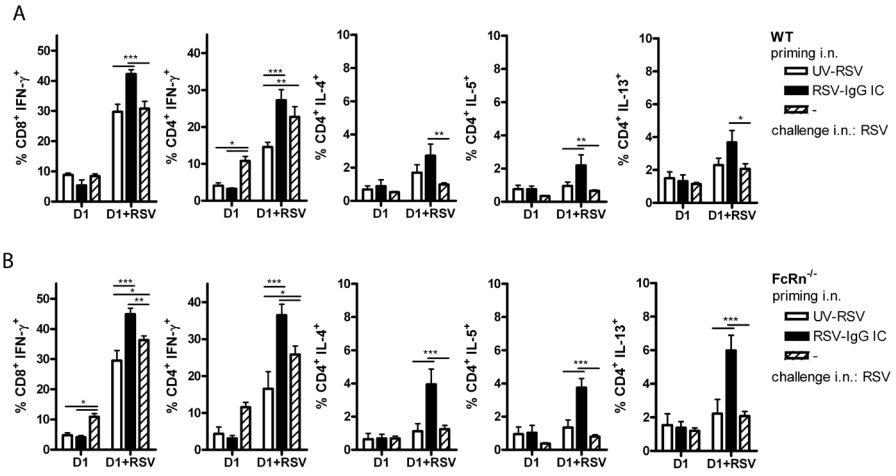


Figure 3. Intranasal inoculation of RSV-IC efficiently primes RSV-specific $CD4^+$ and $CD8^+$ T cell responses in WT and FcRn deficient mice.

A. WT mice and **B.** $FcRn^{-/-}$ mice were inoculated with UV-RSV or RSV-IC at day 0. A third group was left untreated. At day 35 all groups were challenged with RSV and six days after challenge lungs were analyzed for T cell responses. The percentage of cytokine producing $CD8^+$ and $CD4^+$ T cells in lung tissue responding to RSV-infected or uninfected D1 cells is shown. Error bars represent the SEM of 5 individual mice per group. Results are shown for five mice per group of one representative experiment. Significance was calculated using a Two-way ANOVA. This experiment was performed twice with similar results. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

In addition to increasing virus specific T cell responses, opsonization of RSV with palivizumab strongly potentiated RSV-specific IgG2c antibody responses. In FcRn$^{-/-}$ mice antibody responses were slightly lower than in wild type mice at day 28 after priming, probably due to shorter antibody half-life in the knockout mice. However, at day 41, i.e. six days after challenge, virus specific antibody response was robust and of equal magnitude in WT and FcRn$^{-/-}$ mice that had been inoculated with immune complexes (Figure 4).

In conclusion, these experiments showed strong immune priming with opsonized RSV that is negligible when non-opsonized non-infectious RSV was used. The enhanced immune priming was independent of FcRn. The CD4^{+} T cell response upon challenge is dominated by Th1 type responses (IFN-γ^{+}, accompanied by IgG2c antibody responses) although a minor Th2 component (IL-4^{+}, IL-5^{+}, IL-13^{+}) was also present.

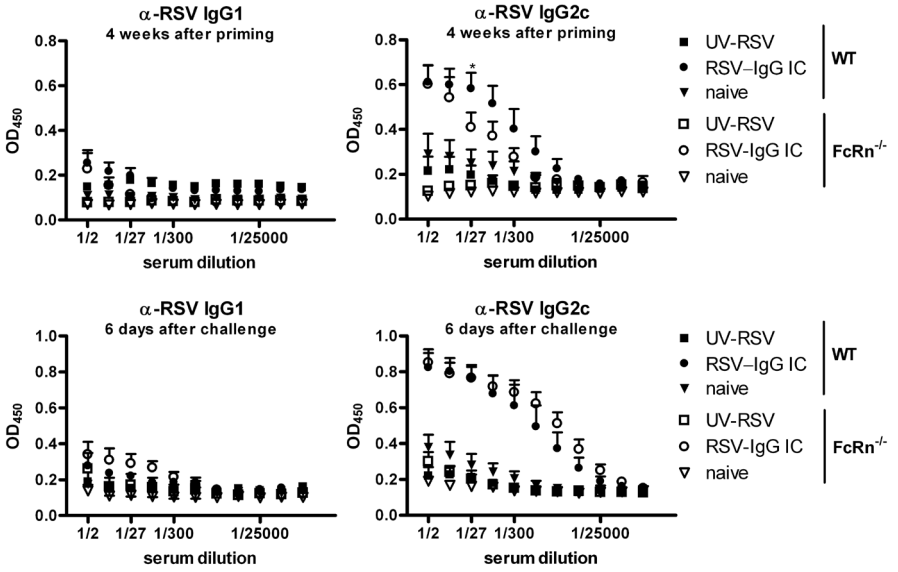


Figure 4. Intranasal administration of RSV-IC efficiently primes RSV-specific IgG2c responses.

WT mice and FcRn$^{-/-}$ mice were inoculated with UV-RSV or RSV-IC 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 ten days prior to RSV challenge and 6 days after challenge. RSV-specific IgG1 and IgG2c levels in serum are shown in a 3 step serial dilution of serum starting at 50%. Error bars represent the SEM of 5 individual mice per group. Results are shown for five mice per group of one representative experiment. Significance was calculated using a Two-way ANOVA. Significance shown concerns data comparing RSV-IC treated WT and FcRn$^{-/-}$ mice ten days prior to RSV challenge. These experiments were performed twice with similar results. *$p<0.05$.

Intranasal administered IgG-opsonized virus particles increase secondary CD8$^+$ T cell responses against subdominant epitopes.

We further specified the CD8$^+$ T cell response by stimulation of lung cells with epitopes derived from different RSV proteins (M, F, G and NP). We observed a shift in epitope hierarchy in RSV-IC treated mice compared to UV-inactivated RSV primed mice and mice with a primary RSV infection. A decreased percentage of responding lung CD8$^+$ INF-γ$^+$ T cells responding to $M_{187\text{-}195}$ was observed in RSV-IC inoculated and RSV challenged mice. In contrast, an increased percentage of CD8$^+$ INF-γ$^+$ T cells was observed when lung cells were stimulated with a mixture of epitopes from the fusion-, attachment- and nucleoprotein from RSV that are subdominant during primary infections and even less abundant in secondary RSV infected mice (28, 34, 35) (Figure 5). This altered epitope hierarchy reflects the distinct antigen processing pathway accessed when virus particles are provided in immune complexes.

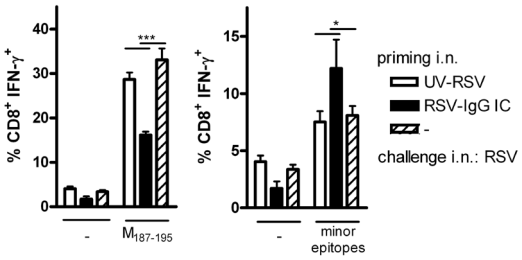


Figure 5. Intranasal administration of RSV-IC primes a CD8$^+$ T cell response to both minor epitopes and the major epitope of RSV.

WT mice were inoculated with UV-RSV or RSV-IC at day 0. A third group was left untreated. At day 35 all groups were challenged with RSV and six days after challenge lungs were analyzed for T cell responses. Percentage of IFN-γ producing CD8$^+$ T cells in lung tissue responding to 1 μg/ml $M_{187\text{-}195}$ or a mixture of $F_{250\text{-}258}$, $F_{433\text{-}442}$, $G_{177\text{-}194}$, $NP_{57\text{-}64}$ and $NP_{360\text{-}368}$ 1 μg/ml each are shown. Error bars represent the SEM of 5 individual mice per group. Results are shown for five mice per group of one representative experiment. Significance was calculated using a Two-way ANOVA. These experiments were performed two times with similar results. *$p<0.05$ and ***$p<0.001$.

Discussion

In the present work we showed that intranasal administration of non-infectious RSV-IC 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 RSV-IC primed mice was independent of FcRn. Therefore, FcRn-mediated transcytosis of these immune complexes across epithelium was not the route by which priming by RSV-IC 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 vitro* using RSV-IC pulsed DCs as antigen presenting cells that also ruled out involvement of FcRn in antigen processing using both FcRn$^{-/-}$ BM-DC (Figure 1) and IgG1 unable to bind FcRn (Figure 2).

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 (17) and a route of immune priming (36). HSV-2 specific T and B cell responses were primed upon i.n. administration of a IgG-Fc-gD fusion protein in a FcRn dependent way, resulting in protection against a lethal dose of an intra-vaginal challenge with HSV-2 (36). These observations might be explained by FcRn mediated transcytosis of gD-Fc across respiratory epithelium. However, it is also possible that FcRn simply increased the half-life of the gD-Fc resulting in increased *in vivo* persistence, thereby prolonging the time for the immune system to respond to the antigen. The findings with respect to the role FcRn in immune priming with gD-Fc fusion proteins and reports showing a role of FcRn in antigen processing of ovalbumin-IC (20) contrast with our observations.

The observed difference between our findings and those published with OVA and gD-Fc, 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 (37). Apparently, the phagosomes in DCs that acquired the immune complexes did not reach the low pH required for IgG binding to FcRn while IC reached phagosomes in macrophages where pH rapidly dropped to <6.5 that did allow FcRn binding. Thus, FcRn independent RSV-IC antigen presentation might be simply due to different mechanisms of viral-IC and protein-IC uptake and presentation, or as described by Liu *et al.* due to the neutral pH in the phagosomes of DCs. In our *in vivo* studies it might be possible that lung DCs efficiently captured RSV-IC via FcR 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 DC (38).

Dendritic cells located underneath the epithelial barrier in lung tissue contribute to the initiation of adaptive immune responses specific for RSV (38). $CD11c^+$ $CD103^+$ DCs express the tight junction proteins ZO-2 and claudin-7 which enable the $CD103^+$ DC to sample the airways (39). DCs internalize antigen via a-specific phagocytosis or when the antigen is opsonized by IgGs via FcγRs. In previous work we showed that antigen presentation and T cell activation of RSV in a murine secondary RSV infection model, i.e. in the presence of acquired antibodies was indeed mediated via FcγRs. Therefore, it might be hypothesized that RSV-IC are sampled by $CD103^+$ DCs in the airways via FcγRs that are present on these cells. Moreover, antigen presentation in the lymph node might depend on DCs that compared to macrophages more readily migrate to the lymph nodes.

In earlier work 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 vitro* and *in vivo* after i.n. RSV challenge (15). This appears to be in contrast with the increase in T cell priming of both $CD4^+$ and $CD8^+$ T cell responses in the present study when mice were i.n. inoculated with RSV-IC (Figure 3). However, in the previous series of experiments we showed that virus-specific IgG acted through

FcγR to decrease $CD8^+$ T cell responses towards live virus compared to non-opsonized live virus. In this study we observed enhanced priming of immune complexes compared to a non-infectious UV-RSV preparation. While live virus infection might be the most effective way of priming $CD8^+$ T cell responses in this mouse model, we demonstrate that pre-existing neutralizing antibodies do not completely block initiation of virus specific $CD8^+$ T responses.
Similar T cell responses were observed after i.n. RSV challenge with- or without prior intranasal priming with UV-RSV (Figure 3A). Thus intranasal exposure with non-infectious virus appears to be an ineffective route of immune priming. However, this can be improved by providing the antigenic material as an immune complex. Indeed, the anti-viral serum IgG responses primed by palivizumab/RSV-IC were robust, resulting in a higher RSV-specific titer.

Material and Methods

Mice

Pathogen-free 6-8 week old C57BL/6cjo wild-type mice were purchased from Charles River (Maastricht, The Netherlands). FcR common-γ-chain$^{-/-}$ ($\gamma^{-/-}$) mice and $FcRn^{-/-}$ mice on a C57BL/6 background (22-24) were bred and maintained at the central animal facility at Utrecht University. The mouse study protocols were approved by the Animal Ethics Committee of the University Medical Center (UMC) Utrecht.

Virus and cell lines

RSV A2 strain was grown in HEp-2 cells (ATCC number: CCL-23), purified by polyethelene glycol precipitation, and stored in liquid nitrogen in 10% sucrose in PBS. Tissue culture infective dose $(TCID)_{50}$ was determined after titration in HEp-2 cells.
HEp-2 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, 21980-065) containing 5% FCS (Gibco, 10270-106) 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 cells, a mouse dendritic cell line derived from C57BL/6 mice (25) used in antigen presentation assays, were maintained in IMDM containing 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin and 50 μM 2-mercapto-ethanol (BioRad, 161-0710) supplemented with 30% conditioned medium from GM-CSF producing R1 cells (mouse fibroblast NIH3T3, transfected with GM-CSF gene, 25).

TNP-labelling of RSV

TNP-labelling was performed as described by Hale *et al.* (26). In short, 200 μg/ml UV-inactivated RSV was incubated with 10 mM TNBS (2,4,6-trinitrobenzene sulfonic acid, Sigma-Aldrich, 92824) in 1 ml

Hank's buffer (Invitrogen, 24020091) for 15 min at 37°C. Unbound TNBS was removed via dialysis with PBS o.n. at 4°C while stirring. Precipitate of the dialysed RSV-TNP was removed by centrifugation at 1500 rpm for 1 min. The concentration stably linked TNP-groups was measured at $OD_{350.}$

Mouse BM-DC cultures

Bone marrow derived dendritic cells (BM-DC) were prepared as described before (27). Bone marrow was depleted for erythrocytes using erythrocyte lysis mix (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA pH 7.4). BM-DCs were enriched for 7 days in RPMI1640 (Gibco, 61870-044) supplemented with 5% hyclone FCS (Perbio, SH30080.03), 1% penicillin/streptomycin, 50 µM 2-mercapto-ethanol and as a source of GM-CSF, 30% culture supernatant from R1 cells (mouse fibroblast NIH3T3, transfected with GM-CSF gene, 25). The percentage of $CD11c^+$ cells (routinely > 70%) was determined by staining with anti-CD11c (BD, clone HL3) and analyzed with a FACS Canto flowcytometer.

***In vivo* RSV infection experiments and tissue sampling**

Mice were lightly anesthetized with isoflurane and intranasally (i.n.) inoculated with $2x10^6$ plaque-forming units (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 read out in the *in vitro* antigen presentation assays. In the RSV challenge experiments mice were i.n. inoculated with RSV-IC or UV-RSV in a volume of 50 µl at day 0. A third group of mice was left untreated. RSV-IC were prepared by pre-incubation of $4,7*10^7$ PFU with 50 µg/ml palivizumab (humanized IgG1 monoclonal Ab, Synagis, 14) for 15 min. in a volume of 200µl. At day 35, all groups of mice were i.n. infected with RSV. Six days after challenge, T cell responses in the lung and IgG levels in serum were analyzed. Mice were sacrificed by i.p. injection of 300 µl pentobarbital. After performing bronchoalveolar lavage (BAL), the lungs were perfused with 10 ml ice cold PBS containing 100 U/ml heparin via the right ventricle. The lungs were removed and cut to 1 mm x 1 mm pieces and incubated with collagenase (2.4 mg/ml, Roche Applied Science, 10103586001) and DNase (1 mg/ml, Roche Applied Science, 10104159001) for 20 min. at 37°C. Single cell suspensions were prepared by processing the tissue trough 70 µm cell strainers (BD Falcon). Single cell suspension of lung cells (10^6) from RSV infected wild type and $FcRn^{-/-}$ mice were stimulated for 5h at 37°C, 5% CO_2, with 2×10^5 RSV infected D1 cells, uninfected D1 cells, 1 µg/ml of the immunodominant peptide $M_{187-195}$ (NAITNAKII) or a mixture of subdominant epitopes: $F_{250-258}$ (YMLTNSELL), $F_{433-442}$ (KTFSNGCDYV), $G_{177-194}$ (SNNPTCWAICKRIPNKKP), NP_{57-64} (ANHKFTGL) and $NP_{360-368}$ (NGVINYSVL) 1µg/ml each (28) in 200 µl IMDM (Gibco, 21980-065) supplemented with 5% FCS, 1 % penicillin/streptomycin, 50 µM 2-mercapto-ethanol and 25 U/mL recombinant hIL-2 (Roche, 11147528001). Brefeldin-A 10 µg/mL (Sigma, B7651) was added for the duration of the stimulus to facilitate intracellular accumulation of cytokines. D1 cells were infected for 48h with RSV m.o.i. 2 before addition to the lung cell suspension. Cytokine production by $CD4^+$ and $CD8^+$ T cells was analyzed by intracellular cytokine staining.

Mouse T cell activation assay

In vitro mouse antigen presentation assays were performed with D1 cells. In experiments to study the role of FcγRs and FcRn, BM-DCs obtained from FcγR or FcRn knockout mice and C57BL/6 wild type mice were used.
To study antigen presentation of RSV immune complexes by BM-DCs or D1 cells, UV-inactivated RSV (UV-RSV) m.o.i. 10, 3, 1, 0.1 and 0 was pre-incubated with either plasma derived from secondary RSV infected mice or naïve mice (pre-immune serum) for 15 min. at 37°C. UV-RSV was used in these experiments to completely rule out infection related effects. In parallel, RSV-TNP m.o.i. 0.1, 0.03, 0.01 and 0 was pre-incubated 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 (29-31). RSV immune complexes were incubated with $5x10^4$ BM-DCs or D1 cells for 24h. After 24h, the antigen presenting cells were incubated with $5x10^5$ total lung cells in the presence of 25 U/ml recombinant hIL-2 (Roche, 11147528001) and 10 µg/ml Brefeldin-A (Sigma, B7651) for 5h at 37°C 5% CO_2. 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 IFN-γ production by lung $CD4^+$ T cells by intracellular FACS staining.

Intracellular cytokine staining

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).

Real-time PCR

RSV-specific RT-PCR: $2x10^5$ A549 cells were incubated with RSV or UV-RSV (m.o.i. 2) in the presence or absence of palivizumab. RSV ($4,7*10^7$ PFU) was pre-incubated with 50 µg/ml palivizumab (humanized IgG1 monoclonal Ab, Synagis, 14) for 15 min. before addition to the cells (14). After 24h total RNA was extracted from these virus exposed cells using MagnaPure LC equipment, cDNA was synthesized and viral loads were determined by real time PCR as recently described (32). In short, RNA extraction was performed using a MagnaPure LC total nucleic acid kit (Roche Diagnostics). Extracted RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems) according to the manufacturer's guidelines. RT inactivation was performed at 95°C for 5 min. Real time PCR was performed with primers specific for the N gene of RSV: 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′-TTCCCTTCCTAACCTGGACATAG-CATATAACATACCT-3′. RT-PCR was performed with 20 µl cDNA, TaqMan universal PCR mastermix (PE Applied Biosystems), primers (RSA 900 nM each, RSB 300 nM each) and fluorogenic probes (58.3 and 66.7 nM for RSA and –B probes, respectively) labelled 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 Applied Biosystems 7900HT Fast Real-Time PCR system for 2 min. at 50ºC, 10 min. at 95ºC and 45 cycles of 15 sec. at 95ºC and 1 min. at 60ºC. Sample Ct values were compared with a standard curve of RSV A2.

FcRn specific RT-PCR: RNA was isolated from cultured BM-DC of WT and $FcRn^{-/-}$ mice by RNAzol (CamproScientific, Veenendaal, The Netherlands), and first-strand cDNA was generated with random primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen) according to the manufacturer's instructions. FcRn-specific mRNA was quantified by subsequent real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis (Taqman), with the forward 5′-GTGGAAGGA-GCCGCCGTCTATG-3′ primer, reverse 5′-TGACCTCCAGCAATGACC ATGCG-3′ primer, and the 5′-ATCGTCATCGGTGTCTTGCTACTCACGG-3′ probe. FcRn expression was normalized for RPL27 expression which is a ribosomal protein.

ELISA

ELISA plates (Nunc, 442404) were coated with denatured RSV lysate from RSV infected HEp-2 cells in PBS for 18h at 4°C. After removal of unbound RSV lysate, plates were blocked in 200 µl 1% bovine serum albumin (BSA) in 0.05% $Tween_{20}$/PBS for 1h at 37°C. Serum of RSV infected 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) or anti-IgG2c (Immunology Consultant Laboratory, GG2c-90P) diluted in PBS supplemented with 0.1% BSA/0.05% $Tween_{20}$ for 2h at RT. 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}.

Statistical analysis

Data were analyzed for statistical significance using a One-way or Two-way ANOVA, 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.

References

1. de Jong, J. M., D. H. Schuurhuis, A. Ioan-Facsinay, M. M. Welling, M. G. Camps, E. I. van der Voort, T. W. Huizinga, F. Ossendorp, J. S. Verbeek, and R. E. Toes. 2006. Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4 T cells upon immune-complex uptake *in vivo*. Immunology 119: 499-506.
2. Hamano, Y., H. Arase, H. Saisho, and T. Saito. 2000. Immune complex and Fc receptor-mediated augmentation of antigen presentation for *in vivo* Th cell responses. J. Immunol. 164: 6113-6119.
3. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 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.
4. Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J. Immunol. 168: 2240-2246.
5. Ponnuraj, E. M., J. Springer, A. R. Hayward, H. Wilson, and E. A. Simoes. 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.
6. Polack, F. P., M. N. Teng, P. L. Collins, G. A. Prince, M. Exner, H. Regele, D. D. Lirman, R. Rabold, S. J. Hoffman, C. L. Karp, S. R. Kleeberger, M. Wills-Karp, and R. A. Karron. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. J. Exp. Med. 196: 859-865.
7. Gimenez, H. B., S. Chisholm, J. Dornan, and P. Cash. 1996. Neutralizing and enhancing activities of human respiratory syncytial virus-specific antibodies. Clin. Diagn. Lab Immunol. 3: 280-286.
8. Ubol, S., and S. B. Halstead. 2010. How innate immune mechanisms contribute to antibody-enhanced viral infections. Clin. Vaccine Immunol. 17: 1829-1835.
9. Brandenburg, A. H., J. Groen, H. A. van Steensel-Moll, E. C. Claas, P. H. Rothbarth, H. J. Neijens, and A. D. Osterhaus. 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.
10. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163: 693-698.
11. 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.
12. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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: 405-421.
13. Piedra, P. A., A. M. Jewell, S. G. Cron, R. L. Atmar, and W. P. Glezen. 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.
14. The IMpact-RSV Study Group. 1998. Palivizumab, a Humanized Respiratory Syncytial Virus Monoclonal Antibody, Reduces Hospitalization From Respiratory Syncytial Virus Infection in High-risk Infants. Pediatrics 102: 531-537.
15. 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.
16. Israel, E. J., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. J. Immunol. 154:

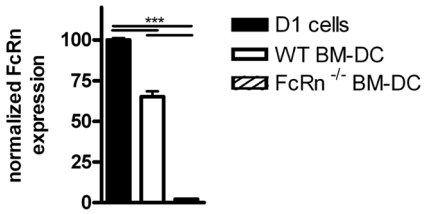
6246-6251.

17. Spiekermann, G. M., P. W. Finn, E. S. Ward, J. Dumont, B. L. Dickinson, R. S. Blumberg, and W. I. Lencer. 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.
18. Yoshida, M., S. M. Claypool, J. S. Wagner, E. Mizoguchi, A. Mizoguchi, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2004. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity. 20: 769-783.
19. Chaudhury, C., S. Mehnaz, J. M. Robinson, W. L. Hayton, D. K. Pearl, D. C. Roopenian, and C. L. Anderson. 2003. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J. Exp. Med. 197: 315-322.
20. Qiao, S. W., K. Kobayashi, F. E. Johansen, L. M. Sollid, J. T. Andersen, E. Milford, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2008. Dependence of antibody-mediated presentation of antigen on FcRn. Proc. Natl. Acad. Sci. U. S. A 105: 9337-9342.
21. Vidarsson, G., A. M. Stemerding, N. M. Stapleton, S. E. Spliethoff, H. Janssen, F. E. Rebers, M. de Haas, and J. G. van de Winkel. 2006. FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis. Blood 108: 3573-3579.
22. Wernersson, S., M. C. Karlsson, J. Dahlstrom, R. Mattsson, J. S. Verbeek, and B. Heyman. 1999. IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. J. Immunol. 163: 618-622.
23. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 76: 519-529.
24. Roopenian, D. C., G. J. Christianson, T. J. Sproule, A. C. Brown, S. Akilesh, N. Jung, S. Petkova, L. Avanessian, E. Y. Choi, D. J. Shaffer, P. A. Eden, and C. L. Anderson. 2003. The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs. J. Immunol. 170: 3528-3533.
25. 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.
26. Hale, A. H. 1980. Molecular requirements for trinitrophenyl recognition by antihapten cytotoxic T lymphocytes. Cell Immunol. 49: 408-414.
27. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 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.
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. Kim, J. K., M. F. Tsen, V. Ghetie, and E. S. Ward. 1994. Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. Eur. J. Immunol. 24: 2429-2434.
30. Kim, J. K., M. Firan, C. G. Radu, C. H. Kim, V. Ghetie, and E. S. Ward. 1999. Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn. Eur. J. Immunol. 29: 2819-2825.
31. Medesan, C., D. Matesoi, C. Radu, V. Ghetie, and E. S. Ward. 1997. Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1. J. Immunol. 158: 2211-2217.
32. 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.
33. Ober, R. J., C. G. Radu, V. Ghetie, and E. S. Ward. 2001. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. Int. Immunol. 13: 1551-1559.
34. Rutigliano, J. A., M. T. Rock, A. K. Johnson, J. E. Crowe, Jr., and B. S. Graham. 2005. Identification of an H-2D(b)-

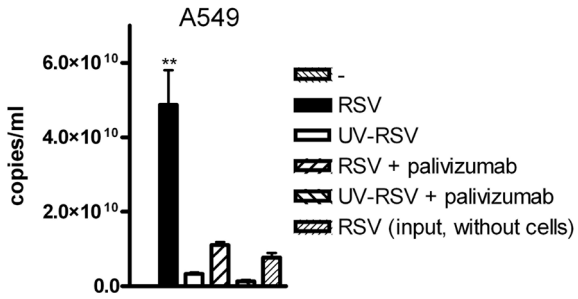
restricted CD8+ cytotoxic T lymphocyte epitope in the matrix protein of respiratory syncytial virus. Virology 337: 335-343.

35. Rutigliano, J. A., T. J. Ruckwardt, J. E. Martin, and B. S. Graham. 2007. Relative dominance of epitope-specific CD8+ T cell responses in an F1 hybrid mouse model of respiratory syncytial virus infection. Virology 362: 314-319.
36. Ye, L., R. Zeng, Y. Bai, D. C. Roopenian, and X. Zhu. 2011. Efficient mucosal vaccination mediated by the neonatal Fc receptor. Nat. Biotechnol. 29: 158-163.
37. Liu, X., L. Lu, Z. Yang, S. Palaniyandi, R. Zeng, L. Y. Gao, D. M. Mosser, D. C. Roopenian, and X. Zhu. 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.
38. 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.
39. 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.

Supplementary figures



Supplementary figure 1. WT BM-DCs and the D1 cell line but not BM-DCs from FcRn$^{-/-}$ mice express FcRn.
FcRn expression levels were analyzed with RT-PCR and normalized for RPL27 expression. Result of one representative culture is shown. Error bars represent the SEM of a duplicate within one experiment. Significance was calculated using a One-way ANOVA. ***p<0.001.



Supplementary figure 2. Palivizumab completely neutralizes RSV.
RSV (4,7*10^7 PFU) was pre-incubated for 15 minutes with 50 µg/ml palivizumab similar to the *in vivo* experiments. UV-RSV was used as a negative control for replication. RSV, RSV-palivizumab, UV-RSV or (UV-)RSV-palivizumab IC were added to A549 cells (equivalent of mo.i. 10) and incubated for 24h. As a control, viral input (without cells) was also included in the PCR. Real time PCR was performed on the RSV-N gene. Results are shown of one representative experiment. Error bars represent the SEM of a duplicate within one experiment. Significance was calculated using a One-way ANOVA. These experiments were performed two times with similar results. **p<0.01.

chap

ter 5

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

Debby Kruijsen[1], Marcel A. Schijf[1,2,], Michaël V. Lukens[1,*], Nathalie O. van Uden[1], Jan L. Kimpen[1], Frank E. Coenjaerts[3] and Grada M. van Bleek[1]*

[1]Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Centre, Utrecht, The Netherlands,
[2]Department of Immunology, Danone Research - Centre for Specialised Nutrition, Wageningen, The Netherlands,
[3]Department of Medical Microbiology, University Medical Centre, Utrecht, The Netherlands.

[*] Marcel A. Schijf and Michaël V. Lukens contributed equally to this work

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 polymorphonuclear 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 (Fig. 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 (Fig. 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 (Fig. 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 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 two fold higher compared to RSV primed mice (Fig. 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 prepara-

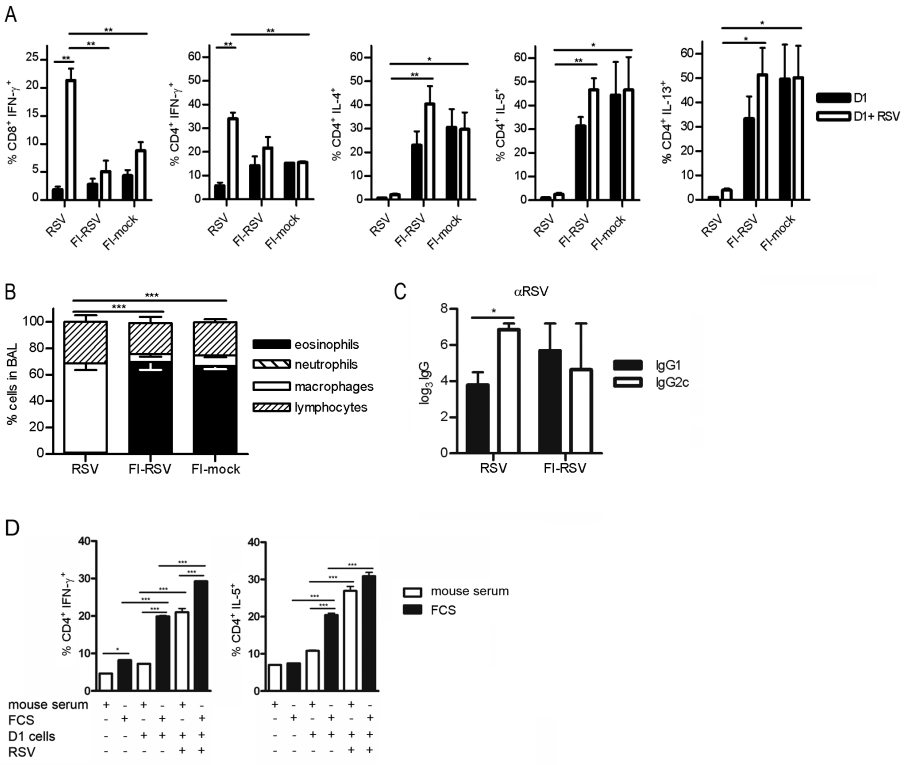


Figure 1. FI-RSV vaccination induces strong Th2 responses in lung tissue upon RSV challenge in C57BL/6 mice. Two groups of mice were vaccinated i.m. with either FI-RSV or FI-mock and a third group was i.n. infected with RSV. All groups were i.n. challenged at day 36 with RSV. Six days after challenge 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 *** $p < 0.001$.

tions 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 (Fig. 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 Fig. 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 (Fig. 2), without a necessary contribution of vaccine characteristic factors like formalin treatment of the mock vaccine (Fig. 2A and B) or the alum adjuvant (Fig. 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.

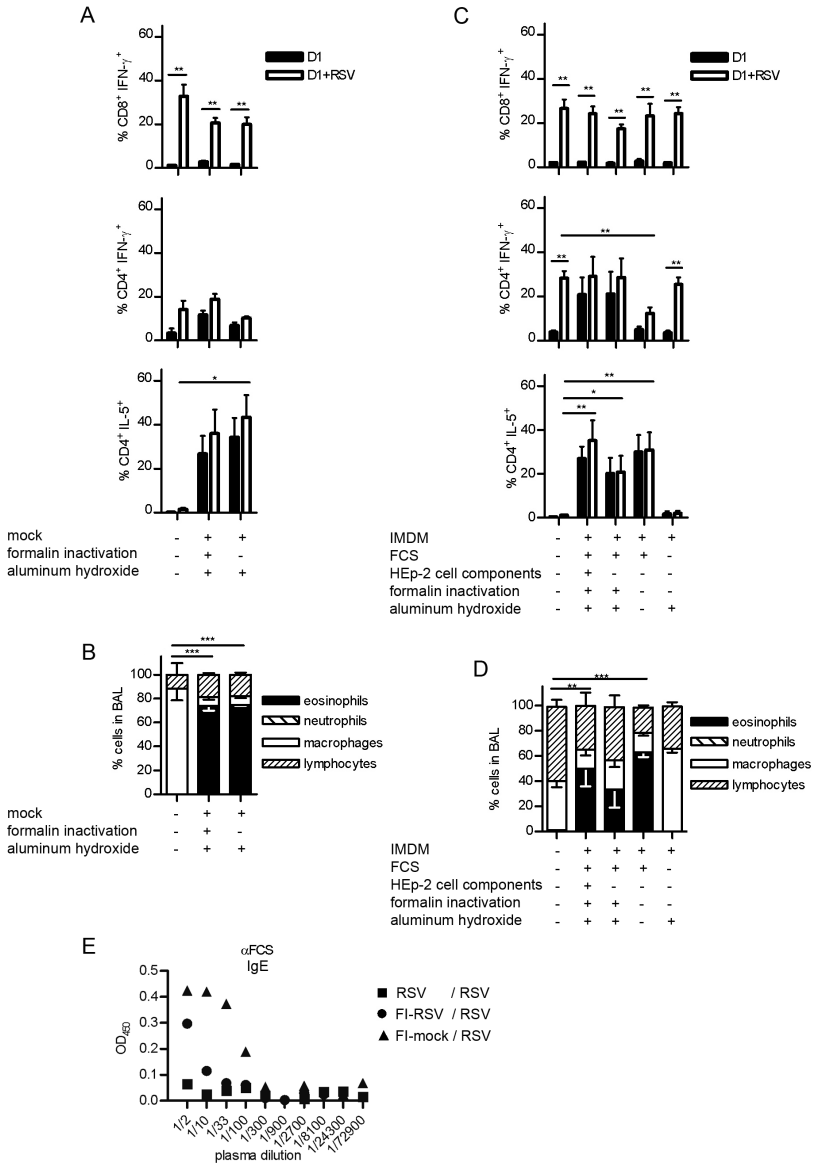
A viral trigger is not necessary during challenge to induce the FCS-specific allergic immune response

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

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 *** $p < 0.001$.

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 (Fig. 3A) and BAL eosinophilia (Fig. 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



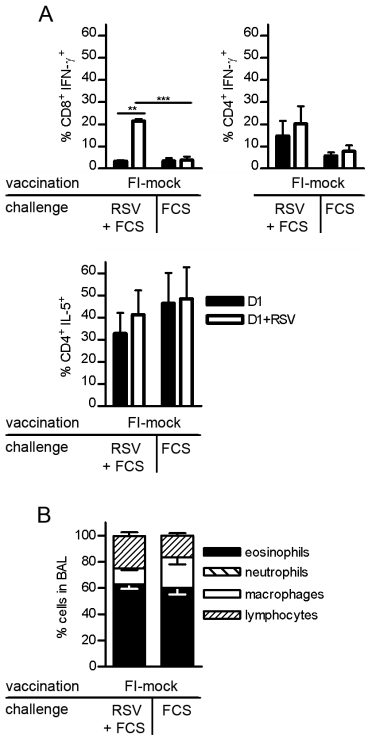


Figure 3. A viral trigger is not required to induce Th2/eosinophilia in FI-mock primed FCS challenged mice.
Two groups of mice were i.m. vaccinated with FI-mock and at day 28 i.n. challenged with FCS or RSV cultured in IMDM + FCS. Eight days after challenge, lung cells were analyzed for T cell responses 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 *** $p < 0.001$.

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 Fig. 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 eosinophila.

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 Fig. 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 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 Fig. 4. In contrast to i.m. mock vaccination, i.n. exposure to mock did not prime a T cell response. 96 h after challenge $CD4^+$ T cell responses became evident in the lungs of FI-RSV-vaccinated and secondary RSV-infected mice (Fig. 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 (Fig. 4A). 96 h 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 48 h after challenge when compared to a secondary and primary RSV infection (Fig. 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 (Fig. 4A and B) or i.n. mock primed mice (Fig. 4A). Both $CD103^+$ $CD11b^{low}$ MHC class II^{high} and $CD103^-$ $CD11b^{high}$ MHC class II^{high} DC contributed to the enhanced influx (Fig. 4A and B; DC gating strategy shown in Supplemented data Fig. 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 96 h 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/eosinophila 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 hyperresponsiveness [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

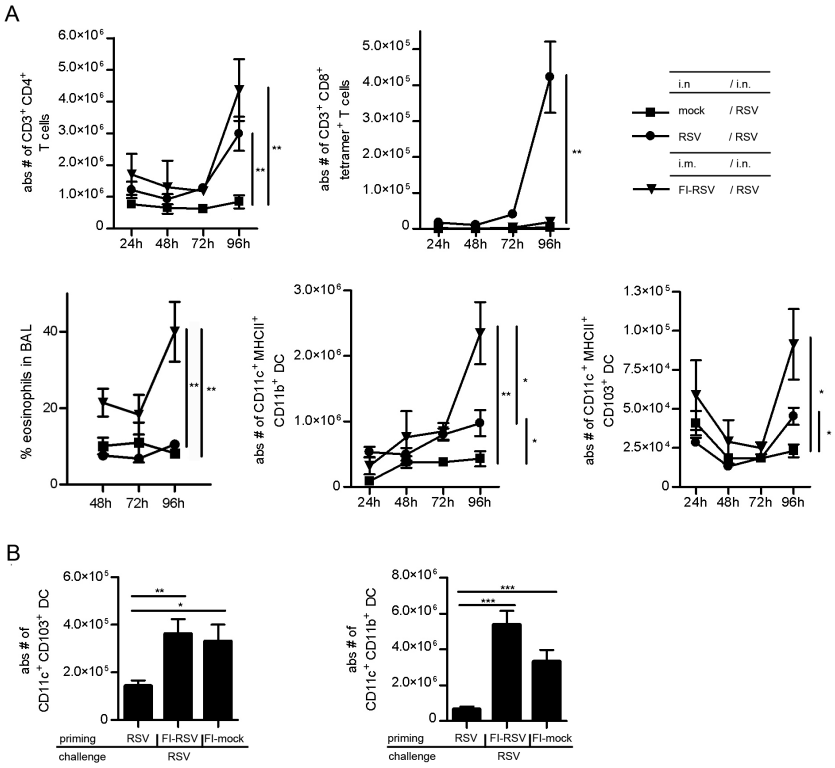


Figure 4. Kinetics of the cellular influx into the lungs during allergic versus non-allergic airway inflammation.
A. Three groups of mice were i.n. infected with RSV, i.n. primed with mock (HEp-2 cell culture supernatant) or i.m. vaccinated with FI-RSV. At day 28, mice were i.n. challenged with RSV. 24 h, 48 h, 72 h and 96 h after challenge lung cells were analyzed for absolute numbers of $CD3^+$ $CD4^+$ T cells, $CD3^+$ $CD8^+$ H-$2D^b$ $M_{187\text{-}195}$ tetramer$^+$ T cells, $CD11c^+$ $MHCII^{high}$ $CD11b^{high}$ DC and $CD11c^+$ $MHCII^{high}$ $CD103^+$ DC. 48 h, 72 h and 96 h 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 96 h after challenge. In B. significance is calculated using a One-Way ANOVA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

(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 (Fig. 5). We found that the total cellular influx into the lungs did not differ upon infection with

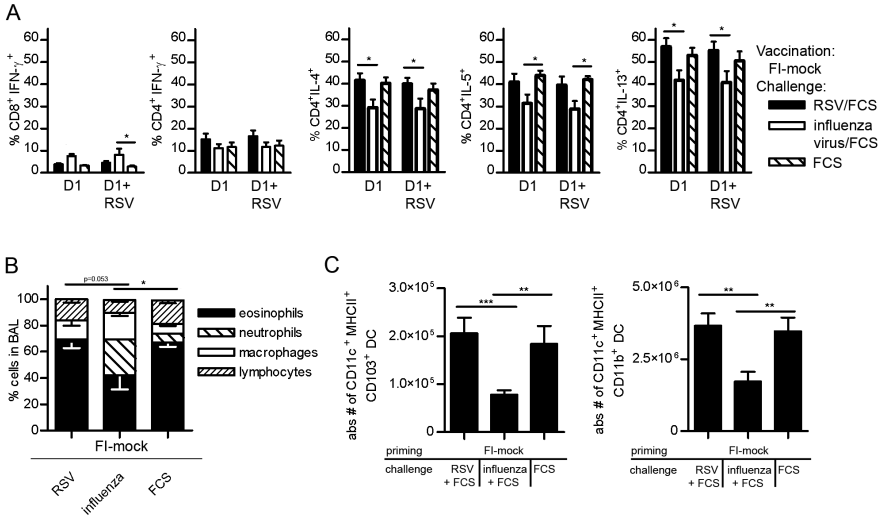


Figure 5. Influenza virus infection during FCS challenge decreases FI-mock primed allergic airway inflammation. Three groups of C57BL/6 mice were vaccinated with FI-mock. At day 28 mice were i.n. exposed to (1) RSV cultured in IMDM + FCS, (2) influenza virus plus FCS or (3) FCS alone. Five days after 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 $CD11c^+$ MHCIIhigh $CD103^+$ 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 *** $p < 0.001$.

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 (Fig. 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 (Fig. 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 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 (Fig. 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 (Fig. 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.

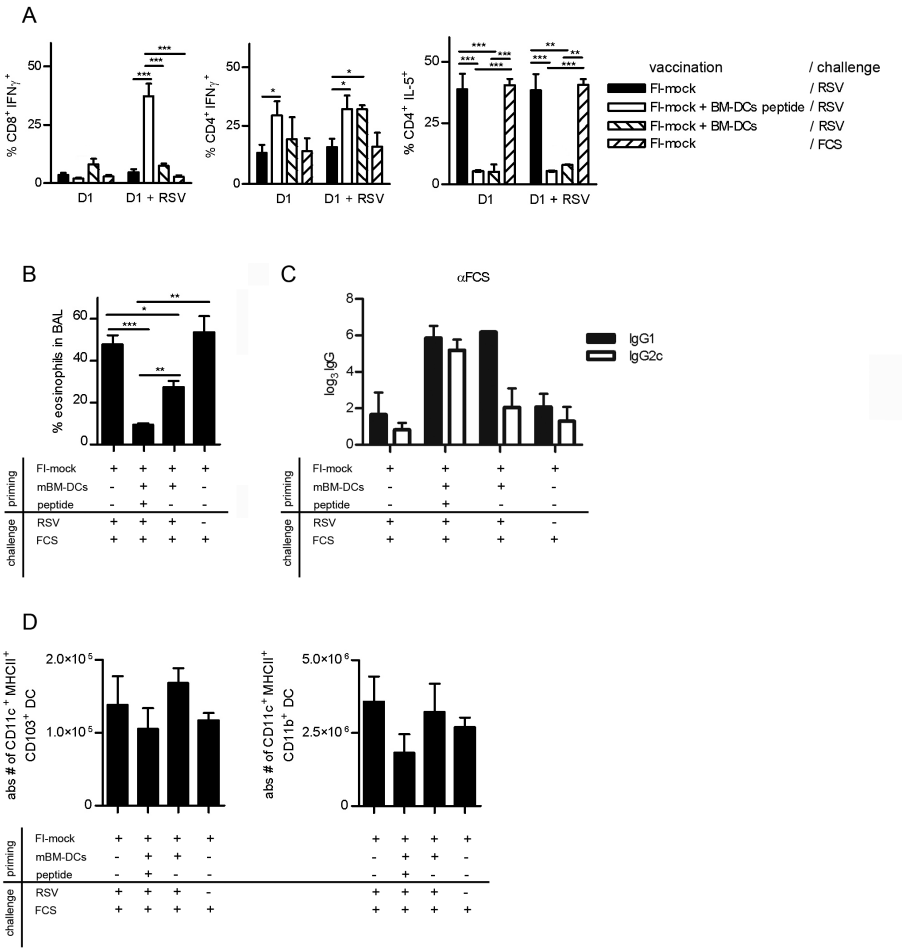


Figure 6. CD4⁺/8⁺ T cell priming by LPS matured BM-DC results in suppression of Th2 responses and lung eosinophilia.

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 *** $p < 0.001$.

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. Fig. 6A shows that mice intravenously injected with lipopolysaccharide (LPS)-matured BM-DC loaded with the dominant RSV epitope $M_{187\text{-}195}$ 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 $M_{187\text{-}195}$ 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Figs. 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, Fig. 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 (Fig. 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 Fig. 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 (24 h) whereas influenza virus mediated type I IFN production was more robust and increased over time (5 days) [54].

Because STAT 1-mediated signaling, a down stream 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 (Fig. 5). Decreased cDC responses in comparison to FI-RSV or FI-mock-vaccinated mice were also observed in secondary or primary RSV-infected mice (Fig. 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 (Fig. 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 (Fig. 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 parachymal $CD11b^{high}$ 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.

Acknowledgements

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

Material 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 (1000 x g, 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 (50,000 x g_{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 (1000 x g, 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 24 h followed by a 1 h incubation with the H-$2D^b$ 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 mm 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 contain-

ing 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 FACSCanto 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 h 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 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. 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 1 h 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 2 h 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 2 h 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.

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 inflam-

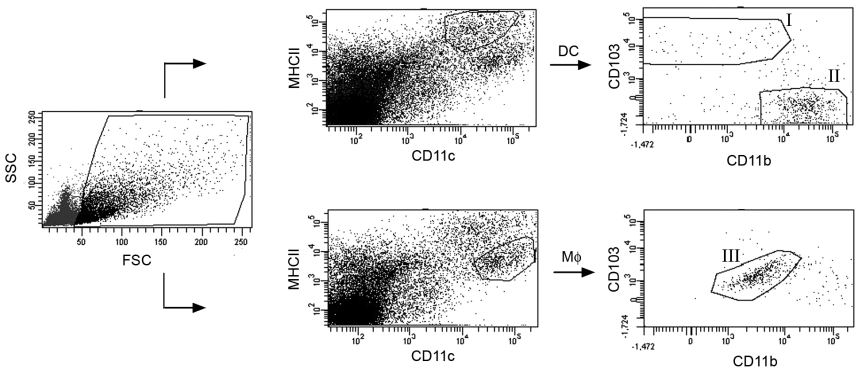
matory site, Int. Immunol. 11 (1999) 979-986.

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 $CD11b^{high}$ 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.

Supplementary figures



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).

chap

ter 6

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

Marcel A. Schijf [1,2], Debby Kruijsen[1], Johan Garssen[2,3], Grada M. van Bleek[1,] and Belinda van't Land[2,*]*

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

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

Submitted

Abstract

Breast feeding reduces the risk to develop severe Respiratory Syncytial Virus (RSV) infections in infants. In addition to maternal antibodies other immune modulating factors in human milk might contribute to this protection. In this study dietary prebiotic oligosaccharides, similar to oligosaccharides present in human milk, were evaluated in a C57BL/6 mouse RSV infection model. During primary RSV infection, an increased percentage of RSV specific $CD4^+$ T cells producing IFN-γ was found in the lungs at day 8 to 10 post infection in mice receiving a diet containing short chain Galacto-oligosacharides, 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 cell numbers in the lung tissue and the magnitude of airway eosinophilia at day 4 and 6 after viral challenge. This was accompanied by a decreased influx of inflammatory DCs ($CD11b^+/CD11c^+$) into the lungs. Moreover, at day 8 after viral challenge IFN-γ producing $CD4^+$ and $CD8^+$ T cell numbers were increased in FI-RSV-vaccinated mice on prebiotic diet. These findings suggest that specific dietary oligosaccharides can influence either the trafficking and/or the effector function of innate immune cell and $CD4^+$ and $CD8^+$ T cell subsets in the lungs of RSV infected mice. The increased systemic Th1 responses potentiated by these prebiotics, 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 (1). 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 (2-4). 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 (5). 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 (6,7).

The mammalian gastrointestinal tract harbors a complex ecosystem. Formation of the microbiota population, starting directly after birth, is shaped during infancy and is unique for each individual throughout life (8,9). The intestinal microbiota composition is important for establishment of gut homeostasis and affects local mucosal immunity (10). Although it has been documented that the host genetic background facilitates a core microbiome (11), factors like caesarian section, diet and reduced microbial pressure in western countries, shape host microbial populations (12,13). 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 (14,15). 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 (16). 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. Undoubtedly additional factors and mechanisms are involved and will be identified in the future.

Breast feeding reduces the risk of severe RSV bronchiolitis (17). In addition to maternal antibodies, human milk contains immune modulating components including prebiotic oligosaccharides. Prebiotic oligosaccharides stimulate the growth of commensal bacteria suggested to be beneficial to the host (18). 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 infections and severity of asthma and it lowers IgE antibody titers in atopic disease (19-21). In addition, in infants receiving scGOS, lcFOS and pectin-derived oligosaccharides (pAOS) a preventive effect was found for development of atopic dermatitis (22).

In this study the effect of dietary intervention with a specific mixture of prebiotic oligosaccharides is evaluated on virus specific lung T cell responses in a C57BL/6 mouse model of primary RSV infection and in a formalin-inactivated (FI)-RSV vaccine model, which is an enhanced disease model with allergic (Th2-type) features.

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 which results in $CD4^+$ and $CD8^+$ T cell responses in the lungs peaking around day 8-10 after viral exposure (26). 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 (Fig. 1A). During the course of infection a significantly ($p<0.05$) lower cellular influx in the BAL was observed in mice receiving prebiotic diet compared to control mice (3.2 ± 0.2 vs. $5.0 \pm 0.8 \times 10^5$ cells) at day 8 post infection. The cellular composition of BAL samples at all time points was similar compared to control (Fig. 1B).

The effect of prebiotic diet 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 (26). 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 prebiotic group as compared to controls at day eight 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, Fig. 1C). Th17 cells were barely detectable in the lungs in both groups. No effect of the diet 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 cells 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 and similar fractions of tetramer positive cells produced INF-γ at all time points measured. These data show that dietary intervention with scGOS/lcFOS/pAOS selectively increases RSV specific $CD4^+$ T cell mediated IFN-γ production in the lungs of RSV infected mice.

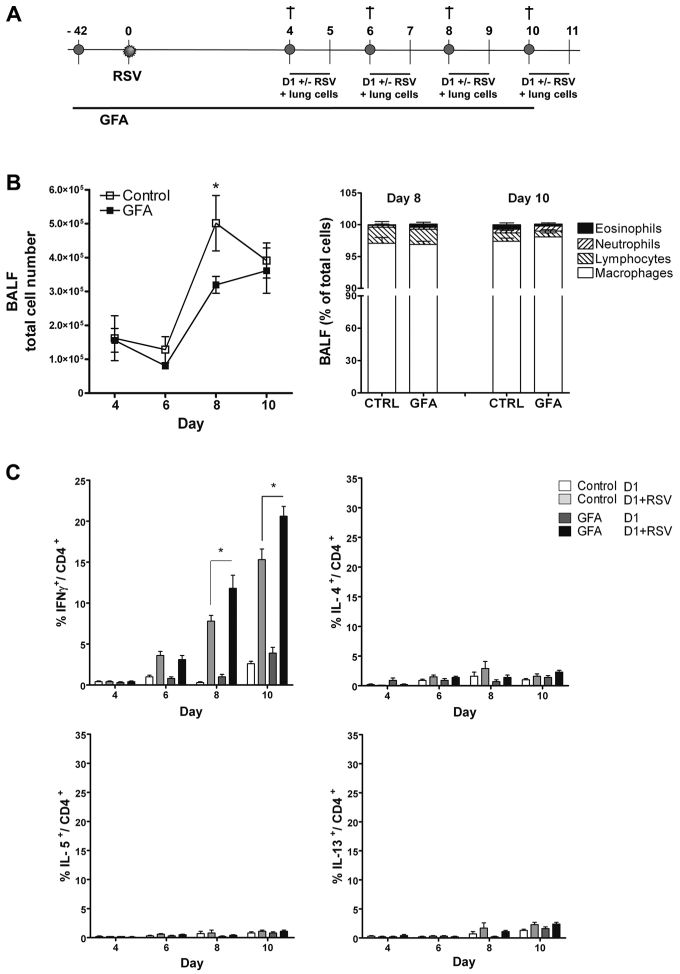


Figure 1. Dietary intervention with scGOS/lcFOS/pAOS (GFA) increases RSV specific CD4⁺ T cell mediated IFN-γ production during primary RSV infection.

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 as a fraction of total CD4⁺ T cells measured after *in vitro* restimulation of lung single 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 (*p<0.05).

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. The formalin-inactivated (FI)-RSV vaccination model, based on the

	% NKG2A ^{POS}/ CD8 ^{POS}		% IFNγ ^{POS}/ CD8 ^{POS} (D1-RSV) §		%TET ^{POS}/ CD8 ^{POS}		% IFNγ ^{POS}/ CD8 ^{POS} (NAITNAKII) §	
	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA	Ctrl	GFA
Day 4	10.0±1.3	9.0±1.2	1.0±0.2	1.0±0.1	0.8±0.1	0.9±0.1	0.9±0.1	1.0±0.2
Day 6	17.4±2.0	18.7±1.5	9.2±1.3	10.0±1.3	7.4±1.0	8.2±0.8	7.1±0.8	8.3±0.9
Day 8	37.1±2.3	39.3±1.2	27.9±1.7	27.2±2.2	14.9±1.6	15.1±1.1	18.6±1.7	17.5±2.0
Day 10	55.4±2.4	60.2±2.3	30.6±1.8	28.0±2.7	26.0±1.8	29.4±2.1	19.4±1.1	21.3±1.1

Table I: ScGOS/lcFOS/pAOS dietary supplement does not affect the RSV-specific lung CD8$^+$ T cell response during primary RSV infection.

C57BL/6 mice received scGOS/lcFOS/pAOS (GFA) supplemented 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 figure shows the mean ± SEM of control or scGOS/lcFOS/pAOS treated mice from 2 individual experiments, n=8 / group.

original vaccine as tested in the 1960's in infants was used (25). 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 single cell suspensions were analyzed for inflammatory cell influx and T cell responses (Fig. 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 ± 0.6 x10^6 cells, compared to control mice). Furthermore, analysis of the BAL fluid cell composition showed that eosinophil influx, characteristic for this model, was significantly 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 (Fig. 2B).

Interestingly, the amount of IL-4, IL-5, and IL-13 producing CD4$^+$ T cells peaked at day 6 after infection while the IFN-γ producing T cells followed a similar pattern compared to the primary infection i.e. they were still increasing at day 8 post infection. Dietary intervention significantly decreased IL-4, IL-5, and IL-13 producing T cell numbers early after challenge at day 4 and 6. Moreover, similar to the primary infection model, a significantly ($p<0.05$) increased number of CD4$^+$ IFN-γ producing cells was measured 8 days after challenge (44.1 ± 4.4 vs. 32.6 ± 3.8 CD4$^+$ IFN-γ $^+$ cells) in mice receiving prebiotic diet compared to control (Fig. 2C).

The development of the CD8$^+$ T cell response against RSV showed no difference in total number of activated (NKG2A$^+$) CD8$^+$ cells between the two diet groups (Table 2). At day 4 p.i. the percentage of recently activated CD8$^+$ T cells was twofold lower (10.0 ± 1.3 versus 19.1 ± 1.5 % NKG2A$^+$ / CD8$^+$ cells) in FI-RSV vaccinated mice compared to a primary RSV infection showing a slower development

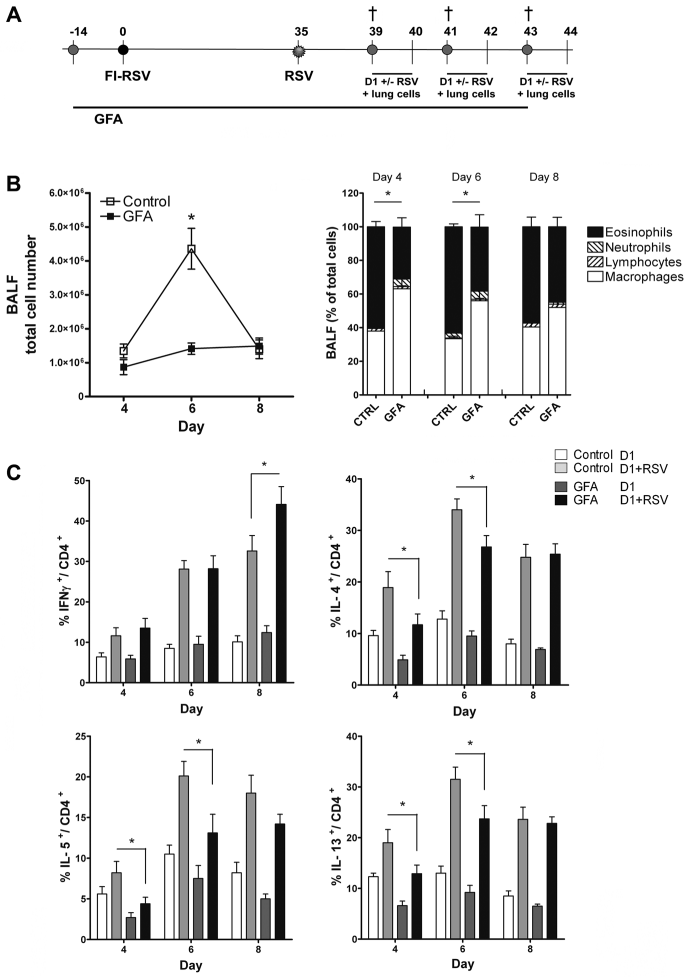


Figure 2. Dietary intervention with scGOS/lcFOS/pAOS (GFA) decreases the Th2 type immune response and lung eosinophilia in FI-RSV vaccinated mice.

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 % of total BAL cells. **C.** percentages of cytokine producing $CD4^+$ T cells of *in vitro* restimulated 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 (*$p<0.05$).

of the $CD8^+$ T cell response in vaccinated mice. The virus specific $CD8^+$ T cell response, measured by the $M_{187–195}$ tetramer staining, showed a significant increase in virus specific $CD8^+$ T cell numbers in the prebiotic diet group 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 treated mice) were measured at this time point.

	% NKG2A ^{POS}/ CD8 ^{POS}		% IFNγ ^{POS}/ CD8 ^{POS} (D1-RSV) §		%TET ^{POS}/ CD8 ^{POS}		% IFNγ ^{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 *

Table II. ScGOS/lcFOS/pAOS dietary supplement increases RSV-specific lung CD8$^+$ T cell response in FI-RSV vaccinated mice.

C57BL/6 mice received scGOS/lcFOS/pAOS (GFA) supplemented 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 figure shows the mean +/- SEM of control or scGOS/lcFOS/pAOS 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.

Reduced airway eosinophila at day 4 and 6 after challenge (Fig. 2B) correlated with significantly decreased numbers of GATA-3 expressing (data not shown) and RSV specific IL-4, -5, -13 producing CD4$^+$ T cells (Fig. 2C) in the lungs of prebiotic treated mice. These data show that dietary intervention with scGOS/lcFOS/pAOS can shift 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.

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 2 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 antigen from airways (30,31). Different DC populations might locally be involved in polarization of Th cell subsets (32,33). In this study the kinetics of lung DC populations present during the FI-RSV induced disease was investigated, (Fig. 3B). 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 treatment 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 fed control

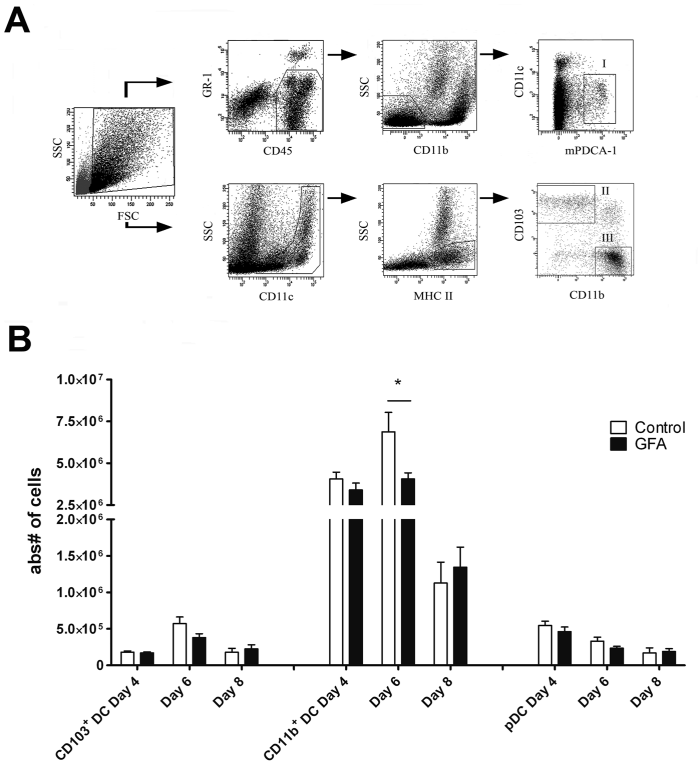


Figure 3. Dietary intervention with scGOS/lcFOS/pAOS (GFA) decreases CD11c$^+$ CD11b$^+$ DC influx in lung tissue of FI-RSV vaccinated mice at day 6 after RSV infection.

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 (*p<0.05).

diet compared to mice receiving prebiotic diet. 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 can affect the lung CD11b$^+$ DC population, a subset known to be important in leukocyte recruitment, at day 6 after RSV challenge in FI-RSV induced disease.

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 (13). Its effect on systemic immunity however, remains unclear and is a growing field of interest. In this study we demonstrate that a specific mixture of orally applied oligosaccharides scGOS/lcFOS/

pAOS, with known prebiotic properties (23) 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 (34-36). In this model the oral administration of scGOS/lcFOS/pAOS reduced lung cell infiltration at day 6 after challenge and airway eosinophila at day 4 and 6 after challenge which correlated with significant decreased numbers of GATA-3 expressing (data not shown) and RSV specific IL-4, -5, -13 producing $CD4^+$ T cells in the lungs of scGOS/lcFOS/pAOS treated mice. Although a slightly different ratio was used in previous studies (9:1:2), these findings extend earlier observations that a prebiotic mixture decreases lower airway hyperreactivity, IgE serum levels and lung inflammatory cell influx in a ovalbumin induced murine asthma model. (37). 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 (19). These studies all underscore the immune modulating and more specifically a Th1 skewing effect of treatment with 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 prebiotic treated animals. Interestingly, the increase in IFN-γ production at day 8 post infection was also seen during primary RSV infection when Th2 response do not play a significant role. This suggests that the reduced Th2 responsiveness mediated by the prebiotic diet is related to a mechanism which favors the development of a Th1 type of response.

The mechanism(s) involved in systemic immune modulation by the prebiotic diet is currently largely 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 (38-41). 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) (42). 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 (16).

In the FI-RSV induced disease model we showed that lung $CD11b^+$ DC populations, T cell and airway eosinophil populations can be controlled by a mechanism induced by the prebiotic diet. Peak DC, Th2 cell and eosinophil concentrations showed similar kinetics and it is currently unclear by which cell type or what mechanism suppression of the Th2 arm of the immune response might be achieved in animals on the prebiotic diet. We are currently checking the possible involvement of regulatory T cells in this mechanism (24). Preliminary data suggest that 4 days after challenge, $FoxP3^+/CD4^+$ cell populations are systemically decreased in mice that received the oligosaccharide diet (data not shown).

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 become systemically available (43). Lectins are known to bind carbohydrate structures and lectins are known to 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) (44,45). A relation between Tregs and dietary oligosaccharides has been found in a mouse influenza vaccination model in which scGOS/lcFOS/pAOS supplementation of the diet was found to affect $CD25^+$ Treg numbers (24).

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 (23,24). 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 $2x10^6$ 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 (25). 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 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).

In vitro re-stimulation

Isolated lung cells were restimulated 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) (26). 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 (27). 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 (28). 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

Cells were first pre-incubated with 2.4.G2 an Fc receptor specific Ab (anti-CD16/32) to reduce non-specific binding. To identify mDC populations in lung tissue, lung single cell suspensions were washed with PBS containing 2% FCS, 2mM 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). For pDC populations cells were incubated with anti-Ly6C (BD, clone RB6-8C5), anti-CD45R (BD, clone RA3-6B2), anti-CD11b (BD, clone M1/70), anti-CD11c (BD, clone HL3) and anti-mPDCA-1(Miltenyi Biotec, clone JF05-1C2.4.1). RSV specific $CD8^+$ T cells were visualized with MHC class I - $M_{187–195}$ tetramer, manufactured as previously described (29). 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 (BD, clone 20d5), anti-CD8 (BD, clone 53-6.7) and anti-CD4 (BD, 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 (BD, clone XMG1.2), anti-IL-5 (BD, clone TRFK5), anti-IL-4 (BD, 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) and differences were considered significant when $p<0.05$.

References

1. 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.
2. Martinez, F. D. 2003. Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. Pediatr. Infect. Dis. J. 22:S76-S82.
3. 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.
4. 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.
5. 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.
6. 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.
7. Olson, M. R., and S. M. Varga. 2008. Pulmonary immunity and immunopathology: lessons from respiratory syncytial virus. Expert. Rev. Vaccines. 7:1239-1255.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. Maslowski, K. M., and C. R. Mackay. 2011. Diet, gut microbiota and immune responses. Nat. Immunol. 12:5-9.
14. 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. 5: 1087-1094
15. Sin, D. D., and E. R. Sutherland. 2008. Obesity and the lung: 4. Obesity and asthma. Thorax 63:1018-1023.
16. 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.
17. 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.
18. 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.
19. 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.
20. 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.

21. 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.
22. 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.
23. Gori, A., G. Rizzardini, B. van 't Land, K. B. Amor, J. van Schaik, C. Torti, T. Quirino, C. Tincati, A. Bandera, J. Knol, K. Benlhassan-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. doi:10.1038/mi.2011.15.
24. van 't Land, B., M. A. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. Beaty, S. R., C. E. Rose, Jr., and S. S. Sung. 2007. Diverse and potent chemokine production by lung $CD11b^{high}$ dendritic cells in homeostasis and in allergic lung inflammation. J. Immunol. 178:1882-1895.
31. 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.
32. 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.
33. 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.
34. 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.
35. 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.
36. 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.

37. 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.
38. 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.
39. 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.
40. 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.
41. 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.
42. 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.
43. 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.
44. 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.
45. 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

chap

ter 7

Summary and general discussion

In this thesis we addressed several aspects concerning RSV infection in the presence or absence of acquired immunity. An important focus was to study the role of antigen presentation, the antigen presenting cells involved and the role of antibodies on RSV specific immunity.

Summary

During a natural infection with RSV mainly respiratory epithelial cells and antigen presenting cells, like alveolar macrophages and dendritic cells (DC) become infected (1-4). Infection of these cells results in initiation of innate immune responses, including the production of interferons. The production of interferons is important for inhibition of viral replication and activation of immune cells. In addition, the production of chemokines and cytokines by innate immune cells triggers an influx of inflammatory cells into the lung. Initiation of the adaptive immune response is predominantly mediated via DCs, which are the professional antigen presenting cells (1). DCs capture antigen either via direct infection or via uptake of viral material through the endosomal route. These cells transport viral material to the lung draining lymph node and present antigenic peptides to $CD4^+$ and $CD8^+$ T cells. Activated T cells migrate back to the site of inflammation to eliminate virus infected cells. We show in **chapter 2** that different subsets of lung DCs present antigenic epitopes of RSV in the context of MHC class I and class II molecules in mice. The $CD103^+CD11b^-$ DCs located directly underneath the airway epithelia and the $CD103^-CD11b^+$ DCs from the lung parenchyma, become infected with RSV. Upon migration to the lung drainig lymph nodes, these DCs present antigens to $CD4^+$ and $CD8^+$ T cells. The activated T cells migrate back to the site of inflammation where they eliminate virus infected cells. Furthermore, also a subset of lymph node resident DCs acquire viral antigens via a non-infectious route and these cells are also capable to activate RSV specific effector/memory $CD4^+$ and $CD8^+$ T cells. It remains to be established whether all these DC types are also able to activate naïve T cells in the lymph nodes. However, to test this T cell receptor transgenic mice are required that are currently not available. After an initial wave of DC migration to the lung draining lymph nodes, the lungs are repopulated with DCs most likely derived from blood monocytes or DC precursors in the lungs (2).

The activation of the adaptive immune response as described above might be different during primary RSV infections and secondary infections, i.e. in the presence of pre-existing immunity like RSV specific T cells and virus specific antibodies. In mouse and ex vivo human studies we addressed the question how antibodies affect (primary) RSV infections and how they modulate the initiation of adaptive immune responses. Antibodies specific for RSV might be obtained by the infant from the mother via the placenta and breast milk or acquired after natural infection. During viral infections antibodies can have different effects on the immune response. Neutralizing antibodies lower the viral load and thereby reduce pathogenesis induced by viral infection. By lowering viral load antibodies can also reduce innate immune responses and inflammation or alter innate immune responses by targeting pathogens to intracellular compartments, where different TLRs are present compared to the cell surface. Both neutralizing and non-neutralizing antibodies can play a role in

the antigen presentation process and determine the level of $CD8^+$ and $CD4^+$ T cell activation (5-8).
We studied the effect of antibodies on the initiation of RSV specific T cell responses in a mouse RSV infection model using wild type C57BL/6 and FcR knockout mice. In **chapter 3** we showed that antibodies critically affected the RSV specific $CD4^+/CD8^+$ T cell balance in the mouse models. We had found earlier that during a primary RSV infection in children admitted to the intensive care unit mainly virus specific $CD8^+$ T cell responses were observed in peripheral blood and hardly any $CD4^+$ T cells (9). In contrast, in healthy adults very low numbers of RSV specific $CD8^+$ T cells were detectable in peripheral blood while $CD4^+$ T cells were more abundant (**chapter 3** and 10-14). We showed *in vitro* using PBMCs that non-neutralizing and neutralizing antibodies enhanced RSV specific $CD4^+$ T cell responses while neutralizing antibodies decreased $CD8^+$ T cell responses. These different effects of neutralizing and non-neutralizing antibodies were also observed in the *in vivo* mouse model. From this observation that neutralizing antibodies decreased the balance of $CD8^+/CD4^+$ virus specific T cells it appears that antibody mediated cross-presentation was presumably a minor route by which $CD8^+$ T cell responses were elicited, while direct infection of APC or possibly cross-presentation of dying epithelial cells might be more important.
The altered balance between RSV specific $CD4^+$ and $CD8^+$ T cell responses in the presence of antibodies suggested an altered route for antigen presentation of RSV opsonized by IgGs. FcγRs are expressed on antigen presenting cells, they bind immune complexes (IC) and promote internalization of the antigen into the endosomal route. Internalization of IC into the endosomal route facilitates MHC class II antigen presentation and subsequent $CD4^+$ T cell activation (15, 16). In wild type mice we showed that RSV-IC were more efficiently presented to $CD4^+$ T cells when activating FcγRs were expressed compared to the sitiuation in FcγR knockout mice, in both experiments *in vivo* and *in vitro* (**chapter 3**). The inhibiting FcγR-IIb did not play such a role. In conclusion, both neutralizing and non-neutralizing antibodies increased the $CD4^+$ T cell response via an FcγR dependent antigen presentation route, while neutralizing antibodies lowered $CD8^+$ T cell responses. It is interesting to speculate that the existing neutralizing antibody response in RSV-immune individuals might favor $CD4^+$ T cell expansion and thus explain the low virus $CD8^+/CD4^+$ T cell ratio for RSV in adults and elderly individuals (17). In contrast to the situation for RSV, influenza virus $CD8^+$ T cells outnumber $CD4^+$ T cells. The difference might be explained by the fact that influenza viruses escape antibody neutralization and therefore efficiently boost $CD8^+$ T cell responses in immune individuals.
In addition to FcγRs, FcRn binds the IgG Fc domain, but at a different site then FcγR. Due to a high binding affinity for IgG at low pH FcRn binds IgG in endosomes, whereas FcγRs bind IgG at the cell surface at neutral pH (18, 19). FcRn plays a role in protecting antibodies from degradation in lysosomes by shuttling endocytosed IgGs back to the cell surface, where they are released (20, 21). In addition to its function in increasing antibody half life, FcRn plays a role in antibody translocation across epithelial barriers. FcRn is located in gut and lung epithelium and facilitates antibody translocation into gut and airways and vice versa (22, 23). FcRn is also important in translocation of IgG from mother to child across the placenta. Recently, FcRn was found to mediate MHC class II mediated antigen presentation of the model antigen OVA, when it was opsonized by IgGs (24). Since

we found that FcγRs played an important role in the antigen presentation in immune mice, we decided to study the role of FcRn in antigen presentation during RSV infection. We showed for RSV that FcRn was not involved in antigen presentation to $CD4^+$ T cells (**chapter 4**). Furthermore, we tested whether FcRn was involved in immune complex transport across the lung epithelial barrier during RSV infections facilitating viral antigen access to APC in the lungs. We showed indeed that increased T cell responses were found when RSV was applied as an immune complex with neutralizing antibodies compared to inoculation of UV-inactivated RSV. However, this enhanced response was FcRn independent (**chapter 4**).

Despite the fact that a (primary) RSV infection results in the induction of $CD4^+$ and $CD8^+$ T cell responses and stimulates the production of neutralizing antibodies, re-infections occur frequently even with genetically similar virus strains (25). The fact that natural immunity is not 100% effective against re-infections is a hurdle for vaccine development. It is important to understand how highly neutralizing antibodies and efficient T cell responses can be induced and maintained via a primary exposure to RSV or an RSV vaccine. Several vaccination approaches have been studied such as formalin-inactivated RSV (FI-RSV), life-attenuated viruses and subunit (protein) vaccines. A clinical trial with FI-RSV performed in the 1960s resulted in a disaster. Children who received FI-RSV intramuscularly and experienced a natural RSV infection, developed severe lower respiratory tract disease compared to control formalin-inactivated parainfluenza vaccine recipients (26-29).
Different animal models were used to study the FI-RSV mediated enhanced disease (30-36). In BALB/c mice FI-RSV vaccination results in a strong Th2 response with a central role for $CD4^+$ T cells and pulmonary influx of eosinophils/neutrophils. This is accompanied by decreased $CD8^+$ T cell responses, poor neutralizing antibody responses, airway hypersensitivity, increased mucus production and weight loss (37). We performed FI-RSV vaccination experiments in C57BL/6 mice (**chapter 5**) to understand the role of different components of the vaccine contributing to the harmful immune response. We found $CD4^+$/Th2 cells dominating the immune responses, similar to the BALB/c model. We showed in our study that contrary to earlier reports the typical Th2/eosinophilic response was independent of formalin-inactivation of the virus (epitope disruption or carbonyl mediated adjuvant effects 38). Also the adjuvant aluminum hydroxide (an adjuvant known to shift the recall response towards Th2 was not crucial 32, 33, 39-42). We and others (36, 39, 43) showed in mice that the strong Th2 response mediated by FI-RSV vaccination was similar to the allergic response observed in mice vaccinated with the control vaccine. Both FI-RSV (cultured in FCS) and FCS alone administered i.m. induced a Th2 response upon i.n. RSV/FCS challenge. Thus specific characteristics of RSV proteins were not responsible for the deleterious immune response as reported earlier, where particularly the G protein was claimed to induce the extreme Th2 response (34, 35).
Inactivated parainfluenza virus, used as the control vaccine in the 1960s trial, did not cause serious lower respiratory tract disease. We therefore reasoned that not only the specific mode of vaccination, but also characteristics of the virus might contribute to the particular type of immune response upon natural virus exposure. We indeed showed that intramuscular priming of mice with FCS and

an intranasal challenge with influenza virus plus FCS resulted in a reduced Th2 response compared to mice challenged with RSV plus FCS. This suggested that different respiratory viruses can locally influence the nature of T cell responses, presumably by the nature of the local innate response triggered by the infecting virus. We hypothesize that different innate signatures of natural respiratory infections could possibly also explain different effects during allergic/asthmatic responses.
In our study described in **chapter 5** we additionally showed that T cell responses in the lung could be manipulated by the way the immune response was primed. Priming of a Th1 skewed $CD4^+$ T cell response or a $CD8^+$ T cell response (in **chapter 5** accomplished by i.v. injection of matured antigen loaded dendritic cells) resulted in decreased Th2 immunity locally in the lungs of RSV/FCS challenged mice.
Thus there are three critical elements in inactivated RSV vaccines that contribute to the characteristic unfavorable immune response; 1. the absence of priming of a $CD8^+$ or Th1-skewed $CD4^+$ T cell response by inactivated RSV, 2. the innate immune response induced by a natural RSV infection in the lungs is inadequate to shift the Th0, Th2 response induced during priming towards a more protective Th1 response, or the innate response creates a situation whereby Th2 cells are preferably attracted, 3. the virus specific antibodies produced are poorly neutralizing, hence not protecting the host against viral damage, but importantly also not efficiently protecting the host against a high antigenic load in the face of an already primed $CD4^+$ T cell response.

Infants who receive breast milk are protected against severe RSV infections. This is partially explained by the neutralizing antibodies present in breast milk that are transferred to the child. However, more compounds present in breast milk might contribute to protection like non-digestible oligosaccharides. These non-digestible oligosaccharides have been attributed immune modulating effects that may be ascribed to their ability to affect the composition of intestinal microbial flora (44). Recent studies on the effect of these compounds in breast milk to prevent atopic diseases were encouraging. It has been shown that oligosaccharides mimicking those compounds present in breast milk can alter the Th2/Th1 balance (45, 46). Because one explanation for severe primary RSV infections could be the relative immaturity and Th2 bias of the infant immune system we hypothesized that oligosaccharide mediated immune modulation might affect the RSV specific immune response. We studied the effect of a specific oligosaccharide diet in a FI-RSV induced Th2 response in our murine vaccination model (**chapter 6**). We indeed observed that mice on a diet with specific non-digestible oligosaccharides showed a reduced percentage of Th2 cytokine producing $CD4^+$ T cells in FI-RSV vaccinated/RSV challenged mice compared to the response in mice that received control diet. The exact mechanism how oligosaccharides influence the immune response needs to be elucidated.

General discussion: New insights for vaccine development

Since the discovery of RSV many attempts have been made to develop a vaccine that prevents seri-

ous lower respiratory tract infections in infants. However, major challenges are; 1. incomplete immune protection after natural infection, 2. inactivated or subunit vaccines tend to induce immune pathology, 3. the virus is highly infectious and infants are often infected in the first months of life. Therefore, intervention is necessary soon after birth, which is challenging due to immaturity of the immune system characterized by the Th2 bias, which appears to be unfavorable for RSV. Moreover, a vaccine needs to be effective in the presence of maternal antibodies.

It is currently not exactly clear what the correlates of protection are for RSV disease. T cell deficient children are not able to clear RSV efficiently, indicating that T cells are required for effective clearance of the virus (47, 48). RSV specific neutralizing antibodies are present in human serum and provide partial protection against severe RSV lower respiratory tract infections. It has been shown that low serum antibodies correlated with the enhanced susceptibility for re-infection (49-51). Because the bioavailability of antibodies in the lumen of the lungs is only 0.5% – 1.3% of the serum antibody levels, high antibody titers are necessary to be locally protective (52). Thus, serum pharmacokinetics and lung bio-availability of the antibodies need to be taken into account. For this reason, current vaccine strategies are focused on the induction of high titer, highly neutralizing antibody responses in vaccinated individuals. At the moment, high risk groups for RSV infection receive prophylactic treatment with palivizumab, a highly neutralizing antibody directed to the fusion protein of RSV. However, this product only protects 50% of treated individuals and higher affinity antibodies developed recently (Motavizumab 53, 54)) unfortunately were not more effective in a phase 2 trial (55). Furthermore, the consequences of prophylactic administered neutralizing antibodies for the induction of virus specific T cell responses have never been studied. We showed in **chapter 3** that neutralizing antibodies skew the $CD4^+/CD8^+$ T cell balance during RSV infections. One might imagine that prophylactic administration of palivizumab in naive infants or maternal antibodies protect the infant from a serious lower respiratory tract infection due to neutralization of the virus, however, induction of anti-viral $CD8^+$ T cell responses might be suppressed. This may not be favorable since $CD8^+$ T cells are required for efficient viral clearance (56, 57). However, cross-presentation of dead cell material of RSV infected cells to $CD8^+$ T cells in the human physiological system might not be excluded as was shown in **chapter 4**, where we showed that opsonized RSV particles administered i.n. can prime $CD8^+$ T cell responses. They appear to prime immune responses against a broad peptide repertoire of the virus, while natural infection is predominantly directed against one major epitope of the virus. It might be an advantage to induce a $CD8^+$ T cell responses against a broad peptide repertoire to prevent viral escape by mutations.

Regulation of viral infection and anti-viral immunity by antibodies.

The first objective of prophylactic antibody treatment is to prevent infection and suppress inflammation resulting from the virus induced immune responses. However, Miao *et al.* found that non-neutralizing antibodies, in the absence of a RSV specific T cell responses, reduced RSV induced pulmonary inflammation in mice (58). Non-neutralizing antibodies increased viral clearance by a

mechanism that depended on FcRs and might involve processes like ADCC and complement activation. Thus FcRs might be necessary to lower viral load when neutralizing capacity of antibodies is low. While non-neutralizing antibodies might be involved in viral neutralization they might also be involved in the induction of enhanced disease. In FI-RSV mouse models it was shown that the presence of non-neutralizing antibodies resulted in IC deposition in the lungs upon RSV challenge (59). These non-neutralizing antibodies might induce enhanced infection or at least not prevent infection upon exposure to RSV. In addition, IC deposition resulted in activation of the classical complement cascade with tissue damage by formation of the cell-lytic complex C5-9. Furthermore, anaphylotoxins can induce bronchoconstriction, mucus secretion, recruitment of inflammatory cells and T cell activation. In mice it was shown that both complement C3 and IgGs were required for induction of bronchoconstriction upon FI-RSV vaccination and challenge. However, cellular influx into the lungs was dependent on either IgG or C3. These processes might have contributed to enhanced disease in the human vaccine trial, because in lung sections of the two FI-RSV deceased children complement deposition was observed (59).
Despite the contribution of the Fc tail in neutralization of the virus, the Fc tail might also affect the T cell responses due to altered antigen presentation. Enhanced $CD4^+$ T cell activation was shown in **chapter 3** when RSV was opsonized by antibodies. This results in a lower $CD8^+/CD4^+$ T cell ratio if the antibodies were highly neutralizing. To avoid those FcR mediated effects, highly neutralizing nanobodies, antibody fragments consisting of a single monomeric variable domain derived from llama's, might be an option as prophylactic treatment. Hultberg *et al.* made multimeric constructs of llama antibodies directed against RSV. Bivalent RSV nanobodies showed a 4000 fold higher neutralizing capacity compared to palivizumab *in vitro* (60).
Thus the FI-RSV vaccination trial and animal models of FI-RSV enhanced disease taught two lessons. Vaccination approaches should aim to elicit highly neutralizing antibodies to prevent infection related damage and exuberated inflammation. Furthermore, Th2 immune responses need to be avoided. Ideally RSV vaccines should prime a Th1 response, whereby the most effective way to regulate the Th2/Th1 balance is via the induction of a (robust) $CD8^+$ T cell response.

The role of different adjuvants in the induction of an efficient immune response.

Initially the production of non-neutralizing antibodies in the FI-RSV vaccine trial was explained as a consequence of formalin-mediated disruption of RSV epitopes. However, Delgado *et al.* showed that lack of TLR stimulation during vaccination with FI-RSV explained the production of non-neutralizing RSV specific antibodies (61). They showed that vaccination with UV-inactivated RSV also induced non-neutralizing antibodies just like FI-RSV. Simultaneous stimulation of TLR4, TLR3 and TLR7 upon UV-RSV vaccination resulted in higher affinity antibody production and protection against enhanced disease in these mice. They further showed that direct TLR stimulation of B cells, induced B cell maturation with clonal expansion, class switching, somatic hypermutation and production of high affinity antibodies. Also other studies showed that addition of TLR ligands during

vaccination skews the Th2 response towards Th1 (62, 63). However, not all TLR ligands are equally effective and the moment of TLR administration is critical. Addition of TLR9 agonist CpG during FI-RSV administration increased the protection against RSV challenge by increased neutralizing antibody titers, altered chemokine and cytokine profiles, reduced weight loss and reduced influx of eosinophils into the lungs (64). However, treatment with TLR7/8 agonists did not affect the FI-RSV induced Th2 immunity. In contrast to these observations, CpG did not affect the Th2 immune response in FI-RSV vaccinated mice when administered during intranasal challenge while TLR7/8 agonists reduced Th2 immunity slightly. A TLR2 ligand incorporated in a non-replicating RSV virosome vaccine also resulted in highly neutralizing antibodies, antigen presentation to $CD4^+$ and $CD8^+$ T cells and a favorable Th1/Th2 response in mice and cotton rats (63).
In addition to incorporating TLR ligands in RSV vaccines, the presence of natural TLR ligands on (intestinal) microbial flora modulated the immune responses during priming (**chapter 6**). A recent study using an influenza virus infection model in mice suggested that gut microbes were required to provide a TLR stimulus for generation of effective virus-specific $CD4^+$ and $CD8^+$ T cell and antibody responses. Local or systemic injection of TLR ligands restored the immune impairment upon depletion of neomycin-sensitive bacteria in the animals. Different TLR ligands are required as adjuvant when live or inactivated antigens are used as vaccine. Microbiota induced TLR stimuli did not affect OVA specific immune responses. Moreover, the nature of the innate immune response triggered by the natural virus might determine the requirements of the microbial sensitization of the immune system of the host, because it was illustrated that gut microbiota did not affect herpes simplex virus type 2 immunity (65). We currently do not know what the mechanisms are of immune modulation with prebiotics in the FI-RSV model described in **chapter 6**. Currently, studies are ongoing to determine the effect the diet has on the composition of gut and oral microbiota.
Clearly, TLR ligands could contribute to a more beneficial response by potentiating a more mature B cell response. Moreover, our work in **chapter 5** where we showed that LPS matured DCs primed a strong Th1 type response suggest that there might be potential inactivated vaccine approaches, if the adjuvant is carefully chosen to increase B cell maturation and strongly polarize Th1 type immunity.

Involvement of antigen presenting cells during priming and in the effector phase of the immune response.

Important factors to keep in mind when making the choice of live versus inactivated vaccines are the particular adjuvant to be used, the route by which the vaccine will be administered and the antigen presenting cells present at a particular location. This was nicely shown in a primary antigen exposure model, where different lung DC subsets contributed differently in the activation of $CD4^+$ and $CD8^+$ T cells when mice were intranasally inoculated with influenza virus or exposed to ovalbumin. $CD11b^+$ DCs presented antigen mainly to $CD4^+$ T cells, while $CD103^+$ DCs were important for the induction of $CD8^+$ T cell responses (3). Intranasal infection of mice with influenza virus resulted

also in antigen presentation by $CD11b^+$ DCs to $CD8^+$ T cells, albeit to a lower extent than $CD103^+$ DCs. During RSV infections in mice, we found that different lung DC subsets acquired viral antigen and were equally efficient in presenting antigen to memory $CD4^+$ and $CD8^+$ T cells when tested ex vivo (**chapter 2**). Thus administration of live or inactivated antigens appear to influence the cell types involved in antigen presentation, the route of antigen processing and the subset of T cells activated. In addition, at different locations different DC subsets might contribute to the priming of an immune response. Therefore, the priming route of a vaccine (intradermal, intranasal, intramuscular or intravenous) can affect the quality of the priming of the immune response.

During the effector phase local DC populations are important regulators of immune responses by producing chemokines and polarizing cytokines. In an OVA allergy model, it was shown that $CD11c^+$ DCs are involved in enhancing allergic lung inflammation (66, 67). Depletion of $CD11c^+$ DCs resulted in a reduced Th2 response (68). 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 (69, 70). In our FCS allergic model we showed that $CD103^+$ and especially $CD11b^+$ DCs (mDC) were recruited to the lungs upon RSV/FCS challenge and were more abundant than during primary RSV infections or in mice primed and challenged with live virus. In the FCS allergic model, respiratory challenge with influenza virus plus FCS reduced the recruitment of mDCs compared to RSV plus FCS challenge. In these experiments the lower influx of mDCs nicely associated with decreased Th2 responses. Also in the prebiotic experiments lower Th2 responses correlated with lower influx of mDCs and eosinophilia. Whether mDC and especially $CD11b^+$ DCs orchestrate the Th2 response needs to be determined. Because in a house dust mite allergic model, GM-CSF, IL-33, TSLP and IL-25 secreted by airway epithelia caused a strong Th2 response with increased $CD11b^+$ DC numbers (71). For different antigens the regulation of the immune response is different depending on the innate response induced by the antigen, cell types present at the location of antigen exposure, the cell types secreting the cytokines, cellular recruitment upon cytokine production and the type of cytokines produced by inflammatory cells. These features also affect the kinetics of cellular influx and efflux in the antigen exposed tissue. In the RSV allergic model we showed that influx of DCs and Th2 cells occurred with similar kinetics (**chapter 5**). The cells involved in initiating the recruitment of both cell types or whether recruitment of one cell type affects the recruitment or polarization/maturation of the other needs to be determined.

Current RSV vaccine approaches

Different strategies for RSV immunoprophylaxis are live vaccines (replication competent or replication defective vectors), protein vaccines (Purified F) and particle vaccines (VLP's or virosomes). For all vaccine strategies researchers have to deal with the fact that the vaccine should elicit a more efficient immune response compared to a natural infection with RSV. At the moment much effort has gone into the development of life attenuated RSV vaccines. The advantage of life attenuated

vaccines is the reduction of infection related pathology, proteins are expressed in their native conformation, and natural innate responses are initiated. Moreover, the vaccine might be administered intranasally for induction of local immunity in the lungs (72-75). However, it has been difficult to achieve the right level of attenuation. Under-attenuation results in residual virulence and over attenuation might result in insufficient immunogenicity.
An additional hurdle in vaccine development is the age of the target populations. Both infants and elderly people suffer from RSV related serious lower respiratory tract infections. Both populations might require different vaccine approaches. In infants maternal antibodies might interfere with the immune response induced by the vaccine. In addition, infants have a Th2 skewed immune response which might not be beneficial for the induction of an anti-RSV immune response. With respect to the elderly population one should deal with the fact that immunity wanes upon increasing age with decreasing antibody levels in blood and decreased T cell activity (17, 76). Vaccination of the elderly might be difficult because of the presence of acquired immunity to RSV. It remains to be established why immunity is not effective in the elderly population and how to boost those components of the immune response that are necessary for protection.

In conclusion, the work presented in this thesis provides important contributions to current thinking with respect to vaccine development. Our research clearly implies the requirement for vaccines to induce a Th1 $CD4^+$ and $CD8^+$ T cell response to RSV, accompanied with high neutralizing antibody titers. Time will tell whether this is best accomplished by life attenuated vaccine (native expression of viral proteins, natural innate responses and intranasal administration), subunit/inactivated vaccines or replication defective gene-based vectors. However, the character and intensity of the innate immune response induced by the attenuated virus, the vector or adjuvant will be crucial.

References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392: 245-252.
2. Beyer, M., H. Bartz, K. Horner, S. Doths, C. Koerner-Rettberg, and J. Schwarze. 2004. Sustained increases in numbers of pulmonary dendritic cells after respiratory syncytial virus infection. J. Allergy Clin. Immunol. 113: 127-133.
3. del Rio, M. L., J. I. Rodriguez-Barbosa, E. Kremmer, and R. Forster. 2007. $CD103^-$ and $CD103^+$ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to $CD4^+$ and $CD8^+$ T cells. J. Immunol. 178: 6861-6866.
4. von Garnier, C., L. Filgueira, M. Wikstrom, M. Smith, J. A. Thomas, D. H. Strickland, P. G. Holt, and P. A. Stumbles. 2005. Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. J. Immunol. 175: 1609-1618.
5. de Jong, J. M., D. H. Schuurhuis, A. Ioan-Facsinay, M. M. Welling, M. G. Camps, E. I. van der Voort, T. W. Huizinga, F. Ossendorp, J. S. Verbeek, and R. E. Toes. 2006. Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4 T cells upon immune-complex uptake *in vivo*. Immunology 119: 499-506.
6. Hamano, Y., H. Arase, H. Saisho, and T. Saito. 2000. Immune complex and Fc receptor-mediated augmentation of antigen presentation for *in vivo* Th cell responses. J. Immunol. 164: 6113-6119.
7. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 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.
8. Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J. Immunol. 168: 2240-2246.
9. Lukens, M. V., A. C. van de Pol, F. E. Coenjaerts, N. J. Jansen, V. M. Kamp, J. L. Kimpen, J. W. Rossen, L. H. Ulfman, C. E. Tacke, M. C. Viveen, L. Koenderman, T. F. Wolfs, and G. M. van Bleek. 2010. A systemic neutrophil response precedes robust $CD8(^+)$ T-cell activation during natural respiratory syncytial virus infection in infants. J. Virol. 84: 2374-2383.
10. Brandenburg, A. H., L. de Waal, H. H. Timmerman, P. Hoogerhout, R. L. de Swart, and A. D. Osterhaus. 2000. HLA class I-restricted cytotoxic T-cell epitopes of the respiratory syncytial virus fusion protein. J. Virol. 74: 10240-10244.
11. Cherrie, A. H., K. Anderson, G. W. Wertz, and P. J. Openshaw. 1992. Human cytotoxic T cells stimulated by antigen on dendritic cells recognize the N, SH, F, M, 22K, and 1b proteins of respiratory syncytial virus. J. Virol. 66: 2102-2110.
12. de Waal, L., Y. Suzer, L. S. Wyatt, K. Sintnicolaas, G. Sutter, B. Moss, A. D. Osterhaus, and R. L. de Swart. 2006. T cell responses to respiratory syncytial virus fusion and attachment proteins in human peripheral blood mononuclear cells. Viral Immunol. 19: 669-678.
13. Isaacs, D., C. R. Bangham, and A. J. McMichael. 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet 2: 769-771.
14. Mbawuike, I. N., J. Wells, R. Byrd, S. G. Cron, W. P. Glezen, and P. A. Piedra. 2001. HLA-restricted $CD8^+$ cytotoxic T lymphocyte, interferon-gamma, and interleukin-4 responses to respiratory syncytial virus infection in infants and children. J. Infect. Dis. 183: 687-696.
15. van Niel, G., R. Wubbolts, and W. Stoorvogel. 2008. Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. Curr. Opin. Cell Biol. 20: 437-444.
16. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nat. Immunol. 5: 685-692.
17. de Bree, G. J., J. Heidema, E. M. van Leeuwen, G. M. van Bleek, R. E. Jonkers, H. M. Jansen, R. A. van Lier, and T. A.

Out. 2005. Respiratory syncytial virus-specific CD8+ memory T cell responses in elderly persons. J. Infect. Dis. 191: 1710-1718.

18. Shields, R. L., A. K. Namenuk, K. Hong, Y. G. Meng, J. Rae, J. Briggs, D. Xie, J. Lai, A. Stadlen, B. Li, J. A. Fox, and L. G. Presta. 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J. Biol. Chem. 276: 6591-6604.
19. Wines, B. D., M. S. Powell, P. W. Parren, N. Barnes, and P. M. Hogarth. 2000. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. J. Immunol. 164: 5313-5318.
20. Chaudhury, C., S. Mehnaz, J. M. Robinson, W. L. Hayton, D. K. Pearl, D. C. Roopenian, and C. L. Anderson. 2003. The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. J. Exp. Med. 197: 315-322.
21. Junghans, R. P., and C. L. Anderson. 1996. The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor. Proc. Natl. Acad. Sci. U. S. A 93: 5512-5516.
22. Yoshida, M., S. M. Claypool, J. S. Wagner, E. Mizoguchi, A. Mizoguchi, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2004. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity. 20: 769-783.
23. Israel, E. J., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a beta 2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. J. Immunol. 154: 6246-6251.
24. Qiao, S. W., K. Kobayashi, F. E. Johansen, L. M. Sollid, J. T. Andersen, E. Milford, D. C. Roopenian, W. I. Lencer, and R. S. Blumberg. 2008. Dependence of antibody-mediated presentation of antigen on FcRn. Proc. Natl. Acad. Sci. U. S. A 105: 9337-9342.
25. Hall, C. B., E. E. Walsh, C. E. Long, and K. C. Schnabel. 1991. Immunity to and frequency of reinfection with respiratory syncytial virus. J. Infect. Dis. 163: 693-698.
26. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89: 422-434.
27. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. 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: 405-421.
28. Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn. 1969. 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: 435-448.
29. Chin, J., R. L. Magoffin, L. A. Shearer, J. H. Schieble, and E. H. Lennette. 1969. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 89: 449-463.
30. de Swart, R. L., T. Kuiken, H. H. Timmerman, G. van Amerongen, 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.
31. Prince, G. A., A. B. Jenson, V. G. Hemming, B. R. Murphy, E. E. Walsh, R. L. Horswood, and R. M. Chanock. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. J. Virol. 57: 721-728.
32. Piedra, P. A., H. S. Faden, G. Camussi, D. T. Wong, and P. L. Ogra. 1989. Mechanism of lung injury in cotton rats immunized with formalin-inactivated respiratory syncytial virus. Vaccine 7: 34-38.

33. Waris, M. E., C. Tsou, D. D. Erdman, S. R. Zaki, and L. J. Anderson. 1996. 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: 2852-2860.
34. Openshaw, P. J., S. L. Clarke, and F. M. Record. 1992. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. Int. Immunol. 4: 493-500.
35. Culley, F. J., A. M. Pennycook, J. S. Tregoning, T. Hussell, and P. J. Openshaw. 2006. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. J. Virol. 80: 4521-4527.
36. Vaux-Peretz, F., J. M. Chapsal, and B. Meignier. 1992. 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: 113-118.
37. Openshaw, P. J., F. J. Culley, and W. Olszewska. 2001. Immunopathogenesis of vaccine-enhanced RSV disease. Vaccine 20 Suppl 1: S27-S31.
38. Moghaddam, A., W. Olszewska, B. Wang, J. S. Tregoning, R. Helson, Q. J. Sattentau, and P. J. Openshaw. 2006. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat. Med. 12: 905-907.
39. Boelen, A., A. Andeweg, J. Kwakkel, W. Lokhorst, T. Bestebroer, J. Dormans, and T. Kimman. 2000. 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: 982-991.
40. Rimaniol, A. C., G. Gras, F. Verdier, F. Capel, V. B. Grigoriev, F. Porcheray, E. Sauzeat, J. G. Fournier, P. Clayette, C. A. Siegrist, and D. Dormont. 2004. Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. Vaccine 22: 3127-3135.
41. Sun, H., K. G. Pollock, and J. M. Brewer. 2003. Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation *in vitro*. Vaccine 21: 849-855.
42. Ulanova, M., A. Tarkowski, M. Hahn-Zoric, and L. A. Hanson. 2001. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect. Immun. 69: 1151-1159.
43. Piedra, P. A., P. R. Wyde, W. L. Castleman, M. W. Ambrose, A. M. Jewell, D. J. Speelman, and S. W. Hildreth. 1993. 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: 1415-1423.
44. Vos, A. P., M. Haarman, J. W. van Ginkel, J. Knol, J. Garssen, B. Stahl, G. Boehm, and L. M'Rabet. 2007. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatr. Allergy Immunol. 18: 304-312.
45. Macfarlane, G. T., H. Steed, and S. Macfarlane. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J. Appl. Microbiol. 104: 305-344.
46. 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.
47. Fishaut, M., D. Tubergen, and K. McIntosh. 1980. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J. Pediatr. 96: 179-186.
48. Hall, C. B., K. R. Powell, N. E. MacDonald, C. L. Gala, M. E. Menegus, S. C. Suffin, and H. J. Cohen. 1986. Respiratory syncytial viral infection in children with compromised immune function. N. Engl. J. Med. 315: 77-81.
49. Bruhn, F. W., and A. S. Yeager. 1977. Respiratory syncytial virus in early infancy. Circulating antibody and the severity of infection. Am. J. Dis. Child 131: 145-148.
50. Lamprecht, C. L., H. E. Krause, and M. A. Mufson. 1976. Role of maternal antibody in pneumonia and bronchiolitis due to respiratory syncytial virus. J. Infect. Dis. 134: 211-217.
51. Ogilvie, M. M., A. S. Vathenen, M. Radford, J. Codd, and S. Key. 1981. Maternal antibody and respiratory syncytial

virus infection in infancy. J. Med. Virol. 7: 263-271.

52. Murphy, B. R., B. S. Graham, G. A. Prince, E. E. Walsh, R. M. Chanock, D. T. Karzon, and P. F. Wright. 1986. Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. J. Clin. Microbiol. 23: 1009-1014.
53. Wu, H., D. S. Pfarr, S. Johnson, Y. A. Brewah, R. M. Woods, N. K. Patel, W. I. White, J. F. Young, and P. A. Kiener. 2007. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. J. Mol. Biol. 368: 652-665.
54. Abarca, K., E. Jung, P. Fernandez, L. Zhao, B. Harris, E. M. Connor, and G. A. Losonsky. 2009. Safety, tolerability, pharmacokinetics, and immunogenicity of motavizumab, a humanized, enhanced-potency monoclonal antibody for the prevention of respiratory syncytial virus infection in at-risk children. Pediatr. Infect. Dis. J. 28: 267-272.
55. Fernandez, P., A. Trenholme, K. Abarca, M. P. Griffin, M. Hultquist, B. Harris, and G. A. Losonsky. 2010. A phase 2, randomized, double-blind safety and pharmacokinetic assessment of respiratory syncytial virus (RSV) prophylaxis with motavizumab and palivizumab administered in the same season. BMC. Pediatr. 10: 38.
56. Cannon, M. J., E. J. Stott, G. Taylor, and B. A. Askonas. 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. Immunology 62: 133-138.
57. Cannon, M. J., P. J. Openshaw, and B. A. Askonas. 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168: 1163-1168.
58. Miao, C., G. U. Radu, H. Caidi, R. A. Tripp, L. J. Anderson, and L. M. Haynes. 2009. Treatment with respiratory syncytial virus G glycoprotein monoclonal antibody or F(ab')2 components mediates reduced pulmonary inflammation in mice. J. Gen. Virol. 90: 1119-1123.
59. Polack, F. P., M. N. Teng, P. L. Collins, G. A. Prince, M. Exner, H. Regele, D. D. Lirman, R. Rabold, S. J. Hoffman, C. L. Karp, S. R. Kleeberger, M. Wills-Karp, and R. A. Karron. 2002. A role for immune complexes in enhanced respiratory syncytial virus disease. J. Exp. Med. 196: 859-865.
60. Hultberg, A., N. J. Temperton, V. Rosseels, M. Koenders, M. Gonzalez-Pajuelo, B. Schepens, L. Itati Ibanez, P. Vanlandschoot, J. Schillemans, M. Saunders, R. A. Weiss, X. Saelens, J. A. Melero, C. T. Verrips, S. Van Gucht, and H. J. de Haard. 2011. Llama-derived single domain antibodies to build multivalent, superpotent and broadened neutralizing anti-viral molecules. PLoS. One. 6: e17665.
61. Delgado, M. F., S. Coviello, A. C. Monsalvo, G. A. Melendi, J. Z. Hernandez, J. P. Batalle, L. Diaz, A. Trento, H. Y. Chang, W. Mitzner, J. Ravetch, J. A. Melero, P. M. Irusta, and F. P. Polack. 2009. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat. Med. 15: 34-41.
62. Boukhvalova, M. S., G. A. Prince, L. Soroush, D. C. Harrigan, S. N. Vogel, and J. C. Blanco. 2006. The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 24: 5027-5035.
63. Stegmann, T., T. Kamphuis, T. Meijerhof, E. Goud, A. de Haan, and J. Wilschut. 2010. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Vaccine 28: 5543-5550.
64. Johnson, T. R., S. Rao, R. A. Seder, M. Chen, and B. S. Graham. 2009. 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 27: 3045-3052.
65. 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.
66. Beaty, S. R., C. E. Rose, Jr., and S. S. Sung. 2007. Diverse and potent chemokine production by lung CD11b^{high} dendritic cells in homeostasis and in allergic lung inflammation. J. Immunol. 178: 1882-1895.
67. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J. Exp. Med. 207: 2097-2111.

68. van Rijt, L. S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H. C. Hoogsteden, and B. N. Lambrecht. 2005. *In vivo* depletion of lung CD11c$^+$ dendritic cells during allergen challenge abrogates the characteristic features of asthma. J. Exp. Med. 201: 981-991.
69. Lambrecht, B. N., M. De Veerman, A. J. Coyle, J. C. Gutierrez-Ramos, K. Thielemans, and R. A. Pauwels. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. J. Clin. Invest 106: 551-559.
70. de Heer, H. J., H. Hammad, T. Soullie, D. Hijdra, N. Vos, M. A. Willart, H. C. Hoogsteden, and B. N. Lambrecht. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. J. Exp. Med. 200: 89-98.
71. Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat. Med. 15: 410-416.
72. Polack, F. P., and R. A. Karron. 2004. The future of respiratory syncytial virus vaccine development. Pediatr. Infect. Dis. J. 23: S65-S73.
73. Karron, R. A., P. F. Wright, R. B. Belshe, B. Thumar, R. Casey, F. Newman, F. P. Polack, V. B. Randolph, A. Deatly, J. Hackell, W. Gruber, B. R. Murphy, and P. L. Collins. 2005. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J. Infect. Dis. 191: 1093-1104.
74. Wright, P. F., R. A. Karron, R. B. Belshe, J. Thompson, J. E. Crowe, Jr., T. G. Boyce, L. L. Halburnt, G. W. Reed, S. S. Whitehead, E. L. Anderson, A. E. Wittek, R. Casey, M. Eichelberger, B. Thumar, V. B. Randolph, S. A. Udem, R. M. Chanock, and B. R. Murphy. 2000. Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. J. Infect. Dis. 182: 1331-1342.
75. Graham, B. S. 2011. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol. Rev. 239: 149-166.
76. Falsey, A. R., P. A. Hennessey, M. A. Formica, C. Cox, and E. E. Walsh. 2005. Respiratory syncytial virus infection in elderly and high-risk adults. N. Engl. J. Med. 352: 1749-1759.

Nederlandse samenvatting
Dankwoord
Curriculum Vitae
List of publications

Nederlandse samenvatting

Het respiratoir syncytieel virus (RSV) is een virus dat luchtweg infecties kan veroorzaken bij de mens. Voor het 2e levensjaar raakt 90% van de kinderen voor de eerste keer geïnfecteerd met RSV. Mensen ondergaan gedurende hun leven meerdere infecties met RSV met vaak milde symptomen van verkoudheid. Kinderen jonger dan 6 maanden, patiënten met een immuun deficiëntie en ouderen kunnen ernstige lagere luchtweg infecties ontwikkelen na een RSV infectie. Dit gaat gepaard met symptomen van ernstige longontsteking of bronchiolitis, zuurstof tekort en verminderde voedselopname bij baby's. Ongeveer 1-3% van de met RSV geïnfecteerde baby's ontwikkelt een ernstige lagere luchtweg infectie waarbij ziekenhuis opname noodzakelijk is. In 10% van deze groep is mechanische beademing en dus opname op de intensive care unit nodig. Gezonde volwassenen kunnen soms al binnen 4 maanden na een RSV infectie opnieuw geïnfecteerd raken met RSV. Dit geeft aan dat het natuurlijk afweersysteem niet voldoende bescherming biedt tegen RSV.

RSV werd in 1956 ontdekt en sindsdien hebben verschillende onderzoeksgroepen een poging gedaan om een vaccin te ontwikkelen tegen RSV. De eerste poging was een formaline geïnactiveerd RSV (FI-RSV) vaccin. Deze techniek werd ook toegepast bij onder andere een parainfluenza vaccin waar voor het bescherming bood tegen een natuurlijke infectie met het virus. In tegenstelling tot het parainfluenza virus vaccin ontwikkelde 80% van de kinderen gevaccineerd met FI-RSV ernstige lagere luchtweg klachten na een natuurlijke infectie met RSV. Twee kinderen zijn zelfs overleden aan de ernstige RSV infectie. Sindsdien zijn er geen nieuwe vaccins op de markt gebracht.

Voor de ontwikkeling van een vaccin gericht tegen RSV is het essentieel om de RSV specifieke immune response te bestuderen en te begrijpen waarom een natuurlijke infectie door RSV geen blijvende bescherming biedt tegen her-infecties met een genetisch identieke RSV stam.
Na een virale infectie wordt de intrinsieke ('innate') en de adaptieve (geheugen) immuun response in werking gezet. De innate immuun respons is een a-specifieke afweer reactie. Immuun cellen die hierbij betrokken zijn reageren op structuren die aanwezig zijn op het virus partikel. Deze cellen gaan door de interactie met het virus interferonen produceren waardoor de anti-virale respons wordt geïnitieerd. Dit resulteert in remming van virale replicatie en activatie van immuun cellen. Daarnaast worden er cytokines en chemokines geproduceerd die er voor zorgen dat andere immuun cellen naar de locatie van infectie migreren. In de tijd dat de innate immuun respons plaats vindt wordt de virus specifieke adaptieve immuun respons geïnitieerd. Deze adaptieve respons is een paar dagen later actief op de locatie van infectie dan de innate immuun respons, maar deze cellen blijven wel aanwezig in het individu om een tweede infectie met het zelfde virus sneller op te ruimen dan na een eerste infectie. Activatie van de adaptieve immuun respons, waaronder T cellen en B cellen, gebeurt door antigeen presenterende cellen (APC). Deze APCs transporteren viraal materiaal naar de lokale lymfeklieren om daar T cellen en B cellen te activeren. APCs kunnen viraal materiaal opnemen door directe infectie met virus of door opname van dood celmateriaal

van geïnfecteerde cellen. Na opname van deze virale eiwitten worden stukjes van deze eiwitten: epitopen, gepresenteerd aan T cellen via MHC moleculen. Geactiveerde T cellen migreren terug naar het geïnfecteerde weefsel om daar de infectie te bestrijden. In de lymfeklieren kunnen twee belangrijke subtypes T cellen geactiveerd worden. De $CD4^+$ en de $CD8^+$ T cellen. $CD4^+$ T cellen zijn helper T cellen en kunnen cytokines produceren waardoor ze de immuun respons kunnen beïnvloeden of sturen. De $CD8^+$ T cellen zijn de killer T cellen. Deze cellen doden virus geïnfecteerde cellen. De B cellen gaan na activatie virus specifieke antilichamen produceren. Deze antilichamen komen in het bloed terecht en zijn pas nuttig tijdens een nieuwe blootstelling aan het zelfde virus. Binding van antilichamen aan een virus partikel zorgt er tijdens de meeste virale infecties voor dat het virus wordt geneutraliseerd en daardoor niet opnieuw kan infecteren.

Na een primaire blootstelling met RSV raken voornamelijk luchtwegepitheel cellen en APCs, gelokaliseerd in de longen, geïnfecteerd. Deze APCs transporteren het viraal materiaal naar de lymfeklieren waar zowel $CD8^+$ als $CD4^+$ T cellen geactiveerd raken. Ook worden de B cellen geactiveerd om RSV specifieke antilichamen te produceren. Er zijn verschillende cellen die behoren tot de APCs waaronder de dendritische cellen (DCs). Dit zijn de professionele APCs. In **hoofdstuk 2** laten we zien dat er tijdens een RSV infectie verschillende subtypes van de DCs een bijdrage leveren aan de $CD4^+$ en $CD8^+$ T cel activatie in de lymfe klieren. Daarnaast beschrijven we ook de kinetiek van de migratie van de verschillende subtypes DCs uit de longen naar de lymfeklieren. Hierbij zien we dat er tegelijkertijd een influx van DCs uit het bloed naar de longen plaatsvindt. Hierdoor verandert de samenstelling van de DCs in de longen na een RSV infectie vergeleken met de situatie voorafgaand aan de infectie. Dit kan mogelijk gevolgen hebben voor het verloop van een herhaalde infectie. Tijdens een herhaalde infectie kunnen de lokale innate respons geïnduceerd door het virus en de verschillende subtypes DCs die in de longen aanwezig zijn en die hierdoor geactiveerd worden mogelijk het karakter van de uiteindelijke T cel response beïnvloeden.

De inductie van een immuun respons tegen RSV kan anders verlopen in de aanwezigheid van antilichamen. Deze antilichamen kunnen afkomstig zijn van de moeder in de eerste maanden na de geboorte, of RSV specifieke antilichamen zijn aangemaakt na een voorgaande infectie met RSV. Deze antilichamen kunnen de immuun respons beïnvloeden doordat ze het virus neutraliseren. Hierdoor vindt er minder infectie plaats en wordt de innate immuun respons ook beïnvloed. Innate immuun processen kunnen verminderen maar ook van karakter veranderen. Mogelijk kunnen antilichamen ook de adaptieve immuun response beïnvloeden. Als antilichamen aan het virus binden (het virus wordt geopsoniseerd door antilichamen) kan de route van antigeen presentatie aan $CD4^+$ of $CD8^+$ T cellen beïnvloed worden. Wanneer een virus door antilichamen wordt geopsoniseerd wordt het een immuun complex genoemd (IC). Een directe virale infectie van APCs resulteert in presentatie van stukjes van RSV eiwitten, epitopen, aan $CD8^+$ T cellen via de MHC klasse I moleculen. Zodra het virus geneutraliseerd is door antilichamen kan het niet meer infecteren en is het in de regel zo dat er ook minder $CD8^+$ T cel activatie plaatsvindt. Het IC wordt wel door de cel opgenomen, maar via

een andere route. Wanneer het IC aan de buitenkant van de cel bindt wordt het door de cel omgeven door een membraan en wordt het door de cel opgenomen in een apart compartiment; een endosoom. Deze route wordt de endosomale route genoemd. Zodra het virus in deze route terecht komt worden de epitopen gepresenteerd in MHC klasse II moleculen. Dit heeft als gevolg dat de $CD4^+$ T cellen geactiveerd worden. Opname van een IC in de endosomale route kan gebeuren door binding van antilichamen in het IC aan moleculen aan het oppervlak van de APC. Deze moleculen worden Fc Receptoren genoemd. In de aanwezigheid van antilichamen kan er dus een verschuiving plaatsvinden in $CD4^+$ of $CD8^+$ T cel activatie.

Voor RSV is het bekend dat antilichamen die verkregen zijn via de moeder bescherming bieden tegen een RSV infectie, maar deze antilichamen nemen binnen enkele maanden in hoeveelheid af. Daarnaast is er een correlatie tussen het niveau RSV specifieke antilichamen in het bloed en de kans op een RSV infectie. Hoe hoger het niveau van RSV specifieke antilichamen in het bloed hoe lager de kans op een RSV infectie. Dus antilichamen kunnen bescherming bieden tegen een RSV infectie wanneer ze in hoge concentratie aanwezig zijn. Om deze reden is palivizumab (Synagis^{tm}) ontwikkeld. Palivizumab is een neutraliserend antilichaam gericht tegen het fusie eiwit van RSV, het eiwit wat betrokken is bij penetratie van de gastheercel. Toediening van palivizumab resulteert in een reductie van RSV gerelateerde ziekenhuisopname in >50% van de behandelde kinderen. Op het moment krijgen alleen hoog risico groepen voor een ernstige RSV infectie profylactische behandeling met palivizumab (Synagis^{tm}) omdat deze behandeling erg duur is en gedurende het winter seizoen maandelijks moet worden herhaald.

Hoe antilichamen de adaptieve immuun respons specifiek voor RSV beïnvloeden hebben we beschreven in **hoofdstuk 3**. In voorgaande studies hebben we laten zien dat kinderen die op de intensive care unit zijn opgenomen vanwege een ernstige RSV infectie voornamelijk een $CD8^+$ T cel respons tegen RSV ontwikkelen. Er zijn in deze kinderen bijna geen RSV specifieke $CD4^+$ T cellen detecteerbaar. Als we de immuun cellen in bloed van volwassen mensen, die meerdere RSV infecties hebben ondergaan, bestuderen dan zien we het tegenovergestelde. Er zijn voornamelijk RSV specifieke $CD4^+$ T cellen aanwezig, maar bijna geen $CD8^+$ T cellen. Om te bestuderen of dit verschil tussen de immuun respons in volwassenen en kinderen wordt veroorzaakt door herhaalde infecties met RSV in de aanwezigheid van RSV specifieke antilichamen, zijn we overgestapt naar een muis model. Hierin laten we zien dat wanneer RSV compleet wordt geneutraliseerd door antilichamen er voornamelijk $CD4^+$ T cellen geactiveerd worden. We hebben daarnaast ook laten zien dat dit via de Fc Receptor wordt gereguleerd. We zien in dit model ook dat wanneer het virus geneutraliseerd is er minder $CD8^+$ T cellen geactiveerd raken. Dus in de aanwezigheid van antilichamen wordt de balans tussen de $CD4^+$ en $CD8^+$ T cel activatie beïnvloed. De activatie van $CD4^+$ T cellen wordt versterkt en van de $CD8^+$ T cellen wordt geremd.

Naast de Fc Receptor is er een tweede receptor die antilichamen kan binden namelijk de neonatale Fc Receptor. Deze receptor komt onder andere tot expressie in de placenta, op luchtweg epitheel cellen en in APCs. In de placenta zorgt de neonatale Fc Receptor er voor dat antilichamen van de moeder naar het kind kunnen worden getransporteerd. In APCs zorgt de neonatale Fc Receptor er

voor dat antilichamen worden gerecycled in het bloed zodat ze niet worden afgebroken. Hierdoor wordt de levensduur van antilichamen in het bloed verlengd. Daarnaast is er aangetoond dat de neonatale Fc Receptor een rol kan spelen in antigeen presentatie aan $CD4^+$ T cellen. Wij laten echter in **hoofdstuk 4** zien dat de neonatale Fc Receptor geen rol speelt in antigeen presentatie van RSV epitopen aan T cellen. Daarnaast is er beschreven dat de expressie van de neonatale Fc Receptor in luchtweg epitheel cellen een belangrijke rol kan spelen in virale infecties. De neonatale Fc Receptor kan er voor zorgen dat antilichamen over het epitheel worden getransporteerd waardoor virus in de luchtwegen kan worden geneutraliseerd. Als gevolg kan de neonatale Fc Receptor ook een rol spelen bij het transport van IC uit de luchtwegen naar longweefsel waar immuun cellen zich bevinden. In een muis model laten we zien dat de T cellen efficiënter worden geactiveerd wanneer RSV als IC in de luchtwegen aanwezig is (**hoofdstuk 4**). Deze efficiëntere T cel activatie is echter niet afhankelijk van de neonatale Fc Receptor. Dus de neonatale Fc Receptor is niet betrokken bij het efficiënt activeren van de adaptieve immuun response wanneer RSV is geopsoniseerd door antilichamen in de luchtwegen. Omdat de concentratie antilichamen in het bloed hoger is dan in de luchtwegen, waar infectie plaatsvindt, zou deze receptor wel een belangrijke bijdrage kunnen leveren in virus neutralisatie door de antilichamen over het epitheel te transporteren, maar dit moet voor RSV nog verder worden onderzocht.

Het is voor vaccin ontwikkeling erg lastig om een manier te vinden om een efficiënte immuun respons te induceren die zowel een sterk neutraliserende antilichaam productie initieert en een anti-virale $CD8^+$ T cel respons aangezien een natuurlijke infectie met RSV dit al niet efficiënt doet. Goed neutraliserende antilichamen zijn vereist om infectie gerelateerde schade in de longen te beperken en een $CD8^+$ T cel respons om RSV geïnfecteerde cellen te doden. Er zijn verschillende pogingen gedaan om een vaccin te ontwikkelen tegen RSV sinds de klinische trials uitgevoerd met FI-RSV. Maar tor dusverre niet met veel succes. Het is essentieel om goed te begrijpen waarom de FI-RSV trial desastreus afliep en waarom dezelfde methode gebruikt voor vaccin ontwikkeling voor parainfluenza wel efficiënte bescherming bood tegen een her-infectie met parainfluenza virus. Om deze reden is FI-RSV vaccinatie bestudeerd in verschillende diermodellen. FI-RSV gevaccineerde muizen ontwikkelen een sterke allergische reactie na een reguliere infectie met RSV. Deze allergische reactie bestaat onder andere uit een versterkte $CD4^+$ T cel activatie en de productie van niet-neutraliserende antilichamen. Er worden hierbij geen $CD8^+$ T cellen geactiveerd. In **hoofdstuk 5** beschrijven we verschillende bevindingen die mogelijk bijdragen aan de sterke allergische reactie na FI-RSV vaccinatie. Allereerst laten we zien dat inactief RSV dat aanwezig is in het vaccin voornamelijk een allergische $CD4^+$ T cel respons activeert. Na blootstelling van de luchtwegen aan levend RSV verergert deze allergische reactie. Door op een andere manier een $CD4^+$ T cel response, een anti-virale (Th1) $CD4^+$ T cel response, te induceren werd de response deels onderdrukt. Deze Th1 $CD4^+$ T cel respons werd gestimuleerd door DCs, gekweekt uit muizen beenmerg, die rechtreeks in de bloedbaan werden geïnjecteerd en door een bacterieel lipopolysaccharide, LPS, geactiveerd werden om de innate immuun respons in de DC te stimuleren. In tegenstelling tot deze methode

werd de allergische (Th2) $CD4^+$ T cel respons geïnduceerd via het injecteren van het vaccin in de spier zonder toediening van LPS, maar met een andere hulpstof (adjuvant) aluminium hydroxide. Er kunnen verschillende redenen zijn waardoor er verschillende $CD4^+$ T cel responsen werden geïnduceerd via de twee methodes. 1. de locatie van injectie van het antigeen waardoor verschillende lymfoide organen worden bereikt voor T cel activatie, 2. de types DCs betrokken bij het activeren van de T cel respons (de beenmerg DCs of de DCs aanwezig in de spier) of 3. de stimulatie van de immuun respons door de twee verschillende adjuvantia; LPS of aluminium hydroxide. Door de uit beenmerg gekweekte DCs ook te beladen met een MHC klasse I epitope van RSV wordt er ook een anti-virale $CD8^+$ T cel respons geïnduceerd, hierdoor werd de allergische reactie compleet onderdrukt. Onze hypothese is dat inactief RSV in het vaccin niet sterk genoeg is om de juiste innate immuun respons te induceren. Daarnaast hebben we ook laten zien dat blootstelling van de luchtwegen aan levend RSV na de vaccinatie ook niet de capaciteit heeft om de allergische reactie te onderdrukken terwijl een intranasale influenza virus infectie dit wel kan. Dus niet alleen de vaccinatie stap maar ook de karakteristieke eigenschappen van het natuurlijke virus zijn mede bepalend voor de uiteindelijk immuun response in de long. Op het moment wordt er veel aandacht besteedt aan het manipuleren van RSV vaccins om de RSV specifieke innate immuun response te versterken en tevens te sturen richting een anti-virale T cel response met een Th1 cytokine profiel en sterk neutraliserende antilichamen.

In de literatuur is beschreven dat kinderen zijn beschermd tegen ernstige RSV infecties doordat er antilichamen van de moeder in het lichaam aanwezig zijn. Deze antilichamen worden overgedragen via de placenta en via moedermelk na de geboorte. Naast het overdragen van antilichamen via de moedermelk worden er echter ook veel andere componenten uit moedermelk overgedragen die een belangrijke bijdrage kunnen leveren aan de efficiëntie van het afweersysteem van het kind. Een categorie van die immuun modulerende componenten zijn specifieke suikers genaamd oligosaccharides. Deze oligosaccharides leveren een belangrijke bijdrage aan de samenstelling van bacteriën in de darmen. Een goede balans in de darm bacteriën zorgt er mede voor dat de inductie van allergische reacties voor verschillende pathogenen of niet-infectieuze allergische stoffen wordt verminderd. In het allergische FI-RSV muizen model laten we zien dat muizen op een dieet van bepaalde oligosaccharides een verminderde allergische reactie ontwikkelen tegen RSV in vergelijking met muizen op een normaal dieet (**hoofdstuk 6**). Het exacte mechanisme moet nog verder worden uitgezocht.

Er wordt veel onderzoek gedaan naar mogelijke vaccins tegen RSV, maar er zijn een aantal aspecten aan de ontwikkeling hiervan die de efficiënte werking van het vaccin kunnen belemmeren. 1. De doelgroep voor vaccin ontwikkeling zijn baby's. Deze kinderen hebben nog antilichamen van de moeder in het bloed. Deze antilichamen kunnen de inductie van een goede adaptieve immuun respons negatief beïnvloeden. Daarnaast is het immuun systeem van het kind nog niet compleet ontwikkeld waardoor het de neiging heeft om allergische immuun reacties te induceren. Het ge-

bruik van de juiste adjuvantia in vaccins zou hierbij een uitkomst kunnen bieden. 2. Een reguliere RSV infectie kan geen efficiënte immuun response induceren, dus het is erg lastig om een vaccin te ontwikkelen wat dit beter kan. 3. Neutraliserende antilichamen bieden bescherming tegen een ernstige RSV infectie, maar de concentratie antilichamen in het bloed neemt snel af. Hierdoor kunnen de antilichamen mogelijk onvoldoende bescherming bieden tegen RSV infecties in de luchtwegen. 4. Inactief RSV als vaccin is geen optie gezien de ernstige resultaten van de FI-RSV vaccin trial. Op het moment wordt er veel onderzoek gedaan naar de ontwikkeling van gemodificeerd RSV. Deze virussen veroorzaken geen ernstige lagere luchtweg infecties, omdat ze zo zijn gemodificeerd dat het virus wel de cel kan infecteren waardoor er viraal eiwit en RNA in de cel komt, maar waar de essentiële eiwitten uit zijn gehaald of gemuteerd die nodig zijn voor efficiënte replicatie. Omdat er wel virale eiwitten in de cel tot expressie komen kunnen deze in voldoende hoeveelheid worden aangemaakt door de gastheercel om een immuun respons te induceren. 5. Naast de jonge kinderen zijn ook ouderen een doelgroep waarvoor vaccin ontwikkeling nodig is, omdat ouderen vaak longontsteking ontwikkelen net als bij influenza virus infecties. Mogelijk heeft dit te maken met een verouderend en minder effectief immuun systeem. In deze doelgroep spelen andere factoren een rol waardoor het lastig is om een vaccin te ontwikkelen. Ouderen zijn al meerdere malen geïnfecteerd geweest met RSV en hebben daardoor ook RSV specifieke T cellen en antilichamen aangemaakt wat de efficiëntie van vaccinatie kan belemmeren. Omdat ook de immuun response bij ouderen afneemt reageren zij ook minder goed op vaccinatie. Hierdoor is het mogelijk dat de immuun respons die geïnitieerd wordt door een vaccin niet van voldoende kwaliteit is om een her-infectie met RSV te voorkomen. Er moet goed worden bekeken op welk punt de immuun response bij ouderen te kort schiet om te kijken welk onderdeel van de immuun response door vaccinatie moeten worden versterkt.

We kunnen concluderen dat RSV-specifieke antilichamen de adaptieve immuun respons sterk kunnen beïnvloeden, maar wel een belangrijke rol spelen in de bescherming tegen een her-infectie. Daarnaast kunnen we concluderen dat in het FI-RSV vaccin model RSV zelf geen efficiënte innate immuun respons kan induceren, maar dat met de toevoeging van de juiste adjuvantia wel een efficiënte immuun respons geïnduceerd kan worden. Voor vaccin ontwikkeling is het daarom van groot belang om de verschillende aspecten van het virus en de mogelijke uitwerkingen van vaccinatie goed in kaart te brengen om een desastreus verloop van een vaccinatie trial in de toekomst te voorkomen. Onze huidige visie is dat een gebalanceerde $CD8^+/CD4^+$ T cel respons en een hoge titer van efficiënt virus neutraliserend antilichamen titer beide nagestreefd moeten worden.

Dankwoord

In dit hoofdstuk wil ik graag diegene bedanken die het mede mogelijk hebben gemaakt om dit proefschrift tot stand te laten komen.

Allereerst natuurlijk Dr. G.M. van Bleek, beste Grada. In de afgelopen 4,5 jaar heb ik ontzettend veel van je geleerd. Niet alleen het goed uitvoeren van wetenschappelijk onderzoek en het beschrijven van de resultaten hieruit, maar ook hoe de selectie van de jeugdelftallen bij de prof clubs tot stand komt in de voetbal wereld. Met dat laatste zal ik zelf waarschijnlijk niet veel te maken krijgen, maar met het eerste hoop ik veel te kunnen doen in mijn verdere carrière. Je had altijd een kritische blik op experimenten en de daarbij behorende resultaten, veel goed onderbouwde theorieën en ideeën waar ik veel van heb geleerd. Hierbij wil ik ook van de gelegenheid gebruik maken om jou heel veel succes te wensen in de nieuwe uitdagingen van je carrière.

Beste paranimfen Hanneke en Mariska. Lieve Mariska, toen ik als pas afgestudeerde in de RSV groep terecht kwam heb jij me op het lab alle fijne kneepjes van het vak en technieken geleerd waar ik nog veel gebruik van heb gemaakt. Daarnaast wil ik je ook erg bedanken voor de gezelligheid die je op het lab wist te creëren. Dit maakte de werkdagen erg aangenaam.
Lieve Hanneke, als bikkelharde co-brabander wil ik jou ook erg bedanken voor de gezellig tijd op het lab en de leuke gezellig uitjes/feestjes buiten het werk waaronder jouw vrijgezellenfeest natuurlijk. Op het lab had je naast het harde werken ook altijd nog ruimte voor een gezellig kopje thee drinken en een luisterend oor voor je collega's. Dames: Bedankt !!

Beste RSV collega's. Michael; Toen ik net als AIO begon in de RSV groep heb ik veel geleerd en vragen mogen stellen over het RSV onderzoek en de bijbehorende technieken, hiervoor bedankt! Ook Marcel bedankt voor je hulp op het lab, de discussies over ons onderzoek en de kennismaking met de wereld van prebiotica. Dit was erg leerzaam. Succes met jouw verdere onderzoek en het uiteindelijk afronden van je proefschrift. Nathalie, je bent een langere tijd collega geweest op het lab en de laatste tijd zelfs een directe collega geweest. Bedankt voor alle hulp op het lab en het uitvoeren van soms complexe en grote experimenten. Ook Leontine bedankt voor de gezellige klets op het lab, niet als directe collega in mijn onderzoek, maar wel als RSV collega. Mirjam, Michiel, Beatrijs, Maarten en Marije: veel succes met jullie verdere carrière.

Tetje en Mark. Ik vond het erg leuk en leerzaam om jullie stages te mogen begeleiden. Het werk dat jullie hebben uitgevoerd is ook deels gepubliceerd. Vele handen maken licht werk en in dit geval met goed resultaat, dus mijn dank daarvoor is groot. Het is leuk om te horen dat jullie nu ook beide OIO zijn geworden. Heel veel succes hierbij.

Jessica en Cindy, jullie waren destijds net iets eerder begonnen met jullie onderzoek voordat ik als AIO begon in het UMC. We hadden niks met elkaars onderzoek van doen (op de longen na dan Jessica), maar we probeerden regelmatig elkaars projecten te begrijpen, soms met succes. Ook naast het werk hebben we gezellig feestjes/kermissen gehad. Bedankt voor de gezelligheid en steun in de afgelopen jaren!

Cora, Elke, Mirjam, Annibal, Niels, Huijing, Lotte, Vanessa, Hilde, Michael, Marijke, Mirjam, Karima, Jitske, Pirkko, Ben, Koos, Will, Gerda, kortom alle collega's op het lab bedankt voor de gezellige klets, lunches, promotie feestjes, koffie pauzes… etc. Ik wil jullie erg veel succes wensen met het afronden van jullie proefschrift of andere lab werkzaamheden en jullie verdere carrière.

Lieve ouders, schoon-ouders en familie. Mede dankzij jullie steun en interesse in mijn onderzoek en het wereldje daar omheen heeft het in de jaren mogelijk gemaakt om door de pieken en dalen van het onderzoek heen te kijken en mijn proefschrift af te ronden. Jullie hebben altijd trots aan kennissen, vrienden en familie verteld wat ik voor onderzoek/opleiding doe en dit boekje kunnen jullie nu als resultaat laten zien.

Ook wil ik vrienden en vriendinnen bedanken voor de steun en interesse in mijn onderzoek. Het was niet altijd te begrijpen wat ik in Utrecht uitgevoerd heb, waarom dat niet dichter bij huis kon, waarom ik in hemelsnaam muizen gebruikte en waarom het telkens zo lang duurde voordat ik resultaten had die gepubliceerd konden worden. Het resultaat is in dit boekje gebundeld.

Lieve Frank! Ook voor jou waren het 4,5 jaar mee genieten van mijn promotie in zowel de pieken en de dalen. Je hebt vaak, waar mogelijk, met me meegedacht over mijn onderzoek en de aspecten er omheen. Je hebt me op de juiste momenten gestimuleerd en gesteund de afgelopen jaren, je hebt me met beide benen op de grond gehouden en nog veel andere dingen die niet in woorden uit te drukken zijn. Bedankt!!!

Houdoe hè !,

Debby

Curriculum Vitae

Debby Kruijsen werd geboren op 28 mei 1983 te Tilburg. Na het behalen van haar HAVO diploma in 2004 aan s.g. Durendael te Oisterwijk werd in het zelfde jaar begonnen met de studie Biochemie en medische microbiologie aan de Fontys hogeschool te Eindhoven. Binnen deze studie werden twee stages doorlopen. De eerste stage werd doorlopen bij Janssen Pharmaceutica te Beerse, België, onder begeleiding van Dr. M. Wouters en Dr. K. Smans. Na de afstudeer stage bij Biomerieux te Boxtel begeleidt door mevrouw L. Nes behaalde ze haar diploma in 2004. In dat zelfde jaar startte ze de opleiding Biomolecular Sciences te Utrecht. Gedurende deze opleiding heeft ze twee wetenschappelijke stages doorlopen waarvan de eerste bij de afdeling Biochemistry of Lipids onder begeleiding van Dr. G. Snoek aan de Universiteit Utrecht. Naar aanleiding van deze stage heeft ze haar tweede wetenschappelijke stage uitgevoerd bij de vakgroep biochemistry and Molecular Biology aan de Medical University of South Carolina, USA, onder supervisie van Dr. C. Luberto en Prof. Dr. Y. Hannun. Na het behalen van haar master in 2006 startte zij als promovendus bij de afdeling pediatrische immunologie van het UMC Utrecht onder begeleiding van Dr. G.M. van Bleek en Prof. Dr. J.L. Kimpen. De resultaten van dat onderzoek staan beschreven in dit proefschrift.

List of publications

Debby Kruijsen, Helga Einarsdottir, Marcel A. Schijf, Frank E. Coenjaerts, Ellen C. van der Schoot, Gestur Vidarsson and Grada M. van Bleek. Intranasal administration of antibody-opsonised respiratory syncytial virus particles efficiently primes virus specific immune responses in mice.
Submitted

Marcel A. Schijf , **Debby Kruijsen**, Johan Garssen, Grada M. van Bleek and Belinda van't Land. Specific dietary oligosaccharides increase Th1 responses in a mouse Respiratory Syncytial Virus infection model. *Submitted*

Marije P Hennus, Riny Janssen, Jeroen LA Pennings, Hennie M Hodemaekers, **Debby Kruijsen**, Nicolaas J Jansen, Linde Meyaard, Adrianus J van Vught, Louis J Bont. Host transcription profiles during mechanical ventilation for respiratory syncytial virus infection. *Submitted*

Debby Kruijsen, Marcel A. Schijf, Michaël V. Lukens, Nathalie O. van Uden, Jan L. Kimpen, Frank E. Coenjaerts and Grada M. van Bleek. Local Innate and Adaptive Immune Responses Regulate Inflammatory Cell Influx into the Lungs After Vaccination with Formalin Inactivated RSV.
Vaccine (2011) 29: 2730-2741

Debby Kruijsen, Mark J. Bakkers, Nathalie O. van Uden, Marco C. Viveen, Tetje C. van der Sluis, Jan L. Kimpen, Jeanette H. Leusen, Frank E. Coenjaerts and Grada M. van Bleek. Serum Antibodies Critically Affect Virus-Specific $CD4^+$/$CD8^+$ T Cell Balance during Respiratory Syncytial Virus Infections.
The Journal of Immunology (2010) 185: 6489-6498

Michaël V. Lukens, **Debby Kruijsen**, Frank E.J. Coenjaerts, Jan L.L. Kimpen and Grada M. van Bleek. 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: 7235-7243