Neonatal innate immunity
A translational perspective

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Contents

Chapter 1

General Introduction

The immune system

Humans live in intimate community with thousands of microbial species that are inhaled, swallowed, or inhabit our skin and mucous membranes. In fact, the human body contains 10-fold more bacteria than human cells[1]. The vast majority of microbes do no harm and actually benefit human health in many ways, including development, nutrition, and protection against disease[2-4]. However, some microorganisms are highly pathogenic and capable of causing severe infection. The human immune system has evolved to allow us to live in symbiosis with these microbes. The main tasks of the immune system are to discriminate beneficial from potentially harmful microbes and to defend our body against potential pathogens. Based on the speed and specificity of the reaction, the immune system can be divided into innate and adaptive immune components[5]. The innate immune system is the first site of contact with invading pathogens. Its main tasks are to decrease the number and virulence of potentially harmful micro-organisms and to orchestrate the adaptive immune response. The innate immune system consists of physical barriers such as skin and mucus, antimicrobial peptides, complement and immune cells, including monocytes, macrophages, neutrophils and natural killer (NK cells). Although the innate immune system provides a rapid (minutes to hours) response to invading pathogens, it lacks specificity and memory. To provide these qualities, innate immune cells secrete several cytokines and chemokines that recruit and instruct B- and T-lymphocytes, which subsequently mount the adaptive immune responses needed for long-lasting protection[5].

Toll-like receptors: Key pattern recognition receptors of the innate immune system

Microbes are recognized by the innate immune system through pattern-recognition receptors (PRR), of which the Toll-like receptor (TLR) family is best characterized[6]. TLRs are evolutionary highly conserved receptors that recognize pathogen-associated molecular patterns (PAMPs) expressed by viruses, bacteria, parasites and fungi. In addition, several endogenously expressed antigens can trigger TLR responses[6]. So far, 10 functional TLRs have been identified in humans (Table 1), each detecting different PAMPs. Based upon their localization and specific agonists, TLRs can be divided into two subgroups. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and mainly recognize microbial membrane components, whereas TLR3, TLR7 and TLR9 are localized in intracellular vesicles (endosomes, lysosomes, the endoplasmic reticulum) and recognize microbial nucleic acids[7-11]. TLR8 is localized primarily intracellular with a small proportion on the cell surface[7, 10]. Receptor localization and resulting availability of ligand and signaling molecules is an important mechanism regulating TLR responses[10].

TLR signalling

Individual TLRs trigger specific biologic responses. For example, TLR3 and TLR4 generate production of both type I interferon and inflammatory cytokines, whereas cell surface TLR1, TLR2, TLR6 and TLR5 induce mainly pro-inflammatory cytokines[6]. These differences are explained by differential recruitment of adaptor molecules and activation of downstream signaling cascades (Figure 1). Adaptor molecules used by TLRs belong to the Toll/Interleukin-1 receptor (TIR)-family and include MyD88, TIR domain containing adaptor protein (TIRAP), TIR-domain containing adaptor-inducing interferon-β (TRIF) and TIR-domain containing adapter molecule 2 (TRAM)[10]. MyD88 is used by all TLRs except for TLR3, and activates NF-κB and mitogen-activated protein

kinases (MAPKs) to induce NF-κB activation and production of inflammatory cytokines[12]. In contrast, TLR3 and TLR4 use TRIF to activate alternative pathways leading to activation of transcription factors NF-κB and interferon-regulatory transcription factor-3 (IRF-3) which induce type I interferon and inflammatory cytokines[12]. TIRAP and TRAM serve as sorting adaptors that recruit MyD88 to TLR2 and TLR4 and TRIF to TLR4, respectively. Thus, TLR signaling pathways can be largely divided into MyD88-dependent pathways, which induce production of pro-inflammatory cytokines, and MyD88-independent/TRIF-dependent pathways, which induce production of type I interferon.

Figure 1: Toll-like receptor signalling
TLR signalling is initiated by ligand binding to its specific TLR, resulting in formation of homodimers (such as TLR4) or heterodimers (TLR1/2 and TLR2/6). All TLRs, except for TLR3, use the MyD88 adaptor protein to induce intracellular signalling. Upon TLR activation, MyD88 associates with the receptor through its TIR domain. This results in the subsequent recruitment and activation of several members of the IL-1 receptor-associated kinase (IRAK) family, which ultimately activate mitogen-activated protein kinases (MAPK) (JNK, p38) and NF-κB to initiate transcription of pro-inflammatory cytokines. The adaptor molecule TRIF is used by TLR3 and TLR4 to induce activation of transcription factor IRF-3 and expression of type I interferon. TIRAP and TRAM serve as sorting adaptors that recruit MyD88 to TLR2 or TLR4 and TRIF to TLR4, respectively.

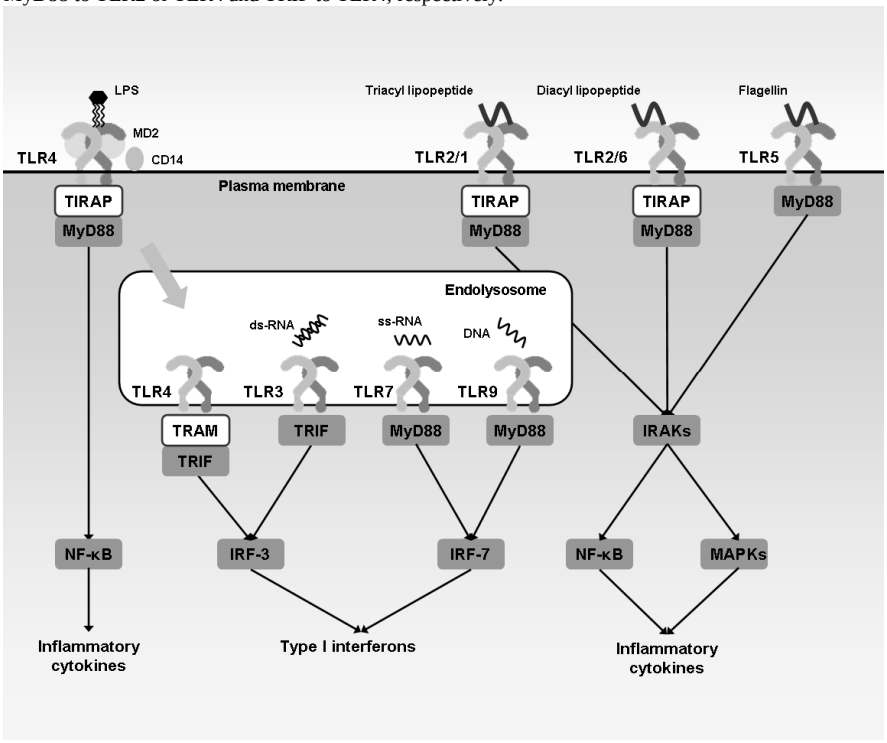

Table 1: Toll-like receptors

	Agonist	Localization	Expression	Adaptor protein
TLR1	- Triacyl lipopeptides on Gram-negative bacteria and mycoplasma.	Cell surface	- Monocytes/macrophages - Dendritic cells (DC) - B-lymphocytes	MyD88
TLR2	- Bacterial lipopeptides - Lipoteichoic acid, lipoarabinomannan on Gram-positive bacteria - Zymosan on fungi - tGPI-mucin from *Trypanosoma cruzi* - Hemagglutinin from measles virus	Cell surface	- Monocytes/macrophages - Myeloid dendritic cells (mDC) - Mast cells	MyD88
TLR3	- double stranded RNA - Polyinosinic-polycytidylic acid (Poly I:C)	Intracellular	- DC - B-lymphocytes	TRIF
TLR4	- Lipopolysaccharide on Gram-negative bacteria - F-protein on respiratory syncytial virus	Cell surface/endosome	- Monocytes/macrophages - mDC - Mast cells	MyD88/TRIF
TLR5	- Bacterial flagellin	Cell surface	- Monocytes/macrophages - DC	MyD88
TLR6	- Diacylated lipopeptides from Gram-positive bacteria and mycoplasma	Cell surface	- Monocytes/macrophages - Mast cells - B-lymphocytes	
TLR7	- Viral single-stranded RNA (ssRNA) - Imidazolquinoline derivates (imiquimod, R-848) - Guanine analogs (loxoribine)	Intracellular	- Monocytes/macrophages - Plasmacytoid dendritic cells (pDC) - B-lymphocytes	MyD88
TLR8	- Viral ssRNA - R-848	Cell surface	- Monocytes/macrophages - pDC - B-lymphocytes	MyD88
TLR9	- Unmethylated CpG DNA motifs on bacteria and viruses	Intracellular	- Monocytes/macrophages - pDC - B-lymphocytes	MyD88
TLR10	- Unknown	Unknown	- Monocytes/macrophages - B-lymphocytes	Unknown

TLR responses shape the adaptive immune system

In addition to their function in activation of the innate immune response, TLR responses are crucial for the successful induction of adaptive immunity. The generation of the adaptive immune response relies on three types of signals provided by antigen presenting cells (APC). Signal 1 is the presentation of antigen in the context of MHC, which activates its specific T-cell receptor. Signal 2 is referred to as 'co-stimulation', and is provided by CD80 and/or CD86 binding to CD28 on T-cells. Signal 3 refers to the signals that direct the differentiation of the naive T-cell towards distinct effector cells (e.g. T-helper (Th)1, Th2, Th17 cells) and is mediated by cytokines, chemokines and membrane-bound ligands[13, 14].

TLR modulate the strength and nature of all three signals. TLR-mediated activation of APC induces expression of MHC class II and costimulatory molecules[15, 16], thus enhancing their capacity to activate T-cells. In addition, TLR signalling induces production of cytokines and chemokines by APC which direct the differentiation of $CD4^+$ T-cells into Th1-, Th2-, Th17- or regulatory T cells[14]. Exposure of naïve T cells to IL-12p70 and IL-10 promotes Th1- and Th2 differentiation, respectively. IL-23 and TGF-β in co-presence of IL-6 support Th17 development, whereas TGF-β by itself is an important inducer of regulatory T cells[17]. In summary, TLR-mediated innate immune activation determines the strength and nature of the signals that induce the ensuing adaptive immune response.

Deregulated TLR signalling results in disease

In line with their central role in innate and adaptive immunity, deregulated TLR responses contribute to the pathogenesis of a wide variety of diseases, including infections, autoimmune and chronic inflammatory diseases[18-27]. Defects in TLR-mediated production of pro-inflammatory cytokines, due to genetic defects or developmental immaturity, compromise host immunity and confer increased risk of infection[18, 19, 28]. Conversely, excessive TLR responses during infection might increase disease severity by hyper induction of pro-inflammatory cytokines[20, 22], by facilitating tissue damage[27] or by impairing adaptive immunity[29, 30]. A key example of the detrimental effects of deregulated TLR signaling is Gram-negative sepsis, in which circulating lipopolysaccharide (LPS), through interaction with TLR4, causes widespread inflammation, multi-organ failure and shock, resulting in 30-50% mortality [26, 31]. Currently, several antagonists of LPS or TLR4 are under clinical development for the treatment of sepsis[32]. In addition to infections, aberrant TLR signaling has been linked to various other diseases, including atherosclerosis[21], diabetes[24], rheumatoid arthritis[33] and allergic airway disease[23]. Thus, despite their crucial role in host defence against infection, TLRs need to be tightly regulated to prevent excessive inflammation.

The neonatal period requires distinct regulation of the TLR system

Birth is one of the major challenges for the regulatory capacities of the immune system. During pregnancy, the fetal immune system is continuously exposed to maternal antigens which might induce harmful alloimmune responses leading to preterm delivery[28]. After birth, the neonatal immune system needs to balance the transition from the sterile intrauterine environment to the outside world full of micro-organisms, allowing for microbial colonization of the skin and mucous membranes, while protecting the neonate from infection[28]. To face these challenges, human neonates are born with a TLR system that is generally biased against the production of pro-inflammatory, Th1-polarizing cytokines[34-36]. Although initially described as 'immature', increasing evidence indicates that decreased neonatal production of pro-inflammatory cytokines reflects a highly regulated response tailored to the distinct requirements of the neonatal environment[28, 37-39]. Accordingly, the neonatal period provides a unique opportunity to study the regulatory mechanisms that keep the TLR system in check. Insight into these mechanisms will identify strategies to enhance host immunity in case of (neonatal) infection and might identify novel targets to restore immune balance in auto-immune and inflammatory diseases.

Environmental determinants of neonatal innate immunity

Immune development is likely driven by continuous reciprocal interactions between the host immune system and its environment. Especially, environmental exposure during the perinatal period may induce long-term changes in the evolving immune system that protect from or predispose to subsequent disease. Several environmental factors may contribute to neonatal immune function. First, birth by caesarean section is associated with increased cord blood levels of IL-13 and IL-10, and with increased risk of atopy and asthma[40]. Second, early life exposure to endotoxin increases PHA-induced production of IFN-γ and IL-13 during childhood[41, 42]. Several other factors, including cigarette smoke exposure, presence of siblings and birth season may impact on neonatal immune development. However, the key determinants of neonatal immune function are yet unclear. Identification of environmental drivers of neonatal innate immunity is of paramount importance if we hope to prevent infections and allergic disease.

Regulation of the neonatal immune system: a role for vitamin D?

Another example of an immune regulatory factor that might determine the risk of subsequent infections and atopy is vitamin D. An essential nutrient and hormone, vitamin D has functions that extend well beyond its classic role in bone metabolism, including modulation of the immune response[43]. Vitamin D inhibits proliferation, IL-2 and IFN-γ production by T-cells and induces the generation of regulatory T-cells[43-45]. In addition, vitamin D suppresses expression of MHCII, CD80 and CD86 and production of IL-12 by dendritic cells[43, 46], while stimulating the production of IL-10. Thus, vitamin D is considered of great importance for regulation of the immune response and maintenance of immune tolerance.

Certain immune modulatory effects of vitamin D may already occur *in utero*, during fetal development. Epidemiologic evidence demonstrates that neonates born from vitamin D deficient mothers have increased risk of immune disease, including type I diabetes, inflammatory bowel disease and multiple sclerosis[47-49]. Accordingly, vitamin D might provide a potential target to modulate early life immune function and to prevent subsequent infections and inflammatory disease.

Aims and thesis outline

In summary, TLRs are gatekeepers of the immune system that function to discriminate between beneficial and potentially harmful microorganisms and to maintain the balance between tolerance and excessive inflammation. This task is especially challenging during the fetal and neonatal period, which is characterized by sudden and overwhelming confrontation to multiple PAMPs that can trigger TLR responses. Decreased neonatal TLR-mediated generation of pro-inflammatory responses is an effective strategy to cope with this challenge, but may confer increased risk of infection. In addition, disrupted development of the TLR system during the neonatal period might contribute to the subsequent development of atopy.

However, little is known on the development of the TLR system in the postnatal period and its consequences for subsequent disease. For the studies described in this thesis, we took the unique opportunity to study neonatal innate immune responses in the healthy humans in the postnatal period.

This thesis aims to characterize the role of early postnatal TLR responses in subsequent susceptibility to infections and atopy.

Specifically, the following questions will be addressed:
- What is the ontogeny of the human TLR system?
- What are the clinical determinants of neonatal TLR function?
- What are the basic mechanisms causing distinct neonatal TLR responses?
- Do distinct neonatal TLR responses increase the risk of respiratory tract infections and atopy during infancy?
- Does early life deficiency of vitamin D predispose to subsequent viral respiratory tract infections?

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Chapter 2

Neonatal innate immunity in allergy development

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Abstract

Purpose of review
The neonate is born with a distinct immune system that is biased against the production of T-helper cell 1 (Th1) cytokines. Birth imposes a great challenge on the neonatal immune system, which is confronted with an outside world rich in foreign antigens. Exposure to these antigens shapes the developing neonatal immune system, inducing Th1- or Th2-polarized responses that may extend beyond the neonatal age and counteract or promote allergic sensitization. This review describes how engagement of the innate immune system might contribute to the development of allergy in children.

Recent findings
The exact role of innate immune stimulation in the development of allergies is a controversial area. Epidemiological literature suggests that microbial exposure in early childhood protects against the development of allergies, whereas a large amount of experimental data demonstrates that innate immune stimulation enhances Th2 responses upon primary and secondary antigen exposure.

Summary
Dose, site, and timing of allergen exposure are likely to modulate the innate immune response, polarizing the maturing neonatal immune system towards Th1- or Th2-type responses, thereby protecting from or predisposing to asthma and allergies. Modulation of neonatal innate immune responses may be a novel approach to prevent asthma and allergies.

Key words: allergy, asthma, neonate, review, toll-like receptor

Introduction

Neonatal immunity is immature. Adaptive immune responses are impaired at birth by lack of preexisting memory and low frequency and impaired function of effector B cells and T cells[1]. Therefore, the neonate is largely dependent on passively acquired antibodies and innate immune responses in the defense against micro-organisms. It is also well documented that neonatal innate immunity is distinct from that of adults in its response to bacterial and viral pathogens[1, 2]. Perinatal life is characterized by massive and constant exposure to new antigens, predominantly in the mucosa of the digestive and respiratory tract. The neonatal impairment in production of proinflammatory/T-helper cell 1 (Th1)-polarizing cytokines is believed to prevent potentially harmful hyperinflammation caused by these antigens. In addition, during this period, tolerance to many new allergens is induced, which is the key mechanism to prevent the development of allergy. This process is orchestrated by cells from the innate immune system. The distinct characteristics of the neonatal innate immune system may be essential in the induction of tolerance. Here, we review the role of the distinct neonatal innate immune system in the development of allergy.

Neonatal innate immunity

Quantitative and qualitative differences between the neonatal and adult innate immune system may contribute to neonatal Th2 polarization. Although cord blood contains a higher number of monocytes than adult venous blood, many phenotypic differences have been described, including lower baseline human leukocyte antigen (HLA)-DR expression and decreased up regulation of CD40 on ex-vivo stimulation with lipopolysaccharide (LPS)[3]. Cord blood also contains a higher ratio of plasmacytoid dendritic cells (pDCs) over myeloid dendritic cells (mDCs) compared with adult blood. Activation of pDCs generally leads to production of Th2-polarizing cytokines, whereas mDCs are associated with Th1-type responses[4, 5]. In addition, neonatal cord blood contains lower numbers of differentiated natural killer T (NKT) cells than adults[6]. Because of their central role in the induction and polarization of the immune response, most studies on neonatal innate immunity have focused on antigen-presenting cells (APCs) and toll-like receptors (TLRs)[7, 8]. Upon stimulation with various TLR agonists, human neonatal cord blood, as well as cord blood-derived monocytes and APCs, generally produces decreased amounts of Th1-polarizing cytokines compared with adults (Table 1)[3, 4, 9-18]. The inability to produce Th1-polarizing cytokines persists throughout the neonatal period[4]. In contrast, cord blood demonstrates similar or increased production of the Th2-polarizing cytokine interleukin (IL)-6 and elevated production of IL-10 upon stimulation with agonists for TLR2, TLR4 or TLR7[4, 15]. An interesting exception is TLR8 agonists, such as single-stranded RNAs and imidazoquinolines, which induce similar magnitude of Th1-polarizing responses in newborn and adult blood, monocytes and APCs[16].

Multiple mechanisms have been reported to contribute to decreased cord blood TLR-agonist induced cytokine production. First, studies investigating neonatal TLR expression report that TLR4 expression in cord blood monocytes from preterm neonates is lower than adult expression but increases during gestational aging[19]. At birth, newborn and adult blood monocytes express similar basal levels for various TLRs[14]. However, a study investigating in vitro stimulus-induced expression of TLR4 reported an elevated LPS-induced expression of TLR4 in cord blood monocytes compared with adult monocytes[20].

The differential regulation of stimulus-induced TLR expression in neonates and adults remains to be determined. Second, neonates demonstrate defects in signaling pathways downstream of TLRs. LPS-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) is decreased in whole cord blood, suggesting that the mechanism for impaired neonatal TLR responses is localized at the level of, or upstream of, kinase phosphorylation[14, 21]. Expression of the MyD88 adapter, which is used by all TLRs except for TLR3, is decreased in cord blood monocytes compared with adults[18]. Cord blood MyD88-independent signaling is also impaired, as illustrated by decreased whole blood cytokine responses to agonists for TLR3 and decreased LPS-induced interferon-regulatory factor 3 (IRF-3)-mediated production of the IL-12p35 subunit in cord blood monocytes[9, 10, 22].

Soluble mediators may induce or modulate innate immune responses. Human breast milk contains several TLR-modulating factors, including soluble CD14 (sCD14), soluble TLR2 and a thus far unidentified ~80 kD protein that inhibits signaling through membrane TLR2 but activates TLR4 in human intestinal epithelial and mononuclear cells[23]. Breast-milk-mediated intestinal immune modulation may be an important factor guiding postnatal immune development, preventing harmful inflammation caused by intestinal colonization, but selectively allowing for pro-inflammatory responses needed for immune maturation. The complement system can be activated by innate immune responses, resulting in generation of the anaphylatoxins C3a and C5a, recruitment of innate immune cells and formation of the membrane attack complex[24]. In the airways, C3a production favors Th2-type responses, whereas C5a can induce both inflammation and tolerance. Concentrations of complement components in neonatal plasma are diminished compared with those in adults[25]. Levels of complement in other organs, including the neonatal lung, and the role of neonatal complement deficiency in allergy development remain to be defined.

Neonatal plasma contains high concentrations of adenosine, an endogenous purine metabolite that induces intracellular cyclic AMP (cAMP), thereby inhibiting TLR2-mediated production of tumor necrosis factor alpha (TNF-α)[15]. Interestingly, cAMP is also known to inhibit the production of several other cytokines that are impaired in newborns (IL-12 and interferon alpha (IFN-α)), while preserving IL-10 and IL-6[26]. Elevated concentrations of adenosine in neonatal blood may thus be a general mechanism underlying distinct polarization of neonatal TLR-mediated cytokine responses.

In summary, neonatal innate immune cells demonstrate immunologic responses distinct from those of adults. In general Th1-polarizing responses to pure TLR agonists are impaired at birth, while production of Th2-polarizing and anti-inflammatory cytokines is preserved. However, it should be noted that under certain circumstances, such as in vitro stimulation with whole group B streptococci and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), human neonatal monocytes and APCs are capable of mounting adult-level responses[26, 27]. Thus, although responses to pure TLR agonists are polarized, neonatal mononuclear cells can mount robust inflammatory/Th1-polarizing responses to certain microbial particles. We will next discuss how innate immune stimulation in the early postnatal period may induce permanent changes that determine immune function in later life and protect from or predispose to asthma and allergies.

Table 1: Evidence for distinct neonatal TLR-mediated cytokine responses.
Cytokine concentrations after in vitro stimulation with TLR agonists are depicted. (↓) decreased compared with adults, (=) similar to adults, (↑) increased compared with adults. mDC, myeloid dendritic cells; moDC, monocyte-derived dendritic cells; PBMC, peripheral blood mononuclear cells; TLR, toll-like receptor.

TLR	Culture system					Ref
	Whole blood	*PBMC*	*Monocytes*	*mDC*	*moDC*	
1	TNF-α (↓)					[14, 15]
2	TNF-α (↓) IL-6 (↑)			IL-23 (=)		[14, 15, 17]
3	IL-12p70 (↓) IL-10 (=)		IL-12p35 (↓)	TNF-α (=) IL-1β (=) IL-6 (=) IL-8 (=)	IL-12p40 (↓)	[4, 13, 17, 76]
4	IL-12p70 (↓)	IL-6 (↑) IL-10 (=) TNF-α (=/↓)	IL-12p35 (↓)	IL-12p35 (↓)	IFN-β (↓)	[3, 4, 9, 11-15, 17, 18]
	IL-10↑ TNF-α (↓/=) IL-8 (=) IFN-γ (=)	IL-23 (↑)	TNF-α (↓) IL-1α (=) IL-12p40 (↓)	IL-12p40 (=) IL-23 (↑) IL-1α (=) TNF- α (↓)	TNF- α (↓)	
6	TNF-α (↓)					[14]
7	IL-10 (=) TNF-α (↓) IFN-α (↓)				IL-12p70 (↓) TNF-α (↓)	[4, 14]
8	TNF-α (=)				IL-23 (↑)	[14, 16, 17]
9	TNF-α (↓) IFN-α (=/↓)					[4, 11]

Evidence that neonatal innate immunity protects against allergy development

Epidemiological studies have suggested that early life exposure to innate immune stimuli, such as endotoxin (TLR4), reduces the risk of allergy development, leading to the hygiene hypothesis[28]. This could already occur prenatally, as several studies suggest that atopic sensitization already occurs prenatally[29, 30]. Many allergens can be transferred transplacentally, and antigen-specific T-cells can be detected in neonatal cord blood. In utero, the presence of high concentrations of sCD14 in amniotic fluid decreases the risk of atopic dermatitis in the newborn[31]. A European study in 922 women living in rural areas showed that maternal exposure to animal sheds during pregnancy decreased the risk of specific immunoglobulin E (IgE) in cord blood[29], possibly by Th1 polarization in the neonate[32]. After birth, children exposed to house dust endotoxin, mould or farm animals have decreased risk of allergic airway disease[29, 33, 34]. Breastfeeding protects against atopic disease[35]. Children fed breast milk that contains low levels of sCD14 have a higher

risk of subsequent allergy[31], indicating that the protective effect of breast milk may be mediated through innate immune mechanisms. Day care attendance, which is related to microbial exposure during early childhood, is associated with decreased total IgE at age of 3 years and decreased risk of asthma at school age[36, 37]. The protective effect of farm living is strongly dependent on the genetic variation in CD14 and TLRs[38], stressing the role of the innate immune system in early life immune polarization. An Australian healthy birth cohort study showed that cord blood mononuclear cells from infants born to allergic mothers have increased Th1-driving responses, such as IL-12 and IFN-γ production, to different TLR agonists[39]. Increased IL-6 and TNF-α production to lipoteichoic acid (TLR2), LPS (TLR4) and flagellin (TLR5) at birth were associated with an increased risk of allergic disease at the age of 1 year. Taken together, epidemiological data suggest that high exposure to microbial products before or after birth protects against the risk of allergic diseases (Table 2). However, the role of timing, dose and interaction with susceptibility genes require further study.

Basic studies have added to the evidence that stimulation of the innate immune system may protect against allergic responses. In a murine model of allergic asthma, the role of synthetic TLR3 or TLR7 agonists during sensitization has been studied. Systemic treatment with poly I:C (TLR3) or R-848 (TLR7) one day before intraperitoneal sensitization with ovalbumin (OVA) prevented eosinophilic airway inflammation, histopathological changes and airway hyperresponsiveness (AHR) upon subsequent allergen challenge[40, 41]. Most of these effects, except bronchoalveolar lavage (BAL) eosinophilia, were dependent on IL-12. The effect of TLR9 ligation on subsequent allergen challenge was tested in an OVA murine model[42]. Subcutaneous injection of CpG-oligodeoxynucleotide (ODN)(TLR9) reduced BAL eosinophilia and AHR by an IL-12 dependent mechanism. In addition, in human adult volunteers with established house dust mite allergy, TLR2 stimulation during antigen exposure decreased the production of Th2-polarizing cytokines[43]. The role of innate immune stimulation in established allergy may be different from the role in allergic sensitization. In allergen-sensitized mice, continuous exposure to LPS (TLR4) decreases allergic airway responses upon allergen challenge[44]. Similar effects on allergen challenge were seen using sterile house dust extracts, although the effects of house dust were only partially TLR4-mediated. In a recent study, the concept that TLR stimulation during sensitization may decrease the eosinophilic airway response during challenge was expanded to a viral model. Footpad inoculation with poly I:C (TLR3), LPS (TLR4) or PolyU (TLR8) during vaccination with formalin-inactivated respiratory syncytial virus (RSV) prevented against eosinophilic airway inflammation and AHR during challenge[45]. It was shown that TLR ligation exerted its effect by increasing maturation of antibody affinity. Although this is not an allergic model, it further supports the concept that systemic use of TLR agonists may modulate the immune response to protect against subsequent allergic sensitization at distant sites.

Evidence that neonatal innate immunity promotes allergy development

Epidemiologic studies have demonstrated that infections with certain pathogens, including *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, RSV and influenza virus, may predispose to asthma or exacerbate pre-existing asthma[46, 47]. In addition, several genetic studies report associations between loss-of-function variants in innate

Table 2: Role of innate immune stimulation in allergy development in mice and humans
Comparison of evidence that innate immune responses promote or prevent the development of allergy. NKT, natural killer T cell; Th1, T-helper cell 1; Th2, T-helper cell 2; TLR, toll-like receptor.

Allergy promotion	Ref	Allergy prevention	Ref
Murine studies Allergen may signal through TLR by molecular mimicry	[55]	*Murine studies* Continuous local innate immune stimulation decreases response to allergen	[44]
Local TLR stimulation enhances concomitant response to allergen	[54, 57]	Repeated nebulization of CpG-ragweed compound reduces airway symptoms by enhancing Th1 response	[74]
TLR4 on airway epithelium is required for house dust mite allergic responses	[56]	Systemic administration of TLR3,4,7,8,9 agonists prevent exaggerated Th2 response to allergen	[40, 42, 44, 45, 79]
NKT cells in the airways promote asthmatic airway inflammation	[63]		
Human studies Loss-of-function TLR mutations are associated with risk of asthma	[48, 50]	*Human studies* Epidemiological negative association between infant endotoxin exposure and asthma risk	[29, 33, 34]
		Perinatal use of probiotics protects against atopic eczema	[69, 70]

immune genes and asthma risk. Polymorphisms inhibiting the function of CD14 or TLR4 have been associated with decreased asthma incidence and disease severity[48]. Inhibitory polymorphisms in TLR1 and TLR6, which form heterodimers with TLR2 to induce cytokine production, also protect against asthma development. This is consistent with experimental data showing that activation of APC by Pam3Cys induces high levels of Th2-polarizing effector molecules, including IL-13 and IL-1β, but low levels of IL-12, IFN-α , IL-18 and IL-27[49]. Finally, mutations that decrease the TLR-inhibitory effect of IL-1 receptor kinase M (IRAK-M) were associated with early-onset asthma[50]. Thus, epidemiologic and genetic studies support a role for innate immune stimulation in promoting allergy development (Table 2).

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Multiple experimental studies have attempted to clarify the mechanisms by which innate immune stimulation promotes allergy development. Studies using purified TLR agonists demonstrate a key role for TLR stimulation in atopic sensitization. Mice deficient in MyD88, a key adapter for TLR signaling, do not develop a specific IgE response, eosinophil airway inflammation and AHR in a HDM allergy model that uses intranasal sensitization[51]. OVA-sensitized mice that lack functional TLR4 exhibit less pronounced airway inflammation upon OVA challenge compared with wild-type mice[52]. Another study confirmed that LPS, administered intranasally during OVA sensitization, can function as an adjuvant to promote OVA-induced airway sensitization[53]. This study reports a dose-dependent effect of LPS, with low doses promoting Th2-type responses, whereas high doses induce a Th1-type response. In this and other studies, LPS-dependent sensitization to intranasal antigen did not occur in TLR4-deficient mice and depended on MyD88-dependent maturation of pulmonary dendritic cells[54]. House dust mite allergen Der p2 has homology to myeloid-differentiation protein-2 (MD-2), a component of the TLR4 receptor complex that binds LPS. It was shown that Der p2 signals through MD-2/TLR4 as a result of molecular mimicry[55]. This study shows that allergen can use TLRs to initiate a robust innate immune response. The role of TLR4 signaling in house dust mite allergy is also supported by the finding that murine airway epithelial TLR4 expression is required for allergic airway inflammation[56]. Double-stranded RNA (dsRNA), a TLR3 agonist, may also protect against allergy development. In a murine OVA model, concomitant mucosal administration of OVA and low-dose dsRNA enhanced allergen-induced lung inflammation in wild type mice but not in mice deficient for TLR3, IL-4 or signal transducer and activator of transcription 6 (STAT6)[57]. Rat pups have altered pulmonary response to macrophage-activating lipopeptide-2 (MALP-2)(TLR2/6) with increased tissue and decreased bronchoalveolar mononuclear cells[58]. Finally, TLRs may modulate direct effects of allergen on nonimmune cells, such as smooth muscle or the neuroendocrine system. It was shown that TLR2 signaling is required for in vitro rat smooth muscle contraction to HDM[59]. Interestingly, the effects of TLR agonists on atopic sensitization appear to be dose-dependent, with low doses of dsRNA and LPS inducing a Th2-type response, whereas high doses promote Th1-biased responses[57, 60]. Of note, most studies used adult mice, and the effect of TLR agonists in neonatal mice remains to be determined.

Non-dendritic cell, non-TLR-mediated mechanisms that affect the development of allergy have also been described. Natural killer (NK) cells interact with dendritic cells to produce IFN-γ and to promote Th1-type responses[61]. Adult asthma patients have lower numbers of IFN-γ producing $CD56^{++}/CD16^{+}$ NK cells, indicating that NK cells may predispose to asthma or exacerbate existing asthma. As neonatal NK cells are phenotypically and functionally mature[62], we hypothesize that NK cells may modulate the asthmatic response in established asthma. The role of NKT cells in asthmatic airway inflammation was demonstrated by showing specific increase in numbers of $CD4^{+}$ NKT-cells in the lungs of asthmatic patients, but not in those with sarcoidosis[63]. In Th1-deficient (T-bet -/-) mice, which spontaneously develop allergic airway inflammation, the essential role of NKT

cells was confirmed[64]. Most $CD4^+$ NKT cells produce IL-4, and a subset expresses the IL-25 receptor IL-17RB. This subset of NKT was shown required for the induction of allergic airway inflammation[65]. As neonatal NKT cells are distinct and are virtually all $CD4^+$ cells, it is conceivable that the neonate is more susceptible to NKT cell-driven allergic responses[6].
In summary, there is evidence that innate immune stimulation of mucosal cells may be a causative factor in the development of asthma. This appears to be a global effect, because similar effects are observed following different agonists of the innate immune apparatus. However, intraspecies differences in the innate immune system prevent direct extrapolation of results from murine studies to humans. Further translational studies on the effect of mucosal immune activation on allergy development will be important. In addition, it is not yet clear to what extent the effect of local innate immune activation on Th1- or Th2-polarization and allergy development is determined by the immunological maturational status of the child.

Protection or Promotion: can both be true?
How can we reconcile apparent contradictory evidence that stimulation of the innate immune response can both prevent and promote allergic sensitization? Clearly, it is difficult to compare the level of evidence for both hypotheses. Whereas clinical evidence mainly points to a protective effect, experimental studies showing the opposite are quite convincing. Murine studies need to be interpreted with caution, as accumulating evidence indicates important differences between the murine and human TLR system[66].
Nevertheless, different response patterns upon innate immune stimulation can be distinguished, leading to either protection or promotion (Fig. 1). The effect of innate immune responses on the development of allergy is determined by the timing, dose and site of stimulation[47]. Both experimental and human observational studies have shown that the magnitude of mucosal innate immune stimulation determines whether innate responses prevent or enhance allergy development[57, 60]. Repeated high-dose administration of TLR agonist in the airways induces a Th1 response upon allergen exposure, whereas exposure to low-dose TLR agonists is associated with a Th2-like immune response. The site of innate immune triggering appears crucial for the effect on allergic sensitization. Mucosal exposure to TLR agonists enhances concomitant or subsequent responses to aeroallergens. Apparently, innate immune agonists prime the mucosal APCs to respond more vigorously to subsequent stimuli. It has been postulated that innate stimuli represent danger signals, which in turn may trigger Th2 responses[67]. This is advantageous in case of pathogen encounter, but may enhance IgE response upon concomitant allergen exposure. Systemic innate immune activation appears to have a global protective effect on allergy development, which is mediated by Th1-driving signals. To our knowledge, no reports exist that systemic innate immune activation has a deleterious effect on allergen exposure at distant sites.

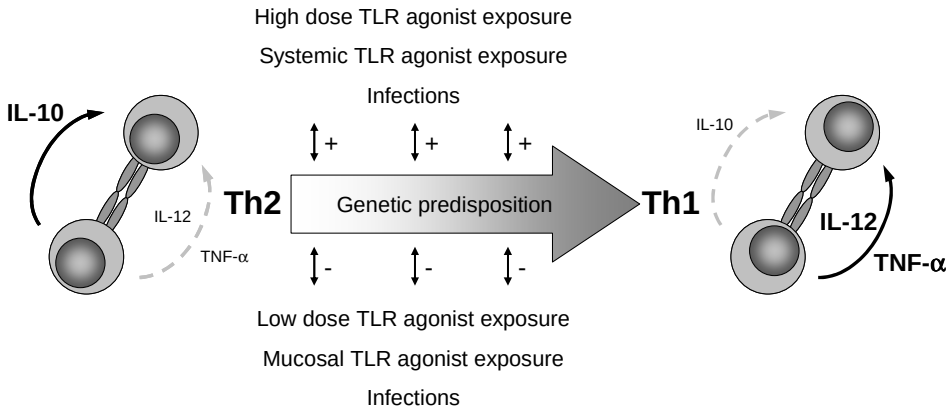
Targets for intervention
Probiotics have been used in attempt to reduce the development of allergic disease. A small randomized controlled trial (RCT) in 2001 showed that treatment of pregnant women during late pregnancy with *Lactobacillus rhamnnosus* GG (LGG) reduced the development of atopic dermatitis in the neonates[68]. Since that publication, many RCTs

have been reported with conflicting results. The largest published RCT (*n*=925) confirmed that treatment with a mixture of Bifidobacterium and Proprionibacterium of pregnant women 2-4 weeks before delivery and of their children during the first 6 months of life prevented the development of eczema (odds ratio 0.74, *P*=0.04)[69]. However, meta-analyses of this and other studies were inconclusive[70, 71]. Taken together, we conclude that the large differences in RCT results do not yet support large-scale supplementation of probiotics to infant formula milk in the general population.

Synthetic TLR agonists are being developed for treatment of oncologic, autoimmune and allergic diseases[72]. In addition, TLR agonists are under biopharmaceutical development as vaccine adjuvants and as immunomodulators in cancer and allergy. Synthetic TLR9 ligands are being considered in the treatment of allergic diseases. Although synthetic TLR ligands used in RCTs have not always proven effective in decreasing symptoms, they are well tolerated in general. ISS 1018 (Dynavax Technologies, Berkeley, California, USA) is a short, synthetic, unmethylated CpG motif-based ODN which induces cell activation through TLR9. Repeated nebulization of this compound in stable mild asthmatics resulted in an increased local expression of IFNγ, but did not affect the expression of Th2 cytokines, airway eosinophilia or AHR upon experimental allergen challenge[73]. Subsequently, this compound was conjugated to ragweed. In an RCT with 25 patients with allergic rhinitis, increasing doses of this allergen-TLR9-agonist conjugate injected for 6 consecutive weeks prior to the ragweed season were well tolerated[74]. In the intervention group, a two-thirds reduction of symptoms was observed compared with placebo-treated patients. Apparently, concomitant administration of TLR9 agonist and allergen can induce tolerance in allergic individuals. However, the precise mechanism remains to be defined. Whether this strategy may be used for primary prevention is not known. Recently, TLR4 agonists have been used to treat allergic patients. A single dose of the chemical compound CRX-675 was well tolerated at different doses in patients with allergic rhinitis 1 day before allergen challenge, although no clear benefit was demonstrated in this phase 1 trial[75]. In 2 ongoing RCTs it is being determined whether the genetic background of patients determines the response to LPS. The inflammatory response in airway tissue in atopic and nonatopic asthmatics is measured 4h after instillation of LPS in the lower airways (ClinicalTrials.gov NCT00644514). The effect of repeated LPS nebulization in healthy volunteers is measured in relation to their TLR4 genotype (ClinicalTrials.gov NCT00671892). In healthy volunteers, the effect of low versus high-dose LPS inhalation on exhaled nitric oxide concentration is being assessed (ClinicalTrials.gov NCT00643058). Finally, administration of a combination of TLR agonists has a synergistic effect on in vitro maturation of human neonatal dendritic cells, suggesting that targeting a combination of TLRs may be a powerful approach to instruct the neonatal immune system towards a protective Th1 response[76]. Taken together, the synthetic TLR agonist may be a novel tool to prevent or treat allergic disease. However, most studies have been performed in adults with established allergy. More information is needed with respect to the effect of these compounds on allergy prevention in newborns.

Finally, the NKT cell is a promising target for immunomodulation. In an allergic mouse model, a single intraperitoneal injection of α-galactosylceramide during allergen challenge prevents eosinophilic airway inflammation and AHR[77]. In humans, α-galactosylceramide is well tolerated[78], but RCT have not yet been performed in atopic individuals.

Figure 1: Effects of allergen exposure on development of allergy: a model.
Neonatal innate immune responses at birth polarize towards a Th2 response, characterized by decreased TLR agonist-induced production of Th1 cytokines such as IL-12 and TNF-α, and increased agonist-induced production of IL-10. After birth, there is a gradual maturation towards Th1-polarizing responses. The degree and speed of this maturational process determine the risk of subsequent allergy development. Exposure to innate immune stimuli generally enhances innate immune maturation. However, the effects of innate immune stimulation are not universal, and depend on host factors, such as genetic predisposition and maturational state of the innate immune system, as well as on the site, dose and timing of exposure. IL, interleukin; Th1, T-helper cell 1; Th2, T-helper cell 2; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.



Conclusion

The role of innate immune responses in the development of allergy is controversial. Recent literature has provided the fundamental insight that magnitude and direction of the effect of innate immune responses is unequivocal. Timing, dose, site of activation and host genetic background are clearly crucial to understanding the interaction between innate immune stimulation and allergy development. Initially, clinical and basic studies showed that repeated systemic innate immune stimulation before birth or during the newborn period redirects the Th1-Th2 balance. However, increasing evidence argues against the general benefit of innate immune stimulation on allergy development, suggesting that host factors, site, dose and timing of exposure play an essential role. Host genetics may modulate the effects of TLR activation on allergy development. Low-dose innate immune stimulation of mucosal cells enhances concomitant sensitization to allergen. Various environmental influences, including cigarette smoke and respiratory viruses, trigger the innate immune system, by which they may enhance responses upon allergen exposure.

On the basis of epidemiologic data, synthetic innate immune stimuli are being studied as a strategy to prevent allergy. A better understanding of the complex interaction between these stimuli and the developing neonatal immune system will be essential to determine the optimal use of these compounds.

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Chapter 3

Prenatal prevention of respiratory syncytial virus bronchiolitis

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Abstract

Respiratory syncytial virus (RSV) is the most important cause of infant lower respiratory tract infection, causing significant morbidity and mortality. Susceptibility to severe RSV infection may already be determined before birth, during fetal development. Accordingly, pregnancy may provide a unique window of opportunity for interventions aimed at preventing severe RSV bronchiolitis. Delayed fetal innate immune maturation may predispose to severe RSV bronchiolitis. Modulation og intrauterine immune development, through maternal nutrition, probiotics or allergen exposure during pregnancy, may protect against RSV bronchiolitis. The association between RSV bronchiolitis and insufficient cord blood concentrations of vitamin D, a nutrient that modulates maturation of the fetal airways and immune system, suggests that strategies aimed at increasing maternal vitamin D intake during pregnancy may prevent infant RSV bronchiolitis. In animal models, maternal RSV immunization during pregnancy increases the titer of RSV-neutralizing antibodies in the offspring and reduces viral replication. However, in humans, clinical development of preventive and therapeutic interventions during pregnancy is hampered by the unique position of pregnant women and their fetuses as research subjects. Ethically acceptable interventions that target the pregnant woman and her fetus are needed to reduce the major burden caused by RSV bronchiolitis during infancy.

Introduction

RSV is the leading cause of acute bronchiolitis in infancy. RSV is estimated to account for 33.8 million new episodes of bronchiolitis in children worldwide and for 66.000-199.000 deaths annually[1]. Currently, there is no effective treatment for RSV bronchiolitis and there is an unmet need for strategies to prevent RSV bronchiolitis. The pathogenesis of severe RSV infection is incompletely understood. Host characteristics, including pre-morbid dysregulated immune function and small airway diameter are thought to contribute to RSV disease. As RSV hospitalization primarily occurs in neonates and infants[1], predisposition to severe RSV infection originates very early in life. In addition to genetic factors, the intrauterine environment modulates the development of the fetal immune system and respiratory tract, thereby determining the subsequent susceptibility to RSV bronchiolitis. Accordingly, pregnancy provides a unique window of opportunity for primary prevention of RSV bronchiolitis.

Prevention of RSV bronchiolitis during pregnancy

Although the importance of fetal development for subsequent health and disease is well recognized, medical-ethical considerations limit the development of preventive or therapeutic interventions that target fetal development. Historic tragedies such as DES and thalidomide illustrate that potential adverse effects of these interventions can be detrimental to the developing fetus[2]. Nowadays, pregnant women are granted special protection from research risks. In the United States, legislative guidelines allow clinical research involving pregnant women or fetuses only for interventions that directly benefit maternal or fetal health[3]. Fetal therapy is indicated for a limited number of potentially life-threatening diseases, such as prenatal corticosteroids to prevent hyaline membrane disease and highly active antiretroviral therapy to prevent transmission of human immunodeficiency virus[4, 5]. Although understandable, our reluctance to intervene with fetal development limits the development of interventions that could benefit fetal health on the long term. For example, polysaccharide vaccines against *Haemophilus influenzae* type B, *Neisseria Meningitidis* and *Streptococcus pneumoniae* are safe and immunogenic in pregnancy, no large-scale phase III clinical trials have been performed[6]. Owing to its widespread incidence in neonates and infants, RSV bronchiolitis may be a key disease that can be prevented by modulation of the intrauterine environment.

Modulation of fetal immune development

Modulation of *in utero* immune maturation may prevent subsequent RSV bronchiolitis. Clinical studies have associated severe RSV infection with multiple presymptomatic differences in the immune system, including impaired Toll-like receptor mediated production of pro-inflammatory cytokines[7, 8], decreased Dicer-mediated production of antiviral micro-RNA sequences[9], and decreased expression of TNFRSF25, a member of the tumor necrosis factor receptor superfamily that activates NF-κB and that potentiates T-cell IFN-γ production[10]. Stimulation of fetal immune development may boost postnatal antiviral defence mechanisms and protect against RSV bronchiolitis. A number of environmental exposures in pregnancy are associated with fetal immune function and may provide targets for intervention, including maternal nutrition, probiotics and maternal allergen exposure. Maternal nutrition is of critical importance for the development of the fetal immune system. In clinical studies, maternal *n*-3 PUFA (found

in oily fish) consumption in pregnancy protected against subsequent allergic disease[11]. In a randomized trial, maternal fish-oil supplementation in pregnancy was associated with decreased allergen-induced production of IL-5, IL-13 and IL-10 and IFN-γ in cord blood, which translated to decreased risk of atopy and asthma during infancy and childhood[12]. The association of RSV bronchiolitis with distinct neonatal cytokine responses[8] and with increased risk of childhood atopy[13] shows that prevention of RSV bronchiolitis may be an additional benefit of maternal fatty acid consumption during pregnancy.

In the past decades, the effect of maternal probiotics supplementation on fetal immune development and subsequent atopic disease has been subject of multiple trials[14-16]. In a randomized controlled trial in 159 pregnant women with a family history of atopy, maternal supplementation of *Lactobacillus GG* starting 2-4 weeks before expected delivery and postnatally for 6 months to their infants resulted in a twofold decreased risk of infant atopic eczema[16]. Protection against atopy may be mediated through modulation of fetal immune development, as maternal use of *Lactobacillus rhamnosus* during pregnancy increased concentrations of IFN-γ in cord blood plasma compared with placebo[15]. However, subsequent trials failed to confirm a preventive effect of probiotics supplementation on subsequent atopy [17, 18]. In fact, one of these trials reported increased incidence of infant wheezing bronchitis in the *Lactobacillus* group[17]. The immunomodulatory effect of maternal probiotics supplementation depends on the type of probiotics, the timing and duration of supplementation. Whereas fetal immune development already starts from 4 weeks of gestational age, no studies have been performed supplementing probiotics before 36 weeks gestational age. Earlier initiation and prolonged administration of probiotics supplementation during pregnancy may confer enhanced protection against subsequent childhood disease, including RSV bronchiolitis.

Vitamin D is an essential nutrient and hormone that may prevent RSV bronchiolitis. Vitamin D affects both innate and adaptive immune responses and also influences fetal airway development. We and others have recently demonstrated that cord blood vitamin D deficiency in healthy neonates is associated with increased risk of severe RSV infection[19, 20]. As cord blood vitamin D concentrations are mainly derived from and correlate with vitamin D levels in maternal plasma, correction of maternal vitamin D status may prevent infant RSV infection. Although the World Health Organization recommends daily supplementation of 400 IU vitamin D to all pregnant women, 46% of newborns in industrialised countries are born with insufficient concentrations of vitamin D[20]. Lack of adherence to these guidelines, reduced sun exposure and increased vitamin D requirement during pregnancy (exceeding the amounts that can be obtained through diet) may account for the high prevalence of vitamin D deficiency among newborns. The optimal dose of vitamin D supplements during pregnancy needed to benefit neonatal birth weight, bone status and risk of childhood asthma is subject of several ongoing studies. Prevention of infant RSV infection may be an additional favourable effect of vitamin D supplementation during pregnancy and should be subject of future clinical trials.

Reduction of maternal allergen exposure during pregnancy may be another strategy to prevent severe RSV bronchiolitis in the offspring. Allergic infants are at increased risk of severe course of disease during RSV infection[21]. Conversely, severe RSV bronchiolitis is not associated with subsequent development of allergic disease, indicating that allergic sensitization precedes development of severe RSV bronchiolitis and may play a causal role in disease pathogenesis[21] In a prospective randomized cohort study in 291 neonates

of atopic parents, reduction of pre- and postnatal allergen exposure was associated with decreased incidence of respiratory symptoms, decreased wheeze with shortness of breath and decreased use of inhalant medication in the first year of life[22]. However, in this study, no viral testing was performed. As RSV is the main cause of infant viral-induced wheeze, prevention of early allergic sensitization may also prevent the development of severe RSV bronchiolitis during infancy.

Maternal vaccination

It is well established that maternal neutralizing antibodies protect neonates against RSV bronchiolitis in the first months of life. However, after the age of 3 months, antibody titers drop below protective levels and incidence of RSV bronchiolitis rises[23]. Animal studies have demonstrated that maternal vaccination may boost maternal antibody titers and increase transplacental transfer of RSV-specific immunoglobulins to the neonate[24]. In addition, maternal vaccination may increase RSV-specific IgA in breast milk, thereby increasing the duration of protection. Munoz and colleagues published the results of a randomized clinical trial in 35 pregnant women demonstrating that maternal vaccination with a purified RSV fusion protein was safe and increased transplacentally acquired RSV-specific IgG in the children up to age 6 months[25]. In this small population, no difference was observed in the frequency or severity of infant respiratory tract infections. After this publication in 2003, no other clinical papers have been published on this subject. Larger clinical studies, either in humans or in non-human primates, are urgently needed to determine whether maternal vaccination reduces the burden of RSV bronchiolitis in infants.

Conclusion

In summary, pregnancy provides a unique window of opportunity to improve fetal health and to prevent diseases in early life, including RSV bronchiolitis. Manipulation of maternal nutrition, probiotics supplementation, adequate intake of vitamin D and maternal vaccination during pregnancy should be explored. However, clinical development of these strategies is hampered by the exempt position of pregnant women as research subjects. Recently, Chervenak and colleagues have proposed a framework for design of clinical trials during pregnancy[3]. In this framework, the fetus is considered an individual patient with future health risks which may be prevented by intervention during pregnancy and need to be balanced against the risks for the mother. Legislative guidelines are highly needed to stimulate the development of strategies that modulate the intra-uterine environment to prevent RSV bronchiolitis during childhood.

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Chapter 4

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

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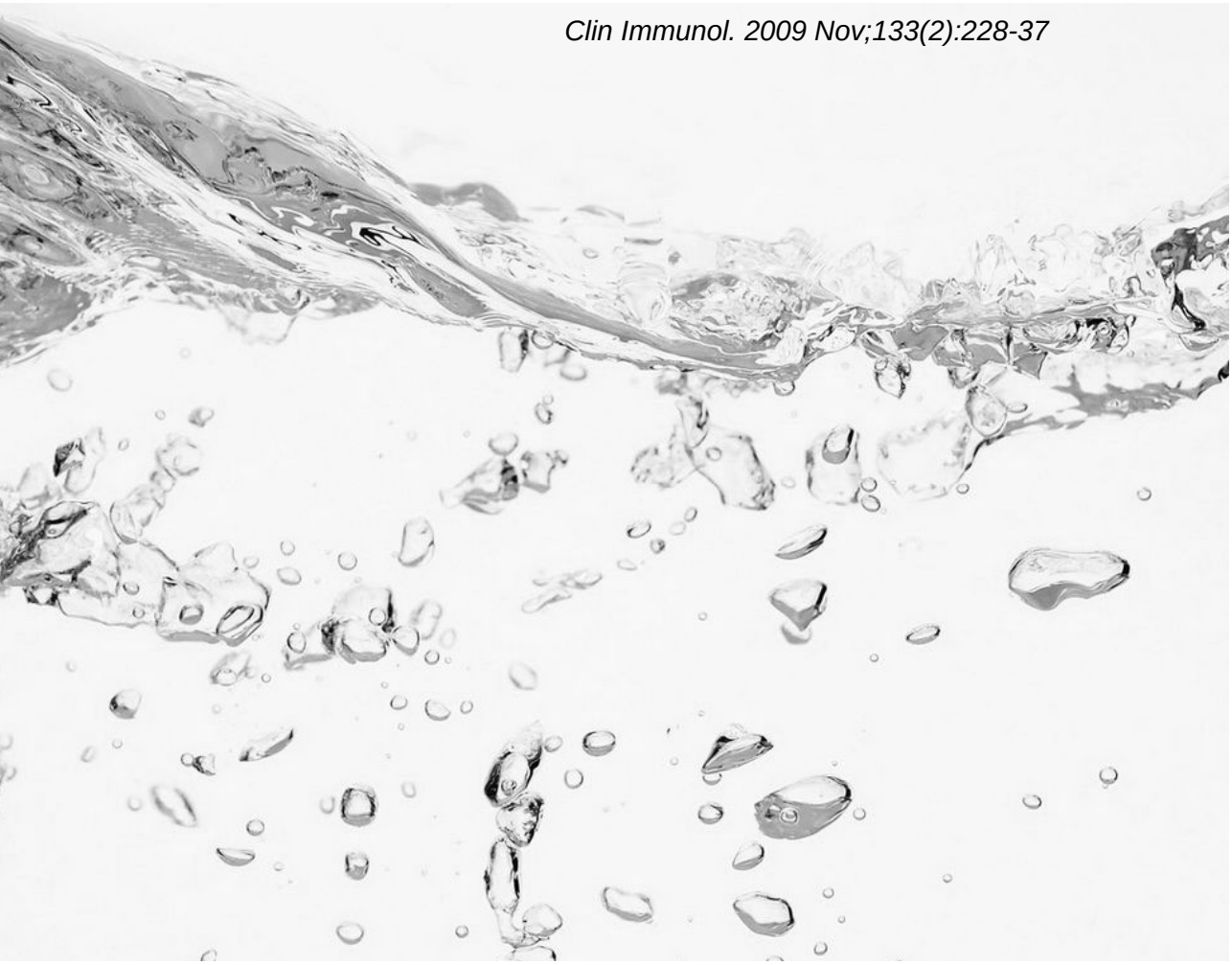


Abstract

Introduction
Newborns are highly susceptible to infectious diseases, which may be due to impaired immune responses. This study aims to characterize the ontogeny of neonatal TLR-based innate immunity during the first month of life.

Methods
Cellularity and Toll-like receptor (TLR) agonist-induced cytokine production were compared between cord blood obtained from healthy neonates born after uncomplicated gestation and delivery (n=18), neonatal venous blood obtained at the age of one month (n=96), and adult venous blood (n=17).

Results
Cord blood TLR agonist-induced production of the Th1-polarizing cytokines IL-12p70 and IFN-α was generally impaired, but for TLR3, 7 and 9 agonists, rapidly increased to adult levels during the first month of life. In contrast, TLR4 demonstrated a slower normalization, with low LPS-induced IL-12p70 production and high IL-10 production up until the age of one month.

Conclusions
Polarization in neonatal cytokine responses to LPS could contribute to neonatal susceptibility to severe bacterial infection.

Introduction

Neonates have an increased susceptibility to infection. The incidence of infections is particularly high in the first weeks of life, and rapidly decreases thereafter[1]. Common causes of infection in neonates include commensal bacteria such as group B streptococci and coagulase negative staphylococci, and Gram-negative organisms like *Escherichia coli*[1].

Susceptibility to infection appears to be due to immaturity of the neonatal immune system. Neonatal adaptive immune responses are hampered by a lack of pre-existing memory and decreased Th1-type responses[2]. In addition, the innate immune system of newborns is also impaired[3]. Toll-like receptors (TLRs) are highly conserved components of the innate immune system and are involved in the recognition of microbial pathogen-associated molecular patterns[4]. TLR activation triggers intracellular signalling cascades, resulting in production of inflammatory mediators that modulate the primary immune response and instruct the adaptive immune system. Thus, TLRs are essential in initiating and orchestrating the immune response. Studies of neonatal cord blood suggest that neonatal responses to multiple TLR agonists are impaired at birth. Neonatal cord blood monocytes demonstrate lower *in vitro* production of tumor necrosis factor-α (TNF-α) after stimulation with several TLR agonists, including bacterial lipopeptides (TLR2) and lipopolysaccharide (LPS; TLR4)[5, 6]. TLR-mediated responses in human cord blood dendritic cells (DC) are also distinct. Upon *in vitro* LPS stimulation, neonatal monocyte-derived DC (moDC) showed a significantly lower expression of activation markers CD40 and CD80 and decreased production of interleukin-12p70 (IL-12p70) and interferon-β (IFN-β) compared to adult moDC[5, 6]. Thus, impairments in the newborn TLR system may predispose for infections. The importance of the TLR system in newborns and infants is exemplified by patients with defects in the TLR-MyD88-IRAK4 pathway, who tend to present with severe infections early in life and clinical disease lessens with age[7-9].

Most studies assessing neonatal TLR responses used cord blood, which is more readily available than neonatal venous blood. However, the rapidly changing physiology at birth leads to significant changes to the blood compartment in the first hours and days of life. Because of the critical role of TLRs in the developing neonatal immune system, insight into the development of TLR function during the first months of life will likely contribute to a better understanding of the host defence against infection during this critical period in life. Here we show that unlike responses to agonists for TLR3, 7 and 9, neonatal responses to LPS are impaired throughout the first month of life, suggesting a TLR-pathway selective impairment that could contribute to susceptibility to particular infections.

Materials and Methods

Blood

The research protocol was approved by the local Medical Ethics Committee of the University Medical Center Utrecht and written informed consent was obtained from parents of all participants. Blood was obtained from healthy newborns participating in an ongoing birth cohort study on the role of neonatal TLR responses in the pathogenesis of respiratory tract infections and asthma. Cord blood was collected directly after uncomplicated vaginal delivery (n=18). Peripheral venous blood was obtained by

venipuncture at the age of 1 month (n=96), or from healthy adult volunteers (n=17). Exclusion criteria for blood collection at birth or at the age of one month were preterm delivery, a complicated obstetric history, perinatal use of antibiotics by mother or child or any type of medical intervention. To investigate the timing of TLR4 maturation, a third group of children was included from whom venous blood was collected 5 days (range 1-7) after delivery (n=22). In the latter group, we allowed for minor medical issues, such as macrosomy or low temperature warranting glucose control. None of the participants had any sign or symptom of infectious disease, such as respiratory tract complaints or fever, in the two weeks prior to sampling. Due to practical considerations, we were unable to obtain repeated blood samples in the same children. Baseline characteristics are shown in Table 1. Blood was collected in sterile tubes and anticoagulated with EDTA for differential blood count, or with sodium heparin for flow cytometry and in vitro TLR stimulation assays. Limited volume and technical issues prevented us from performing all measurements in all subjects. The exact *n* for each experiment can be found in Supplementary Table 1.

Flow cytometry

Expression of cell surface antigen was determined by incubating whole blood samples with fluorescence-labeled monoclonal antibodies for 15-30 minutes. Antibodies were conjugated to fluorescein isothiocyanate (FITC) (CD8, CD14, CD45RA, CD56, lineage cocktail), phycoerythrin (PE) (CD5, CD16, CD45RO, CD62L, CD123), allophycocyanin (APC) (CD3, CD11c, CD19) or peridinin-chlorophyll-protein complex (PerCP) (CD4, HLA-DR). All antibodies were obtained from Becton and Dickinson Biosciences, Franklin Lakes, NJ.

After incubation, red blood cells were lysed using 1x lysing solution (BD Biosciences). Cell pellets were washed in phosphate-buffered saline and fixed using 1% paraformaldehyde. Flow cytometry was performed using the FACS Calibur system (BD Biosciences) and data were analyzed using CellQuest pro software (BD Biosciences). Whole blood concentrations of lymphocytes and neutrophils were determined by total and differential leukocyte count using the Cell-Dyn Sapphire haematology analyzer (Abbott diagnostics, Abbott Park, IL). Manual leukocyte differential was performed in case of abnormal cell morphology. Myeloid dendritic cells (mDC) were identified as HLA-DR$^+$, lineage- and CD11c$^+$, plasmacytoid dendritic cells (pDC) as HLA-DR$^+$, lineage$^-$ and CD123$^+$. Natural killer (NK)-cells were marked by CD3$^-$, CD16$^+$ and CD56$^+$, and monocytes were identified as HLA-DR$^+$ and CD14$^+$. Absolute numbers of mononuclear cells were calculated by multiplying the percentage of cells in the lympho-monocyte gate (as determined by flow cytometry) with the concentrations of lymphocytes and monocytes from the differential leukocyte count.

TLR agonists

TLRs were stimulated using polyinosinic:polycytidylic acid (poly I:C, TLR3), ultrapure LPS from *E. coli* (TLR4), loxoribine (TLR7) and CpG oligonucleotide type A (ODN CpG 2216, TLR9), all from InvivoGen (San Diego, CA). For co-stimulation, recombinant IFN- γ was purchased from PeproTech Inc. (Rocky Hill, NJ).

Cell stimulation

In vitro TLR stimulation was performed using optimal concentrations of TLR agonists and incubation times for cytokine measurements, as titrated in pilot experiments (data not shown). Accordingly, blood samples were stimulated with LPS (100 ng/ml) + IFN-γ (20 ng/ml), poly I:C (200 μg/ml), ODN CpG (30 μg/ml) or loxoribine (1 mM). For mononuclear cell stimulation in plasma exchange assays, lower concentrations of stimuli were used (50 ng/ml LPS and 20 ng/ml IFN-γ). For cytokine protein measurements in culture supernatant, blood samples were diluted 1:14 in RPMI medium containing 2.0 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin prior to *in vitro* TLR stimulation. This dilution allowed us to study cytokine responses to multiple TLR agonists in limited blood volume. After 24h incubation at 37ºC and 5% CO_2, samples were centrifuged at 1000 x *g* for 5 min. Supernatants were collected and stored at -80°C until further analysis. For RNA studies, stimulations were performed in undiluted blood using a 5h incubation time optimized for RNA detection. Upon stimulation, blood was collected in PAXgene reagent (PreAnalytiX GmbH, Hombrechtikon, Germany) and stored at -80°C until further processing.

Plasma studies

The effect of plasma on TLR-agonist induced cytokine production by PBMC was studied according to previous reports[6]. Plasma was prepared by centrifugation of heparinized blood at 1000 *g* for 10 minutes. Fresh adult peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient separation, and stimulated with LPS (50 ng/ml) and IFN-γ (20 ng/ml) in the presence of 10% heterologous adult or neonatal plasma (24 h; 37ºC; 5% CO_2). For each experiment, plasma derived from 8 to16 different one-month old neonatal or adult donors was used.

Cytokine ELISA

Cytokine concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions: IL-10 (Sanquin/CLB, Amsterdam, Netherlands), IL-12p70 (Diaclone Research, Besançon, France) and IFN-α (Bender Medsystems, Burlingame, CA, USA). Internal controls were used to minimize inter-assay variations. Lower limits of detection were 1.0 pg/ml (IL-10), 2.0 pg/ml (IL-12p70) and 2.7 pg/ml (IFN-α). For samples with cytokine concentrations below the detection limit, the concentration was arbitrarily defined as half of the detection limit.

RNA measurements

RNA was extracted using the PAXgene Blood RNA kit (PreAnalytix GmbH), according to a modified protocol optimized for small blood volumes[10]. RNA was subsequently purified and concentrated using the RNAeasy mini-elute kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentrations in the purified samples were measured using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and cDNA was prepared using the RT^2 First Strand Kit (SA Biosciences, Frederick, MD). qRT-PCR was performed according to manufacturer's instructions, using a customized PCR array including 28 different TLR-related transcripts (SA

Biosciences). SYBR-Green (SA Biosciences) was used for detection and fluorescence was read on the ABI Prism 7300 Sequence Detector (Applied Biosystems, Foster City, CA). Resulting mRNA levels were normalized to housekeeping genes and compared using the ΔCT method.

Statistical analysis
All data were analyzed in the Statistical Package for Social Sciences (SPSS) version 15.0 software. The distribution of variables was checked for normality using the Kolmogorov-Smirnov test. Cytokine and mRNA concentrations after TLR stimulation and flow cytometry data were logarithmically transformed, and geometric means between groups were compared using Student's *t* test, or one-way ANOVA with post-hoc analysis (Bonferroni test for multiple comparisons). Correlations between LPS-induced and LPS+IFNγ-induced release of IL-10 and IL-12p70 were calculated using Pearson correlation on logarithmically transformed data. All *p* values are two-sided and were considered significant when $p < 0.05$.

Results

Different cell composition in neonatal and adult venous blood
To determine whether differences in innate immune cell numbers may contribute to the pattern of neonatal TLR-mediated responses, whole blood leukocyte differentials were performed by flow cytometry. Cord blood and neonatal venous blood were highly cellular compared to adult venous blood, containing higher concentrations of monocytes (Fig. 1A) and equal concentrations of mDC, pDC and NK-cells (Figs. 1B-D). Consistent with previous studies, neutrophil concentrations at the age of one month were lower than those in cord blood and adult blood (Fig. 1E)[11].

Cord blood TLR agonist-induced cytokine release is distinct
To assess the ability of neonatal innate immune cells to mount an immune response to microbial products, we tested *ex vivo* whole blood responses to a panel of TLR agonists. Cytokine concentrations in unstimulated control samples were below the limit of detection for all cytokines (data not shown). Cord blood TLR agonist-induced cytokine responses were significantly different from adult TLR responses (Figs. 2 and 3). Cord blood hemocytes demonstrated similar (TLR3 and TLR7) or increased (TLR4 and TLR9) levels of TLR agonist-induced IL-10 production. In contrast, cord blood production of Th1-polarizing cytokines IL-12p70 and IFN-α in response to agonists for TLR3, TLR4 and TLR7 was decreased compared to adults.

Table 1: Characteristics of participants

Characteristic	Group		
	Birth (n=18)	1 wk (n=22)	1 mo (n=96)
Gender, male, *n* (%)	10 (56)	13 (59)	49 (51)
Gestational age, wk, mean (95% CI)	40.0 (39.3-40.6)	39.9 (39.3-40.6)	39.8 (39.5-40.0)
Birth weight, g, mean (95% CI)	3650 (3443-3857)	3643 (3354-3931)	3583 (3487-3678)
Mode of delivery, *n* (%)			
Vaginal	18 (100)	6 (27)	67 (70)
Caesarean section	0 (0)	16 (73)	29 (30)
Siblings, *n* (%)	13 (72)	16 (73)	59 (61)

Figure 1: Whole blood concentrations of innate immune cells. Whole blood leukocyte differential was performed in cord blood (n=18), neonatal venous blood obtained at the age of one month (n=96) and adult venous blood (n=17). Cell percentages were determined by FACS and multiplied by the total concentration of lymphocytes and monocytes from the complete blood count. Data are represented as geometric mean + 95% CI. *; $p<0.05$.

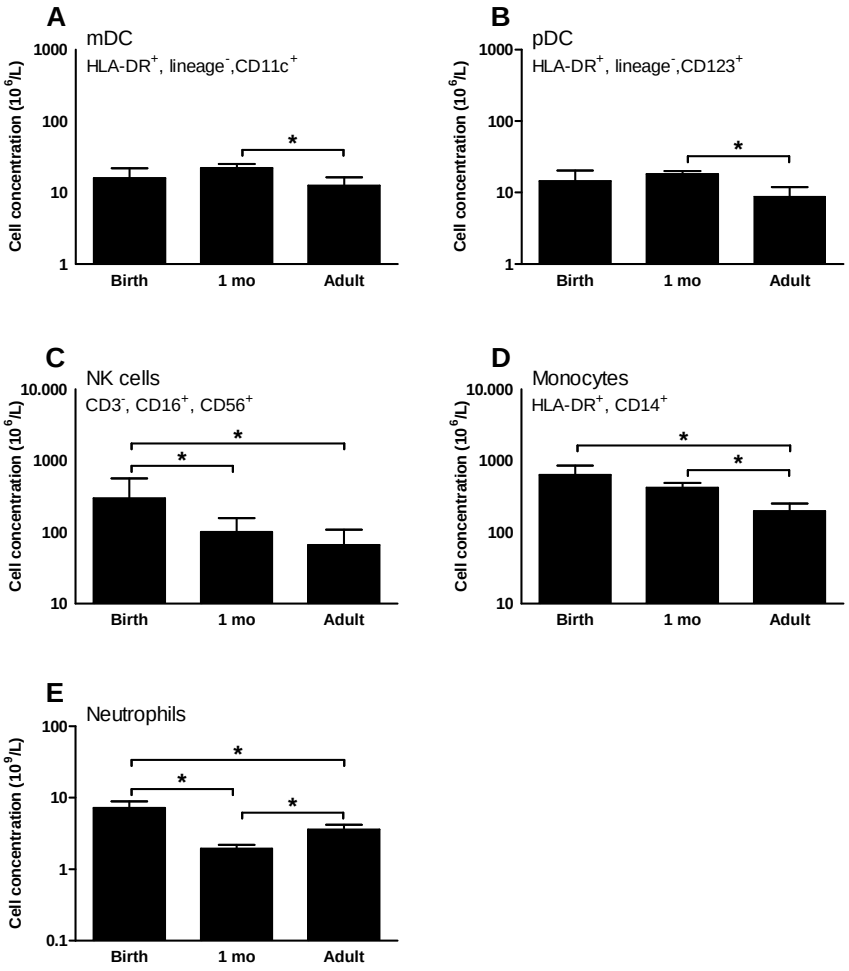
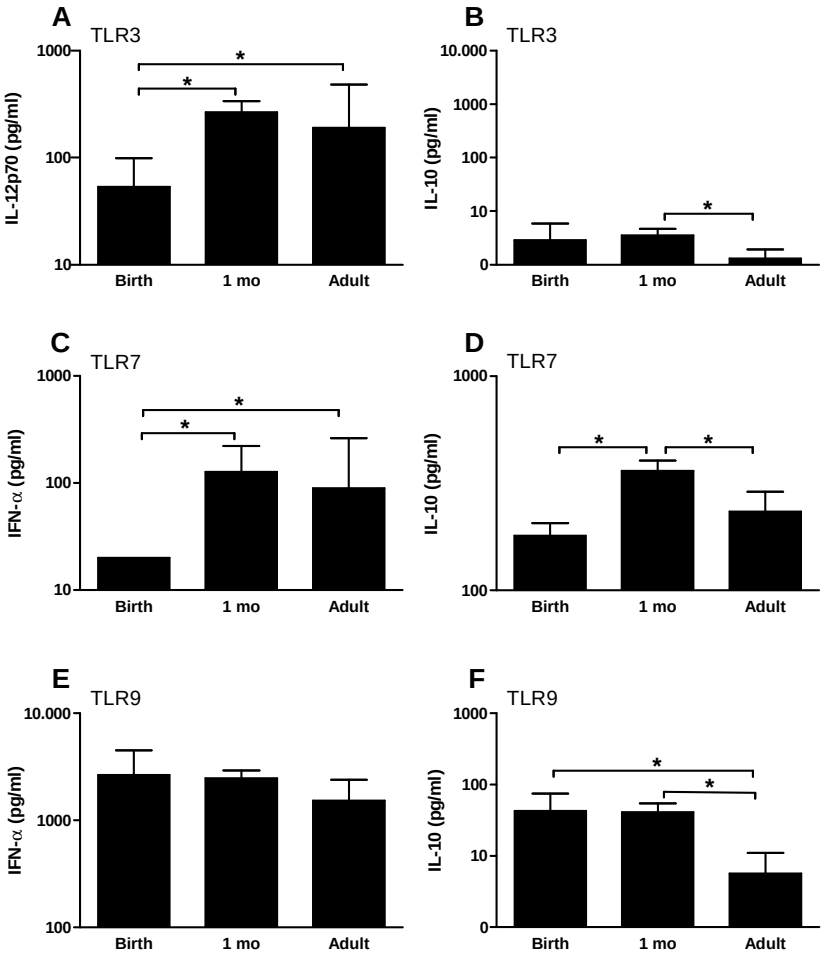


Figure 2: Rapid maturation of TLR responses in healthy newborns. Cytokine production in cord blood (n=18), neonatal venous blood at age one month (n=96) and adult venous blood (n=17) was measured after a 24h-incubation with poly I:C (TLR3, panels A+B), loxoribine (TLR7, C+D) and ODN CpG (TLR9, E+F). Neonatal ability to produce Th1-type cytokines upon TLR-agonist stimulation was impaired at birth, but rapidly increased to adult levels. Data are represented as geometric means + 95% CIs. *; p<0.05.



TLR agonist-induced cytokine responses differentially mature during the first month of life

To determine whether the distinct patterns of neonatal TLR-mediated responses persist beyond the immediate peripartum period, we next determined TLR agonist-induced cytokine release in healthy newborns at the age of one month. Neonatal capacity to produce Th1-type cytokines IL-12p70 or IFN-α in response to agonists of TLR3, TLR7 and TLR9 rapidly increased to adult levels within the first month of life (Fig. 2). In marked contrast, TLR4-mediated production of IL-12p70 remained significantly decreased compared to adults (mean 47 pg/ml, 95%-CI 29-76 pg/ml vs 437 pg/ml, 166-1129 pg/ml, $p<0.05$) (Fig. 3). 1 month old neonates demonstrated increased production of IL-10 in response to stimulation with agonists for TLR3, TLR4, TLR7 and TLR9. However, although differences in TLR3-mediated IL-10 production were significant, these results should be interpreted with caution because poly I:C induced very little IL-10 in all populations. At age one month, there was a modest inverse correlation between TLR4-mediated IL-10 and IL-12p70 production (ρ -0.167, $p<0.05$).

TLR-4 responses gradually mature during the first month of life

To study whether neonatal TLR4-mediated cytokine responses are impaired all throughout the first month of life, LPS+IFN-γ-induced cytokine production was studied in venous blood obtained from neonates during the first week of life (Fig. 3). LPS+IFN-y stimulation induced similar amounts of IL-10 in cord blood (1192 pg/ml), venous blood drawn at the age of one week (741 pg/ml) and at the age of one month (988 pg/ml). LPS+IFN-γ-induced IL-12p70 production at age one week was significantly higher than in cord blood (53 pg/ml vs 6,5 pg/ml, $p<0.01$), similar to levels at age one month (47 pg/ml, pg/ml), and lower than in adults (433 pg/ml, $p<0.01$). These findings suggest that the ability of neonatal blood to produce Th1-type cytokines in response to LPS remains impaired at least until the age of one month. Previous studies have shown that perinatal events influence cord blood TLR agonist induced cytokine production[6, 12]**.** In our study, both univariate and multivariate analyses did not reveal any association between gestational age, sex, maternal parity or mode of delivery and TLR agonist-induced cytokine production in neonatal venous blood drawn at the age of one month (data not shown). This study was not sufficiently powered to investigate the clinical determinants of neonatal TLR responses in cord blood or venous blood at the age one week.

Distinct neonatal responses to LPS are associated with differences in cytokine transcription

We next examined whether the distinct neonatal TLR4-mediated IL-12p70 and IL-10 protein responses are also evident on mRNA level (Fig. 4). Stimulation with LPS or LPS+IFN-γ induced high levels of IL-12A (p35) mRNA in adults. In contrast, cord blood and neonatal venous blood demonstrate impaired LPS-induced and LPS+IFN-γ-induced up regulation of IL-12A mRNA (Fig. 4A), with significantly lower levels of LPS-induced IL-12A compared to adults. Although differences between neonatal venous blood and adult venous blood were not significant, IL-12B demonstrated similar patterns (Fig. 4B). This suggests that that differences in mRNA levels may underlie the observed differences in LPS+IFN-γ-induced IL-12p70 protein production between neonates and adults.

In contrast, despite higher LPS-induced IL-10 protein production in cord blood (Fig. 3), LPS-induced levels of IL-10 mRNA were similar in all age groups (Fig. 4E). However, small but significant differences may have remained undetected because of a relatively small number of participants for this part of the study. As previous studies on neonatal TLR responses mainly focused on TNF-α and IL-6[6, 13, 14], we determined LPS- and LPS+IFN-γ-induced mRNA levels of these cytokines. Consistent with existing literature, TLR4-mediated production of TNF-α mRNA trended lower in cord blood and at the age of one month compared to adults (Fig. 4C), whereas TLR4-mediated transcription of IL-6 was similar in all age groups (Fig. 4D).

Effect of recombinant IFN-γ on LPS-induced cytokine production

Stimulation with LPS only (i.e. without IFN-γ) resulted in low/undetectable levels of IL-12p70 in 77% of all samples. Addition of exogenous IFN-γ has been shown to increase transcription of IL-12p35 and IL-12p40, and to increase LPS-induced release of IL-12p70[15, 16]. Therefore, recombinant IFN-γ (rIFN-γ) priming was used to optimize LPS-induced IL-12p70 production. To verify that the observed patterns were not solely the result of a newborn-specific effect of IFN-γ, we compared cytokine production in samples stimulated with LPS only and LPS with IFN-γ (Supplementary Fig. 1). Production of IL-12p70 production showed similar developmental patterns, with low/undetectable levels at birth and higher levels at the age of one month or in adults. Interestingly, although there was a strong correlation between LPS-induced and LPS+IFN-γ-induced IL-10 production (ρ=0.809), LPS-only stimulation resulted in high-levels of IL-10 with no differences between age groups. This suggests that the effect of IFN-γ may be age-dependent, selectively inhibiting LPS-induced IL-10 production in one-month olds and adults, but not in cord blood.

Figure 3: Distinct TLR4 responses up until the age of one month. Cytokine production in cord blood (n=18), neonatal venous blood at age one month (n=96) and adult venous blood (n=17) was measured after a 24h-incubation with LPS+IFN-γ. Cord blood TLR4 responses were characterized by low levels of IL-12p70 (A) and high levels of IL-10 (B). Although LPS+IFN- γ-induced production of IL-10 and IL-12p70 gradually decreased or increased respectively, TLR4-mediated cytokine responses at the age of one month remained significantly impaired compared to adult responses. *, p<0.05.

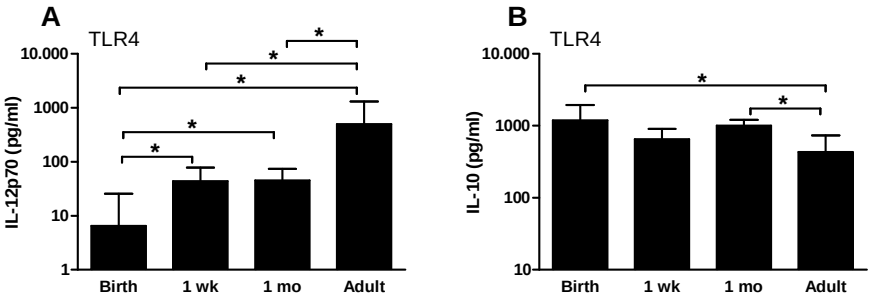


Figure 4: Distinct neonatal TLR4-mediated cytokine mRNA responses
Cytokine mRNA concentrations in cord blood (n=7), neonatal venous blood at age one month (n=7) and adult venous blood (n=7) were measured after a 5h-incubation with medium, LPS or LPS+IFN-γ. TLR4-mediated transcription of IL-12A and IL-12B and TNF-α was impaired both in cord blood and in venous blood at age one month, compared to adult venous blood. In contrast, despite higher medium-induced levels in cord blood, TLR4 agonist-induced production of IL-6 and IL-10 was similar in all age groups. *; $p<0.05$.

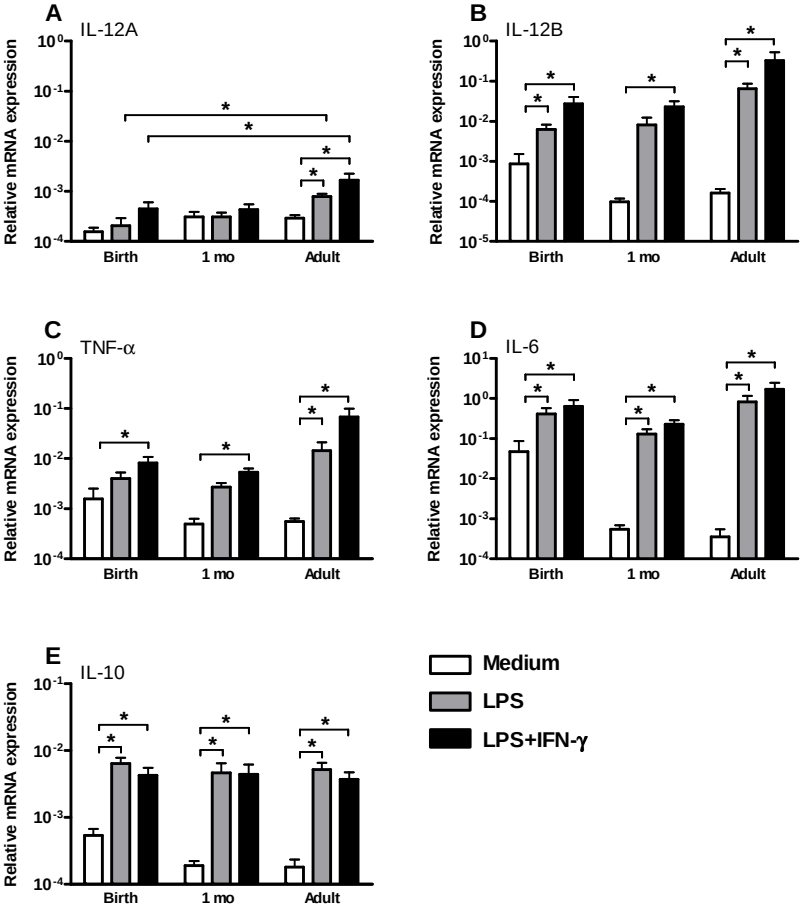
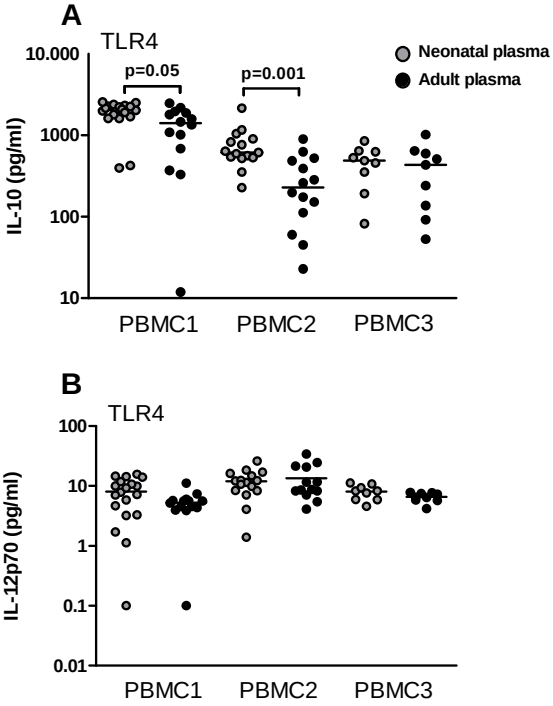


Figure 5: Donor- and cytokine specific effect of neonatal plasma on LPS-induced cytokine production.
Adult PBMC derived from heparinized blood from three separate donors were stimulated with LPS and IFN-γ in the presence of 10% heterologous adult or neonatal plasma. Each dot represents a different plasma donor. After 24h incubation, the extracellular medium was collected for IL-10 and IL-12p70 measurements. Bars represent median values.



Neonatal LPS-induced production of IL-10 but not IL-12p70 is influenced by a soluble factor

The maturation lag of TLR4 responses prompted us to further explore the underlying mechanism of distinct neonatal LPS-induced cytokine production. To determine whether differences in TLR4 complex-mediated production of IL-10 and IL12p70 are due to cellular or soluble factors, we compared LPS-induced IL-10 and IL-12p70 release from adult PBMC cultured in heterologous adult and neonatal plasma obtained at the age of one month (Fig. 5). Neonatal plasma conferred diminished release of IL-10 in two out of three PBMC donors ($p<0.05$), but did not affect IL-12p70 release. The magnitude of modulation of IL-10 release by plasma was smaller than the differences observed in our whole blood assay. In addition, the donor-specificity and lack of plasma-mediated effect on IL-12p70 release suggest that differences in neonatal and adult LPS-induced cytokine production may not be solely modulated by soluble factors.

Discussion

Human neonates are highly susceptible to infections, which is generally ascribed to transient developmental deficiencies in the innate and adaptive immune system. Here, we demonstrate that despite similar or higher concentrations of innate immune cells, neonatal ability to produce Th1-type cytokines upon TLR stimulation is impaired compared to adults. The neonatal TLR system undergoes rapid and differential development during the first month of life. Whereas the ability to produce Th1-type cytokines in response to agonists for TLR3, TLR7 and TLR9 rapidly increases to adult levels during the first month of life, TLR4 mediated responses remain impaired at least up to one month of age. Age-dependent impairments in the TLR system may contribute to the high neonatal susceptibility to infection.

Our results are consistent with previous studies, demonstrating that neonatal innate immune system is distinct and polarized towards Th2-type responses[5, 6, 17]. This polarization is thought to play an important role in the prevention of harmful maternal-fetal alloimmune reactions leading to preterm labour and delivery[3]. However, the bias against Th1-cell polarizing cytokines leaves the newborn susceptible to infection. Insight into the kinetics and factors modulating neonatal TLR development may result in new strategies to prevent and/or treat infections and allergic diseases.

Studies investigating postpartum TLR development describe gradual maturation of Th1-cell polarizing capacity from infancy to childhood[12, 18, 19]. The importance of establishing the immune status in early life is underscored by several studies showing that variations in early immune development have long-term sequelae with regard to the prevalence of many diseases[20-23]. To our knowledge, this is the first study to investigate postpartum TLR development during the first month of life in a large cohort of healthy newborns. The striking maturation of neonatal TLR responses identifies the first month of life as an essential period in the development of this part of the innate immune system.

Studies investigating the cause of impaired neonatal TLR responses have mainly focused on impaired production of Th1-type cytokines such as TNF-α and IL-12[5, 6, 13, 14, 24, 25]. Different mechanisms have been suggested to explain decreased cord blood LPS-induced IL-12p70 production. First, plasma factors, such as LPS binding protein, have been shown to modulate LPS-delivery to the TLR4 receptor complex[26, 27]. Adenosine, present in neonatal plasma, increases intracellular levels of cyclic adenosine monophosphate (cAMP), thereby inhibiting TLR-mediated TNF-α production[14]. Interestingly, increased levels of cAMP also decrease IL-12 production while increasing production of IL-10, exemplifying its immunopolarizing potential[3]. In our assay, neonatal plasma showed a modest increase in LPS-induced release of IL-10 in two out of three adult PBMC donors, and had no effect on the production of IL-12p70. Because of limited volume, we were only able to use 10% plasma, whereas previous studies used 100% plasma. In addition, instead of cord blood mononuclear cells, we used adult PBMC, which may be less sensitive to the TLR-modifying effects of neonatal plasma. We hypothesize that TLR4-mediated cytokine production is modulated by a complex and dynamic interplay between soluble factors and other (cell intrinsic) factors. The concentration of soluble mediators and target cell sensitivity will be subject of further studies.

Second, differences in LPS-induced cytokine production may be due to differences in expression of the TLR4 complex, consisting of TLR4, CD14 and MD-2. The relative

level of TLR4 expression in neonates compared to adults is still a matter of debate, which is further complicated by methodological differences between studies[6, 19, 28]. However, while differences in TLR4 complex expression or soluble factors may explain a general increase or decrease in cytokine release, they are unlikely to explain the polarization observed in our study.

Third, IL-10 has been shown to negatively modulate IL-12p70 production[29, 30]. Indeed, in our samples, there was a modest but significant negative correlation between LPS+IFN-γ induced IL-10 and IL-12p70 release. This supports the notion that TLR4-mediated cytokine production is tightly regulated to maintain the balance between Th1 and Th2. From our results, we cannot conclude whether polarized neonatal TLR4 responses are primarily due to increased production of IL-10, decreased production of IL-12p70, or to a common factor influencing both cytokines.

Fourth, signaling downstream of TLR4 also differs between newborns and adults. A microarray study comparing cord blood and adult LPS-activated monocytes showed significant differences in expression of several signal transduction factors and transcription factors, including JunB and STAT4[31]. LPS-induced mRNA levels of both subunits of the IL-12p70 heterodimer by cord blood monocytes has been shown to be decreased due to decreased half-life (IL-12p40) or defective nucleosome remodelling, resulting in impaired transcription (IL-12p35)[32-35]. Our data indicate that, in a whole blood assay, defective IL-12p70 production at birth is due to impaired transcription of both IL-12A (encoding the IL-12p35 subunit) and IL-12B (IL-12p40), and that these impairments are maintained up until the age of one month.

This study has potential limitations. First, in vitro study of whole blood represents a minimally perturbed system, yet may not reflect patterns of response *in vivo*[36]. Second, repeated sampling in the same children would have been the optimal strategy to address maturation of TLR responses during the neonatal age. This was not feasible for practical reasons. Third, the whole blood stimulation assay limits conclusions on cell-specific mechanisms underlying impairments in neonatal TLR function. Similar limitations would have been encountered using isolated PBMC, because PBMC composition changed markedly during the neonatal age. We believe that the whole blood stimulation model is a relevant representation of the immunological status of newborns and infants that correlates with susceptibility to infection[37-39].

In conclusion, neonatal TLR responses are distinct from those of adults, and differentially mature during the first month of life. The propensity towards high IL-10 but low IL-12p70 production in response to TLR4 agonists during the first month of life might contribute to neonatal susceptibility to pathogens that are recognized by TLR4, such as Gram-negative bacteria and RSV[40]. Finally, future studies aimed at identifying the factors influencing postpartum TLR development will further delineate the age-dependent maturation of this key aspect of innate immunity and may identify new strategies to modulate the maturation of the neonatal innate immune system, to prevent infections and/or allergy during this vulnerable age.

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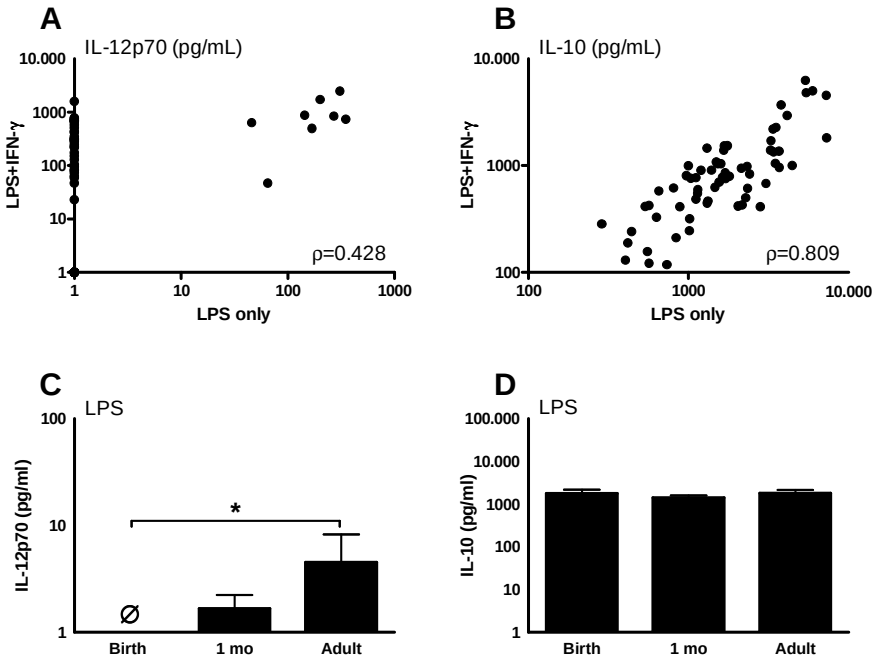
Supplementary information

Supplementary figure 1: Effect of IFN-γ on LPS-induced cytokine production
Cytokine production in cord blood (n=17), neonatal venous blood at age one month (n=29) and adult venous blood (n=16) was measured after a 24h-incubation with LPS only or LPS+IFN-γ. Concentrations of IL-12p70 after LPS-only stimulation were often below the detection limit of the ELISA: 0% of cord blood samples were within detectable range, vs 10% of one-month and 30% of adult samples. Addition of rIFN-γ resulted in measurable levels of IL-12p70 in 33%, 77% and 95% of samples respectively. Of note, although there was a strong correlation in IL-10 production in samples stimulated with LPS only and LPS+IFN-γ (ρ=0.809), the age-specific patterns observed after LPS+IFN-γ stimulation were lost, indicating that the effect of IFN-γ on LPS-induced IL-10 may be age-dependent.



Supplementary table 1: Number of participants for innate immune measurements

Experiment	N			
	Birth	1 wk	1 mo	Adult
Flow cytometry				
mDC	17	n/a	84	18
pDC	15	n/a	85	15
Monocytes	14	n/a	89	15
NK cells	17	n/a	32	17
Neutrophils	17	n/a	85	17
RNA measurement upon TLR stimulation	7	7	7	7
Cytokine measurements upon in vitro TLR stimulation				
TLR3, IL-10	18	n/a	93	18
TLR3, IL-12p70	18	n/a	93	18
TLR4, IL-10	18	18	94	18
TLR4, IL-12p70	18	16	93	18
TLR7, IL-10	18	n/a	72	16
TLR7, IFN-α	13	n/a	29	14
TLR9, IL-10	14	n/a	92	16
TLR9, IFN-α	10	n/a	90	13

Chapter 5

Breast feeding modulates neonatal innate immune responses: A prospective birth cohort study

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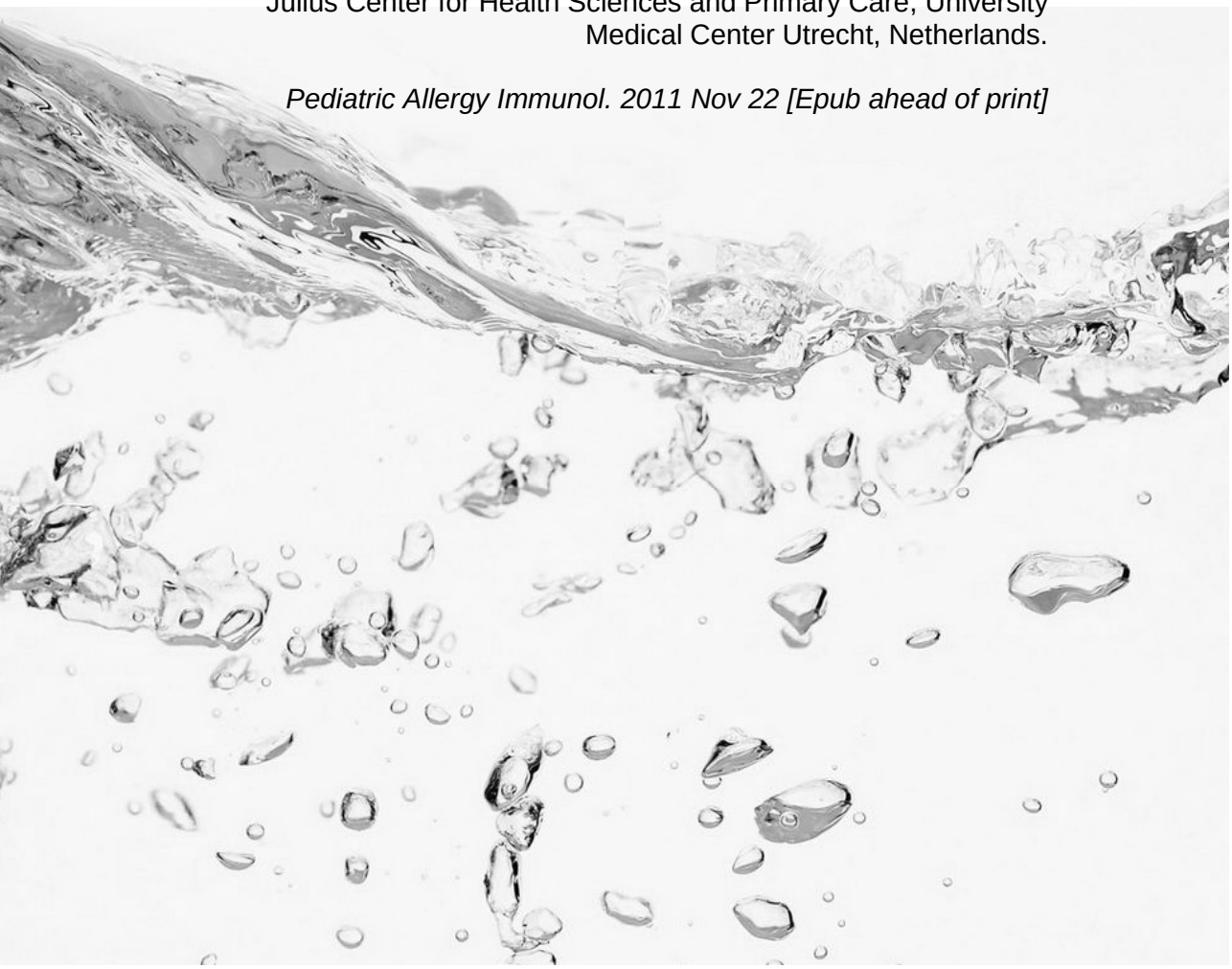


Abstract

Neonatal Toll-like receptor (TLR) responses are biased towards Th2-polarizing responses at birth, and rapidly mature towards more balanced responses during the first month of life. To determine the influence of environmental exposure on postnatal TLR function, a prospective birth cohort study was performed in 291 healthy term neonates. Mode of delivery, breastfeeding, birth month, siblings, pets and parental smoking were analyzed in relation to neonatal innate immune parameters at the age of one month. Whole blood concentrations of innate immune cells were measured by flow cytometry and in vitro TLR-mediated cytokine production was determined by ELISA. Breast feeding was the major determinant of neonatal innate immunity, associating with 5 (31%) of neonatal innate immune parameters, of which the association with TLR7-mediated IL-10 production was most significant (76 pg/mL in breastfed neonates versus 293 pg/mL in formula-fed neonates, p=0.001). Of innate immune variables, TLR3-mediated IL-12p70 production was highly associated with environmental exposures (pets, breastfeeding and mode of delivery), whereas TLR9-mediated cytokine responses were not associated with any environmental factor. We conclude that the protective effect of breastfeeding against subsequent infections and atopy might be explained by its innate immune modulatory effects in the first month of life.

Introduction

The first month of life is a challenging period for the development of the immune system. Born from a sterile intra-uterine environment, the neonatal immune system is suddenly exposed to a world full of foreign antigens. To prevent potentially harmful immune reactions towards maternal antigens in utero and to allow for bacterial colonization after birth, the neonatal immune system is generally biased against the generation of pro-inflammatory responses. However, this bias renders the neonate highly susceptible to infection. Infections are the second most common cause of neonatal mortality world wide, accounting for 1 million annual deaths[1]. In addition, increased neonatal Th2-polarizing responses towards food and aeroantigens might induce the patterns that are associated with childhood atopy[2-4].

We have previously demonstrated that neonatal Toll-like receptor responses rapidly change during the first month of life, maturing from a Th2-polarizing phenotype to increased production of Th1-polarizing cytokines[5]. The first month of life might thus provide a window of opportunity for intervention. Indeed, early life exposure to environmental factors, for example cigarette smoke[6], has been associated with increased risk of asthma or atopy, while other factors, such as siblings[7, 8], pets[9] and breastfeeding [10], are protective. Although in vitro studies and animal models have shown that many of these factors have immune modulatory functions[11-13], only few studies have investigated the effects of early life environmental exposure on neonatal innate immune function in humans[14, 15]. The current study aims to provide quantitative evidence of environmental factors influencing neonatal innate immune cellularity and TLR-mediated cytokine production at the age of one month.

Methods

Study design and recruitment criteria

A prospective birth cohort study was performed in two urban hospitals in Utrecht, The Netherlands[5]. Eligible were healthy neonates born after uncomplicated pregnancy of ≥37 weeks after either vaginal delivery or elective caesarean section. Excluded were neonates with major congenital anomalies and neonates whose parents had insufficient knowledge of the Dutch or English language to comply with the research protocol. To avoid extensive counselling of parents just after delivery, a two-step inclusion system was used (Figure 1). The first consent interview took place around childbirth, during which parents were informed about the study but no decision on study participation was made. One to three weeks after delivery, parents were recontacted. During this second interview, informed consent was obtained and a visit to the hospital was scheduled. The study was approved by the ethical review boards of the Utrecht University Medical Center Utrecht and the Diakonessen Hospital. All parents provided written informed consent for study participation.

Clinical characteristics

Clinical characteristics were collected from the hospital charts and from standardized parental questionnaires performed by a physician or trained research nurse[16]. Determinants assessed were: mode of delivery (elective caesarean section vs vaginal delivery), exclusive breastfeeding during the first month of life, presence of siblings or

household pets and parental smoking. As a surrogate marker for exposure to seasonal antigens (e.g. respiratory viruses, pollen[17], indoor endotoxin [18]), birth month was used, either as an ordinal variable (values 1-12, for seasonality analysis), or dichotomized into birth in April to September versus birth in October to March, for regression analysis[19]. Although not directly related to environmental exposure, parental atopy was included into analyses as a marker of genetic background and because of its reported association with cord blood TLR responses[20]. Parental atopy was defined as physician-diagnosed asthma, hay fever or atopic dermatitis in either parent.

Blood

At the age of one month, 0.5-3 mL of neonatal blood were obtained by venipuncture and collected in sterile sodium heparin tubes. None of the participants had any sign or symptoms of infectious disease (e.g. runny nose, fever) in the two weeks prior to sampling.

Innate immune cellularity

Whole blood concentrations of innate immune cells were determined as previously described (Supplementary table 1 and [5]). Whole blood concentrations of neutrophils, eosinophils and basophils were determined by total and differential leukocyte count (Cell-Dyn Sapphire haematology analyzer, Abbott diagnostics, Abbott Park, IL). Percentages of myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), monocytes and natural killer (NK)-cells were measured by flow cytometry, using markers depicted in supplementary table 1 and figure headings. Percentages were converted to absolute concentrations by multiplying the percentage of cells in the lympho/monocyte gate (as determined by forward-sideward scatter properties) by the total numbers of lymphocytes and monocytes from the differential leukocyte count.

TLR stimulation

In vitro TLR stimulation was performed in 1:14 diluted whole blood, using optimal concentrations of TLR agonists and incubation times for cytokine measurements, as titrated in pilot experiments and described previously (Supplementary table 2 and [5]). Cytokines were arbitrarily chosen based upon their pro-inflammatory (TNF-α, IFN-α, IL-12p70) or regulatory properties (IL-10). TNF-α production was only measured upon TLR2-stimulation. We were unable to detect any TLR2-mediated IL-10 production.

Due to technical reasons, data on TLR2 and TLR7-mediated cytokine responses were available for part of the cohort (n=95 and n=128, respectively). For most environmental determinants, this did not affect statistical power substantially. However, none of the neonates with data on TLR2-mediated TNF-α production were born after caesarean section, and no comparisons were made for this outcome parameter.

After a 4h (TNF-α) or 24h (IFN-α, IL-12p70, IL-10) incubation at 37°C and 5% CO_2, samples were centrifuged at 1000×g for 5 min, and supernatants were collected and stored at −80°C until further analysis. Cytokine concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions: TNF-α, IL-10 (both from Sanquin/CLB, Amsterdam, The Netherlands), IL-12p70 (Diaclone Research, Besançon, France) and IFN-α (Bender Medsystems, Burlingame, CA, USA). The lower limits of detection were 1.0 pg/mL

(TNF-α and IL-10) and 2.0 pg/mL (IL-12p70 and IFN-α). For samples in which the cytokine concentration was below the detection limit, the concentration was arbitrarily defined as half of the detection limit.

Statistical analysis

Baseline characteristics were compared between participants and non-participants using the Student's *t*-test (gestational age, birth weight), or the X^2-test (gender, mode of delivery, birth season, presence of siblings). Innate immune variables were normalized by means of logarithmic transformation, and geometric means were compared between neonates with and without exposure to the environmental determinant of interest, using the Student's *t*-test. Linear regression was used to assess the weighted effects (expressed as the regression coefficient β and 95% confidence interval of β) of multiple variables on continuous outcome measures (e.g. the relative influence of breastfeeding or siblings on TLR3-mediated IL-12p70). In addition to environmental determinants, parental atopy was included into the regression analysis as a marker of genetic predisposition. To correct for multiple testing, the Bonferroni method was used, setting the threshold for statistical significance at $p<0.0005$.

Seasonality of innate immune variables was tested by fitting logarithmically transformed cell concentrations or cytokine levels to a sine function with a period of 12 months in a nonlinear regression model. Statistical significance of this seasonal distribution was determined using the F-test. Because this type of analysis has an inherent risk of over fitting, a more stringent cut off was used for statistical significance ($p<0.01$). To address the possibility that seasonal patterns were due to methodological factors (e.g. differences in reagents or machinery during the study period), sensitivity analyses were performed, analyzing each birth year in our cohort separately. All reported p-values are two-sided. Analyses were performed in SPSS 15.0.

Results

Study population

From March 2006 to February 2010, 3032 neonates were born in participating hospitals (Figure 1). Parents of 1168 (39%) of these neonates were asked to participate, of which 291 (25%) gave written informed consent for study participation. There were no differences in gender, birth weight, gestational age, siblings or birth season between participating and non-participating neonates (Supplementary table 3). Participating neonates were more likely to be born through elective caesarean section compared to non-participants (14 versus 9%, $p=0.04$). Complete perinatal data were available for all newborns, and 284 (98%) provided information on exposure during the first month of life. Characteristics of the study population are shown in table 1.

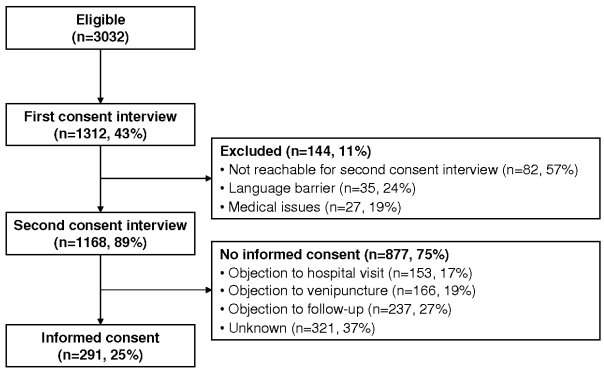
Table 1: Characteristics of study population

Characteristic	N=291
Gender, n (%)	
Male	141 (48)
Female	150 (52)
Gestational age, wks (SEM)	40.0 (0.06)
Birth weight, g (SEM)	3611 (27)
Mode of delivery	
Vaginal	251 (86)
Caesarean section	40 (14)
Birth season	
Spring	70 (24)
Summer	76 (26)
Fall	72 (25)
Winter	73 (25)
Any siblings, n (%)	186 (64)
Exclusive breastfeeding, n (%)	154 (53)
Pets, n (%)	92 (32)
Parental smoking, n (%)	73 (25)
Parental atopy, n (%)	160 (55)

Determinants of neonatal innate immunity

To determine the major postnatal determinants of neonatal innate immunity at the age of one month, univariable analysis and multivariable logistic regression analysis were performed. In univariable analyses (Figures 2-4), 6 environmental determinants were analyzed in relation to 16 measures of neonatal innate immunity: whole blood concentrations of 7 different innate immune cells (Figure 2) and 9 TLR-mediated cytokine responses at the age of one month (Figure 3). Associations with birth month are depicted in Figure 4. Results of multivariable analyses are shown in table 2 and 3 (only significant associations are shown). Out of 96 combinations tested, 18 (19%) significant univariable associations were found. Breast feeding, birth month and mode of delivery were the major determinants of neonatal innate immunity, affecting 5 (31%), 4 (25%) and 3 (19%) of neonatal innate immune parameters, respectively.

Figure 1: Flow chart of study population.



Breast feeding
Exclusive breastfeeding during the first month of life was associated with decreased whole blood concentrations of monocytes ($3.3x10^6$/L vs $4.5x10^6$/L, p=0.03, Figure 2a) and neutrophils ($1.6x10^9$/L vs $2.0x10^9$/L, p=0.01, Figure 2b), 2-fold decreased TLR2-mediated TNF-α (p=0.04, Figure 3a), 2-fold increased TLR3-mediated IL-12p70 (p=0.002, Figure 3b) and 4-fold decreased TLR7-mediated IL-10 (p=0.001, Figure 3g) compared formula or mixed feeding. Except for TLR3-mediated IL-12p70, all associations were independent (Table 3). The association with TLR7-mediated IL-10 remained significant after correction for multiple testing. In addition, the amount of TLR7-mediated IL-10 production per pDC was significantly lower in breastfed neonates compared to formula-fed neonates ($12.5x10^3$ vs $22.5x10^3$ pg/mL/pDC, p=0.013).

Mode of delivery
Birth via elective caesarean section was associated with 8-fold lower concentrations of NK-cells (p<0.001) (Figure 2e), with 2-fold decreased TLR3-mediated IL-12p70 (p=0.001, Figure 3b) and 2-fold increased TLR4-mediated IL-12p70 production (p=0.05, Figure 3d) as compared to vaginal delivery. Associations with concentrations of NK-cells (Table 2) and TLR3-mediated IL-12p70 were independent, and remained significant after multiple testing correction.

Birth month
Seasonal patterns by birth month were observed for whole blood concentrations of NK-cells and mDC, and for cytokine responses to TLR3 and TLR7 (Figure 4). Birth in winter months was associated with low TLR-3 mediated IL-12p70 production (p<0.001), and high production of IL-10 upon stimulation of TLR7 (p<0.001). Sensitivity analyses showed the same seasonal effects when each inclusion year was analyzed separately (data not shown). Except for mDC, all associations were independent (Table 2), and the association between birth month and TLR7-mediated IL-10 remained significant after correction for multiple testing. In multivariable regression analysis, two additional significant associations were identified, demonstrating that birth in winter months was associated with increased concentrations of monocytes (p<0.001) and pDC (p=0.02) compared to birth in summer months (Table 2).

Siblings, pets and parental smoking
No significant associations were found between presence of siblings, household pets and parental smoking and any parameter of neonatal immune function at the age of one month, neither in univariable analysis (Figures 2 and 3), nor in multivariable logistic regression analysis.

Figure 2: Associations between environmental factors and concentrations of innate immune cells at the age of one month.
Neonatal venous blood was obtained at the age of one month (n=291). Concentrations of neutrophils, eosinophils and basophils were determined by differential leukocyte count. Percentages of monocytes, NK cells, pDC and mDC were determined by flow cytometry and multiplied by the total concentration of lymphocytes and monocytes from the complete blood count. Data are represented as geometric means and 95% confidence intervals derived from log-transformed variables. NK-cells, Natural killer cells; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells.

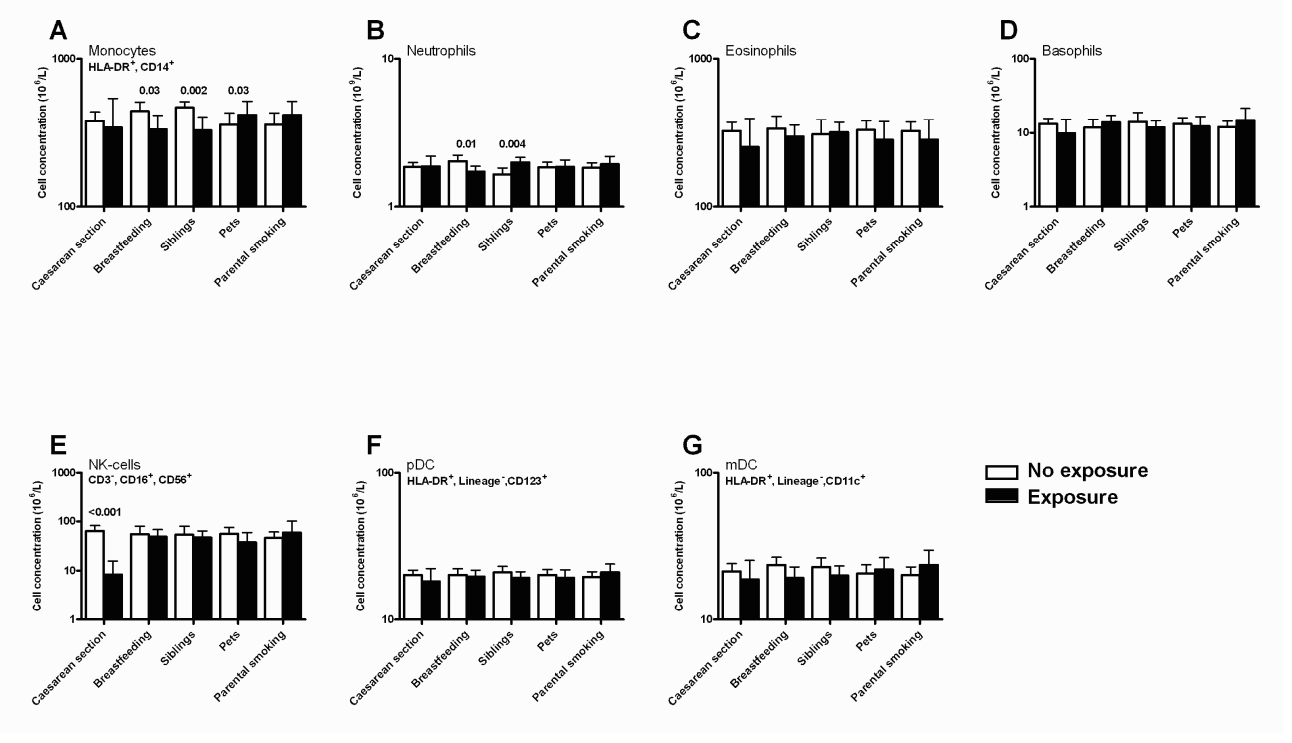


Figure 3: Associations between environmental factors and TLR-mediated cytokine responses at the age of one month.
Cytokine production in neonatal venous blood at age one month (n=291) was measured after a 4h-incubation with Pam_3Cys SSNA (TLR2, panel A), or a 24h incubation with poly I:C (TLR3, B+C), LPS+IFN-γ (TLR4, D+E), loxoribine (TLR7, F+G) and ODN CpG (TLR9, H+I). Data are represented as geometric means and 95% confidence intervals derived from log-transformed variables.

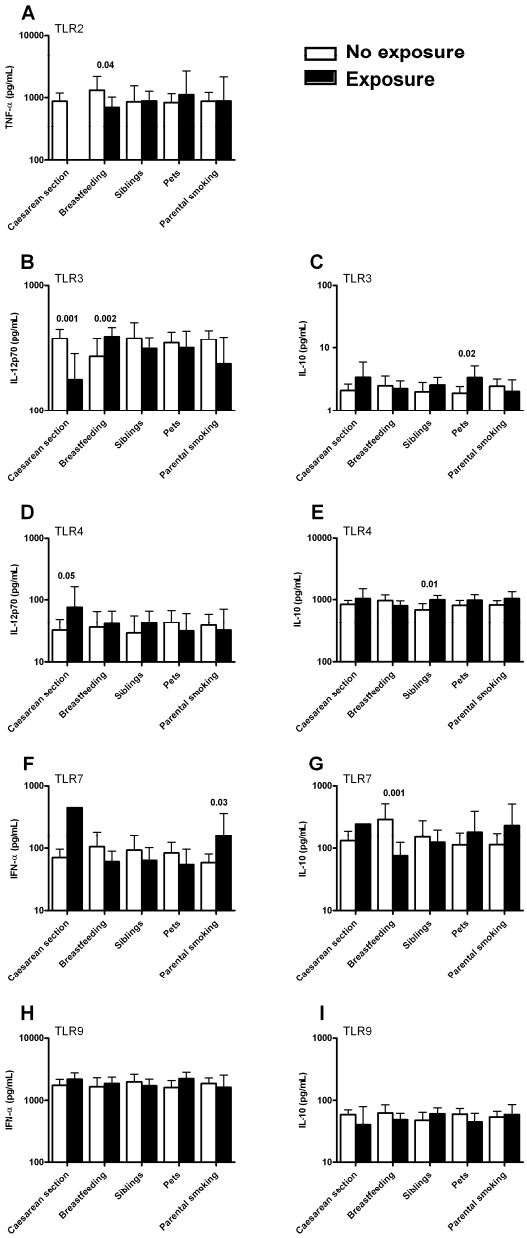


Table 2: Independent associations between environmental exposure and whole blood concentrations of innate immune cells at the age of one month. Variables included into regression analysis were: parental atopy, birth in April-September, mode of delivery, breastfeeding, siblings, pets and parental smoking. Significant associations are shown.
*; Remains statistically significant after correction for multiple testing.

a

Monocytes	**β (95% CI)**	**p-value**
Birth Apr-Sep	-0.24 (-0.35 to -0.12)	<0.001*
Siblings	-0.14 (-0.26 to -0.17)	0.03

b

Neutrophils	**β (95% CI)**	**p-value**
Breastfeeding	-0.17 (-0.13 to -0.02)	0.006
Siblings	0.19 (0.03 to 0.14)	0.002

c

NK-cells	**β (95% CI)**	**p-value**
Mode of delivery (CS)	-0.34 (-1.14 to -0.55)	<0.001*
Birth Apr-Sep	0.14 (0.04 to 0.44)	0.02

d

pDC	**β (95% CI)**	**p-value**
Birth Apr-Sep	-.16 (-0.14 to 0.02)	0.01

Prenatal determinants of neonatal innate immunity

To determine the role of the prenatal environment on neonatal immune function, characteristics of study participants at birth, including birth weight, gestational age and parental atopy were related to concentrations of innate immune cells and TLR-mediated cytokine responses at the age of one month. Birth weight and gestational age were not associated with neonatal innate immune cell concentrations, nor with TLR-mediated cytokine responses. Parental atopy was associated with decreased TLR3-mediated IL-12p70 (280 pg/mL versus 425 pg/mL, p=0.02, both in univariate analysis (Supplementary table 3) and in multivariate analysis (Table 3). However, this association lost significance after correction for multiple testing.

Neonatal concentrations of innate immune cells and TLR responses are differentially sensitive to environmental exposure in the first month of life

When the collective effect of environmental exposure on innate immune responses at the age of one month was analyzed, distinct patterns emerged. For example, concentrations of monocytes were sensitive to 3 environmental factors (negatively associated with breastfeeding and siblings, positively associated with pets) (Figure 2a), whereas concentrations of eosinophils, basophils and mDC were relatively inert. For TLR responses, high sensitivity to environmental influences was found for TLR3-mediated IL-12p70 (birth month, cesarean section, breastfeeding) (Figure 3b), which was also associated with parental atopy (Supplementary table 4). In contrast to TLR3-mediated IL-12p70 production, TLR9-mediated production of IFN-α and IL-10 was not associated with any environmental factor (Figure 3h-i).

Table 3: Independent associations between environmental exposure and TLR-mediated cytokine responses at the age of one month. Variables included into regression analysis were: parental atopy, birth in April-September, mode of delivery, breastfeeding, siblings, pets and parental smoking. Significant associations are shown.
*; Remains statistically significant after correction for multiple testing.

a

TLR2	**TNF-α**	
	β (95% CI)	**p-value**
Breastfeeding	-0.25 (-0.52 to -0.11)	0.04

b

TLR3	**IL-12p70**			**IL-10**	
	β (95% CI)	**p-value**		**β (95% CI)**	**p-value**
Parental atopy	-0.16 (-0.31 to -0.03)	0.02	Pets	0.20 (0.05 to 0.46)	0.01
Mode of delivery (CS)	-0.22 (-0.54 to -0.14)	0.001			
Birth Apr-Sep	-0.22 (-0.37 to -0.10)	0.001			
Parental smoking	-0.15 (-0.35 to -0.03)	0.02			

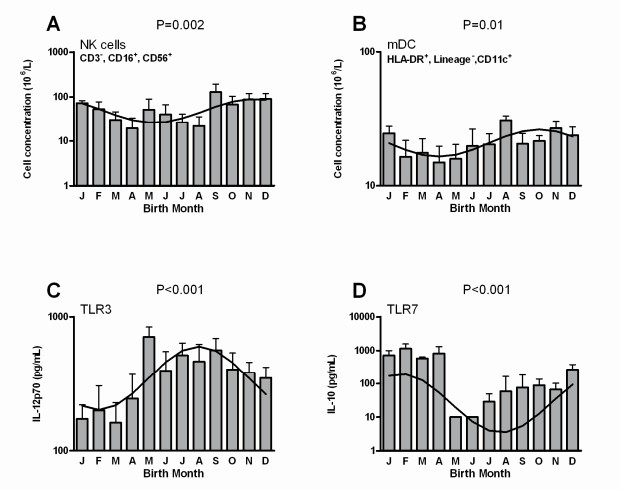
c

TLR4	**IL-12p70**			**IL-10**	
	β (95% CI)	**p-value**		**β (95% CI)**	**p-value**
Parental atopy	0.13 (0.00 to 0.66)	0.05	Siblings	0.17 (0.04 to 0.29)	0.01

d

TLR7	**IFN-α**			**IL-10**	
	β (95% CI)	**p-value**		**β (95% CI)**	**p-value**
Parental atopy	0.18 (0.004 to 0.54)	0.05	Birth Apr-Sep	0.35 (0.32 to 0.92)	<0.001*
Parental smoking	0.24 (0.10 to 0.72)	0.009	Breastfeeding	-0.32 (-0.87 to -0.27)	<0.001*

Figure 4: Seasonal patterns of neonatal whole blood concentrations of innate immune cells and TLR-mediated cytokine responses according to birth month. Seasonality was assessed by fitting the data to a sinusoidal curve, and statistical significance was calculated using the F-test. Bars represent geometric means and 95% confidence intervals derived from log transformed variables.



Discussion

This study demonstrates that whole blood concentrations of innate immune cells and TLR-mediated cytokine responses are modulated by environmental exposure in the first month of life. Breastfeeding had the highest influence on neonatal innate immune cellularity and TLR responses. Concentrations of monocytes and TLR3-mediated IL-12p70 were most sensitive to environmental modulation, whereas responses to TLR9 were largely inert.

Previous birth cohort studies have demonstrated that dysregulated innate immune responses at birth predispose to the subsequent risk of childhood atopy. Low TLR4-mediated IL-12p70 in cord blood is associated with enhanced allergen-induced Th2-type responses in the postnatal period[2]. Moreover, neonates who subsequently develop atopic dermatitis already have increased TNF-α and IL-6 in response to TLR2, TLR3, TLR4 and TLR5 at birth[20]. Parental characteristics are likely to explain part of the association between cord blood TLR responses and atopy. Two recent prospective studies in high-risk neonates have demonstrated that maternal atopy is associated with distinct cord blood TLR expression and function[20, 21]. These associations are likely partially explained by genetic factors[22, 23]. In addition, increasing evidence indicates that the intrauterine environment importantly impacts on fetal immune development, thus predisposing to subsequent infections and atopy[24-26].

We have previously identified the first month of life as a period of rapid maturation of the neonatal TLR system, offering a window of opportunity for interventions aimed at modulating innate immune development and preventing atopy. During this important period, TLR mediated cytokine responses rapidly mature from a Th2-biased profile towards increased Th1-cell polarizing responses characteristic of later life[5]. Early life exposure to TLR (ant)agonists, including microbial products, is thought to drive postnatal TLR maturation[27]. TLR responses at the age of one month thus likely reflect previous exposures that have modulated neonatal innate immune maturation. Here, we provide a comprehensive overview of environmental factors guiding postnatal TLR maturation. Our observations show that neonatal TLR responses at the age of one month are sensitive to environmental exposure, suggesting that strategies aimed at modulating early immune-environment interactions might be effective to prevent subsequent allergic disease.

We identify several potentially modifiable factors that modulate postnatal innate immune function.
First, innate immune responses at the age of one month were markedly different between neonates who received exclusive breastfeeding during the first month of life and those who did not. Several explanations may account for the association between breastfeeding and low TLR7-mediated IL10 production. Breast milk contains multiple immune modulatory compounds that directly influence TLR-mediated immune responses, including immunoglobulins, nucleotides, oligosaccharides and antimicrobial proteins/peptides[28, 29]. *In vitro,* breast milk increases monocyte production of IL-10 while decreasing production of IFN-γ in response to LPS, mitogen and allergen[30]. Immune modulation by breast milk is TLR-specific, as breast milk suppresses IL-8 production to

agonists for TLR2 and TLR3, while enhancing responses to TLR4 and TLR5[11]. To date, no studies have investigated the direct effects of breast milk on responses to agonists for TLR7. We propose that differential modulation of TLR-mediated IL-10 by breast milk might serve to promote neonatal tolerance to bacterial colonization, while suppression of TLR7-mediated IL-10 production may promote neonatal defense against viral infections. In addition, decreased TLR7-mediated IL-10 production and increased TLR3-mediated IL-12p70 production in breastfed infants may also reflect more rapid transition to a Th1-polarized innate immune system. Accordingly, promotion of early life innate immune maturation might be one of the mechanisms by which breast feeding protects against subsequent allergic disease[31-33]. However, breastfeeding did not affect cytokine responses to TLR4 and TLR9. More insight into the effects of breast milk on the developing immune system is needed for the generation of immune modulatory strategies that use breastfeeding to prevent subsequent allergy.

Second, mode of delivery may provide a modifiable target to modulate neonatal immune function. Consistent with findings in cord blood[34], neonates born after caesarean section had decreased NK-cells and decreased TLR3-mediated IL-12p70 at the age of one month. Decreased neonatal pro-inflammatory responses may contribute to the increased risk of childhood asthma in this population[35].

Third, birth month significantly influences subsequent neonatal innate immune function. Decreased TLR3-mediated IL-12p70 and increased TLR7-mediated IL-10 in neonates born in winter potentially indicate increased Th2-polarization, which might predispose to subsequent allergy and asthma[20, 36]. Interestingly, a recent study in cord blood demonstrates that birth in winter is associated with general hyporesponsiveness of T-cell mediated production of Th1-type (IFN-γ) and Th2-type (IL-5) and regulatory (IL-10) responses, suggesting that part of the differences in immune responses induced by birth month are determined before birth[37]. Many seasonal factor(s) might be responsible for this effect, including respiratory viruses, indoor endotoxin, seasonal allergens and vitamin D[20, 38-41]. Future studies will be needed to identify these factors and to determine their possible effects on the health of children later in life.

Finally, exposure to pets, siblings or cigarette smoke in the first month of life all left their imprint on the neonatal immune system. However, no consistent pattern towards Th1- or Th2 polarization was observed. Future studies will be needed to further delineate the interactions between environmental factors and the developing neonatal immune system, and the consequences for disease.

The effect of environmental exposure may not only depend on the type and amount of exposure, but also on the relative maturational state of the innate immune system at the time of exposure. We have previously demonstrated that cytokine responses to TLR3 and TLR4, rapidly mature during the first month of life, whereas responses to TLR9 are already mature at birth[5]. In the current study responses to TLR3 and TLR4-mediated IL-12p70 were most sensitive to environmental modulation, whereas TLR9-mediated responses were inert. Thus, sensitivity to environmental triggers might differ between individual TLRs and/or cytokines, with highest sensitivity for those systems that undergo most rapid maturation after birth.

This study has several strengths. First, we included a large, unselected cohort of healthy newborns, whereas previous birth cohort studies selected children from atopic parents[13, 20]. Second, we used an unbiased approach, investigating the effect of a broad panel of environmental determinants on neonatal innate immune function. Third, in addition to concentrations of innate immune cells, we also studied their functionality by measuring TLR-mediated cytokine production.

Potential limitations also deserve discussion. First, this study included multiple comparisons and is thus prone to false discovery. To minimize the possibility of type I error, we focused on general patterns that were consistent among different determinants. The high number of significant associations and the consistency with previous cord blood studies indicates that the majority represent real associations. Future studies will be needed to validate these findings. Second, the innate immune parameters measured in our study only represent part of the wide spectrum of genes that is activated upon TLR signalling. Third, we did not measure neonatal immune function at birth, and we cannot exclude any prenatal effects of environmental factors on neonatal immunity. However, due to the rapid development neonatal immunity in the first month of life, we propose that the effect of the environmental determinants primarily affects postnatal immune development.

In conclusion, this study demonstrates that neonatal innate immune are susceptible to multiple environmental exposures, of which breastfeeding is the most important. Breastmilk-mediated modulation of the developing innate immune system might create the patterns that protect from subsequent disease. Interventions aimed at modulating early life immune-environment interactions, including breast feeding, may be effective in the prevention of infections, asthma and/or atopy.

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Supplementary information

Supplementary table 1: Markers used to identify innate immune cells by flow cytometry

Cell type	Marker	Fluorochrome	Clone
Monocytes	HLA-DR$^+$	PerCP	L243
	CD14$^+$	FITC	MoP9
NK-cells	CD3$^-$	APC	SK7
	CD16$^+$	PE	B73.1
	CD56$^+$	FITC	NCAM16.2
Plasmacytoid DC	HLA-DR$^+$	PerCP	L243
	CD123$^+$	PE	9F5
	Lineage$^-$	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
Myeloid DC	HLA-DR$^+$	PerCP	L243
	Lineage$^-$	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
	CD11c$^+$	APC	S-HCL-3

Supplementary table 2: Agonists used for *in vitro* TLR stimulation

TLR	Agonist	Concentration	Manufacturer
2	Pam_3Cys SSNA	1 μg/mL	Bachem Biosciences, Weil am Rhein, Germany
3	Polyinosinic:polycyticylic acid (Poly I:C)	200 μg/mL	Invivogen, San Diego, CA
4	Ultrapure lipopolysaccharide derived from *E. Coli*	100 ng/mL	Invivogen
	Interferon-γ (IFN-γ)	20 ng/mL	Preprotech Inc, Rocky Hill, NJ
7	Loxoribine	1 mM	Invivogen
9	CpG oligodeoxynucleotide (CpG ODN)	30 μg/mL	Invivogen

Supplementary table 3: Characteristics of study participants and non-participants

Characteristic	Participants (n=291)	Non-participants (n=1021)	p-value
Gender, n (%)			0.43
Male	141 (48)	520 (51)	
Female	150 (52)	497 (49)	
Gestational age, wks (SEM)	40.0 (0.06)	40.0 (0.03)	0.97
Birthweight, g (SEM)	3611 (27)	3567 (36)	0.53
Mode of delivery			
Vaginal	251 (86)	922 (91)	0.04
Caesarean section	40 (14)	95 (9)	
Birth season			0.93
Spring	70 (24)	249 (25)	
Summer	76 (26)	263 (26)	
Fall	72 (25)	251 (25)	
Winter	73 (25)	254 (25)	
Any siblings, n (%)	186 (64)	626 (63)	0.68

Supplementary table 4: Effect of parental atopy on neonatal whole blood concentrations of innate immune cells and TLR-mediated cytokine responses at the age of one month. Parental atopy was defined as physician-diagnosed asthma, hay fever or atopic dermatitis in either parent. Values represent geometric means and 95% confidence intervals derived from log-transformed variables.

Innate immune parameter	**Parental atopy (n=160)**	**No parental atopy (n=131)**	**p-value**
Whole blood cell concentrations (10^6/L)			
Monocytes	379 (314-459)	377 (304-468)	0.97
Neutrophils	1825 (1666-1999)	1884 (1725-2057)	0.63
Eosinophils	315 (268-371)	315 (251-395)	0.99
Basophils	14.5 (11.5-18.3)	11.2 (8.7-14.4)	0.13
NK-cells	52.9 (37.4-74.7)	45.8 (31.8-65.9)	0.57
pDC	19.6 (18.1-21.2)	20.2 (17.9-22.9)	0.66
mDC	19.6 (16.8-23.0)	22.9 (19.7-26.7)	0.17
TLR-mediated cytokine responses (pg/mL)			
TLR2; TNF-α	925 (633-1351)	822 (480-1405)	0.72
TLR3; IL-12p70	280 (219-358)	425 (341-530)	0.02*
TLR3; IL-10	2.3 (1.7-2.3)	2.4 (1.7-3.3)	0.99
TLR4; IL-12p70	53.6 (32.5-88.3)	25.2 (14.2-44.6)	0.05
TLR4; IL-10	846 (691-1036)	887 (732-1075)	0.75
TLR7; IFN-α	99.1 (62.8-156)	54.0 (34.9-83.5)	0.06
TLR7; IL-10	158 (93.8-264)	98.6 (54.0-180)	0.24
TLR9; IFN-α	1750 (1365-2245)	1789 (1310-2442)	0.91
TLR9; IL-10	48.3 (37.4-62.5)	65.1 (50.7-83.6)	0.11

Chapter 6

Neonatal plasma polarizes TLR4-mediated cytokine responses towards low IL-12p70 and high IL-10 production via distinct factors

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Abstract
Human neonates are highly susceptible to infection, which may be due in part to impaired innate immune function. Neonatal Toll-like receptor (TLR) responses are biased against the generation of pro-inflammatory/Th1-polarizing cytokines, yet the underlying mechanisms are incompletely defined. Here, we demonstrate that neonatal plasma polarizes TLR4-mediated cytokine production. When exposed to cord blood plasma, mononuclear cells (MCs) produced significantly lower TLR4-mediated IL-12p70 and higher IL-10 compared to MC exposed to adult plasma. Suppression by neonatal plasma of TLR4-mediated IL-12p70 production, but not induction of TLR4-mediated IL-10 production, was maintained up to the age of 1 month. Cord blood plasma conferred a similar pattern of MC cytokine responses to TLR3 and TLR8 agonists, demonstrating activity towards both MyD88-dependent and MyD88-independent agonists. The factor that increased TLR4-mediated IL-10 production was heat-labile. In contrast, inhibition of TLR4-mediated IL-12p70 production by cord blood plasma was resistant to heat inactivation or protein depletion and was independent of IL-10. We excluded that the neonatal plasma factor causing decreased IL-12p70 production was vitamin D or prostaglandin E2. In conclusion, human neonatal plasma contains at least two distinct factors that suppress TLR4-mediated IL-12p70 production or induce IL-10 or production. Further identification of these factors will provide insight into the ontogeny of innate immune development and might identify novel targets for the prevention and treatment of neonatal infection.

Introduction

Toll-like receptors (TLRs) are key pathogen recognition receptors of the innate immune system that recognize highly conserved structures expressed by micro-organisms, called pathogen-associated molecular patterns (PAMPs)[1]. TLR-mediated recognition of microbial products or endogenous "danger" signals results in production of cytokines and chemokines that initiate the innate immune response and instruct the adaptive immune system[2]. The balance between the production of pro- and anti-inflammatory cytokines is tightly regulated, presumably to allow efficient immune responses while protecting the host from excessive inflammation[3]. Birth imposes major challenges on the regulatory capacity of the immune system, including prevention of harmful allo-immune reactions to maternal antigens *in utero*, and balancing the transition from a sterile intra-uterine environment to the microbe-rich outside world[4]. To face these demands, neonatal TLR responses are physiologically biased against the production of pro-inflammatory cytokines[5-7]. However, this bias leaves the newborn highly susceptible to infections[8]. Recent studies indicate that despite impaired production of Th1-type cytokines, the neonatal TLR system is not generally depressed. In fact, neonatal innate immune cells, including monocytes and dendritic cells (DC), demonstrate increased TLR-mediated production of certain cytokines (i.e. IL-10, IL-23)[7, 9-13]. Together, this suggests the existence of regulatory mechanisms that actively polarize the neonatal TLR system towards decreased production of Th1-polarizing cytokines while Th2- and Th17-polarizing responses are relatively preserved. Insight into the mechanisms underlying this polarization is of great interest, as a biological phenomenon and to identify potential targets for the prevention and treatment of neonatal infections.
Despite many studies describing impaired TLR-mediated production of Th1-polarizing cytokines by cord blood innate immune cells[3, 6, 9, 12, 14-16], the underlying mechanisms are incompletely characterized [12, 13, 17, 18]. In addition, it is as yet unclear whether the mechanisms that suppress specific cytokine production in cord blood extend into the neonatal and infant age. We have previously demonstrated that whole blood TLR4-mediated cytokine responses at birth and at the age of one month are distinct from adult responses and characterized by decreased production of IL-12p70 and increased production of IL-10[16]. In the current study, we aimed to determine whether soluble factors in neonatal plasma contribute to distinct neonatal TLR4-mediated production of IL-12p70 and IL-10.

Materials and Methods

Blood
The research protocol was approved by the Medical Ethics Committee of the University Medical Centre Utrecht and written informed consent was obtained from parents of all participants. Blood was obtained from healthy newborns participating in an ongoing birth cohort study on the role of neonatal TLR responses in the pathogenesis of respiratory tract infections and asthma[16]. Cord blood was collected immediately after uncomplicated term vaginal delivery, but before delivery of the placenta. Neonatal venous blood was collected from healthy newborns at the age of one month (4-7 weeks, here referred to as 'neonate') and adult blood was obtained from healthy volunteers. None of the participants had signs or symptoms of infectious disease, such as respiratory tract complaints or fever, in the two weeks prior to sampling. Blood was collected in sterile, heparin-coated collection tubes (BD Biosciences, Franklin Lakes, NJ). Plasma was prepared within 24h from blood collection via centrifugation (1000*g*, 10 min, room temperature), aliquoted into cryovials (Nalge Nunc International, Rochester, NJ) and stored at -20°C prior to use.

Immune agonists
In vitro stimulation was performed using optimal concentrations of immune agonists and incubation times for cytokine measurements, as titrated in pilot experiments (data not shown). Accordingly, adult peripheral blood mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMCs) were stimulated with polyinosinic:polycytidylic acid (poly I:C; 200 μg/mL; Invivogen, San Diego, CA), ultrapure LPS from *E. Coli* (100 ng/mL; InvivoGen), or CL-075 (10 μg/mL, InvivoGen). For co-stimulation, recombinant IFN-γ was used (20 ng/mL; PeproTech Inc, Rocky Hill, NJ).

Cell isolation and stimulation
Adult PBMCs and CMBCs were obtained by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient separation. Cells were washed twice in sterile PBS and seeded in 96-well polystyrene culture plates (Nalge Nunc International, Rochester, NJ) at $1x10^6$/mL in RPMI medium containing 2.5 g/L D-glucose, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES and 300 mg/L L-glutamine (Invitrogen, Breda, NL). MCs were pre-incubated for 30 minutes in plasma prior to addition of TLR agonists. After 24 h incubation at 37°C and 5% CO_2, supernatants were collected for ELISA, or cells were collected for intracellular cytokine staining. Unless stated otherwise, experiments were performed in 10% plasma.

Cytokine measurement
Concentrations of IL-12p70 and IL-10 in culture supernatants were determined by ELISA according to manufacturer's instructions (eBioscience, San Diego, CA). Intracellular IL-12p40 production was detected on a 10-color flow cytometer (LSR II, BD Biosciences Franklin Lakes, NJ), using monoclonal antibodies directed against CD14, CD3, CD56, CD16, HLA-DR or, IL-12p40 (for specific fluorochromes, antibody clones and manufacturer, see supplementary table 1). Isotype controls were used to correct for non-specific staining.

Plasma heat-inactivation and protein depletion
To characterize the plasma factor responsible for impaired neonatal TLR responses, several approaches were used. Heat-inactivation was performed by heating plasma for 30 min at 56°C. To deplete plasma proteins, plasma was diluted 2-fold in serum-free RPMI medium and boiled for 10 min at 100°C. Afterwards, set precipitate was removed by centrifugation at 13000 *g* for 10 min. To ensure complete depletion/denaturation of plasma proteins, the supernatant was subjected to another cycle of boiling and centrifugation. Efficacy of protein depletion was verified by BCA assay (Pierce, Rockford, IL). As plasma contains several proteins that are needed for LPS-induced cytokine release, all experiments using heat-inactivated or protein-depleted or modified plasma were performed on a background of 5% fetal calf serum (FCS). Addition of FCS up to a concentration of 20% did not affect the differential modulation of LPS-induced cytokine release between cord blood and adult plasma (data not shown).

IL-10, PGE_2 and vitamin D experiments
To determine the role of IL-10 as a mediator of TLR4-mediated IL-12p70 production by cord blood plasma, we blocked IL-10 by incubating plasma for 30 min in anti-IL-10 antibody (1 μg/mL, EBioscience, clone JES3-9D7) or isotype control (eBioscience), prior to running the *in vitro* stimulation assay. The effect of PGE_2 and 1,25-dihydroxy vitamin D (1,25-OHD) on TLR4-mediated cytokine responses was assessed by determining IL-12p70 and IL-10 production in presence of 10% FCS supplemented with recombinant PGE_2 or 1,25-OHD (both obtained from Sigma Aldrich, Zwijndrecht, The Netherlands). Plasma concentrations of PGE_2 were measured by ultra sensitive enzyme immunoassay according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Plasma concentrations of 25-OHD (the stable form of vitamin D) were measured with the Modular E170 analyzer (Roche)[19].

Statistical analyses
Comparisons were made using the Student's t-test. When multiple groups were compared (e.g. age-dependent effects of plasma on TLR4-mediated cytokine responses, concentrations of PGE_2), one-way ANOVA with post-hoc Bonferroni correction was used. Correlations between TLR4-mediated intracellular IL-12p40 MFI and concentrations of IL-12p70 in culture supernatant and between IL-12p70 production and plasma PGE_2 concentration were assessed by Pearson correlation. All *p*-values are two-sided and were considered significant if $p<0.05$.

Results

Differences in TLR4-mediated cytokine production between neonates and adults are due to cellular and soluble factors

We have previously reported that whole blood TLR4-mediated cytokine responses at birth (i.e. in cord blood) and in neonatal venous blood obtained at the age of one month are distinct from those in adult blood, and characterized by decreased production of IL-12p70 and increased production of IL-10[16]. To determine whether the differences in TLR4-mediated production of IL-12p70 and IL-10 are due to differences in the cellular or soluble fraction of the blood, we isolated CBMCs and adult PBMCs, resuspended them to equivalent concentrations ($1x10^6$) and stimulated them with LPS in the presence of cord blood or adult plasma (Fig 1). CMBC failed to produce any IL-12p70, independent of the source of plasma (Fig 1A). Remarkably, adult PBMCs readily produced IL-12p70, but only when stimulated in the presence of adult plasma. This demonstrates that the differences in whole blood TLR4-mediated IL-12p70 production are due to differences in both the cellular and the soluble fraction of the blood.

To further explore modulation of TLR4-mediated cytokine production by soluble factors in plasma, we stimulated PBMCs from three different adult donors in the presence of adult or cord blood plasma (Fig 1B). Although there were differences in cytokine production and plasma sensitivity between PBMC donors, cord blood plasma conferred decreased TLR4-mediated IL-12p70 and increased IL-10 for all plasmas and in all PBMC donors tested.

Potential explanations for the differential modulation of TLR4-mediated cytokine production by cord blood plasma and adult plasma include the lack of factor(s) in cord blood plasma that stimulate LPS+IFN-γ-induced IL-12p70 production (and inhibit IL-10), or the presence of factor(s) that inhibit IL-12p70 (and induce IL-10). To distinguish these possibilities, we added increasing concentrations of cord blood or adult plasma to PBMC cultured in 5% FCS. Addition of either cord blood or adult plasma dose-dependently inhibited TLR4-mediated IL-12p70, while increasing TLR4-mediated IL-10 (Fig 1C). Inhibition of TLR4-mediated IL-12p70 and induction of IL-10 were more pronounced in the presence of cord blood plasma, with statistically significant stronger polarization of TLR4-mediated cytokine responses compared to adult plasma across plasma concentrations of 1-50% (v/v). Differential modulation of TLR4-mediated IL-12p70 and IL-10 production by cord blood plasma and adult plasma was maintained in the presence of up to 20% FCS (data not shown). These results indicate that human plasma contains one or multiple factors that polarize TLR4-mediated cytokine production towards low IL-12p70 and high IL-10 and that these factors are present at increased concentrations in cord blood plasma.

Figure 1: Human plasma suppresses TLR4-mediated production of IL-12p70 while inducing IL-10. A; TLR4-mediated release of IL-12p70 and IL-10 by cord blood mononuclear cells (CBMC) or adult peripheral blood mononuclear cells (PBMC) stimulated in the presence of 10% cord blood plasma or adult plasma. Bars represent mean+SEM of five independent cord blood or adult plasma donors. B; TLR4-mediated release of IL-12p70 and IL-10 by PBMC from three different adult donors stimulated in the presence of 10% cord blood plasma or adult plasma. C; Dose-response curves showing TLR4-mediated release of IL-12p70 and IL-10 by adult PBMC in the presence of increasing concentrations of cord blood plasma or adult plasma. Each dot represents mean ± SEM of five independent cord blood or adult plasma donors. Data are representative of three independent experiments. *; $p<0.05$, **; $p<0.01$.

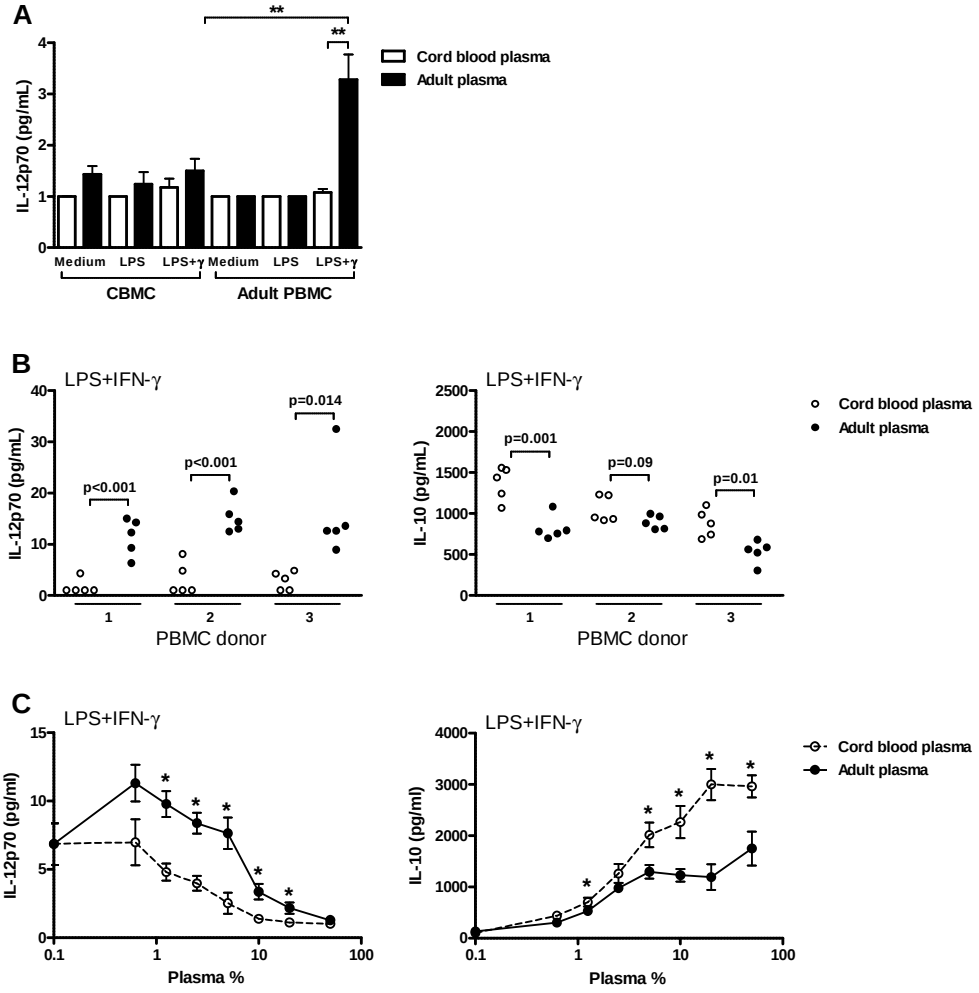
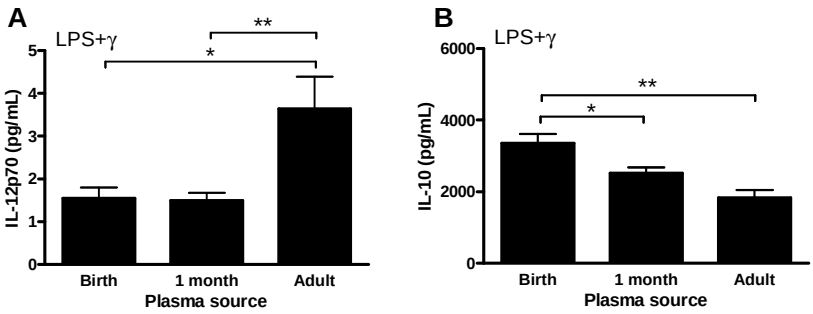


Figure 2: Suppression of TLR4-mediated IL-12p70, but not induction of IL-10, by neonatal plasma is maintained up until the age of one month.
Adult PBMC were stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) in the presence of 10% plasma derived from cord blood, healthy newborns aged one month, or adult volunteers. After 24h incubation, supernatants were collected and concentrations of IL-12p70 (A) and IL-10 (B) were determined by ELISA. Bars represent mean+SEM of five different plasma samples. *; $p<0.05$, **; $p<0.01$.



Suppression of TLR4-mediated IL-12p70, but not induction of IL-10, by neonatal plasma persists up to the age of one month

We have previously shown that neonatal whole blood TLR4-mediated cytokine responses are biased towards low IL-12p70 and high IL-10 throughout the first month of life[16]. To determine whether modulation of TLR4-mediated responses by plasma contributes to distinct neonatal cytokine production at the age of one month, we determined TLR4-mediated production of IL-12p70 and IL-10 by adult PBMC stimulated in the presence of 10% plasma derived from cord blood, from healthy neonates aged one month or from adult volunteers. Results showed similar patterns to those previously observed in whole blood (Fig 2)[16]. TLR4-mediated production of IL-12p70 was significantly decreased in the presence of both cord blood plasma and neonatal plasma obtained at the age of one month compared to adult plasma (Fig 2A). In contrast, TLR4-mediated IL-10 production in presence of plasma of one month old neonates was significantly reduced compared to cord blood plasma and not statistically different from IL-10 production in presence of adult plasma (Fig 2B). This suggests that TLR4-mediated IL-12p70 and IL-10 are regulated through distinct plasma factors that are differentially present throughout the first month of life.

Cord blood plasma suppresses TLR4-mediated IL-12p40 production by primary monocytes

Next, we aimed to identify the cell type(s) responsive for the suppressive effect of neonatal plasma on TLR4-mediated IL-12 production (Fig. 3). Among PBMCs, monocytes and mDC were the main producers of TLR4-mediated IL-12p40, accounting for (mean ± SEM 62.2 ± 1.9 and 22.0 ± 1.8% of IL-12p40 positive cells, respectively (Fig 3A). Cord blood plasma conferred decreased production of TLR4-mediated IL-12p40 (assessed by mean fluorescent intensity, MFI) by monocytes, but not by mDC (Fig 3B-C). Although we were unable to detect any IL-12p35, there was a strong correlation ($R=0.75$, $p<0.01$) between the effects of individual plasma samples on monocyte IL-12p40 MFI and PBMC-mediated production of IL-12p70 (Fig 3D), suggesting that

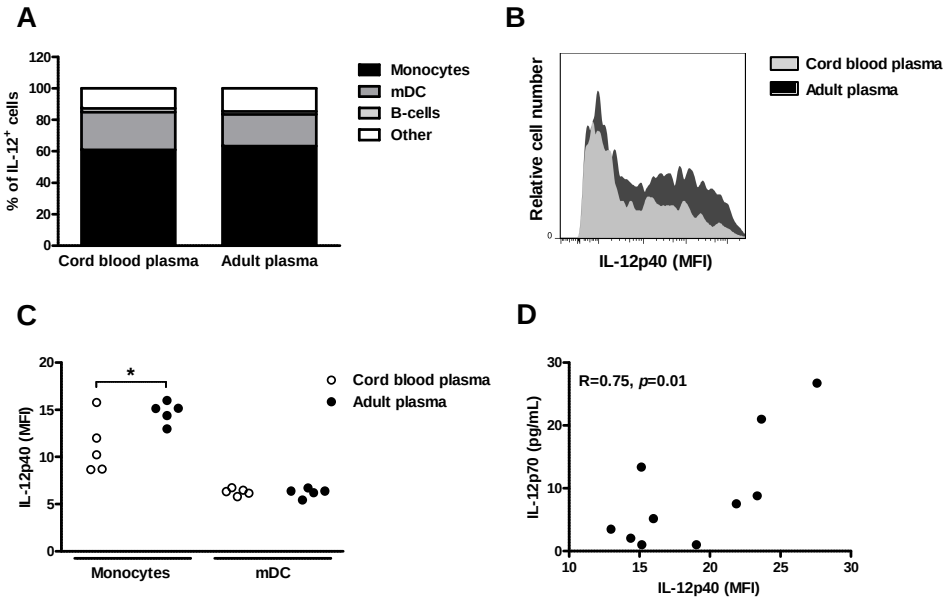
monocyte intracellular IL-12p40 is a reliable marker for extracellular IL-12p70 release. These findings confirm that the majority of TLR4-mediated IL-12p70 is produced by monocytes. Furthermore, they indicate that cord blood plasma suppresses TLR4-mediated IL-12p70 via suppression of monocyte IL-12p40 production.

Cord blood plasma modulates TLR4-mediated cytokine production independent of MyD88

Among TLRs, TLR4 is unique in its capacity to signal through both MyD88-dependent and MyD88-independent pathways[2]. To determine whether one of these pathways is differentially affected by plasma, we determined the effects of cord blood plasma and adult plasma on responses to TLR3, which signals through MyD88-independent/TRIF dependent pathways, and TLR8, which signals MyD88-dependently (Fig 4).

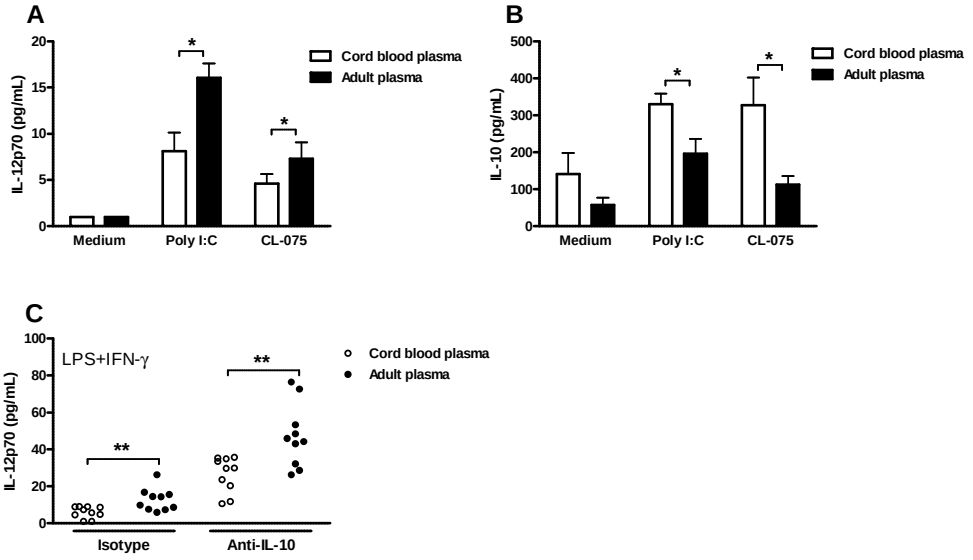
Figure 3: Cord blood plasma suppresses TLR4-mediated IL-12p40 production by primary monocytes.

Adult PBMC were stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) in the presence of 10% cord blood plasma or adult plasma. After 24 hours, intracellular levels of IL-12p40 in monocytes (CD14+/HLA-DR+), myeloid dendritic cells (mDC, CD3-/CD16-/CD56-/CD14-/HLA-DR+/CD11c+) and B-cells (CD3-/CD16-/CD56-/CD19+) were determined by flow cytometry. A; Proportion of monocytes, mDC and B-cells among the total population of IL-12p40^{+} cells. Bars represent means of five different plasma samples. B; Representative histograms of monocyte IL-12p40 production upon TLR4-stimulation in the presence of cord blood plasma or adult plasma. C; Mean fluorescence intensity (MFI) of IL-12p40 in monocytes and mDC upon TLR4-stimulation in the presence of cord blood plasma or adult plasma. Each dot represents one individual plasma sample. D; Correlation between TLR4-mediated monocyte IL-12p40 MFI and IL-12p70 protein production by PBMC. Each dot represents one individual plasma sample. All data are representative of three independent experiments. *; $p<0.05$.



Cord blood plasma significantly suppressed IL-12p70 production to both agonists (Fig 4A), while increasing the production of IL-10 (Fig 4B). This indicates that cord blood plasma polarizes TLR-mediated cytokine production through mechanisms independent of MyD88. To exclude the possibility that suppression of IL-12p70 by cord blood plasma was secondary to increased basal concentrations of IL-10 or to its increased release, we stimulated PBMC in presence of IL-10 neutralizing antibody. Blocking IL-10 increased TLR4-mediated IL-12p70 production (Fig 4C), but failed to neutralize the difference between cord blood and adult plasma, indicating that suppression of IL-12p70 by cord blood plasma is not mediated via IL-10.

Figure 4: Cord blood plasma polarizes cytokine responses to MyD88-dependent and independent TLR agonists.
A-B; Agonist-induced production of IL-12p70 (A) and IL-10 (B) by adult PBMC stimulated in the presence of 10% cord blood plasma or adult plasma. PBMC were stimulated for 24h with medium, poly I:C (TLR3, 200 μg/mL) or CL-075 (TLR8, 10 μg/mL). After 24h, supernatants were collected and cytokine concentrations were determined by ELISA. Bars represent mean+ SEM from five different plasma samples. C; TLR4-mediated production of IL-12p70 by adult PBMC stimulated in the presence of 10% cord blood plasma or adult plasma, with or without IL-10 neutralizing antibody (1 μg/mL). Each dot represents one individual plasma sample. Data are representative of three independent experiments. *; $p<0.05$, **; $p<0.01$.



Plasma regulates TLR4-mediated production of IL-12p70 and IL-10 through distinct factors

We next aimed to characterize the factor(s) responsible for the polarization of TLR4-mediated cytokine production by cord blood plasma. When assessing the stability of this/these factor(s), we found that heat-inactivation differentially affected TLR4-mediated production of IL-12p70 and IL-10 (Fig 5). While heat-inactivation did not affect plasma-mediated suppression of IL-12p70 production (Fig 5A), the differential effect of cord blood plasma and adult plasma on TLR4-mediated IL-10 production disappeared (Fig 5B). To investigate whether the factor suppressing TLR4-mediated IL-12p70 production is a protein, we depleted plasma proteins by repetitive boiling. This approach resulted in a robust reduction of plasma protein content (mean ± SEM; 59.2 ± 3.7 mg/mL vs 7.8 ± 0.3 mg/mL, $p<0.0001$) (Fig 5C) and eliminated the induction of TLR4-mediated IL-10 by cord blood plasma (Fig 5E). However, protein-depletion of plasma did not abrogate the differential effect of cord blood plasma and adult plasma on TLR4-mediated IL-12p70 production (Fig 5D). This confirms regulation of TLR4-mediated cytokine production by distinct heat labile (IL-12p70) and heat-stable (IL-10) factors in plasma. Furthermore, failure of protein-depletion to neutralize the differential effect of cord blood plasma and adult plasma on TLR4-mediated IL-12p70 production (Fig 5D) indicates that the IL-12p70 suppressive factor(s) in cord blood plasma is not a protein.

The cord blood plasma factor suppressing TLR4-mediated IL-12p70 is not PGE_2 or vitamin D

Vitamin D and PGE_2 are lipid molecules that are known to have IL-12p70 suppressive potential[20, 21]. Indeed, 1,25-OHD (the active form of vitamin D) dose-dependently suppressed TLR4-mediated IL-12p70 production by PBMC. At high concentrations, 1,25-OHD also suppressed IL-10 production compared to vehicle control. However, concentrations of 25-OHD were similar in cord blood plasma and adult plasma (Fig 6F), suggesting that suppression of TLR4-mediated IL-12p70 by cord blood plasma is not mediated by vitamin D. Similarly, PGE_2 conferred dose-dependent inhibition of TLR4-mediated IL-12p70 production and at high concentrations, stimulated TLR4-mediated IL-10 production (Fig 6D-E). Inhibition of IL-12p70 was observed for concentrations physiologically present in plasma[22], suggesting that plasma PGE_2 might contribute to suppression of TLR4-mediated IL-12p70 production.

However, concentrations of PGE_2 were elevated in cord blood plasma, but not in plasma from neonates at the age of one month compared to adult plasma (mean ± SEM; 2921 ± 534 pg/mL; 369 ± 105 pg/mL and 469 ± 78 pg/mL, respectively, $p<0.001$) (Fig 6F). In addition, there was no correlation between PGE_2 concentrations in individual adult plasma samples and suppression of TLR4-mediated IL-12p70 production ($R=-0.02$, $p=0.84$) (Fig 6G). Because no TLR4-mediated IL-12p70 was produced in presence of cord blood plasma or plasma from neonates aged one month, no correlations with plasma PGE_2 concentrations could be calculated for these age groups. Together, these data suggest that PGE_2 is not the major cord blood plasma factor causing exaggerated suppression of TLR4-mediated IL-12p70 production over adult plasma.

Fig 5: Cord blood plasma modulates TLR4-mediated production of IL-12p70 and IL-10 via distinct factors.
A-B; TLR4-mediated production of IL-12p70 (A) and IL-10 (B) by adult PBMC stimulated in the presence of heat-inactivated plasma (30 min at 56°C) or control plasma. C-E; Effect of protein-depleted plasma on TLR4-mediated cytokine responses. Plasma proteins were depleted by two consecutive cycles of boiling and centrifugation and protein concentrations were measured by BCA protein assay (C). Adult PBMC were stimulated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) in the presence of protein-depleted plasma or control plasma, and production of IL-12p70 (D) or IL-10 (E) were measured by ELISA. Each dot represents one individual plasma sample. All data are representative of at least three individual experiments. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

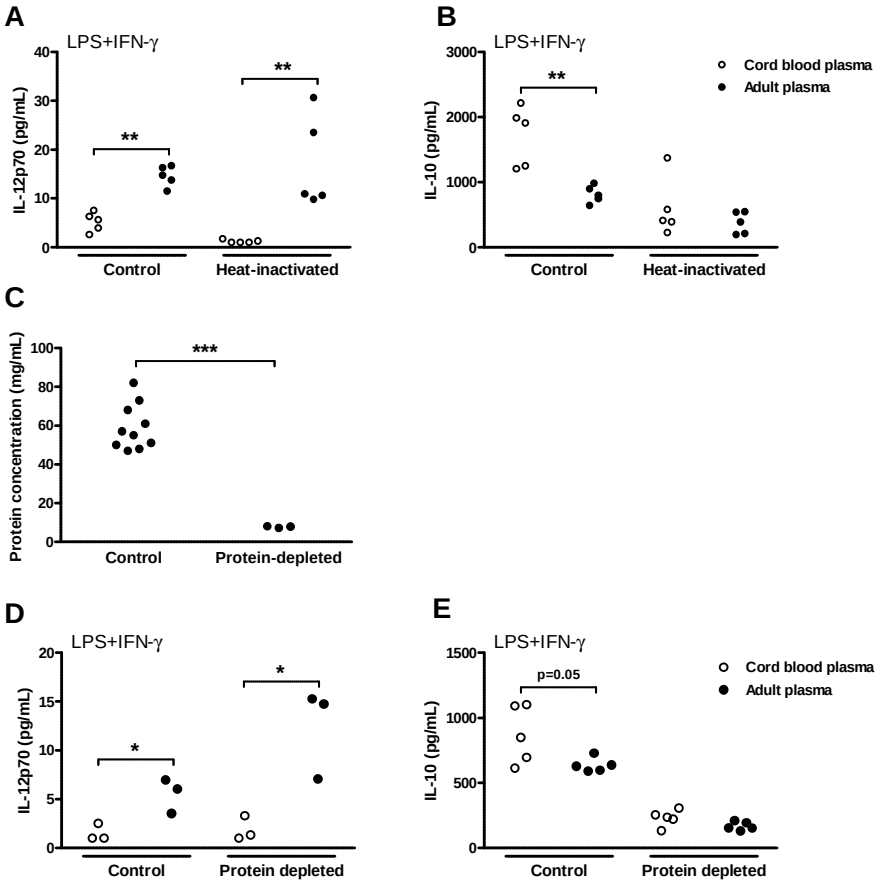
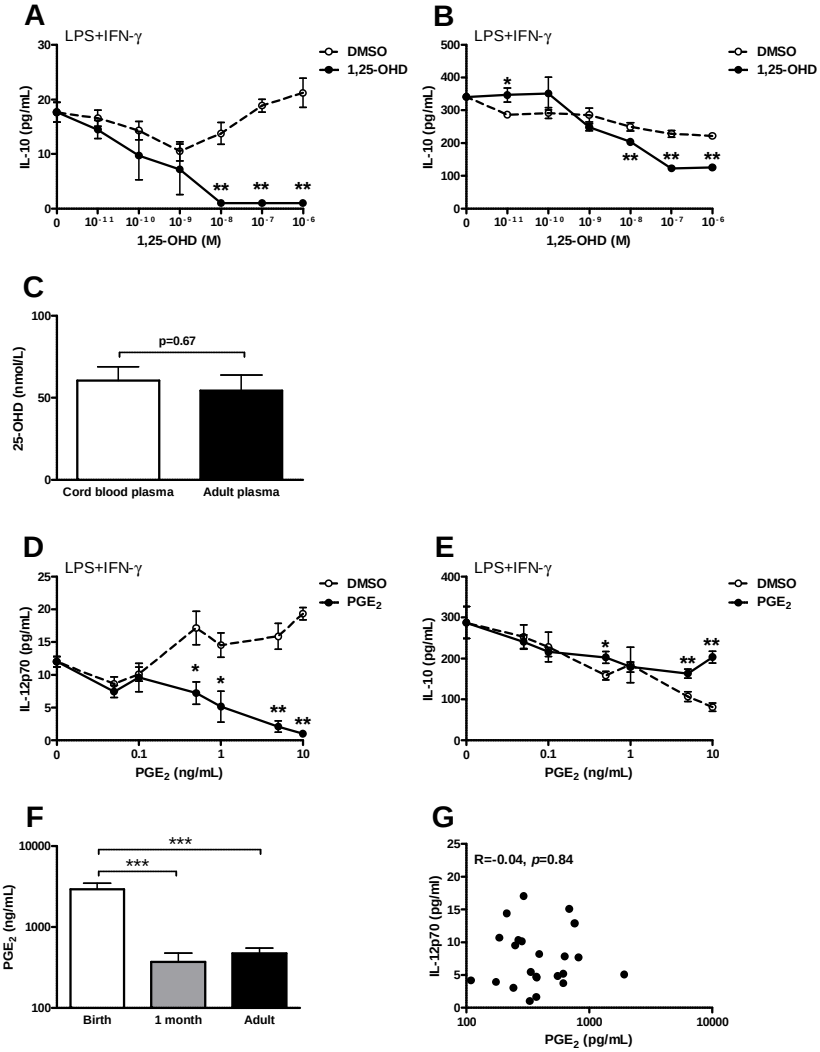


Fig 6: Effect of prostaglandin E_2 and vitamin D on TLR4-mediated IL-12p70 production.
A-B, TLR4-mediated production of IL-12p70 (A) and IL-10 (B) by adult PBMC stimulated in the presence of increasing concentrations of 1,25-dihydroxyvitamin D (1,25-OHD). C, Concentrations of 25-hydroxyvitamin D (25-OHD) in cord blood plasma and adult plasma, determined by EIA. D-E, TLR4-mediated production of IL-12p70 (D) and IL-10 (E) by adult PBMC stimulated in the presence of increasing concentrations of prostaglandin E_2 (PGE_2). F, Concentrations of PGE_2 in cord blood plasma and adult plasma. G, Correlation between plasma concentration of PGE_2 and its capacity to suppress TLR4-mediated IL-12p70 production. Data are presented as the mean ± SEM of five to eight individual plasma donors (A-F) and are representative of three independent experiments. In panel G, each dot represents one individual plasma donor. *; $p<0.05$, **; $p<0.01$; ***; $p<0.001$.



Discussion

Despite increasing awareness of the importance of the TLR system in the neonatal defence against infections[4], much remains to be learned about the mechanisms regulating neonatal TLR responses. Here, we demonstrate that cord blood plasma polarizes TLR-mediated cytokine responses towards low production of IL-12p70 and high production of IL-10. We show that this polarizing effect is present throughout the first month of life, thereby potentially contributing to high neonatal susceptibility to infection. Although to a lesser extent, adult plasma also inhibited TLR4-mediated IL-12p70 production and induced IL-10 production, suggesting that human plasma is a physiologic regulator of TLR responses throughout life.

The strength of our model, namely the use of a single PBMC donor to study the effects of multiple plasmas, allowed us to analyze the effect of plasma on TLR responses while eliminating any contribution of cellular differences between neonates and adults. Of note, adult plasma enhanced LPS-induced IL-12p70 production only in adult PBMC (Fig 1A), indicating that in addition to the pivotal soluble plasma factors, cellular factors also contribute to the difference in TLR-mediated IL-12 production between neonates and adults.

Previous studies have identified multiple cellular mechanisms that account for different TLR responses between neonates and adults. An elegant study by Goriely et al. demonstrate that decreased TLR4-mediated IL-12p70 production by cord blood dendritic cells is due to impaired nucleosome remodelling, resulting in decreased accessibility of the IL-12p35 promoter[17, 23]. In addition, despite similar basal levels of CD14[12], neonatal monocytes might express lower levels of MyD88[24, 25], and demonstrate lower TLR4-mediated p38 phosphorylation[12].

In addition to cellular defects, the current study indicates that differences in the soluble fraction of the blood play a pivotal role in polarization of the neonatal TLR system against T1-polarizing responses. The relatively easy accessibility of plasma and the existence of multiple plasma-replacement strategies (e.g. dialysis, filtration) clinically approved for the treatment of neonatal kidney failure, renders plasma a highly interesting target for immune modulatory strategies aimed at treating neonatal infection.

Our results demonstrate that polarization of TLR4-mediated cytokine responses by cord blood plasma is not due to a single factor, but mediated by multiple distinct factors that independently suppress production of IL-12p70 or induce production of IL-10 (Fig 5). Its resistance to heat-inactivation and protein depletion suggests that the IL-12p70 suppressive factor in cord blood plasma is not a protein.

First, vitamin D is an essential nutrient with multiple immune modulatory functions, including inhibition of IL-12p70 production by dendritic cells[21] and PBMC (Fig 6). However, concentrations of 25-OHD, the stable form of vitamin D, were similar in cord blood plasma and adult plasma. Thus, although vitamin D may contribute to suppression of TLR4-mediated IL-12p70 by cord blood plasma and adult plasma, it is unlikely to account for the enhanced suppression of TLR4-mediated IL-12p70 by cord blood. Of note, we did not exclude the possibility that increased neonatal conversion of 25-OHD to

1,25-OHD might result in higher plasma concentrations of bioactive vitamin D and increased suppression of TLR4-mediated IL-12p70 production[26].
Second, PGE_2 is an important lipid mediator that can suppress IL-12p70 production by LPS-stimulated dendritic cells[20]. In figure 6, we confirm that PGE_2 is a potent suppressor of TLR4-mediated IL-12p70 which is present in increased concentrations in cord blood plasma. However, the observation that PGE_2 concentrations in plasma from neonates aged one month were similar to adult levels, and the lack of correlation between concentrations of PGE_2 in individual plasma samples and the extent to which they suppresses TLR4-mediated IL-12p70 production, suggests that PGE_2 is not the major plasma factor that suppresses neonatal TLR4-mediated IL-12p70 production.
In addition to vitamin D and PGE_2, plasma contains many other non-protein factors capable of suppressing TLR4-mediated IL-12 production, including metabolites [27, 28], polyunsaturated fatty acids[29] and carbohydrate structures[30]. Selective depletion and separation strategies are needed to identify factors that suppress neonatal TLR4-mediated IL-12p70 production.
In contrast to suppression of TLR4-mediated IL-12p70 production, the factor in cord blood plasma that induces IL-10 is heat-labile and lost upon protein depletion (Fig 5). A hallmark example of heat-sensitive immune modulatory factors in plasma is the complement family. Although multiple studies have shown that complement factors can suppress TLR-mediated production of pro-inflammatory cytokines[31, 32], their effect on IL-10 is still subject of debate. Whereas C3b and C5a have increase IL-10 production in response to allergen[33] or TLR stimulation[34] respectively, C1q suppresses IL-10 production by human DC[35]. Moreover, neonatal plasma concentrations of complement are diminished, ranging from ~10-70% of adult levels[36, 37]. Thus, complement is unlikely to account for the enhanced induction of TLR4-mediated IL-10 by cord blood plasma. Other plasma factors capable of enhancing TLR4-mediated IL-10 production include low-density lipoproteins[38], retinoic acid[39], and adenosine[40]. The relative contribution of these and other factors to induction of TLR4-mediated IL-10 by whole plasma will require further investigations.

Our results raise an important aspect of experimental designs for study of TLR responses in that the extracellular medium may significantly affect results. Studies investigating neonatal TLR responses have used different soluble fractions, [7, 11, 13, 25, 41] ranging from 5% FCS[41] to 100% autologous plasma[13]. In this respect, a recent study by Kollmann et al. is of specific interest. These authors compared neonatal and adult TLR responses by isolated monocytes or dendritic cells (DC), stimulated in whole blood or 10% human AB serum[10]. In this study, intracellular TNF-a and IL-12p40 in neonatal monocytes and myeloid DC were significantly decreased compared to adults when cells were stimulated in whole blood, but not in 10% human serum, suggesting the differential presence of a soluble modulator in neonatal or adult plasma. Interestingly, this context-dependent effect was not observed for pDC, which responded similarly as single-cell suspension or in whole blood. A second methodological issue relates to the in vitro preparation of plasma. Plasma is a very complex fluid, full of potentially bioactive molecules. Differences in anticoagulant, centrifugation speed, temperature, and additional steps such as filtration or freeze-thawing, may affect the presence of these molecules, thereby significantly

affecting assay results. Overall, the current study urges cautious interpretation of studies investigating TLR-mediated responses using human plasma components.

In summary, we demonstrate that human newborn plasma contained increased concentrations of soluble factors that independently suppress IL-12p70 or increase IL-10 production to various TLR agonists, thereby potentially contributing to compromised neonatal innate immune responses. In addition, the concentration-dependent polarization of TLR responses by adult plasma suggests that plasma might contain multiple physiologic regulators of TLR responses throughout life. Further identification of these plasma factors and the mechanism by which they mediate their effect might provide novel therapeutic targets to promote immune responses in case of infection, to enhance responses to vaccination, and to limit excessive inflammation in patients with auto-immune disease.

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Supplementary material

Supplemental table 1: Antibodies used for flow cytometric detection of intracellular IL-12p40

Epitope	Fluorochrome	Manufacturer	Clone
CD3	PE	Biolegend	UCHT1
CD11c	PE-Cy7	eBioscience	3.9
CD14	PerCP-Cy5.5	Biolegend	HCD14
CD16	PE	Sanquin Reagents	CLB-FcR gran/1, 5D2
CD19	Pacific blue	Biolegend	HIB19
CD56	PE	Sanquin	NKI-nbl-1 B159
HLA-DR	FITC	eBioscience	LN3
IL-12p40	APC	BD Biosciences	C11.5

Chapter 7

Low neonatal Toll-like receptor 4-mediated interleukin-10 production is associated with subsequent atopic dermatitis

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Abstract

Background
Atopic dermatitis (AD) and respiratory syncytial virus lower respiratory tract infection (RSV LRTI) are common diseases during early life. Impaired Th1-cell polarizing Toll-like receptor (TLR) responses play an important role in the pathogenesis of both diseases. Neonatal TLR-mediated production of Th1-type cytokines is decreased at birth but rapidly increases during the first month of life.

Objective
To determine whether decreased TLR-mediated production of Th1-polarizing cytokines at the age of one month predisposes to subsequent AD or RSV LRTI.

Methods
A prospective healthy birth cohort study was performed. Whole blood concentrations of innate immune cells and TLR-mediated cytokine responses were measured at the age of one month in 291 neonates. AD was determined by physician questionnaire at the age of one year and RSV LRTI was defined as parent-reported respiratory symptoms and presence of RSV RNA in a nose-throat specimen.

Results
Of participating neonates, 45 (15%) developed AD and 41 (14%) developed RSV LRTI. Risks of AD and RSV LRTI were not associated (X^2, p=1.00). AD was associated with decreased concentrations of basophils (7.6 versus $14.0x10^6$/mL, p=0.002) and plasmacytoid dendritic cells (17.0 versus $20.5x10^6$/mL, p=0.04), increased concentrations of NK-cells (79.7 versus $45.1x10^6$/mL, p=0.03), and 2-fold lower TLR4-mediated IL-10 production (p=0.001). In contrast, RSV LRTI was neither associated with neonatal concentrations of innate immune cells, nor with TLR-mediated TNF-α, IL-12p70, IL-10 or IFN-α production.

Conclusion
AD, but not RSV LRTI, is associated with distinct presymptomatic differences in the innate immune system. We hypothesize that decreased neonatal IL-10-mediated immune regulation during early life might play a causal role in the initiation of AD.

Introduction

Toll-like receptors (TLR) are key pathogen recognition receptors of the innate immune system that initiate the early antimicrobial response and orchestrate the ensuing adaptive response[1]. We and others have shown that neonatal TLR responses are characterized by decreased production of Th1-polarizing cytokines (IL-12p70, TNF-α, IFN-α) and increased production of the regulatory cytokine IL-10 compared to adult responses[2-6]. Polarized neonatal cytokine responses are thought to prevent potentially harmful allo-immune responses towards maternal antigens in utero, and to facilitate microbial colonization after birth[3]. However, impaired generation of Th1-polarizing responses leaves the neonate highly susceptible to infection[7]. In addition, bias towards Th2-type immune responses at the neonatal and infant age has been associated with increased risk of allergic disease in later life[8-10].

Atopic dermatitis (AD) is a chronic inflammatory skin disorder, with a lifetime prevalence of 20% in westernized countries[11]. AD occurs in the first 6 months of life in 45% of children and before the age of one in 60%[11]. Hyperreactive Th2-type immune responses play a central role in the pathogenesis of AD[12, 13]. Acute eczematous skin lesions are infiltrated with Th2-cells expressing high levels of IL-4, IL-5 and IL-13[14]. In addition, patients with AD have elevated IL-13-expressing T-cells in the peripheral blood[15], increased eosinophils and high serum IgE[14]. This Th2-bias might be dictated by the innate immune system, as both genetic and functional defects in TLRs and their downstream signaling molecules are associated with AD[16-18].

Respiratory syncytial virus (RSV) is the most common cause of childhood acute lower respiratory tract infection worldwide, affecting 90% of children before the age of two years[19, 20]. RSV disease severity varies from mild upper respiratory tract infection to lower respiratory tract infection (LRTI) necessitating hospitalization. Decreased TLR-mediated induction of pro-inflammatory responses might contribute to severe RSV disease. In vitro, RSV activates the immune system through TLR2, TLR4, and potentially TLR3 and TLR7[21-24]. TLR4-deficient mice demonstrate decreased RSV-induced IL-12 production by bronchoalveolar lavage cells and delayed viral clearance[25]. In humans, genetic studies have demonstrated clear associations between severity of RSV disease and polymorphisms in innate immune genes, including TLR4 and its co-receptor CD14[23, 26, 27].

Several studies suggest that specific patterns of TLR-mediated cytokine responses might impact on disease severity[28-30]. Whereas most respiratory viruses induce robust Th1-type immune responses, immune responses in infants with acute RSV infection are characterized by low levels of Th1-type cytokines IFN-γ and IL-12p70[28, 29]. In addition, compared to upper respiratory tract infection, infants with RSV bronchiolitis have a higher IL-10/IL-12 mRNA ratio in nasal lavage fluid and elevated IL-4/IFN-γ mRNA ratios in nasal lavage fluid and in stimulated PBMC[30]. Of note, decreased serum concentrations of IFN-γ were only observed in RSV-infected infants, whereas high serum IL-4 levels were also observed in infants infected with parainfluenzavirus and adenovirus[29], suggesting that insufficient production of pro-inflammatory or Th1-type cytokines might contribute to severe RSV infection[31, 32].

We have previously identified the first month of life as a critical period for the development of the TLR system. During this period, TLR-mediated cytokine responses rapidly mature from a Th2-biased profile towards increased Th1-type responses characteristic of later life[33]. Postnatal maturation of TLR responses is likely guided by exposure to innate immune stimuli[34], which might thus modulate the risk of subsequent disease. In the current study, we hypothesized that neonates with delayed maturation of TLR responses during the first month of life, resulting in persistent impairments in Th1-immunity, may have increased susceptibility to severe RSV infection and atopic dermatitis in infancy and childhood. We therefore studied the impact of different TLR responses at the age of one month on the subsequent risk of AD and RSV LRTI in the first year of life in a healthy birth cohort.

Methods

Study design and population

A prospective observational birth cohort study was performed in two urban hospitals in Utrecht, The Netherlands, from March 2006 to February 2010. Study design and inclusion criteria have been published previously[35]. Eligible were healthy neonates born after uncomplicated pregnancy of ≥37 weeks through either vaginal delivery or elective caesarean section. Clinical characteristics were collected from hospital charts and standardized parental questionnaires[36]. At the age of one month, neonatal venous blood was obtained in sterile sodium heparin tubes. None of the participants had any signs or symptoms of infectious disease (e.g. runny nose, fever) in the two weeks prior to sampling. The study was approved by the ethical review boards of the University Medical Center Utrecht and the Diakonessen Hospital Utrecht. All parents provided written informed consent for study participation.

Innate immune cellularity

Whole blood concentrations of innate immune cells were determined as previously described[33]. Concentrations of neutrophils, eosinophils and basophils were obtained from differential leukocyte count. Concentrations of monocytes, natural killer (NK)-cells, myeloid dendritic cell (mDC) and plasmacytoid dendritic cells (pDC) were determined by flow cytometry. Monocytes were identified as HLA-DR$^+$/CD14$^+$, NK-cells as CD3$^-$/CD16$^+$/CD56$^+$, mDC as HLA-DR$^+$/lineage$^-$/CD11c$^+$ and pDC as HLA-DR$^+$/lineage$^-$/CD123$^+$ (supplementary table 1)[6]). To obtain absolute concentrations, the percentage of cells in the lymphocyte/monocyte gate (as determined by forward-sideward scatter properties) was multiplied with the total numbers of lymphocytes and monocytes from the differential leukocyte count.

TLR stimulation

In vitro TLR stimulation was performed in 1:14 diluted whole blood, using optimal concentrations of TLR agonists and incubation times for cytokine measurements, as titrated in pilot experiments and described previously (supplementary table 2)[6]. After a 4h (TNF-α) or 24h (IFN-α, IL-12p70, IL-10) incubation at 37°C and 5% CO_2, samples were centrifuged at 1000×g for 5 min. Supernatants were collected and stored at −80°C until further analysis.

Cytokine protein detection

Cytokine concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions: TNF-α, IL-10 (both from Sanquin/CLB, Amsterdam, The Netherlands), IL-12p70 (Diaclone Research, Besançon, France) and IFN-α (Bender Medsystems, Burlingame, CA, USA). The lower limits of detection were 1.0 pg/mL (TNF-α and IL-10) and 2.0 pg/mL (IL-12p70 and IFN-α). For samples in which the cytokine concentration was below the detection limit, the concentration was arbitrarily defined as half of the detection limit.

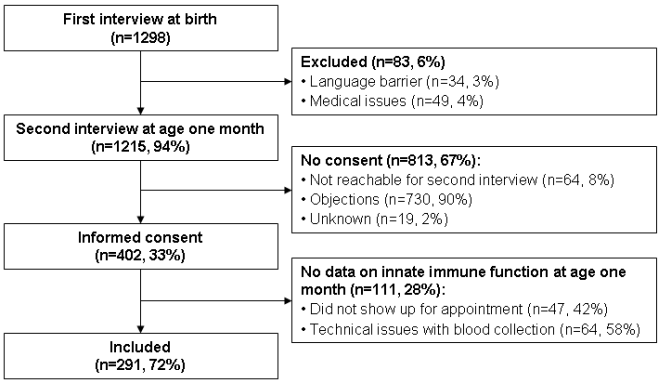
Clinical outcomes

The primary outcome RSV LRTI was defined as (1) parent-reported LRTI symptoms, and (2) simultaneous presence of RSV RNA in a nose-throat specimen. Throughout the first year of life, parents completed prospective logs to record presence and severity of respiratory symptoms. LRTI was defined by two independent researchers using strict criteria: moderate or severe cough or wheeze of any severity lasting for at least two days. At every respiratory episode, parents obtained a nose-throat swab[37]. Samples were sent to the researchers in viral transport medium and upon receipt frozen at -80^{o}C. The presence of RSV RNA was determined by real-time polymerase chain reaction (PCR) as described previously[38]. Secondary outcomes were 'physician-attended RSV infection' and 'atopic dermatitis'. Physician-attended RSV infection was defined as an episode of respiratory tract symptoms for which the general practitioner or pediatrician was visited, and simultaneous presence of RSV RNA in a nose-throat swab. At the age of one year, general physicians of all participants were contacted to answer a standardized, four-item questionnaire. Atopic dermatitis assessed by the question "Was this participant diagnosed with one or multiple episodes of "atopic eczema" (International classification of primary care code S87) in his/her first year of life?" For sensitivity analysis, parent-reported atopic dermatitis was derived from parental questionnaire at the age of one year, defined as a positive answer to the question "Did your child experience any eczema or pruritic skin rash in the first year of life?".

Statistical analysis

Means of logarhythmic transformed variables were compared between groups using Student's *t*-test, or one-way ANOVA with post-hoc analysis (Bonferroni). Differences between dichotomic variables were determined using the X^2-test. Logistic regression analysis was used to determine the effect of innate immune determinants on outcome variables, while adjusting for possible confounding factors. Based on results from univariate analysis, 'birth weight', 'birth season' and 'day care' were considered potential confounders for the association between innate immune variables and RSV LRTI. Similarly, 'birth season', 'day care' and 'parental atopy' were considered potential confounders for the association between innate immune variables and AD.

Figure 1: Flow chart of study population.



Results

During the study period, 1298 neonates were eligible for study participation (figure 1). Of these, 402 gave informed consent. Due to missed appointments (n=47) or technical reasons (e.g. failure in blood collection, blood clotting, n=64), innate immune parameters at the age of one month were not available for 111 (28%) of participants, resulting in a final cohort of 291 neonates. Baseline characteristics did not differ between participating neonates and non-participants (supplementary table 3). At the age of one year, 45 participants (15%) had AD (table 1). Baseline characteristics were similar between neonates with and without AD. In addition, 41 (14%) participants had experienced an episode of RSV LRTI (table 1), and twenty-two participants (7%) had physician-attended RSV infection. Neonates who developed RSV LRTI had a higher birth weight and gestational age compared to those who did not, as observed previously[35]. There was no relation between risk of AD and RSV LRTI (X^2, p=1.00).

AD

Distinct neonatal concentrations of innate immune cells are associated with subsequent AD

Whole blood concentrations of innate immune cells at the age of one month were compared between neonates with and without subsequent AD (figure 2). Neonates with subsequent AD had decreased concentrations of basophils (7.6 vs $14.0 \cdot 10^6$/L, p=0.002) and pDCs (17.0 vs $20.5 \cdot 10^6$/L, p=0.04), and increased concentrations of NK-cells (79.7 vs 45.1×10^6/L, p=0.03) compared to neonates without subsequent AD. All associations remained significant after adjustment for potential confounders (table 2) and an additional borderline significant association was found between whole blood concentrations of eosinophils and subsequent risk of AD (p=0.05).

Decreased TLR4-mediated IL-10 production is associated with increased risk of subsequent atopic dermatitis

We next investigated whether TLR-mediated cytokine responses at the age of one month were associated with prospective susceptibility to AD (figure 3). Neonates who developed physician-diagnosed atopic dermatitis had 1.8-fold lower TLR4-mediated IL-10 responses at the age of one month compared to those who did not (533 pg/mL vs 954 pg/mL, p=0.003). Logistic regression analysis, adjusting for birth season, parental atopy and daycare as potential confounders, showed similar results (B=0.13 (95%-CI 0.04-0.41), p<0.001) (table 2). Sensitivity analyses using 'parent-reported atopic dermatitis' (defined as eczema or pruritic skin rash, derived from parental questionnaire at the age of one year) rendered similar findings, both in univariate analysis (588 pg/mL vs 995 pg/mL, p=0.001) and in multivariate analysis (B=0.24 (0.10-0.62), p=0.003). No significant correlations were found between concentrations of NK-cells, pDC, basophils, eosinophils and TLR4-mediated IL-10 production.

Table 1: Characteristics of study population

	RSV LRTI			Atopic dermatitis		
	Yes (n=41)	**No (n=250)**	**p-value**	**Yes (n=45)**	**No (n=246)**	**p-value**
Gestational age, wks (SEM)	40.3 (0.2)	39.9 (0.1)	0.01	40.1 (0.1)	39.9 (0.1)	0.27
Birth weight, g (SEM)	3834 (73)	3574 (29)	0.001	3653 (71)	3603 (29)	0.52
Gender, n (%)			0.61			0.87
Male	18 (44)	123 (49)		21 (47)	120 (49)	
Female	23 (56)	127 (51)		24 (53)	126 (51)	
Delivery mode, n (%)			0.09			1.00
Vaginal	39 (95)	212 (85)		39 (87)	212 (86)	
Elective caesarean section	2 (5)	38 (15)		6 (13)	34 (14)	
Birth season, n (%)			0.06			0.12
Winter	8 (20)	62 (25)		5 (12)	65 (26)	
Spring	9 (22)	67 (27)		16 (37)	60 (24)	
Summer	17 (41)	55 (22)		11 (26)	61 (25)	
Fall	7 (17)	66 (26)		13 (30)	60 (24)	
Siblings, n (%)	25 (61)	161 (66)	0.73	29 (64)	157 (64)	1.00
Pets, n (%)	12 (29)	80 (33)	0.72	16 (36)	65 (33)	0.73
Feeding, n (%)						
Ever breastfed	24 (85)	211 (87)	0.81	37 (84)	209 (85)	0.63
Exclusive breastfeeding >1 mo	24 (59)	130 (54)	0.61	20 (46)	134 (54)	0.25
Age at start formula, mo (SEM)	4.3 (0.8)	4.2 (0.3)	0.93	4.8 (0.9)	4.1 (0.3)	0.38
Day care attendance, n (%)	30 (79)	128 (64)	0.09	31 (78)	127 (51)	0.07
Maternal atopy[†], n (%)	12 (29)	93 (38)	0.30	15 (34)	90 (37)	0.74
Parental atopy[†], n (%)	23 (56)	137 (56)	1.00	24 (55)	136 (55)	0.87
Maternal atopic dermatitis, n (%)	10 (24)	46 (19)	0.40	9 (20)	47 (19)	0.84
Parental atopic dermatitis, n (%)	68 (28)	14 (34)	0.46	17 (39)	65 (26)	0.15
Parental smoking, n (%)	4 (10)	69 (28)	0.10	8 (18)	65 (26)	0.26

[†]Parental atopy was defined as parent-reported physician-diagnosed asthma, atopic dermatitis or hay fever.
SEM; standard error of the mean.

RSV LRTI

Neonatal whole blood innate immune cells are not associated with RSV LRTI

The primary outcome RSV LRTI in the first year of life was analyzed in relation to neonatal whole blood concentrations of innate immune cells (figure 2). There were no differences in concentrations of mDCs, pDCs, neutrophils and basophils at the age of one month between neonates with and without subsequent RSV LRTI. Neonates who developed RSV LRTI had decreased concentrations of monocytes (363 vs $468 \cdot 10^6$/L,

p=0.05) and eosinophils (363 vs 437·10^6/L, p=0.05) compared to those without RSV LRTI, but significance was lost after adjustment for potential confounders. No differences in cell concentrations were found in relation to the secondary outcome physician-attended RSV (data not shown). Analyses using cell percentages instead of absolute concentrations (e.g. eosinophils as a percentage of total leukocytes) rendered similar results (data not shown).

Neonatal TLR-mediated cytokine responses are not associated with RSV LRTI

No differences in cytokine responses to agonists for TLR2, TLR3, TLR4, TLR7 and TLR9 were found between neonates who developed RSV LRTI in the first year of life and those who did not (figure 3). The secondary outcome 'physician-attended RSV infection' was associated with higher TLR3-mediated IL-12p70 production at the age of one month (676 pg/mL vs 316 pg/mL, p=0.015). This remained statistically significant after adjustment for potential confounders (*B*=7.48 (1.67-33.4, p=0.021)).

Figure 2: Atopic dermatitis is associated with distinct concentrations of innate immune cells at the age of one month. Whole blood concentrations of innate immune cells at the age of one month were measured by flow cytometry and compared between neonates with subsequent atopic dermatitis (n=45) and those without (n=246) (panel a-g), or between neonates with subsequent RSV LRTI (n=41) and those without (n=250) (panel h-n). Bars represent geometric means and 95% confidence intervals derived from log transformed data.

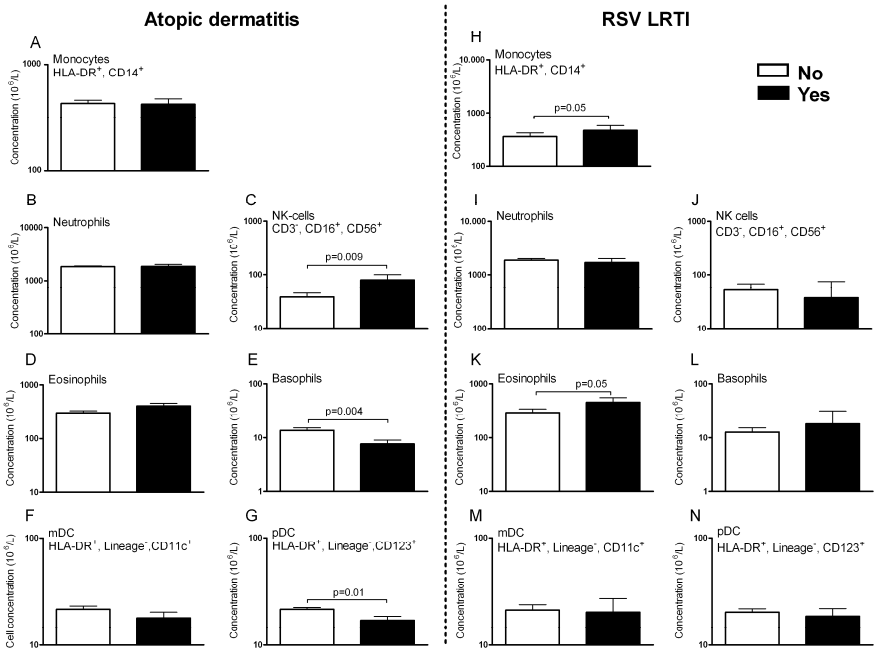
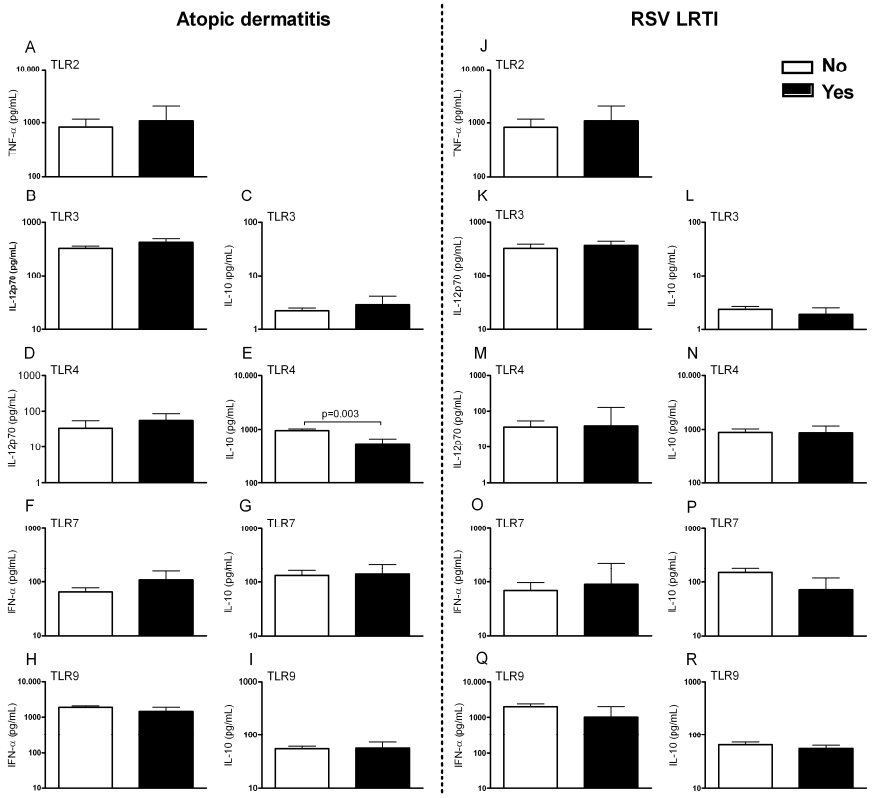


Table 2: Independent associations between concentrations of innate immune cells and TLR-mediated cytokine responses at the age of one month and the subsequent risk of atopic dermatitis. Logistic regression analysis was performed comparing concentrations of innate immune cells and TLR-mediated cytokine responses between neonates with subsequent atopic dermatitis (n=45) and those without (n=246), while adjusting for birth season, parental atopy and daycare as potential confounders. Only significant associations are shown.

	OR (95%-CI)	p-value
pDC	0.19 (0.40 to 0.91)	0.04
NK cells	1.83 (1.12-3.02)	0.02
Eosinophils	3.00 (1.00 to 8.97)	0.05
Basophils	0.28 (0.12 to 0.71)	0.01
TLR4-mediated IL-10	0.13 (0.04 to 0.41)	<0.001

Figure 3: Decreased TLR4-mediated IL-10 production in neonates with subsequent atopic dermatitis. Whole blood TLR-mediated cytokine production at the age of one month was measured by ELISA and compared between neonates with subsequent atopic dermatitis (n=45) and those without (n=246) (panel a-i), or between neonates with subsequent RSV LRTI (n=41) and those without (n=250) (panel j-r). Bars represent geometric means and 95% confidence intervals derived from log transformed data.



Discussion

This prospective unselected birth cohort study demonstrates that AD is preceded by marked differences in the innate immune system at the age of one month. Neonates who subsequently developed AD had decreased TLR4-mediated IL-10 production, decreased concentrations of basophils and pDC and increased concentrations of NK-cells compared to those who did not. By contrast, except for a modest association with increased TLR3-mediated IL-12p70 production, RSV LRTI was associated with presymptomatic differences neither in innate immune cellularity, nor in TLR-mediated cytokine responses at the age of one month,

TLR-mediated cytokine responses play an important role in the pathogenesis of AD. Genetic studies have associated polymorphisms in TLR2 (R753Q and A1694T) and the TLR9 promoter (C1237T)[39, 40] to increased risk and severity of AD. However, the mechanism by which these polymorphisms contribute to AD pathogenesis remains to be defined. In vitro, monocytes from AD patients carrying the TLR2 R753Q polymorphism produce significantly more IL-6 and IL-12 upon TLR2 stimulation than monocytes from patients carrying the wild-type TLR2 variant[41]. In contrast, macrophages from patients with AD express less TLR2 and produce less TLR2-mediated production of pro-inflammatory cytokines (IL-6, IL-8, IL-1β) compared to macrophages from healthy donors[42]. In addition, no differences in TLR expression or TLR2-, TLR3- and TLR4-mediated production of IL-12 and TNF-α were observed between monocyte-derived DC from highly atopic individuals and healthy controls[43]. Thus, although TLR responses are evidently associated with AD, their exact role in AD pathogenesis remains to be defined. Whether TLR signalling predisposes to or protects from AD likely depends on the specific TLR, cytokine and cell type, and might differ between AD initiation and propagation of existent disease.

Multiple studies suggest that increased TLR-mediated production of pro-inflammatory cytokines might contribute to the initiation of AD[44]. Cord blood mononuclear cells (CBMC) from atopic mothers produce higher TLR2-, TLR3- and TLR4-mediated IL-12 and IFN-γ compared to those from non-atopic mothers[44]. In addition, in high-risk neonates, increased production of TLR2-, TLR4- and TLR5-mediated IL-6 and TNF-α production by CBMC was associated with increased risk of childhood AD[44]. These associations suggest that early-life TLR hyper responsiveness might play a causal role in the pathogenesis of AD.
Disrupted postnatal development of the TLR system might also contribute to AD pathogenesis. Lack of early life exposure to innate immune stimuli, including the TLR4 agonist endotoxin, contributes to the subsequent development of asthma, allergic sensitization and AD[34, 45-47]. Additionally, in contrast to the age-dependent down regulation of Th2-type immune responses observed in non-atopic children, atopic children demonstrate persistent Th2-skewed allergen-specific immune responses (i.e. increased IL-4, IL-13, decreased IFN-γ) between birth and the age of two years[48, 49]. Persistent Th2-deviated adaptive immune responses might be due to lack of Th1-polarizing innate immune signals, as atopic children demonstrate an inverse pattern of innate immune maturation from non-atopic children, decreasing, rather than increasing,

TLR-mediated production of TNF-α, IL-12, IL-6 and IL-1β from birth to the age of five[50].
Together, these studies suggest that altered early life TLR responses, either due to genetic predisposition or to disrupted postnatal maturation, might result in persistent dysfunction of innate and adaptive immunity, thus predisposing to atopy. Previously, we identified the first month of life as a period of rapid development of the TLR system, providing a potential window of opportunity for immune modulatory interventions aimed at preventing atopy[6]. The association between TLR4-mediated IL-10 at the age of one month and subsequent risk of AD stresses the importance of the first month of life as a critical period in the development of the immune system and might suggest that strategies aimed at increasing IL-10 production during this highly sensitive period might restore the immune balance and protect from subsequent AD.

Several explanations may account for the association between low neonatal TLR4-mediated IL-10 production and AD. First, IL-10 is an important regulatory cytokine[51-54]. In mice, intradermal injections of IL-10 plasmid DNA suppress the development of AD skin lesions[55]. In apparent contrast, human AD skin lesions express increased IL-10 compared to psoriatic and healthy skin[56, 57]. This increase is accompanied by a parallel increase in pro-inflammatory cytokines[56, 57]. As the balance between inflammatory and regulatory cytokines, rather than their absolute concentrations determines the ensuing adaptive immune response[58], elevated concentrations of IL-10 in AD do not rule out its relative insufficiency. In support of a beneficial role of IL-10, subcutaneous allergen-specific immunotherapy increases IL-10 expression in AD skin, coinciding with clinical improvement[59]. Thus, deficient IL-10-mediated immune regulation might predispose to AD. As a second potential explanation, decreased neonatal IL-10 production to TLR4 stimulation might reflect a general dysregulation of responses mediated by TLR4, including responses to allergens associated with AD[60]. Several allergens associated with AD[61] are structurally homologous to components of the TLR4 receptor complex[62-64]. Moreover, one of these allergens, Der p 2, also showed functional homology to the MD-2 part of TLR4, initiating TLR4-signaling in MD-2 deficient mice[64]. Thus, similarities in the structure and biological functions of LPS and allergens might account for the association between neonatal TLR4-mediated IL-10 and AD. No association was found between TLR4-mediated IL-12p70 production and AD, suggesting that other mechanisms also play a role. Third, differences in microbial flora might account for the association between neonatal innate immune responses and AD. Early life microbial colonization drives innate immune maturation[3]. This process might be different in AD, as AD patients have distinct microbial flora of their skin[65] and gut[66] compared to non-atopic subjects. Interestingly, differences in gut colonization precede the development of clinical AD[66], suggesting that dysregulated microbial-induced immune development might play a causal role in the initiation of AD[34]. However, differences in microbial flora might also be the result, rather than the cause of dysregulated immune responses associated with AD, and future studies will be needed to address this issue.
AD was also associated with decreased whole blood concentrations of basophils and (in regression analysis) increased concentrations of eosinophils. This might be due to the distinct cytokine environment in AD neonates, which might preferentially drive differentiation of eosinophil/basophil precursor cells towards the eosinophil lineage. One

of the key factors driving eosinophil differentiation is IL-5[67]. A key Th2-cytokine, IL-5 plays an important role in the pathogenesis of AD[68], as illustrated by its increased expression in peripheral blood T-cells and skin biopsies of AD patients[69, 70]. In addition, increased IL-5 production during the neonatal and infant period is associated with elevated concentrations of eosinophils and total IgE[71] and increased risk of atopy at the age of one year[72].

Why do neonatal innate immune responses associate with AD, but not RSV LRTI? The lack of associations between neonatal innate immune cell concentrations or TLR-mediated cytokine responses measured in this study and subsequent RSV LRTI challenges the concept that decreased Th1-type immune responses during and after severe RSV infection are due to predisposed innate immune dysfunction[28-30]. Instead, it might suggest that these distinct immune responses are the result, rather than the cause of severe RSV disease.
Several studies indicate that RSV virulence factors might actively suppress host Th1-type immune responses. First, *in vitro*, compared to influenza or parainfluenza, DC infected with RSV in vitro produce decreased IL-12p70, and increased amounts of IL-10, IL-11 and PGE_2, consistent with a decreased Th1-type response[73, 74]. Second, both RSV and measles virus, another member of the *Paramyxoviridae* family, are capable of blocking TLR7- and TLR9-mediated IFN-β secretion by pDC[75]. Third, in murine models, IFN-γ receptor knockout mice experience aggravated RSV-induced pulmonary pathology[76]. Interestingly, this was not observed in IL-4 deficient mice, suggesting that insufficient Th1-type responses, rather than enhanced Th2-polarization predispose to severe RSV infection. Fourth, RSV-infected infants have lower TNF-α in nasopharyngeal aspirates compared to infants with influenza A infection[77] and decreased serum concentrations of IFN-γ compared to infants with influenza, parainfluenza or adenovirus infection[29], supporting the idea that suppressed Th1-type immune responses are not a general consequence of severe respiratory tract infection, but might be specific for RSV.
Of note, when studying the role of T-cell polarization in RSV infection, one has to take into account that the causal role of adaptive immune cells in RSV pathogenesis is still controversial. The majority of immune cells in RSV infected airways are neutrophils, and T-cells only occur at later time points, coinciding with recovery[32, 78]. In light of this controversy, our results suggest that impaired TLR-mediated production of Th1-type cytokines is not the cause of severe disease, or at least indicate that this impairment is not a predefined characteristic of a susceptible host.

This study has several strengths, including 1) we were able to follow a large cohort of healthy neonates born at term after uncomplicated gestation and delivery prospectively; 2) venous blood was collected at the age of one month, preventing any potential artefacts present in cord blood (e.g. hypoglycaemia, low pH, circulating maternal cells[79] and 3) all respiratory symptoms suspected to arise from RSV infection were confirmed by PCR. Potential limitations also deserve discussion. First, we did not identify the cell type responsible for TLR-mediated cytokine production. Consequently, the observed association between TLR4-mediated IL-10 and AD might be secondary to differences in innate immune cell concentrations. However, the cell types that were relatively decreased in atopic neonates either lack TLR4 (pDC)[80] or are incapable of responding to LPS *in*

vitro (basophils)[81]. Furthermore, adjustment for concentrations of innate immune cells rendered a similar significant association between TLR4-mediated IL-10 and AD (data not shown). In the future, flow cytometry-based studies will be required identify the cell type responsible for the decreased IL-10 production in AD. Second, the *in vitro* whole blood model is likely very different from the *in vivo* response in AD skin or RSV-infected airways[78]. Third, our comprehensive characterization of neonatal innate immunity includes multiple comparisons. To minimize the risk of false discovery, we focused on associations that were highly significant, that remained significant after adjustment for potential confounders and that were present for multiple related outcomes (e.g. physician-diagnosed AD and parent-reported AD). No attempt was made to correct for multiple testing, and future studies will be needed to validate our findings.

In summary, this study demonstrates that atopic dermatitis, but not RSV LRTI, is associated with presymptomatic decreased TLR4-mediated IL-10 at the age of one month. Future studies aimed at characterizing the basic mechanism and clinical factors modulating neonatal TLR responses might identify potential targets for prevention of AD.

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Supplementary material

Supplementary table 1: Markers used to identify innate immune cells by flow cytometry

Cell type	Marker	Fluorochrome	Clone
Monocytes	HLA-DR$^+$	PerCP	L243
	CD14$^+$	FITC	MoP9
NK-cells	CD3$^-$	APC	SK7
	CD16$^+$	PE	B73.1
	CD56$^+$	FITC	NCAM16.2
Plasmacytoid DC	HLA-DR$^+$	PerCP	L243
	CD123$^+$	PE	9F5
	Lineage$^-$	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
Myeloid DC	HLA-DR$^+$	PerCP	L243
	Lineage$^-$	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
	CD11c$^+$	APC	S-HCL-3

Supplementary Table 2: Agonists used for *in vitro* TLR stimulation

TLR	Agonist used	Concentration	Manufacturer
2	Pam_3Cys SSNA	1 μg/mL	Bachem Biosciences, Weil am Rhein, Germany
3	Polyinosinic:polycyticylic acid (Poly I:C)	200 μg/mL	Invivogen, San Diego, CA
4	Ultrapure lipopolysaccharide derived from *E. Coli*	100 ng/mL	Invivogen
	Interferon-γ (IFN-γ)	20 ng/mL	Preprotech Inc, Rocky Hill, NJ
7	Loxoribine	1 mM	Invivogen
9	CpG oligodeoxynucleotide (CpG ODN)	30 μg/mL	Invivogen

Supplementary Table 3: Baseline characteristics of study population compared to all eligible neonates

	Included in study population (n=291)	**Not included in study population (n=1007)**	**p-value**
Birth weight, g (SE)	3611 (27)	3571 (36)	0.56
Gestational age, wks (SE)	40.0 (0.06)	40.0 (0.03)	0.94
Siblings, n (%)	186 (64)	622 (62)	0.63
Male gender, n (%)	141 (48)	517 (51)	0.39
Birth Season, n (%)			0.93
Winter	70 (24)	248 (25)	
Spring	76 (26)	246 (24)	
Summer	72 (25)	263 (26)	
Fall	73 (25)	250 (25)	

Chapter 8

Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis

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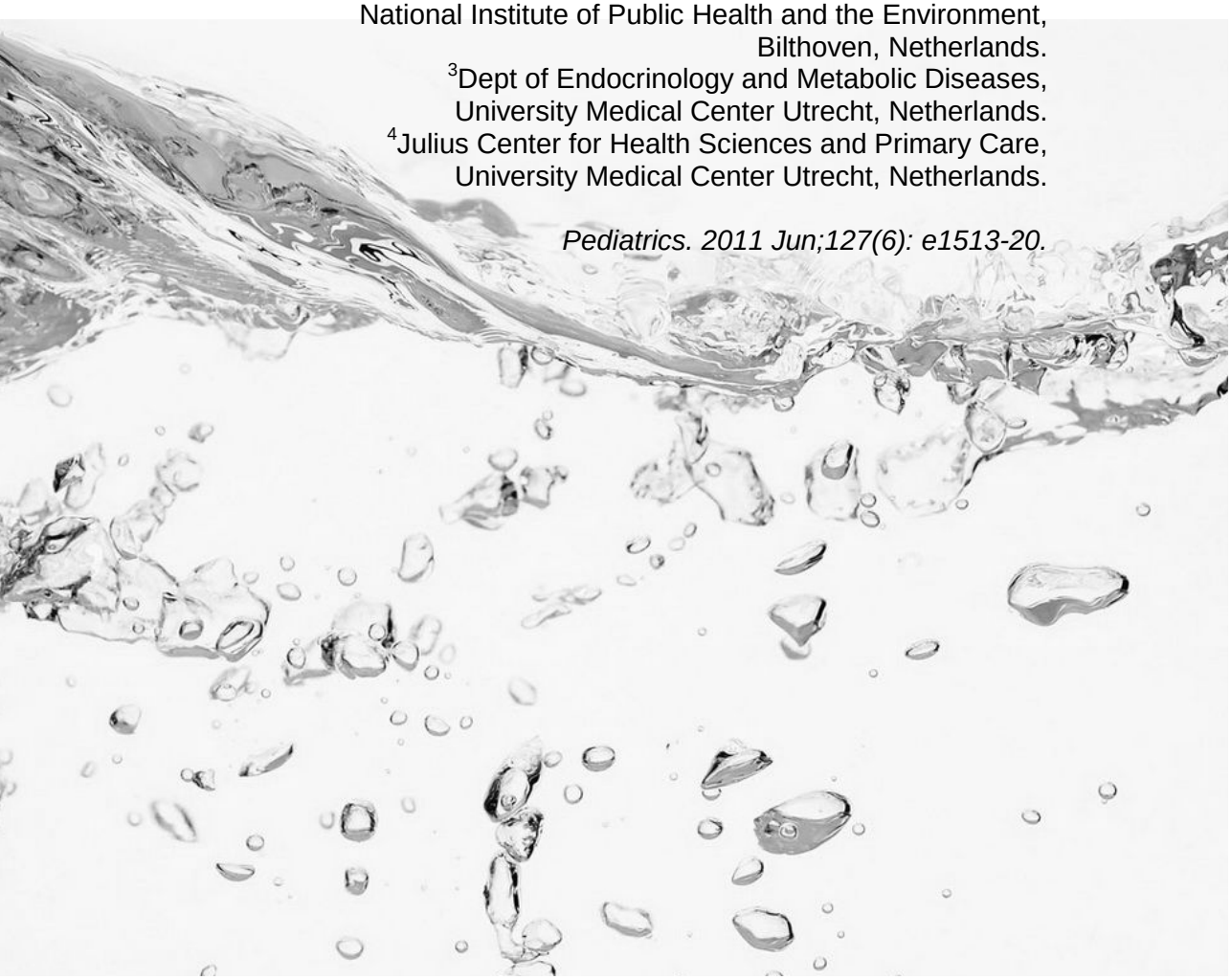


Abstract

Background
Respiratory syncytial virus (RSV) is the most important pathogen causing severe lower respiratory tract infection (LRTI) in infants. Epidemiological and basic studies suggest that vitamin D may protect against RSV LRTI.

Objective
To determine the association between plasma vitamin D concentrations at birth and the subsequent risk of RSV LRTI.

Methods
A prospective birth cohort study was performed in healthy term neonates. Concentrations of 25-hydroxyvitamin D (25-OHD) in cord blood plasma were related to RSV LRTI in the first year of life, defined as parent-reported LRTI symptoms in a daily log, and simultaneous presence of RSV RNA in a nose-throat specimen.

Results
The study population included 156 neonates. Eighteen (12%) developed RSV LRTI. The mean plasma 25-OHD concentration was 82 nmol/L. Overall, 27% of neonates had 25-OHD concentrations <50 nmol/L, 27% had 50-74 nmol/L and only 46% had 25-OHD ≥75 nmol/L. Cord blood 25-OHD concentrations were strongly associated with maternal vitamin D3 supplementation during pregnancy. Concentrations of 25-OHD were lower in neonates who subsequently developed RSV LRTI compared with those who did not (65 nmol/L versus 84 nmol/L, p=0.009). Neonates born with 25-OHD concentrations <50 nmol/L had a sixfold (95% confidence interval 1.6-24.9, p=0.01) increased risk of developing RSV LRTI in the first year of life compared to those with 25-OHD concentrations ≥75 nmol/L.

Conclusion
Vitamin D deficiency in healthy neonates is associated with increased risk of RSV LRTI in the first year of life. Intensified routine vitamin D supplementation during pregnancy may be a useful strategy to prevent RSV LRTI during infancy.

Introduction

Respiratory syncytial virus (RSV) is the most important respiratory pathogen in young children, in whom it may cause significant morbidity. Although >90% of all infants encounter RSV before the age of 2 years, only 10% develop a severe lower respirator tract infection (LRTI). Several risk factors for RSV LRTI have been described, but th majority of infections occur in infants without any known risk factors [1, 2]. Insight into the factors that predispose infants to RSV LRTI may result in new strategies to prevent infection.

Vitamin D is an essential nutrient, with functions that extend beyond its classical role in bone metabolism. Vitamin D regulates >1000 human genes, with receptors present in most cells throughout the body[3]. In westernized countries, 40% of pregnant women and 50% of newborns and infants have vitamin D insufficiency[4-8]. Concentrations of vitamin D in the fetus and newborn are dependent on and correlate with maternal serum 25-hydroxyvitamin D (25-OHD) concentrations[9-12]. Accordingly, maternal vitamin D insufficiency has been related to many diseases in the offspring, including type I diabetes[13], multiple sclerosis[14], schizophrenia[15], infant wheeze[16] and acute respiratory infections[12, 17].

Basal and epidemiologic evidence suggests that vitamin D may protect against severe RSV LRTI. *In vitro*, vitamin D decreases the inflammatory response of airway epithelial cells to RSV infection, without jeopardizing viral clearance[18]. In humans, RSV occurs in a seasonal pattern with peaks in winter, when serum concentrations of vitamin D are lowest[19]. Genetic polymorphisms in the vitamin D receptor are associated with hospitalization for acute LRTIs, predominantly RSV bronchiolitis, in infancy[20]. Furthermore, several studies have demonstrated that plasma concentrations of 25-OHD are lower in infants hospitalized for acute LRTI compared with healthy controls[10-12, 21, 22]. However, these studies were cross-sectional and included a limited number of subjects.

In this healthy birth cohort study, we aimed to determine the association between cord blood vitamin D status and the subsequent risk of RSV LRTI in the first year of life.

Methods

Study design and recruitment criteria

This study was part of a prospective birth cohort study on early life determinants of RSV LRTI performed in 2 medical centers in Utrecht, Netherlands. Study design and recruitment criteria have been published previously[23]. Eligible study participants were healthy newborns, born after uncomplicated gestation of ≥37 weeks. To avoid extensive counseling of parents just after delivery, a 2-step approach was used (Fig 1). The first consent interview took place soon after delivery. During this interview, parents were informed that cord blood had been collected and were provided with oral and written information on the study. A second interview was scheduled when the infant was 1-3 weeks, during which we provided further information and obtained informed consent. The study was approved by the ethics review boards of the University Medical Center Utrecht and the Diakonessen Hospital Utrecht, and parents of all participants provided written informed consent for study participation.

Clinical characteristics
Data on baseline characteristics and use of vitamin D supplements were collected from hospital charts and standardized parental questionnaires[24]. Maternal ethnicity was defined as caucasian or other on the basis of country of birth. Season of birth was designated as: winter (December, January, February), spring (March, April, May), summer (June, July, August) or fall (September, October, November). To assess the relation between cord blood 25-OHD levels and sun exposure, monthly hours of sunshine in The Netherlands during the study period were obtained from the archives of the Dutch Royal Meteorological Institute[10].

Plasma vitamin D measurement
Cord blood was collected directly after delivery and anticoagulated by use of sodium heparin. Plasma was prepared by centrifugation (10 minutes at 500 x *g)*, and stored at -80°C. Plasma 25-OHD concentrations (nmol/L, to convert to ng/mL divide by 2.496) were measured with the Modular E170 analyzer (Roche, Basel, Switzerland). Interassay variability for pooled serum analyses was 19% at 33 nmol/L 25-OHD, 12% at 62 nmol/L and 10% at 99 nmol/L. Plasma concentrations of 25-OHD were analyzed both as a continuous variable and divided into quartiles (<25 nmol/L, 25-49 nmol/L, 50-74 nmol/L and ≥75 nmol/L)[7]. Because of the low number of neonates in the <25 nmol/L group (n=7), for outcome analyses the lower quartiles (<25 and 25-49 nmol/L) were pooled.

Primary and secondary outcome
The primary outcome was defined as parent-reported RSV LRTI, which was defined as (1) LRTI symptoms, and (2) simultaneous presence of RSV RNA in a nose-throat specimen. Parents were instructed to record presence and severity of respiratory symptoms during the first year of life in a daily log[23]. LRTI symptoms were defined by 2 independent researchers who used strict criteria: moderate or severe cough or wheeze of any severity lasting for at least 2 days. At the second day of every respiratory episode, parents obtained a nose-throat swab specimen. Samples were sent to the researchers in viral transport medium and frozen at -80^{o}C[25]. The presence of RSV RNA was determined by real-time polymerase chain reaction as described previously[23].
The secondary outcome 'physician-attended RSV LRTI' was defined as (1) respiratory illness for which the general practitioner or paediatrician was visited, and (2) simultaneous presence of RSV RNA in a nose-throat swab.

Statistical analysis
Cord blood plasma 25-OHD concentrations were normally distributed, and means were compared by using Student's t-test. X^2 analysis was used to test associations between categorical variables. Seasonality of 25-OHD was tested by fitting the data to a sine function with a period of 12 months in a nonlinear regression model. Statistical significance of seasonal distribution was determined by comparing the resulting sinusoidal model with the best fitting linear model, using the F-test. Logistic regression analysis was performed to determine the effect of cord blood 25-OHD concentrations on the risk of subsequent RSV LRTI adjusted for potential confounders. Because of the limited number of cases, only a restricted number of potential confounders could be analyzed. The variables birth month, birth weight and maternal ethnicity showed the

highest association with both cord blood 25-OHD concentration and risk of RSV disease in single-variable analyses and were therefore included in regression models. To adjust for birth month, 2 approaches were used. In the first approach, we used 'deseasonalization' of 25-OHD concentrations (Supplemental Figure 1)[26]. In this approach, the predicted 25-OHD concentrations for each subject, derived from the sinusoidal model, were subtracted from the actual observed value. Subsequently, the overall mean was added and the resulting deseasonalized 25-OHD concentrations were analyzed using logitistic regression analysis, also adjusting for maternal ethnicity and birth weight. In the second approach, we used regression analysis in which we adjusted for birth ±10 weeks from the start of the RSV season (yes versus no), next to maternal ethnicity and birth weight. Analyses were performed in SPSS 15.0 (SPSS Inc, Chicago, IL).

Results

Population characteristics

From November 2006 to December 2009, 1007 neonates were eligible for study participation (Fig 1). Of these, cord blood was collected from 481 neonates (48%), and for 161 (33%) of these infants the parents agreed to their participation in follow-up. Because of technical reasons, plasma 25-OHD concentrations were not measured in 5 (3%) of participating neonates, which resulted in a final cohort of 156 neonates. Baseline characteristics did not differ between participating subjects and non-participants (Supplemental table 1). Of the participating neonates, 18 (12%) developed RSV LRTI in their first year of life, of whom 10 neonates had a physician-attended RSV LRTI. Neonates who subsequently developed RSV LRTI had a higher birth weight (3903 versus 3523 g, p=0.001), and trended toward higher gestational age (40.4 versus 39.9, p=0.06) compared with those who did not, as observed previously (Supplemental table 2)[27]. There were no differences in birth season, number of siblings, maternal ethnicity, mode of feeding and use of vitamin D supplements between neonates who did and those who did not develop RSV LRTI.

Figure 1: Flow chart of study population.

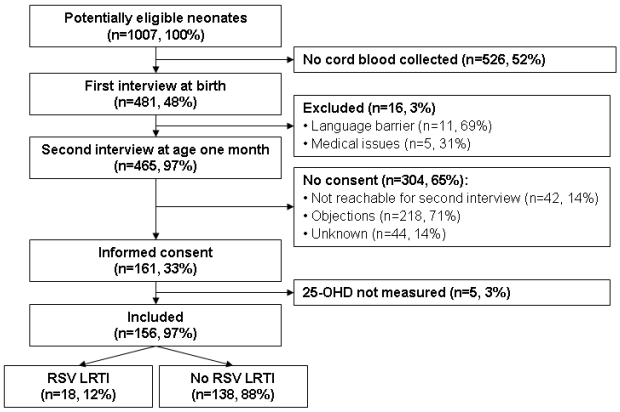


Table 1: Characteristics of study population according to cord blood vitamin D status.

	Cord blood 25-OHD concentration				
	<25 nmol/L (n=7)	25-49 nmol/L (n=29)	50-74 nmol/L (n=48)	≥75 nmol/L (n=72)	*p*-value
Birth weight, g (SE)	3237 (167)	3529 (90)	3604 (74)	3574 (50)	0.246
Gestational age, wks (SE)	39.8 (0.41)	40.1 (0.21)	40.2 (0.14)	39.8 (0.13)	0.345
Any siblings, n (%)	0 (0)	12 (41)	18 (38)	31 (43)	0.17
Male gender, n (%)	4 (57)	13 (45)	24 (50)	30 (42)	0.75
Birth Season, n (%)					0.012
Winter	3 (43)	11 (38)	16 (33)	13 (18)	
Spring	1 (14)	6 (21)	7 (15)	18 (25)	
Summer	1 (14)	3 (10)	7 (15)	28 (39)	
Fall	2 (29)	9 (31)	18 (38)	13 (18)	
Maternal Ethnicity, n (%)					
Caucasian	2 (29)	15 (52)	33 (69)	63 (88)	<0.001
Other	5 (71)	14 (48)	15 (31)	9 (13)	
Ever breastfed, n (%)	6 (100)	19 (79)	33 (87)	51 (81)	0.56
Maternal vitamin D supplement use during pregnancy, n (%)	0 (0)	9 (32)	18 (38)	51 (71)	0.005
Neonatal vitamin D supplement use, n (%)	4 (67)	12 (63)	30 (81)	46 (75)	0.50

High prevalence of vitamin D deficiency in healthy newborns

The mean cord blood plasma 25-OHD concentration among healthy newborns was 82 nmol/L (SE: 3.5 nmol/L). Overall, 4% of neonates had 25-OHD levels of <25 nmol/L and 23% had levels <50 nmol/L; 27% had 25-OHD levels of 50-74 nmol/L, and only 46% had 25-OHD levels of 75 nmol/L or higher.

Use of vitamin D supplements during pregnancy increases cord blood 25-OHD concentrations

Of participating women, 46% reported use of vitamin D-containing supplements during pregnancy. The majority of these (97%) were multivitamin preparations that contained a daily dose of 400 IU (10 µg) vitamin D3. Of these women, 74% used supplements during the first trimester, 86% during the second trimester, and 81% during the third trimester. In total, 54% of participating women used vitamin D supplements throughout pregnancy. Maternal use of vitamin D supplementation during pregnancy was associated with increased concentrations of 25-OHD in cord blood (73 versus 96 nmol/L, p=0.003). After birth, 75% of all neonates received vitamin D supplements (daily recommended dose 400 IU vitamin D3) during the first month of life. Characteristics of participants according to vitamin D status at birth are shown in Table 1.

25-OHD concentrations show a seasonal pattern

To explore the seasonal variation in the concentrations of 25-OHD, cord blood 25-OHD concentrations and birth month were fitted to a sinusoidal model. Cord blood concentrations of 25-OHD showed a seasonal distribution with a baseline level of 84 nmol/L and an amplitude of 15 nmol/L (p=0.001, Fig 2a). Maximum fitted concentrations of cord blood 25-OHD were observed in neonates born in July, and concentrations reached their nadir in January. Of neonates born in winter, 33% had cord blood 25-OHD concentrations <50 nmol/L and 70% had concentrations <75 nmol/L, compared with 10%

and 28% of neonates born in summer (Fig 2b, X^2 p=0.012). Seasonality of 25-OHD concentrations was present for all birth years in our cohort (data not shown). We also related cord blood 25-OHD levels to monthly hours of sunshine during the study period, according to the Royal Netherlands Meteorological Institute[10], and found a strong correlation between cord blood 25-OHD levels and monthly sun hours (Fig 2c, R=0.196, p=0.01).

Cord blood vitamin D concentrations are associated with RSV LRTI in the first year of life

Plasma concentrations of 25-OHD at birth were related to the risk of RSV LRTI in the first year of life (Fig 3). Neonates who subsequently developed RSV LRTI had 1.3-fold lower cord blood concentrations of 25-OHD compared with those who did not (65 ± 7 versus 84 ± 11 nmol/L, p=0.009) (Fig 3a). Logistic regression analysis, with correction for birth month, birth weight and maternal ethnicity as potential confounders, demonstrated that cord blood 25-OHD concentrations were independently associated with subsequent risk of RSV LRTI (B=0.978 (95% CI: 0.959-0.997), p=0.024). Sensitivity analyses, corrected for birth ± 10 weeks of the start of the RSV season, maternal ethnicity, and infant birth weight, also revealed a significant negative association between cord blood 25-OHD concentrations and risk of RSV LRTI (B=0.976 (95% CI: 0.957-0.996), p=0.018). With the use of the secondary outcome physician-attended RSV LRTI, a similar trend was observed (63 vs 83 nmol/L, B=0.983 (95% CI: 0.962-1.004), p=0.117).

We also analyzed the risk of RSV LRTI in neonates who were born with cord blood 25-OHD levels <50 nmol/L, 50-75 nmol/L and ≥75 nmol/L (Fig 3b). Compared with neonates with cord blood 25-OHD levels ≥75 nmol/L, the adjusted relative risk of RSV LRTI was 6.2 (95%-CI: 1.6-24.9, p=0.01) in neonates with 25-OHD levels <50 nmol/L.

Figure 2: High prevalence of vitamin D deficiency in healthy newborns. Concentrations of 25-OHD in cord blood plasma (n=156) were measured with the Modular E170 analyzer, and related to birth month (a) or birth season (b). Seasonality of cord blood 25-OHD was assessed by fitting the data to the best fitting linear and sinusoidal model. C, monthly hours of sun during the study period were obtained from the archives of the Dutch Royal Meteorological Institute[10], and related to cord blood 25-OHD by using Pearson correlation. Bars represent mean + SE of the mean (a and c), or percent of subjects (b).

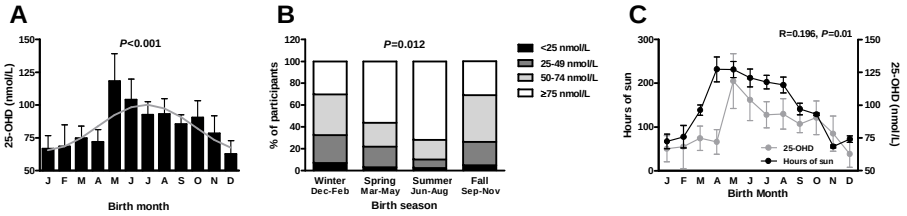
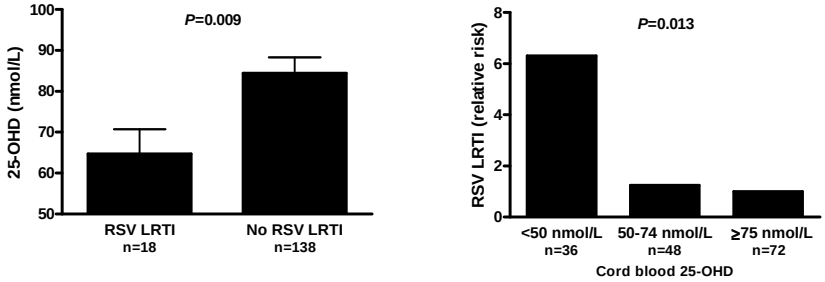


Figure 3: Association between cord blood vitamin D concentrations and RSV LRTI in the first year of life. (a) Cord blood concentrations of 25-OHD in neonates who subsequently developed RSV LRTI (n=18) and those who did not (n=138). (b) Risk of RSV LRTI per quartile of 25-OHD levels. Because of the limited number of cases, the lower quartiles (<25 nmol/L, n=7 and 25-49 nmol/L, n=29) were pooled. Bars represent mean + SE of the mean (a), or risk of RSV LRTI relative to neonates with 25-OHD ≥75 nmol/L (b).



Discussion

In this prospective birth cohort study, we demonstrate that 54% of healthy newborns in the Netherlands are born with insufficient 25-OHD concentrations required for maximum health[28, 29], and that low plasma concentrations of 25-OHD at birth are associated with increased risk of RSV LRTI in the first year of life.

RSV is the most important respiratory pathogen in infancy, yet the mechanisms responsible for severe RSV disease are incompletely understood. Although antibody therapy is recommended for children at high risk of severe infection, the majority of infections occur in children without any known risk factors[30], for whom no preventive strategies are currently available. Micronutrient supplementation to pregnant women and their newborns could be an easy and affordable strategy to prevent RSV LRTI.

The prevalence of vitamin D deficiency in our cohort was comparable to reported prevalences in other Westernized countries[6, 28, 29, 31]. Cord blood vitamin D concentrations demonstrated a seasonal pattern, with maximum concentrations in neonates born in July and lowest concentrations in neonates born in December. This vitamin D concentration peak, which was relatively early compared with peaks observed in previous cohort studies[11, 32] may have occurred because pregnant mothers experienced extraordinarily high sun exposure in spring months during the study period (Fig 1c). In addition, the partial association between hours of sunshine and cord blood 25-OHD levels indicates that other factors, including time spent outdoors, use of sun protection[33, 34], and nutritional intake of vitamin D by the mother might contribute to cord blood vitamin D status.

To our knowledge, this is the first longitudinal study to demonstrate a relationship of plasma 25-OHD concentrations at birth and the subsequent risk of RSV LRTI. Previous cross-sectional studies have related low plasma concentrations of 25-OHD to increased severity of respiratory tract infection[12, 17, 22]. In Turkey and rural Bangladesh, plasma 25-OHD concentrations during infection were observed to be lower in children hospitalized with acute LRTI compared with age-matched healthy controls[12, 22], and subclinical vitamin D deficiency was associated with acute respiratory tract infection in Indian

children[17]. In addition, a recent cohort study in 284 Finnish children hospitalized for acute wheezing demonstrated a significant association between plasma vitamin D levels and risk of viral coinfection, specifically coinfections with RSV, rhinovirus, or both[8]. In contrast, studies in Canada failed to show a difference in plasma 25-OHD between children with and without respiratory tract infection[21, 35].

Several explanations may account for the protective effect of vitamin D at birth against subsequent RSV LRTI, which we observed in our study. Severe RSV infection is thought to arise from an interplay between the host immune response, airway anatomy, and RSV viral load. All these factors may be affected by vitamin D.

Vitamin D has immune modulatory properties that may influence the development of the fetal and neonatal immune system. Low vitamin D intake during pregnancy is associated with increased incidence of diseases related to immune dysfunction in the offspring, including type I diabetes, asthma and allergic rhinitis [16, 36, 37]. In vitro, vitamin D has many immune modulatory functions, including induction of tolerogenic dendritic cells[38], development of $CD4^+CD25^+Foxp3^+$ regulatory T-cells[39], activation of T-cell signaling[40] and elaboration of tolerizing and anti-inflammatory cytokines, including interleukin 10[39, 41, 42]. Moreover, results of a recent study demonstrate that maternal vitamin D intake during pregnancy increases expression of tolerogenic genes in cord blood[43], which suggests that the immune modulatory function of vitamin D may already be occurring prenatally.

In addition, vitamin D may modulate early lung development. In animal models, vitamin D has been shown to promote lung development and surfactant production [44-46]. In humans, $1,25(OH)_2D$, the biologically active form of vitamin D, promotes surfactant production[47], and downstream effectors of vitamin D have been detected in fetal lungs as early as 14 weeks' gestational age[48]. Vitamin D might thus accelerate fetal lung development, thereby potentially protecting against RSV disease.

Vitamin D also has many antimicrobial properties that may result in decreased viral load during infection[42]. Neonates who are born vitamin D deficient may also have lower serum concentrations during the neonatal period and infancy, which may confound the demonstration of any association between cord blood vitamin D and RSV LRTI during infancy. We did not measure vitamin D concentrations during RSV infection. However, 75% of neonates and infants in our cohort received vitamin D supplements after birth (400 IU per day), and there was no association between postnatal vitamin D supplement use and cord blood vitamin D levels (data not shown) or risk of RSV LRTI (Supplemental table 2).

Our results suggest that strategies aimed at improving maternal vitamin D status during pregnancy might decrease the risk of RSV disease in the offspring. In agreement with recommendations of the American Association for Pediatrics and the World Health Organization, the Dutch Health Council recommends daily supplementation of 400 IU (10 μg) vitamin D to all pregnant women and breastfed newborns[49]. However, the optimal dose of vitamin D supplementation is still under debate. Especially during pregnancy, doses up to 4000 IU per day may be needed to obtain optimal maternal and neonatal health[29, 42, 50-52]. In addition, adherence to the current guidelines is generally poor[53]. In our cohort, only 46% of women reported use of vitamin D-containing supplements during pregnancy. Although vitamin D supplementation during pregnancy resulted in increased cord blood 25-OHD concentrations, we did not find a significant

association with risk of RSV LRTI. However, the current study was insufficiently powered to answer this question. The association between cord blood 25-OHD concentrations and subsequent RSV LRTI suggests that larger clinical trials should be conducted to investigate the effect of vitamin D supplementation during pregnancy on the susceptibility to RSV LRTI in the offspring.

Potential limitations of this study warrant discussion. The sample size was relatively low and the number of cases in our cohort was relatively small. Limited statistical power particularly affected analyses that used cord blood 25-OHD quartiles, which resulted in wide confidence intervals. Nevertheless, despite the low number of cases, we were able to demonstrate significant differences in RSV risk. In addition, lack of parental compliance may have caused misclassification of infants who did experience RSV LRTI, but whose parents forgot to take a nose-throat swab or fill out the diary. However, because of the low incidence of RSV LRTI, we do not think that this limitation had a significant effect on conclusions. Another limitation was that detailed information on sun exposure and dietary habits was not available for analysis. As a surrogate marker of sun exposure, birth month was included into our analysis. Similar results were found, which indicated that cord blood 25-OHD is an independent predictor of RSV LRTI.

In conclusion, vitamin D deficiency was highly prevalent in Dutch newborns, and cord blood 25-OHD concentrations were associated with susceptibility to subsequent RSV LRTI. Increased awareness of vitamin D status of pregnant women and intensified routine vitamin D supplementation may help prevent RSV LRTI during infancy. Randomized trials are required to address this question.

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Supplemental information

Supplemental table 1: Baseline characteristics of the study population compared to all eligible.

	Included in study population (n=156)	Not included in study population (n=851)	*p*-value
Birth weight, g (SE)	3568 (37)	3583 (43)	0.88
Gestational age, wks (SE)	40.0 (0.09)	40.0 (0.04)	0.37
Siblings, n (%)	95 (61)	536 (64)	0.59
Male gender, n (%)	71 (46)	425 (50)	0.34
Birth Season, n (%)			0.77
Winter	43 (28)	239 (28)	
Spring	62 (21)	186 (22)	
Summer	39 (25)	181 (21)	
Fall	42 (27)	245 (29)	
Maternal Ethnicity, n (%)			0.32
Caucasian	106	541	
Other	50	310	

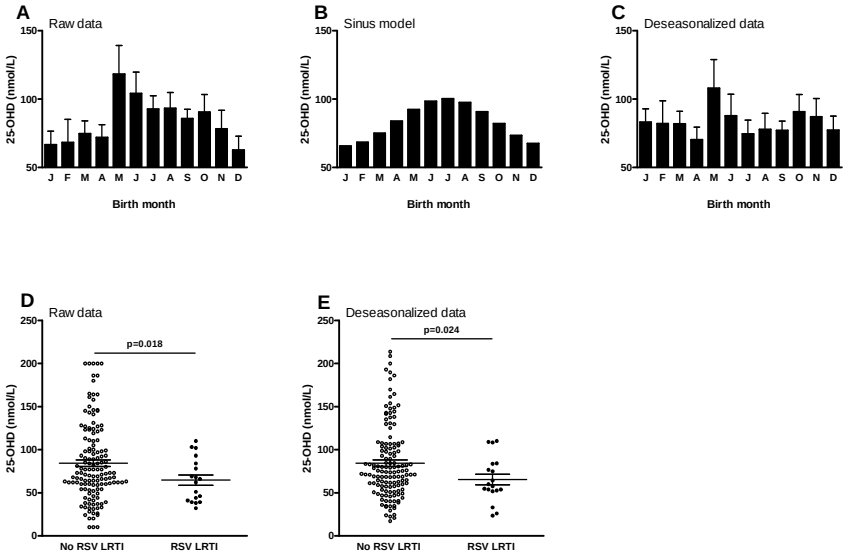
Supplemental table 2: Characteristics of study population according to RSV LRTI

	RSV LRTI (n=18)	No RSV LRTI (n=138)	*p*-value
Birth weight, g (SE)	3903 (374)	3523 (457)	0.001
Gestational age, wks (SE)	40.4 (0.25)	39.9 (0.09)	0.06
Siblings, n (%)	13 (72)	82 (59)	0.30
Male gender, n (%)	7 (39)	64 (46)	0.55
Birth Season, n (%)			0.54
Winter	3 (17)	40 (29)	
Spring	3 (17)	29 (21)	
Summer	5 (28)	34 (25)	
Fall	7 (39)	35 (25)	
Maternal Ethnicity, n (%)			0.15
Caucasian	15 (83)	92 (67)	
Other	3 (17)	46 (33)	
Ever breastfed, n (%)	15 (83)	94 (83)	1.00
Maternal vitamin D supplement use during pregnancy, n (%)	6 (43)	51 (47)	0.78
Neonatal vitamin D supplement use, n (%)	10 (71)	82 (75)	0.76

Supplemental figure 1: Strategy for deseasonalization of cord blood 25-OHD concentrations.
A sinusoidal model was fitted to cord blood 25-OHD concentrations and birth month (t):

$$25\text{-OHD} = A + B * \sin\left(C + \frac{2\pi t}{12}\right)$$

The predicted values derived from this model (b) was subtracted from the actual 25-OHD concentration for each subject (a), and the overall mean was added to obtain deseasonalized 25-OHD concentrations (c). Regression analysis, performed with either uncorrected 25-OHD (d) or deseasonalized levels (e), showed similar results. Bars represent mean + SEM.



Chapter 9

Summary

The neonatal innate immune system is immature

Human newborns are highly susceptible to infections. The incidence of infections is particularly high in the first weeks of life, and rapidly decreases thereafter. Common causes of infection in neonates include viruses, commensal bacteria such as group B streptococci and coagulase negative staphylococci, and Gram-negative organisms like *E. coli*. High neonatal susceptibility to infection appears to be due to immaturity of the neonatal immune system. Toll-like receptors (TLRs) are key pathogen-recognition receptors of the innate immune system, which serve to initiate the early antimicrobial response and to instruct the ensuing adaptive immune response. Neonatal Toll-like receptor mediated immune responses at birth are distinct, and characterized by decreased production of Th1-polarizing cytokines, whereas production of Th2-polarizing and regulatory cytokines is increased. This bias is thought to prevent potentially harmful allo-immune reactions towards maternal antigens in utero, and to facilitate microbial colonization after birth. However, impaired generation of pro-inflammatory responses renders the neonate highly susceptible to infections. In addition, mounting evidence indicates that dysregulated innate immune responses at birth play an important role in the pathogenesis of atopy and asthma.

Impairments in neonatal innate immune responses at birth have been well characterized. However, little is known on the early postnatal development of the human innate immune system and its consequences for health and disease. The immunology part of the Netherlands Amniotic Fluid Study is a prospective birth cohort study that aims to characterize the ontogeny of the neonatal immune system during the first month of life and its consequences for subsequent infections and atopy. In the current thesis, we aim to address the following questions:

- What is the ontogeny of the human TLR system?
- What are the clinical determinants of neonatal TLR function?
- What are the basic mechanisms causing distinct neonatal TLR responses?
- Does delayed postnatal TLR development increase the risk of respiratory tract infections and atopy during infancy?

Postnatal ontogeny of the neonatal TLR system

First, we aimed to characterize the early postnatal development of the human innate immune system (**Chapter 4**). To this, we compared whole blood numbers of innate immune cells and TLR-mediated cytokine responses between cord blood obtained from health newborns directly after uncomplicated gestation and delivery, neonatal venous blood obtained at the age of one month and adult venous blood. Cord blood TLR-mediated production of key Th1-polarizing cytokines IL-12p70 and IFN-α was generally impaired compared to adult responses, whereas production of IL-10 was increased. At the age of one month, TLR3-mediated IL-12p70 and TLR7 and TLR9-mediated IFN-α production had matured to adult levels. In contrast, TLR4 demonstrated slower maturation, with low IL-12p70 and high IL-10 production up until the age of one month. Together, these findings indicate that the neonatal TLR immune system rapidly changes during the first month of life.

Breast feeding is the major environmental determinant of neonatal TLR function
The rapid development of the innate immune system suggests that the impact of environmental exposure to immune modulatory factors may be highest in the first month of life. To identify the major environmental factors modulating the neonatal immune system, we analyzed the effects of mode of delivery, breastfeeding, birth month, siblings, pets and parental smoking on whole blood numbers of innate immune cells and TLR-mediated cytokine responses at the age of one month (**Chapter 5**). Breast feeding was the major determinant of neonatal innate immunity. Especially, the association between breast feeding and TLR7-mediated IL-10 production was highly significant. The protective effects of breastfeeding against infections might be due to its capacity to modulate early life immune maturation.

Neonatal TLR4-mediated IL-10 and IL-12p70 production are differentially modulated by distinct soluble factors in plasma
We next aimed to characterize the basic mechanisms causing distinct neonatal Toll-like receptor responses. Based on its relatively slow maturation during the first month of life (**Chapter 4**), we chose to focus on TLR4-mediated production of IL-12p70 and IL-10. In **Chapter 6**, we demonstrate that neonatal plasma polarizes TLR4-mediated production of IL-12p70 and IL-10 via distinct factors. Compared to adult plasma, whole plasma derived from cord blood significantly suppressed TLR4-mediated production of IL-12p70, while inducing production of IL-10. This pattern was also observed for TLR3- and TLR8-mediated IL-12p70 and/or IL-10 production, demonstrating that plasma modulates responses to MyD88-dependent and MyD88-independent agonists. We also found that the ontogeny of these factors was distinct. Whereas plasma-mediated IL-12p70 suppression persisted up until the age of one month, IL-10 stimulation disappeared by one month of age. These results indicate that human plasma contains distinct factors that induce IL-10 and that suppress TLR-mediated IL-12p70 production. These factors are present at increased concentrations in neonatal plasma, thereby potentially predisposing to microbial infections and atopy. Identification of these factors might provide insight into the ontogeny of innate immune development as well as novel targets for the prevention and treatment of neonatal infections and atopy.

Low TLR4-mediated IL-10 production is associated with atopic dermatitis
We hypothesized that delayed postnatal TLR maturation, resulting in impaired Th1-polarizing responses at the age of one month, predisposes to microbial infections and to atopy. To address this hypothesis, in **Chapter 7**, TLR-mediated cytokine responses at the age of one month prospectively related to the risks of RSV lower respiratory tract infections or atopic dermatitis in the first year of life. Neonates with subsequent atopic dermatitis had lower concentrations of basophils and plasmacytoid dendritic cells, higher concentrations of natural killer cells, and twofold lower Toll-like receptor 4-mediated IL-10 compared to neonates without subsequent atopic dermatitis. No differences in Toll-like receptor-mediated cytokine production, nor in whole blood concentrations of innate immune cells were observed between neonates with and without subsequent RSV LRTI. This might indicate important differences in the pathogenesis of AD and RSV LRTI. Differences in TLR-mediated cytokine responses observed during AD may reflect pre-existent host immune dysfunction that predisposes to allergy. In contrast, differences in

TLR-responses during and after severe RSV infection may be the result, rather than the cause of severe disease. Insight into the clinical and basic factors guiding postnatal maturation of the neonatal TLR system might identify new targets for the prevention of AD.

Cord blood vitamin D deficiency predisposes to RSV bronchiolitis

Vitamin D is an essential nutrient and hormone with extensive immune modulatory functions. Basic and clinical studies suggest that vitamin D may protect against RSV infection. In **Chapter 8,** we hypothesized that vitamin D might exert its protective effects during fetal development of the airways and immune system. To address this hypothesis, we prospectively related cord blood concentrations of 25-hydroxyvitamin D (25-OHD) to the risk of RSV LRTI. Cord blood vitamin D insufficiency was highly prevalent, affecting 54% of newborns. Concentrations of 25-OHD in cord blood plasma were significantly lower in the neonates who subsequently developed RSV LRTI compared to those who did not. Neonates born with 25-OHD concentrations <50 nmol/L had six fold increased risk of RSV LRTI in the first year of life compared with those with 25-OHD concentrations ≥75 nmol/L. We conclude that vitamin D deficiency is highly common in healthy term neonates and is associated with increased risk of RSV LRTI in the first year of life. Intensified routine vitamin D supplementation may be an effective strategy to prevent RSV LRTI during infancy.

Conclusions and discussion

Reflecting on the research in this thesis within the context of related literature, our main findings are that early life immune development provides a unique opportunity for interventions aimed at preventing atopy and infectious diseases. Suggested strategies include promotion of breast feeding, intensified vitamin D supplementation during pregnancy, and plasma-directed therapies. However, the studies presented in this thesis are observational, and randomized clinical trials are urgently needed to establish the feasibility of early life immune modulation in the prevention of subsequent disease. Advancement of these trials is hampered by the unique position of pregnant women and their offspring as research subjects. Novel guidelines for translational research during the perinatal period are urgently needed to increase our insight into early life immune development and to utilize this important period in the prevention of subsequent disease.

Chapter 10

General Discussion
Plasma: directing the immune orchestra

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Introduction

The immune system is like an orchestra. All sections of the orchestra contribute to the beauty of the symphony, yet no player can create a masterpiece on his own. Whether it is a peaceful ballad or a furious tango, the quality of the final piece depends on continuous interactions between the sections. If one or multiple sections are out of rhythm or tune, the quality of the music is greatly affected. In order to achieve such harmony, the director coordinates the interplay between the sections, determines the pace and dynamics of the orchestra, and tailors this to the specific requirements of the audience. In this final chapter, we will attempt to integrate our findings on the neonatal immune orchestra with pre-existent knowledge on its players and their mutual interactions. Finally, we will speculate on a potential role for plasma as the director of the neonatal immune orchestra.

The orchestra

The neonatal immune system is markedly distinct from the adult innate immune system (reviewed in **Chapter 2**). In general, neonatal innate immune responses are biased against production of pro-inflammatory, Th1-type immune responses. This bias is an effective strategy to cope with the unique challenges of the neonatal period, including maintaining tolerance to maternal antigens in utero and balancing the transition from the sterile intrauterine environment to the antigen-rich outside world[1]. However, decreased generation of Th1-type immune responses leaves the neonate highly susceptible to infection and limits responses to vaccination at neonatal and infant age[2].

Identification of the factors that contribute to decreased neonatal Th1-type immune responses might result in new strategies to protect this vulnerable population. Moreover, insight into the mechanisms that maintain neonatal immune tolerance in its highly challenging environment might inspire novel therapies for auto-immune and inflammatory diseases in later life.

The players

Innate

Multiple differences have been identified between neonatal and adult innate immune cells, including neutrophils and antigen-presenting cells (APC).

At birth, human neonates have a higher proportion of immature neutrophils with decreased expression of complement receptor 3 (CR3) and L-selectin[3, 4]. Neonatal neutrophils are impaired in many functions, including chemotaxis, rolling adhesion, transmigration and lamellipodia formation[5]. In addition, neonatal neutrophils have reduced capacity to form neutrophil extracellular traps (NETs), and contain decreased amounts of the antimicrobial peptides lactoferrin and bactericidal-permeability increasing factor (BPI), which correlates with their decreased activity against Gram-negative bacteria[6].

Neonatal APC are also distinct from their adult counterparts. At birth, cord blood APC, including monocytes, conventional dendritic cells (cDC) and plasmacytoid DC (pDC) produce less IL-12p70, IFN-α and TNF-α compared to adult APC upon *in vitro* stimulation with most TLR agonists[7, 8]. In addition, cord blood myeloid DC (mDC) display impaired TLR3- or TLR4-mediated phenotypic maturation, failing to increase membrane expression of the co-stimulatory molecules CD40, CD80 and HLA-DR[9, 10].

Together, impairments in neonatal innate immunity contribute to the decreased neonatal capacity to generate pro-inflammatory immune responses needed for defense against pathogens.
In **Chapter 4**, we aimed to characterize the postnatal development of the neonatal innate immune system. We demonstrate that neonatal whole blood TLR-mediated cytokine responses differentially mature during the first month of life. Whereas TLR3-mediated IL-12p70 production and TLR7- and TLR9-mediated IFN-α production reach adult levels before the age of one month, TLR4-mediated responses are polarized towards low IL-12p70 and high IL-10 production throughout the first month of life. Decreased neonatal propensity to produce Th1-type cytokines upon TLR4-stimulation might contribute to infections with pathogens that are recognized by TLR4, including Gram-negative bacteria and RSV[11].

Adaptive
Neonatal adaptive immune responses are also distinct from adult responses. Neonates have decreased whole blood $CD4^+$ and $CD8^+$ T cell numbers, which have a more naïve phenotype compared with adult T cells[12]. Functionally, both neonatal $CD4^+$ and $CD8^+$ T cells produce decreased amounts of IFN-γ and TNF-α upon bacterial stimulation compared to their adult counterparts[12]. In addition, cord blood contains increased amounts of $CD4^+CD25^+$ regulatory T-cells, with increased inhibitory potential compared to adult regulatory T cells[13].
Multiple differences between neonatal and adult B-cells have also been described. Neonates have increased percentages of naïve B-cells that fail to up regulate expression of CD86 and MHCII in response to antigen receptor ligation[14, 15]. Additionally, neonatal antibody responses are delayed in onset, reach lower peak levels, are of shorter duration and differ in the distribution of IgG isotypes compared to adult responses[14].

Neonatal immunity: impaired or regulated?
Together, distinct neonatal innate and adaptive immune responses have led to the consideration of the neonatal immune system as 'generally impaired'. However, production of certain cytokines, including IL-6, IL-10 and IL-23 by neonatal antigen presenting cells actually exceeds that of adult cells[8, 16, 17]. In addition, multiple findings indicate that under certain circumstances, neonatal immune cells are capable of mounting adult-like responses. First, when stimulated in vitro with agonists of TLR8 or with whole group B streptococcus, cord blood mononuclear cells (CBMC) produce similar or increased amounts of cytokines (including IL-1β, IFN-y and TNF-α) compared to adult peripheral blood mononuclear cells (PBMC)[17-19]. Second, certain vaccines, including *Mycobacterium bovis* bacillus Calmette-Guérin, induce memory Th1-type responses of similar magnitude when given at birth or later in life[20, 21]. Third, in chapter 4, we demonstrate that in contrast to the marked impairment in TLR7-mediated IFN-α production, cord blood TLR9-mediated IFN-α responses are already comparable to adult level. Together, these findings indicate that the neonatal immune system is not generally impaired, but rather suggest that suppression of pro-inflammatory immune responses reflects a highly regulated system tailored to the unique challenges of the neonatal environment.

Table 1: Anti-inflammatory factors in human plasma

Factor	Effect	Reference
Cytokines		
IL-4	↓ monocyte/macrophage pro-inflammatory cytokine production	73
IL-10	↓ monocyte activation, ↓ pro-inflammatory cytokine production	74
IL-13	↓ monocyte/macrophage pro-inflammatory cytokine production	73
TGF-beta	↓ Th1-type cytokine responses, ↑ regulatory T-cells	75
Antagonists of specific cytokines		
IL-1 receptor antagonist	↓ IL-1 mediated cytokine synthesis	76
Soluble IL-1 receptor	↓ IL-1 mediated cytokine synthesis	77
Soluble IL-2 receptor (sCD25)	↓ IL-2 mediated immune activation	78
Soluble IL-4 receptor	↓ IL-4 mediated immune activation	79
Soluble IL-7 receptor (sCD127)	↓ IL-7 mediated immune activation	80
Soluble TNFα receptor	↓ TNF-α mediated immune activation	81
Soluble gp130	↓ IL-6 mediated immune activation	82
Soluble human IL-23 receptor α	↓ IL-23 mediated immune activation	83
Immune globulins		
IgG*	↓ Fc receptor expression and function; ↓ DC activation, ↓ Th1-type cytokines, ↓ NK cell activation, ↑ Treg function	34, 84
IgA*	↓ Fc-αR-mediated TNF-α and IL-6; ↓TLR4-mediated IL-6 and TNF-α, ↑ IL-1RA	85, 86
Hormones		
β-endorphin	↓ neutrophil respiratory burst; ↓ T-cell mediated IL-2 and IFN-γ	87, 89
Cortisol	↓ IL-1, IL-6 and TNF-α by APC; ↓ Th1-type responses	90, 91
Estrogen	↓ TNF-α, IFN-γ, IL-12, ↑ Th2-type responses	92
Testosterone*	↓ macrophage activation, ↑ regulatory T-cells	93, 94
Metabolites		
Adenosine	↓ TLR-mediated TNF-α, IL-12.	95, 96
ATP	↑ IL-23; ↓ IL-12 (however, also pro-inflammatory capacity)	97, 98
Lipids		
Apolipoprotein A1	↓ chemotaxis, ↓ adhesion, ↓ NF-κB activation	99
HDL	↓ intracellular reactive oxygen species; ↓ proteasome activation; ↓ NF-κB activation	100
LDL-lnhibitor	↓ lymphocyte activation	101, 102
N-3 polyunsaturated fatty acids	↑ TLR4-mediated IL-10 and ↓ IL-12p70 production by DC, ↓ T-cell activation	103, 104

Factor	Effect	Reference
Carbohydrates		
Mannose*	↑ NF-κB activation, ↓ TLR4-mediated DC maturation, ↑ TLR4-mediated IL-10, IL-12p35, IL-12p40, IL-6.	54, 105
Fucose	↑ TLR4-mediated IL-10, ↑ TLR4-mediated IL-12p35, IL-12p40, IL-6.	105
Vitamins		
Vitamin A	↓ Th1-cell cytokines; ↑ Th2-type responses; ↑ IL-4 gene expression.	106
Vitamin D	DC: ↓ maturation, ↓ expression of MHCII, CD40, CD80 and CD86, ↓ IL-12, ↑ IL-10. T-cells: ↓ Th1 and Th17-cell responses, ↑ Treg generation. B-cells: ↓ proliferation, ↓ plasma-cell differentiation and ↓ IgG production.	106
Minerals		
Zinc	↑ cAMP, ↓ NF-kB activation, ↓ LPS-induced TNF-α (also pro-inflammatory effects)	107
Adipokines		
Adiponectin	↓ granulopoiesis; ↓ NK-cell cytotoxicity; ↓ phagocytosis, ↓ TNF-α; ↓ IFN-γ; ↑ IL-10; ↑ IL-1RA	108
Other		
Activin	↓ DC maturation	109
Albumin	↓ TLR-4 mediated TNF-α	31
BPI	↓ LPS-mediated immune activation	110, 111
C5a	↓ monocyte TLR-mediated IL-12, IL-23, IL-27	112
Clara cell protein	↓ IFN-γ production and function	113
CRP	↑ IL-10, ↑ Treg, macrophage reprogramming	114
Exosomes	↓ profliferation of activated $CD4^+$ T-cells	115
Hepatocyte growth factor	↓ DC antigen presentation	116
Histamine*	↓ Th1-type and ↓ Th2-type responses through interaction with HR2	117
Inhibin	↓ DC maturation	109
Lysozyme	↓ neutrophils chemotaxis; ↓ lymphocyte activation and proliferation; ↓ complement activation	118, 119
Nitric oxide	↓ T-cell mediated IFN-γ	120
P-selectin	↑ tolerogenic DC; ↑ regulatory T-cells	121
Prostaglandin E2	↓ IL-12; ↓ IL-6; ↓ TNF-α; ↑ IL-10	122
Soluble CD8	↓ cytotoxic T-cell activation	123
Soluble CD40 ligand	↓ TLR4-mediated IL-12 (however, also pro-inflammatory capacity)	124
Vascular Endothelial Growth Factor	↓ DC maturation; ↓ T-cell development	125, 126

Table 2: Clinical trials using plasma therapy for immune-mediated diseases

Disease	Study Phase	Design	Treat-ment	Regimen	Concomittant treatment	Results	Ref
ANCA-associated vasculitis	III	RCT (aimed n=500)	PEX	7 cycles	Glucocorticoids	Ongoing (NCT 00987389)	[127]
Multiple sclerosis	I/II	Randomized trial (n=40)	PP	3 cycles in 3 mo	Mitoxantrone	Ongoing (NCT 01214317)	[127]
Idiopathic crescentic glomerulonephritis	I/II	Prospective randomized trial (n=32)	PEX	Not reported	Methylprednisolone, prednisone, azathioprine	No effect	[128]
Polyneuropathy associated with monoclonal gammopathy of undetermined significance	III	Double-blind placebo-controlled RCT (n=39)	PEX	6 cycles in 3 wks	None	Improvement in clinical symptoms	[129]
Systemic lupus erythematosus	III	Randomised trial (n=20)	PEX	6 cycles in 2 wks	None	Reduction in serum Ig, no difference in clinical improvement	[130]
Guillain-Barré syndrome	n/a	Meta-analysis of existent trials	PEX	n/a	Supportive care	Shorter time to recovery, improvement in clinical symptoms	[131]
Multiple myeloma with acute renal failure	III	RCT (aimed n=280)	PEX	7 cycles in 2 wks	Dexamethasone, chemotherapy	Ongoing (NCT 00416897	[127]
Nephrogenic fibrosing dermopathy/nephrogenic systemic fibrosis	IV	RCT (aimed n=32)	PP	One to three 5-daily courses	None	Ongoing (NCT 01078987)	[127]
Acute liver failure	II	RCT (aimed n=183)	PEX	3 cycles in 3 days	Standard medical treatment	Ongoing (NCT 00950508)	[127]
Maternal red cell alloimmunization	n/a	Retrospective multicenter case series (n=9)	PP	3 cycles in 6 days	IVIG	100% survival, significant reduction in maternal antired cell titres.	[132]

Disease	Study Phase	Design	Treat-ment	Regimen	Concomittant treatment	Results	Ref
Proliferative Lupus nephritis	II	RCT (n=18)	PP	1-2 weekly cycles for 6 months	Corticosteroids	Reduction in proteinuria and number of urinary podocytes	[133]
Acute immune thrombocytopenic purpura	II	RCT (n=13)	PEX	7 cycles in 7-10 days	Prednisone	Reduced anti-platelet antibody in 10/12 pts. No difference in response rate	[134]
Hepatitis C genotype I virus infection	III	RCT (n=10)	PP	5 cycles in 10 days	Pegylated Interferon-α, Ribavirin	20% sustained viral response	[135]
	IV	RCT (aimed n=116)	PP	5 cycles in 10 days	Pegylated Interferon-α, Ribavirin	Ongoing (NCT 00977054)	[127]
Idiopathic pulmonary fibrosis	I/II	Non-randomized feasibility study (aimed n=10)	PEX	Single exchange of 1.5x estimated plasma volume	Rituximab, corticosteroids	Ongoing (NCT 01266317)	[127]
Myasthenia gravis	III	RCT (n=87)	PEX	5 cycles in 10 days	None	Ongoing (NCT 01179893)	[127]
Inflammatory demyelinating disease	III	Double-blind RCT (aimed n=22)	PEX	7 cycles in 14 days	None	Ongoing (NCT 00004645)	[127]
Childhood Guillain-Barre Syndrome	I/II	RCT (aimed n=170)	PP	5 cycles in 10 days	None	Ongoing (NCT 00004833)	[127]
Thrombotic microangiopathiy	n/a	Retrospective analysis (n=17)	PEX	Daily exchange until normalizat io of platelet count	None	13/17 complete remissions, 2/17 partial remissions, 2 no response	[136]
Critical care patients	I/II	RCT (n=70)	PT	Not reported	Standard medical treatment	Ongoing (NCT 00302965)	[127]

RCT: Randomized controlled trial; PP: plasmapheresis; PF; plasma filtration.

The director

Plasma-mediated regulation of the immune system
Previous studies aimed at characterizing the mechanisms regulating the neonatal immune system have mainly focused on differences between neonatal and adult immune cells[22-27], and the contribution of the soluble fraction of the blood has been relatively underappreciated. Because all blood leukocytes are suspended in plasma, continuous reciprocal interactions between these cells and their environment are likely to exist. Plasma is a complex mixture of numerous factors with immune modulatory potential. The distinct composition of neonatal plasma might thus play a key role in suppression of the immune system.

Plasma-mediated suppression of the TLR system
In **Chapter 6**, we studied the effect of neonatal plasma on TLR-mediated cytokine responses. We demonstrate that mononuclear cells (MC) suspended in neonatal plasma produce decreased TLR4-mediated IL-12p70 and increased IL-10 compared to MC suspended in adult plasma. This was not specific for TLR4, as neonatal plasma also suppressed IL-12p70 production and induced IL-10 production in response to agonists for TLR3 and TLR8. Through heat-inactivation and protein-depletion, we show that neonatal plasma modulates TLR4-mediated IL-12p70 and IL-10 production through distinct factors. Increased plasma concentrations of soluble factors that suppress TLR-mediated pro-inflammatory cytokine production might contribute to the high neonatal susceptibility to infection. Moreover, the observation that adult plasma also conferred concentration-dependent inhibition of TLR4-mediated IL-12p70 and induction of IL-10 production suggests that human plasma contains factors that suppress TLR-mediated production of pro-inflammatory cytokines throughout life.

One of the first studies demonstrating an anti-inflammatory effect of plasma on TLR-mediated immune responses was performed by Levy et al, who showed that neonatal plasma confers decreased production of TNF-α in response to TLR1/2, TLR2/6, TLR4 and TLR7[28]. In this study, titration of cord blood plasma to adult hemocytes suspended in 10% autologous plasma dose-dependently inhibited TLR1/2 mediated TNF-α production. Contrary to our observations for TLR4-mediated IL-12p70, these authors report that titration of adult plasma resulted in a dose-dependent increase in TNF-α production[29], suggesting that under certain circumstances, plasma (derived from adult donors) can support pro-inflammatory responses.
Additional (although indirect) evidence for an immunosuppressive role of plasma on the TLR system may be provided by the study of Kollmann et al[7], who aimed to provide a comprehensive overview of differences between the neonatal and adult TLR system. In this study, *in vitro* TLR stimulation was performed in whole blood (which essentially consists of cells in 100% plasma) and in mononuclear cells suspended in medium containing 10% AB serum. Although no formal comparison of these two models was made, distinct patterns can be observed. Compared to mononuclear cells in 10% serum, whole blood responses were characterized by decreased TLR3-, TLR7- and TLR9-mediated IFN-α, decreased TLR3-mediated IFN-y and decreased TLR3-, TLR4- and TLR8-mediated IL-12p70, consistent with a Th1-suppressive effect of plasma. Evidently,

next to plasma concentrations, many other differences between the whole blood and PBMC models (e.g. presence of neutrophils, red blood cells, platelets) may contribute to these observations. However, this study, together with the findings by Levy et al and our own finding that plasma dose-dependently inhibits TLR4-mediated IL-12p70 production (**Chapter 6**), strongly hints towards an anti-inflammatory effect of plasma on TLR-mediated cytokine responses.

Immune suppression by plasma: beyond TLRs

In addition to suppression of TLR-mediated pro-inflammatory cytokine production, preliminary findings in our laboratory (data not shown) demonstrate that plasma also dose-dependently suppresses T-cell mediated IL-2 production. This suggests that plasma might have a more general inhibitory effect on the immune system, suppressing both innate and adaptive immune responses. Indeed, suppressive effects of plasma have been described on many immune cells, including neutrophils, NK-cells, macrophages, dendritic cells, Th1-, Th17- and cytotoxic T-cells (Figure 1). In addition, plasma contains multiple factors that stimulate the proliferation and function of regulatory T cells, thereby further contributing to its immune suppressive capacity.

Relatively little is known on the effects of soluble factors in plasma on Th2 cells, eosinophils and basophils. In **Chapter 6**, we demonstrate that plasma dose-dependently induces TLR4-mediated production of IL-10, suggesting that plasma might stimulate Th2 type immune responses. However, we were unable to detect any T cell mediated IL-4 in our assays (unpublished data). The effect of plasma on adaptive immune responses will be subject of further studies.

Plasma is a complex mixture of anti-inflammatory factors

The observation that plasma has anti-inflammatory capacity has triggered numerous studies characterizing individual plasma factors causing this effect. To generate an overview of plasma factors with potential immune suppressive activity, we performed a systematic literature search, searching PubMed for synonyms of “plasma” and “anti-inflammatory” (for search strategy, see supplementary figure 1). The resulting immune suppressive factors and their mode of action are shown in Table 1. Below, we will elaborate on the immune suppressive effects of the most abundant plasma proteins, lipids and carbohydrates.

Anti-inflammatory effects of plasma proteins

Proteins represent the most commonly studied immune modulatory constituents of human plasma. With an average plasma concentration of 3.4-5.4 g/dL, *albumin* is the most abundant protein in human plasma, accounting for ~50% of the total protein content[30]. In a mouse endotoxemia model, infusion of phosphate-buffered saline (PBS) containing 4% albumin significantly reduces mortality compared to PBS only[31]. In humans, albumin-containing solutions are commonly used in the resuscitation of critically ill patients[32]. In addition to increasing intravascular oncotic pressure, suppression of pro-inflammatory innate immune responses might be one of the mechanisms by which albumin mediates its beneficial effect. In vitro, albumin inhibits TLR4-mediated TNF-α production by human umbilical vein endothelial cells in vitro[31]. Moreover, whereas albumin solutions are not superior to crystalloid solutions for treatment of patients with burns or hypovolemia, they

do decrease mortality in patients with sepsis[33]. Thus far, no studies have addressed the direct effect of albumin on immune cells in vitro or in vivo, and this should be subject of further investigation.
The second most abundant protein in plasma is *immune gamma-globulin* (IgG), which has a concentration of 0.74 mg/dL (11% of total protein content)[30]. IgG has a dual role in the immune system. One the one hand, it has important immune-enhancing effects, including agglutination, opsonization and neutralization of microbial pathogens[34]. On the other hand, increasing evidence indicates that IgG has important immune suppressive effects[34]. Initially used as a replacement therapy for immune deficient patients, intravenous immunoglobulin (IVIG) was noted to improve concomitant idiopathic thrombocytic purpura (ITP). In a subsequent study in children with ITP, high-dose IVIG resulted in a dramatic increase in platelet counts and often resolution of clinical disease[35]. Currently, IVIG infusions are a cornerstone in the treatment of a variety of inflammatory and autoimmune disorders, including idiopathic thrombocytopenic purpura, chronic inflammatory demyelinating polyneuropathy and Kawasaki disease[34]. Multiple mechanisms have been proposed by which IVIG mediates its immune suppressive effects, including Fc receptor blockade, inhibition of complement deposition, enhancement of regulatory T cells, accelerated clearance of auto antibodies, modulation of adhesion molecules and cell receptors and activation of regulatory macrophages through the FcγRIIb receptor (reviewed in [34]). Many of these mechanisms are likely to act in concert and additional mechanisms continue to be discovered[36]. For now, it can be concluded that immune suppression is an important strategy by which IgG limits excessive inflammation.

Anti-inflammatory effects of plasma lipids
Increasing evidence indicates that the lipid fraction of plasma has important immune suppressive effects. Patients with cholera or polymicrobial sepsis have grossly lipemic blood and high serum levels of *triglycerides*, leading to the term 'lipemia of sepsis'[37, 38]. Several in vitro and in vivo studies suggest that elevation of plasma lipids is an active immunomodulatory mechanism that serves to protect the host from excessive inflammation. In vitro, *chylomicrons* and *VLDL* bind endotoxin and inhibit its activity[39, 40]. In vivo, induction of hyperlipoproteinemia, through infusion of exogenous lipoproteins[41], synthetic emulsions[42], recombinant lipoproteins[43] or diet [44] protects mice against LPS-mediated toxicity. Moreover, hypolipidemic animals suffer increased LPS-induced mortality, which is reversed when serum lipids are restored to physiological range[41]. In humans, however, the effects of plasma lipids on LPS-induced toxicity are less clear. Although addition of a fat-rich emulsion inhibits LPS-induced TNF-α production in human whole blood in vitro[45], induction of hypertriglyceridemia by lipid infusion in healthy adult volunteers failed to reduce LPS-induced fever, leukocytosis or TNF-α release[46]. This lack of effect might be due to the abrupt bolus administration of LPS (which might saturate plasma lipids), to differences in metabolism or to differential effects of various lipid substances (e.g. saturated versus unsaturated phospholipids) on the immune system. In addition to endotoxin, serum lipoproteins also inhibit pro-inflammatory responses to bacterial lipopeptides[47], viruses[48-50] and even parasites[51, 52], suggesting that lipid-mediated immune suppression is a general mechanism to prevent excessive inflammation in response to a broad range of microbial stimuli.

Table 3: Immune indications for intravenous immune globulin (IVIG) therapy. Depicted are approved indications for IVIG according to the United States Food and Drug Administration[137] or recommended indications according to the United Kingdom Department of Health clinical guidelines[138]

FDA-approved indications	Off-label indications
Idiopathic thrombocytopenic purpura	Graft versus host disease following allogeneic bone marrow transplant or hematopoietic stem cell transplantation
Kawasaki disease	Polymyositis
Chronic inflammatory demyelinating polyneuropathy	Acute disseminated encephalomyelitis
Kidney transplant with high antibody recipient or an ABO incompatible donor	Autoimmune diabetic proximal neuropathy
Alloimmune thrombocytopenia	Central nervous system vasculitis
Autoimmune hemolytic anemia	Polymyositis
	Atopic dermatitis
	Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection
	Systemic vasculitides
	Acute antibody-mediated rejection following solid organ transplantation
	Systemic lupus erythematosus

Anti-inflammatory effects of plasma carbohydrates

Plasma *carbohydrates* and related substances significantly impact on the immune system. A specific family of pattern recognition receptors, the C-type lectin receptors (CLR), recognizes *mannose*, *fucose* and *glucan structures*. Although most studies on CLR focus on their interactions with pathogen-expressed oligosaccharides, many CLR ligands are also present in human plasma. For example, mannose and fucose, the main ligands for the CLR DC-SIGN, are highly present in human plasma[53] and are capable of suppressing TLR4-mediated production of IL-12p40 and IL-6[54]. In addition, fucose also increases TLR4-mediated production of IL-10[54]. Based on these data, signaling through CLR is thought an important evasion strategy for pathogens to escape the immune system[55]. However, the evolutionary conservation of these structures among many species suggests that CLR function is highly important for host survival[56-58]. Maintenance of immune tolerance in plasma might be one of these functions.

Immune suppression by plasma: functional considerations

The anti-inflammatory properties of human plasma may be understood in relation to its biological function. Plasma is an essential transport medium, supplying oxygen, nutrients to all cells of the body and allowing for communication between distant cells or organ systems. During infection, plasma serves to transmit signals from the affected organ throughout the body, alerting it to danger and initiating its defense mechanisms. Communication through plasma has multiple advantages over cell-cell contact. First, production of soluble factors allows for one cell to rapidly reach multiple target cells localized throughout the body. Second, whereas cell-cell contact is hampered by biological barriers[59], certain soluble factors, especially small lipophilic molecules, readily cross these barriers[60], thus providing a mechanism for immune signaling to shielded organs. Third, while cell-cell contact only transmits signals from a single cell to another, the presence in plasma of many factors produced by multiple organ systems provides a

cell with a more integrated picture of the host condition, allowing it to tailor its immune response in more detail.
Thus, plasma has the important task of rapidly distributing the signals that initiate host defense mechanisms during infection. However, the ubiquitous presence of plasma and the highly toxic nature of many of these signals[27] render this a highly dangerous task, which, if left unchecked, might result in lethal inflammation[61]. Therefore, suppression of pro-inflammatory immune responses might be a key function of plasma, allowing for the transport of danger signals throughout the body, without causing systemic inflammation to the host.

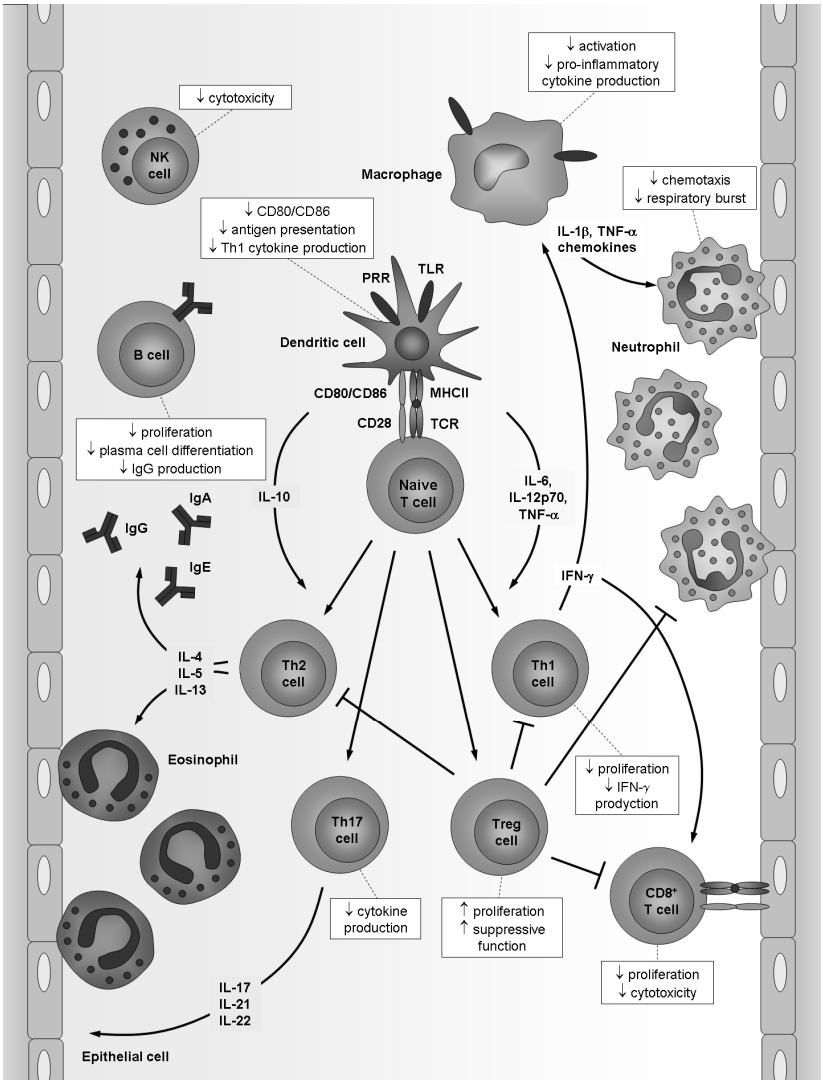
The symphony

The widespread presence of plasma and its interactions with virtually all organ systems in the body render the effects of plasma on the immune system highly relevant, not only as a biological phenomenon, but also as a therapeutic target. The notion that plasma has anti-inflammatory activity might provide a mechanistic explanation for the beneficial effect of various plasma products that are currently used in the treatment of multiple immune-mediated diseases. Moreover, plasma might provide an attractive target for prevention and/or treatment of infections and inflammatory disease.

Anti-inflammatory effects of plasma: culprit of neonatal infection
Enhanced suppression of pro-inflammatory cytokine production by neonatal plasma might contribute to the high neonatal susceptibility to microbial infection. Accordingly, reversal of plasma-mediated immune suppression might be an effective strategy to prevent and/or treat neonatal infection. One example of such an approach would be to transfuse neonates with adult plasma, assuming that dilution of the already present plasma reduces its inhibitory effect. In a case-report, treatment of a neonate with disseminated echovirus infection with seventeen transfusions of 10 ml/kg maternal plasma from day 6 to day 21 of life resulted in complete recovery[62]. Although the authors primarily attributed this to echovirus-specific antibody in maternal plasma, reduction of plasma-mediated immune suppression might be an additional mechanism for this beneficial effect. Despite its obvious limitations, this study provides the first evidence that transfusion with adult plasma might offer a feasible and safe strategy to treat neonatal infection.

Anti-inflammatory effects of plasma: useful in the treatment of inflammatory disease
In inflammatory or auto-immune disease, the anti-inflammatory capacity of plasma might be overruled, resulting in excessive inflammation. In these diseases, restoration of plasma-mediated immune suppression might have a beneficial effect on disease outcome. Indeed, advantageous effects of plasma therapy have been reported for multiple diseases that are caused by excessive inflammation (Table 2). Moreover, IVIG, a plasma product consisting of the pooled IgG of over one thousand donors, is FDA-approved for the treatment of idiopathic thrombocytopenic purpura, Kawasaki disease, and chronic inflammatory demyelinating polyneuropathy and is used off-label for many other immune-mediated diseases (Table 3).

Figure 1: Targets for plasma-mediated immune suppression.
Human plasma suppresses pro-inflammatory immune responses at multiple levels. Plasma contains multiple factors that inhibit innate immune antimicrobial mechanisms, including NK-cell cytotoxicity, neutrophil proliferation, chemotaxis and respiratory burst. In addition, plasma suppresses expression of MHC class II and costimulatory molecules and production of pro-inflammatory cytokines by antigen presenting cells. Adaptive immune cells are also responsive to the anti-inflammatory effects of plasma. Multiple plasma factors serve to decrease proliferation and effector mechanisms of Th1-, Th17 and cytotoxic T-cells. In contrast, generation and function of regulatory T cells (Treg) is induced. Finally, vitamin D in plasma suppresses proliferation, activation and immune globulin production by B-cells.
NK-cell: natural killer cell; PRR: Pattern-recognition receptor; TLR: Toll-like receptor.



An example for the use of plasma therapy to treat excessive inflammation is thrombotic thrombocytic purpura (TTP). The majority of patients with TTP have auto antibodies against von Willebrand factor (vWF)-cleaving protease, resulting in ultra large vWF-fragments which attract and activate platelets and induce a vicious cycle of widespread thrombosis, systemic endothelial injury and inflammation. Plasma therapy (plasmapheresis, plasma exchange or plasma transfusion) is highly successful in the treatment of TTP, decreasing mortality from 90% to 10%[63]. However, controversy still exists whether this effect is due to removal of toxic substances or to repletion of a deficient factor[64, 65]. In light of this controversy, although plasma exchange is considered the most effective treatment of TTP, simple transfusion of plasma (i.e. without removing any patient plasma) is also beneficial, suggesting that repletion of an immune suppressive factor might account for its effect.[66]. In summary, the example of TTP might provide proof-of-principle evidence that plasma therapy can be used in the treatment of immune disease. As plasma is affordable and readily available worldwide, the potential of plasma to inhibit pro-inflammatory responses is of substantial interest as a novel immune suppressive therapy for a wide variety of diseases.

Cord blood might provide a highly potent source of plasma for treatment of immune disease

In chapter 6, we demonstrate that cord blood plasma confers increased inhibition of TLR4-mediated IL-12p70 and increased induction of IL-10 compared with adult plasma. Based upon this observation and on previous studies showing that cord blood plasma inhibits of TLR-mediated pro-inflammatory cytokine production[8, 28], we propose that the immune suppressive effects of plasma therapy can be enhanced when cord blood plasma is used instead of adult plasma.

In addition to enhanced immune suppression, cord blood plasma transfusion may have additional advantages over adult plasma transfusion. First, some of the complications associated with adult plasma transfusions (e.g. transmission of infections, alloimmunization) may be less evident when cord blood is used as a plasma source[67]. Second, with the advent of cord blood banking, the collection and storage of cord blood plasma for autologous therapy in subsequent life has become a feasible goal. However, for now, the plasma volume required for transfusion (1 to 1.5 times the circulating volume, i.e. 3-4 L plasma for a 70 kg adult), necessitates the use of pooled plasma of multiple donors. In future, optimization of plasma collection and transfusion strategies will be needed to enhance the feasibility of this approach. In addition, clinical trials are needed to investigate whether the anti-inflammatory effect of cord blood plasma *in vitro* can be translated *in vivo* to enhance the efficacy of plasma therapy in patients with immune disease.

Potential risks of plasma therapy

Despite its widespread clinical use, there are several risks associated with plasma therapy. In clinical trials, the most common adverse events are mild to moderate nausea and dizziness, which occur in 10% of patients and are self-limiting[68]. More serious complications of plasma therapy include hypotension, bradycardia, haematoma, coagulation abnormalities, alloimmunization and infection[69]. Some of these adverse events (hypotension, coagulation abnormalities) are due to removal of a large volume of

plasma in plasma exchange therapy and are less likely to occur with plasma infusion. Theoretically, the use of cord blood plasma instead of adult plasma could reduce the risk of infection and alloimmunization. Large scale clinical trials are required to evaluate the effectiveness and potential side effects of plasma therapy prior to its widespread application in clinical practice.

Conclusions and directions for future research

In conclusion, in addition to previous studies demonstrating immune suppressive effects of whole neonatal plasma[28, 29] or of individual plasma components[70-72], the results in this thesis demonstrate the age-dependent presence in plasma of multiple factors that collectively serve to suppress stimulus-induced production of pro-inflammatory cytokines while inducing production of IL-10.

In neonates, enhanced plasma-mediated immune suppression might predispose to microbial infection. In adults, insufficient plasma-mediated immune suppression might result in excessive inflammation or auto-immune disease. Unraveling the interactions between plasma and the cellular part of the immune system is highly relevant, to enhance our understanding of the basic mechanisms regulating immune balance and for the development of novel anti-inflammatory therapies. What are the immune suppressive factors in plasma? Where are they produced? By which mechanism do they mediate their effect? How do these factors impact on host susceptibility to infection and auto-immunity? Can we use plasma as an immune-suppressive therapy for the treatment of inflammatory disease? Future translational studies aimed at answering these questions might result in new therapeutic strategies to prevent and/or treat infections and inflammatory disease.

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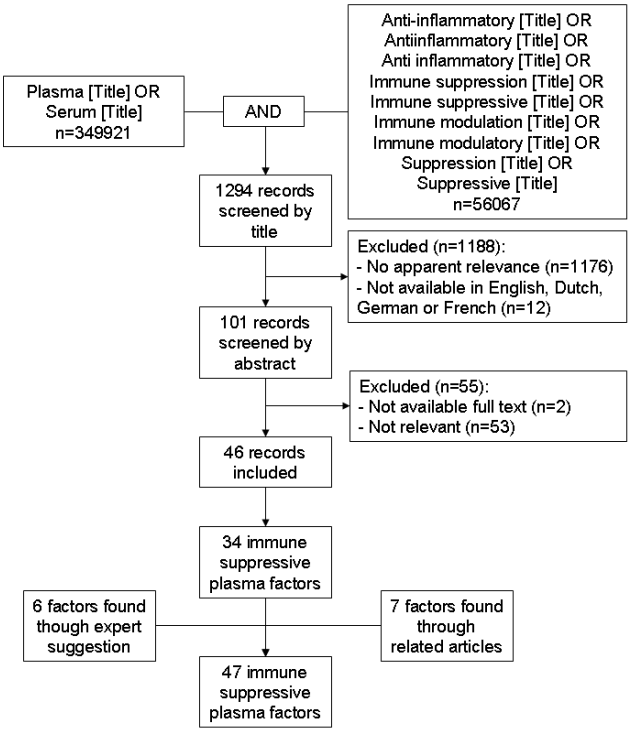
Supplementary information

Supplementary figure 1: Search strategy used to identify immune suppressive factors in plasma
A systematic Pubmed search was performed for articles with the key words "plasma" and "anti-inflammatory" or related synonyms in their title. In total, 1294 records were identified, which were subsequently screened by title. Excluded were papers with no apparent relevance (no immune suppressive factor or one that is not present in plasma) or papers written in a language other than English, Dutch, German or French. Abstracts of the resulting 101 articles were read, of which 46 articles were included, identifying 34 immune suppressive factors in plasma. Through related articles and expert's opinion, an additional 13 factors were found, resulting in 47 immune suppressive plasma factors described in table 1.



Chapter 11

Nederlandse samenvatting voor niet-ingewijden

Het afweersysteem van pasgeborenen is onrijp
Pasgeborenen (neonaten) hebben vaker en ernstiger infecties dan volwassenen. Dit komt door onrijpheid van het neonatale afweersysteem, dat ons beschermt tegen virussen en bacteriën. Toll-like receptoren zijn ‘sensoren’ van het aangeboren afweersysteem. Met een Toll-like receptor scant een afweercel continu zijn omgeving, op zoek naar lichaamsvreemde eiwitten die kenmerkend zijn voor virussen of bacteriën. Er zijn 10 verschillende Toll-like receptoren, die elk een ander viraal of bacterieel eiwit herkennen. Wanneer de afweercel met zijn Toll-like receptor een virus of bacterie vindt, dan start hij een afweerreactie, gericht op het vernietigen van het virus/de bacterie. Toll-like receptoren vormen zo onze eerste verdediging tegen infecties.

Afweer tegen lichaamsvreemde stoffen kan echter ook schadelijk zijn. Ieder mens heeft bijvoorbeeld miljoenen bacteriën in zijn darm, die helpen om onze voeding te verteren. Afweer tegen deze onschadelijke bacteriën leidt tot ontsteking van de darm. Zwangerschap is een tweede voorbeeld waarin afweer mogelijk schadelijk is. Tijdens de zwangerschap wordt de ongeboren foetus continu blootgesteld aan allerlei lichaamsvreemde eiwitten van de moeder. Afweer op deze eiwitten kan leiden tot ontsteking in de baarmoeder en tot vroeggeboorte. Overmatige afweer speelt bovendien een belangrijke rol in het ontstaan van vele ziektes, waaronder allergie en astma. Toll-like receptoren hebben dus de moeilijke taak om gevaarlijke indringers te onderscheiden van onschuldige prikkels. De mate van Toll-like receptor activatie en de resulterende balans tussen stimulerende en remmende signalen bepaalt of er een afweerreactie wordt gemaakt of niet.

Neonatale Toll-like receptor reacties zijn bij de geboorte onrijp en worden gekenmerkt door lagere productie van afweer stimulerende signalen en hogere productie van signalen die de afweer remmen. Onrijpheid van het neonatale afweersysteem is gunstig tijdens de zwangerschap: Het voorkómt vroeggeboorte ten gevolge van afweerreacties tegen maternale eiwitten en het bevordert bacteriële kolonisatie van de huid en de darm. Na de geboorte wordt de neonaat echter blootgesteld aan een buitenwereld vol met mogelijk schadelijke bacteriën en virussen. De verminderde afweer is nu nadelig en geeft een hogere kans op (ernstige) infecties. Bovendien is aangetoond dat afwijkende Toll-like receptor reacties bij de geboorte later een verhoogde kans geven op astma en allergie.

Er is veel bekend over de onrijpheid van neonatale Toll-like receptor reacties bij de geboorte. Daarentegen is er veel minder bekend over de ontwikkeling van neonatale Toll-like receptor reacties ná de geboorte en de gevolgen voor latere gezondheid en ziekte. Wij veronderstelden dat ontwikkeling van Toll-like receptor reacties al heel vroeg in het leven plaatsvindt, en dat verstoorde vroege ontwikkeling van het Toll-like receptor systeem een verhoogde kans geeft op latere infecties en allergie.

In dit proefschrift worden de volgende vragen beantwoord:
- Hoe verloopt de rijping van Toll-like receptor reacties na de geboorte?
- Welke (omgevings)factoren beinvloeden neonatale Toll-like receptor rijping?
- Wat is de basale oorzaak voor onrijpheid van het neonatale Toll-like receptor systeem?
- Geeft vertraagde rijping van neonatale Toll-like receptor reacties een verhoogde kans op virale luchtweginfecties en allergie op de kinderleeftijd?
- Geeft vitamine D tekort bij de geboorte een hoger risico op virale luchtweginfecties in het eerste levensjaar?

De eerste levensmaand is essentieel voor de rijping van het neonatale Toll-like receptor systeem

In **hoofdstuk 4** beschrijven we de vroege ontwikkeling van het Toll-like receptor systeem. Hiertoe vergeleken we aantallen afweercellen en Toll-like receptor reacties in navelstrengbloed van gezonde neonaten, veneus bloed van één maand oude neonaten en veneus bloed van volwassen vrijwilligers. Toll-like receptor gemedieerde productie van afweerstimulerende factoren (IL-12p70, IFN-α) in navelstrengbloed was lager dan in volwassen bloed, terwijl de productie van afweerremmende factoren (IL-10) juist hoger was. Op de leeftijd van één maand waren neonatale Toll-like receptor 3, 7 en 9 reacties al vergelijkbaar met volwassen reacties. Toll-like receptor 4 ontwikkelde zich echter veel langzamer, met aanhoudend lage productie van IL-12p70 en hoge productie van IL-10 tot op de leeftijd van één maand. Deze bevindingen tonen aan dat de eerste maand een essentiële periode is voor de ontwikkeling van het Toll-like receptor systeem. Inzicht in de klinische en basale determinanten van neonatale Toll-like receptor ontwikkeling biedt mogelijk nieuwe aangrijpingspunten voor preventie en behandeling van neonatale infecties en atopie op de kinderleeftijd.

Borstvoeding beïnvloedt neonatale Toll-like receptor reacties

In de eerste levensmaand wordt de pasgeborene blootgesteld aan vele factoren die de ontwikkeling van het Toll-like receptor systeem (en daarmee mogelijk het risico op infecties en allergie) beïnvloeden. Om te bepalen welke omgevingsfactoren de grootste invloed hebben op het neonatale Toll-like receptor systeem, onderzochten wij het effect van keizersnede, borstvoeding, geboortemaand, aanwezigheid van broertjes of zusjes, huisdieren en roken door ouders op neonatale afweercellen en Toll-like receptor reacties op de leeftijd van één maand (**Hoofdstuk 5**). Borstvoeding had de grootste invloed op het neonatale afweersysteem. Borstgevoede neonaten hadden viermaal lagere TLR7-gemedieerde IL-10 productie dan flesgevoede neonaten. Het beschermende effect van borstvoeding tegen infecties en allergie wordt mogelijk verklaard via beïnvloeding van neonatale Toll-like receptor ontwikkeling.

Neonatale TLR4-gemedieerde afweerreacties worden beïnvloed door plasma

In **Hoofdstuk 6** onderzochten we de basale oorzaak voor onrijpheid van het neonatale Toll-like receptor systeem. Is er een intrinsiek defect van neonatale afweercellen, of wordt hun functie geremd door signalen van buiten? Vanwege de relatief trage ontwikkeling in de eerste levensmaand (**Hoofdstuk 4**), concentreerden we ons op TLR4-gemedieerde productie van IL-10 en IL-12.

Dit hoofdstuk toont aan dat neonatale TLR4 responsen worden beïnvloed door signalen vanuit het plasma, de vloeistof die alle afweercellen in bloed omgeeft. Afweercellen omgeven door navelstrengbloed plasma produceerden meer TLR4-gemedieerde IL-12p70 (afweer stimulerend), en minder IL-10 (afweer remmend) dan diezelfde afweercellen omgeven door volwassen plasma. De invloed van plasma op TLR4 responsen was leeftijdsafhankelijk: Remming van IL-12p70 hield aan tot op de leeftijd van één maand, terwijl inductie van IL-10 op de leeftijd van één maand verdwenen was. De IL-12p70 remmende factor in plasma was ook aanwezig in eiwitvrij plasma, terwijl de factor die IL-10 stimuleert verdwenen was. Dit toont aan dat plasma meerdere factoren bevat, die gezamenlijk bijdragen aan de onrijpheid van het neonatale afweersysteem.
Manipulatie van deze factoren biedt nieuwe mogelijkheden voor de preventie en behandeling van neonatale infecties en atopie.

Lage neonatale TLR4-gemedieerde IL-10 productie is geassocieerd met atopisch eczeem
We veronderstelden dat verstoorde vroege ontwikkeling van het Toll-like receptor system een hoger risico geeft op virale luchtweginfecties en atopie. Om dit te toetsen relateerden we Toll-like receptor responsen op de leeftijd van één maand aan het risico op atopisch eczeem en op lagere luchtweginfecties door het respiratoir syncytieel virus (RSV) in het eerste levensjaar (**Hoofdstuk 7**). Neonaten met atopisch eczeem hadden op de leeftijd van één maand lagere aantallen basofiele granulocyten en plasmacytoide dendritische cellen, hogere aantallen natural killer cellen en tweemaal lagere TLR4-gemedieerde IL-10 productie dan neonaten zonder eczeem. Er waren geen verschillen in aantallen afweercellen, of in Toll-like receptor gemedieerde afweerreacties, tussen neonaten met en zonder RSV luchtweginfecties. De verschillen in afweerfunctie tussen neonaten met en zonder eczeem waren aanwezig vóór het ontstaan van huiduitslag, wat suggereert dat verstoorde afweer op de leeftijd van één maand een oorzaak is van eczeem.

Vitamine D tekort in navelstrengbloed geeft een hogere kans op ernstige RSV infectie
Vitamine D is belangrijk voor de botopbouw en voor een goede functie van ons afweersysteem. Klinisch en basaal onderzoek suggereert dat vitamine D beschermt tegen ernstige RSV luchtweginfecties. **Hoofdstuk 8** toont aan dat deze bescherming al vóór de geboorte ontstaat, tijdens de ontwikkeling van de foetale luchtwegen en het afweersysteem. In dit hoofdstuk bepaalden we vitamine D concentraties in navelstrengbloed en relateerden deze aan het risico op RSV luchtweginfecties in het eerste levensjaar. Vitamine D tekort kwam vaak voor: 54% van alle neonaten had onvoldoende vitamine D. Neonaten met een vitamine D tekort bij de geboorte hadden een zes keer hogere kans op ernstige luchtweginfectie met het RS-virus.
Vitamine D in navelstrengbloed is afkomstig van moeder. De gezondheidsraad adviseert dagelijks gebruik van vitamine D door alle zwangere vrouwen, maar slechts 46% volgt dit advies. Onze resultaten suggereren dat hoge inname van vitamine D tijdens de zwangerschap ernstige RSV luchtweginfecties in het eerste levensjaar kan voorkómen.

Conclusie en discussie

Dit proefschrift toont aan dat beïnvloeding van Toll-like receptor ontwikkeling in de eerste levensmaand unieke kansen biedt ter preventie van latere infecties en allergie. Voorgestelde strategieën zijn bevordering van borstvoeding, intensivering van vitamine D suppletie tijdens de zwangerschap en plasma-gemedieerde therapie. De studies in dit proefschrift zijn observationeel en gerandomiseerde trials zijn nodig om te bepalen of beinvloeding van vroege Toll-like receptor ontwikkeling daadwerkelijk ziekte voorkómt. Deze studies worden echter gehinderd door de uitzonderingspositie van zwangere vrouwen en hun ongeboren vrucht als proefpersonen. Nieuwe richtlijnen voor wetenschappelijk onderzoek in de perinatale periode zijn vereist om ons inzicht in vroege ontwikkeling van het afweersysteem te vergroten, en om deze unieke periode te benutten voor de preventie van latere ziektes, waaronder infecties en atopie.

About the author

Curriculum vitae

Mirjam Elisabeth Belderbos werd op 24 januari 1983 geboren te Doorn. In 2001 behaalde zij cum laude haar VWO diploma aan het Revius Lyceum aldaar. In datzelfde jaar ging zij geneeskunde studeren aan de Universiteit Utrecht. Tijdens haar studie deed zij acht maanden onderzoek in het laboratorium van Dr. Roberto Pili, Johns Hopkins University Baltimore, naar de rol van bloedplaatjes in het ontstaan van kanker. Dit onderzoek werd bekroond met de junior prijs van de Saal van Zwanenbergstichting. In 2008 rondde zij cum laude haar studie af. Hierna werkte ze drie maanden als ANIOS algemene kindergeneeskunde in het Wilhelmina Kinderziekenhuis te Utrecht. In oktober 2008 startte zij bij de afdeling immunologie van het Wilhelmina Kinderziekenhuis met het promotieonderzoek dat resulteerde in dit proefschrift. Tijdens dit onderzoek werd zij begeleid door dr. L. Bont, dr. G. van Bleek, prof.dr. J. Kimpen, en prof. dr. L. Meyaard. Als onderdeel van haar promotietraject werkte zij zes maanden in het laboratorium van dr. O. Levy, Harvard University, Boston. Dit promotietraject werd gefinancierd door het Nederlands Astma Fonds en door een persoonlijke onderzoeksbeurs van de Alexandre Suerman stichting van het Universitair Medisch Centrum Utrecht. Vanaf 1 juni 2011 werkt Mirjam in het Universitair Medisch Centrum Groningen, waar zij zich specialiseert tot kinderarts.

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Dankwoord

Promoveren is topsport. Na een korte warming up beklim je het startblok. Je kan nog net je brilletje en badmuts goed doen voordat het startschot klinkt. De eerste baantjes gaan soepel en snel. Dan het loodzware middenstuk, waarin de finish eindeloos ver weg lijkt. Na lang zwoegen komt uiteindelijk de finish in zicht. Een laatste eindsprint en voldaan tik je aan. Het was een mooie race…

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De coaches

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Het water

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Het thuisfront

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Mirjam, 2011