Lost in transition?

Immune regulatory mechanisms in early life

Sanne Birgit Elisabeth Anne Hoeks

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Lost in transition? Immune regulatory mechanisms in early life

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Address of correspondence
S.B.E.A. Hoeks
Diakonessenhuis, Department of Pediatrics,
PO Box 80250, 3508 TG Utrecht

E-mail: shoeks@diakhuis.nl

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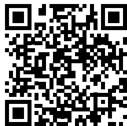

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Promotor:

Prof. dr. A.B.J. Prakken

Copromotoren:

Dr. I.M. de Kleer

Dr. W. de Jager

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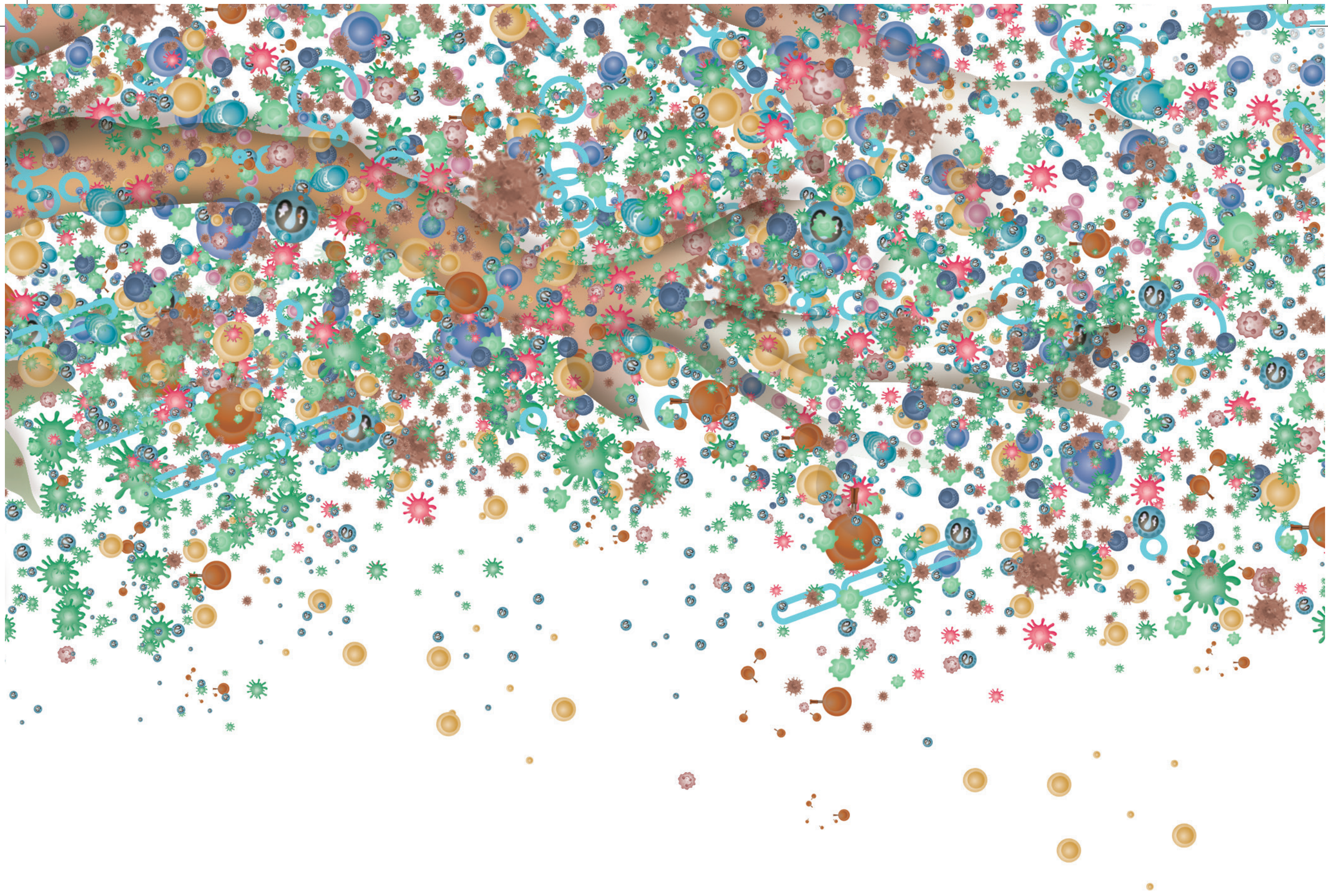


GENERAL INTRODUCTION

Based on
The origin of allergic diseases and the early development of the immune system.
Hoeks SB, de Kleer IM

Ned Tijdschr Allergie & Astma 2012;12:145-154

GENERAL INTRODUCTION

The prenatal and early postnatal immune system is highly dynamic and fundamentally different from the adult immune system.[1] The perinatal immune cells seem remarkably adapted to the different demands of their environment, the maternal-fetal interface and early postnatal life. Upon birth a neonate is suddenly exposed to thousands of new, mainly harmless, antigens present in food and in the microbiome colonizing the mucosae and skin. This new environment requests the active induction of immune tolerance on the one hand and the suppression of pro-inflammatory responses towards these harmless antigens on the other hand. This indicates that the initial dogmatic view of the neonatal immune system as immature and therefore deficient compared to that of an adult seems inadequate. The vulnerability of neonates to infections might simply be the price that needs to be paid by this highly advanced immune system that has priority in the effective induction of immune tolerance.

Although challenging throughout the entire lifespan, especially newborns are highly pressured by the balance of immune tolerance and inflammation. Once tolerizing memory to most harmless environmental antigens is established, demands on the infant's immune system shifts from a preferred tolerant state towards an immune system with priority in fighting harmful pathogens. This immune development from birth, throughout infancy, into adulthood is considered to be programmed but highly influenced by genes, environment and the interaction between them, so called epigenetics. Especially in the first weeks/months after birth this development is reflected in major shifts in immune cells.[2, 3]

Of importance, evidence is also mounting that immune system programming that starts in early life influences the risk of developing conditions such as allergic, autoimmune, reproductive, and neuropsychiatric disorders in later life. In addition, immune regulation early in life might serve as a model to identify new therapeutic strategies to restore immune tolerance upon immune dysregulation in clinical conditions like autoinflammation, autoimmunity and allergy.

HISTORICAL PERSPECTIVE

A successful pregnancy is a unique result of immune regulatory mechanisms in both the pregnant mother and her offspring. However, survival is not the only successful outcome parameter. The nine months in utero might be seen as one of the most critical periods in a person's life, shaping future capacities and health trajectories.[4] Two milestone publications set the stage for this theory: one on acquired immunological tolerance by Medawar (1963) and one on fetal programming by Barker (1989).

In 1963 Medawar demonstrated that in utero inoculation of donor strain cells will lead to acceptance of tissue grafts later in life (Fig 1).[5] This effect was specific for the inoculated strain as grafts from other strains were rejected.[6] These experiments proved that the immune system is not pre-programmed to distinguish between self and non-self but is shaped to a tolerant state as a result of exposure to self-molecules during early development.[7] This concept was called acquired immunological tolerance and newer research now suggests that his observations are applicable in a lot of other domains, amongst which are reproductive technology, inflammatory diseases, neonatal infections and vaccination strategies further underscoring the translational implications of this research.[8]

In 1989, Barker initiated the Developmental Origins of Health and Disease (DOHaD) hypothesis (Fig 2, *A*) with his report on the fetal environment in utero as a significant determinant of risk for adult-onset diseases.[9, 10] In studying coronary artery disease rates in the early twentieth century, it became apparent that birth weight was inversely correlated with increased early death secondary to coronary heart disease (Fig 2, *B*). Furthermore, rates of growth in the first two years of life has been associated with adult onset hypertension and type 2 diabetes.[11]

Medawar's immune tolerance concept and Barkers DOHAD hypothesis stress that the immune system in early life is functionally distinct and responsive to programming that persists into adulthood. During the perinatal period the immune system is shaped for an entire lifespan, "a foundation for a life time".

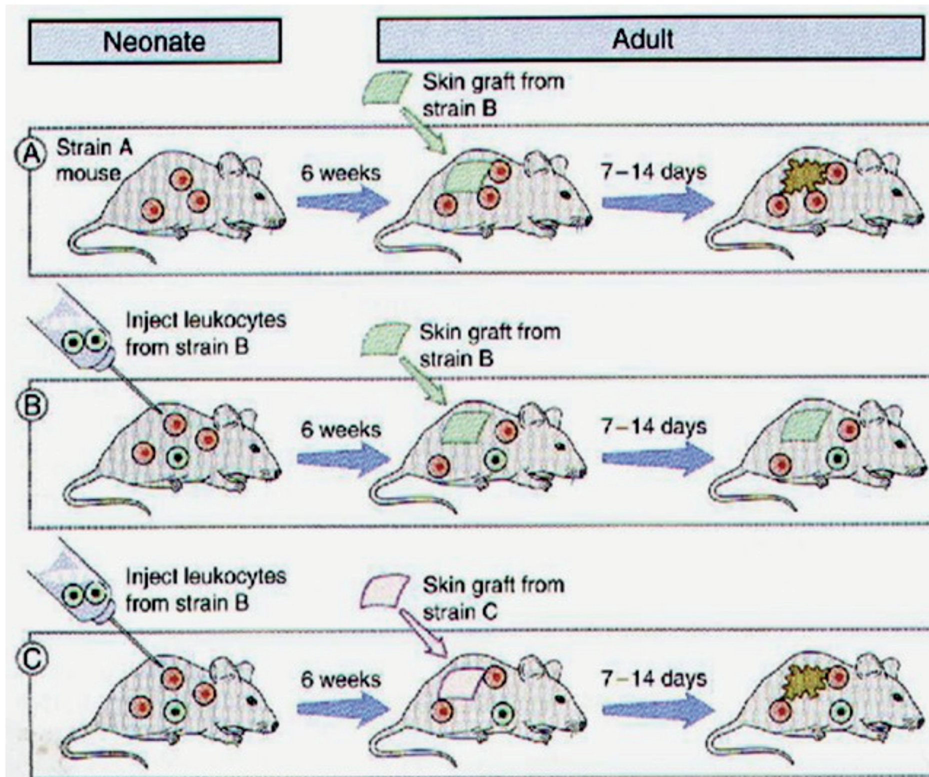


FIGURE 1. Induction of immunological tolerance in tissue transplants. A, An adult strain A mouse rejects a graft from a strain B mouse. B, If a neonatal strain A mouse is injected with strain B leucocytes (shown in red), when the mouse becomes an adult it fails to reject a strain B graft. C, This neonatally injected strain A mouse rejects grafts from other strains (e.g. strain C) indicating that tolerance is immunologically specific.

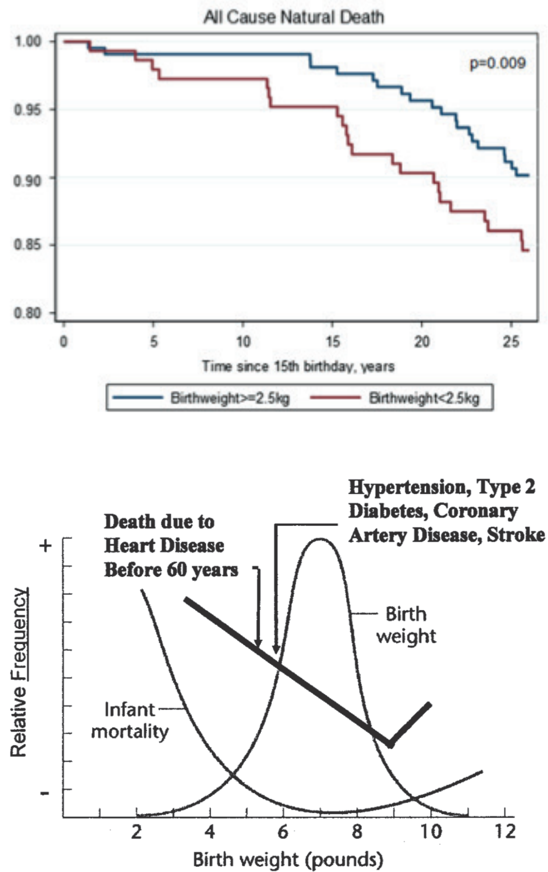


FIGURE 2. A, Kaplan–Meier curves for all-cause natural in first 25 years of adult life, adjusted for year of birth and sex, and categorized by low birth weight (<2.5 kg) versus higher. Adapted from: The Barker hypothesis confirmed: association of low birth weight with all-cause natural deaths in young adult life in a remote Australian Aboriginal community. *Journal of Developmental Origins of Health and Disease* 2019;10(1):55–62; Hoy WE and Nicol JL. B, Relation of birth weight to infant mortality and complex adult-onset disease. Adapted from: The Barker hypothesis: how pediatricians will diagnose and prevent common adult-onset diseases. *Transactions of the American clinical and climatological association 2009;120; Dover, GJ*

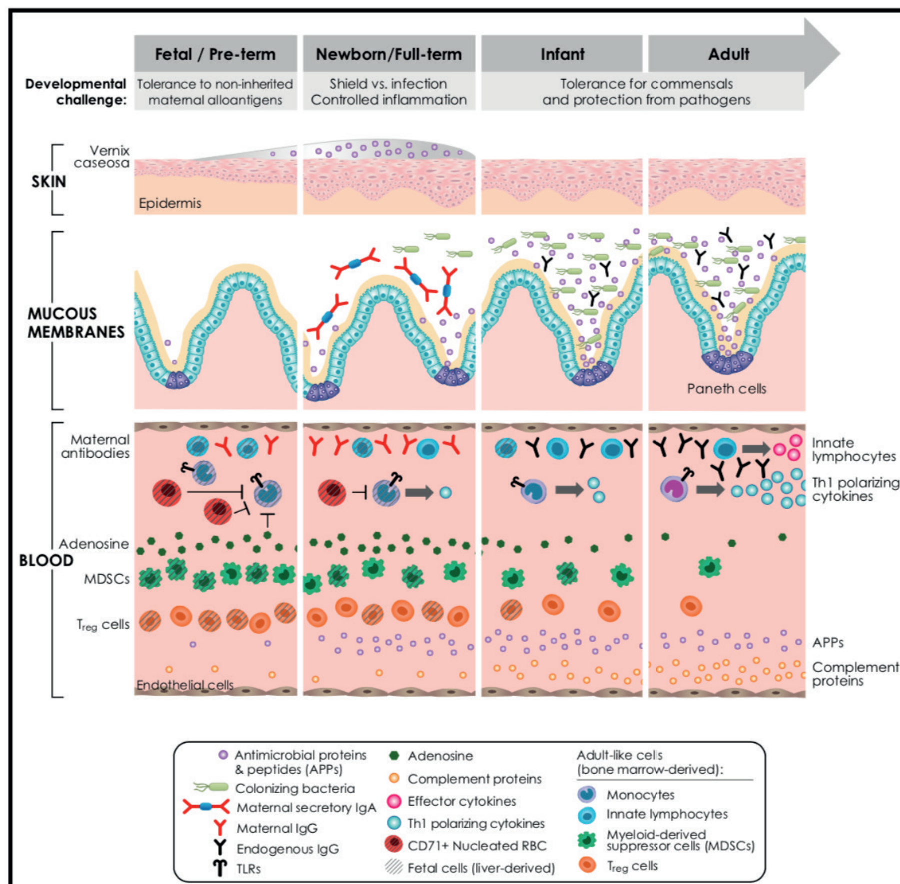
CHARACTERISTICS OF THE PERINATAL IMMUNE SYSTEM: SOPHISTICATED BUT VULNERABLE

Maintaining the delicate balance between immune tolerance against newly encountered but harmless antigens on the one hand and induction of potent immune responses against pathogens on the other hand seems to be an exceptionally complicated challenge of the neonatal immune system. Specific characteristics of perinatal immune cells are involved in this task. In the first place, neonates harbor an immune phenotype with complex immune regulatory mechanisms that match the unique environmental pressure and challenges during pregnancy and in the first days of life.[12] Myeloid-derived suppressor cells (MDSC) are present at high frequencies in early human life and could therefore play an important role in immune homeostasis. Soluble regulatory mediators are produced at high levels. These regulatory mediators, like adenosine, Transforming Growth Factor (TGF)β and prostaglandin E2 together with progesterone from the yellow body, encourage T_H2 cytokine production.[13-15] Additionally, fetal and neonatal T cells have a strongly regulated interferon (IFN)γ gene expression and are highly sensitive for interleukin (IL)-4.[16] Besides this T_H2 polarization, high circulating concentrations of IL-10 and high frequency of forkhead box P3 (FOXP3) regulatory T cells (Tregs) with corresponding immune suppressive capacities, are present respectively in neonatal blood and at mucosal sites.[17, 18] Infants have limited exposure to antigens in utero. As a consequence, neonatal T- and B-cell populations consist of mainly naive cells.

The sophisticated regulated perinatal immune responses come with a price. The neonatal immune system is shown to be less effective to clear infections upon microbial invasion. Intracellular pathogens cause more severe disease (e.g. disseminated herpes infections and tuberculosis in neonates) or replicate at higher levels in the fetus and young child as compared to the adult. Especially premature infants are at much higher risk for infection and this risk is inversely related to the gestational age. This is exemplified by infections with coagulase-negative staphylococci (CoNS), which are most common in infants born at gestational age below 30 weeks irrespective of confounding clinical factors and explained by the developmental difference in activity of toll-like receptor (TLR)-2.[19, 20] Most of the fetal and neonatal T-cells and B-cells are in a naive state and not equipped for a fast and adequate immune response. Furthermore, innate immunity although relatively high in numbers, is functionally not as effective as the adult innate immune system.[21] Antimicrobial immune recognition and antigen presentation are significantly impaired because of reduced numbers of dendritic cells (DC) and other antigen presenting cells (APC), as well as reduced receptor expression and reduced intracellular signaling in these cells.[22, 23] Neonatal DCs express less MHC class I and II, have decreased co-stimulatory capacity and an impaired IL12p70 production.[24-26] Components promoting pro-inflammatory T cell responses such as the complement system, are produced at lower levels in early life. In general fewer cytokines

per individual cell are produced.[27] Natural killer (NK) cells have a poor cytotoxicity, due to absence of adequate levels of activating cytokines. Neutrophils have a reduced chemotactic ability and a decreased capacity for adherence and extravasation from the bloodstream.[28] Moreover, functional assays have shown that the complement system exhibits at birth lower opsonizing capacity, relatively impaired chemotaxis and reduced lytic function.[29, 30]

To help initial immune responses, evolutionary conserved mechanisms like the production of superoxide are employed for effectively assisting in bacterial killing. Furthermore, neonates have obtained maternal immunoglobulins (Ig): transplacental transported IgG in the last trimester of pregnancy and maternal IgA secreted in human milk. However, these systems are not always sufficient enough to compensate for the ineffectiveness of the neonatal innate immune system to fight infections. Perinatal characteristics of immunological responses are summarized in figure 3.



◄ **FIGURE 3.** Ontogeny of Fetal, Neonatal, and Infant Host Defense. Host-protective barrier functions include physical, chemical, and functional components of the epithelial of skin and mucous membrane of the fetus, neonate (birth to 28 days of age), and infant (1 month – 1 year of age). These have to be understood in the context of age-specific developmental challenges as outlined near the top of the figure. A, Skin: While physical and chemical barriers are reduced early in life, especially in the preterm, the vernix caseosa and skin epithelia of full-term newborns robustly expresses antimicrobial proteins and peptides (APPs). B, Mucous membranes: In parallel with and induced by an increasingly complex microbiota, the newborn intestinal mucosal epithelium rapidly changes structurally with increase in crypts, and crypt-based Paneth cells, as well as functionally with increasing APP expression. C, Blood: The composition of neonatal blood is distinct, with relatively low concentrations of complement components and APPs and high concentrations of the immunosuppressive purine metabolite adenosine. Plasma also contains maternal antibodies transferred beginning mid-gestation, and supplemented by postnatal factors derived from breastmilk. Innate immunity is detectable from the end of the first month of gestation, with changes driven largely by the increasing exposure to environmental microbes. Neonatal APCs such as blood monocytes express pattern recognizing receptors (PRRs) (e.g., TLRs) with distinct functional responses including limited T_H1-polarizing cytokine production to most stimuli. Age-dependent differences in activity of interferon response factor (IRF) transcription factors as well as epigenetic changes contribute to this cytokine ontogeny. Adaptive immunity develops from 4 weeks of gestation onwards, with changes driven by an evolving chimerism reflecting fetal (liver-derived, shaded cells) Treg-rich lymphocytes and more adult-like (bone marrow- derived, not shaded cells) lymphocytes with distinct epigenetically encoded functional programs. Adapted from: Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny *Immunity* 2017;46:350-63; Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, and Levy O

PERINATAL TO ADULT IMMUNE DEVELOPMENT

Most research on the neonatal immune system has been performed with cord blood (CB) cells as a representative for the neonatal immune system. Though, this approach ignores the dynamic changes after birth but several ethical issues hamper research on children and especially neonates since withdrawal of blood is invasive, painful and a relatively large volume of the blood is required for functional immunological tests (10 ml of blood is 3,5% of the total blood volume in a term born baby). Therefore blood of (healthy) new-born babies and infants must be proportional to these issues, especially in repetitive blood sampling, which is needed to address questions on (functional) immune maturation.

In this thesis, we describe two alternative methods to address these questions on immune maturation. First, we followed newborns with a lip and/or palate cleft into their first year of life. This diagnosis is usually antenatally made and these infants need repetitive surgery at fixed time points in their first year of life. Therefore we were able to draw blood from the cord and subsequently three preoperative samples when the intravenous line was placed before surgery. We hypothesized that these infants represent healthy infants since their immune system is considered healthy. Despite some unsolved questions like the influence of anesthetics and perioperative antibiotics on immune maturation, this method generated new data about physiological immune maturation. Subsequently, we evaluated functional immune maturation in newborns with a viral infection. In this group of infants we generated ex vivo data of immune characteristics as a model for ultimate stress on the immune system.

The immune system undergoes major alterations after birth: it matures and gradually a better functional protection from invasive pathogens is established. The total DC number shows an inverse correlation with age across the lifespan, and so do the absolute numbers of circulating myeloid DCs (mDCs) and plasmatocytoid DCs (pDCs).[31] It has been observed that at birth pDCs outnumber mDCs, so that the pDC-to-mDC ratio is inversed in comparison to adults.[32, 33] Major functional maturation of circulating monocytes and DCs occurs during the first years of life, reflected by changes in the responsiveness to TLR stimulation, although the pattern of TLR expression by APCs has been reported to be similar between neonates and adults and among children of different ages.[34-37] The highest counts of NK cells are found in cord blood but they decline significantly (by 2–3 times) during the first postnatal days. Their levels further decrease progressively throughout infancy and early childhood, to reach adult levels around the fifth year of age.[38] There are no significant changes of the NK cell circulating subsets, CD56 bright, and CD56dim, until adulthood, apart from a slightly higher proportion of CD56bright NK cell numbers in neonates. It was shown that the responses of NK cells are determined by the balance between activating and inhibitory signals from relative cell surface receptors. Several phenotypic changes on NK cells regarding the expression of these receptors have been reported during the first years of life.[39] C8 and especially C9 are the most markedly reduced at birth.[30] No data on functional maturation of basophils and eosinophils exist.

IMMUNE PROGRAMMING, ENVIRONMENTAL STIMULI AND EPIGENETICS

The drastic changes across immune components after birth seem to follow a stereotypic pattern. This was first shown in a model using preterm pigs. Irrespective of postconceptional age, immune maturation started after birth and the postnatal age was the main predictor for the stage of immune maturation.[40] This finding was confirmed in human in more recent studies using multiplex techniques.[41, 42] Of interest, immune developmental pathways are shared by children of different levels of maturity and postnatal environmental conditions.

The microbiome and the environment contribute to the development of immunity after birth. The quick colonization by microbiota directly upon birth is a crucial trigger to set of immune developmental pathways. The important role of microbiota-derived factors, particularly in immune development and maturation, is best illustrated in animals raised in germ-free environments.[43] Germ-free mice exhibit a number of significant differences in their immune system, including the cellular composition and maturation status at mucosal sites. Interestingly, the phenotype of germ-free mice is only partially rescued by bacterial colonization after weaning, demonstrating the critical role of early-life exposure for life-long immune homeostasis.[44]

Consequently an altered microbiome or microbial deprivation increases the risk for various immune dysregulation and inflammatory diseases.[45, 46] For example, birth by caesarean section increases the risk for asthma, particularly in children of allergic parents.[47] Microbial dysbiosis during the first 100 days of life was associated with the development of asthma in a human birth cohort,[48] a finding that is in line with the hygiene hypothesis.[49] Studies in neonatal mice have shown that antibiotic treatment in the perinatal period alters intestinal microbiota, results in smaller numbers of Tregs cells, elevated serum IgE and increased susceptibility to allergic asthma in adulthood.[50, 51] Well-known are the epidemiological studies that show that growing up on a dairy farm protects children from allergy, hay fever, and asthma. High endotoxin concentrations in animal feces and the farm environment were identified as functional triggers of regulatory mechanisms and immune homeostasis. The strongest protective effect of such exposure was exerted during fetal and early postnatal life.

The molecular substrate of the environmental effects on immune development is found in epigenetic changes in gene expression of immune cells. Epigenetic changes in gene expression are functional changes without changes in basepair order of the DNA.[52, 53] This plasticity in gene expression enables fast phenotypical adaptations upon environmental changes and these adaptations can be passed on to new cells to preserve these epigenetic

changes in next generations. Genes that are sensitive for epigenetic changes are for example genes coding for enzymes involved in detoxing effects of oxidative stress (like cigarette smoke and pollution) and genes coding for proteins involved in metabolic and immunological processes.

Already before birth both maternal and paternal environmental factors like cigarette smoke, nutrients, medication and maternal disease influence the development of the fetal immune system via epigenetic changes. Some of these maternal factors are known to induce stable epigenetic changes in gene expression and to pass these adaptations on to their offspring in next generations.[54, 55] Therefore, the epigenome can be conceived of as a series of switches that cause various parts of the genome to be expressed or not.[56] The fetal and neonatal period may be particularly important for setting these switches and meddling factors like infectious pathogens, nutrients, chemicals but also the presence of commensals might induce long lasting structural or functional effects in this crucial developmental period. These negative influences may set switches wrongly and lead to morbidities later in childhood or adulthood.

THIS THESIS

It is clear that immune ontogeny is an emerging field. Fetal life and early infancy are increasingly recognized as a critical period to shape the immune system for life. Slowly the neonatal immune system changes its regulatory responses into effector responses. Genes, environmental factors and the epigenome will influence this maturation process and different developmental trajectories might be initiated leading to disease pathways. This concept has been recognized in the 1000 days campaign and the WHO's Every Newborn Action Plan but pathophysiological mechanisms supporting this theory are mostly unidentified.

In this thesis, we chose to study Tregs because of their indispensable role in the establishment and maintenance of immunological tolerance as shown in devastating autoimmune diseases in human and mice caused by null mutations in the X-linked Foxp3 gene.[57, 58]

We explored how FOXP3 Treg contribute to antigen specific immune tolerance shortly after birth when the skin and mucosa of the child is quickly colonized by tons of (harmless) microbes and when the child is exposed to food antigens and inhaled antigens (**chapter 2**). Next, we questioned the functionality of neonatal T_H17 cells since RORC and FOXP3 are counterbalanced (**chapter 3**).

For the studies in chapter 2 and 3 we used cord blood. Since we recognized that cord blood resembles the relatively sterile prenatal situation before immune cells have been exposed to foreign antigens but also reflects an acute phase response due to the highly stressful event of birth. Therefore we also performed a follow up study on Treg and T_H17 cells in healthy children in their first year of life (**chapter 4**). To overcome ethical issues in retrieving blood samples from healthy children, we needed to be inventive and we collected samples from infants in need of repetitive surgery in their first year of life and samples of newborns with a viral infection (see intermezzo). In **chapter 5** we questioned whether neonatal immune cells are defective or respond to strong stimuli in vivo. In the last chapter, we discuss the role of system biology in future studies (**chapter 6**). It is clear that in overcoming the ethical issues in research on newborns and infants, we need to be inventive in sample collection but also in research methods and technical solutions.

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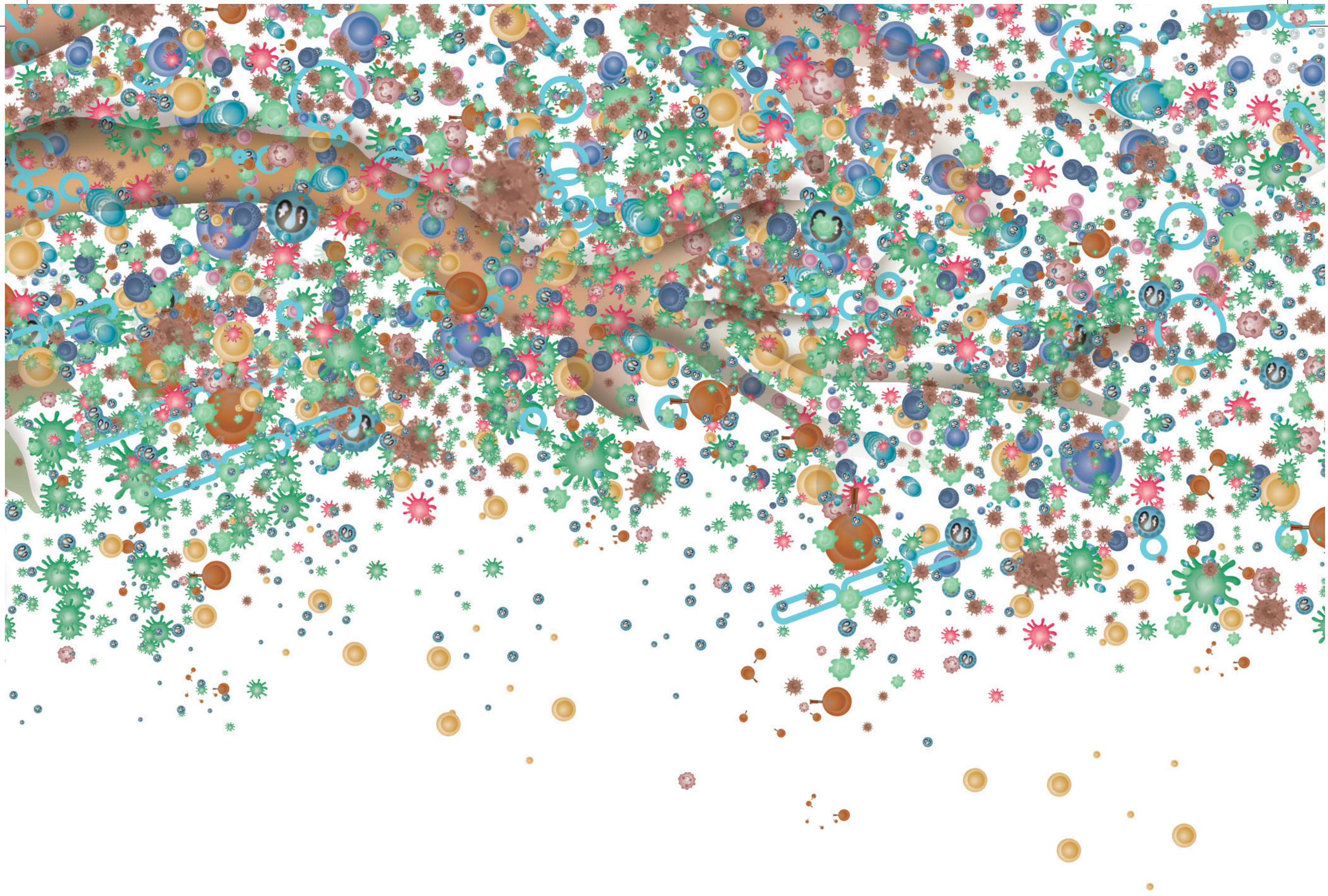

Critical role for programmed death 1 signalling and protein kinase B in augmented regulatory T-cell induction in cord blood

Hoeks SB*, de Roock S*, Meurs L, Steur A, Hoekstra MO, Prakken BJ, Boes M, de Kleer IM
*Both authors contributed equally

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INTRODUCTION

In newborns the induction of productive immune responses is generally blunted in comparison with adults, resulting in tolerogenic immune reactivity.[1] This immune status results in an increased potency to engraft neonatal animals, ineffective vaccination responses in newborns, and reduced occurrence of graft-versus-host disease when cord blood (CB) derived allografts are used.[2, 3] We hypothesized that forkhead box protein 3 (FOXP3)–positive regulatory T (Treg) cells are pivotal in this phenomenon because these cells are key players in immune homeostasis.[4] When comparing the number of FOXP3$^+$ cells in human CB and adult peripheral blood (APB), we found less FOXP3$^+$ cells in CB than APB (Fig 1, *A*, ex vivo, uncultured cells). However, when naive (CD25$^-$CD45RO$^-$) T cells were activated by plate-bound anti-CD3, significantly more FOXP3$^+$ T cells were induced from CB precursors. Remarkably, we only observed this difference between CB and APB when viable antigen-presenting cells (APCs; T cell–depleted CB or APB mononuclear cell fraction) were included in the culture; replacement with irradiated APCs abrogated the effect, whereas co-stimulation by soluble anti-CD28 showed a less pronounced difference (Fig 1, *A*). Induced CB FOXP3$^+$ T cells (sorted as CD4$^+$CD25$^+$CD127^{low} cells) were able to suppress dose dependently the proliferation of both CD4 and CD8 effector T cells when cultured together in different ratios (not shown), confirming the Treg nature of these cells. Thus on the first activation, CB T cells have a tendency to become functional FOXP3$^+$ Treg cells.

To substantiate the role of CB APCs in the induction of high percentages of Treg cells, we activated naive T cells in an alloreaction with APCs. Again we observed a higher number of Treg cells when CB T cells were cultured. CB APCs were able to induce higher numbers of Treg cells than APB APCs from both CB and APB precursor T cells (Fig 1, *B*). Nevertheless, CB T cells always contained higher percentages of Treg cells, indicating a T-cell intrinsic mechanism as well.

We investigated the percentages of different APC populations because the APCs used in our previous experiments consisted of a mixture of different cell types. In both APB and CB, we found comparable numbers of conventional dendritic cells (HLA-DR$^+$CD11c$^+$) and plasmacytoid dendritic cells (HLA-DR$^+$CD123$^+$), with slightly more CD14$^+$ monocytes in APB (not shown). When we sorted different subsets as APCs and cultured them with anti-CD3–activated T cells, we observed that all APC subsets induced more Treg cells from CB than from APB precursor cells (Fig 1, *C*).

The augmented Treg cell induction was not correlated with a reduced proliferation of CB T cells, which we showed to be identical in CB and APB by using carboxyfluorescein succinimidyl ester dilution assays (data not shown). Moreover, we found no difference in the kinetics of FOXP3 upregulation when we measured the percentage of FOXP3$^+$ cells daily; both APB and

CB showed a peak of FOXP3⁺ cells around 4 days of culture, after which the percentage of FOXP3⁺ cells decreased to stable expression at day 6 (Fig 1, *D*). Both APB and CB cells had a similar stable FOXP3-expressing population compared with the initial peak around day 4; 65% of the FOXP3⁺ T cells at day 4 remain stable FOXP3-expressing cells during the 8-day culture.

The induction of Treg cells in the periphery on activation can be mediated by several factors. Cytokines like TGFβ and IL-2 are known to induce FOXP3,[5] whereas inflammatory cytokines prevent this. To test whether possible differences in secreted mediators, such as cytokines and chemokines, can explain the difference in Treg cell induction, we exchanged culture supernatants between CB and APB every day during the 6-day culture period of naive T cells with APCs and anti-CD3. This approach did not result in altered differences between CB and APB Treg cell induction, making a role for secreted mediators unlikely (not shown).

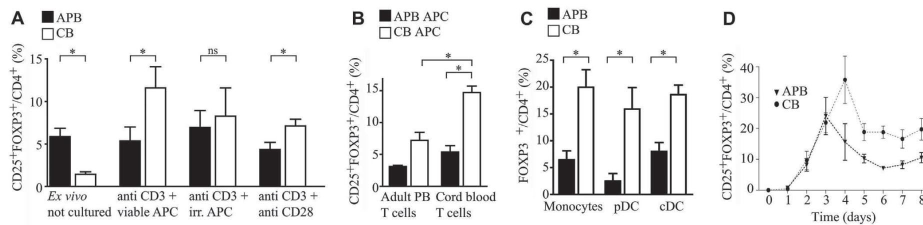

FIGURE 1. CB APCs stimulate Treg induction. A, FOXP3⁺ Treg cell percentages ex vivo or after 6-day naive (CD4⁺CD25⁻CD45RO⁻) T-cell stimulation, as indicated in the figure (left to right, n= 3, 6, 4, and 10). ns, Not significant. B, Treg cell percentage after 6-day alloreaction between naive T cells and APCs (n= 4). C, Treg cell percentage of anti-CD3–activated naive T cells after 6-day culture in the presence of sorted indicated APCs (left to right, n= 5, 3, and 3). cDC, Conventional dendritic cells; pDC, plasmacytoid dendritic cells. D, Time course of Treg cell expansion of anti-CD3–activated naive T cells cultured with viable APCs (n= 4). *P ≤.05.

Another factor influencing FOXP3 induction is the strength of the T-cell receptor (TCR) signal and costimulation.[6, 7] In our settings the TCR signal was standardized by plate-bound anti-CD3, but co-stimulation is provided by the APCs. High co-stimulation through CD28 signaling prevents the upregulation of FOXP3. We therefore investigated the role of the CD28 ligands CD80 and CD86 on APCs. We observed no difference in the expression of these molecules. When blocking antibodies toward CD80, CD86, or both were added to the cultures, this did not result in a difference in FOXP3 expression (Fig 2, *A*). This finding was confirmed by adding increasing concentrations of cytotoxic T lymphocyte–associated antigen 4-immunoglobulin (CTLA4- Ig) to the cultures, blocking both CD80 and CD86, which had no obvious effect (data not shown).

Inhibition of downstream signaling pathways of TCR and CD28 on T-cell activation is able to promote FOXP3 induction as well. The phosphoinositide 3-kinase/protein kinase B (PKB) pathway is central in T-cell differentiation.[8] Prevention of activation of this pathway by limited co-stimulation or signaling through inhibitory molecules, such as programmed death 1 (PD-1), promotes FOXP3 upregulation.[7, 9] We hypothesized that differential downstream signaling on activation might be pivotal to the high levels of FOXP3 found on day 6 of activation and onward (Fig 1, *D*). We therefore investigated the role of PD-1 and PKB in the induction of FOXP3 in CB-naive T cells. On activation, CB $CD4^+$ T cells have an increased expression of the PD-1 molecule compared with APB (Fig 2, *B*). When we blocked the interaction between PD-1 and its ligand PD-L1 by including a blocking mAb to PD-L1 in the culture, a significant reduction in the percentage of $FOXP3^+$ T cells was observed (Fig 2, *C*), isotype mAb had no effect (not shown). A PD-1–blocking mAb showed comparable results. As a consequence, the phosphorylation status of PKB on activation by different concentrations of anti-CD3 was lower for CB than APB cells (Fig 2, *D*). Additionally, CB T cells required approximately 10-fold more TCR triggering than APB T cells before an increase in PKB phosphorylation was observed.

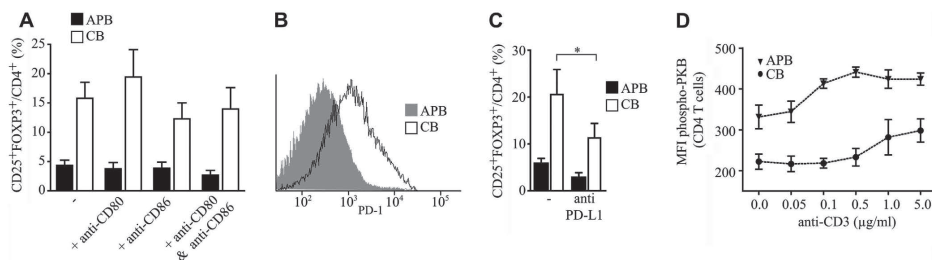

FIGURE 2. PD-1/PD-L1 interaction stimulates Treg cell induction. A, Treg cell percentage after 6-day stimulation of naive T cells in the presence of anti-CD3/viable APCs with blocking mAb as indicated (n= 6). B, PD-1 expression on 6-day anti-CD3–activated T cells in the presence of viable monocytes. C, Treg cell percentages of anti-CD3–activated naive T cells with viable monocytes in the presence of blocking mAb (CB, n= 11; APB, n= 3). D, Relative phosphorylated PKB levels in $CD4^+$ T cells cultured with a graded amount of plate-bound anti-CD3 (n= 3). *P ≤.05.

Shortly after birth, the immune system of the newborn encounters all kinds of neoantigens. The low percentages of Treg cells found ex vivo in CB are not likely to play a major role in maintaining tolerance to these neoantigens. However, we here show that PD-1 signaling in CB T cells facilitates their differentiation into induced functional $FOXP3^+$ Treg cells through a mechanism involving reduced PKB signaling. This phenomenon might well represent a mechanism that is developed to ensure active tolerance in the neonatal immune system.

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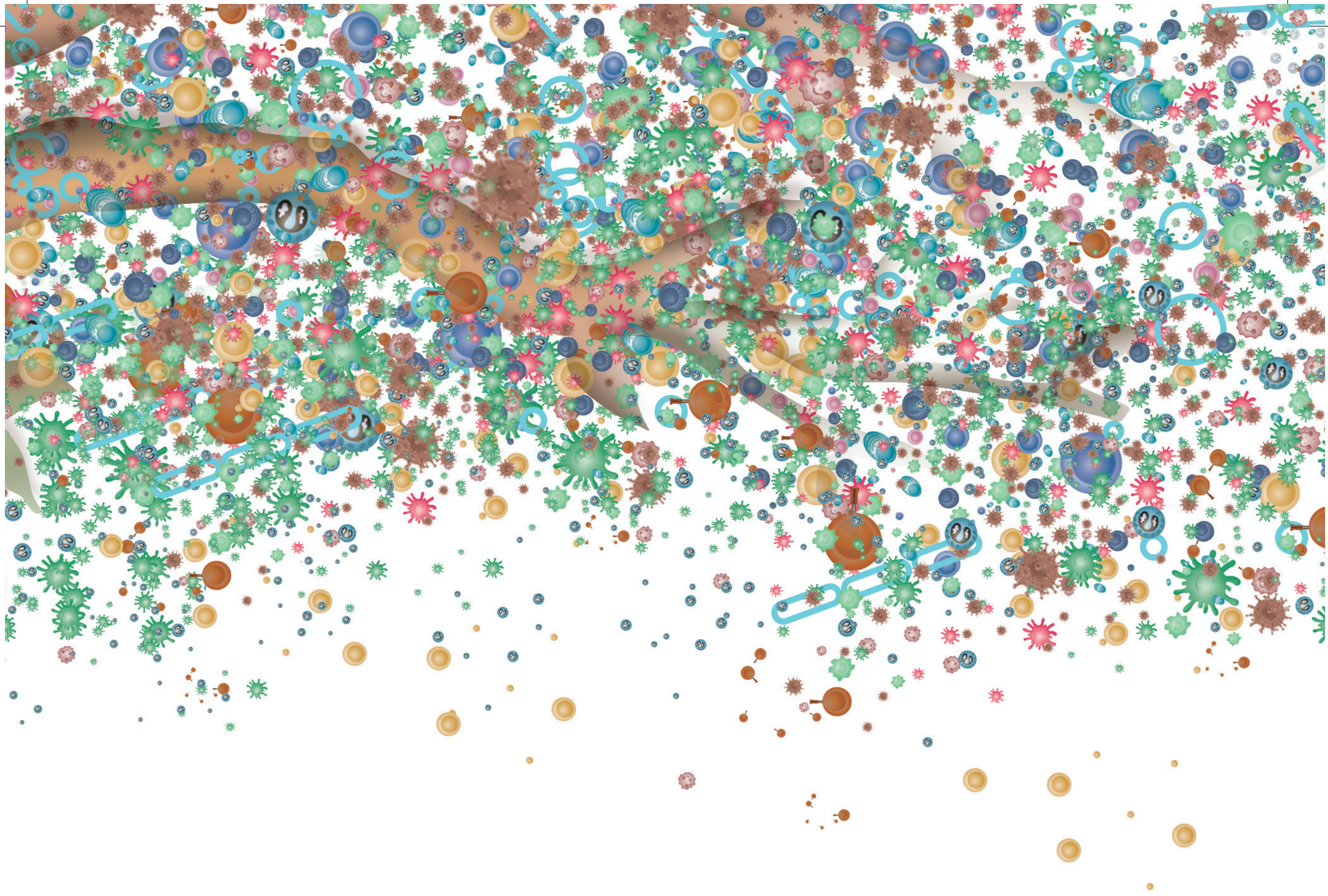

Chapter 3

Defective T_H17 development in human neonatal T cells involves reduced RORC2 mRNA content

de Roock S*, Stoppelenburg AJ*, Scholman R, Hoeks SB, Meerding J, Prakken BJ, Boes M
*Both authors contributed equally

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INTRODUCTION

The neonatal immune system shows a different response on activation when compared with the adult system.[1] Several mechanisms contribute to this phenomenon, which involves both functioning of the innate system[2] and adaptive mechanisms of the immune system[3] of which the T_H2 bias has been investigated intensively. We previously showed an important role for human regulatory T (Treg) cells[4] in modulation of immune activation in the neonatal setting, which is induced by the interaction between antigen-presenting cells (APCs) and naive T cells. Especially programmed death-1 ligation by programmed death-L1 contributes significantly to the induction of Treg cells from human cord blood (CB)-derived precursors.[4]

3

T_H17 and Treg cells have a reciprocal development pathway.[5] Both cells play an important role in the induction and perpetuation of inflammatory responses during infection, and also in the pathogenesis of autoimmune and allergy-related diseases. On T-cell activation in the presence of TGF-β, both signature transcription factors for T_H17 and Treg-cell lineage are upregulated: retinoid acid–related orphan receptor (ROR) γT for T_H17 cells and forkhead box (FOXP) 3 for Treg cells. In the absence of proinflammatory cytokines, FOXP3 dominates RORγT function and prevents T_H17 development. Because of the propensity of Treg-cell induction in CB, we hypothesized there to be a regulatory mechanism in CB that inhibits T_H17 cell development in neonatal T cells derived from CB cells as compared with adult peripheral blood (APB) cells. Activation of $CD4^+CD25^-CD45RO^-$ T cells in the presence of APCs (see this article's Methods section) shows increased numbers of $FOXP3^+$ cells in the CB (shown to be functional Treg cells earlier[4]) than in the APB (Fig 1, *A* and *D*). IL-17 production, however, was not observed in the CB (Fig 1, *A* and *B*). Replacement of APCs by anti-CD28 antibody prevented T_H17 induction in the APB (see Fig E1, *A*, in the Online Repository). The lack of IL-17, IL-21, IL-22, and GM-CSF (Fig 1, *C*) in the supernatants of CB cell cultures confirms that T_H17 phenotype is not induced in the CB after TCR stimulation. Other key cytokines, including IFN-γ and IL-13, were also reduced in CB cell cultures than in APB cells (Fig E1, *B*), confirming earlier work.[6] IL-21 and IL-22 in the APB were only partly derived from IL-17–producing cells (Fig E1, *C*).

T_H17 cells may derive from any naive T cell when activated in a conductive cytokine milieu, or from a defined precursor cell. In humans, $CD161^+CD4^+$ T cells are thought to be such precursors to the T_H17 cell lineage.[7] It has also been suggested that T_H17 cells more readily develop from $CD45RO^+$ memory T cells.[8] Therefore, we tested whether memory cell contamination within our naive cells at the start of culture accounts for the increased ability of APB to yield T_H17 cells. Fluorescence-activated cell sorting–sorted populations of $CD25^-CD45RO^-CD161^-$ cells from the APB can still develop into T_H17 cells, whereas the same population in the CB develops into Treg cells (Fig E1, *D*). Thus, in our culture system with viable APCs present, CB-naive T cells cannot differentiate into T_H17.

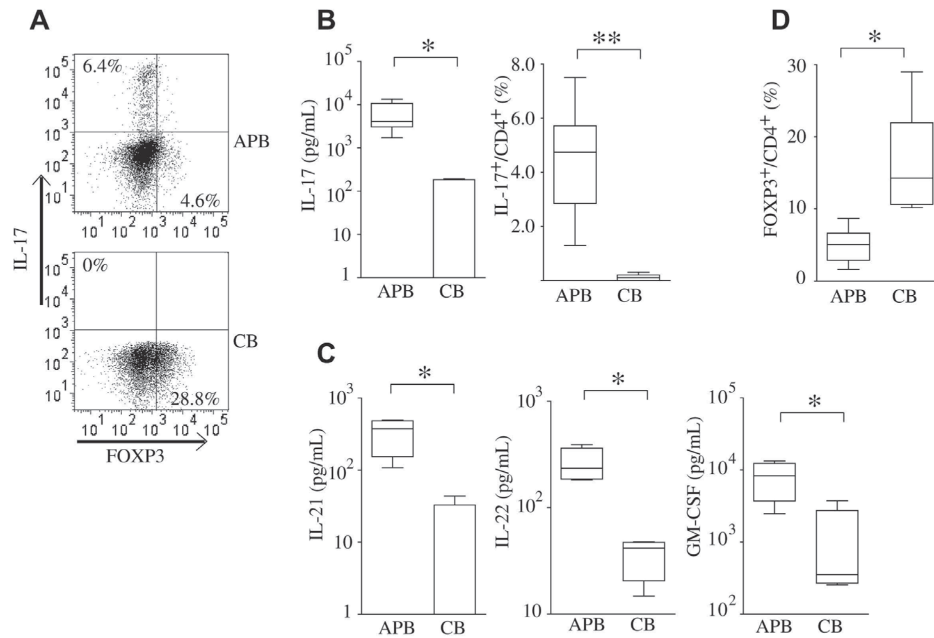

FIGURE 1. CB T cells are deficient in T_H17 development but do upregulate FOXP3. APB and CB naïve T cells were activated by plate bound anti-CD3 in the presence of viable APC for 6 days. IL-17⁺ and FOXP3⁺ CD4⁺ T cell numbers were assessed by flow cytometry. A, Representative cytometry plots. B, Supernatant IL-17 concentration (n=8) and percentage of IL-17⁺ CD4 cells (n=8 APB, n=5 CB). C, Supernatant IL-21, IL-22 and GM-CSF (n=4 APB, n=5 CB). D, Percentage of FOXP3⁺ CD4⁺cells (n=8). *p<0.05, **p<0.01, Mann Whitney U Test.

FIGURE E1. A, Culture supernatant IL-17 concentration after culture for 6 days with anti-CD3 and APCs or with APCs replaced by anti-CD28. n = 4 for APB and 5 for CB. B, IL-10, IFN-γ, and IL-13 concentration in APB and CB cell culture supernatants. APB and CB-naive T cells were cultured with APCs and plate-bound anti-CD3 for 6 days. Supernatants were subsequently analyzed for cytokine. n = 4 for APB and 5 for CB. C, Flow cytometric analysis of IL-17 and IL-21 or IL-22 production in APB cells. Naive T cells were cultured with APCs and plate-bound anti-CD3 for 6 days, restimulated with phorbol 12-myristate 13-acetate, ionomycin, and monensin and subsequently analyzed for production of IL-17, IL-21, and IL-22. Representative for n = 5. D, CD161 expression does influence T_H17 development in CB. APB and CB T cells were sorted as $CD25^-CD45RO^-CD161^-$ or $CD4^+CD161^+$ and subsequently activated by anti-CD3 in the presence of viable APCs without addition of recombinant cytokines. Left plot, APB CD161⁺ cells become IL-17 producers rather than APB CD161⁻ cells. CB cells do not produce IL-17 regardless of their CD161 expression. Right plot, CB T cells upregulate FOXP3 independently of CD161 expression. n = 3. *P < .05 and **P < .01, Mann-Whitney U Test. ►

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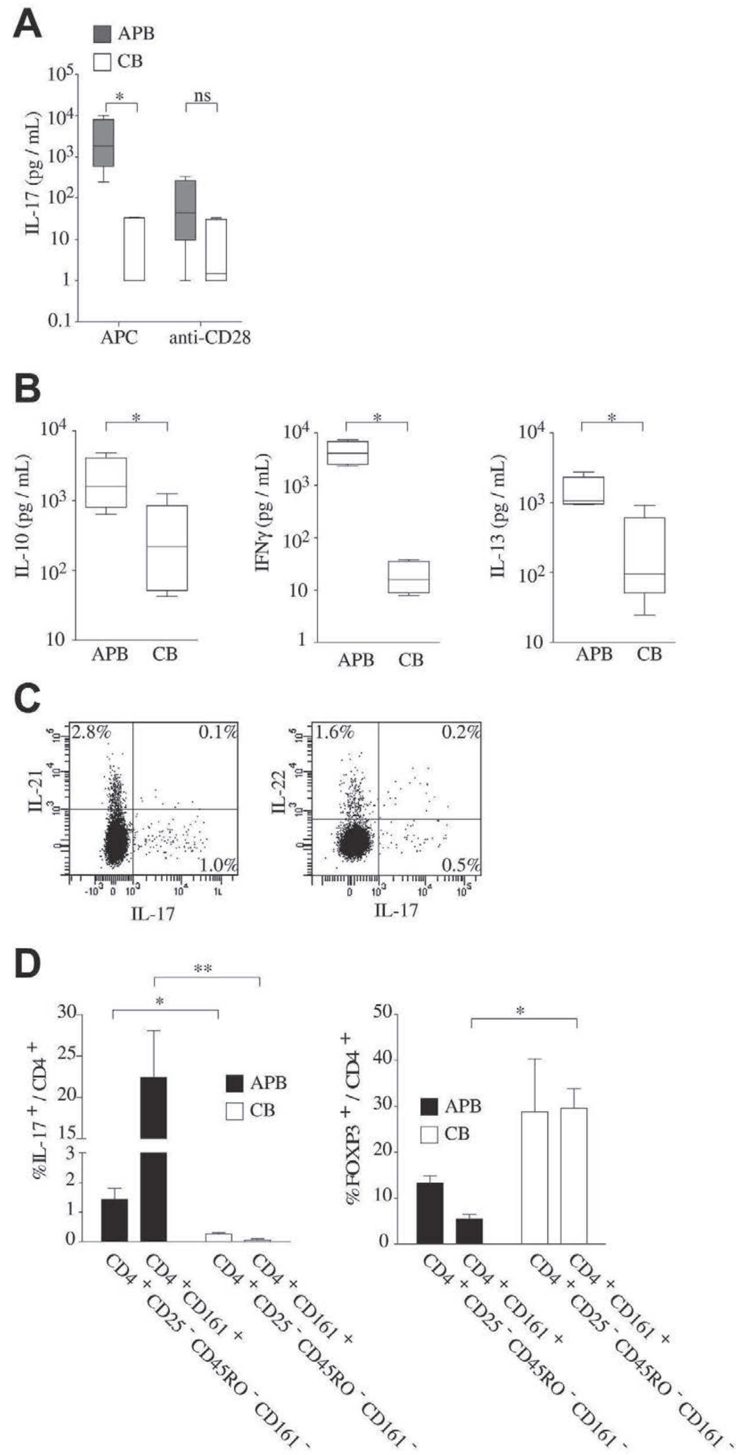
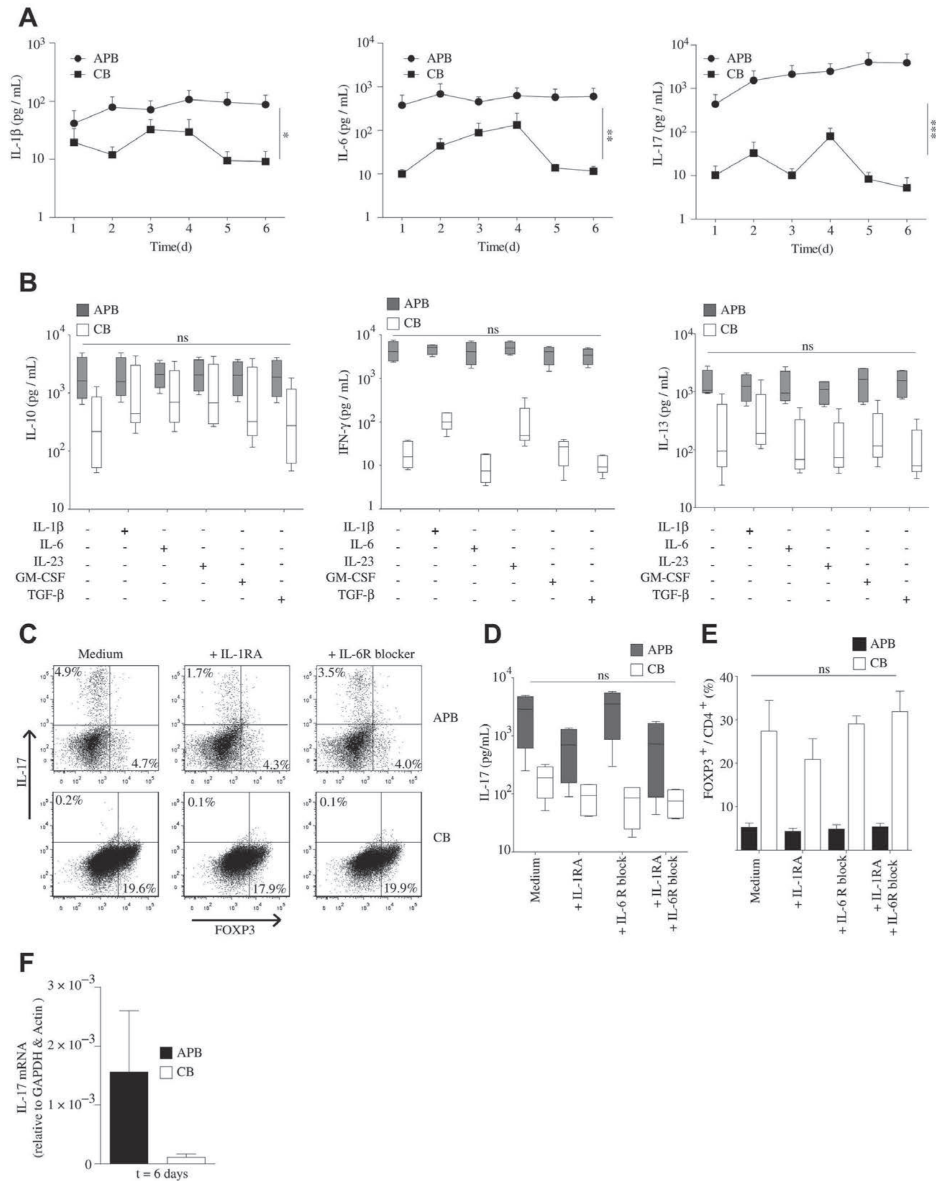




A
APB
CB
IL-1β (pg / mL)
IL-6 (pg / mL)
IL-17 (pg / mL)
Time(d)
*
**
***
B
IL-10 (pg / mL)
IFN-γ (pg / mL)
IL-13 (pg / mL)
ns
IL-1β
IL-6
IL-23
GM-CSF
TGF-β
C
Medium
+ IL-1RA
+ IL-6R blocker
4.9%
4.7%
1.7%
4.3%
3.5%
4.0%
0.2%
19.6%
0.1%
17.9%
0.1%
19.9%
IL-17
FOXP3
D
IL-17 (pg/mL)
+ IL-6 R block
+ IL-1RA + IL-6R block
E
FOXP3 + / CD4 + (%)
F
IL-17 mRNA (relative to GAPDH & Actin)
t = 6 days

◂ **FIGURE E2.** A, CB cell cultures produce low amounts of proinflammatory cytokines. APB and CB-naive T cells were activated by anti-CD3 in the presence of viable APCs. Culture supernatants were harvested from separate wells daily and analyzed for IL-1β, IL-6, and IL-17 concentration. n = 4. B, Addition of recombinant human cytokines does not influence APB cell culture supernatant concentration of IL-10, IFN-γ, and IL-13, but does so in CB cell cultures. n = 4 for APB and 5 for CB. C, Blockade of proinflammatory cytokine signaling in CB cells does not affect FOXP3 upregulation. APB and CB-naive T cells were activated by anti-CD3 in the presence of viable APC and blocking antibodies toward IL-1β (1 µg/mL anakinra) and IL-6 (1 µg/mL tocilizumab) signaling. After 6 days, IL-17–producing and FOXP3-expressing cells were assessed by flow cytometry. Representative cytometry plots for n = 4. D, IL-17 concentration in the culture supernatants after 6 days of culture, n = 4. E, Percentage of FOXP3-expressing CD4 T cells. n = 4. F, IL-17 mRNA content is lower in CB cells than in APB cells after 6 days of culture. n = 4. *P < .05, **P < .01, ***P < .001, Student t test of the area under the curve (Fig E2, A), Kruskal-Wallis with Dunn post hoc (Fig E2, B and D, comparing the effect of blocking antibodies to medium control) and Student t test (Fig E2, F).

3

When IL-6 (mouse) or IL-1β, IL-6, and IL-23 (human) are present during T-cell activation, FOXP3 is downregulated and RORγT enforces T_H17 development. Neonatal innate cells differ in their activation-induced cytokine production, compared with APB cells.[2] Does this biased cytokine production profile account for the difference in T_H17 development capacity? Indeed, culture supernatants of CB cells show reduced concentrations of both IL-1β and IL-6 during the culture period of 6 days as compared with APB cultures where these cytokines were readily produced. APB cultures yield an increased concentration of IL-17 at the end of the culture (see Fig E2, *A*).

To further investigate whether the absence of these cytokines in CB cell cultures had a role in the disability of T_H17 development, we added recombinant human cytokines to the cultures of naive APB and CB $CD4^+$ T cells and monitored again both IL-17 production and FOXP3 expression. Addition of IL-1β, IL-6, and IL-23 resulted in an increased number of IL-17–producing APB T cells without affecting the number of cells that upregulated FOXP3 (Fig 2, *A-C*). Only the combination of IL-1β, IL-6, and IL-23 induced IL-17 production in CB cells but never to the extent observed in APB cells. The number of $FOXP3^+$ cells was reduced when these cytokines were included alone or in combination in the cultures. Addition of GM-CSF or TGFβ did not clearly affect IL-17 production alone, nor in combination with the inflammatory cytokines (Fig 2, *B*). The combination of inflammatory cytokines and these cytokines did show a reduced FOXP3 expression in APB cells. Thus, CB T cells respond to the cytokines as shown by reduced FOXP3 expression and the increase in IL-10 and IFNγ production (Fig E2, *B*), suggesting that the lack of T_H17 differentiation in these experiments is not due to the absence of the specific receptors for these cytokines.

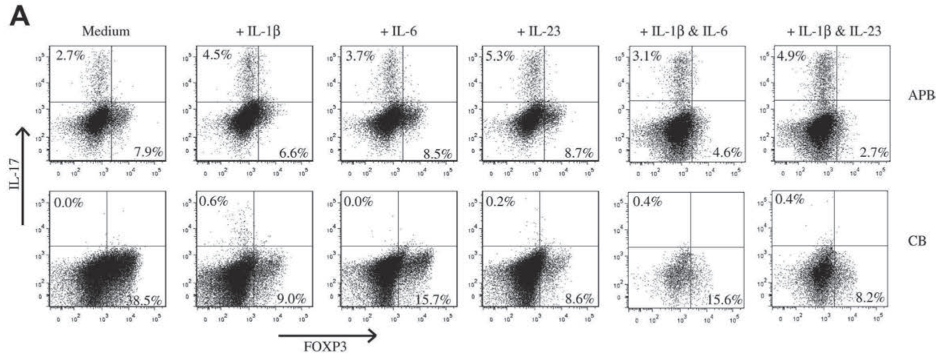
As the development of T_H17 cells inversely correlates to the development of Treg cells,[5] we considered that the induction of T_H17 cells in the APB could be responsible for the diminished induction of Treg cells in APB cells as compared with CB cells. Pharmacologic inhibition of proinflammatory cytokine signaling, through the addition of IL-1RA– or IL-6R– blocking antibody (anakinra and tocilizumab, respectively), decreased the number of IL-17–producing T cells and lowered concentrations of supernatant IL-17 (Fig E2, *C-E*). Nevertheless, the number of APB cells expressing FOXP3 after 6 days of culture remained stable and was not affected by these treatments. Because CB cell cultures did not produce IL-1β and IL-6, blockade of the signaling pathways did not affect the percentages of IL-17$^+$ or FOXP3$^+$ T cells.

Finally, as CB precursor cells did not form T_H17 cells, while we did see a signaling effect of inflammatory cytokines, we hypothesized that the blockade in T_H17 differentiation should be upstream of the transcription of IL-17 but downstream of cytokine signaling. We therefore analyzed the T_H17 defining transcription factor RORγT, in humans encoded by transcript variant 2 of the *RORC* gene.[9, 10] Indeed, quantitative PCR of *RORC2* mRNA after 3 and 4 days of activation shows significantly lower *RORC2* mRNA content in CB as compared with APB T cells (Fig 2, *D*), resulting in a reduced IL-17 mRNA (Fig E2, *F*) and prevention of IL-17 production.

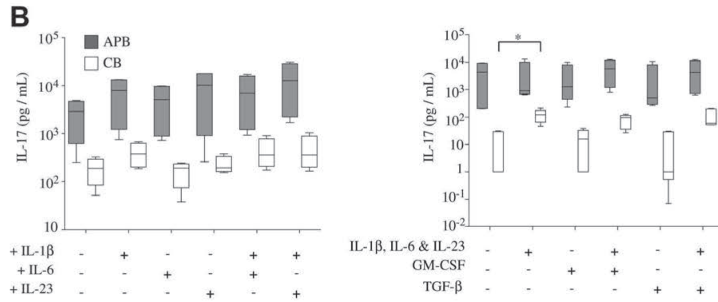
We propose that neonatal T cells have an intrinsic mechanism that prevents T_H17 differentiation through the regulation of RORγT expression. This may be of functional relevance to prevent adverse immune reactions against self or nonpathogenic microbes during the establishment of T-cell repertoire and bacterial colonization. Exact mechanisms preventing *RORC* transcription in CB cells are not known yet but might be found in upstream signaling or DNA methylation and histone acetylation. Appropriate activation, for example, via innate signaling, can overcome this deficiency.[1]

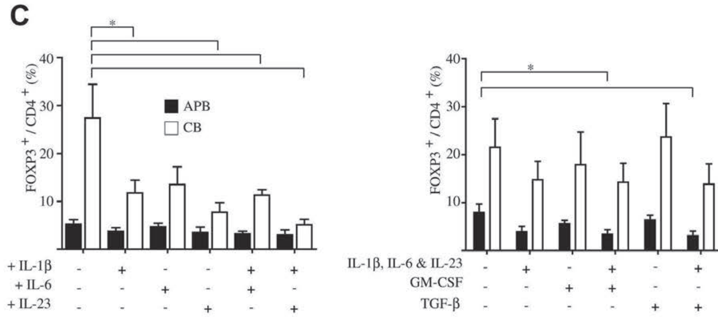
We thank Dr Wilco de Jager for technical assistance in the cytokine measurements.

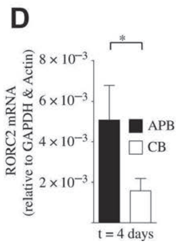
FIGURE 2. CB T cells cannot be forced to Th17 development and do not upregulate RORC. APB and CB naïve T cells were activated by plate bound anti-CD3 in the presence of viable APC and recombinant cytokines as indicated for 6 days. A, Representative cytometry plots. B, IL-17 in the culture supernatants (n = 6 left plot and n = 4 APB, n = 5 CB right plot). C, Percentage of FOXP3$^+$ CD4$^+$ cells (n = 6 APB, n = 5 CB left plot, n = 4 APB, n = 5 CB right plot). D, Relative RORC2 mRNA expression (n=4). *p<0.05, *P < .05, Kruskal-Wallis with Dunn post hoc (Fig 2, B, C) and Student t test (Fig 2, D). ►


A
Medium
+ IL-1β
+ IL-6
+ IL-23
+ IL-1β & IL-6
+ IL-1β & IL-23
APB
CB
IL-17
FOXP3
2.7%
7.9%
4.5%
6.6%
3.7%
8.5%
5.3%
8.7%
3.1%
4.6%
4.9%
2.7%
0.0%
38.5%
0.6%
9.0%
0.0%
15.7%
0.2%
8.6%
0.4%
15.6%
0.4%
8.2%

3


B
APB
CB
IL-17 (pg / mL)
+ IL-1β
+ IL-6
+ IL-23
IL-1β, IL-6 & IL-23
GM-CSF
TGF-β


C
APB
CB
FOXP3 + / CD4 + (%)
+ IL-1β
+ IL-6
+ IL-23
IL-1β, IL-6 & IL-23
GM-CSF
TGF-β


D
RORC2 mRNA (relative to GAPDH & Actin)
APB
CB
t = 4 days

METHODS

CB samples were obtained from normal deliveries of full-term neonates (approved by the Medical Ethical Committee of the University Medical Center Utrecht). Following ethical guidelines of our hospital, no further information (eg, exact gestational age or sex) on the CB samples was provided, which limited the selection criteria for these samples. Adult samples were collected from healthy volunteers, aged between 21 and 35 years. Mononuclear cells from CB and APB were isolated by using Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). Naive T cells and APCs were isolated by using magnetic cell sorting (CD4-T-cell enrichment and $CD25^{+}CD45RO^{+}$ T-cell depletion and $CD3^{+}$ cell-depletion, respectively; BD Bioscience, San Jose, Calif, cell purity after magnetic cell sorting >95%) or by fluorescence-activated cell sorting by using ARIA II. A total of 50,000 T cells were cultured for 6 days with 100,000 autologous, nonirradiated (viable) APCs or 1 µg/mL anti-CD28 (eBioscience, San Diego, Calif), activated with 1 µg/mL plate-bound anti-CD3 (OKT3, eBioscience) and 30 units recombinant human IL-2/mL (Novartis, Arnhem, The Netherlands) in RPMI 1640 (Gibco, Breda, The Netherlands) supplemented with 10% human AB serum (Sanquin, Amsterdam, The Netherlands), 1% l-glutamine (Gibco), and 0.5% penicillin-streptomycin (Gibco), restimulated with phorbol 12-myristate 13-acetate (20 ng/mL) and ionomycin (1 µg/mL) in the presence of monensin (Golgistop, BD Bioscience) for 4 hours and subsequently analyzed for IL-17 (eBio64DEC17, eBioscience, phycoerythrin-conjugated) and FOXP3 (PHC101, eBioscience, eFluor450-conjugated) expression by using flow cytometric analysis. In other experiments, IL-21 (clone 3A3-N2) and IL-22 (clone 22URTI) production was also analyzed by flow cytometry.

Recombinant IL-1β (Miltenyi Biotec, Bergisch Gladbach, Germany; 10 ng/mL), IL-6 (50 ng/mL), IL-23 (BD Bioscience, 10 ng/mL), GM-CSF (Immunotools, Friesoythe, Germany; 500 units/mL), and TGFβ (Peprotech, NJ, 5 ng/mL) were added to the cultures. Signaling of IL-1β and IL-6 was blocked with IL-1RA anakinra (Kineret) or IL-6 receptor blocker tocilizumab (RoActemra), both added as 1 µg/mL.

Culture supernatants were analyzed for cytokine production by multiplex analysis, and cultured cells were analyzed for *RORC2*and *IL-17* mRNA expression. Detection limits for luminex were (lower limit-upper limit, pg/mL) as follows: IL-1β (0.6-4141), IL-6 (2.4-22643), IL-13 (0.43-10173), IL-17 (0.57-9940), IL-21 (12.2-87363), IL-22 (0.8-2294), IFNγ (6.24-20733), and GM-CSF (2.62-40413).

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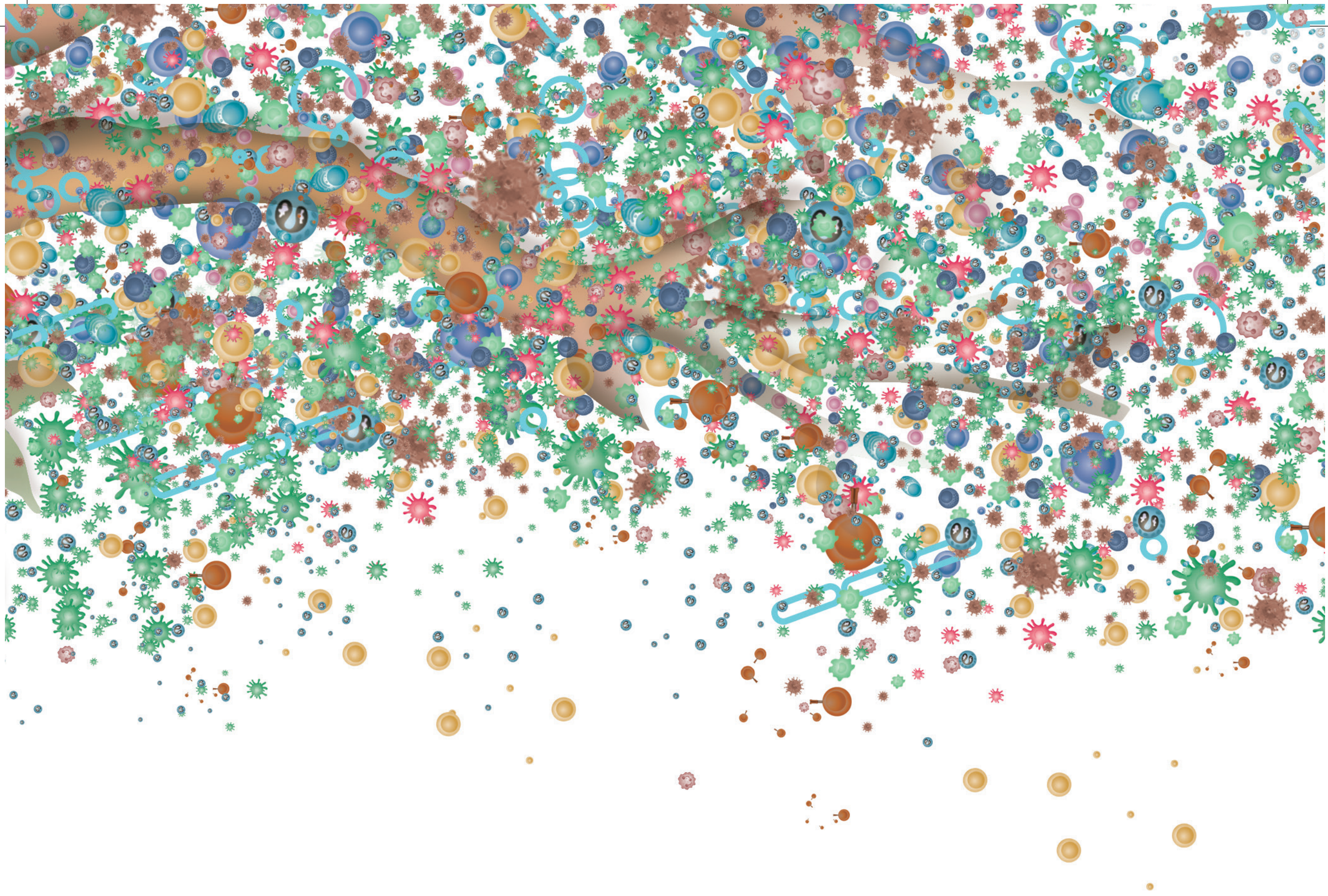

T_H17 differentiation capacity develops within first three months of life

Hoeks SB*, Dijkstra KK*, Prakken BJ, de Roock S
*Both authors contributed equally

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INTRODUCTION

The neonatal immune system has been shown to respond differently when compared with the adult system. Most research on this phenomenon has been performed with cord blood (CB) cells as a representative for the neonatal immune system, showing differences in cytokine production,[1] receptor expression,[2] and cell differentiation capacity.[3] Because of ethical restrictions, it is hard to obtain cells from healthy newborns and infants. Children prenatally diagnosed with an orofacial cleft will have several surgical corrections in the first year of life, providing the opportunity to study immune maturations with longitudinal samples (see Table E1 and methods section).

To provide an insight into the changes in the composition of peripheral blood cells after birth, we analyzed the relative fractions of T cells, monocytes, and B cells of all infants at different ages. Although the total number of T cells did not change, the ratio between CD4 and CD8 cells decreased after birth. The number of monocytes gradually decreased over the first year of life but was higher again in adults. B cells showed an inverse pattern (see Fig E1, *A-E*).

We further characterized subtypes of $CD4^+$ T-helper cells. Most of these cells had a naive ($CD45RA^+$) phenotype in CB (Fig 1). Although we hypothesized that colonization of the mucosal tissues early after birth would result in a rapid increase in cells with a memory ($CD45RO^+$) phenotype, we observed only a slight change over the first year of life (Fig 1, *A*). No differences were found in the percentages of Forkhead box protein 3 ($FOXP3^+$) regulatory T (Treg) cells, but we did observe differences in the ratios of subpopulations as described by Miyara et al.[4] Resting Treg cells in population I are described as potent suppressors that are not in cell cycle. These cells were significantly more abundant throughout the first year of life. Activated Treg cells are not only potent suppressors but also express proliferation markers. These cells were present in equal proportions in all age groups. Population III has been described as consisting of less stable Treg cells with regard to the DNA methylation status of the FOXP3 locus and containing IL-17–producing cells while their suppressive capacity is under debate.[4, 5] These cells were significantly more abundant in adult samples.

Next, we assessed the cytokine production of isolated PBMCs after activation by phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Very low concentrations of T-cell cytokines IL-17, IL-13, and IL-10 were found, without age-dependent differences (not shown). IL-1β, IL-6, and TNFα were significantly lower in CB samples as shown before6 and increased with age (Fig 1, *C-E*). IFNγ was the only T-cell cytokine observed in considerable amounts in adult samples, but not in CB and 3-month samples (Fig E2). So, the ex vivo phenotype of all infant samples showed a more naive and regulatory phenotype and an increasing capacity of inflammatory cytokine production with age.

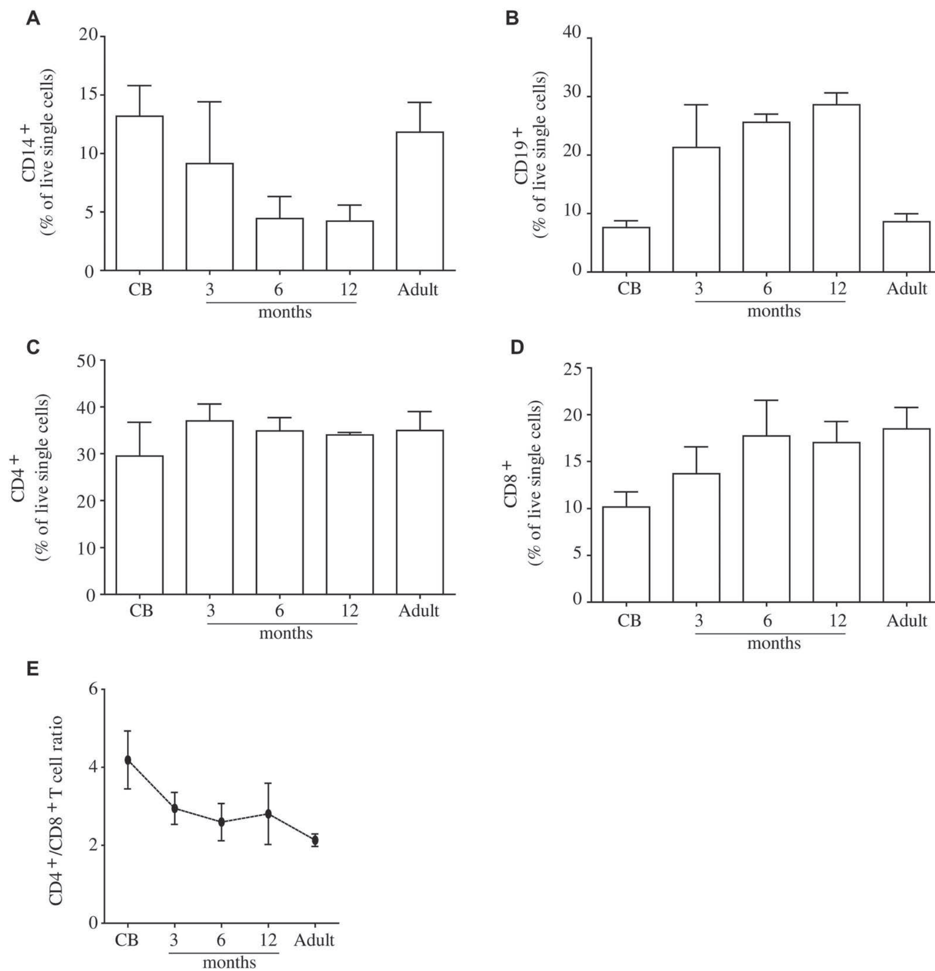

FIGURE E1. Differences in PBMC composition ex vivo. After thawing, PBMC were stained for CD3, CD4, CD8, CD14 and CD19 and analyzed by flow cytometry. A, $CD14^+$ monocytes, B, $CD19^+$ B cells, C, $CD3^+$ $CD4^+$ T helper cells, D, $CD3^+CD8^+$ cytotoxic T cells as a fraction of live single cells, and E, $CD4^+$/$CD8^+$ ratio. CB n=6, 3 months n=4, 6 months n=3, 12 months n=3, adult n=6 for all graphs; none of the differences were statistically significant. Data as mean ± SEM.

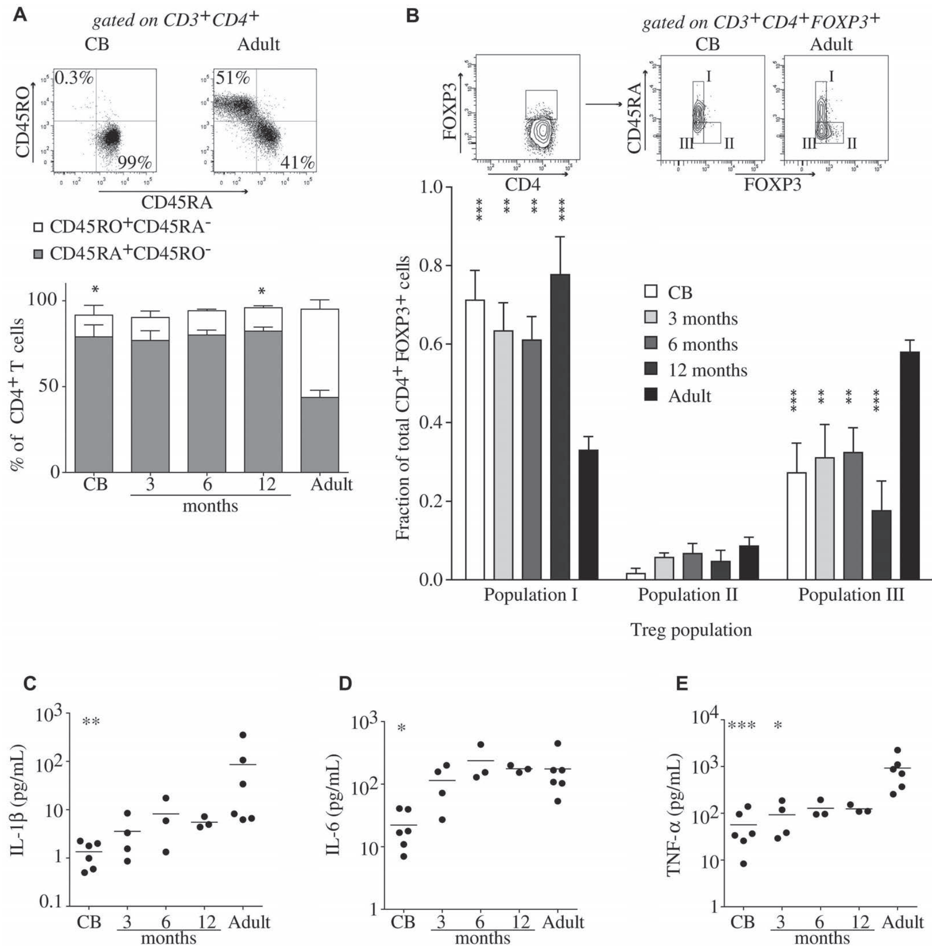

FIGURE 1. Until age 12 months, most TH cells remain naive and most FOXP3+ cells are resting Treg cells found in population I. After thawing, PBMCs were stained for CD3, CD4, CD45RA, CD45RO, and intracellularly for FOXP3. A, Representative plots for CD45RA+/CD45RO+ CD4+ T-cell distribution and fraction of CD45RA+CD45RO- and CD45RA-CD45RO+CD4+ T cells. B, Distribution of Treg-cell populations according to Miyara et al[4] for cells derived at different ages. C, IL-1β, D, IL-6, and E, TNFα concentration in the supernatant after 4 hours incubation of PBMCs with phorbol 12-myristate 13-acetate and ionomycin. Data as mean ± SEM. *P < .05, **P < .01, ***P < .001, compared with adult.

4

FIGURE E2. T cell subsets ex vivo. A, $CD4^{+}FOXP3^{+}$ cell percentages ex vivo. B, PBMC were stimulated with PMA and Ionomycin. Supernatants were collected and analyzed for IFNg. *P < .05.

To investigate the capacity of T cells to become activated and differentiate into different T-cell subsets, we cultured PBMCs for 6 days with anti-CD3. We focused on Treg cells and T_H17 cells because it has been shown that CB cells differ from adult cells in their capacity to differentiate into these cells.[2, 6] In addition, the gut microbiota has an important role in the development of these cells and colonization occurs soon after birth.[7-9] We therefore hypothesized that the infant samples would reflect an adult phenotype with regard to T_H17 and Treg-cell differentiation.

First, we assessed the proliferation of PBMCs on anti-CD3 activation by 3H-labeled thymidine incorporation. Cells derived from adults showed the highest proliferation, although only significantly different from the samples obtained at 3 months (Fig E3, *A*). The inability of CB cells to produce IL-17 was confirmed in these assays (Fig 2, *A* and *B*). The number of T cells producing IL-17 was already increased at age 3 months, and the amount of IL-17 in the supernatant of these cell cultures gradually increased from CB to adulthood. On activation, CB cells easily upregulated FOXP3 when compared with adult cells (Fig 2, *C*). In contrast to the differences in T_H17 development, this phenomenon was still observed in samples derived from children aged 3 to 12 months. So, the propensity of CB T cells to become Treg cells remained until at least age 1 year, while the inability to become T_H17 is overcome within the first months of life.

Furthermore, we investigated the production of other T-cell–derived cytokines in these cultures. No difference in IL-13 production was found. The assumed T_H2 bias in infants was mainly present in the form of a significantly reduced IFN-γ production, corresponding with a lower IFNγ/IL-13 ratio compared with that in adults (see Fig E3, *B*-*D*).

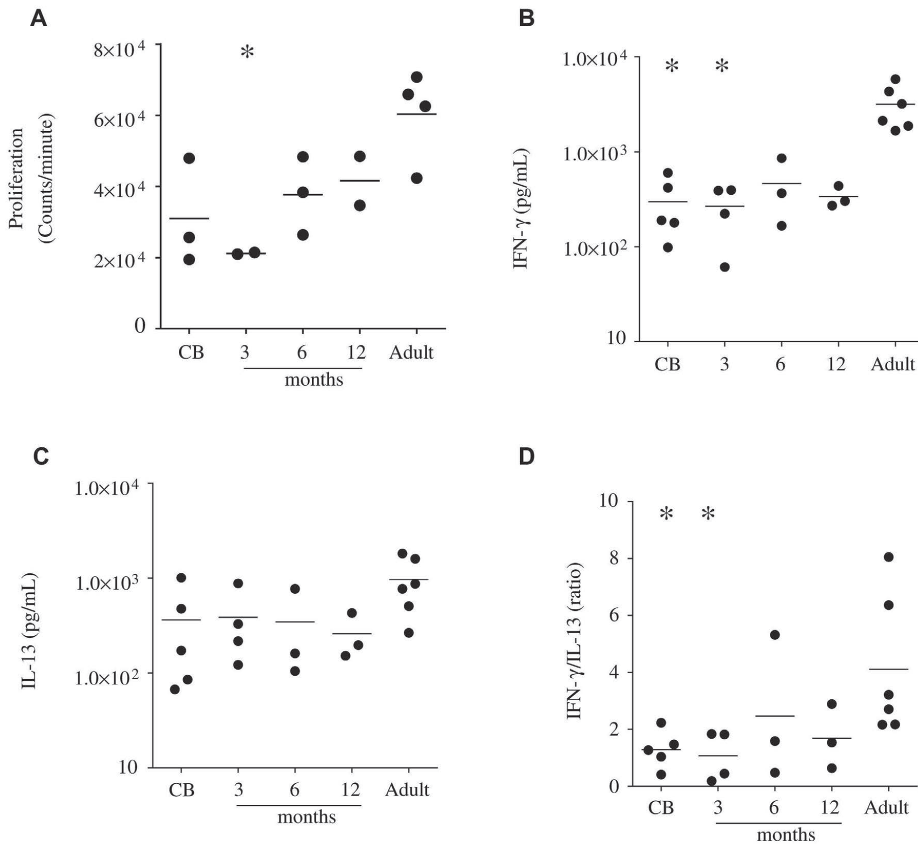

FIGURE E3. Proliferation and cytokine production after activation with anti-CD3. PBMCs were activated by plate-bound anti-CD3. A, Proliferation was measured after 4 days of culture by 3H-labeled thymidine incorporation for 16 hours. B and C, After 6 days, culture supernatants were harvested and analyzed for IFNg and IL-13 production, respectively. D, ratio between IFNg and IL-13 in the culture supernatant. *P < .05.

4

Antigen-presenting cells (APCs) in CB have an important role in the induction of Treg cells from naive T cells. Alloreactions between naive T cells and APCs show higher numbers of Treg cells when CB-derived APCs are used.[2] We wondered whether this capacity of CB APCs is also observed in infant-derived APCs. We first tested the number of induced Treg cells when fluorescence activated cell sorting–sorted $CD4^{+}CD45RA^{+}CD45RO^{-}$ naive T cells were activated with autologous APCs (Fig 2, *D*). In contrast to CB samples, all other samples had Treg-cell numbers comparable to adult samples. Next, we activated naive T cells from samples from donors aged 3 to 12 months with autologous APCs from CB and vice versa. Because of low cell numbers, we had to pool results from all infant samples. CB APCs induced more $FOXP3^{+}$ T cells than did APCs derived from older donors (Fig 2, *E*). Nevertheless, CB T cells

always showed a higher percentage of FOXP3$^+$ cells than did T cells from 3- to 12-month-old donors. So, the propensity of CB naive T cells to become Treg cells was dependent on the interaction between T cells and APCs.

We conclude that neonatal T cells develop the capacity to differentiate into T_H17 cells before the age of 3 months and retain a propensity to become Treg cells until the age of at least 12 months. So, immunity against pathogens is being raised while at the same time the immune system remains to have a regulatory profile. This latter fact might be important in the maintenance of tolerance toward allergens and food antigens encountered for the first time.

We thank all of the infants and their parents who took part in this study, Dr L. Pistorius and Dr G. T. R. Manten for their help with the inclusion of the participants, and Mariska van Dijk for performing the Luminex assay.

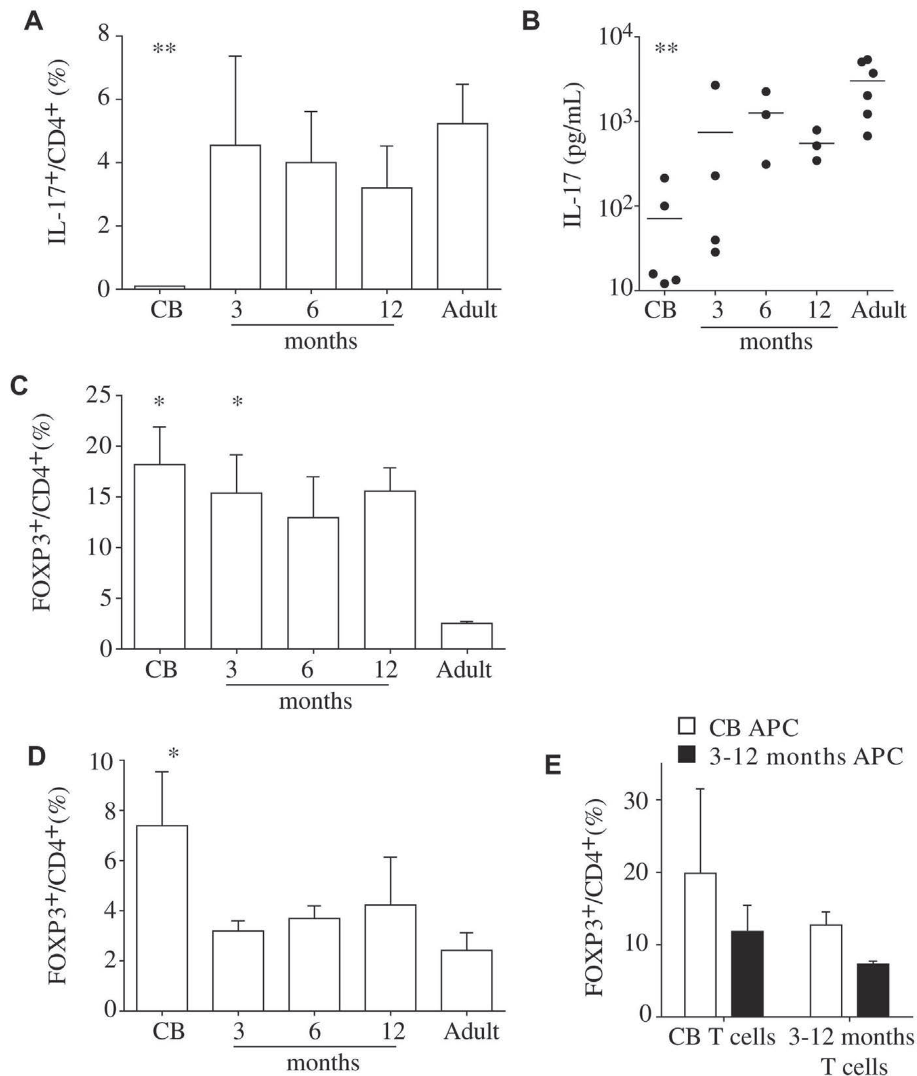

FIGURE 2. T_H cells have a high propensity to become FOXP3+ Treg cells in the first 12 months of life, but T_H17 cells can already be induced at 3 months. PBMCs were activated by plate-bound anti-CD3. A, IL-17–producing T_H cells were assessed after 6 days of culture and 4 hours of restimulation with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin. B, The concentration of IL-17 in the supernatants of PBMCs cultured for 6 days. C, FOXP3 expression was assessed by using flow cytometry with intracellular staining after 6 days of PBMC culture. D, FOXP3 expression after 6 days of culture of CD4+CD25-CD45RA+CD45RO- T cells with CD3- APCs. E, FOXP3 expression in CD4 T cells cultured with APCs from the same donor but isolated at a different donor age. A and B, CB n= 5, 3 months n= 4, 6 months n= 3, 12 months n= 3, adult n= 6; C, CB n= 5, 3 months n= 4, 6 months n= 3, 12 months n= 3, adult n= 6; D, CB n= 5, 3 months n= 2, 6 months n= 2, 12 months n= 3, adult n= 6; E, CB n= 2, 3 months n= 3, 6 months n= 3, 12 months n= 3. *P <.05, **P <.01, compared with adult. Data as mean ± SEM.

METHODS

Sample collection

Between June 2011 and January 2013, 7 children were enrolled in the study. Infant characteristics are presented in Table 1. All mothers were healthy and had uncomplicated pregnancies. All children were prenatally diagnosed with a different severity of orofacial cleft that required 1 or multiple chirurgical corrections. Blood samples were drawn from the umbilical cord directly after birth and from peripheral blood during surgical interventions that took place at regular intervals after birth. Additional control samples were taken by venipuncture from 6 unrelated healthy volunteers. This study was approved by the local medical committee of the University Medical Center Utrecht (08-331). Written informed consent was obtained from all parents before inclusion in the study. All procedures adhered to the principles of the Declaration of Helsinki.

Infant characteristics	
Mode of delivery	
- Vaginal (%)	71
- Cesarean section (%)	29
Sex	
- Female (%)	29
- Male (%)	71
Prematurity (%, GA below 37 wk)	14 (GA 34^{+4} wk)
Birth weight (g), mean ± SD	3307 ±819
Infant samples	
CB (%)	86
3 mo (%)	57
6 mo (%)	43
12 mo (%)	43

TABLE E1. Infant and sample characteristics. GA, gestational age.

Cells and culture conditions

After collection of the blood samples, CBMCs or PBMCs were isolated within 24 hours by using Ficoll-Isopaque density gradient centrifugation (Ficoll-Isopaque, Pharmacia, Upsalla, Sweden). Cells were washed and frozen in 90% FCS and 10% dimethyl sulfoxide at −80°C. Cells stored for more than 1 month were transferred to −150°C.

All samples from one donor were thawed simultaneously together with a sample from an adult donor. Cells were subsequently aliquoted for different experiments.

For *ex vivo* cytokine production measurements, 1×10^5 PBMCs were cultured in culture medium (RPMI 1640 supplemented with 1% l-glutamine and 100 mg/mL streptomycin and 100 U/mL penicillin; Gibco, Breda, The Netherlands) with 20 ng/mL phorbol myristate acetate and 1 µg/mL ionomycin at 37°C. Then, either after 4 hours, supernatants were harvested for cytokine analysis, or after 30 minutes, monensin (Golgistop, BD Bioscience, San Jose, Calif) was added and cells were cultured for another 3.5 hours and stained for IL-17 production.

For measurement of proliferation, differentiation, or cytokine production on immune activation, 1×10^5 PBMCs were activated by plate-bound anti-CD3 (1 µg/mL, clone OKT3; eBioscience, San Diego, Calif) at 37°C. After 4 days, ^{3}H-labeled thymidine was added for another 16 hours for the measurement of proliferation. After 6 days of culture with plate-bound anti-CD3, supernatant was harvested for cytokine measurement and cells were used for flow cytometric analysis after restimulation with 20 ng/mL phorbol 12-myristate 13-acetate and 1 µg/mL ionomycin for 4 hours in the presence of monensin.

4

Naive T cells and APCs were fluorescence activated cell sorting–sorted on an ARIA II (BD Bioscience) as $CD4^+CD45RA^+CD45RO^-$ and $CD3^-$ fractions, respectively. A total of 5×10^4 naive T cells and 5×10^4 APCs were activated by 1 µg/mL plate-bound anti-CD3 for 6 days and subsequently analyzed for FOXP3 expression by using flow cytometry.

Flow cytometric analysis

After thawing, an aliquot of cells was immediately stained with antibodies directed against CD3, CD4, CD8, CD14, and CD19 to quantify relative numbers of CD4 and CD8 T cells, monocytes, and B cells, respectively. Naive and memory cells were quantified by staining with CD45RA and CD45RO. For Treg-cell quantification, cells were permeabilized by using the FOXP3 staining kit from eBioscience and subsequently stained with an antibody against FOXP3 (clone PCH101, eBioscience). IL-17 (clone eBio64DEC17, eBioscience) was stained after restimulation with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin and permeabilization with the FOXP3 staining kit. After culture, cells were stained with a fixable viability dye (eBioscience) to discriminate between dead and viable cells.

Cytokine analysis

Supernatants of cell cultures were harvested and frozen at −80°C until analysis. Cytokine concentrations were measured by using Multiplex analysis (Luminex, Austin, Tex) as described elsewhere.[10]

Statistics

Significance of the differences observed was tested using Kruskal Wallis test and Dunn's Multiple comparisons test using GraphPad Prism 5.03 for Windows. CB and infant samples were compared to samples derived from adult controls.

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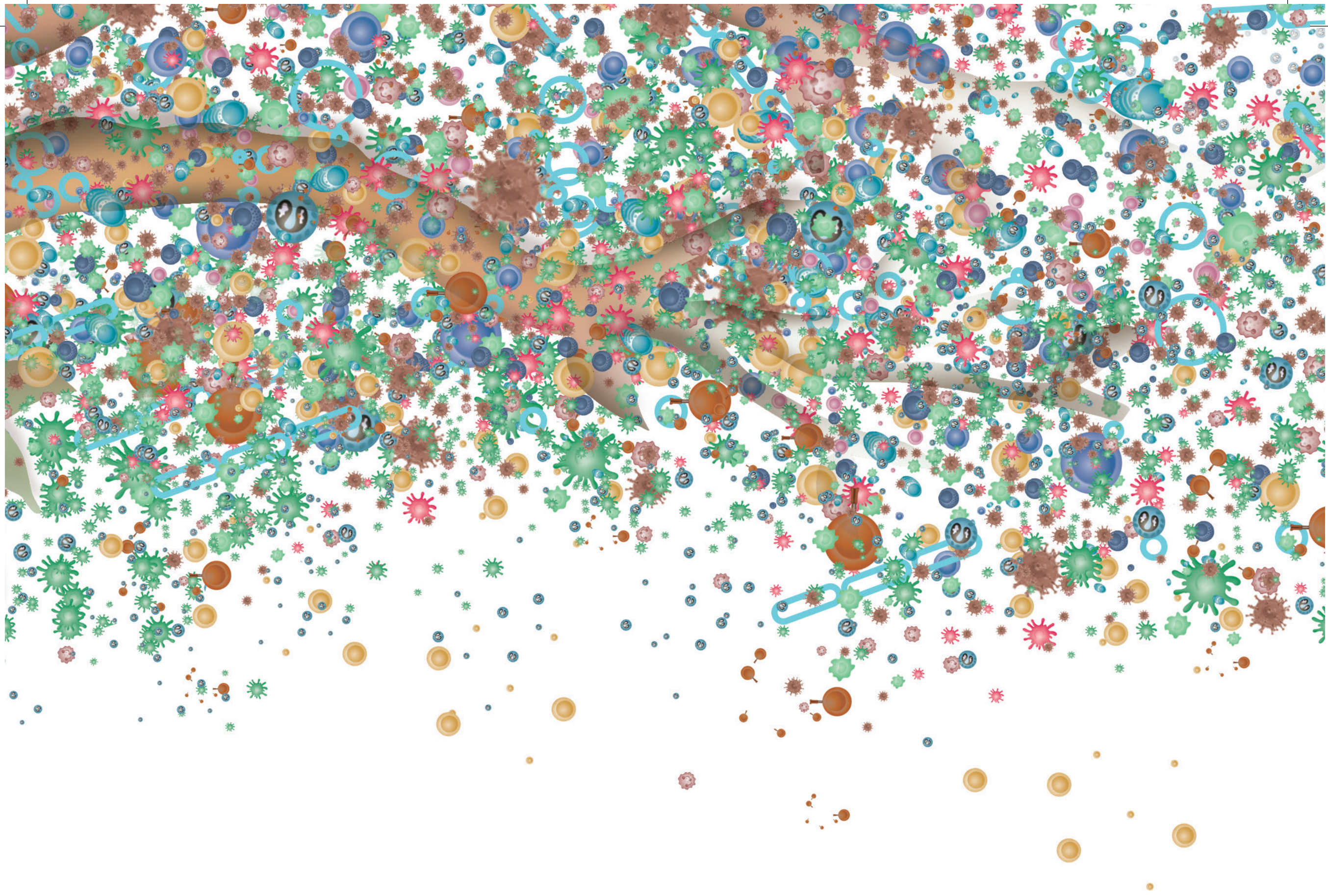

White matter injury in neonatal viral encephalitis is associated with restrained systemic inflammation

Hoeks SB, Groenendaal F, de Vries LS, de Jager W

Submitted

ABSTRACT

Objective: Viral infections of the central nervous system (CNS) in neonates are associated with seizures and cerebral white matter injury (WMI), which are strong predictors of neurodevelopmental impairment. Especially neonates suffer from severe clinical symptoms and experience long-term consequences after neonatal viral encephalitis. In animals, activated immune cells in viral CNS infections contribute to neuropathology. However, the neonatal immune system is equipped with extensive immune regulatory mechanisms to support the unique demands for intrauterine survival and early postnatal life. Therefore, we studied the immune signature and its correlation with the development of WMI in neonatal viral encephalitis.

Methods: We performed a multiplex immunoassay for 33 immune related proteins in cerebrospinal fluid (CSF) and plasma of 17 neonates with a viral encephalitis caused by Enterovirus (EV), Human Parechovirus (HPeV), or Herpes Simplex virus type I (HSV) confirmed with PCR. Neurodevelopmental outcome was assessed with the Griffiths Mental Development Scales and neuroimaging was performed with cerebral ultrasound and/or MRI. According to their neurodevelopmental outcome and abnormalities on neuroimaging, infants were categorized in three groups: favorable, uncertain and severely affected.

Results: Frontal white matter injury characterizes EV and HPeV encephalitis. These neurotropic viruses also share similar serum immune profiles though in all neurotropic viruses, an impaired systemic immune activation is associated with severe white matter injury and a compromised neurodevelopmental outcome.

Interpretation: We hypothesize that this failure to mount a proper immune response might be related to physiological adaptations of neonatal immune cells, virulence factors and/or immune escape mechanisms of neurotropic viruses. The complexity of the immune network ensuing viral invasion of the CNS and the vulnerability of neuronal and axonal cells suggest that more insight in physiological mechanisms that induce immune maturation in neonates may reveal strategies to diminish white mater injury following viral encephalitis in neonates and improve clinical outcome.

INTRODUCTION

Viral infections of the central nervous system (CNS) in neonates are associated with seizures and cerebral white matter injury,[1-4] which is a strong predictor of neurodevelopmental impairment.[5,6] Enterovirus (EV), Human Parechovirus (HPeV) and Herpes Simplex virus (HSV) are common causes of neonatal viral encephalitis and can be associated with severe white matter injury,[7-10] while pathophysiological mechanisms remain unclear.[8] In vitro and animal studies show that activated immune cells and soluble proteins in viral CNS infections contribute to neuropathology and induce long-term cellular damage or death.[11-14] Especially neonates suffer from severe clinical symptoms and possibly experience long-term consequences after neonatal viral encephalitis.

The neonatal period is characterized by important processes of brain growth and maturation. The neonate encounters a pathogen in a period of critical brain development, especially for white matter structures that induces susceptibility for injury.[15] Furthermore, the neonatal immune system is equipped with age-specific characteristics that reflect adaptation to the specific demands placed on the immune system in early life.[16,17] Neonatal immune responses are generally dampened compared to adults and more potent inflammatory responses develop only gradually during the first year.[18,19] Moreover, pathogens continuously evolve mechanisms to evade or inhibit the immune system and might even hijack immune mechanisms to facilitate infection and survival.[16] For example, fetuses and newborns are most severely affected by the ZIKA virus since it activates autophagy in neural stem cells, which in turn inhibits normal neurogenesis during development.[20] Based on these properties, pathogen-host interactions in neonates are supposed to be different from adults and might explain differences in clinical symptoms and long-term consequences after neonatal viral encephalitis.

However, ethical and practical issues in studies on human newborns hamper experiments to address their state of immunity and potential connection with neuropathology. With the emergence of innovative technology, it is possible to simultaneously investigate a broad spectrum of immune proteins in minimal amounts of bodily fluids and therefore these methods are especially suitable for neonates. Soluble proteins produced by immune cells and by the local tissue of the inflamed brain tissue and endothelium, mediate inflammatory processes by activating and recruiting immune cells and therefore play key roles in the regulation of the immune response. Detection of these proteins in both cerebral spinal fluid (CSF) and in blood might serve as a biomarker to assess disease severity and may predict long-term neurological consequences. In this retrospective study, we aim to evaluate neonatal immune responses, cerebral injury and subsequent neurodevelopmental outcome in neonatal viral encephalitis. Therefore, we assessed a broad panel of 33 soluble immune-related proteins in CSF and plasma of neonates diagnosed with a viral encephalitis caused by EV, HPeV or HSV. Magnetic resonance imaging (MRI) of the brain and long-term neurodevelopmental follow-up data were used to categorize the impact of abnormalities on neurological development.

METHODS

Subjects

Between January 2003 and January 2013 patients with clinical signs of encephalitis and a positive viral specific polymerase chain reaction (PCR) in blood and/or cerebral spinal fluid (CSF), admitted to the neonatal intensive care at the University Medical Centre, Utrecht, the Netherlands, were eligible for the study. Encephalitis was defined as sepsis-like disease (e.g. rash, problems in thermoregulation, apnoea and/or feeding) and neurological symptoms (e.g. lethargy, irritability and/or seizures). Bacterial cultures remained negative. Residual samples of plasma and/or CSF of 17 infants were stored at -80°C until analysis.

Neurodevelopmental outcome was assessed at 6, 12 and 24 months. Infants were seen in the follow-up clinic by the neonatologist, physiotherapist and psychologist. A full neurological assessment, as well as the Griffiths Mental Development Scale (GMDS) or Bayley Scales of Infant and Toddler Development (BSID, 2nd or 3rd edition) were obtained by a certified pediatric physiotherapist to assess cognitive and motor development.[21, 22] We defined moderate delay as a GMDS score and BSID cognitive or motor scale scores < −1 to –2 SDS, and severe delay as scale scores < −2 SDS.

Serial cranial ultrasound (cUS) was performed, using a high-resolution (5-8 MHz) transducer (Toshiba). Magnetic resonance imaging (MRI) was performed using a 3 tesla Philips scanner (Achieva, Philips Medical Systems, Best, The Netherlands). MRI included sagittal, axial and/or coronal T1- and T2-weighted images and diffusion weighted images (DWI). Slice thickness varied between 4 and 1.2 mm.

According to abnormalities on cerebral imaging and neurodevelopmental outcome, infants were categorized in three groups: normal, uncertain or severe (table 1). Infants with normal imaging results and scores on neurodevelopmental tests within the normal range were classified as normal. Infants with extensive white matter lesions but a favorable neurodevelopmental outcome were indicated as uncertain affected. Patients were labelled as severe when patients died or white matter injury was extensive and combined with an adverse neurodevelopmental outcome with epilepsy, cognitive- and motor developmental delay and/or hearing problems.

Multiplex immunoassay

CSF and EDTA were centrifuged and stored at -80°C until analysis. Measurements of 33 proteins related to inflammation were performed using an in-house developed and validated (ISO9001:2015 certified) multiplex immunoassay based on Luminex technology (xMAP, Luminex corporation Austin TX USA) as described previously.[23] Interleukin (IL)-1RA, IL-1α, IL-1β, IL-6, IL-7, IL-8 (CXCL8), IL-12, IL-13, IL-17, IL-18, IL-23, IL-25, IL-27, IL-29, IL-33, Interferon (IFN)α,

IFNβ, IFNγ, chemokine (C-C motif) ligand (CCL)2 or monocyte chemoattractant protein (MCP)-1, CCL3 or Macrophage Inflammatory Protein (MIP-1α), CCL4 or MIP-1β, CCL5 or Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES), CCL7 or MCP-3, CCL19 or MIP-3β, chemokine (C-X-C motif) ligand (CXCL)9 or Monokine Induced by Gamma interferon (MIG) and CXCL10 or Interferon gamma-induced protein (IP)10.

Aspecific heterophilic immunoglobulins were pre-absorbed from all samples with HeteroBlock (Omega Biologicals, Bozeman MT, USA). Acquisition was performed with the FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT Software, version 4.2 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (BioRad).

Statistical analysis

Basic descriptive statistics were used to describe the patient population. All statistical analyses were performed using either GraphPad Prism 6 or SPSS Statistics 21 (IBM). Values below the lower limit of detection were set to the lowest detected value. Hierarchical cluster analysis was performed using Omniviz 6.1.2 (Instem scientific). P values less than 0.05 were considered significant.

RESULTS

Seventeen infants were included in this study. Eight infants were infected with EV, five with HPeV and the remaining four with HSV. The gestational age ranged from $26^{1/7}$ to $40^{6/7}$ weeks, the median age at disease onset was eight days after birth, ranging from 2 to 98 days (table 1). Two infants died.

	EV (n=8)	HPeV (n=5)	HSV (n=4)
Gestational Age, weeks	38 (29-41)	39 (26-40)	40 (38-40)
Birth weight, grams	3115 (730 - 3950)	3100 (960 - 3840)	3515 (2485 - 3960)
Age at onset, days	7 (4-65)	8 (6-98)	10 (2-27)
CSF, days after onset	1 (0-4)	0 (0-1)	1 (0-3)
Blood, days after onset	3 (0-5)	2 (1-2)	1 (1-3)
CSF cells, .10^6/L	106 (1-2120)	2 (0-4)	6 (3-68)
CSF protein, g/L	1 (0.61-1.85)	0.75 (0.64-0.98)	0.73 (0.53-0.99)
CRP, mg/L	11.5 (1-17)	11 (4-17)	12 (2-32)
Leucocytes, 10^9/L	11.7 (6.5-14.6)	4.5 (2.3-7.6)	9.6 (3.8-27.2)
Inotropic support	1 (12,5%)	1 (20%)	1 (25%)
Seizures			
- Clinical	1 (12,5%)	1 (20%)	3 (75%)
- aEEG	1 (12,5%)	1 (20%)	3 (75%)
US abnormalities	6 (75%)	4 (80%)	4 (100%)
MRI abnormalities	3 (37,5%)	3 (60%)	3 (75%)
Adverse clinical outcome	1 (12,5%)	1 (20%)	4 (100%)
Severity groups			
- Normal	7 (87,5%)	1 (20%)	0 (0%)
- Uncertain	0 (0%)	3 (60%)	0 (0%)
- Severe	1 (12,5%)	1 (20%)	4 (100%)

TABLE 1. Characteristics of patients. Values are the median (range) and absolute numbers (percentages); EV = Enterovirus, HPeV = Human Parechovirus, HSV = Herpes Simplex Virus; CSF = Cerebral Spinal Fluid; CRP = C-Reactive Protein.

Neuroimaging findings

Cerebral MRI including diffusion weighted imaging (DWI) was performed 1-7 days (median 5 days) after disease onset if the cranial ultrasound (cUS) examination showed abnormalities or if clinical and/or subclinical seizures were present. In five infants (4 EV and 1 HPeV), cUS was normal and no seizures occurred. One infant with HSV died of severe pulmonary infection and MRI was not performed. One infant had normal MR imaging. MRI showed diffusion restriction in ten out of eleven infants with cystic evolution in three. Both in EV and HPeV infections, distribution of white matter injury was highly symmetrical and six out of eight infants had white

matter lesions in the frontal and parietal lobe whereas in three infants with HSV distribution of white matter lesions was asymmetrical and present in the parietal and temporal lobes, in the deep grey matter and optic radiation (Fig 1).

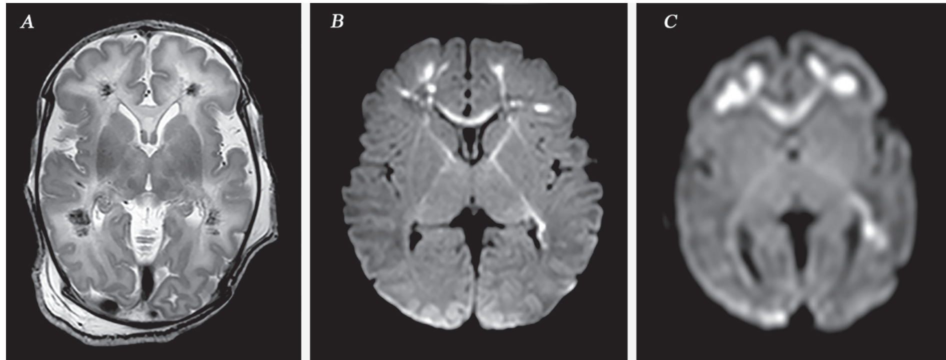

FIGURE 1. MRI, axial plane in an infant with EV (A), HPeV (B) and HSV (C) infection. In A, a T2 sequence is used, in B and C, DWI. The distribution of abnormalities in the white matter is comparable. In the infant with EV infection there is a hemorrhagic component. In the infants with HPeV and HSV infection, involvement of the corpus callosum and optic radiation (left side) is seen as well and in the infant with HPeV infection the internal capsule shows also restricted diffusion.

Neurodevelopmental outcome

Infants with EV and HPeV encephalitis were noted to have better cognitive and motor scores at follow up visits compared to infants with HSV encephalitis. Six of eight infants with EV encephalitis had a favorable clinical outcome at a median follow-up of 60 (range 3-96) months. Follow-up was discontinued during the first year in three infants without clinical seizures and without MRI abnormalities. One infant died due to redirection of care because of severe, extensive cerebral damage seen on MRI. Following HPeV encephalitis, one infant had mild behavioral problems and in the other four infants a normal outcome was observed at a median age of 60 (range 2-60) months. None of the infants with EV and HPeV developed epilepsy and the GMDS assessment scores were within the normal range with a median developmental quotient (DQ) of 105 (range 97-109). One of the four infants with HSV died, two developed epilepsy and showed neurodevelopmental delay. One of these infants scored within the normal range on the GMDS with DQ 89 at 24 months but with a speech and language disorder and 93 on the WPPSI at 6 years of age. The other child scored within the normal range on GMDS with DQ 98 at 42 months but the WPPSI-II showed a total IQ of 80 at 6 years of age. One infant with HSV developed hypsarrythmia and cerebral palsy and was too severely affected to be formally assessed.

Relation neuro-imaging and outcome

Infants were allocated to three groups; Eight out of seventeen patients had no abnormalities on imaging and a favorable neurodevelopmental outcome (6 EV and 2 HPeV); Three out of seventeen patients were categorized as uncertain affected and showed mild cerebral injury on neuroimaging associated with a favorable early outcome (1 EV and 2 HPeV); Six out of seventeen patients had severe neuro-imaging abnormalities with an adverse clinical outcome with psychomotor retardation or death (1 EV, 1 HPeV and 4 HSV).

Three infants were born preterm (2 EV and 1 HPeV) and two of them were infected at (late) preterm age. MRI showed punctate white matter injury in one infant (HPeV) and was normal in the other infant (EV). All preterm born infants had a favorable neurodevelopmental outcome.

Immunoprofiles and association with imaging and outcome

All measured proteins and their concentration are listed in table 2. We compared the concentration of immune related proteins in two specific matrices, CSF and plasma, between neonates with encephalitis caused by either EV, HPeV or HSV. CSF was available in fourteen infants, plasma was available in thirteen infants. Reciprocal changes in the concentration of different immune related proteins show resemblance across the different viruses in both plasma and CSF, as indicated by spider plots (Fig 2, *A and B*). In plasma in EV, HPeV as well as in HSV encephalitis, a relatively low concentration of MIP-3β was correlated to a relatively high concentration of IL-8. One exception was the relatively high concentration of IFNa in plasma of infants with a HSV encephalitis compared to infants with an EV or HPeV infection (Fig 2, *A*). This pattern with identical reciprocal relations in the concentration of immune related proteins in different viruses, could also be observed in CSF but was less consistent (Fig 2, *B*).

Next, we performed a hierarchical cluster analysis, comparing cytokine profiles in plasma at time of diagnosis and without (I), those with uncertain (II) and those with severe (III) neurological sequelae or extensive abnormalities on neuroimaging. This analysis revealed a clear separation in 12 immune-related proteins in plasma specific for an impaired neurodevelopmental outcome or severe injury after neonatal viral encephalitis, as depicted in figure 3. In CSF, multiple clusters of immune related proteins were identified and these clusters were neither related to the virus nor prognostic for neurodevelopmental outcome whereas cell influx in CSF was directly related to the expression of proteins (data not shown).

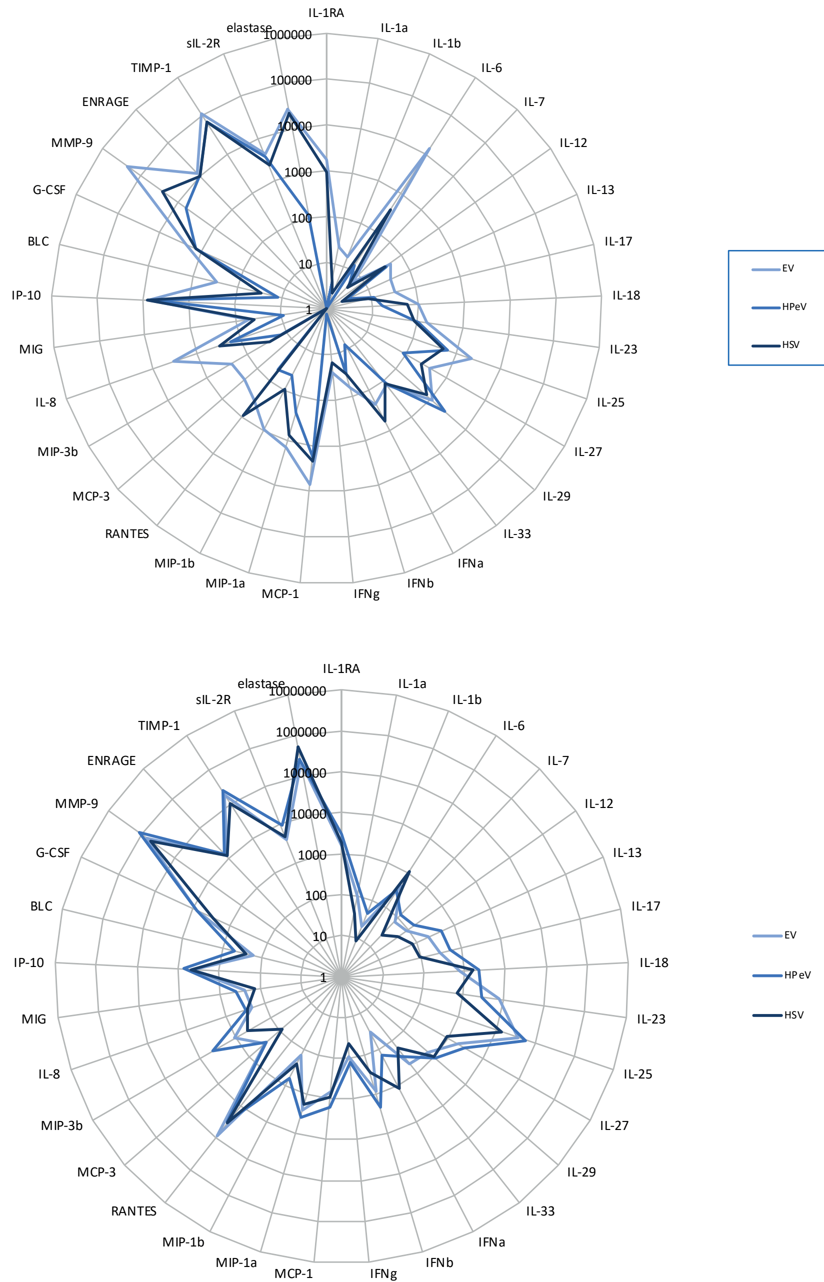

FIGURE 2. Radarplot of profile of immune-related proteins in neonatal encephalopathy with enterovirus (EV), human parechovirus (HPeV) and herpes simplex virus (HSV). Means of EV (light grey), HPeV (dark grey) and HSV (black) are represented on log10 scale. Levels of concentration of cytokines and chemokines (A) in EDTA and (B) in CSF.

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Markers (pg/ml)	EV	
	CSF	Blood
IL-1RA	325 (1-4974)	1794 (921-2182)
IL-1a	9 (4-59)	83 (14-216)
IL-1b	2 (1-53)	18 (7-38)
IL-6	617 (19-41549)	148 (75-1151)
IL-7	4 (1-15)	72 (29-123)
IL-12	42 (32-79)	69 (39-147)
IL-13	5 (1-117)	190 (60-384)
IL-17	11 (2-98)	261 (43-577)
IL-18	40 (15-268)	587 (351-1411)
IL-23	109 (69-307)	3205 (681-30350)
IL-25	771 (148-6306)	33236 (6672-62659)
IL-27	294 (10-745)	1711 (712-3217)
IL-29	1175 (624-2296)	521 (299-1423)
IL-33	129 (114-142)	305 (157-1000)
IFNα	12 (1-845)	29 (13-73)
IFNβ	39 (25-143)	913 (230-1506)
IFNγ	4 (1-56)	74 (29-178)
MCP-1	6983 (1117-10000)	596 (233-1401)
MIP-1α	466 (1-4128)	2517 (1038-3755)
MIP-1β	101 (49-3661)	137 (72-194)
RANTES	87 (19-1325)	69674 (2937-240923)
MCP-3	2 (1-848)	362 (65-479)
MIP-3β	122 (1-835)	822 (288-2196)
IL-8	1198 (101-9232)	191 (158-341)
MIG	25 (5-110)	232 (142-363)
IP-10	8121 (1368-9379)	4270 (1068-7919)
BLC	97 (1-848)	158 (129-223)
G-CSF	1571 (1048-4016)	8410 (1686-12071)
MMP-9	50829 (7949-789096)	356971 (204465-1820000)
ENRAGE	10471 (10232-14468)	11552 (9688-15050)
TIMP-1	72414 (45843-216057)	121487 (59399-516211)
sIL-2R	3968 (2816-7604)	3495 (1614-6657)
elastase	4158 (62-68455)	34933 (25612-1000000)

TABLE 2. Concentration of cytokines and chemokines in CSF and plasma of neonates with EV, HPeV and HSV encephalitis are shown (EV, enterovirus; HPeV, human parechovirus; HSV, herpes simplex virus). Data are displayed as mean (range).

	HPeV		HSV	
	CSF	Blood	CSF	Blood
	1 (1-1)	2919 (2637-3201)	997 (541-2162)	1563 (292-4021)
	4 (3-5)	168 (131-205)	4 (4-5)	26 (8-82)
	1 (1-1)	47 (33-61)	3 (1-4)	5 (3-21)
	11 (90-21)	304 (249-358)	106 (85-1239)	390 (19-3754)
	5 (2-8)	125 (114-136)	1 (1-1)	18 (3-66)
	40 (30-45)	149 (118-180)	36 (34-36)	46 (34-67)
	1 (0-10)	457 (325-588)	1 (1-1)	35 (11-232)
	11 (7-17)	528 (391-664)	7 (6-10)	70 (27-204)
	16 (12-20)	2329 (793-3865)	63 (54-70)	1578 (297-2944)
	112 (68-124)	2931 (2090-3771)	80 (80-82)	515 (152-1553)
	627 (338-882)	55656 (43389-67923)	521 (271-559)	12414 (1667-27091)
	82 (39-144)	2979 (2512-3446)	223 (92-511)	933 (259-1468)
	2050 (1264-4847)	1100 (750-1450)	694 (569-731)	678 (518-1821)
	123 (115-131)	296 (250-342)	116 (111-121)	147 (134-207)
	7 (3-13)	145 (107-183)	90 (50-2162)	571 (12-3419)
	34 (13-39)	2168 (1624-2711)	25 (23-50)	264 (95-457)
	1 (1-2)	127 (94-159)	18 (1-41)	41 (10-83)
	1766 (1559-1977)	1557 (1039-2075)	948 (600-4650)	697 (222-2094)
	288 (1-382)	3722 (3162-4283)	1038 (447-1050)	2067 (543-2314)
	44 (22-63)	618 (426-810)	52 (33-247)	97 (51-762)
	41 (20-96)	32045 (11668-52422)	88 (19-1325)	25453 (4131-84607)
	1 (1-1)	273 (238-307)	1 (1-1)	59 (37-192)
	16 (7-21)	4199 (3935-4462)	42 (3-50)	483 (76-800)
	167 (118-189)	307 (292-323)	245 (104-499)	247 (60-494)
	9 (7-11)	420 (318-522)	48 (36-55)	121 (45-279)
	7030 (3894-7882)	7354 (6866-7842)	8258 (7796-8734)	5504 (426-8267)
	7 (5-32)	489 (262-717)	31 (9-59)	101 (68-728)
	1330 (1072-1545)	8074 (6593-9556)	1254 (1158-1573)	2259 (1600-5188)
	5194 (4245-8691)	1164894 (204465-1970000)	6557 (5403-19184)	278328 (269722-1310000)
	10227 (10144-11017)	13914 (13853-13974)	9947 (9860-10307)	12027 (10397-12879)
	68510 (57345-81159)	245355 (156128-334581)	71076 (60583-71506)	115828 (46173-139420)
	3986 (2490-4353)	9099 (5958-12241)	2668 (2464-3024)	5358 (6865-2138)
	118 (36-198)	249090 (106796-391384)	3963 (3031-4963)	512234 (9425-1000000)

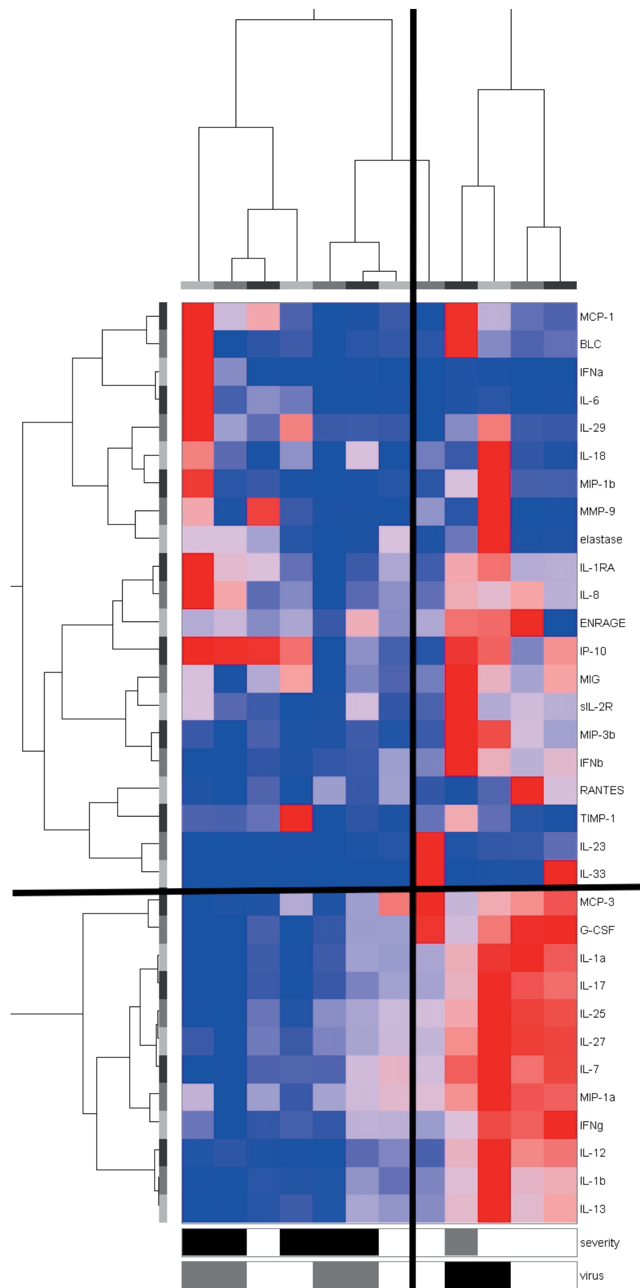

FIGURE 3. Neurological outcome was analyzed in white (normal), grey (uncertain affected) or black (severely affected). Hierarchical cluster analysis of proteins in plasma of patients with neonatal viral encephalitis identified two clusters of molecules that predicted neurodevelopmental outcome. Data were normalized for each mediator individually. Blue indicates minimal expression; red indicates maximal expression.

DISCUSSION

Viral encephalitis in neonates is associated with increased risk for impaired neurodevelopmental outcome and it was suggested that innate immune responses might be involved in development of white matter injury.[8] Still, the effect of viral infections on the host immunity and its association with cerebral damage is not well investigated. In this pilot study, we demonstrated that neonates are able to generate a substantial systemic immune response upon viral invasion. Different neurotropic viruses elicit a similar pattern of immune-related proteins in plasma of neonates (Fig 2*A*). This study shows that the distribution and location of WMI following neonatal viral encephalitis shows also striking similarities in EV and HPeV (Fig 1) which is moreover corresponding with cerebral damage after other neurotropic viruses as Rotavirus and Chikungunya virus.[24, 25] Despite these similarities in systemic immune responses and in distribution of WMI, long-term neurodevelopmental outcome is variable. In this study, we showed that the concentration of immune-related proteins in blood after EV, HPeV and HSV encephalitis in neonates appears to be inversely correlated to the severity of cerebral injury and long-term neurodevelopmental outcome (Fig 3). We could identify clusters of immune proteins in plasma, which were associated with long term neurodevelopmental outcome and/or abnormalities on cerebral imaging (cUS and/or MRI). These clusters reveal that a low concentration of various immune-related proteins in plasma is associated with severe cerebral injury and a compromised clinical outcome.

Our data suggest that impaired immune activation is associated with white matter injury after viral encephalitis in neonates. Immune responses of neonates are often indicated as impaired or immature since neonatal immune responses deviate from infants and adults. However the ex vivo data described in this study indicate that neonates are able to induce potent immune related protein profiles. The neonatal immune system is characterized by the induction of immune regulatory pathways and have unique features to induce immune tolerance.[26-29] On the other hand, they suffer from these intrinsic adaptations dampening their immune responses, as these adaptations make them vulnerable for infections. Preterm infants are affected even more than term born babies as they have a smaller pool of monocytes and neutrophils, they lack maternal antibodies and have a reduced ability to detect viruses and kill pathogens with a lower production of cytokines limiting T cell activation.[30] This might explain why preterm infants with EV and HPeV encephalitis more often suffer from severe white matter injury with cystic evolution,[31] whereas white matter injury after viral CNS infections is not seen in older infants nor in adults.[32]

Furthermore, neonates show a fast brain development that continues after birth. Interfering events like infections disturb brain development especially in the last trimester since the growth velocity of the brain volume accelerates after 25 weeks.[33] Therefore preterm born

babies might have an increased vulnerability for WMI. In this study, three preterm born infants were studied however they were infected at late preterm or term equivalent age. They demonstrated a systemic immune response upon viral infection similar to term born infants. They did not develop cysts in the white matter.

To the best of our knowledge this study is the first to show that a complex network of immune-related proteins shapes the neonatal immune response to invasive viral infections. This immune response is not characterized with a classical IFN or T_H1 signature but it reveals the importance of a good functioning complex network of immune proteins. In the present study, we demonstrate in all studied neurotropic viruses similar connections between immune-related proteins (Fig 2*A*). This finding implies that immune-related proteins act in cooperation rather than alone.

We could not identify in CSF similar correlations of immune-related proteins and neurodevelopmental outcome. This compartment might not representative for the injured cerebral tissue. Furthermore, rapid and tightly controlled immune responses are required to restrain the viral infection within the CNS while limiting tissue damage.[34] However, it is not clear which pathophysiological pathways are involved in development of WMI after viral encephalitis. Since most cells of the CNS are non-regenerative, preferentially immune responses that combat the virus and simultaneously promote cell survival are first applied.[35] Different ratio and type of cytokines will affect barrier integrity,[36, 37] which subsequently may lead to cerebral injury.[38] Different immune mechanisms in CNS infections have been described, besides peripheral immune cells that cross the BBB, CNS neurons and glia actively regulate macrophage and lymphocyte responses and have the ability to direct neuroprotective lymphocyte responses.[39-41] In addition to cytokine secretion, T cells kill infected T cells through perforin- and/or granzyme-mediated mechanisms. Secretion of granzymes in some CNS infections does not lead to lysis but prevents viral replication while sparing the infected neuron.[42] Lastly, IP-10 and MIG are IFN-inducible CXC chemokines and potent chemoattractants for activated T-cells, memory T-cells and NK cells by signaling through the CXCR3 receptor.[43] IP-10 enhances the BBB and early expression within the CNS after viral infection might be important in initiating and maintaining a protective immune response.[44] Exaggerated activation of IP-10 and MIG has been proposed to be associated with adverse outcomes of viral infections.[45-47] In the present study we could not associate the concentration of IP-10 and MIG in CSF with the development of WMI or with an impaired neurodevelopmental outcome. Altogether, both disturbance of CNS barrier integrity and the local immune responses in brain parenchyma in response to viral infections, might explain the absence of a connection between the concentration of immune proteins in the CSF and the development of WMI.

Besides similarities in systemic immune responses in different viral CNS infections described in this study, shared characteristics of distribution of WMI in different viral CNS infections are remarkable. A diffuse distribution of white matter injury with a frontal predominance characterizes CNS infections caused by EV and HPeV but also by Chikungunya virus and Rotavirus.[48] In HSV asymmetrical lesions with both grey and white matter injury has been reported, noticeably different from injury after the other neurotropic viruses.[10] Cerebral development progresses after birth and irreversible neuronal damage or different developmental pathways may be induced upon interference in this development. Remarkably, infants with WMI after EV or HPeV infections, without cystic evolution and without involvement of the central grey nuclei will have a better long term neurodevelopmental outcome than expected based on their MRI abnormalities.[49, 50]

As the study design is of retrospective exploratory nature, the number of include patients was limited, and therefore not from all subjects both plasma an CSF was available. Furthermore, as expected, no references MRIs of healthy newborns are available due to ethical restrictions. Four infants were doing so well during admission (no convulsions, normal cerebral imaging) that follow up for neurodevelopmental outcome was discontinued during the first year. Despite these limitations associated with the study population, the present data enhances the understanding of neonatal immune responses upon viral invasion of the CNS.

To conclude, our data indicate that different neurotropic viruses provoke rather identical immune responses and EV and HPeV share rather identical distribution of subsequent white matter injury upon viral invasion of the CNS but severity of cerebral injury seems to be dependent on the degree of immune activation. The complexity of the immune network ensuing viral invasion of the CNS and the vulnerability of neuronal and axonal cells suggest that more insight in biological improvement of immunity in neonates may reveal strategies to diminish white mater injury after viral encephalitis in neonates.

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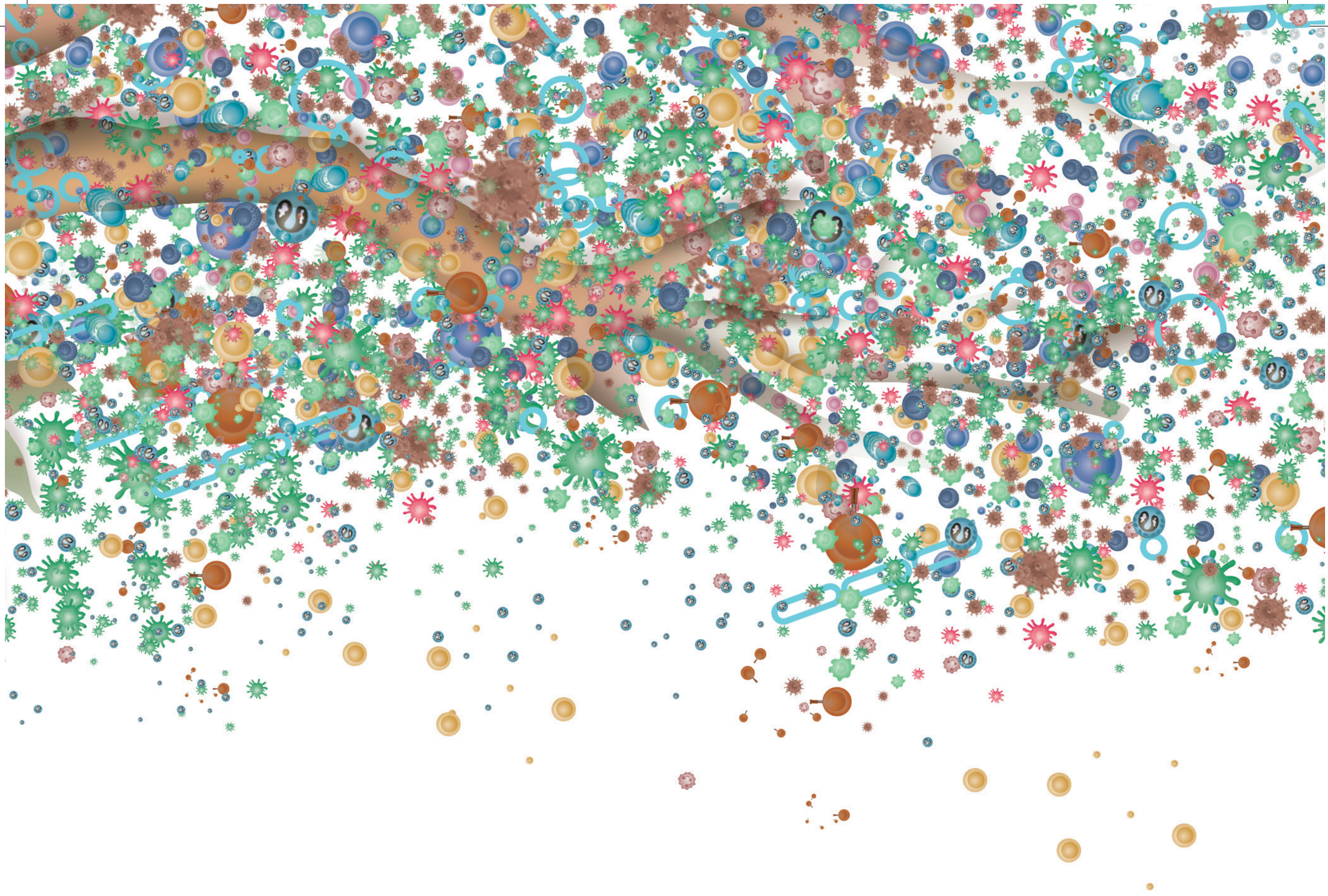

Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples

Keustermans GC, Hoeks SB, Meerding JM, Prakken BJ, de Jager W

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ABSTRACT

Cytokines are key components of the innate and adaptive immune system. As pivotal players in the progression or regression of a pathological process, these molecules provide a window through which to monitor diseases and can thus act as biomarkers. In order to measure cytokine levels, a plethora of protocols can be applied. These methods include bioassays, protein microarrays, high-performance liquid chromatography (HPLC), sandwich enzyme-linked immunosorbent assay (ELISA), Meso Scale Discovery (MSD) electrochemiluminescence and bead based multiplex immunoassays (MIA). Due to the interaction and activity of cytokines, multiplex immunoassays are at the forefront of cytokine analysis by allowing multiple cytokines to be measured in parallel. However, even with optimized protocols, sample standardization needs to occur before these proteins can optimally act as biomarkers. This review describes various factors influencing the levels of cytokines measured in plasma, serum, dry blood spots and tissue biopsies, focusing on sample collection and handling, long term storage and the repetitive use of samples. By analyzing how each of these factors influences protein levels, it is concluded that samples should be stored at low temperatures in order to maintain cytokine stability. In addition, within a study, sample manipulations should be kept the same, with measurement protocols being chosen for their compatibility with the research in question. By having a clear understanding of what factors influence cytokine levels and how to overcome these technical issues, minimally confounded data can be obtained and cytokines can achieve optimal biomarker activity.

INTRODUCTION

Cytokines are small protein, glycoprotein or peptide molecules that, through cell signaling, allow intricate cellular communication. These molecules are produced by cells of various embryological origin and are classified according to structure or function.[1] Structural classification is used mainly for cytokines that do not display a high level of redundancy, allowing these members to be separated into distinct groups. In general, however, cytokines are divided into functional families based on the enhancement of immunological responses, these include interleukin-1 (IL-1), IL-6, IL-10, IL-12, IL-17, TNF, IFN, TGF, PDGF, gamma chain (IL-2, IL-4, IL-7, IL- 9, IL-15, TLSP), and beta chain (IL-3, IL-5) families and chemokines (C, CC, CX and CXC3). They exhibit a wide variety of activities but are most often seen as effector molecules that can alter the behavior of the immune system instantly during an immune response. Cytokines can act in an autocrine, paracrine, endocrine or juxtacrine manner (Fig. 1). Upon binding with their cell surface receptor, these molecules initiate an intracellular signaling cascade that may result in the up regulation and/or down regulation of gene expression or may take on transcription factor activity all subsequently inducing further cytokine production or curbing their own activity via feedback inhibition. The effect exerted by a cytokine depends on its extracellular levels, the expression of its complementary receptor and the type of signaling cascade initiated by this receptor binding, with cell type particularly influencing receptor expression and downstream signaling. These molecules exhibit redundancy in their activity as well as dual functionality. This is exemplified by their ability to act in one case as a receptor ligand and in another as a transcription factor.[2] Cytokines produced by cells of the innate immune system play an essential role in influencing the immune response towards protective immunity and act as a key link between the innate and adaptive immune system. In response towards pathogens these molecules are secreted by immune cells and act as first danger signals, alerting, and initiating immunological pathways. Disturbance in this balance, however, plays a role in chronic disease progression by influencing auto-inflammatory or auto-immune pathways.[3, 4]

The specific role of cytokines in these pathological disorders has been the subject of extensive research over the past few decades with molecular cloning, specific blockage of activity, gene deletion and receptor identification as well as the testing of recombinant cytokines illustrating how essential cytokines are to disease pathogenesis.[5-7] As a result of this work, the use of new drugs which modulate the inflammatory processes of the immune system for many disease types in the field of oncology and immunology have been shown to result in major changes in the downstream cytokine milieu. The most elegant illustration of the functionality of cytokines comes from the blockage of TNFα in rheumatoid arthritis, this specific blockage results in the effective reduction of disease activity and joint destruction.[8, 9] Contradictory to these positive intervention strategies, in 2006, a phase 1 clinical trial involving a CD28 receptor monoclonal

antibody agonist TGN1412 resulted in a severe cytokine storm in healthy volunteers.[10,11] These positive and negative cytokine mediated events illustrate that cytokines are key components of effector phase immunity. Thus, monitoring these molecules can provide key insight into disease progression or regression, in essence, cytokines can be looked at as biomarkers of a disease process. Per definition, biomarkers are "A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[12] Biomarkers can be discovered and isolated locally (biopsies) or systemically (e.g. blood or urine) and can either be drug-related or disease-related with cytokines falling into the latter of these categories. Disease related biomarkers provide insight into the possible effect of treatment on a patient (predictive marker), the disease state of a patient (diagnostic marker) or future disease development with regards to a certain outcome but irrespective of treatment (prognostic marker).[13] In order for cytokines to take on this biomarker role however, a key hurdle needs to be overcome, namely that of a lack of standardization in cytokine analysis. This standardization process is independent of cytokine structure or biology but dependent on protocols and sample handling. This review aims to provide a comprehensive overview on current methods for cytokine analysis in an effort to provide a means to standardization that will be applicable to biomarker discovery and validation in a clinical context.

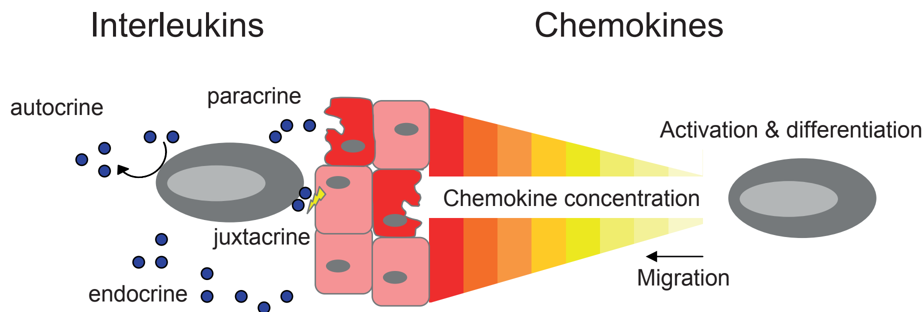

FIGURE 1. General function of cytokines. When secreted cytokines (interleukins) can act on cells or tissue that secrete them (autocrine), surrounds them (paracrine), travel to distant cells (endocrine) or remain cell bound and activate neighboring cells (juxtacrine). On the other hand chemokines are regulatory proteins which induce migration, activation and differentiation of cells to inflamed or damaged tissue.

SAMPLE STANDARDIZATION

Various methodologies are being optimized and designed to allow the precise measurement of cytokine levels within biological fluids however, even in the presence of assay improvement, a key factor needs to be monitored to achieve accurate cytokine measurements, that of sample standardization. The fact cytokines have a short-half life, are released by cells during storage as well as the possibility of degradation during sample handling make it of vital importance that we understand how to treat and store these samples for optimal analysis.[14] As a whole, unless standardization is achieved, data produced by these analytical protocols will remain incomparable and will not represent the true nature of the biological processes at hand.

Various types of samples have been used for cytokine analysis. For most patients, these samples can comprise of blood (plasma or serum),[15] urine,[16] saliva,[17] synovial fluid,[15] cerebrospinal fluid,[18] bronchoalveolar fluid,[19] aqueous humour eye fluid,[20] intestinal fluid,[21] exhaled breath condensates,[22] middle ear effusion[23] or lysed biopsies of a diseased organ[24, 25] thus, for each of these sample types, standardization needs to occur. The following section will illustrate which factors influence cytokine levels within common clinical sample types (blood and biopsies), outlining which conditions allow maximum cytokine measurement, stability and representation within a biological process.

Sample collection and handling

Sample handling and storage are pivotal in biomarker discovery as mishandling of these samples can drastically alter experimental outcomes and produce data that are not reflective of the biological situation. It has been shown for plasma collected in EDTA, sodium heparin (heparin) and sodium citrate (citrate) tubes (see de Jager et al. for methodology[26]) that, in healthy donors, cytokines are expressed at low levels while chemokines are expressed at higher levels (Fig. 2). In addition, data did not seem to indicate a difference in cytokine levels between anticoagulant effects, as cytokine levels were expressed in a similar range. However, following a consecutive measurement from the same donor (matched data), the general total recovery of spiked cytokines was more stable for heparin and EDTA tube samples, with EDTA tubes exhibiting decreased recovery of IL-15 and IL-18.[26] Citrate tubes showed lower levels of recovery for IL-1β, IL-2 and IL-6 while serum samples had significantly higher levels of CXCL8 and lower IL-6.[26] In addition to this data, it is known that platelet associated chemokines CCL3, CCL5, CXCL4 and TGF- β increase in serum as a result of ex vivo platelet degranulation, this activity could explain the higher levels of CXCL8 recorded in serum samples.[27, 28] Along with platelet degranulation, white blood cells release IL-1β during the clotting process resulting in increased levels of this cytokine in serum samples.[29]

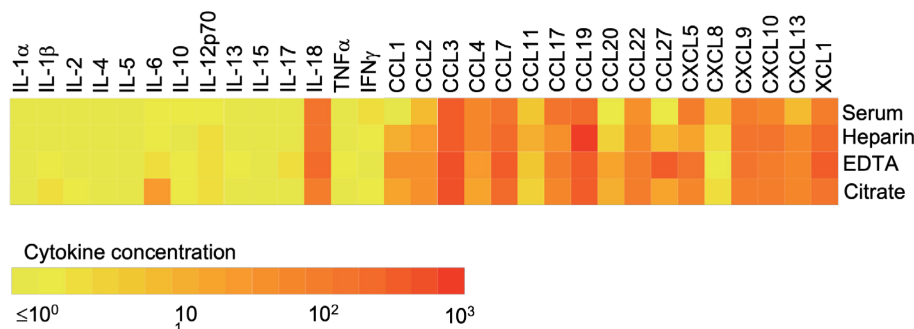

FIGURE 2. Cytokine expression in various blood drawing tubes. Blood samples were obtained from healthy individuals using various blood collection tubes. After centrifugation cell free plasma (sodium heparin, citrate EDTA) and serum was measured using a multiplex immunoassay as described elsewhere.[15] Color profiles were generated using geometric mean values as previously described.[30] In general all different tubes types can be used for this kind of assay, though slight variations are observed between various blood collection tubes.

For many clinical trials, experiments do not require the separation of blood components thus, most sample handling errors occur during the culturing of whole blood samples. To prevent the introduction of manipulation errors under these conditions a standardized culture system such as the TrueCulture© syringe can be used.[31] In this culture model, blood is collected directly from donors into a specially designed syringe tube. These tubes can be stored stably at -20 °C and can easily be thawed prior to use. The culture system creates a sealed environment in which freshly drawn blood can mix with a pre-prepared nutrient and stimulant solution. Samples can easily be incubated at 37 °C and once this incubation period is over, sedimented cells can be separated rapidly from the supernatant, preventing any further contact between the two environments. The syringes can be stored at -20 °C until further analysis, further minimizing analyte degradation. Data shows that cytokine levels measured using this system have a high degree of stability, with similar levels being measured in consecutive healthy donor blood draws.[31] Thus, in a clinical setting, this system facilitates stable and comparable culture conditions with minimal methodological error.

Temperature and time delay of processing

It is known that artifacts in cytokine measurements are affected by the duration of contact between serum or plasma and blood cells.[32] When plasma or serum cannot be isolated from whole blood or, if there is a delay in this separation procedure, it is important to understand cytokine dynamics under these conditions as changes here may greatly affect the experimental outcome and data analysis. For whole blood it has been shown that cytokine production can already occur within a 2-h period following blood draw.[33] When whole blood is stored at 4 °C, room temperature (RT) and 35 °C before separation into serum and plasma, some poignant changes in cytokine levels can be seen.[34] From a panel of inflammatory markers, keeping

blood at 4 °C before the removal of plasma was sufficient to maintain cytokine levels when compared to control plasma samples (plasma immediately isolated and store at frozen until analysis). After 4h at 4 °C however, cytokine levels already began to increase.[34] Samples stored at RT before separation showed a greater increase in cytokine levels than those seen at 4 °C. After 4h at RT most analytes significantly increased and levels continued to rise significantly over the 48 h. In order to expand on these data, we chose to analyze the effect of temperature, time and tube type on the level of a set of cytokines during a 48 h period. Our analysis revealed that plasma storage temperature (RT or 4 °C), time and collection tube type have an influence of total cytokine levels. In general, it was seen that at 4 °C there is less fluctuation in cytokine levels with minimal variation between plasma collection tubes while serum exhibited a partially higher total marker level compared to plasma samples stored at 4 °C. The data seems to indicate that there is no difference in anticoagulant tube effect or time at 4 °C even after 48 h (Fig. 3). The partially higher levels seen in serum samples can be explained by higher chemokine levels known to occur in serum samples when compared to plasma.[26] At RT data showed that, over a 48 h period, cytokine levels increase drastically with heparin tubes showing the greatest increase (89 ± 28%) followed by serum (56 ± 16%), EDTA (45 ± 13%) and finally citrate (30 ± 12%). The substantial increase in heparin tubes illustrates that although this sample tube type is ideal in multiple measurements for recovery, it is less than optimal when used to collect samples that are left at RT for longer than 4 h. If donor plasma samples are to be left at RT for up to 48 h citrate tubes would be the best for blood collection, showing the smallest change over time. The general increase in analyte levels after 48 h at RT can be explained by the fact that at RT, soluble receptors may begin to release bound analytes into solution resulting in an increase in the measurable amounts of these compounds.[35] It has been shown that blood samples stored at 35 °C have a lower mean concentration of inflammatory markers than those stored at RT however, these levels are higher than those measured in samples stored at 4 °C.[34] Serum isolated from clotted blood stored at 4 °C shows the lowest increase in inflammatory marker concentration while at 35 °C samples have the greatest increase, exhibiting a mean fold increase of 320 when compared to control serum samples. At RT most inflammatory markers are significantly increased and after 48 h general levels are significantly larger ranging from a 15 to 1700 fold increase. As a whole, serum samples do not show a significant decrease in analyte concentration. In addition to this, it is known that activation of the coagulation cascade occurs in serum tubes and that above RT, large amounts of chemokines are released from the blood cells.[34, 36]

Taking all this data into consideration, it is of vital importance that whole blood be kept 4 °C until separation into plasma or serum and that this separation occur within 4 h in order to prevent the absorption, release or degradation of cytokines.[37] If procedure specific tube types cannot be used then, any tube can be used as long as it is the same type within an experiment. Healthy donor samples that are stored at the same temperature and for an equal amount of

time as patient samples should also be used as comparative controls. Samples should also be collected at the same time of day as, it is known that analyte levels are influenced by circadian rhythms. This is exemplified in Rheumatoid arthritis where proinflammatory cytokines (TNF and IL-6) released at night contribute to morning disease related symptoms.[38] If all these steps can be taken then artificial analyte changes can be circumvented.

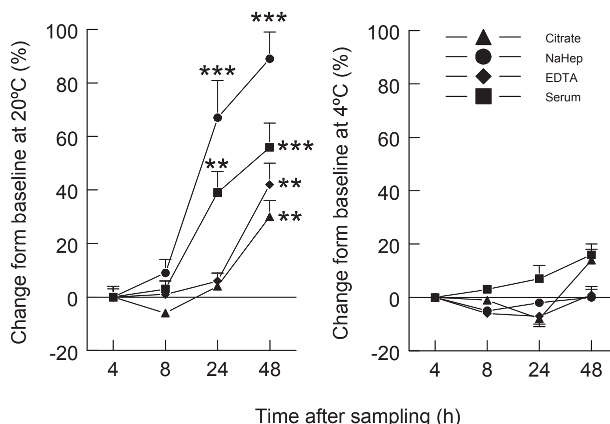

FIGURE 3. Influence of time and temperature after blood draw. Blood was collected from healthy donors in EDTA (EDTA), Sodium Heparin (NaHep), Sodium Citrate (Citrate) and clotting (serum) tubes. Subsequent to centrifugation, cell free plasma or serum was collected and stored for 4, 8, 24 and 48 hours at room temperature or at 4°C. All the plasma samples where then analyzed by multiplex immunoassay. as described elsewhere.[15] The figure illustrates the level of inflammatory cytokines for various tube types when samples were left at room temperature (left) and 4 degrees (right). *p<0.05, ** p<0.01, *** p<0.001.

Long term stability and repetitive use

Previous work of our group has shown that most cytokines remain stable within a two year period however degradation of some cytokines can already be seen within one year of storage at -80 °C.[26] In general a decrease over time is seen losing approximately 10–20% each year after 2 years of storage (Fig. 4). Eventually, a five- fold increase or decrease in different cytokine levels can occur after 5 years as a result of cross-reactivity between protein epitopes.[39] In addition to this storage effect, repetitive freeze thawing cycles also influence cytokine recovery. The levels of these molecules can either be stable, increased or decreased after multiple freeze– thawing cycles, depending on each cytokine. In general, cumulative levels significantly decrease (Fig. 3). Thus, in order to optimally measure cytokine levels, samples should be analyzed within 2 years and undergo minimal freeze–thawing procedures so as to maintain stable cytokine levels.[26, 39, 40] If the two year time period is however, not conducive to the experimental set up, individual changes in cytokine/chemokine levels should be assessed before conducting these long term storage studies, or biobanking.

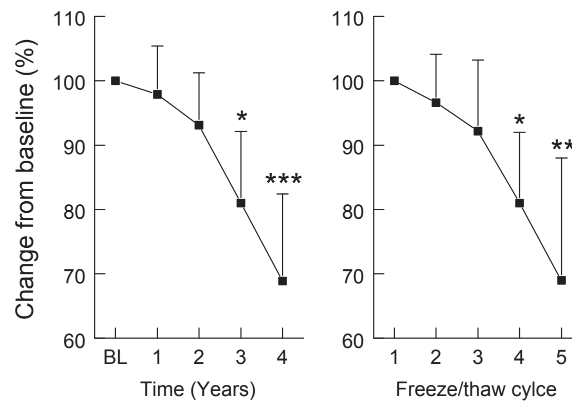

FIGURE 4. Influence of long time storage and multiple freeze thawing cycles. Blood samples of healthy individuals were stored at -80°C and measured at baseline and various time points. As shown in the left panel cytokine levels are subject to change even frozen at -80°C after approximately 2 years of storage. Next blood samples were repeatedly thawed and cytokine profiles were assed. After several cycles cytokine levels are subjected to breakdown, resulting in lower levels (right panel). * $p< 0.05$, ** $p<0.01$, *** $p<0.001$.

Several long term stored samples used in experiments or housed in biobanks are, in some cases, not originally collected for the experiments they are used in. A prime example of this is the use of dried blood spots (DBS) in neonatal screening. Excess DBS samples from these procedures are often stored in biobanks for use in other research as the Guthrie card on which samples are stored is an easy and appropriate medium for transport and long term storage.[34, 35, 41] The use of these samples in research and particularly inflammatory marker research means that a comprehensive understanding of sample storage, handling and analyte stability is required. Skogstrand et al. performed experiments in which DBS were stored for several days at various temperatures. Analysis of these samples revealed that the levels of inflammatory markers remained stable for up to 7 days when compared to control DBS samples stored at -20 °C. Any changes seen in analyte levels at this stage were smaller than those observed in plasma and serum from whole blood stored for shorter periods of times.[34] After 30 days however, some pro-inflammatory markers showed a significant change in concentration. In addition to this, at RT or 35 °C, there was no specific trend for individual inflammatory markers but rather a trend towards increased analyte levels with protection of samples against humidification at 4 °C having no positive influence. Even though measurable levels of pro-inflammatory markers were lower in DBS, these levels were more stable than those observed for liquid blood samples. Sample storage for several years, however, still results in varied levels of individual compounds. It is suggested in this study that faster drying of DBS and storage at low temperatures would be the optimal method by which to conserve analyte levels.[34] DBS thus seem to be a possible reliable alternative method for whole

blood, serum or plasma sample storage. Requiring a small volume of blood, (approximately 15 µl), on a stable and portable filter paper,[42] these samples are less vulnerable to biological degradation. However, even with higher analyte stability, the sample extraction process for DBS results in the dilution of low abundant proteins. This dilution factor greatly influences the cytokine panel being measured and results in the loss of delicate disease associated cytokines.[43]

Lysed tissue (biopsies)

In order to analyze diseased tissue, biopsies must be taken. Due to the fact that not all cytokine mediated pathological responses can be measured systemically, measuring cytokine levels within the diseased tissue can provide a better understanding of the cellular pathological process. In addition to this, cytokine levels that can be measured systemically only provide a reflection of what is occurring at the sight of inflammation whilst biopsies provide a key primary representation of the tissue specific diseases process. As with all samples, the processing of this tissue is important. When analyzing cytokine levels in whole cells, samples need to initially be lysed. We thus performed an experiment to determine if the type of lysis buffer used could alter cytokine levels measured due to increased background levels. For these experiments, peripheral blood human mononuclear cells where used to mimic biopsy tissue due to the fact that tissue samples where not readily available. Cells were stimulated with LPS (4 h, 37 °C). Following stimulation, cells were lysed with various lysis buffers; Roche Complete Lysis M, Cell Signaling Technology (CST) or Bio-Rad Lysis buffers. Data showed that the Roche lysis buffer had the highest noise/ background levels and resulted in the lowest cytokine measurements, Bio-Rad background levels were lower than those of Roche with partially higher cytokine levels while the CST lysis buffer resulted in the least background activity and produced the highest cytokine measurements, resulting in a better signal to noise ratio (Fig. 5). Our data clearly indicates that depending on the lysis buffer used, cytokine levels measured within tissue can be drastically altered, indicating that lower lysis buffer background levels result in higher cytokine measurements. It is thus important to understand the functionality of any lysis buffer used in tissue sample analysis as this may influence the discovery of cytokine networks that cannot be measured systemically but rather have a localized tissue specific role.[24]

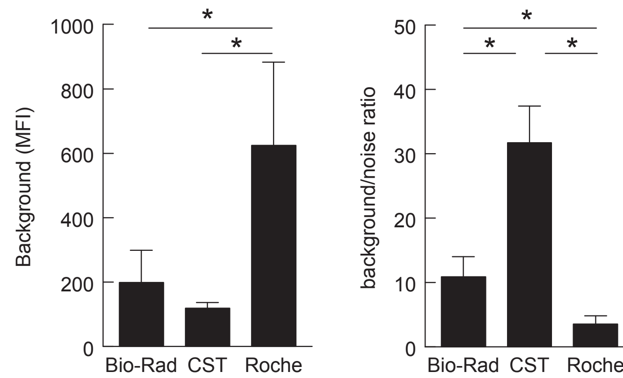

FIGURE 5. Influence of lysis buffer on assay performance. Peripheral blood human mononuclear cells from healthy donors were isolated using Ficoll-gradient centrifugation. Cells were then stimulated with a concentration 10 ng/ml of LPS for 4hours. Following the incubation period supernatant was removed and cells were washed with PBS. Next cells where lysed according to the lysis protocols for Complete lysis M (Roche), Cell signaling Technologies lysis buffer (CST) and Bio-Rad. The samples where then analyzed by multiplex immunoassay for various markers. The graphs illustrate (left) background levels, only lysis buffer and (right) background - noise ratio for the complete cytokine panel. * indicates all p values <0.05.

METHODS TO ANALYZE CYTOKINES

Cytokines can be measured at various levels. mRNA can be detected by real time PCR[44] and intracellular proteins can be detected by flowcytometric assays of permeabilized cells.[45] Due to the range and location of cytokine activity, there are a plethora of methods by which to measure cytokine levels. Irrespective of the chosen methodology, several factors are key to creating the optimal protocol by which to analyze cytokines as biomarkers. Parameters should include (a) a high specificity, affinity and sensitivity (b) make use of a simple protocol (c) be reproducible and reliable (d) be time and cost effective and (e) allow the detection of multiple cytokines within a small sample volume.[46, 47] This review focuses on the measurement of cytokines at a protein level describing various techniques that can be used for their detection. Pro's and con's of the various methodologies are summarized in Table 1.

Bioassays

Bioassays look at cytokines and their activity within a system by focusing on their biological activity and using this as a read out. In a bioassay the activity of a sample is tested on a sensitive cell line and the results of this activity are compared to a standard cytokine preparation. This assay is highly sensitive and permits the detection of bioactive molecules however, it is also semi-quantitative, low in specificity, shows a narrow analytical range, is time consuming and requires a large sample size.[46] In addition to these factors, cell lines respond to different molecules present within a sample thus, any changes observed may be the result of one or multiple compounds.[48] This combination of disadvantageous factors leads to the innovation of more specific and sensitive assays by which to measure cytokines directly.

Protein Microarrays

Protein microarrays analyze the interaction, function and activity of proteins on a large scale.[49] This sensitive, high-throughput method allows large numbers of proteins to be measured rapidly, economically and in parallel.[50] The protein chip used in this procedure is comprised of a support surface to which a range of capture proteins are bound. Fluorescently labeled probe molecules are then added to the array and upon interaction with the bound capture protein, a fluorescent signal is released and read by a laser scanner.[51] Analytical (capture) microarrays use antibodies, aptamers or affibodies bound to the chip surface in order to bind the specific and desired protein within a complex protein solution, commonly cell lysate. The subsequent protein interactions provide information on the expression levels, binding affinity and or specificity of the proteins within the solution permitting comparisons of protein expressions between various solutions. The protein microarray system is applicable for biomarker detection by allowing protein expression profiling however, some challenges do exist for this procedure. Downfalls come in the form of difficulties manufacturing chips with stable proteins holding the necessary primary or

tertiary structure as these are vital in their interactive ability and biological activity. Protein array shelf life is relatively short due to protein denaturation and difficulties still exist in finding and isolating capture molecules for the wide range of proteins within the human genome.[52] A complex balance between quantifying amounts of bound protein, maintaining sensitivity and reducing background noise is difficult to obtain especially due to the low affinity or low specificity of capture agents. Most poignant however, is the inability of the chip to provide a complete view of the proteome with abundant proteins overpowering the detection of less abundant proteins whose levels are also key in therapeutic analysis.[53]

High-performance liquid chromatography

Unlike protein microarrays, high-performance liquid chromatography (HPLC) does not identify cytokines using protein–protein interactions but rather, as compounds with specific weights, hydrophobicity, protonating abilities, ligand affinity and ion exchange.[54] In short, HPLC uses the specific chemistry of each compound as a method of identification and separation. This method allows the quantification and purification of compounds by loading a sample onto a separation column containing solid particles under pressure. The sample is then separated into individual compounds according to their interaction with the column particles. The separation is in itself influenced by the liquid solvent condition and the chemical interactions between sample and solvent. HPLC has successfully been used to purify and separate cytokines such as IL-1 derived from various cells such as macrophage and epidermal cells.[54] However, even with the ability to achieve better separation than ordinary liquid chromatography, HPLC is a less than optimal method of analysis. Some disadvantages of this process include a high cost and complexity, the coelution of compounds with similar structure and polarity,[55] the irreversible absorption of compounds which then remain undetected and the low sensitivity of the apparatus to certain compounds as a result of the speed of the process.[56]

Sandwich antibody assays

It is known that several parameters must be met in order for a protocol to be optimal for cytokine biomarker discovery. As seen in bioassays, protein microarrays and HPLC, parameters are often either suboptimal or conflicting within an assay. Brining each of these factors close to or within optimal range will, however, give way to the perfect assay. There are two types of sandwich antibody assays, those that are plate-based and those that are bead-based. Plate-based assays such as Sandwich Enzyme-linked immunosorbent assay (ELISA) and Meso Scale Discovery electrochemiluminescence (MSD) as well as bead-based assays such as multiplex immunoassays (MIA) are currently on the forefront of achieving the parameter goals required for cytokine biomarker discovery. In essence, these sandwich assays work with the principle of sandwiching a cytokine between two specific antibodies that intern bind to two none competing epitopes of that cytokine.[46] For bead based analysis, the antibodies are

either coated to a solid carrier (bead), acting as the capture antibody and or, in the case of the second antibody, bound to a labeled reporter. In plate-based assays however, the capture antibodies are bound in distinct positions within the wells of 96 well plate.[57]

	Pro's	Con's
Bioassay	Sensitive detection of bioactive molecules	Semi-quantitative Narrow analytical range Low specificity Time consuming Large sample size
Protein microarray	Sensitive High through put Rapid Parallel measurement of multiple proteins	Low Protein detection system stability (denaturation) Non-specific activity of capture protein Masking of low protein levels by higher protein levels Matrix/Heterophlic (auto-) antibody interference
HPLC	Relatively rapid Low false positives	High cost and complexity Co-elution of compounds Irreversible absorption of compounds Low sensitivity
ELISA	High specificity High sensitivity Wide analytical range Reproducibility	Unable to distinguish between bioactive and inactive molecules Varying binding affinity of antibodies Large sample volume High reagent costs Narrow dynamic range Only measure one protein at a time Matrix/Heterophlic (auto-) antibody interference
MSD	Quantitative and qualitative analysis High sensitivity Low background Detection of multiple cytokines	Unable to distinguish between bioactive and inactive molecules Performance of assay in various matrices is unknown
Bead based multiplex immunoassay	High specificity High sensitivity Broad analytical and dynamic range Reproducibility Rapid Small sample volume	Unable to distinguish between bioactive and inactive molecules Matrix/Heterophlic (auto-) antibody interference

TABLE 1. Pro's and con's of various assays

Enzyme-linked immunosorbent assay

The ELISA procedure encompasses the detection of an analyte within a liquid sample in a liquid environment within a reaction chamber. In lieu with heterogeneity of the assay, the desired component is separated from the analytical mixture by binding to an immobilized solid phase, usually the bottom of a transparent plate. Following this binding, substrate is added which is enzymatically converted, resulting in an optical change (colored or fluorescent) that allows the quantitative and qualitative measurement of the desired compound. The ELISA protocol allows for high specificity and sensitivity as well as a wide analytical range and reproducibility, all of which are dependent on the type of biological fluid and cytokine being measured.[58, 59] Downfalls in this procedure, however, include the inability to distinguish between bioactive and inactive compounds, varying binding affinity of antibodies as a result of differences in the internal structure of recombinant proteins used to generate these antibodies, large sample volumes, high reagent costs, a narrow dynamic range and the fact the protocol only permits the measurement of one cytokine at a time in a specified sample volume.[59] In order to overcome the inability to detect multiple cytokines simultaneously, the ELISA protocol was advanced to include a sequential ELISA analysis and ELISPOT assays however, these assays are time consuming, laborious and limited in their ability to detect a spectrum of cytokines.[46] Taking into account the complex interaction between multiple cytokines during a disease process, an assay had to be developed that combated one of the major pitfalls of the ELISA protocol, that of an inability to sufficiently and accurately measure multiple cytokines. At present, some of the most common multiplex assays used are the bead-based Luminex multi-analyte profiling (xMAP) technology [Luminex, Austin Texas, USA] and the cytometric bead array (BD Biosciences, San Diego California, USA)[58] or, the plate-based MSD array (Meso Scale Discovery, Gaithersburg, USA).[57]

Meso scale discovery

The principle of MSD is based on a reaction in which an electron transfer in electrochemically generated intermediates causes these molecules to enter an excited state. Once excited these molecules can emit a photon of light when re-entering a lower energy level.[60] Initially, capture antibodies are coated onto the surface of a plate. Samples are then incubated on the plate followed by the addition of an electrochemiluminescence tagged antibody. Analysis of this plate reveals fluorescent regions in which specific interactions have occurred between the antibodies and analyte, allowing both a quantitative and qualitative analysis of the desired compound. This method is highly sensitive, has low background, does not incorporate washing steps and most importantly, allows the detection of multiple analytes at the same time. What remains unknown however is, how this assay will perform in various sample matrices.[57]

Multiplex immunoassays

In bead-based multiplex immunoarrays, identifiable bead sets are stably coated with desired and specific capture antibodies. These beads are then incubated with a small sample volume allowing the capture of the analyte that binds specifically to the capture antibody. Following this, labeled detection antibodies bind to the analyte- capture antibody-bead complex to make a four member solid phase sandwich that when passed through the detection system allows the identification and quantification of the desired compound. In comparison to ELISA, multiplex assays in general are more sensitive, show a broad analytical and dynamic range – measuring a few pg/ml, are highly specific, are rapid, require smaller sample volumes[58] and allow the simultaneous measurement of up to 500 different proteins (xMAP technology, Luminex, Austin Texs, USA). Even in the presence of all these advantageous features however, these multiplex assays, similar to other antibody based technologies, are affected by the presence of heterophilic and auto-antibodies. These antibodies cause false positive and false negative signals by binding to either the capture antibody, detection antibody or to the antigen.[35] In order to combat this phenomenon three methods can be undertaken. The first involves heterophilic/auto-antibody blockage with animal serum (ineffective when antibody titers are high as seen in a diseased state).[58] The second is through the use of internal assay markers that allow the interfering antibodies to be monitored. These internal markers can clearly indicate when heterophilic or auto-antibodies are influencing data and can thus allow the exclusion of unfit samples during assay analysis.[30, 61] The third and most preferable method is the removal of these antibodies by incubating samples with either protein-L,[30] or with antibodies cocktail such as Hetero-Block.[62]

CONCLUSIONS

By understanding how factors can manipulate cytokine levels and plugging in the necessary controls or methodology, an objective view of cytokine levels within a disease process can be reached, enabling clear results and definitions that will all culminate in achieving optimal biomarker function of these molecules. We recommend that, especially in the context of clinical trials, sample handing and processing should be done in a standardized way with all individuals undergoing identical practical and handling procedures. In general, different blood sampling tubes can be used however, the tube type within a study should remain the same. Furthermore, directly after sample collection, we recommend keeping samples on ice or at 4 °C before sample separation. The process of sample separation should then be performed as quickly as possible and the products of this separation should be stored at -80 °C for long term storage. When performing cytokine analyses, depending on the sample origin, the matrix interference can be dealt with by removing impeding substances, such as auto antibodies, or by choosing reagents which result in the lowest interference during the assay. By following this general outline, data produced within a study will provide a clearer representation of cytokine levels in patients and allow maximum data mining to be achieved.

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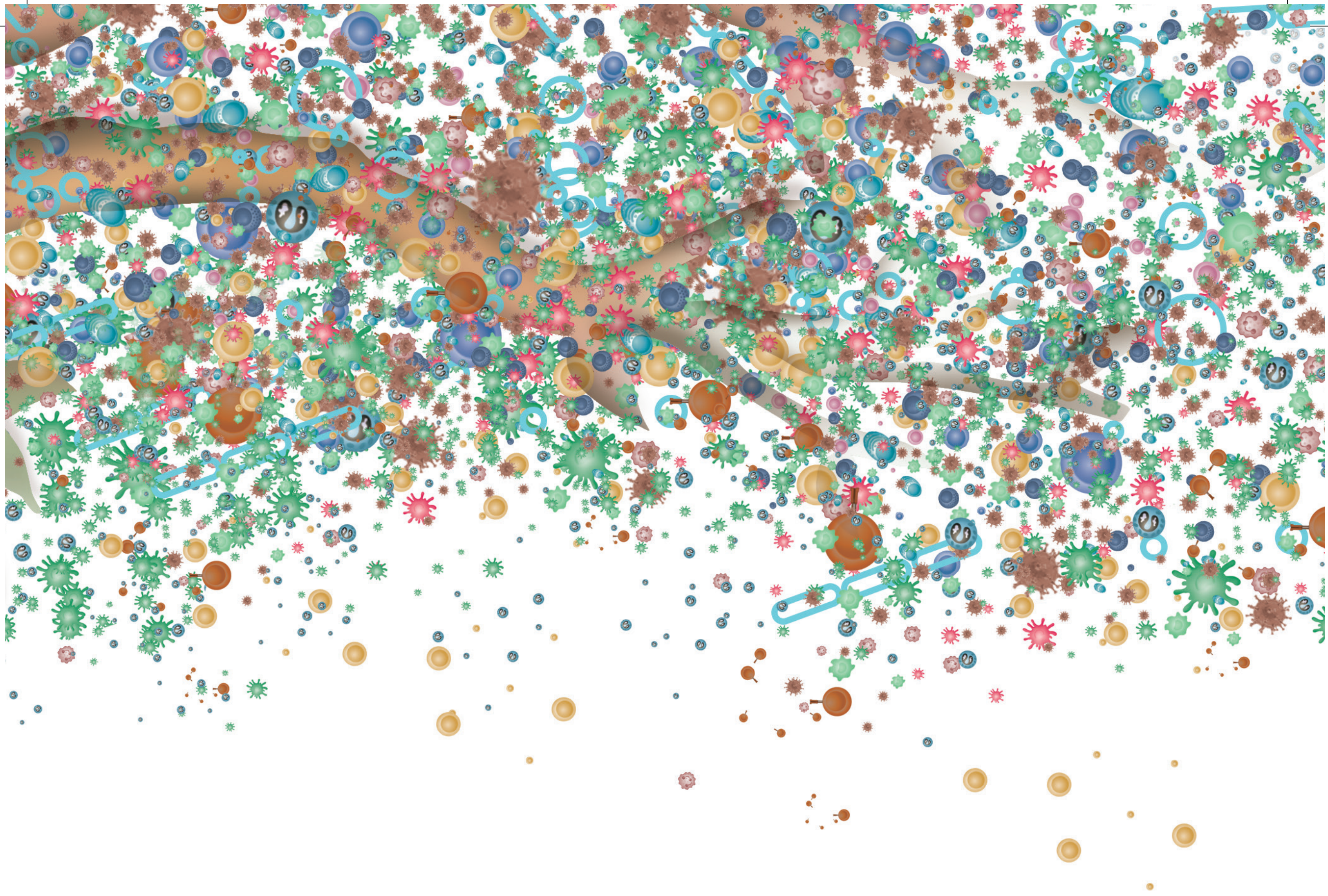

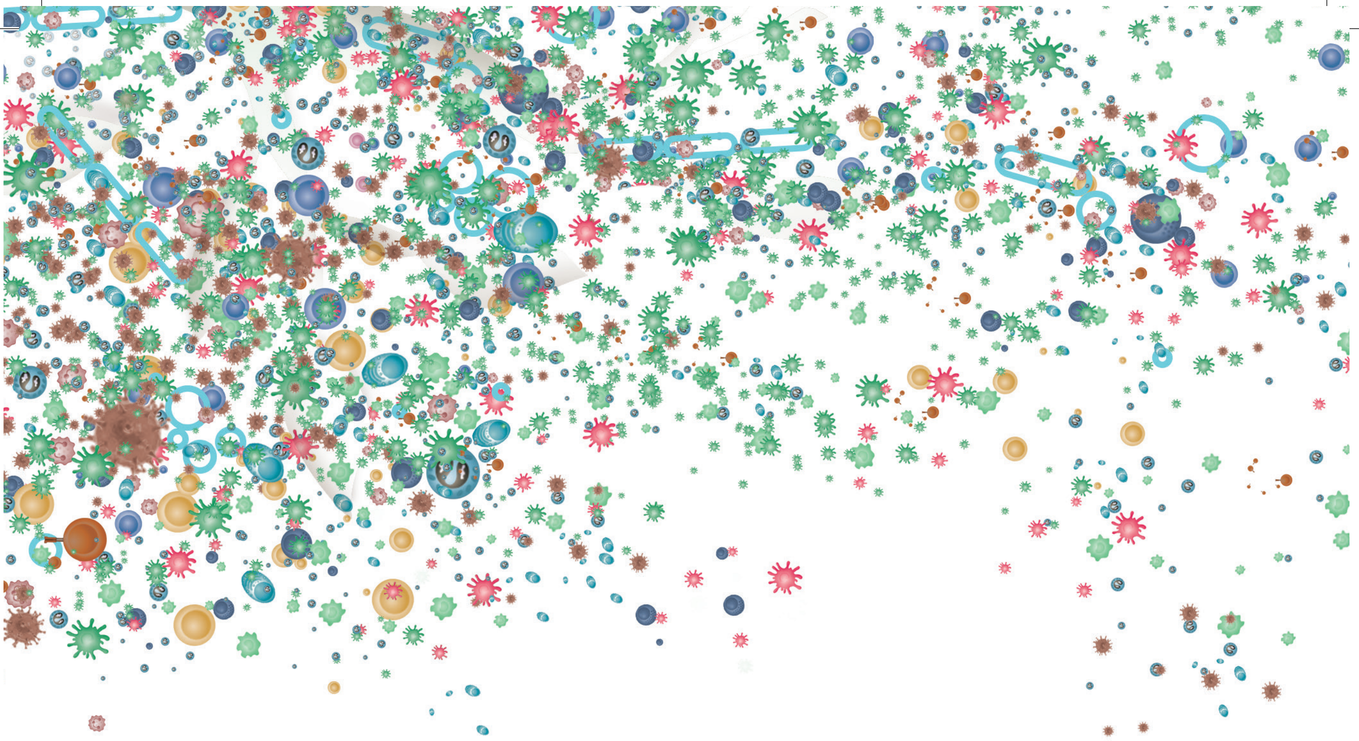

Chapter 7

GENERAL DISCUSSION

GENERAL DISCUSSION

It is clear that immune ontogeny is an emerging field. Fetal life and early infancy are recognized as a critical period to shape the immune system for life. Slowly the neonatal immune system changes its regulatory responses into effector responses. Genes, environmental factors and the epigenome will influence this maturation process and different developmental trajectories might be initiated leading to disease pathways. This concept has been recognized in the 1000 days campaign and the WHO's Every Newborn Action Plan but pathophysiological mechanisms supporting this theory are mostly unidentified.

THE NEONATAL IMMUNE SYSTEM: A BALANCING ACT

Increased adaptive immune tolerance in early life: programmed naive T cells and a role for PD-1/PD-L1 T cell-APC interactions

To secure the developmental trajectory of the immune system, the road to adult immunological wellness, and to prevent long term risk of immune-mediated diseases, knowledge of neonatal immune development and especially the drivers of this immune development like nutritional components, microbes and host-cell interactions is cardinal. In this thesis, it is pitched that immune homeostasis in early life is (partly) achieved by the increased ability of neonatal immunity to induce adaptive immune tolerance and that innate cells play an important role in driving this mechanism. Indeed, the predilection of naive $CD4^+$ T cells to differentiate into $FOXP3^+$ Tregs described in **chapter 2**, confirmed a profound programmed development of naïve T cells upon activation. Besides a clear inborn default of naïve T cells to differentiate into Tregs, cross experiments illustrated that neonatal APC were more potent in driving Treg development due to increased expression levels of PD-L1. The interaction between the negative signaling receptor PD-1 on the T cell and PD-L1 on the APC is one of the several physiological feedback control mechanisms which serve to block T cell proliferation under chronic antigenic stimulation[1], which starts immediately after birth on the skin, mucosa in the gastrointestinal tract and in the airways. Of interest, PD-1/PD-L1 interactions also play a role in tumor immunology and PD-1/PD-L1 inhibitors can be very effective, unlocking anti-tumor activity in tumor-specific T cells and macrophages. We now showed that PD-1/PD-L1 interactions also play a role in Treg induction and thus the development of immune tolerance in early life.

Differentiation into FOXP3 Treg cells involves reduced PKB signaling

PD-1 signaling in CB T cells facilitated their differentiation into induced functional $FOXP3^+$ Treg cells through a mechanism involving reduced PKB signaling (**chapter 2**). CB T cells required approximately 10-fold more TCR triggering than APB T cells before an increase in PKB phosphorylation was observed. Our date fit those of Huygens and Kollmann who showed that newborn effector T cells are limited by a more rapid onset of functional exhaustion compared to adult T cells.[2, 3] Expression of PD-1 by T cells is reported to be one of the hallmarks of exhaustion.[1, 4] Therefore it is not surprising that PD-1 on neonatal T cells has been proposed as a potential target to reverse neonatal T cell exhaustion and to initiate a more robust inflammatory response.[1, 4] Note however, that our data showing a crucial role for PD-1 in FOXP3 induction indicate that such therapy may interfere with neonatal tolerance and therefore may have detrimental side effects.

T_H17 differentiation in CB is blocked at the level of the lineage transcription factor, RORC2.

In **chapter 3**, we hypothesized that the propensity of Treg cell induction in cord blood (CB) could be explained by a regulatory mechanism that inhibits T_H17 cell development. Treg and T_H17 cells have a reciprocal developmental pathway. They also play a reciprocal role in

the induction and perpetuation of inflammatory responses during infection, and also in the pathogenesis of autoimmune and allergy-related diseases. Remarkably, we were not able to differentiate naïve T cells in cord blood into T_H17 cells in contrast to naïve T cells derived from adult blood. T_H17 differentiation in CB is blocked at the level of the lineage transcription factor, RORC2. Significantly lower RORC2 mRNA content was present in cord blood compared to T cells derived from adult blood. Neonatal T cells developed the capacity to differentiate into T_H17 cells during the first 3 months and retained a propensity to become Treg cells until the age of at least 12 months (**chapter 3**). These data give an explanation for the observations that in neonates immunity against pathogens is established within the first 3 months while at the same time the immune system remains to have a regulatory profile. This latter fact is important in the maintenance of tolerance toward allergens and food antigens encountered for the first time.

Strong danger signals can overrule the regulatory default of neonatal immunity

For both Treg and T_H17 development appropriate activation by innate cells is important (**chapter 2 and 3**). CB derived APC were much more efficient in inducing FOXP3 than adult APC. In addition, the low RORC2 content and impaired T_H17 development in CB could be explained by decreased TGFβ, IL-1β and IL-6 production by CB derived innate cells. Thus, besides intrinsic differences between neonatal and adult T cells, innate immunity (APC) plays a crucial role in balancing Treg and T_H17 cells.

In **chapter 5** we questioned whether the deficiency in IL-17 (and IL-6, TNFα, IFNγ and others) is absolute or can be overcome once danger signals are strong enough. We hypothesized that severe infection is a delicate model to put the neonatal immune system under ultimate stress and to provide in vivo activation of immune cells. Cerebral spinal fluid and serum samples were obtained from newborns infected with one of the neurotropic viruses (**chapter 5**). Neonatal viral encephalopathy is often accompanied with severe white matter injury irrespective of the involved virus and with uncertain long term clinical implications. White matter injury has been considered for many years as a bystander effect of the activated immune system in viral CNS infections. Indeed, our approach showed that IL-17, as well as high levels of IL-6 and TNFα are produced in vivo during severe neonatal infections, when strong pro-inflammatory stimuli are present. These data fit previous findings in CB derived from neonates exposed to chorioamnionitis.[5-8] High cord blood frequencies of progenitor (pT_H17, $CD4^+CD161^+$) and mature (mT_H17, $CD4^+CD161^+CCR6^+$) T_H17 cells were found in these patients. Furthermore, IL-17$^+$Tregs were present in CB of preterm neonates exposed to chorioamnionitis as well as T cells with effector memory phenotype that co-expressed T_H17-type surface antigens. Simultaneously, regulatory T cells were decreased in infants exposed to chorioamnionitis relative to age-matched controls.[9] Thus, deficient innate and effector responses in neonates are partial and can be overcome once innate stimuli are strong enough.[10]

TRAINED IMMUNITY

As illustrated by our data on APC-T-cell interactions in **chapter 2 and 3**, innate cells undergo important developmental changes during the first weeks-months postnatally. These developmental changes in expression of costimulatory factors like PD-L1 and changes in PKB signaling clearly determine the quality and quantity of T effector responses postnatally. Regarding the immune developmental steps postnatally, the phenomenon “trained immunity” seems applicable. Trained immunity is explaining that innate immune cells are able to memorize encounters with pathogens or stimuli and respond in a sensitized and nonspecific manner to restimulation.[11-14] For example, neonates given a bacillus Calmette-Guérin (BCG) vaccination which has toll like receptor 2 (TLR2), TLR4, TLR8 and TLR9 agonist activity, experienced a non-specific reduction in neonatal mortality over neonates who did not receive BCG.[15-18] Thus trained immunity boosts the frontline of host defense in neonates and these adaptations of APC to environmental requests will also influence their interaction with T cells. The enhanced immune response that develops upon reencounter with the antigen is associated with a profound change in the intracellular metabolism and regulated by epigenetic changes at the level of histone modifications.[12, 19, 20] The capacity for trained immunity to confer broad immunological protection has sparked speculation that this process can be exploited to improve vaccination strategies.[21] On the other side, unresolved inflammation drives and exacerbates structural changes of tissue. For example in monocytes, promotors of pro-inflammatory genes such as IL-6, IL-8 and TNFα, acquire regulatory changes in histone methylation when exposed to training stimuli that, upon rechallenge, support enhanced gene activation and cytokine production.[21] The deleterious effects of trained immunity on the developing tissue are not restricted to cytokine production, but also include the epigenetic reprogramming of genes encoding for functional proteins, scavenger receptors involved in host defense and chemokines responsible for recruiting circulating immune cells.[21] In conclusion, the interaction between cellular players and the concept of trained immunity illustrates the capacity of the neonatal immune system to respond to environmental inputs in a flexible and adjustable manner. The concept definitely needs further exploitation to enhance immune defense against pathogens and vaccination strategies in neonates without damaging the developing tissues.

WINDOW OF OPPORTUNITY

A non-redundant priming period of the innate and adaptive immune system after birth sets the stage for immune homeostasis and host–microbial interaction.[22] This neonatal window of opportunity provides an explanation for the long-known fact that early environmental influences can have lasting consequences.[23, 24] Of interest immune cell compositional changes after birth follow a stereotypic pattern of development in all children, despite their differences in both maturity and postnatal environmental conditions.[25] During this developmental trajectory, additional exposures and adaptive changes are required to eventually reach an adult-like immune state. For example, epidemiological studies show a strong correlation between early microbial exposure to allergens and a reduced risk of immune-mediated diseases later in life.[26, 27] In contrast, microbial dysbiosis during the first 100 days of life was associated with the development of asthma in a human birth cohort.[27] The fact that these first 100 days also represent a critical period in the development of B cells, natural killer cells (NK), and dendritic cells (DC), as these cell populations reach adult-like phenotypes during this period suggests that environmental influences imprinting on these cells in this period will have long-term consequences. T cell populations do not reach adult-like phenotypes during the first 100 days.[25] Thus such time window for T cells does either not exist or follows a different path than B cells, NK cells and DCs, requesting longer follow up studies.

In **chapter 4** is described that induced regulatory T cells are still highly present at the age of twelve months in comparison with adults. This presence of induced Tregs might partially explain the supposed window of opportunity to prevent immune-mediated diseases later in life. In a mouse model, it has been shown that exposure to Helicobacter pylori in early life protected the mice from asthma and other inflammatory conditions.[28, 29] Systemic depletion of Tregs abolished this protection from asthma whereas adoptive transfer of purified Treg cell populations from an infected donor to an uninfected recipient, was sufficient to restore protection. To investigate if Tregs are in humans also important to create a window of opportunity to induce immune tolerance, the phenotypical and functional classification of Tregs into three subpopulations have been used.[30] Human $FOXP3^{+}CD4^{+}$ Tcells can be divided into resting Tregs ($CD45RA^{+}FOXP3^{low}$), activated Tregs ($CD45RA$-$FOXP3^{high}$) and non-Tregs ($CD4^{+}CD45RA^{-}FOXP3^{-}$). Both resting and activated Tregs have suppressive functions and predominantly activated Tregs are present in the early neonatal period.[31] These Tregs have been activated in an antigen-specific manner to become subsequently highly suppressive in an antigen-nonspecific manner.[32, 33] Moreover Tregs, induced after exposure to S. Aureus, have the ability to convert neonatal conventional $CD4^{+}$ T cells into Tregs via the PD-1/PD-L1 axis[34] and activation of neonatal v-omega-2 lymphocytes will lead to upregulation and prolonged expression of PD-1 that will effectively dampen proinflammatory responses after engagement to its ligand PD-L1. Additionally, an increase in the frequency and stability of Tregs was associated with more favorable clinical outcomes in children who underwent oral immunotherapy for peanut allergy.[35]

It can be concluded that different T cell populations develop uniquely with age. During the first year, T cell differentiation occurs under supervision of Tregs which are still highly present at twelve months of age. The capacity to differentiate into effector T cells like T_H17 increases after the first three months of life together with the change in adult-like phenotype of antigen-presenting cells and other strong effector immune responses. This might implicate that the concept of immune development within a window of opportunity must be implemented into a model with a place for subsequent phases that might depend on each other to facilitate the establishment of a mature, homeostatic but vigilant immune system.[36]

LAYERED IMMUNITY AND IMMUNE COMPUTATION

This vigilant immune system is mandatory when the neonatal period is complicated by an invasive infection and demands adaptation of neonatal immune responses. Neither a single marker nor a binary distinction in the immune response (innate vs adaptive responses; suppressors vs effectors; T_H1 vs T_H2; etc) reflects this adaptation. Therefore, two theoretical concepts about immune development in mammals are proposed: layered immunity and immune computation.

Layered immunity

Layered immunity was originally developed to explain immune cell ontogeny but it may provide a broader framework to understand the maturation of the innate and adaptive immune system as a whole.[36, 37] For example, a fetal immune response to exogenous antigens is actively suppressed by antigen-specific Tregs, and these fetal Tregs are derived from a fetal-specific lineage of T cells, a lineage generated by an HSPC that is distinct from that found in adults. Fetal and adult T cells derive from hematopoietic stem cells that originate in different tissues (respectively, liver and bone marrow) and have distinct properties: Fetally derived cells exhibit higher proliferative capacity and are more prone to a tolerogenic response. Also, the induction of peripheral tolerance to self-antigens may have to precede the tolerance to microbial and dietary antigens at mucosal sites.[38-43] This would suggest that the immune system is comprised of distinct waves, present at different time points in life.[44] Clearly, developmental mechanisms contribute significantly to the initial adaptation to microbial exposure. Prematurity might reduce the ability to undergo innate immune tolerization and reprogramming, therefore increasing the risk to develop necrotizing enterocolitis (NEC), a condition in which incidence and severity rises with lower gestational age at birth. Consistent with this theory, an enhanced innate immune receptor expression and susceptibility to microbial ligands have been demonstrated in mouse fetal intestinal epithelium.[45] The immune profiles for babies born at earlier gestations change more dramatically over a similar time period to those born at later gestations, which suggests that extremely preterm babies are capable of rapid progression to ‘catch up’ immune function.[46] Moreover, the layered development of the immune system is evolutionarily driven and follows a cost–benefit principle. The maintenance of an elaborate immune system may come at a price and may have to be adapted to the availability of energy resources that at this time-point might rather be dedicated to growth and development.[43]

It is undefined which events are involved in progression to the next phase of immune development. It is proposed that the immune system is equipped with an experience-based machine learning strategy which is analogous to that deployed by supervised machine-learning algorithms.[47] However, this theory is still hypothetical and future experiments are necessary to clarify proof of concept.

7

Immune computation

It is proposed that each immune cell receives random signals. Dependent on its array of receptors; each cell then responds to transform (compute) its input information into an output of signal molecules, receptors, metabolic reactants, antibodies, or other products that comprise an inflammatory output. The response of the cell and its outputs are determined by the state of the individual computing cell; this state reflects the cell's differentiation and its history, along with the input to the cell from other cells and molecules.[47] An ordered immune response emerges from the way a collective of cells integrate their behaviors, this is called crowd wisdom[48] comparable to the functioning of a school of fish. Co-respondence is an integral part of immune computation of the body state: the informed few who see the antigens arouse a cohort of "bystander" cells to help mediate the immune response.[47]

Altogether, the complexity of the immune system must be respected to find novel strategies to influence immune maturation to the good and to improve patients health outcomes. Two existing hypothetical theories will be further explored in the future. In this thesis (**chapter 6**), an important role for systems immunology analyses is suggested in humans, especially in early life, to support further research on proper immune maturation: the way to adult immunological wellness.

THE ADULT IMMUNOLOGICAL WELLNESS PROFILE: HOW TO GET THERE?

The Wellness Profile hypothesis proposes that healthy individuals share an immune signature whereas in people with a chronic immune-mediated disease a deviation of this immune signature can be identified – a type of illness profile.[25, 47] According to the standard paradigm, health is a given; health is freedom from pathogenic agents such as bacteria, viruses or malignant cells.[49] The discovery of the DNA genetic code has added mutant or abnormal DNA to the causes of disease. Immune machine learning suggests that immunological wellness is not merely the absence of a specific disease but a particular body state, one that must be learned during early immune repertoire development.[47] This wellness theory suggests that a chronic or recurrent disease might arise from replacement of a healthy reference set of immune body data with an aberrant reference set. Treatment of an auto-immune disease might aim at immune re-education toward a healthy reference profile rather than primarily at suppression of autoreactivity.

This concept fits within the window of opportunity as a deviation of the programmed immune development by early-life exposures influences the risk of developing immune-mediated diseases.[25, 50, 51] This proposed window of opportunity further implies that the human immune system is a sensory system for intrinsic and extrinsic environmental factors. It depends on sensory inputs during its development and specific interactions between the developing immune system and the microbes colonizing the intestine, skin, and airways of a newborn child have been suggested as important.[25] Systems-level analyses in humans have shown that environmental influences explain most of the overall variation among healthy individuals[52-55] and that such environmental influences from infections, vaccines, nutrition, and the microbiome exert a cumulative influence over the course of life.[25, 52, 56]

Exposome and the microbiome

The complexity of environmental exposures over time is termed the exposome.[43, 57] Many of the exposomal conditions and factors operate in a longitudinal fashion over time and have a strong effect on the development of microbial communities at the skin and mucosal sites.[43] It has now been recognized that the major environmental signals driving normal postnatal maturation of immunocompetence in all mammalian species originate from the microflora of the gastrointestinal tract (GIT)[43, 58] and early gut dysbiosis is associated with a perturbation of the stereotypic developmental trajectory.[25] The evolving paradigm is now that colonization of the infant GIT with an appropriate microbiota within crucial developmental time windows is required to optimally drive immune function maturation.[59] Contact with antigens directly after birth is not only pivotal to gain unspecific suppressive regulatory functions that results in tolerance to environmental antigens but

also to control programmed development of other immune cell populations.[25] It is crucial for long term health outcomes to protect and secure this programmed, age-dependent immune development. Therefore, unnecessary (longtime) antibiotic treatment should be avoided since this will disturb proper immune development by means of destruction of the composition of the microbiome.[60-62]

CLINICAL IMPLICATIONS IN NEONATAL CARE

Antibiotics in early life: lifesaving or devastating?

Current clinical practice comprises that antibiotic treatment is initiated in neonates directly after birth based on a risk assessment for early onset neonatal sepsis (EONS) that is composed of maternal factors and clinical symptoms. The results of the blood culture, the golden standard to diagnose sepsis, are awaited for at least 36 hours. Prevalence of a culture proven EONS is 0.1%, antibiotic treatment is initiated in 4-7% of the newborns,[63-66] which indicates a huge overtreatment. On top of that, multiple (clinical) reasons to continue antibiotic treatment despite a negative blood culture are reported and it is not unusual in the neonatal ward to continue treatment for (a random) seven days.[67]

However, neonatal sepsis often presents with aspecific symptoms and can progress extremely fast if not treated immediately. The molecular adaptations of immune cells described in **chapter 2 and 3** underscore that immune regulating mechanisms are importantly programmed into immune cells in this phase of life. Strategies to boost the immune system of newborns have failed and future research is in need of a systems immunology approach in contrast to a stand-alone immune modulator like some specific cytokine. Focus of future research should aim for better diagnostic tools like the clinical use of early markers for sepsis.

At present, sufficiently robust decision tools to disentangle (sensitivity versus specificity) infectious events from other commonly observed events in neonates are still lacking.[68] Neonatal care is highly in need of new methods to diagnose sepsis accurately, discriminative and fast. Not only to treat patients for potential life threatening sepsis but also to prevent newborns from unnecessary and also potential harmful exposure to antimicrobial therapies.[69-71]

New diagnostic approaches for early identification of patients suffering from infection together with the identification of the causative microbes, are therefore urgently needed to reduce the incidence and mortality of neonatal sepsis. Two new promising tools are: Procalcitonin (PCT) as a negative predictor for sepsis and Next Generation Sequencing (NGS) to identify pathogens during sepsis.

Procalcitonin (PCT)

PCT is an inflammatory marker that can be used to exclude sepsis.[72] A large randomized control trial showed that serial low PCT values make it possible to safely stop antibiotic treatment in newborns within 24 hours after birth (NeoPInS). Furthermore, serial PCT measurements are able to guide doctors daily in the questionable need for antibiotic therapy in an individual patient (NeoPInS). Antibiotic therapy will not only address micro-organisms in blood, it will also affect the microbiota. To prevent long term consequences by disrupting the proper formation of the microbiome and subsequently disrupting the programmed development of the immune system, unnecessary antibiotic treatment must especially be avoided in this age group. Therefore, PCT is now implemented in clinical guidelines to prevent and treat neonatal early onset sepsis. A limitation of PCT guided therapy is that it is only applicable for infants with a medium or low risk for early onset sepsis (EONS), high risk infants are often treated with antibiotics for long time despite a negative bloodculture.

To reduce duration of antibiotic treatment is one thing, not to start antibiotics and to exclude sepsis would be the best. PCT has also been shown as a promising marker to rule out sepsis in cord blood.[73, 74] The limitation of early PCT measurement is that it does not allow the detection of late infections due to vertical transmission, furthermore, it has not been tested in late-onset neonatal sepsis. These limitations request an innovative technique that would be able to identify sepsis fast, reliable and is discriminative for different pathogens like bacteria, viruses and fungi.

Next Generation Sequencing

It has been shown in adults that circulating cell-free DNA (cfDNA) from blood plasma of adult septic patients is a more sensitive and specific analyte to identify pathogens by NGS compared to the traditional golden standard, the blood culture. This technique is not new, however a bioinformatics workflow coupled with statistical tests now reveals the clinical relevance.[75] NGS-based diagnosis offers many advantages: First, it is an open platform, providing the opportunity to detect bacterial, fungal and viral pathogens in a single assay. Second, it is quantitative by counting sequence reads and calculating statistical significances. It can therefore potentially discriminate between unspecific colonization, contamination or infection. Third, this technique might in the future be applied at bedsite. The clinical applicability of NGS is currently investigated in late onset neonatal sepsis as LOS is associated with higher chance to find a causative agent in the bloodculture and therefore LOS is a better model to evaluate the clinical use of this technique than EONS.

Diagnostic algorithm

Sepsis is a syndrome that is a result from the complex interplay between an infectious trigger and the host response. Accordingly, diagnosis and prognosis including stratifying patients for personalized treatment requires in addition to the identification of the causative agent the corresponding determination of the host response. This data has so far been unused in the diagnostic algorithms.

The use of an EOS calculator resulted in a decrease in blood culture use and empirical antibiotic administration from 14.5 to 4.9% and 5.0 to 2.6%, respectively, without apparent negative effects.[76] Clinical decision algorithms can also be used to evaluate the effectiveness and safety of withholding antibiotics in specific subgroups,[76] which could include those who appear to be well at the time of assessment or who only have a low-grade fever.[77, 78]

While individual interleukins showed reasonable sensitivities and specificities, the combination of several markers was shown to be superior in several studies.[79] This observation potentiates a personalized diagnostics approach by strategies of both protein levels, gene expression data from NGS and a combination of the two data types. This approach will have the power to alleviate the severe problem of early recognition of septic neonates, and will be particularly useful in assessing the immune response patterns of the neonatal patients and providing thus clues on the infectious or non-infectious nature of the inflammation.

CONCLUSION

To conclude, the neonatal period is an indisputably important and highly dynamic period since sensory inputs influence developmental trajectories in this non-redundant timeframe and will have long term health consequences.

In this thesis, we studied T cell regulatory mechanisms during this crucial period and it is pitched that immune homeostasis in early life is (partly) achieved by the increased ability of neonatal immunity to induce adaptive immune tolerance and that innate cells play an important role in driving this mechanism. Above, we discussed the broader context of our data. Postnatal maturation of immunocompetence in all mammalian species originates from the microbiome of the gastrointestinal tract and early gut dysbiosis is associated with a perturbation of the stereotypic developmental trajectory. Therefore, destruction of the composition of the microbiome with unnecessary antibiotic treatment should be avoided since this will disturb proper immune development.

The overarching aim of future research is to provide an alternative technology for a timely and untargeted identification of pathogens causing sepsis coupled with a stratification strategy and search for predictive biomarkers on the host side in order to tailor treatment strategies to the neonates needs.

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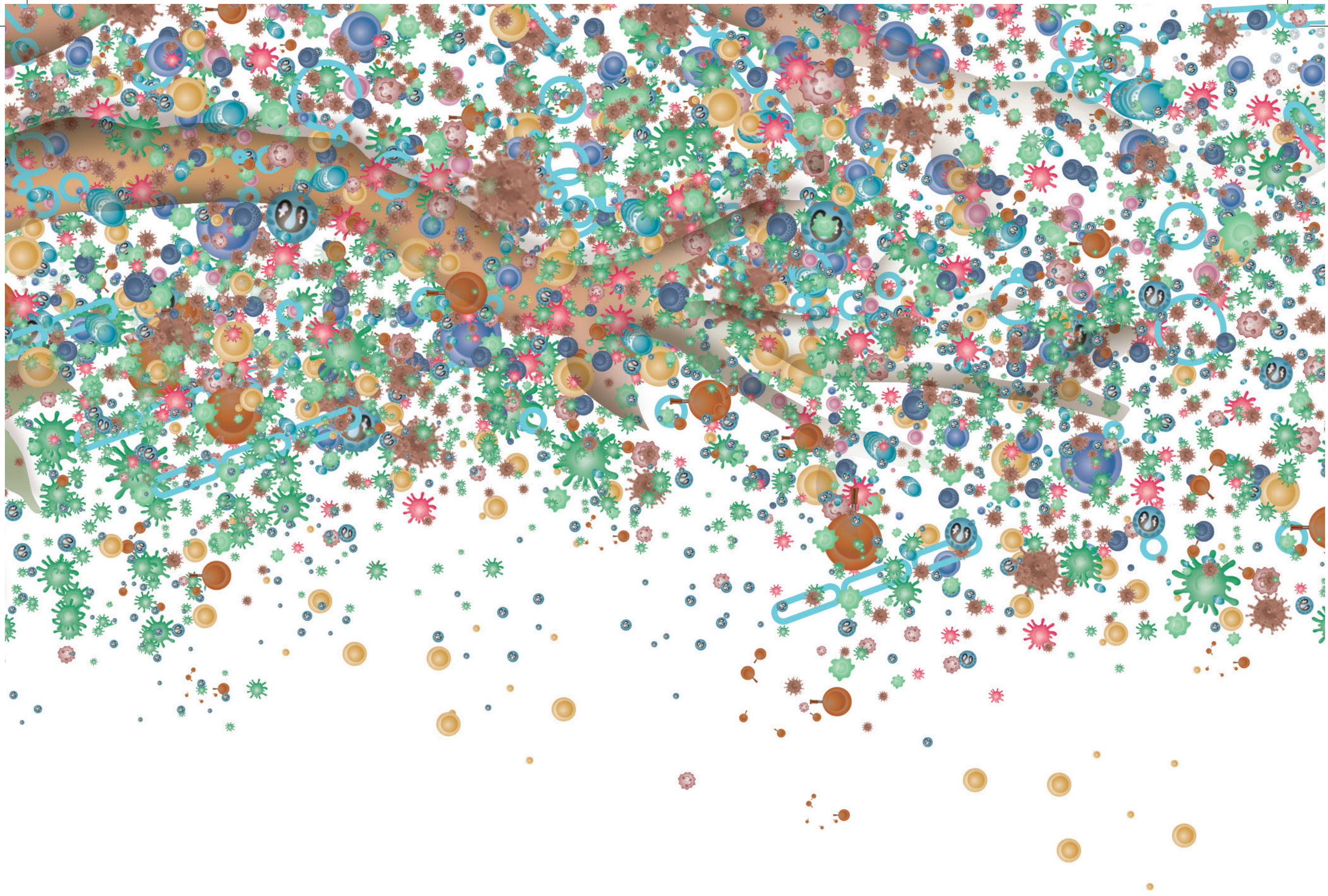
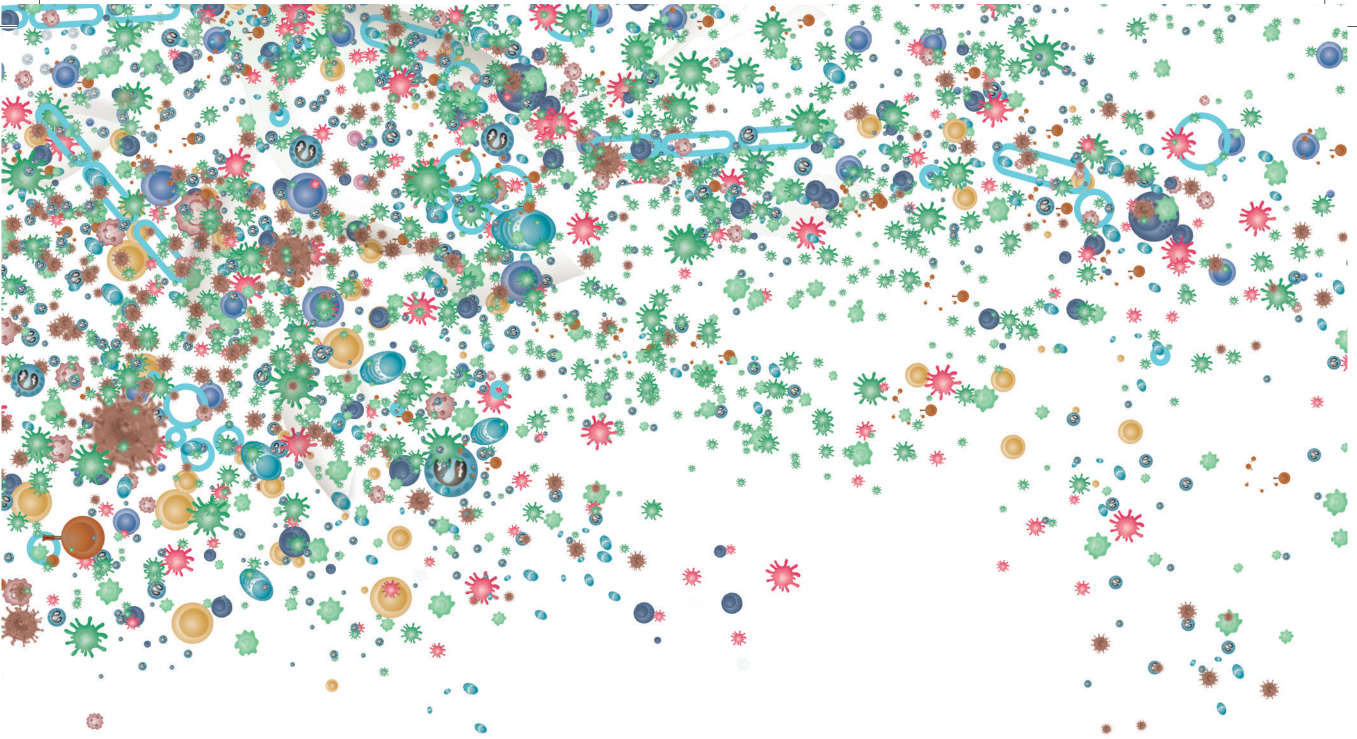




Chapter 8

SUMMARY

SAMENVATTING

SUMMARY

Fetal life and early infancy are recognized as a critical period to shape the immune system for life. Slowly the neonatal immune system changes its regulatory responses into effector responses. Genes, environmental factors and the interaction between both, the epigenome, will influence this maturation process and different developmental trajectories might be initiated leading to disease pathways. This concept has been recognized in the 1000 days campaign and the WHO's Every Newborn Action Plan but pathophysiological mechanisms supporting this theory are mostly unidentified. In this thesis, it is pitched that immune homeostasis in early life is (partly) achieved by the unique increased ability of neonatal immunity to induce adaptive immune tolerance and that innate cells play an important role in driving this mechanism.

Indeed, the predilection of neonatal naive CD4+ T cells to differentiate into FOXP3+ Tregs, in which PD-1/PD-L1 interactions play a role through a mechanism involving PKB signaling (**chapter 2**), and the inability to differentiate into T_H17 cells, which is blocked at the level of RORC2 (**chapter 3**), confirmed a profound programmed development of naïve T cells upon activation and their role in the development of immune tolerance in early life. Neonatal T cells develop the capacity to differentiate into T_H17 cells during the first 3 months and retained a propensity to become Treg cells until the age of at least 12 months (**chapter 4**). However, IL-17, as well as high levels of IL-6 and TNFα are produced in vivo during severe neonatal infections, when strong pro-inflammatory stimuli are present. Thus, deficient innate and effector responses in neonates are partial and can be overcome once innate stimuli are strong enough (**chapter 5**). System immunology, provided that it is standardized in use (**chapter 6**), enables identification of divergent patterns in immune maturation related to long term health outcomes.

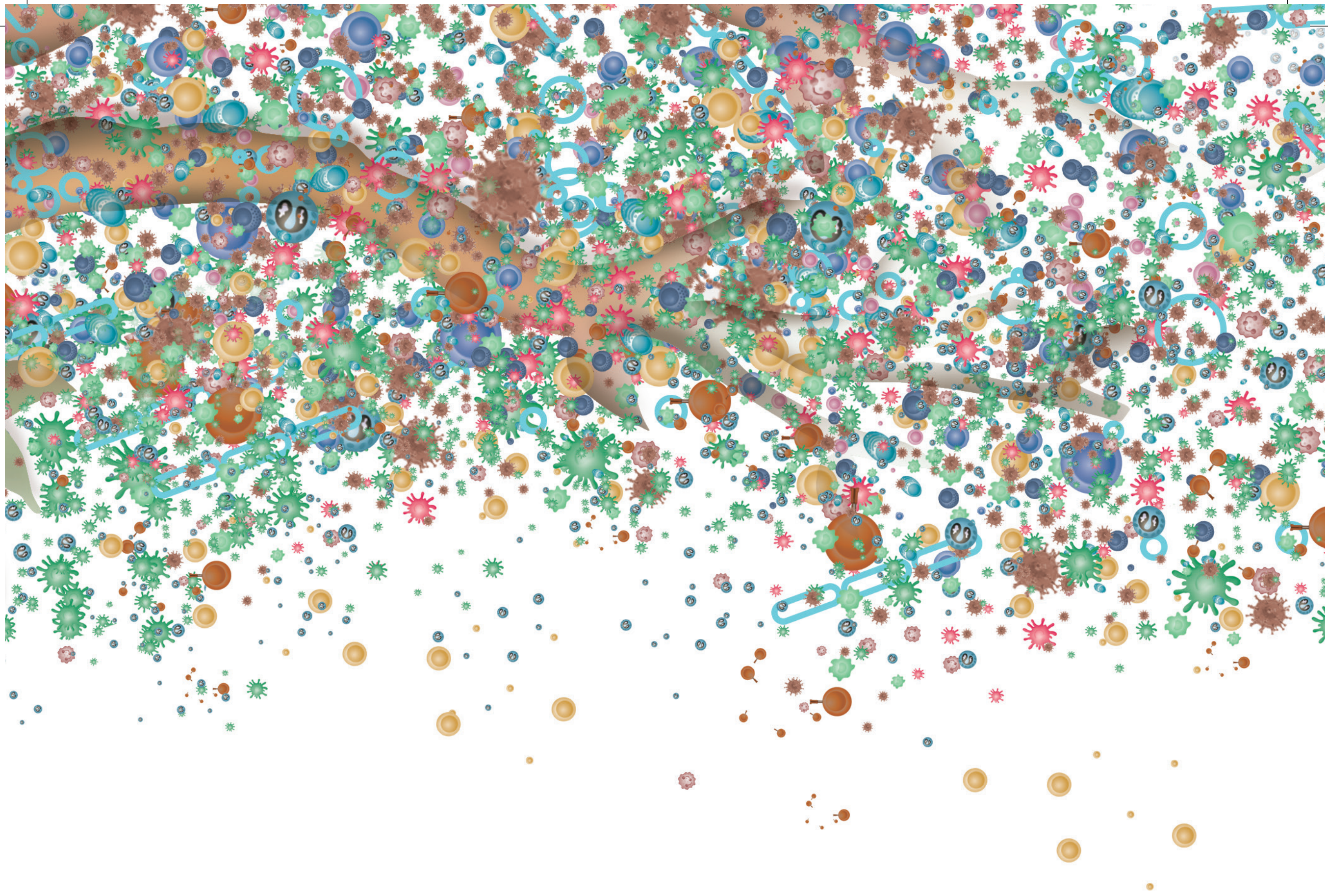
It is crucial for long term health outcomes to protect and secure this programmed, age-dependent and stereotypic immune development. Therefore, unnecessary (longtime) antibiotic treatment should be avoided since this will disturb proper immune development by means of destruction of the composition of the microbiome and subsequently affects proper immune maturation. Development and clinical implementation of better diagnostic tools to identify or exclude neonatal sepsis are mandatory to secure future health outcomes.

SAMENVATTING

De periode voor en kort na de geboorte wordt beschouwd als een kritische periode waarin het immuunsysteem voor het leven wordt gevormd. Het immuunsysteem past zich aan een nieuwe omgeving aan en voor de neonatale periode kenmerkende immuunregulatoire reacties op stimuli veranderen geleidelijk in effectieve immunologische reacties. Genen, omgevingsfactoren en de interactie tussen beiden, het epigenoom, beïnvloeden dit maturatieproces en kunnen alternatieve ontwikkelingstrajecten initiëren welke later in het leven tot ziekte kunnen leiden. Dit concept wordt erkent in “de eerste 1000 dagen” campagne en het “Every Newborn Action Plan” van de WHO maar de pathofysiologische onderbouwing hiervoor ontbreekt nog grotendeels. In dit proefschrift wordt beschreven hoe immunologische homeostase vroeg in het leven (deels) bereikt wordt door het vermogen van neonatale T cellen om immunologische tolerantie te induceren, dat deze immunologische karakteristieken kenmerkend voor deze levensfase zijn en dat naast T cellen, neonatale innate immuuncellen een belangrijke rol spelen in de aansturing van dit mechanisme.

Dat neonatale naïve T cellen bij activatie differentiëren tot regulatoire FOXP3^{+} T cellen, waarbij PD-1/PD-L1 interacties een rol spelen door inhibitie van PKB pathway (**chapter 2**) en daarnaast het onvermogen om te differentiëren tot T_H17 cellen, doordat dit geblokkeerd wordt op het niveau van de transcriptiefactor, RORC2 (**chapter 3**), illustreert hoe immunologische regulatie geprogrammeerd is in deze vroege ontwikkelingsfase. Gedurende het eerste jaar treedt er een verschuiving op waarbij T cellen de capaciteit ontwikkelen om te differentiëren tot T_H17 cellen maar ook het vermogen behouden om in grote aantallen regulatoire T cellen aan te maken (**chapter 4**). Ondanks deze gefaseerde en geprogrammeerde immunologische reacties vroeg in het leven, blijkt het neonatale immuunsysteem zich aan te kunnen passen aan de input van omgevingsfactoren. Sterke pro-inflammatoire stimuli kunnen de deficiënte effectieve immunologische reacties in neonaten modificeren zoals blijkt uit de aanwezigheid van IL-17 en hoge concentraties van IL-6 en TNFα in vivo bij een ernstige neonatale infectie (**chapter 5**). De toepassing van systeem immunologie analyse mits gestandaardiseerd gebruikt (**chapter 6**), maakt het identificeren van afwijkende patronen in immunologische maturatie gerelateerd aan gezondheidsuitkomsten op lange termijn mogelijk.

Vroege verstoring van de compositie van het microbioom is geassocieerd met verstoring van de stereotype immunologische maturatie en kan leiden tot immuun-gerelateerde ziekten zoals allergie, diabetes en auto-immuun aandoeningen. Verstoring van het microbioom kan worden voorkomen door onnodige behandeling met antibiotica te vermijden. De ontwikkeling en de klinische implementatie van betere diagnostische methoden om een perinatale infectie aan te tonen of uit te sluiten, zijn dan ook noodzakelijk om de neonatale periode de basis voor een optimale gezondheid, groei en ontwikkeling te laten zijn.



LIST OF PUBLICATIONS

ABOUT THE AUTHOR

ACKNOWLEDGEMENTS

LIST OF PUBLICATIONS

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Critical role for programmed death 1 signalling and protein kinase B in augmented regulatory T-cell induction in cord blood
J Allergy Clin Immunol 2011 Dec; 128(6):1369-71 Hoeks SB*, de Roock S*, Meurs L, Steur A, Hoekstra MO, Prakken BJ, Boes M, de Kleer IM *Both authors contributed equally

The origin of allergic diseases and the early development of the immune system. Ned Tijdschr Allergie & Astma 2012;12:145-154 Hoeks SB, de Kleer IM

Defective T_H17 development in human neonatal T cells involves reduced RORC2 mRNA content
J Allergy Clin Immunol 2013 Sep;132(3):754-756; de Roock S*, Stoppelenburg AJ*, Scholman R, Hoeks SB, Meerding J, Prakken BJ, Boes M *Both authors contributed equally

Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples
Methods 2013 May 15; 61(1):10-7; Keustermans GC, Hoeks SB, Meerding JM, Prakken BJ, de Jager W

T_H17 differentiation capacity develops within first three months of life
J Allergy Clin Immunol 2014 March; 133(3):891-4; Hoeks SB*, Dijkstra KK*, Prakken BJ, de Roock S *Both authors contributed equally

Aminoacyl-tRNA synthetase deficiencies in search of common themes
Genet Med. 2018;21(2):319-30; Fuchs SA, Schene IF, Kok G, Jansen JM, Nikkels PGJ, van Gassen KL, Terheggen-Lagro SW, van der Crabben SN, Hoeks SE, Niers LE, Wolf NI, de Vries MC, Koolen DA, Houwen RH, Mulder MF, van Hasselt PM

Antenatal volvulus
NTOG 2019;132:38-41; Klootwijk A, Hoeks SB, Manten GT, Tytgat, SH

White matter injury in neonatal viral encephalitis is associated with restrained systemic inflammation
Submitted; Hoeks SB, Groenendaal F, de Vries LS, de Jager W

Chapters in books:

Development of the immune system in the foetal and perinatal period
In: Neonatology and Blood Transfusion. Chapter I-IInd. Book 39 of Developments in Hematology and Immunology; Rijkers GT, Niers T, de Jager W, Janssens P, Gaiser K, Wiertsema S, Hoeks S, van de Corput L, Sanders EA.

Awards

2013 Young Investigators Award Neonatal Update "The science of newborn"

Oral presentations

2017 8th Dutch neonatal fellow meeting. Amsterdam, the Netherlands
2013 Neonatal Update "The science of newborn care". Imperial College London, United Kingdom
2009 Dutch Society Pediatrics. JOD. Annual meeting. Veldhoven, the Netherlands
2009 European Academy of Allergology and Clinical Immunology. Winterschool. Davos, Switzerland
2005 Dutch Society Pediatrics. Neonatology section. Annual meeting Leiden, the Netherlands

Poster presentations

2011 European Academy of Allergology and Clinical Immunology. Winterschool. Davos, Switzerland
2009 Allergy and Asthma symposium: bridging innate and adaptive immunity. Brugge, Belgium.
2009 European Congress of Immunology. Berlin, Germany.
2009 Dutch Society Pediatrics. Annual meeting. Veldhoven, the Netherlands
2008 Dutch research school Allergology. Young investigators meeting. Soesterberg, the Netherlands
2008 World immune regulation. Davos, Switzerland
2008 Dutch Society Pediatrics. Young investigators meeting. Veldhoven, the Netherlands
2004 Dutch Society Pediatrics. Annual meeting. Veldhoven, the Netherlands
2004 European Society Pediatric Infectious Diseases 22th Annual meeting. Tampere, Finland

ABOUT THE AUTHOR

Sanne Hoeks was born on March 21, 1978 in 's-Hertogenbosch, The Netherlands. She completed secondary school at the Gymnasium Beekvliet in Sint Michielsgestel. A gapyear was used to graduate a ninth subject (physics), to gain work experience in the hospital (she was very dedicated to serve food to patients with pulmonary conditions) and to play volleyball (a lot!). In 1997 she was able to start Medical School at the Radboud University Nijmegen. During her study, she became intrigued by Neonatology after a research project investigating the role of mannose binding lectin in neonatal sepsis (under supervision of Leo Gerards, Tannette Krediet, Andre Fleer and Ger Rijkers), an elective and a final internship at the Neonatal ward (Wilhelmina's Childrens Hospital UMC Utrecht and Radboud hospital Nijmegen, respectively). After graduation in 2003, she began her training in Pediatrics at the Wilhelmina's Children's Hospital UMC Utrecht (under supervision of prof. dr. J.L.L. Kimpen and prof. dr. J. Frenkel), Gelre Hospital in Apeldoorn (under supervision of dr. G.T. Heikens) and Tygerberg Hospital in Cape Town, South-Africa (under supervision of prof. dr. P. van der Merwe). In 2007, she started her PhD and alternated between work in the laboratory and clinical rotations. She finished her pediatric training in 2011 and after two more years of research, she started her fellowship in Neonatology at the Wilhelmina's children's Hospital UMC Utrecht (under supervision of prof. dr. F. van Bel and dr. W.B. de Vries). She worked as a consultant in Neonatology in the Maxima Medical Center in Veldhoven from 2018 until she started as a consultant in Pediatrics and Neonatology in the Diakonessenhuis Utrecht medio 2019.

Sanne is married to Jochem de Gruijter. They have four children, Nila (2011), Teun and Juule (2014), Cato (2016).

OVER DE AUTEUR

Sanne Hoeks werd geboren op 21 maart 1978 in 's-Hertogenbosch, Nederland. Ze behaalde in 1996 haar diploma aan het Gymnasium Beekvliet in Sint Michielsgestel. Een tussenjaar werd gebruikt om natuurkunde als negende eindexamenvak te behalen, om werkervaring in het ziekenhuis op te doen (ze was erg toegewijd om longpatiënten eten te serveren) en om (veel!) volleybal te spelen. In 1997 is ze begonnen met de studie geneeskunde aan de Radboud universiteit Nijmegen. Tijdens haar studie raakte ze gefascineerd door het domein van de neonatologie en heeft ze een wetenschapsstage gedaan waarbij naar de rol van mannose-binding lectin in neonatale sepsis werd gekeken (onder supervisie van Leo Gerards, Tannette Krediet, Andre Fleer en Ger Rijkers). Aansluitend volgde een keuze- en afsluitend coschap op de afdeling neonatologie (respectievelijk in het Wilhelmina's Kinderziekenhuis UMC Utrecht en Radboud ziekenhuis Nijmegen) waarna ze haar artsendiploma behaalde in 2003.

In 2004 begon ze aan haar opleiding tot kinderarts in het Wilhelmina's Kinderziekenhuis UMC Utrecht (onder supervisie van prof. dr. J.L.L. Kimpen en prof. dr. J. Frenkel) en in het Gelre ziekenhuis in Apeldoorn (onder supervisie van dr. G.T. Heikens). In 2006 werkte zij in het Tygerbergziekenhuis in Kaapstad (onder supervisie van prof. dr. P. Van der Merwe). In 2007, begon het PhD traject dat resulteerde in deze thesis. In 2011 ronde ze haar opleiding tot kinderarts af en na twee jaar onderzoek, begon ze in 2013 aan haar fellowship Neonatologie in het Wilhelmina's kinderziekenhuis UMC Utrecht (onder supervisie van prof. dr. F. van Bel en dr. W.B. de Vries). Ze werkte als neonatoloog in het Maxima Medisch Centrum in Veldhoven vanaf 2018 tot ze medio 2019 aan de slag ging als kinderarts-neonatoloog in het Diakonessenhuis Utrecht.

Sanne is getrouwd met Jochem de Gruijter. Ze hebben samen vier kinderen, Nila (2011), Teun en Juule (2014), Cato (2016).

TIJD

Tijd – het is vreemd, het is vreemd mooi ook
nooit te zullen weten wat het is

en toch, hoeveel van wat er in ons leeft is ouder
dan wij, hoeveel daarvan zal ons overleven

zoals een pasgeboren kind kijkt alsof het kijkt
naar iets in zichzelf, iets ziet daar
wat het meekreeg

zoals Rembrandt kijkt op de laatste portretten
van zichzelf alsof hij ziet waar hij heengaat
een verte voorbij onze ogen

het is vreemd maar ook vreemd mooi te bedenken
dat ooit niemand meer zal weten
dat we hebben geleefd

te bedenken hoe we nu leven, hoe hier
maar ook hoe niets ons leven zou zijn zonder
de echo's van de onbekende diepten in ons hoofd

niet de tijd gaat voorbij, maar jij, en ik
buiten onze gedachte is geen tijd

we stonden deze zomer op de rand van een dal
om ons heen alleen wind

Rutger Kopland

Herfst 2020

Soms staat de tijd even stil.

Het huis is stil, een volle auto is net vertrokken naar een speurtocht ter ere van de verjaardag van neefjes. Het manuscript ligt bij de beoordelingscommissie. Een raar moment, een laatste punt werd gezet.

Het proefschrift dat voor u ligt is er een die in tijd met mij is meegereisd, waar de input van velen in kan worden herkend en dat eindigt met een missie: kinderen aan de start van hun leven helpen aan een leven lang gezondheid.

Ik ben dan ook een heel groot aantal mensen zeer dankbaar dat zij mij op (een deel van) dit pad hebben willen vergezellen. Ik heb van hen geleerd, zij hebben mij gevormd als mens, als kinderarts en als neonatoloog.

Allereerst waren daar de kinderen en hun ouders, gezonde kinderen die zelf geen baat hadden bij de uitkomsten van het onderzoek. Het was bijzonder om binnen enkele uren nadat een kind geboren was, het afgenomen navelstrengbloed bij hen (thuis) op te halen. Om hen enkele maanden later op een volgend spannend moment, vlak voor de eerste operatie, terug te zien. Er volgden later in het jaar nog meer ingrepen waarbij er extra samples werden afgenomen. Zij hebben een belangrijke en volkomen onbaatzuchtige bijdrage geleverd om meer inzicht te krijgen in hoe het afweersysteem verandert in het eerste jaar.

Professor dr. A.B.J. Prakken. Berent, jij hebt ervoor gezorgd dat de mogelijkheid er was om naar deze onderzoeksvragen te kijken en hebt me altijd aangespoord om dwarsverbanden te zoeken, de vertaalslag naar de kliniek te maken en de mens achter de patiënt te zien. Wat een fantastische onderzoeksgroep heb je neergezet, een zeer talentvolle groep van biologen, biomedische wetenschappers en artsen die werd opgeleid om samen te werken, elkaar te ondersteunen en elkaar beter te maken om de wetenschap vooruit te helpen en de zorg voor patiënten te verbeteren. De diversiteit van onderzoekslijnen die daaruit voortgekomen zijn, is kenmerkend voor jou. Dank voor alles wat je mogelijk hebt gemaakt!

Dr. I.M. de Kleer. Ismé, gedeelde idealen en interesses vormden de basis voor onze samenwerking. Jij begon als kinderarts en PI, waar ik als AIOS getormenteerd door klinische vraagstukken om de hoek kwam kijken en mijn eerste stappen in het lab zette. Je positieve instelling, je enthousiasme en inzet is altijd aanstekelijk en ik waardeer het enorm hoe je altijd betrokken bent gebleven, tijd maakte om mee te kijken, de richting en de ondersteuning gaf die nodig waren. Ik was je zelfs bijna gevolgd naar Gent om aan neonatale muismodellen te

werken. De afronding van dit proefschrift gebeurde ook in jouw (en die van je gezin..) avond- en nachturen. Nu we beiden geland zijn op een geweldige plek en dit project tot een goed einde hebben gebracht, is dit wellicht het moment voor een volgend gezamenlijk project?

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