Viral respiratory infections and the maturation of nasal immune responses in infants: the VIGALL study

Viral respiratory infections and the maturation of nasal immune responses in infants: the VIGALL study

Virale respiratoire infecties en de ontwikkeling van de
immuunrespons in de neus van jonge kinderen: de VIGALL studie

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Chapter 1

Introduction

The prevalence of allergic disease as asthma, allergic rhinitis and atopic dermatitis has rapidly increased during past few decades in children. The correct maturation of the immune response in newborns is considered important for the prevention of the development of allergic disease and asthma. This thesis focuses on the potential role of viral (upper) respiratory tract infections on both the maturation of the immune system in the nose and the expression of allergic disease during the first two years of life.

In the general population the cumulative prevalence of allergic disease (asthma, atopic dermatitis, allergic rhinitis) in childhood is around 25-30% [213, 245]. In western countries, the prevalence of allergic disease has doubled during the last 25 years [155, 120] and therefore allergic disease has become a serious threat to public health. During infancy, allergic disease mainly expresses as atopic dermatitis [21], while allergic rhinitis and asthma generally become manifest after the second year of life [123]. The development of allergic disease is caused by an interaction of multiple genetic and environmental factors (figure 1.1). Allergic disease in the family (genetic predisposition), allergen sensitisation and exposure to tobacco smoke are some of the predictive markers for the development of allergic disease [54, 57, 174, 45]. Recently the hygiene hypothesis proposed that insufficient stimulus by early childhood infections of the immune system may lead to an enhanced risk to develop allergic disease [221, 222]. This idea has been linked to the rapid rise in prevalence of allergic disease last few decades. One of the fundaments of this hypothesis is that changes in lifestyle may have contributed to a reduction in the exposure to micro-organisms of individual children, resulting in a diminished maturation of the child immune system and increased risk of allergic disease. This thesis focuses on the potential role of viral (upper) respiratory tract infections on both the maturation of the immune system in the nose and the expression of allergic disease during the first two years of life.

1.1 Immunity in early childhood

Newborns are more susceptible to respiratory infections than older children and adults. Where in adult life the average individual suffers from 2-4 respiratory infections per year, this number is significantly higher in newborns. On average, during the first two years of life, a child suffers from 6-8 respiratory infections per year, which slowly decreases to 2-4 from 15 years and older [145]. Several factors contribute to this high susceptibility of newborns. Most important are intrinsic differences between the immune response of newborns and that of adults. Firstly, newborns will be exposed to pathogens for the very first time. Therefore the child's immune system has to initiate the immune response from scratch and cannot depend on a memory-type of immune response. Antibodies directed against pathogens and specialized memory T and B-lymphocytes, that due to their

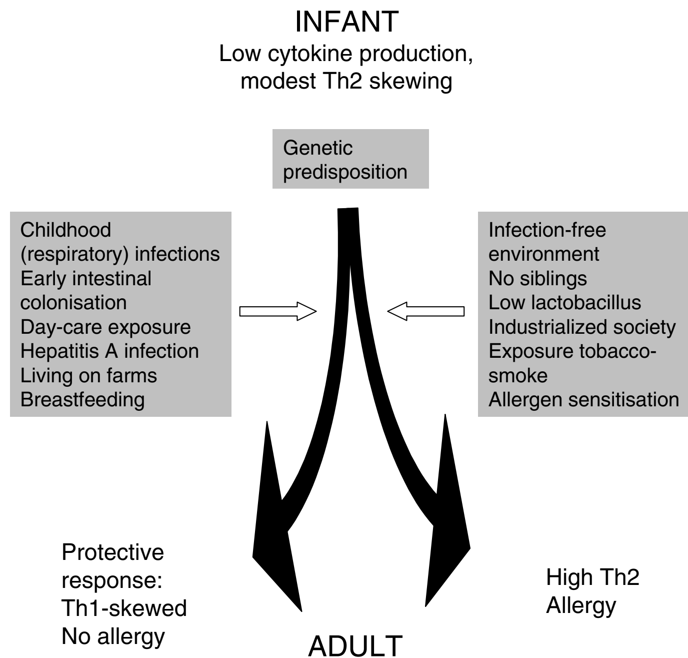

Figure 1.1: Risk and protective factors during early life affecting the development of allergic disease.

presence prior to infection play such an important role in the immune response to pathogens in adults, are virtually absent in newborns [209, 16]. Persisting maternal antibodies that have entered the newborn's circulation via the placenta at the foetal stage or that are taken up via breast milk in breastfeeding newborns mitigate the effects of the absence of the memory response [265]. Secondly, the immune system in newborns is also less efficient in inducing the T helper 1 (Th1)-type cytokine responses, which is the predominant type of cytokine in adults and necessary for an effective eradication of infections [35, 46]. These low Th1 responses may result in less efficient eradication of pathogens in infants. Through the existing equilibrium of Th1 and Th2-type immune responses and the lower level of the Th1-responses in infants, the newborn's immune response is Th2-skewed. As allergy and asthma manifest themselves as a Th2-type disease, the correct maturation of the immune response in newborns from Th2-skewed at birth to Th1 in adulthood is thought to contribute to the prevention of the development of allergic disease and asthma.

Differences in cytokine responses between newborns and adults

Much of our understanding of the neonatal immune system comes from in vitro experiments where peripheral blood cells are triggered with aspecific polyclonal or

infection related stimuli. The amounts and types of cytokines produced by neonatal immune cells are different from those produced by adult immune cells. These differences in mediators combined with differences in the cellular interaction during the immune response reflect the immature status of the immune system at birth.

Pathogens enter the human body via the epithelial cells of the mucosal surfaces. The epithelial cells are then activated to produce a wide variety of cytokines and chemokines (signalling molecules that respectively activate and attract various inflammatory cells), by which the inflammatory response is initiated. The central players in immunity seem to be dendritic cells (DCs). Upon recognition of the pathogen, immature resting DC, which are present in large numbers underneath the epithelium, are activated and a DC maturation programme is triggered. Maturating DCs migrate to secondary lymphoid organs where they stimulate T lymphocytes to develop towards e.g. Th1, Th2 or regulatory T lymphocytes (Tr) (Figure 1.2). Th and Tr lymphocytes mediate the relative balance of cytokine production and thereby mediate important biological processes, as cell growth, cell activation, inflammation, and immunity. Based on their presumed role in the immune response, cytokines have been tentatively divided in Th1-class, mainly involved in the eradication of bacterial or viral infections, and a Th2-class, mainly involved in the eradication of parasitic infections [189]. Besides a Th1 versus Th2 classification, cytokines have also been grouped according to their general function during inflammation, as pro-inflammatory, anti-inflammatory, or regulatory cytokines [131, 92].

In vitro experiments using aspecific polyclonal (PHA, PMA, concavalin A, or ionomycin) or infection related (LPS) stimuli revealed important differences between the immune response of immune cells isolated from newborns compared to adults. Cord blood T lymphocytes and mononuclear cells (CBMC, containing about 85% lymphocytes), showed lower cytokine production levels for Th1-related cytokines (IL-2, IL-12, TNFα, IFNγ) and Th2-related cytokines (IL-3, IL-4, IL-5) than adult peripheral blood T lymphocytes or mononuclear cells (PBMC) [197, 127, 170, 41, 40, 50, 118]. Besides that lower amounts of most cytokines are produced, the immune response in newborns also seems to be skewed towards Th2 cytokine production. Although high production of Th2 cytokines has not been reported for humans *in vivo* as has been for the mouse system [2], some human *in vitro* studies do suggest Th2 skewing in infants [258, 178]. A recent study by Ribeiro-do-Couto and colleagues showed that T lymphocytes from cord blood stimulated by αCD28+αCD3+rIL-2 produced about twice as much Th2 cytokine IL-13 than adult T lymphocytes [56]. Moreover, in the same study the amount of Th1 cytokine IFNγ produced was only one third of that produced by adult T lymphocytes. This resulted in the predominance of Th2 cytokine IL-13 production over that of Th1 cytokine IFNγ. The preferential production of Th2 cytokines, that

in general also has an anti-inflammatory action by suppressing the Th1 response, could well be a consequence of the need to restrict the immune response during pregnancy [53, 194]. As will be discussed later, pregnancy is an immunological challenging problem whereby an immune reaction leading to rejection of the foetus by the mother must be prevented.

The data presented above showed that T lymphocytes of infants behave differently from those of adults when it concerns cytokine production. In addition to the lower cytokine production and the skewing towards a Th2 response, there is ample evidence that also cellular interactions between antigen presenting cells as DCs and T lymphocytes are different in infants compared to adults [96, 126]. In general, DCs play a central role in stimulating T lymphocytes to become either Th1 or Th2 cytokine producing cells. The stimulation of T lymphocytes by DCs is achieved by a complex interplay between cell surface (signalling) molecules and cytokines. Naive Th0 lymphocytes recognize antigen bound to MHC class II molecules on DC by their T cell receptor in conjunction with CD4. Besides this antigen-receptor interaction, interaction of costimulatory molecules and the type of cytokine micro-environment is of importance in T lymphocyte stimulation. For example, high IL-12 producing DCs stimulate Th1 lymphocyte development while DCs expressing low levels of IL-12 stimulate Th2 lymphocyte development [125, 108].

Several parts of this DC mediated T lymphocyte stimulation seem immature in infants. When DCs were cultured in the presence of concavalin A and adult T lymphocytes, neonatal DCs were found to be less efficient than adult DCs in stimulating adult T lymphocyte proliferation [96]. In line with these findings, both unstimulated and LPS stimulated adult DCs were able to activate more adult CD4+ T lymphocytes to produce the Th1-inducing cytokine IFNγ than cord blood DCs [126]. Thus, the interaction between DCs and T lymphocytes seems immature in neonates. Some of the reasons for the impaired interaction between both cells could be a reduced expression of MHC class II molecules on DCs and costimulatory molecules on both neonatal DCs (CD40, CD80, CD86) [135, 83, 96] and T lymphocytes (CD3, ICAM-1, CD11a, CD18, CD58) [93, 52, 133, 110]. A less efficient antigen presentation, co-stimulation, and lower cytokine production in infants compared to adults, could all contribute to a less efficient induction of the T lymphocyte responses.

With regard to the cytokines, attention has especially been focussed on the low production of IL-12 by DCs and of IFNγ by T lymphocytes in newborns as these cytokines play a pivotal role in the host immune response against infections (figure 1.2). An impaired IL-12 cytokine production was found by neonatal DCs after stimulation with LPS compared to adult DCs, a cytokine which is essential for differentiation of precursor T lymphocytes into IFNγ producing Th1 cells [126, 83]. Interestingly, when IL-12 was added to a culture of purified neonatal T lymphocytes, adult-like IFNγ responses could be achieved [159], hinting that

low production of IL-12, at least in part, underlies the immaturity of the infant's immune system. Although neonatal T lymphocytes can respond to IL-12 by the production of IFNγ, the additive effect of pro-inflammatory IL-18 on the IL-12 induced IFNγ production, which is normally observed in adult PBMCs, is less pronounced in infants. Stimulation of neonatal T lymphocytes with a combination of IL-12 and IL-18 does lead to a further increase in IFNγ production but levels are not as high as in adult T lymphocytes cultures [159]. Furthermore, the impaired production of IL-12 by LPS stimulated DCs was upregulated to adult levels by the addition of IFNγ [83]. Thus a low production of IFNγ synthesis by neonatal immune cells, as for example described above for T lymphocytes, could result in suboptimal stimulation and impaired IL-12 production by DCs in neonates. However, whether impaired IL-12 production by neonatal DCs results in suboptimal stimulation of IFNγ synthesis by T lymphocytes or whether a defective IFNγ production by neonatal T lymphocytes results in a suboptimal activation of DCs is not yet understood.

Besides DCs and T lymphocytes, also several other cells of the immune system show an immature cytokine response. A lower production of IL-1 and TNFα proteins by LPS stimulated peripheral blood monocytes from neonates compared to adults has been reported [166, 134]. This was also observed for alveolar macrophages. LPS stimulated alveolar macrophages of children younger than 2 years produced lower levels of IL-1 and TNFα protein than alveolar macrophages of 2-17 year old children [84]. Not all aspects of cytokine production are immature in macrophages as IL-6 protein production was comparable between both groups of children. Moreover, cord blood and adult blood natural killer (NK) cells produced nearly the same levels of IFNγ protein without stimulating the cells. After stimulating the cells with a combination of IL-12 and IL-18, production of IFNγ protein increased for both neonatal and adult NK cells and the production was even higher in infants than adults [159].

The immature immune system in newborns is furthermore characterized by the difference in production of the regulatory cytokine IL-10 between newborns and adults. Although some *in vitro* studies have suggested a lower production of IL-10 in infants compared to adults by LPS stimulated CBMC and PBMC [42] or by T lymphocytes stimulated with αCD3, αCD28, and rIL-2 [56], these observations are not in line with *in vivo* studies suggesting high levels of IL-10 in newborns. A recent study did find that stimulation of naive CD4+ T lymphocytes, which comprise the majority of T lymphocytes in infants, with αCD3, αCD28, and rIL-2 leads to higher production levels of IL-10 in newborns than in adults [182]. This high production of IL-10 was also found when CBMC were stimulated with infection related stimuli (*Bordetella pertussis* toxin) [228]. The reason for the discrepancy with the older studies showing a lower production of IL-10 in PBMC remains unclear.

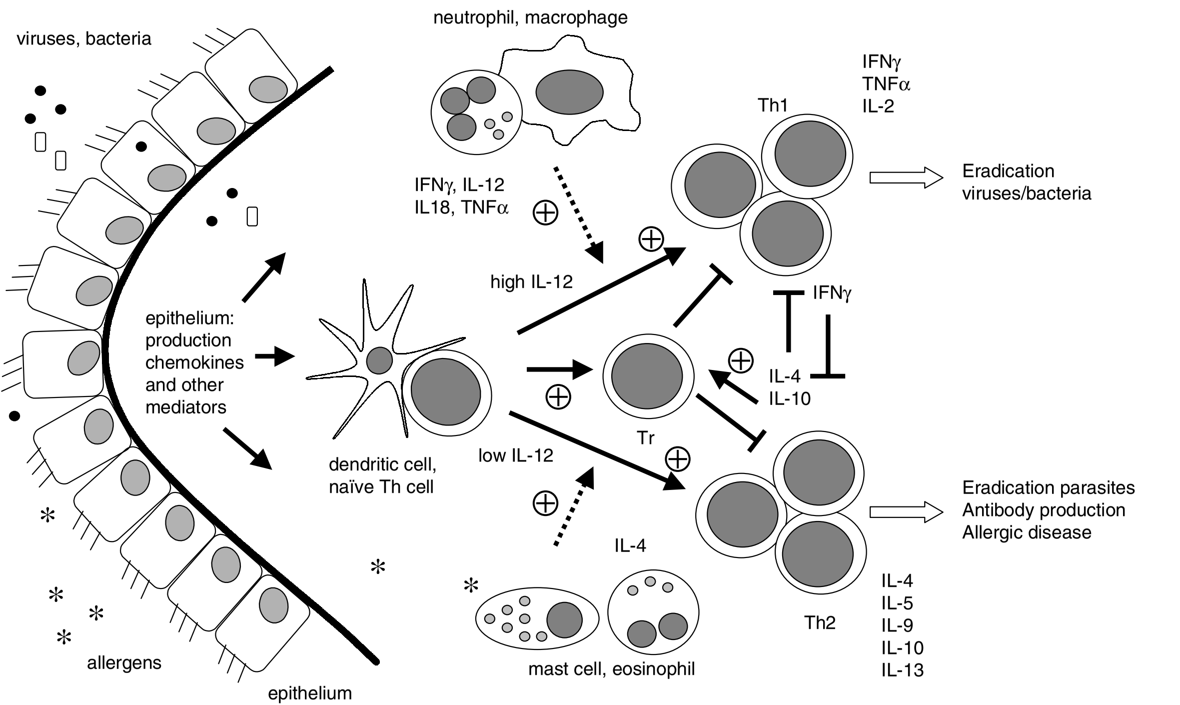

Figure 1.2: Basic immunological mechanisms underlying responses to various antigens entering the body via epithelial tissue.

The importance of IL-10 in infancy has been emphasized by data from *in vivo* observations. For example, high levels of IL-10 are transferred from mother to child by breast milk and suggests an active role of IL-10 in the neonate [71]. High IL-10 responses in infants could result from an IL-10 rich environment that prevails during pregnancy [167]. A high production of regulatory and Th2 cytokines displaying immunosuppressive properties (IL-10, IL-4 respectively) and a low production of Th1 cytokines (TNFα, IFNγ) seems to be necessary for maintaining a succesful pregnancy [53, 194]. This is illustrated by the observation that a low IL-4 and IL-10 production and a high TNFα and IFNγ production is frequently observed by peripheral blood T lymphocytes and by T lymphocytes present at feto-maternal interface of women with recurrent abortions [168, 138]. IL-10 may play a crucial role in the regulation of a balanced Th1 and Th2 maturation during early childhood as it can both downregulate Th1-related and pro-inflammatory (IFNγ, IL-2, IL-12, TNFα and IL-18) [160, 139, 207], and Th2-related cytokines (IL-5) [199]. Furthermore, IL-10 plays an important role in the induction of tolerance [132, 86]. In newborns, tolerance-induction against the numerous harmless antigens, which the infant newly encounters frequently after birth, is essential to prevent the child from initiation excessive harmful inflammatory reactions. The high capacity of newborns to induce tolerance is shown by the high success rate of cordblood transplantation in adult recipients. In these patients the incidence of graft-versus-host-disease was lower after cordblood transplantation than after adult bone marrow transplantation [81].

Thus a considerable amount of data showed that peripheral blood immune responses are immature in infants. As the respiratory epithelium is the first line of defence against various pathogens and an important source of various cytokines [176], particularly this local part of the immune system plays a central role in immunity. However, whether immune responses in the nose are mature in infancy is not fully understood as only a limited number of studies examined this issue. In this thesis we therefore concentrated on the type of nasal immune responses during infancy.

Maturation of the infant's immune system

In vitro experiments with stimulated PBMC reveal an age-related maturation of the immune response from Th2-skewed at birth towards a Th1 cytokine response later in life. This maturation seems to be hampered in children who are allergic or who will become allergic.

The immature and Th2-skewed immune responses during infancy and early childhood need to develop with age into more adult-like immune responses. As adults predominantly induce Th1 responses, which are indispensable for a proper host immune response upon infection, it has been hypothesized that Th1 responses

from birth will strongly increase with age. Such immune maturation has been observed in cross-sectional studies. For example, Elsasser-Beile and colleagues showed that protein levels of Th1 cytokines IFNγ, IL-2, and TNFα produced by polyclonally (PHA + pokeweed mitogen) stimulated whole blood cell cultures increased gradually during childhood and adolescence, although at the age of 17 they still had not reached adult levels [61]. Comparable maturation patterns were found for IL-12p70 protein, when PBMCs were first incubated with IFNγ and further stimulated with LPS [233]. In a recent prospective birth cohort study Buck and colleagues showed that percentages of T lymphocytes producing IFNγ (Th1) and IL-4 (Th2) within mitogen stimulated whole blood samples (PHA+ionomycin) increased from 2 to 12 months but remained lower than percentages found in adults [35]. Furthermore, although no evidence for Th2 skewing was found in these infants, Th1/Th2 ratios in infants were 5-10 times lower than in adults. This reveals that the Th1/Th2 ratio is still immature at 12 months of age and needs to develop to adult ratios.

In vitro data suggest that maturation of the immune system in children who have allergic disease or who will become allergic during later life, show a different pattern of maturation than that of healthy non-allergic children. In general, allergic disease is characterized by the production of allergen specific IgE antibodies which is accompanied by the overproduction of Th2 cytokines (IL-4 and IL-5) and low production of Th1 cytokines (IFNγ) [174, 198]. Van der Velden and colleagues compared Th1 and Th2 cytokine maturation in a birth cohort study during the first year of life in children who did and did not develop allergic disease during the first year of life [238]. Contrary to the unchanged production levels of IL-4, IL-5, and IL-13 observed during the first year of life in mitogen stimulated CBMC or PBMC from non-allergic children, production levels of these Th2 cytokines increased gradually during the first year of life in allergic infants. Largely comparable cytokine maturation patterns were also found in a recent cross-sectional study, which showed that the protein production of Th2 cytokines IL-4 and IL-5 by house dust mite stimulated PBMCs in children with atopic dermatitis (AD) increased rapidly from birth until the age of 12 months, remaining at this elevated level up to 8-15 years of age. However, the production of both cytokines was undetectable in healthy controls until age 2, only reaching levels comparable to patients with AD at age 8-15 years for IL-4 and age 3-7 years for IL-5 [112]. Moreover, while the IFNγ (Th1 cytokine) protein production increased with age in these healthy children, production remained low and unaltered in patients with AD [112]. Thus, although techniques used to stimulate lymphocytes differ considerably between both studies, these studies showed that a rapid increase in Th2 cytokine responses was only observed in children with allergic disease, with a possible concomitant impaired maturation of the Th1 cytokine response.

1.2 Stimulation of immune maturation

Repeated childhood infections or exposure to infection-related stimuli have been hypothesized to enhance the immune maturation in newborns and to prevent the development of allergic disease.

The maturation of the child's immune system from Th2-skewed during infancy to Th1-skewed during adulthood may be the sole consequence of a natural age-related development programme. However, it has been suggested that repeated childhood infections stimulate this immune maturation through the successive induction of Th1 responses. In 1989 Strachan and colleagues observed an inverse relation between the prevalence of hay fever and the numbers of older children in the household. He suggested that unhygienic contacts with older siblings facilitated the transmission of respiratory viral infections resulting in the decreased risk of allergic disease [221]. This hypothesis of an inverse relation between childhood infections and prevalence of allergic disease was linked to the rapid rise in allergic disease during the past few decades. Improvement of health care nowadays have led to the eradication and reduction of many common childhood infections by extensive vaccination program and the widespread use of antibiotics [254]. Although these epidemiological studies cannot differentiate between the contribution of bacterial or viral infections, they do suggest that the overall reduction in the number of childhood infections may have weakened immune maturation, thereby triggering an increase in allergic disease. During following years this idea developed into the world-wide studied 'hygiene hypothesis'.

Several years later, underlying immunological mechanisms of the hygiene hypothesis were formulated. Proposals were based on the observation that, in general, Th1 cytokine immune responses are induced upon bacterial and viral infections [51]. Therefore, repeated infections during childhood may stimulate the Th1 maturation (Figure 1.2). Since Th1 cytokines suppress the production of Th2 cytokines, an increase in Th1 cytokine responses with age would prevent or limit an increase in production of Th2 cytokines with age. In the hygiene hypothesis this concept has been linked to the development of allergic disease. If children would encounter only few infections during childhood, the normal Th1 maturation would be hampered. As a consequence, Th2 cytokine production is not or only poorly suppressed and may even increase with age. This increase in Th2 cytokine production may then enhance the risk of developing Th2 mediated allergic disease.

Different pathogens could potentially stimulate immune maturation and limit the risk for a child to develop allergic disease. The following sections of this paragraph focus on studies that have shown possible contributions of viruses, bacteria, or parasites.

Viruses

Initial suggestions that viral infections may protect against allergic disease were given by a study performed in Guinea-Bissau, which showed that measles infections during childhood were inversely related to the risk of allergen sensitisation in young adulthood [204]. However, later studies could not confirm these results. Two larger studies in Denmark and Finland, contrary to the first study, observed a negative association between measles infection and development of allergic disease and atopy [165, 12]. More consistent results have suggested a protective effect of hepatitis A virus infection on the development of allergic disease. Matricardi and colleagues found that aero-allergen sensitisation, asthma, and allergic rhinitis were less common among hepatitis A virus seropositive than among seronegative Italian male students [140]. Recently, these results were confirmed in a group of ∼34.000 US residents aged 6-59 years, where a 2-4 fold lower life-time prevalence of hay fever, asthma, and sensitisation to airborne allergens was found in subjects seropositive for hepatitis A virus [142]. The same study suggested that this effect might well be pathogen specific. Although the same protective effect was observed in subjects seropositive for herpes simplex virus type 1, the inverse relation between infection and asthma was not found for herpes simplex virus type 2, or hepatitis B and C viruses.

A protective role has also been suggested for upper respiratory tract infections (URTI). Illi and colleagues showed an inverse relation between the number of URTI episodes during the first 3 years of life and the prevalence of asthma at age 7 [101]. Other viral infections during childhood like rubella, varicella, chickenpox, or mumps were not inversely related to the prevalence of atopy (specific IgE production)[13, 141] and in the case of rubella, mums and varicella even showed a trend for a positive relation with asthma, eczema, and hay fever [24]. Thus the effect of viral infections on the child's immune maturation and risk of allergic disease varies and probably depends on the type of virus the child encounters, the type of immune response that is elicited and/or the age at which the child is infected.

Bacteria

The first indication that bacterial infections could have a protective effect on the development of allergic disease came from studies on tuberculosis in Japan [208], although later European studies could not confirm the initial observations [87, 223, 5]. The Japanese study observed an inverse relation between a positive tuberculin responses at 6 and 12 years and the prevalence of asthma, rhinitis, atopic dermatitis, and serum IgE levels at the age of 12 in children who were vaccinated with attenuated bovine *M. tuberculosis* (bacillus Calmette-Guérin (BCG)) [208]. This suggested that the BCG vaccination and, by inference, probably also infection with bacteria as *Mycobacterium tuberculosis*, could have a protective effect on allergic disease. An alternative explanation would be that allergic individuals

were uncapable of mounting tuberculin responses due to a defect in Th1 cytokine production by these patients. Later, more direct evidence for a protective role of bacterial infections on the development of allergic disease was obtained by studies of Matricardi and colleagues. In a cohort of Italian military cadets a lower prevalence of respiratory allergy was found in individuals who had been exposed to *Helicobacter pylori* [141].

Not only bacterial infection which can induce clinical disease, but also relatively harmless bacteria colonising the gut could be involved in the pathogenesis of allergic disease. This was first suggested by Björkstén and colleagues who found *Lactobacilli* and *Eubacteria* more frequently in the intestinal microflora of infants in Estonia, where the prevalence of allergic disease was low, while *Clostridia* was more frequently found in Swedish infants where high prevalence rates of allergic disease have been reported [203]. The same investigators then showed that 2 year old allergic children (atopic dermatitis with a positive skin prick test to egg and/or cow's milk) had a lower count of *Lactobacilli* and higher counts of aerobic bacteria (coliforms and *Staphyococcus aureus*) than non-allergic children [22]. This observation suggested a protective effect of *Lactobacilli* colonising the gut on the development of allergic disease. To test these observations, in a recent Finish intervention study, infants were given probiotics, i.e. cultures of potential beneficial bacteria. The frequency of atopic eczema at 2 years was halved when *Lactobacilli* were given to the mother during the last 2-4 weeks before expected delivery and to the infant until the age of 3-6 months [109, 184]. These results thus more directly suggested a protective effect of *Lactobacilli* on the development of allergic disease.

Now the question raises whether the protective effect on the development of allergic disease is due to infection with bacteria per se or whether particular components of bacteria can induce this protective effect. During recent years, studies among farmer's children have suggested an active role of bacterial endotoxins as lipopolysaccharide (LPS) in immune maturation and protection from allergic disease. A low prevalence of allergen sensitisation, asthma, and allergic rhinitis was observed among farmers children [32, 111, 113], especially those who had been exposed to stables [188], compared to non-farming children. It was hypothesized that exposure to bacterial endotoxins would account for this effect as endotoxins are found in house dust and agricultural dust [246]. This idea was strengthened by findings that the risk of atopic eczema in infants at 6 months of age was $\sim$50% lower in children exposed to high levels of endotoxin present in the mothers mattresses compared to children exposed to low levels [74]. Thus early exposure to bacterial endotoxins may stimulate the neonatal immune maturation towards Th1 cytokine production and thereby reduce the risk of developing allergic disease.

This immunological concept has recently been studied by Gereda and colleagues who found that in infants (9-24 months) with at least three episodes of wheezing, the proportion of T lymphocytes producing the Th1 cytokine IFNγ af-

ter mitogen stimulation (PMA, ionomycin, brefeldin A) increased with increasing levels of endotoxins exposure [75]. However, Braun-Fahrlander and colleagues showed a decrease in production of Th1-related (TNFα, IFNγ, IL-12) and regulatory cytokines (IL-10) by LPS stimulated whole blood cultures with increasing endotoxin load in the mattresses of 6-13 year old children [33]. These opposite cytokine responses could be due to differences in the age of children in both study-groups. Exposure to endotoxins during infancy may stimulate immune maturation and reduce the chance to develop allergic disease as suggested by Gereda and colleagues [75]. However, exposure to endotoxins, even at low doses, during later childhood and adulthood could lead to exacerbation of disease in individuals with allergies and asthma [163]. The high cytokine responses observed in the study of Braun-Fahrlander and colleagues in children exposed to low levels of endotoxins, could thus well result from the fact that in these children a high prevalence of allergic disease was found and that the cytokine responses observed could be related to exacerbation of allergic disease. Thus it seems that a correct timing of infections during infancy may well be important for an optimal immune stimulatory effect.

Parasites

Besides bacterial and viral infections (Th1-inducing), also infection with helminths (Th2-inducing) has been suggested to protect from developing allergic disease. This idea emerged from the observation that the prevalence of allergic disease as asthma is considerably lower in developing countries, where a high parasite burden is found, compared to more Western and developed areas, where only few parasite infections occur [7]. For example, in Brazilian children an inverse relation was found between *Schistosoma mansoni* infection and aero-allergen sensitisation [8], as well as between infection with intestinal helminths (*Ascaris*, *Trichuris* and hookworm) and house dust mite sensitivity in South American children [90]. However, these are just observed correlations and carefully followed birth cohort studies are needed to establish a protective effect of helminth infections on the development of allergic disease.

Besides the hypothesis that balanced Th1 and Th2 cytokine maturation is stimulated by repeated viral and bacterial infection during childhood by their Th1 stimulating properties (figure 1.2), an alternative proposal centered around IL-10 has been put forward to explain immunological mechanisms responsible for the protection against allergic disease due to parasite infections. A recent study by Van den Biggelaar showed higher schistosome-antigen-specific IL-10 production by PBMCs of children who were infected with *Schistosoma mansoni* parasite, while the prevalence of a positive skin reaction to house dust mite in these children was low compared to non-infected children [236]. This suggested a suppressive role of IL-10, induced in chronic schistosomiasis, on atopy. The authors proposed a model for the allergy protective effect of helminth infections, which was based on

the induction of a strong anti-inflammatory and regulatory network, characterized by elevated IL-10 and TGFβ production [260]. As IL-10 can inhibit both Th1 and Th2 responses, this anti-inflammatory and regulatory cytokine production could not only help to prevent the induction of Th2-mediated allergic disease, but also the induction of Th1-mediated autoimmune diseases. Although outside the direct scope of this thesis, an increase in Th1-mediated autoimmune disease has been observed in the Western world [177], which would argue against a simple dogma of changes in the Th1/Th2 ratio as the sole explanation for an increase in Western-life style associated disease. Conversely, also childhood viral or bacterial infections may not only affect the Th1/Th2 balance, but analogous to the mechanisms proposed for helminth infections, could also stimulate a pro- versus anti-inflammatory axis that could contribute to the prevention of Th1 and Th2-mediated disease.

1.3 Respiratory viruses and allergic disease

In the present thesis we will focus on the role of viral respiratory tract infections, in particular of the upper respiratory tract, on the maturation of the infant immune system and development of allergic disease. These infections are the most frequently occurring infections in humans and may thus stimulate immune maturation considerably during early life.

Respiratory tract infections are among the most common and frequently occurring illnesses in humans and therefore are a source of significant morbidity and carry a considerable economic burden. Infants and young children are relatively susceptible to respiratory infections. On average, during the first two years of life, a child suffers from 6-8 respiratory infections per year, which slowly decreases to 2-4 from 15 years and older [145]. Respiratory tract infections in general are limited to the upper respiratory tract and manifest as relatively harmless and self-limiting illnesses characterized by rhinorrhoea, nasal obstruction, sore throat, cough, fever, malaise and/or myalgia which last up to 7 days (common cold) [253]. The majority of common colds are caused by rhinovirus and coronavirus [137]. Infants and young children are relatively susceptible to develop lower respiratory tract infections (LRTI: bronchitis, bronchiolitis and pneumonia). Infection with respiratory syncytial virus (RSV) is the most important cause of severe LRTI in infants below six months of age. At the age of two years virtually all infants have been infected with RSV at least once [211]. Although the majority of RSV infections manifest as a mild common cold, in an estimate of 10-40% the lower respiratory tract is involved. An estimated 0.5-2% of all infants are hospitalized with an RSV infection in their first year of life [205, 241].

The effect that viral respiratory tract infections may have on allergic disease should probably be separated into two distinct situations. As described in the previous paragraphs, respiratory viral infections in infancy may stimulate immune

maturation and thereby reduce the risk of developing allergic disease. On the other hand, in children and adults with established allergic disease, viral respiratory tract infections are important triggers of exacerbation of disease, as is also described for exposure to endotoxins. In children and adults with asthma, 80-90% of exacerbations are accompanied by a respiratory infection [104, 154]. Although patients with asthma are at high risk to develop exacerbation of disease during viral infection, patients are not more susceptible for respiratory tract infections than healthy individuals [47]. Immunological mechanisms underlying enhanced symptomatology during viral respiratory tract infections in patients with allergic disease are thought to involve delayed viral clearance and inappropriate virus-induced immune responses, for example persistent eosinophilia and excessive inflammatory cytokine production, possibly as a result of pre-existing Th2 skewed immune responses [144]. This may further result in immunopathology and tissue damage, and induction or amplification of allergic inflammation.

In addition to the timing of the infection, the severity of infection may also be relevant to whether viral infections prevent or rather promote symptoms of allergic disease in infants and young children. In contrast to a protective effect of URTI on the development of allergic disease in infants, more severe infection in which also the lower respiratory tract is involved (LRTI; bronchiolitis) has long been thought to increase the risk of developing allergic disease. This idea has risen from the observation that LRTIs in infants and young children are frequently accompanied by symptoms of wheezing, which could be suggestive of asthmatic disease [59]. For example, Sigurs and colleagues showed an increased risk of asthma and allergic sensitisation at age 7 in children who needed hospitalisation following RSV-induced LRTI during childhood [210]. Others have concluded that the number of lower respiratory tract infections during the first year of life were positively related to the development of symptoms of allergic disease (asthma, wheeze, bronchial hyperreactivity) at ages ranging from 4 to 7 years of age [151, 39, 101]. Underlying immunological mechanisms were hypothesised to comprise excessive Th2 cytokine activation during LRTI, which could subsequently lead to Th2 mediated allergic disease. Indeed several studies observed Th2 skewed cytokine responses (i.e. increased IL-4 and decreased IFNγ production) by polyclonally stimulated PBMCs or T lymphocytes of children with RSV induced-LRTI [190, 26, 18, 128]. However, the risk of developing symptoms of wheezing following severe LRTI diminishes with age. Although Stein and colleagues found an increased risk of recurrent wheezing in 7-year-old children following bronchiolitis, no increased risk of wheezing was found at age 14 anymore and no association was found with subsequent atopic status [217]. Thus it seems that the risk of asthma-like symptoms like wheezing following LRTI is transient and do not represent symptoms of allergic disease. These non-allergic symptoms during and after RSV bronchiolitis are thought to result from an overproduction of Th1 and pro-inflammatory cytokines. For example, during RSV-induced LRTI high levels of IFNγ were produced by

RSV stimulated peripheral blood T lymphocytes [30] and high levels of IFNγ were found in nasal pharyngeal samples [73, 240]. Furthermore, overproduction of chemokines (RANTES, MIP-1α, MIP-1β) was induced by RSV stimulated peripheral blood T lymphocytes of infants with bronchiolitis [231]. Production of these cytokines could well result in excessive inflammatory reactions and mucus production, which may easily lead to non-allergic airway narrowing and wheezing in infants which already have a small airway-size.

Thus viral infections can trigger severe respiratory symptoms in patients with established allergic disease and can induce symptoms suggestive of asthmatic disease in young children when infecting the lower respiratory tract. However, the majority of respiratory viral infections are limited to the upper respiratory tract. These URTI episodes in infants and children are rather considered to be beneficial for the maturation of their immune responses. As already mentioned, this was recently shown by Illi and colleagues, who found that the risk of a diagnosis of asthma or wheeze at age 7 was reduced by $\sim$40% in children with two or more reported episodes of runny nose during the first year of life [101]. On the other hand, others found a twofold higher risk of asthma or recurrent wheezing at age 4 when the children had had any common cold within the first year of life compared to children without common colds [151]. Results of both studies must be interpreted with care as numbers of runny nose episodes and common colds are relatively low, which could have resulted from a reporting bias induced by the retrospective study-design. However, discrepancies between the studies indicate that a possible protective effect of URTIs on the development of allergic disease, probably depends on various host- and infection-related factors, for example the severity of infection, the type of virus, the age at which the child is (first) infected, the type of immune response induced by the virus, the genetic risk to develop allergic disease and the frequency of respiratory infection.

As the respiratory epithelium is the first line of defence against invading pathogens, this site is likely to play a central role not only in inducing protective host-immune responses, but also in stimulating immune maturation in infants. Although immune maturation patterns have been studied in peripheral blood in infants, no data are available on immune maturation in local tissue of nose and lungs. Furthermore, hypothetical mechanisms that URTI would stimulate immune maturation by repeated induction of Th1 responses has until now not convincingly been verified by *in vivo* data from nasal immune responses. Only one study by Noah and colleagues found an upregulation of Th1 and pro-inflammatory (IL-1, IL-6, TNFα) cytokine levels in nasal lavage samples during common cold in 0-3 year old children [156]. These data indicate that infants and young children are indeed able to induce Th1 responses upon infection. It is however unclear whether this immune response is mature and whether it depends on the type of viral pathogen, the severity of infection and the family history of allergy. Therefore in this thesis we will describe investigations on nasal immune responses upon viral respiratory

infection in 0-2 year old children, related to several host- and infection-related factors. Furthermore, we examined the effect of repeated respiratory infections on nasal immune maturation.

Chapter 2

Aim of the study and study-design

2.1 Aim of the study

In this thesis we will examine immune responses in the nose of infants (0-2 year old) during (upper) respiratory tract infections and the possible role of these infections on the maturation of the nasal immune system during early life. With this knowledge we intend to estimate the relative importance of respiratory tract infections on the development of allergic disease as described in the hygiene hypothesis [222]. These questions were examined in a prospective birth cohort study, the VIGALL study (Dutch abbreviation for 'virally mediated allergy').

In the introduction (**chapter 1**) we describe our current knowledge on maturation patterns of the infant's immune system. Various aspects of the immune system are immature after birth and will develop with age. Repeated childhood infections are hypothesized to stimulate immune maturation and thereby reduce the risk to develop allergic disease (hygiene hypothesis). Currently two immunological theories have been put forward to explain underlying mechanisms. Firstly, it has been proposed that viral infections can stimulate Th1 immune maturation and thereby reduce the risk of developing Th2 mediated allergic disease (Th1/Th2 paradigm). An alternative hypothesis has recently been put forward and suggests a major role for infection-induced IL-10 in regulating both Th1 and Th2 maturation by its anti-inflammatory and regulatory properties (anti-inflammatory theory). In this thesis we will try to find immunological evidence for the Th1/Th2 paradigm.

The immune stimulatory effect of respiratory viral infections may differ according to the type of viral pathogen and the severity of infection. To estimate the relative contribution of individual viruses on the infant's immune maturation, in **chapter 3**, we examined what types of viral pathogens can induce respiratory symptoms in infants. As rhinovirus turned out to be the most prevalent viral pathogen during infancy, we next examined (**chapter 4**) what type of immune response was induced in the nose of children with rhinovirus-induced upper respiratory tract infection (URTI). These nasal immune responses were compared with immune responses induced by the second prevalent respiratory pathogen, respiratory syncytial virus (RSV). Furthermore, we examined whether the type of nasal immune response did depend on the severity of rhinovirus-induced infection.

As we found that respiratory viruses were indeed able to induce Th1 immune responses in the nose of infants during URTI, we questioned whether repeated respiratory tract infections could influence immune maturation. In **chapter 5** we examined the natural immune maturation patterns of nasal cytokine responses in healthy infants as well as in infants with URTI. Furthermore, the numbers of respiratory tract infections a child had experienced were related to these maturation patterns in order to estimate whether repeated infections display an immune stimulatory effect.

In contrast to URTI, infection of the lower respiratory tract (LRTI; bronchiolitis) is thought to trigger development of allergic disease rather than protect against

it. In **chapter 6** we examined whether nasal immune responses differed between infants with RSV-induced bronchiolitis and infants with RSV-induced URTI. Differences in immune responses could give insight in the mechanisms underlying the potential differences in immune pathology between both types of RSV disease.

Viral infections can easily trigger exacerbations of disease in children or adults with established asthma or allergy. In **chapter 7** we examined whether nasal immune responses during common cold differed between allergic and non-allergic adults. Results could explain whether the increased susceptibility for allergic reactions in these patients is based on particular immunological mechanisms.

Finally, in the **chapter 8**, we have discussed the data in the context of relevant literature, with emphasis on the role of viral respiratory tract infections on immune maturation in infants and development of allergic disease.

2.2 Study-design

Selection of participants

From August 1996 to November 1998, 126 infants were included in the VIGALL study between birth and 6 months of age. Eighty-six children had a family history of atopy (FHA), which was defined as having at least one parent with allergic disease. Allergic disease in one or both parents was established with a validated screening questionnaire on asthma and allergy [124]. Parents with self-reported asthma, hay fever, house dust mite allergy, or pet allergy were considered to be allergic and their children were defined as high-risk children (positive FHA). Parents who did not report these symptoms were considered to be non-allergic, and their children were defined as low-risk children (negative FHA). Fifty children with a positive FHA (40%) in the VIGALL study also participated in the PIAMA study [116].

Study-design

The VIGALL study is a prospective birth cohort study (figure 2.1). Children were examined during routine-visits at 6, 12, 18 and 24 months of age. During each visit an interview was taken, physical examination was performed, and nasal brush and blood samples were collected. During the physical examination and interview, symptoms of upper and lower respiratory tract (as runny nose, wheeze, cough, fever), general malaise, and symptoms suggestive of atopic dermatitis (skinrash) were recorded. Additionally, once in the first and once in the second year, parents were asked to visit the study-doctor when their child had symptoms of an upper respiratory tract infection (a runny nose and at least one of the symptoms fever, malaise, sleeping difficulties or loss of appetite). During these sick-visit and about

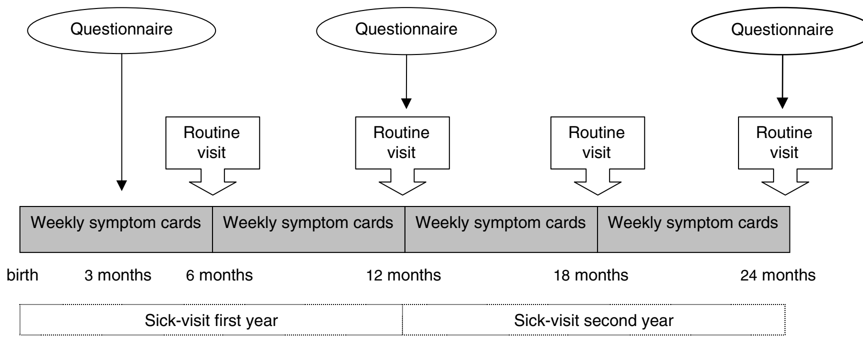

Figure 2.1: Design of the VIGALL birth cohort study.

two weeks later (convalescence) again an interview was taken, physical examination was performed, and nasal brush and blood samples were taken.

At the age of 3, 12 and 24 months, parents filled in questionnaires on various genetic and environmental characteristics of the child (e.g. number of siblings, day-care, breast-feeding, allergic disease in the family) as well as symptoms suggestive of allergic disease in the child. During the whole study-period, parents filled in weekly symptom cards on which symptoms of fever, respiratory tract disease (runny nose, wheezing), gastrointestinal disease, and skin rash were recorded. From these weekly symptom cards, cumulative incidence of respiratory tract infections were calculated. At the age of 12 and 24 months, a diagnosis of atopic dermatitis was made, based on the criteria of the UK Working Party for atopic dermatitis [255].

During routine- and sick-visits, nasal brush and blood samples were taken. In nasal brush samples, cytokine responses were measured as well as the presence of viral pathogens. At the age of 12 and 24 months, levels of total IgE [216] were measured as well as specific IgE against cow's milk, cat and dog dander, hen's egg and house dust mite [201].

Forty-two of 126 children (33%) dropped out for various reasons. Fifteen children dropped out at 12 months of age, 22 children at 18 months and 5 children at 24 months of age. No differences were found between the children who dropped out and those who did not in terms of family history of atopy, day-care attendance, numbers of respiratory infections, and development of atopic dermatitis in the preceding follow-up period.

Chapter 3

Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants

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3.1 Abstract

Respiratory infections in infancy may protect from developing Th2 mediated allergic disease (hygiene hypothesis). To estimate the relative contribution of particular viruses to the development of the immune system and allergic disease, we investigated longitudinally the prevalence of respiratory viral infections in infants.

Hundredtwentysix healthy infants were included in this prospective birth cohort study in their first year of life. Physical examination was performed and nasal brush samples were taken during routine visits every 6 months and during upper respiratory tract infection (URTI) (sick visits). The prevalence of respiratory viral infections in infants with URTI, infants with rhinitis without general malaise and infants without nasal symptoms was studied.

Rhinovirus was the most prevalent pathogen during URTI and rhinitis in 0-2 year-old infants (∼40%). During URTI, also respiratory syncytial virus (∼20%) and coronavirus (∼10%) infections were found, which were rarely detected in infants with rhinitis. Surprisingly, in 20% of infants who did not present with nasal symptoms rhinovirus infections were also detected. During routine visits at 12 months, a higher prevalence of rhinovirus infections was found in infants who attended day-care compared to those who did not. We did not observe a relation between breast-feeding or smoking by one or both parents and the prevalence of rhinovirus infections. The family history of atopy was not related to the prevalence of rhinovirus infection, indicating that the genetic risk on allergic disease does not seem to increase the chance on rhinovirus infections.

In conclusion, rhinovirus infection is the most prevalent respiratory viral infection in infants. It may therefore affect the maturation of the immune system and the development of allergic disease considerably.

3.2 Introduction

The human immune system can respond in various ways to environmental factors. Two of the main responses are the production of T helper 1 (Th1) cytokines during viral and bacterial infections and Th2 cytokine production upon parasite infection [77, 64, 97]. Particularly in the Western world, there has been an increase in the number of subjects who respond to common allergens such as grass pollen and house dust mite, a response which is characterised by Th2 cytokine production [121]. A change in exposure to pathogens may underlie this increased prevalence of allergic disease.

The infant immune system differs from that of adults as it is skewed towards a Th2 response following birth, developing into a normal adult Th1 cytokine profile in subsequent years [179]. As postulated in the hygiene hypothesis [221], frequent infections during childhood may affect this maturation process. Th1 cytokine production during infection may skew infant immune response from Th2 to Th1. As a consequence, infants suffering from many infections may have a diminished risk of developing Th2 mediated allergic disease.

Several types of infection may account for the reduced risk of allergic disease. *Lactobacilli* colonising the gut immediately after birth and bacterial infection or exposure to bacterial products during childhood may reduce the risk of allergic disease [22, 141]. Since viruses induce the majority of respiratory tract infections, these pathogens have been most extensively studied in relation to allergic disease [70]. Epidemiological studies have demonstrated a reduced risk of allergic disease in children with older siblings and children attending day-care, who are known to be all heavily exposed to viral infections [117, 14]. Recently, the first direct evidence for this protective effect was provided by a study of Illi and colleagues, who found a twofold reduction of asthma in children of school age with repeated viral respiratory tract infections in the first 3 years of life [101].

Some data show that viral respiratory tract infections can trigger the development of allergic disease. The high risk of wheezing symptoms following bronchiolitis in infants induced by respiratory syncytial virus (RSV) has suggested the possibility of the subsequent development of asthma. Indeed, Sigurs and colleagues were able to show that children with bronchiolitis had an increased risk of allergic disease at age 7 [210]. But others were not able confirm these observations. Although Stein and colleagues found an increased risk of recurrent wheezing in 7-year-old children following bronchiolitis, no increased risk of allergic disease was found at age 14 [217].

The VIGALL (Dutch abbreviation for Virus mediated allergy) birth cohort study aims to investigate the effect of upper respiratory tract infections (URTI) on the development of allergic disease and the maturation of the infant immune system. Both the prevalence of a particular virus and the host cellular anti-viral immune response probably determine the general contribution of the virus infection

on the maturation of the immune system. Initially, then, we need to investigate the prevalence of viruses during URTI in infants. In adults and children of school age, rhinovirus is the most prevalent inducer of respiratory pathology, which is found in up to 80% of patients with common cold [137, 10] and during the majority of asthma exacerbations [104]. In infants, viruses have mainly been studied during bronchiolitis, but they have hardly been studied at all during URTI. RSV, and occasionally parainfluenzavirus (PIV) and influenzavirus infections, have mainly been found in bronchiolitis [102, 187, 183]. Only recently, rhinovirus infections have been reported in common colds in infants [242]. In this paper we analysed the prevalence of respiratory viruses in 0-2 year-old infants in relation to severity of symptoms, day-care attendance and the age of the child.

3.3 Methods

Participants

The aim of the VIGALL study was to investigate the relation between viral URTI and the development of allergy during childhood. Hundredtwentysix healthy infants, living in the Rotterdam area (Netherlands), were included in this prospective birth cohort study and were followed until two years of age. Recruitment of children took place at the department of obstetrics (Sophia's Children Hospital), at health centers, and among participants of the PIAMA birth cohort study[115]. Eighty-six infants (68%) were selected who had a family history of atopy, defined as allergic disease in one or both parents. Parents with self-reported asthma, hay-fever, house dust mite allergy, or pet allergy were considered to be allergic. This was established with a validated screening questionnaire[124]. Forty infants were selected with a negative family history of atopy, infants of whom both parents reported not to have allergic disease. The Erasmus University Medical Ethics Committee in Rotterdam approved the study design and parents of all infants gave informed consent.

Data collection

Information was collected on birth characteristics (duration of pregnancy, gender, birth weight, numbers of siblings, season of birth), indoor environmental and lifestyle factors (smoke exposure, breast feeding, day-care attendance) using questionnaires completed by the parents when the child reached 3, 12 and 24 months. General symptoms of illness such as rhinorrhoea, cough, fever, and symptoms of allergic disease such as skin rash and wheezing were scored by the parents on weekly symptom cards.

Parents were asked to contact the study doctor when their child had signs of an URTI, defined as rhinorrhoea and fever, general malaise, sleeping difficulties

or loss of appetite. During the subsequent sick visits, infants were considered to have a common cold and were assigned to the 'URTI group'. Physical examination was performed and nasal brush samples were taken. A medical history was taken on general illness symptoms (fever, general malaise, loss of appetite), upper respiratory tract disease (rhinorrhoea, sore throat), lower respiratory tract disease (wheeze, cough, dyspnoea), skin rash and numbers of upper respiratory tract infections in the preceding follow-up period. During sick visits, infants were grouped around 6 (2-9), 12 (10-15), 18 (16-21) and 24 (22-29) months of age.

Children visited the Sophia's children hospital for routine visits at the age of 6, 12, 18 and 24 months. Physical examination was performed, and a medical history and nasal brush sample were taken. During routine visits, infants were assigned to two groups. Infants with rhinorrhoea, without signs of fever, general malaise, sleeping difficulties or loss of appetite were assigned to the 'rhinitis group'. Children without rhinorrhoea were considered not to have upper respiratory tract disease and were assigned into the 'nasal-symptom-free group'.

Parentally reported episodes of rhinorrhoea

The numbers of rhinorrhoea episodes that infants at 12 and 24 months of age had suffered from in the preceding year of life were calculated from the weekly symptom cards. Missing data was obtained from medical histories taken at 6, 12, 18 and 24 months of age. Infants were classified into three groups: (1) infants with 0-3 episodes a year, (2) infants with 3-8 episodes a year and (3) infants with continuous rhinorrhoea, defined as rhinorrhoea for more than 16 weeks a year.

Day-care facilities

Infants were considered to attend day-care when they were cared for regularly by relatives or foster parents and have contact with small numbers of children (< 5 children) other than their siblings, and when infants attended large day-care facilities (usually > 10 children).

Viral diagnostics in nasal brushes

Cells were harvested from the nasal cavity with a cytobrush (Medscand Medical, Sweden) and processed as previously described [82]. Cells were collected in 7 ml of RPMI 1640 medium (Life Technologies, Netherlands). After centrifugation, 4 ml of supernatant was used for the isolation of influenzavirus, parainfluenzavirus, RSV, adenovirus, cytomegalovirus (CMV), enterovirus and echovirus and 3 ml was frozen and stored at $-80\,^{\circ}$C. Cells were stained with fluorescent labelled antiviral antibodies to detect RSV, influenzavirus, parainfluenzavirus and adenovirus. Rhinovirus and coronavirus were detected by the isolation of viral RNA from 0.5 ml frozen nasal brush supernatant using the MagnaPure LC Instrument (Roche

Applied Science, Penzberg, Germany) and amplification by RT-PCR followed by hybridisation with either rhinovirus- or coronavirus-specific radiolabeled probes [172].

Statistical analysis

For the purposes of a simultaneous evaluation of the prevalence of individual viruses, logistic regression was used (GEE estimation using the GENMOD module from SAS), allowing for differences between, as well as within, individuals. The age of the child, symptom severity, season of sampling (winter: October to March, summer: April to September), a family history of atopy and day-care attendance were the factors examined. Fisher's exact test was used to evaluate the relation between rhinovirus prevalence and several genetic and environmental factors at 12 and 24 months separately. The McNemar test was used to compare prevalences of different viruses within symptom and age groups. Kaplan-Meier survival analysis was used to examine differences between children who dropped out at 12, 18 or 24 months compared to those who completed the study. Differences were considered statistically significant if $p \leq 0.05$.

3.4 Results

Patient characteristics

One hundred and twenty-six infants were followed for the first 2 years of life and examined during routine and sick visits. Table 3.1 shows the number of visits as well as the clinical symptoms of the child during the visit. None of the infants was admitted to hospital due to severe lower respiratory tract infection. Forty-two children (33%) dropped out for various reasons. Fifteen children dropped out at 12 months of age, 22 children at 18 months and 5 children at 24 months of age. No differences were found between the children who dropped out and those who did not in terms of family history of atopy, day-care attendance, numbers of episodes of rhinorrhoea, and development of atopic dermatitis in the preceding follow-up period (data not shown).

Prevalence of respiratory viruses in infants

The prevalence of viral respiratory pathogens was examined in infants with URTI, with rhinitis or without nasal symptoms separately around 6, 12, 18 and 24 months of age (Table 3.2). During URTI, a viral infection was diagnosed in 58% (6 months) to 80% (24 months) of the infants. At 6 months of age, rhinoviruses (27%), RSV (18%) and coronaviruses (21%) were mainly found during URTI. In children of 12 months and older, the most prevalent pathogen during URTI was

Table 3.1: Clinical symptoms in infants (%) during routine and sick visits.

	URTI	**Rhinitis**	**Nasal-symptom free**
N	80	133	221
Rhinorrhoea	80 (100%)	133 (100%)	0 (0%)
Loss of appetite	47 (59%)	0 (0%)	4 (2%)
General malaise	66 (83%)	0 (0%)	4 (2%)
Fever	40 (50%)	0 (0%)	0 (0%)
Wheeze	14 (18%)	13(10%)	5 (2%)
Cough	68 (85%)	76 (57%)	41 (19%)

URTI: Upper respiratory tract infection

rhinovirus (43% at 12 months to 60% at 24 months). In infants with rhinitis, a positive virus diagnosis was found in 34% (18 months) to 58% (12 months) and in infants without nasal symptoms in 16% (24 months) to 33% (6 months). These were mainly rhinoviruses (85% and 75% of virus infections respectively). At 6 months of age in the nasal-symptom-free group, in addition to rhinovirus, significantly more CMV was detected compared to PIV, influenzavirus, adenovirus, and enterovirus. Multiple-virus infections were diagnosed in 15% of infants with URTI, 8% of infants with rhinitis and 3% of infants without nasal symptoms. Rhinovirus and coronavirus infections were mostly found in combination with any other virus.

Viral prevalence in relation to symptomatology and age of the child

Multiple logistic regression was conducted to investigate whether the age of the child and the severity of symptoms affected viral prevalence in infants. Age-related and severity-related differences in viral prevalences did not depend on day-care attendance, a family history of atopy and season of sampling.

Rhinovirus

Rhinovirus was the most prevalent inducer of respiratory infection in all symptom groups (Figure 3.1A, Table 3.2). The prevalence of rhinovirus in the URTI group increased with age from 27% at 6 months to 60% at 24 months and remained unchanged in the rhinitis group (31% at 18 months - 52% at 12 months) and in infants without nasal symptoms (14% at 24 months - 28% at 12 months). No statistically significant differences were found between age categories in any symptom groups. We did observe significantly more rhinovirus in the URTI group and

Table 3.2: Prevalences of respiratory viruses in infants with URTI, with rhinitis or infants without nasal symptoms at 6, 12, 18 and 24 months of age. Prevalences were calculated as a percentage of total numbers of infants (N) per age and symptom group.

	N	Any virus detected (%)	Rhinovirus (%)	RSV (%)	Corona-virus (%)	PIV (%)[a]	Influenza-virus (%)[b]	CMV (%)	Other viruses (%)[c]
URTI									
6 months	33	19 (58)	9 (27)	6 (18)	7 (21)	2 (6)	2(6)	1(3)**	2(6)*
12 months	14	11 (79)	6 (43)	3 (21)	0(0)*	2 (14)	1(7)	0(0)*	0(0)*
18 months	28	20 (71)	15 (54)	3 (11)**	1 (4)**	0 (0)**	1(4)**	0(0)**	2(7)**
24 months	5	4 (80)	3 (60)	2 (40)	0 (0)	0(0)	0(0)	0(0)	0(0)
Rhinitis									
6 months	46	25 (54)	19 (41)	1 (2)**	3(7)**	3 (7)**	0(0)**	3(7)**	1(2)**
12 months	33	19 (58)	17 (52)	1 (3)**	1 (3)**	0 (0)**	0(0)**	0(0)**	1(3)**
18 months	29	10 (34)	9 (31)	1 (3)*	0 (0)**	0 (0)**	0(0)**	2(7)*	1(3)**
24 months	25	13 (52)	12 (48)	1 (4)**	1 (4)**	0 (0)**	0(0)**	0(0)**	0(0)**
Nasal-symptom-free									
6 months	70	23 (33)	12(17)	2 (3)*	3 (4)*	1 (1)**	1(1)**	9(13)#	2(3)**
12 months	64	20 (31)	18 (28)	0 (0)**	0(0)**	0 (0)**	0(0)**	3(5)**	1(2)**
18 months	38	12 (32)	10 (26)	0 (0)**	0(0)**	0 (0)**	0(0)**	2(5)*	0(0)**
24 months	49	8 (16)	7 (14)	0(0)* 1(2)	0 (0)*	0(0)*	0(0)*	0(0)*	0(0)*

URTI: Upper respiratory tract infection, RSV: Respiratory syncytial virus, PIV: parainfluenzavirus, CMV: cytomegalovirus

[a] one PIV1, 2 PIV2 and 5 PIV3 infections

[b] 4 influenzavirus A and 1 influenzavirus B infection

[c] adenovirus and enterovirus

# $p < 0.01$ CMV versus PIV and versus influenzavirus, $p < 0.05$ versus "other viruses"

** $p < 0.01$ versus rhinovirus

* $p < 0.05$ versus rhinovirus

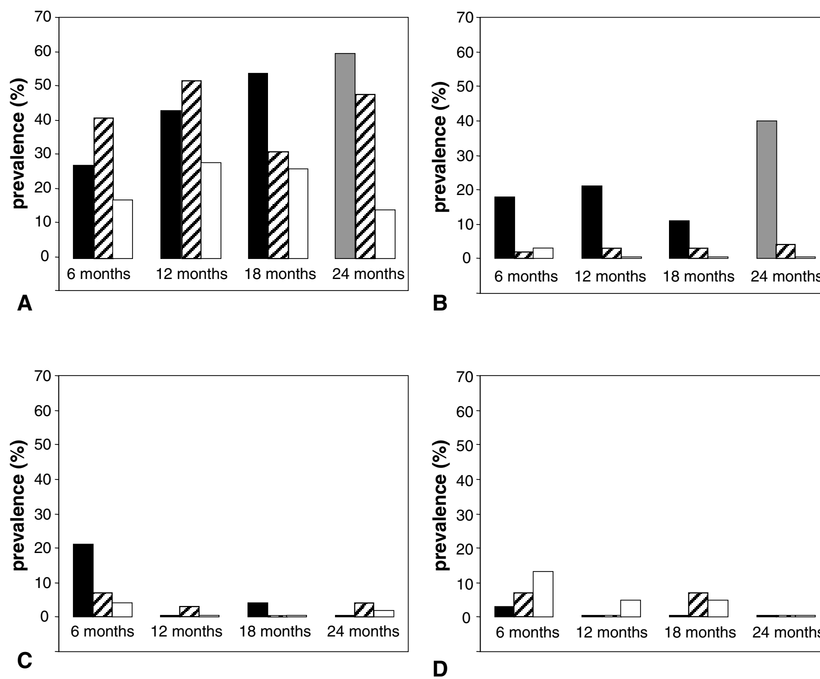

Figure 3.1: Prevalence of rhinovirus(A), RSV (B), coronavirus (C) and CMV (D) in infants with URTI (black bars), rhinitis (cross-hatched bars) or without nasal symptoms (white bars) related to the age of the child. Data represented by the gray bar is based on only a few infants (N=5).

rhinitis group than in infants without nasal symptoms ($p < 0.001$ and p=0.001 respectively), whereas the prevalence of rhinovirus did not differ between the URTI and rhinitis groups.

RSV

A significantly higher prevalence of RSV was found in infants with URTI than in infants with rhinitis or in infants without nasal symptoms (p=0.001 and $p < 0.001$ respectively; Figure 3.1B, Table 3.2). No differences in prevalence of RSV were found between age groups.

Coronavirus

Coronavirus was predominantly found during URTI episodes in 6-month-old infants (21%), decreasing to 0% at 24 months of age (p=0.02; Figure 3.1C, Table 3.2). Significantly more coronavirus infections were found in the URTI group than in the rhinitis and nasal-symptom-free groups (p=0.05 and p=0.004 respectively).

CMV

Some CMV infections were detected in children without nasal symptoms. Prevalences decreased gradually with age from 13% at 6 months to 0% at 24 months (p=0.001; Figure 3.1D, Table 3.2). CMV infections were significantly more prevalent in the nasal-symptom-free group compared with the URTI group (p=0.01) but did not differ from infants in the rhinitis group.

Genetic and environmental factors in rhinovirus prevalence

During routine visits (rhinitis and symptom-free groups), the majority of the viruses found were rhinoviruses. We therefore examined the relationship between several genetic and environmental factors and the prevalence of rhinovirus during routine visits at 12 and 24 months of age separately.

At the age of 12 months, a higher prevalence of rhinovirus was found in children in day-care (51%) compared to other children (19%; p=0.01; Table 3.3). No relation was found between the presence or absence of siblings and rhinovirus prevalence. We were able to confirm the reliability of parental symptom scores since the prevalence of rhinovirus increased from 21% in children with 0-3 episodes of rhinorrhoea to 60% in children with continuous rhinorrhoea during the preceding year of life (p_{trend}=0.01). Simultaneous evaluation using logistic regression of the significant factors day-care attendance and rhinorrhoea episodes showed that both these factors are significant predictors of increased rhinovirus prevalence. A trend for similar relations was observed between day-care attendance and episodes of rhinorrhoea and rhinovirus prevalence at the age of 24 months, but this did not attain statistical significance (data not shown). Breast feeding, smoking parents, season of birth, gender and a family history of atopy did not affect the prevalence of rhinovirus during routine visits at 12 and 24 months. Comparable results were obtained when genetic and environmental factors were related to a positive diagnosis for any virus in infants at 12 and 24 months of age (data not shown).

3.5 Discussion

In the VIGALL birth cohort study, we found that rhinovirus was the most prevalent viral pathogen in infants from 0 to 2 years. Rhinovirus was found in $\sim$40% of children with URTI and also in $\sim$40% of children with rhinitis without general malaise. Rhinovirus was even detected in $\sim$20% of infants without nasal symptoms.

Table 3.3: Prevalence of rhinovirus at 12 months of age in relation to genetic and environmental factors.

		Rhinovirus positive/N (%)[a]	**p-value**
Day-care attendance	Yes	31/61 (51%)	
	No	4/21 (19%)	0.01
Siblings	Yes	19/42 (45%)	
	No	17/47 (36%)	0.40
0-3 episodes of rhinorrhoea		7/34 (21%)	
3-8 episodes of rhinorrhoea		17/40 (43%)	
Continuously rhinorrhoea		12/20 (60%)	0.01[b]
Breast-feeding	Yes	12/22 (55%)	
	No	21/61 (34%)	0.13
Smoking parents	Yes	7/22 (32%)	
	No	29/58 (50%)	0.21
Girls		19/46 (41%)	
Boys		18/51 (35%)	0.68
Born in winter[c]		12/27 (44%)	
Born in summer		25/70 (36%)	0.49
Family history of atopy	Yes	26/70 (37%)	
	No	11/26 (42%)	0.81

[a] N not always 97 due to occasional missing data
[b] test for trend
[c] winter: October-March, summer: April-September

RSV and parainfluenzavirus have usually been considered the most important viral pathogens in infants because these viruses have been found to induce lower respiratory tract infection (bronchiolitis) [102, 187, 183]. However, the importance of rhinovirus in infants has been underestimated. It is only in recent years that PCR techniques have become available. These techniques can detect rhinoviruses more accurately than the viral culture methods used previously [99]. In adults, rhinovirus has already been shown to be the most prevalent viral pathogen during URTI [137, 10]. We have now shown that rhinovirus is the most frequently-detected viral pathogen during URTI and rhinitis in infants as well. This is in accordance with recent data from the Finnish Otitis Media Cohort Study that observed similar high rates of rhinovirus infection in infants during episodes of URTI and acute otitis media [23, 242]. It can therefore be concluded that rhinovirus frequently infects both infants and adults.

Increased prevalences of rhinovirus were found in children attending day-care. Epidemiological studies indicated that frequent respiratory tract infection in infants, associated with day-care attendance or the numbers of siblings, may reduce the risk of developing allergic disease [101, 14, 117]. On the basis of extensive

virus diagnostics, this study found prevalences of viral infections in infants in day-care, in particular rhinovirus, which were ∼2.7 times higher than those for other children. We therefore concluded that the indirect indications of protection by recurrent respiratory infections in epidemiological studies, such as by day-care attendance, are probably rhinovirus infections to a large extend. A well known protective factor for lower respiratory infections during childhood is breast-feeding whereas a parental history of allergic disease and exposure to tobacco smoke have been described as risk factors [76, 48, 115]. Remarkably, in contrast to lower respiratory tract infections, the same studies showed that breast-feeding, a parental history of allergic disease and exposure to tobacco smoke did not relate upper respiratory tract infections. In line with these reports, we also did not observe a relation between a family history of atopy, breast-feeding or smoking by one or both parents and the prevalence of rhinovirus infections during routine visits at 12 months.

At the age of 6 months, in addition to rhinovirus, RSV and coronaviruses were also found in infants with URTI. Only a few coronavirus infections were detected in children of 12 months and older. Below the age of 24 months, the prevalence of RSV remained ∼20%. This is likely to decrease during later childhood because prevalences up to 13% have been reported in adults [263]. At the age of 18 months, most children (75%) will have been infected with RSV at least once [94]. The prevalence of rhinovirus increases slowly to more than 50%. In adults, a prevalence of 80% has been reported for rhinovirus during common cold [10]. This indicates that rhinovirus prevalence found in infants in the present study may increase further. Why does the prevalence of rhinovirus remains unaltered or even increase gradually, whereas the prevalence of RSV decreases with age? For RSV, which has few different serotypes, humoral and cellular responses may possibly protect the child from frequent reinfections. By contrast, over 100 different serotypes of rhinovirus are present [239]. Reinfection with rhinovirus is therefore likely to occur with a new serotype for which the child does not yet have induced humoral and cellular responses.

Frequent viral infections are believed to be important modulators of the natural maturation of the infant immune system from Th2- to Th1-skewed and may affect the development of allergic disease [101, 179]. This maturation is likely to result from repeated stimulation by pathogens, rather than from a single viral infection. The general effect of a particular virus is dependent on both the prevalence of the virus and on the type of host immune response upon infection. RSV is capable of inducing a general inflammatory response in infants which largely induces an immune response towards Th1 [30]. Since now rhinovirus has been found to be more prevalent than RSV in infants, the question raises to what extend rhinovirus infections are able to influence the infant immune maturation and modulation. We are currently investigating what type of immune response a rhinovirus infection can induce in infants and whether this type of immune response differs between

infants with URTI and rhinitis (manuscript in preparation).

Interestingly, rhinovirus was also detected in infants without nasal symptoms (∼20%). It is unlikely that the high prevalence of rhinovirus results from RT-PCR detection that is too sensitive, because this assay is not designed to detect a single viral particle. Rhinovirus detection could indicate imminent or past infection since ∼70% of the infants had symptoms of URTI or rhinitis during the week before or after sampling. In the other children without nasal symptoms, rhinovirus colonised the nasal mucosa without inducing symptoms. This is in accordance with a recent Finish study that detected picornavirus in 20% of nasopharyngeal samples from children without any past or recent respiratory infection [157]. As the nasal mucosa is colonised by bacteria, it is likely that it can also harbor respiratory viruses [80]. These rhinovirus infections may perhaps only induce clinical symptoms when the host immune system is temporarily compromised. It is unclear whether these subclinical rhinovirus infections may also affect immune maturation in infants.

In conclusion, rhinovirus infection has been found to be the most prevalent respiratory viral infection, in ∼40% of infants with URTI and rhinitis, and it may well stimulate the maturation of immune system and prevent the development of allergic disease. More information is subsequently needed on the immune modulating capacity of rhinovirus infections in this context.

3.6 Acknowledgement

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Chapter 4

Reduced nasal IL-10 and enhanced TNFα responses during rhinovirus and RSV-induced upper respiratory tract infection in atopic and non-atopic infants

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4.1 Abstract

Rhinovirus and respiratory syncytial virus (RSV) are the most prevalent inducers of upper respiratory tract infections (URTI) in infants and may stimulate immune maturation. To estimate the amount of immune stimulation, we examined nasal immune responses during rhinovirus and RSV-induced URTI in infants.

Nasal brush samples were taken from infants (2-26 months; 57% atopic family) with rhinovirus-induced URTI (N=20), with RSV-induced URTI (N=7), and with rhinovirus-induced rhinitis (N=11), from children with asymptomatic rhinovirus infection (N=7) and from eight non-infected children. Numbers of nasal brush cells positive for Th1-, Th2-, regulatory and pro-inflammatory cytokines were measured by immunohistochemistry or by measuring protein levels using a cytometric bead array analysis.

During rhinovirus and RSV-induced URTI, fewer regulatory cytokine IL-10 positive cells were found compared to non-infected children. This fall was accompanied by an increase in levels of the Th1 cytokine TNFα. IL-10 responses were inversely related to TNFα responses. No enhanced responses were observed for IFNγ, IL-12, and IL-18. Cytokine responses were comparable in children with rhinovirus-induced URTI and in children with rhinitis, while responses in asymptomatic rhinovirus-infected children were located between those for symptomatic and asymptomatic rhinovirus-infected children. Cytokine responses did not depend on the age of the child or atopy in the family.

In conclusion, reduced nasal IL-10 responses during URTI in infants could facilitate the induction of a TNFα response. TNFα in turn could replace the immature production of IL-12, IL-18 and IFNγ during URTI to induce an effective clearance of the viral infection and which could stimulate the maturation of Th1 cytokine production in infancy.

4.2 Introduction

The nasal mucosa is the first interface that interacts with viral respiratory pathogens. As respiratory infections may stimulate the maturation of the immune system in infants, we are interested in the type of nasal immune response induced upon upper respiratory tract infections (URTI) during early life. Earlier work by our group (manuscript in press) and others has identified rhinovirus and respiratory syncytial virus (RSV) as the most common inducers of viral respiratory tract infections during the first two years of life [242]. In this study, we therefore focused on the nasal immune response during URTI elicited by these viruses in infants and young children.

Infants and young children are prone to respiratory tract infections. This is largely due to the immaturity of the child immune system, exemplified by the low levels of protective antibody production against common pathogens in infants [29]. While a strong Th1-mediated response is required for the efficient eradication of respiratory pathogens [162], *in vitro* studies showed not only that cord-blood cells in infants produce lower levels of Th1 and Th2 cytokines after stimulation compared to peripheral blood cells in adults [197, 127, 40], but also that infants favour the production of Th2 cytokines over Th1 cytokines [178, 56]. As a possible consequence, children in the first two years of life suffer on average from 6-8 respiratory infections a year. With the maturation of the immune system, this gradually declines to 3-4 a year in school-age children[70].

Rhinovirus is the most prevalent inducer of mild URTI in adults [137]. In infants and young children also, the majority of URTIs are induced by rhinovirus (manuscript in press) [242]. At the age of 2 years, 91% of the children were found to have rhinovirus-specific antibodies. This implies that they had already suffered from at least one rhinovirus infection [23]. RSV is the second most prevalent pathogen during URTI in infants and young children, and antibodies against RSV are present in virtually all children by the age of 3 years [232].

There are very few studies examining immune responses in young children during URTI. Noah and colleagues found increased levels compared to baseline of Th1 and pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα in nasal lavages of children aged 0-3.5 years with clinically defined URTI [156]. Others have shown that the immune response could depend on the type of viral pathogen. Hospitalised children younger than 18 months with symptoms of URTI induced by influenza A virus infection had higher concentrations of the Th1-related cytokine TNFα in nasal samples and lower serum concentrations of the Th2 cytokines IL-4 and IL-5 than children of the same age with an RSV infection [225]. We are not aware of any study comparing immune responses in children during URTI induced by rhinovirus or RSV.

In this study, we looked at the type of nasal immune response induced upon URTI in infants and whether these responses differed according to the type of viral

pathogen or the severity of respiratory symptoms in order to estimate the amount of stimulatory effect of these infections on immune maturation. To this end, Th1-related (IL-2, IL-12, IFNγ, TNFα), Th2-related (IL-4, IL-5), pro-inflammatory (IL-18) and regulatory (IL-10) cytokine responses were measured in nasal brush samples of infants (aged 2-26 months) with a rhinovirus or RSV infection.

4.3 Methods

Participants study I: RSV- and rhinovirus-induced URTI

To study nasal immune responses during upper respiratory tract infection (URTI) in infants, we selected infants (aged 2-26 months) from the VIGALL (Virus mediated allergy) birth cohort study [114] who suffered from an RSV-induced URTI (N=7) or from a rhinovirus-induced URTI (N=20). URTI was defined as a runny nose and at least one of the symptoms fever, malaise, sleeping difficulties or loss of appetite. The study was confined to children in whom a single type of virus was detected during URTI. Five out of 7 children with RSV-induced URTI and 11 out of 20 children with rhinovirus-induced URTI had a positive FHA (family history of atopy; one or both parents had allergic disease). Parents with self-reported asthma, hay fever, house dust mite allergy, or pet allergy were considered to be allergic. This was established using a validated screening questionnaire [124]. When both parents reported not having allergic disease, the children were classified as FHA-negative.

As a control group, 8 children were selected (matched for FHA, age and gender) who were completely free of any symptoms (no runny nose, fever, malaise, cough, wheeze, sleeping difficulties, and loss of appetite) in the two weeks spanning the visit. These children had no virus infection during sampling, and had not suffered from atopic dermatitis before the age of 2 as determined using the UK Working Party's Diagnostic Criteria for Atopic dermatitis [255]. These children are referred to as non-infected children.

Participants study II: severity of rhinovirus infection

To study whether nasal immune responses during rhinovirus infection were dependent on the severity of infection, we only selected children infected by rhinovirus who suffered from URTI (N=20), from rhinitis (N=11), or who were asymptomatic during rhinovirus infection (N=7). Definitions of the symptom groups based on severity of rhinovirus infection are shown in Table 4.1. 'URTI'was defined as described in the previous section. 'Rhinitis' was defined as having symptoms of a runny nose only, but without fever, general malaise, sleeping difficulties, and loss of appetite. Children were classified as 'asymptomatic' when they had no symptoms of URTI or rhinitis, with one child having a non-specific cough. Immune

Table 4.1: Definition of symptom groups.

	Virus positive	**Runny nose**	**General illness symptoms** [a]
URTI	+	+	+
rhinitis	+	+	−
asymptomatic	+	−	−
non-infected	−	−	−

URTI: Upper respiratory tract infection
[a] General malaise, fever, loss of appetite and/or sleeping difficulties

responses of children during infection were compared to those of 8 non-infected children.

Data collection and nasal brush sampling

Each visit included a physical examination and nasal brush sampling. A medical history was taken for general illness symptoms (fever, general malaise, loss of appetite, sleeping difficulties), upper respiratory tract disease (runny nose, sore throat), and lower respiratory tract disease (wheeze, cough, dyspnoea) in the two weeks prior to the visit. General symptoms such as runny nose, cough, fever, and symptoms of allergic disease such as skin rash and wheezing were scored by the parents on weekly symptom cards. The Medical Ethics Committee of the Erasmus Medical Centre Rotterdam approved the study design and all parents gave informed consent.

Viral diagnostics

Cells were harvested from the nasal cavity with a cytobrush (Medscand Medical, Sweden) and collected in 7 ml of RPMI 1640 medium (Life Technologies, Netherlands). In nasal brush samples, viruses were detected by three different techniques. After centrifugation, the supernatant was used to detect influenzavirus, parainfluenzavirus, RSV, adenovirus, cytomegalovirus, enterovirus and echovirus. For that purpose, nasal brush supernatant was cultured on Hep-2 cells and, after one week of culture, viruses were detected using immunofluorescence [193]. Additionally, nasal brush cells from the pellet were stained with fluorescent-labelled antiviral antibodies to detect RSV, influenzavirus, parainfluenzavirus and adenovirus. Rhinovirus and coronavirus were detected by the isolation of viral RNA from nasal brush supernatant using the MagnaPure LC Instrument (Roche Applied Science, Penzberg, Germany) and amplification of picornavirus-specific RNA by RT-PCR, followed by hybridisation with either rhinovirus- or coronavirus-specific radiolabelled probes [172].

Immunohistochemical staining of IL-18 positive cells

Cytospin preparations of nasal brush cells were fixed in acetone and placed in a semi-automatic stainer (Sequenza, Shandon, Amsterdam, The Netherlands). Immunohistochemical staining was performed as previously described [82]. In brief, slides were pre-incubated with 10% v/v normal goat serum (CLB, The Netherlands) (10 minutes) and subsequently for 60 minutes with mouse anti-human monoclonal antibodies directed against IL-18 (20 μg/ml, clone 500-M87, Peprotech, United Kingdom) diluted in PBS supplemented with 1% blocking reagent (w/v) (Boehringer Mannheim, Germany). After incubation for 30 minutes with biotinylated goat anti-mouse Ig serum, slides were incubated with polyclonal goat anti-biotin antibody for 30 minutes. After incubation with New Fuchsin substrate (Chroma, Kongen, Germany), sections were counterstained with Gill's haematoxilin and mounted in glycerin-gelatin. Control staining was performed by the substitution of primary monoclonal antibody with isotypic control antibody. The numbers of positive cells, which showed a red cytoplasmic staining, were counted per 1000 nasal brush cells and were represented as percentages of positive cells.

Tyramide signal amplification (TSA) staining for IL-4, IL-10, and IL-12

The super-sensitive alkaline phosphatase staining method [34] was used to detect IL-4, IL-10 and IL-12 positive cells in nasal brush samples. Cytospin preparations of nasal brush cells were incubated with mouse anti-human monoclonal antibodies directed against IL-4 (12 μg/ml, clone1-41-1, Novartis, Switzerland), IL-10 (10 μg/ml, clone IC25-471, Instruchemie, The Netherlands), IL-12p70 (5 μg/ml, clone 24945.11, R&D systems, United Kingdom) or an isotypic control antibody for 60 minutes. After incubation with biotinylated goat anti-mouse Ig serum, endogenous peroxidase was blocked using azide (0.2% w/v), hydrogen peroxide (0.02% v/v) and methanol (50% v/v) in PBS. Slides were then subsequently incubated with streptavidin-conjugated peroxidase (30 minutes) (NEN Inc., Boston, MA, USA), biotinyl tyramide in Tris/HCL buffer (10 minutes) for amplification of the staining signal, and with alkaline-phosphatase conjugated goat-anti-biotin (30 minutes). After incubation with New Fuchsin substrate (Chroma, Kongen, Germany), sections were counterstained with Gill's haematoxilin and mounted in glycerin-gelatin. The numbers of positive cells, those with red cytoplasmic staining, were counted per 1000 nasal brush cells and were represented as percentages of positive cells.

Protein levels of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ

Protein levels of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ were measured in a supernatant of nasal brush samples using the Cytometric Bead Array (CBA) system

(BD Biosciences, San Diego, USA). This multiplexed bead assay allows the detection of six cytokines simultaneously in one sample and was performed according to the manufacturer's protocol. In brief, a mixture of 10 μl of each of the 6 different bead suspensions (in each suspension, beads were coated with antibodies directed against one of the cytokines) was incubated with 50 μl of sample (nasal brush supernatant) and 50 μl of PE-conjugated antibody (PE Detection Reagent) for 3 hours. The fluorescence intensity was measured on a flow cytometer (BD FACScanTM) and was proportional to the cytokine concentration in the sample. The sensitivity of the assay was 2.4 pg/ml for IL-5, 2.6 pg/ml for IL-2 and IL-4, 2.8 pg/ml for IL-10 and TNFα, and 7.1 pg/ml for IFNγ. Values lower than the sensitivity level of the assay were recorded as 0. Total protein concentrations were measured in nasal brush samples using the Bradford method [28] with Coomassie brilliant blue G-250 (Merck, Germany) as the indicator. Absorbance was read at 595 nm and compared with a bovine serum albumin standard curve.

Statistical analysis

To allow for inter- and intrapatient variations, relations of cytokine responses with the type of virus or severity of disease were investigated using regression analysis for repeated measurements (using the 'proc mixed' module from SAS 6.12 for Windows), in which a family history of atopy and the age of the child were considered to be confounding factors. In order to obtain approximately normal distribution in these analyses, all evaluated outcomes were transformed logarithmically. Fisher's exact test was used to examine differences in symptoms of disease and patient characteristics between patient groups. Differences were considered statistically significant when the p-value (two-sided) was ≤ 0.05.

4.4 Results

Symptomatology and patient characteristics

Infection-related symptoms and patient characteristics in each group of children are shown in Table 4.2. Children with either rhinovirus- or RSV-induced URTI suffered by definition from a runny nose with additional infection-related symptoms (see methods section). Although symptomatology is comparable in rhinovirus- or RSV-induced URTI, with high percentages of children suffering from cough (70% and 100% respectively) and general malaise (90% and 100% respectively), there is one clear difference. Three out of 7 children with an RSV-induced URTI displayed wheezing symptoms, whereas this was true for only 1 out of 20 children with rhinovirus-induced URTI (p=0.05). None of the children with an asymptomatic rhinovirus infection showed symptoms of wheezing. Coughing is equally prevalent in children with rhinovirus-induced rhinitis (73%) compared to children with

Table 4.2: Prevalence of symptoms and patient characteristics in infected and non-infected children.

	RSV URTI (N=7)	Rhinovirus URTI (N=20)	Rhinovirus rhinitis (N=11)	Rhinovirus Asymp-tomatic (N=7)	Non-infected (N=8)
Runny nose	7 (100%)	20 (100%)	11 (100%)	0 (0%)	0 (0%)
Fever	2 (29%)	9 (45%)	0 (0%)	0 (0%)	0 (0%)
Cough	7 (100%)	14 (70%)	8 (73%)	1 (14%)	0 (0%)
Wheeze	3 (43%)	1 (5%)	0(0%)	0 (0%)	0 (0%)
Loss of appetite	4 (57%)	13 (65%)	0 (0%)	0 (0%)	0 (0%)
General malaise	7 (100%)	18 (90%)	0 (0%)	0 (0%)	0(0%)
Positive FHA	5 (71%)	11 (55%)	5 (46%)	5 (71%)	4(50%)
Gender (boy)	2 (29%)	6 (30%)	6 (55%)	3 (43%)	5(63%)
Age (months)[a]	10 (2-18)	15 (3-22)	12 (4-23)	12 (6-18)	13(6-26)

URTI: Upper respiratory tract infection, FHA: Family history of atopy
[a] Median (range)

rhinovirus-induced URTI, and hardly ever observed in asymptomatic rhinovirus-infected children (14%). No significant differences were observed in the family history of atopy between the groups of children.

Decrease in numbers of nasal IL-10 positive cells during rhinovirus- and RSV-induced URTI

A Th1-mediated host immune response has been shown to be necessary to eradicate viral pathogens effectively [46], but this response appears to be immature in infants. To examine what type of cytokine response children have upon respiratory infection, we determined the number of Th1-related (IL-12), Th2-related (IL-4), regulatory (IL-10) and pro-inflammatory (IL-18) cytokine positive cells in nasal brush samples of children with URTI caused by rhinovirus or RSV as compared to responses in non-infected children.

The numbers of IL-10 positive cells in the nasal brush samples were related to either rhinovirus- or RSV-induced URTI, as shown in Figure 4.1A. During a rhinovirus infection, numbers of nasal IL-10 positive cells were significantly reduced. Numbers decreased from median percentages of 33% in non-infected children to 19% in rhinovirus-infected children (p=0.03). A similar reduction in numbers of IL-10 positive cells to a median of 15% was observed during RSV-induced URTI ($p < 0.01$).

In contrast, no differences in cell numbers were observed between either rhinovirus or RSV-induced URTI for the Th2-related cytokine IL-4 (Figure 4.1B), the Th1-related cytokine IL-12 (Figure 4.1C), or the pro-inflammatory cytokine IL-18

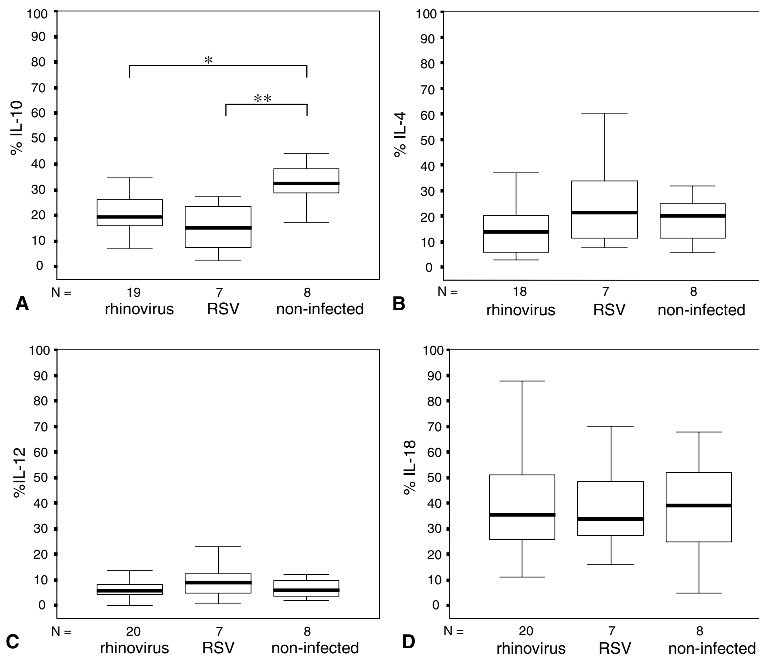

Figure 4.1: Percentages of nasal brush cells positive for IL-10 (A), IL-4 (B), IL-12 (C), and IL-18 (D) in children with rhinovirus- or RSV-induced URTI compared to non-infected children. * $p < 0.05$, ** $p < 0.01$

(Figure 4.1D) compared to non-infected children.

Increased nasal expression of TNFα during rhinovirus- and RSV-induced URTI

As functional differences in cytokine production can be examined when actual protein levels of cytokines are measured, we also determined protein levels for Th1-related (TNFα, IFNγ, IL-2), Th2-related (IL-4, IL-5), and regulatory (IL-10) cytokines in nasal brush supernatant taken during rhinovirus and RSV-induced URTI.

Unfortunately, for the majority of cytokines, levels in a large number of samples were too low for analysis (IL-2 median: 4.2 pg/ml, range 0-11 pg/ml; IL-4 median: 0 pg/ml, range 0-4.1 pg/ml; IL-5 median: 0 pg/ml, range 0-4.8 pg/ml; IL-10 median: 4.8 pg/ml, range 0-24 pg/ml).

Only absolute levels of IFNγ and TNFα were high enough for analysis (IFNγ median: 59 pg/ml, range 0-279 pg/ml; TNFα median: 21 pg/ml, range: 0-794 pg/ml). Cytokine levels were expressed per mg total protein (median 138 μg/ml, range 23-834 μg/ml) measured in each individual sample. Children with

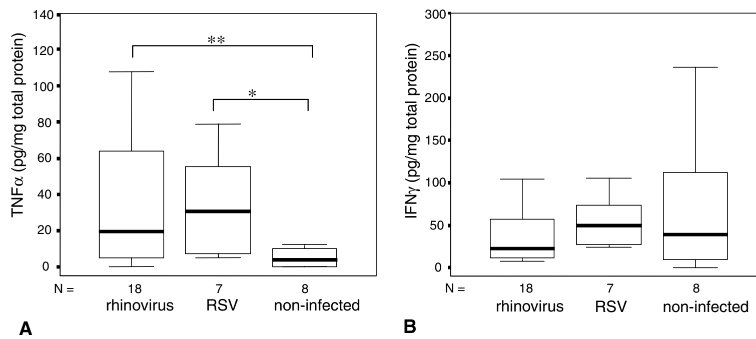

Figure 4.2: Protein levels of TNFα (A) and IFNγ (B) in nasal brush samples of children with rhinovirus- or RSV- induced URTI compared to non-infected children.* $p < 0.05$, **$p < 0.01$

rhinovirus-induced URTI produced significantly higher levels of TNFα protein (median 20 pg/mg total protein) than non-infected children (median 3.5 pg/mg total protein) (Figure 4.2A) ($p < 0.01$). In addition, during RSV-induced URTI, an increase in levels of TNFα was observed (median 30 pg/mg total protein) (p=0.01), which did not differ significantly from rhinovirus-induced URTI. In contrast to TNFα, no effect of viral infection could be detected on IFNγ responses (median rhinovirus 22 pg/mg total protein, RSV 49 pg/mg total protein, non-infected 39 pg/mg total protein) (Figure 4.2B).

Nasal IL-10 and TNFα responses are unrelated to severity of symptoms

To examine whether the type of nasal immune response during rhinovirus infection was dependent on the severity of respiratory symptoms, we examined rhinovirus-infected children with different severities of respiratory disease and compared these to non-infected children (Tables 4.1 and 4.2).

Figure 4.3A shows that a comparable reduction in IL-10 positive cells is observed during rhinovirus-induced URTI (median 19%) and rhinovirus-induced rhinitis (median 13%) compared to non-infected children (median 33%) (URTI: p=0.03; rhinitis: $p < 0.001$). Even in rhinovirus-infected asymptomatic children, the numbers of IL-10 positive cells were lower than in non-infected children (23% versus 33% respectively). The numbers of IL-10 positive cells in the asymptomatic group are located between those in non-infected children and rhinovirus-infected children with URTI or rhinitis, but numbers did not differ statistically from either group.

A comparable situation was observed for TNFα responses upon rhinovirus infection (Figure 4.3B). Children with rhinovirus-induced URTI and rhinitis had

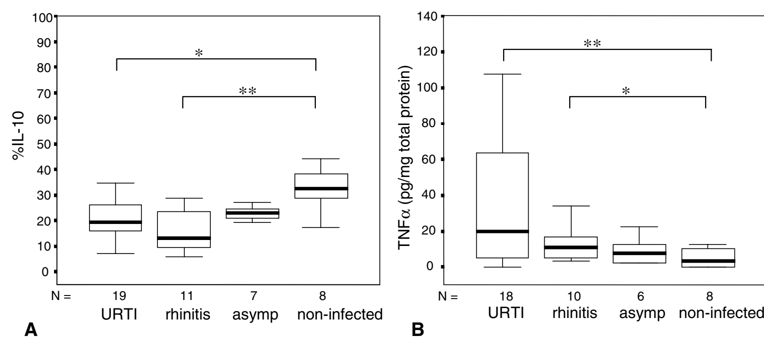

Figure 4.3: Percentages of cells positive for IL-10 (A), and protein levels of TNFα (B) in nasal brush samples of rhinovirus-infected children with URTI or with rhinitis, and asymptomatic rhinovirus-infected children (asymp) compared to non-infected children. * $p < 0.05$, **$p < 0.01$

higher levels of TNFα (median 20 pg/mg total protein and 11 pg/mg total protein respectively) than non-infected children (median 3.5 pg/mg total protein) (URTI: $p < 0.01$; rhinitis: p=0.02). Levels of TNFα did not differ significantly between children with rhinovirus-induced URTI and rhinitis. Levels of TNFα in children with an asymptomatic rhinovirus infection (median 8 pg/mg total protein) were located in between those of non-infected children and those with symptoms of disease, but did not differ significantly from either group.

In rhinovirus-infected children with URTI, with rhinitis, and in asymptomatic children, we also examined numbers of nasal IL-4, IL-12, and IL-18 positive cells as well as protein levels of IFNγ in nasal brush samples, and compared these to responses in non-infected children. However, we could not discern any differences between the groups of children.

Correlation between nasal IL-10 and TNFα responses

As IL-10 has been implicated in the downregulation of various cytokines, including TNFα [207], we investigated a possible relationship between the decreasing numbers of IL-10 positive cells in nasal brush samples and the increasing protein levels of TNFα. To examine this relation, we plotted, in figure 4.4, the individual values for both variables at each sampling moment. An inverse relation was observed between IL-10 and TNFα responses (p=0.04).

4.5 Discussion

This study shows that in infants (2-26 months), a nasal Th1-like response is induced during rhinovirus or RSV-induced URTI. This response is characterised by

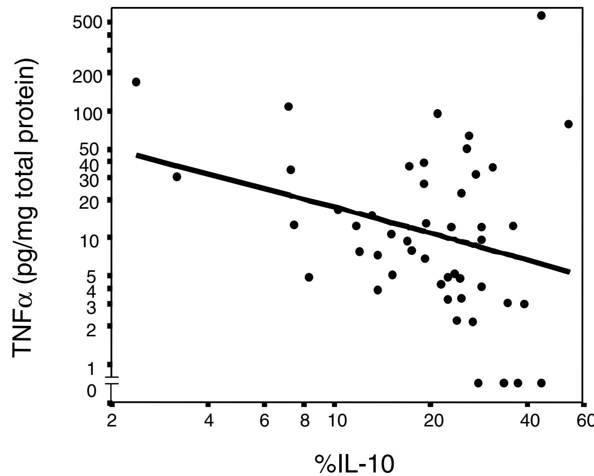

Figure 4.4: Inverse relation between percentages of IL-10 positive cells and protein levels of TNFα in nasal brush samples.

the production of increased levels of TNFα protein. In contrast to adult responses during URTI [46], we did not observe any response in children involving the Th1-related cytokines IFNγ and IL-12, or pro-inflammatory cytokine IL-18. The immaturity of the *in vivo* IFNγ and IL-12 response is probably a reflection of *in vitro* data showing a low IFNγ and IL-12 response in neonatal dendritic cells and T lymphocytes after polyclonal stimulation [126, 40]. Our data suggest that the Th1 response in infants, in the absence of normal adult-like IFNγ and IL-12 responses, largely depends on the increase in TNFα during URTI and that immune responses are only partially mature. The induction of a TNFα response during URTI is likely to result from the concomitant reduction in numbers of cells positive for the regulatory cytokine IL-10 during URTI. The inverse relation between IL-10 and TNFα responses suggested the alleviation of a suppressive effect of IL-10 on the expression of TNFα during URTI. Furthermore, we were able to show that the amount of IL-10 and TNFα response was unrelated to the severity of upper respiratory tract symptoms since comparable responses were observed in children with rhinovirus-induced URTI or rhinovirus-induced rhinitis.

The reduction in numbers of IL-10 positive cells during URTI in children is clearly different from the increase in levels of this cytokine observed in adults with common cold [46]. This could be a consequence of differences in the natural expression of this cytokine between infants and adults. Moreover, it suggests a different role of IL-10 during adulthood than during infancy. IL-10 plays a pivotal role in various immune responses and displays many distinct modes of action [248]. IL-10 is a regulatory cytokine with anti-inflammatory and Th2-stimulating

properties that is mainly produced by monocytes, macrophages, and (regulatory) T lymphocytes. This cytokine can directly inhibit the production of a wide range of cytokines, such as the Th1-related cytokines TNFα, IFNγ, IL-2, and IL-12 [160, 207], the pro-inflammatory cytokine IL-18 [139], and the Th2-related cytokine IL-5 [199]. In this capacity, IL-10 can regulate immune responses by either preventing an inflammatory response or by limiting excessive ongoing inflammation [3]. Therefore, in adults, the induction of IL-10 during infection probably reflects the deactivation of inflammatory responses. This is supported by the observation that low production levels of IL-10 generally lead to more severe disease [149].

In infancy, high numbers of IL-10 positive cells were observed in the noses of non-infected children, while numbers were reduced during URTI. High natural expression of IL-10 in infants was also shown by a study of Rainsford and colleagues, which found that levels of IL-10 in newborns were higher than in adults as measured after stimulating cord or peripheral blood cells [182]. These high levels of IL-10 during infancy could be a reflection of the IL-10 rich environment that prevails during pregnancy [167]. Suppression of the maternal and foetal immune responses by this cytokine seems to be necessary to maintain pregnancy and ensure survival of the foetal allograft. Additionally, an active role for IL-10 during infancy is suggested by the presence of IL-10 in breast milk [71]. The high natural expression of IL-10 in infancy could limit immune responses to foreign antigens to which the child is newly exposed to after birth. In this way, tolerance is induced against relatively harmless antigens, preventing chronic inflammation after every single antigen encounter [132, 86].

In addition to inducing tolerance, it has also been suggested that IL-10 in infants regulates the balanced maturation of Th1 and Th2 lymphocyte development [260]. This idea was raised in a recent study by Van den Biggelaar and colleagues, who found high schistosome-antigen-specific IL-10 production by PBMCs of children infected with *Schistosoma mansoni* parasite, while the prevalence of a positive skin reaction to house dust mite in these children was low compared to non-infected children [236]. This inverse relation between parasite-specific IL-10 production and expression of Th2-mediated allergen sensitisation suggested a suppressive role of IL-10 on Th2 cytokine production in infants.

Regulation of IL-10 production in adults is poorly understood and a mechanism by which high IL-10 responses in these young children is down-regulated during URTI is possibly even more difficult to postulate. Type I interferons have been described as inhibiting IL-10 production by activated human monocytes. On the other hand, these cytokines also stimulate IL-10 production in T lymphocytes [63]. As increased IFNα production is common in adults with naturally acquired influenzavirus infection [107] and in infants with lower respiratory tract infections [150], up-regulation of this cytokine during URTI in children may explain the suppression of IL-10 responses. Alternatively, TGFβ has also been shown to inhibit

IL-10 mRNA synthesis in monocytes [224]. Although, in general, TGFβ production is upregulated during influenza virus infection in mice [200], it is not known whether this is also the case in infants during respiratory infection and could therefore explain the downregulation of IL-10 responses.

The high natural expression of IL-10 in infants may underlie their high susceptibility to respiratory infections and infection-related wheezing episodes. This has already been suggested by Bont and colleagues, who found that high levels of IL-10 produced by monocytes after bronchiolitis correlated with high numbers of wheezing episodes following infection [27]. In order to initiate an effective inflammatory Th1 response upon infection, infants would need to deal with the natural anti-inflammatory character of their immune system, which could be achieved by actively reducing the high number of IL-10 producing cells during infection. As a consequence, at higher IL-10 levels, infections may persevere, leading to more frequent wheezing episodes.

A decrease in numbers of IL-10 positive cells in the noses of these children during URTI would favour the production of Th1 and pro-inflammatory cytokines. This was indeed shown by an increase in levels of the Th1-stimulating cytokine TNFα during rhinovirus or RSV-induced URTI. This resembles the situation in adults, where IL-10 has been identified as a negative regulator of TNFα [207]. TNFα in turn may activate the anti-viral host immune response through the stimulation of functional activities of cytotoxic T lymphocytes, NK cells, and macrophages [229] and the recruitment of inflammatory cells to the site of infection [202]. Furthermore, together with IL-12, TNFα can promote the development of Th1 lymphocytes [206]. Our data showing elevated levels of TNFα in infants are in line with previous studies showing a similar increase of TNFα in nasal lavage samples of children aged 0-3.5 years during URTI [156].

In adults, in addition to a TNFα response, there is also an increase in levels of Th1 cytokine IFNγ during URTI [46]. Other studies have found an increased production of Th1-related cytokines IL-12 and IFNγ protein when adult PBMCs were infected with rhinovirus [162], and an increased production of pro-inflammatory cytokine IL-18 by peripheral macrophages infected by influenza A virus [195]. Neonatal cord-blood dendritic cells are also capable of producing IL-12 after stimulation with influenza virus [15]. We had therefore expected to find a similar increase in these cytokine responses during URTI in infants. However, we were not able to detect any differences in cell numbers for IL-12 or IL-18 positive cells or IFNγ protein levels in nasal brush samples of rhinovirus- or RSV-infected children compared to non-infected children. Data from others suggest that the lack of IL-12, IL-18, and IFNγ responses during URTI could be a direct reflection of the immaturity of the child's immune system [44]. LPS stimulated neonatal DCs fail to produce IL-12p70 or induce levels of IFNγ production by T lymphocytes comparable to those in adults [126]. Interestingly, in the same experiment, levels of IL-10 and TNFα production by LPS-stimulated neonatal DCs were directly

comparable with those of adult DCs. Similarly, foetal and neonatal mononuclear cells are not able to produce IL-2, IL-4, or IFNγ, while IL-10, IL-6, and TNFα was secreted spontaneously and enhanced after polyclonal stimulation [264]. Our results are directly in line with these *in vitro* data that point to a largely immature immune system in infants that is able to mount correct IL-10 and TNFα responses upon infection, but fails to induce proper IL-12, IL-18 and IFNγ responses. It could therefore be the case that the mature production of TNFα during URTI replaces immature IL-12, IL-18 and IFNγ responses in infants in order to induce a host immune response that is capable of clearing the viral infection.

Could these viral URTIs influence the maturation of the immune system in infants? As postulated in the hygiene hypothesis, childhood infections may stimulate immune maturation from neonatal Th2 cytokine responses towards adult-like Th1 responses by repeated Th1 stimulation [222]. As we found that rhinovirus and RSV were capable of inducing Th1 host immune responses, characterized by increased TNFα production, repeated URTIs may indeed stimulate immune maturation. Cytokine responses did not differ according to the severity of rhinovirus infection and this suggests that the immune stimulatory effects of URTI and rhinitis are comparable. Furthermore, even an asymptomatic rhinovirus infection may stimulate immune maturation, as a trend towards decreased IL-10 and increased TNFα responses was observed during this type of infection in infants. Whether Th1 stimulation by respiratory infections is optimal is unknown, as infants lack the ability to produce Th1-related and the pro-inflammatory cytokines IFNγ, IL-12 and IL-18. Further study is needed to determine whether upper respiratory tract infections can indeed stimulate neonatal immune maturation.

In conclusion, infants (2-26 months) showed a clear decrease in numbers of cells positive for the regulatory cytokine IL-10 during rhinovirus and RSV-induced URTI. This was accompanied by an increase in levels of the Th1-stimulating cytokine TNFα. However, no enhanced responses were observed for the Th1-related and pro-inflammatory cytokines IL-12, IL-18 and IFNγ during URTI. This may indicate that immature responses of IL-12, IL-18 and IFNγ during URTI in infants are replaced by mature TNFα responses in order to induce a host immune response that can clear the viral infection effectively and can stimulate Th1 immune maturation.

4.6 Acknowledgement

We would like to thank all parents and children for their participation in the VIGALL study and Frank Kalthoff (Novartis) for kindly providing the αIL-4 antibody. This study was supported by the Netherlands Asthma Foundation, the Netherlands Organisation for Health Research and Development and the Foundation 'Vereniging Trustfonds Erasmus Universiteit Rotterdam' in the Netherlands.

Chapter 5

Age- and infection-related maturation of the nasal immune response in 0-2 year old atopic and non-atopic children

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5.1 Abstract

In infants, peripheral blood immune responses are immature compared to adults. In this study we examined whether an age-related and/or an infection-related maturation was observed in nasal immune responses in atopic and non-atopic children aged 0-2 years.

Children were studied during rhinovirus (N=20) or RSV (N=7) induced upper respiratory tract infections (URTI), and during disease-free periods (baseline) (N=40). Nasal brush samples were taken in which numbers of macrophages, T lymphocytes IL-4, IL-10 and IL-12 positive cells were determined. The cumulative incidence of respiratory infections was recorded on weekly symptom cards, which showed that children on average suffered from 4 episodes a year.

Numbers of macrophages were higher during URTI compared to baseline, and increased with age during URTI. Numbers of T lymphocytes increased with age during URTI and baseline, and were higher during URTI at all ages. Additionally, an age-related decrease in the numbers of IL-4 and IL-10 positive cells was observed in children at baseline, while the number of IL-12 positive cells remained unchanged. T lymphocyte and IL-4 responses were stronger related to the age of the child than the numbers of respiratory infections, while the opposite was true for macrophage responses.

In conclusion, in infants, we found an infection- and age-related increase respectively for nasal macrophages and T lymphocytes during URTI. Furthermore, numbers of IL-4 and IL-10 positive cells decreased with age. Whether this maturation reflects a natural age-related maturation, the degree of exposure to respiratory infections, or possibly both, could not be resolved and needs further study.

5.2 Introduction

The immune system in infants is not fully developed. This is reflected by the high susceptibility of children to respiratory infections during the first few years of life [70]. In general, host immune responses to viral pathogens are mediated by Th1 cytokines [146]. As the production of Th1 cytokines can be inhibited by cytokines secreted by Th2 lymphocytes [164], an adequate balance of Th2 and Th1 cytokines is essential for the efficient eradication of pathogens.

The high risk of infections in infants may be a consequence of an immature cellular host immune response. *In vitro* experiments with peripheral and cord blood cells have shown that cells from infants respond less well to infection-related stimuli than those from adults. This is characterised by a lower production of both Th1- (IL-2, IL-12, IFNγ) and Th2-related cytokines (IL-5)[119, 127, 171]. Furthermore, infants favour the production of Th2 cytokines over Th1 cytokines [56].

The development of the child's immune system towards adult-like Th1 cytokine production progresses over time. Several studies on peripheral blood T lymphocytes and mononuclear cells showed that Th1-related cytokine production (IL-2, IL-12, IFNγ, TNFα) increased with age after stimulating cells *in vitro* with polyclonal or infection-related stimuli [35, 61]. Furthermore it has been suggested that maturation of the immune system in children who will become allergic during later life, shows a different pattern than that of healthy non-allergic children. For example, in a previous study we have shown that contrary to the unchanged production levels of Th2 cytokines IL-4, IL-5, and IL-13 during the first year of life in polyclonally stimulated cord and peripheral blood mononuclear cells from non-allergic children, production levels of these Th2 cytokines increased gradually during the first year of life in allergic infants [238].

Hardly anything is known about factors affecting or regulating the maturation of the immune system. Although one could envisage a simple age-related maturation process of the immune system, it has been suggested that recurrent infections themselves could contribute to the maturation process. The induction of successive immature Th1 responses may steer the child's immune system towards a mature Th1 response in adulthood. In the hygiene hypothesis [222], this idea has been linked to the observation that the prevalence of Th2-mediated allergic disease has increased over the last few decades. One of the fundamentals of this hypothesis is that changes in lifestyle may have contributed to a reduction in the exposure to micro-organisms of individual children, resulting in a diminished maturation of their immune system. The Th2-skewed immune response in infants may therefore persevere and predispose a child to the later development of a Th2-mediated allergic disease. Recently, Illi and colleagues showed an inverse relation between the number of upper respiratory infections during the first 3 years of life and the prevalence of asthma at age 7 [101].

No data are available about whether postulated changes in the developing immune system of young children are also reflected in the nasal mucosa, the first interface to become exposed to respiratory pathogens. In our study, we therefore examined whether the magnitude of nasal macrophage and T lymphocyte responses as well as Th1-driving (IL-12), Th2-driving (IL-4) and regulatory cytokine responses (IL-10) during upper respiratory tract infections (URTI) in 0-2 year old atopic and non-atopic children were affected by age and/or the history of respiratory tract infections.

5.3 Methods

Participants and data collection

In all, twenty-four young children (2-22 months, 8 boys) participating in the VIGALL birth cohort study [114], were examined during 27 episodes of URTI either caused by rhinovirus (N=20) or by respiratory syncytial virus (RSV) (N=7). URTI was defined as a runny nose and at least one of the symptoms fever, malaise, sleeping difficulties or loss of appetite. The study was confined to children in whom a single type of virus was detected during URTI. Sixteen children had a positive family history of atopy (FHA; one or both parents had allergic disease) and 11 children had a negative FHA (no allergic disease in the parents). Parents with self-reported asthma, hay fever, house dust mite allergy, or pet allergy were considered to be allergic. This was established with a validated screening questionnaire [124].

Immune responses in infected children were compared to responses at baseline. Therefore another 40 visits (36 children; age 6-26 months, 18 boys) were selected from the same birth cohort study (matched for FHA, age and gender), during which the children were completely free of any symptoms (no runny nose, fever, malaise, cough, wheeze, sleeping difficulties, or loss of appetite) in the two weeks spanning the visit. The children had no virus infection during sampling, and had not suffered from atopic dermatitis before the age of two (determined using the UK Working Party's Diagnostic Criteria for Atopic dermatitis) [255].

During each visit, a physical examination was performed and nasal brush samples were taken. A medical history was taken for general illness symptoms (fever, general malaise, loss of appetite), upper respiratory tract disease (runny nose, sore throat), and lower respiratory tract disease (wheeze, cough, dyspnoea) in the preceding follow-up period. General symptoms of illness such as runny nose, cough, fever, and symptoms of allergic disease such as skin rash and wheezing were scored by the parents on weekly symptom cards during the first two years of life. The Medical Ethics Committee of the Erasmus Medical Centre Rotterdam approved the study design and all parents gave informed consent.

Cumulative incidence of respiratory infections

The number of respiratory infections experienced by children from birth to each visit, and the total duration of those infections, were calculated on the basis of runny nose symptoms registered on weekly symptom cards. If more than 30% of the weekly symptom cards filled in prior to sampling were incomplete, data were not analyzed for that child. When 70 to 100% of the weekly symptom cards were scored correctly (53 of 67 visits), the numbers of respiratory infections and their total duration from birth up to the visit were extrapolated from the available data. We considered children to be continuously infected when they had 4 or more episodes or 8 or more weeks of respiratory infections per 6 months of life.

Viral diagnostics

Cells were harvested from the nose with a cytobrush (Medscand Medical, Sweden) and collected in 7 ml of RPMI 1640 medium (Life Technologies, Netherlands). After centrifugation, the supernatant was used to detect influenzavirus, parainfluenzavirus, RSV, adenovirus, cytomegalovirus (CMV), enterovirus and echovirus. For this purpose, nasal brush supernatant was cultured on HEp-2 cells and, after one week, viruses were detected using immunofluorescence. Additionally, nasal brush cells from the pellet were stained directly with fluorescent-labelled antiviral antibodies to detect RSV, influenzavirus, parainfluenzavirus and adenovirus [193]. Finally, rhinovirus and coronavirus were detected by the isolation of viral RNA from nasal brush supernatant using the MagnaPure LC Instrument (Roche Applied Science, Penzberg, Germany) and the amplification of picornavirus-specific RNA by RT-PCR, followed by hybridisation with either rhinovirus- or coronavirus-specific radiolabelled probes [172].

Immunohistochemical detection of macrophages and T lymphocytes

An immunohistochemical staining procedure was performed as described elsewhere [82] to detect macrophages (αCD68, 4.6μg/ml, clone EBM11, DAKO, Glostrup, Denmark) and T lymphocytes (αCD3, 4.2 μg/ml, clone T3-4B5, DAKO, Glostrup, Denmark) in nasal brush samples with an isotypic antibody as negative control. The numbers of positive cells with red cytoplasmic and/or cell membrane staining were counted per 1000 nasal brush cells and stated as percentages of positive cells.

Immunohistochemical detection of IL-4, IL-10, and IL-12 positive cells

The super-sensitive alkaline phosphatase staining method [34] was used to detect cytokine positive cells in nasal brush samples. Cytospin preparations of nasal brush cells were incubated with mouse anti-human monoclonal antibodies directed against IL-4 (12 μg/ml, clone 1-41-1, Novartis, Basel, Switserland), IL-10 (10 μg/ml, clone IC25-471, Instruchemie, Delfzijl, The Netherlands) or IL-12p70 (5 μg/ml, clone 24945.11, R&D Systems, Abingdon, United Kingdom) or an isotypic control antibody for 60 minutes. The numbers of positive cells with red cytoplasmic staining were counted per 1000 nasal brush cells and were stated as percentages of positive cells.

Statistical analysis

The relations between the numbers of effector cells and cytokine-positive cells on the one hand and age and family history of atopy on the other hand were investigated with regression analysis for repeated measurements (using the 'proc mixed' module from SAS 6.12 for windows, Cary, North Carolina, USA). In order to obtain approximate linear relations in these analyses, the outcomes were transformed logarithmically. The same method was used to evaluate the effects of numbers of respiratory infection episodes. Differences were considered statistically significant when the p-value was ≤ 0.05.

5.4 Results

Age-related macrophage and T lymphocyte responses during URTI

Macrophages and T lymphocytes are important effector cells involved in the eradication of pathogens from the body. To examine to what extent macrophage and T lymphocyte responses during infection mature with age,we determined cell numbers in nasal brush samples from children with either rhinovirus- or RSV-induced URTI, and compared them to samples from children at baseline.

All children with URTI had, apart from a runny nose, symptoms of general malaise (93%), cough (78%), fever (41%), and/or loss of appetite (63%). During four episodes of URTI, children had symptoms of wheezing (3/7 (43%) during RSV-induced URTI and 1/20 (5%) during rhinovirus-induced URTI).

In children sampled during baseline, the macrophage numbers were low (range 0-2%) and did not depend on the age of the child. During URTI, we observed a marked increase of macrophages into the nose in children at every age compared

to baseline ($p < 0.001$) (Figure 5.1A). Moreover, the number of macrophages attracted to the nose during URTI increased 2.7 fold per 12 months of age (p=0.02; Table 5.1). A URTI episode in six-month-old children resulted in an increase in the median percentage of macrophages from 1% at baseline to 3% during infection, whereas this percentage increased to 10% in children aged 22 months. We did not find differences in numbers of macrophages between RSV and rhinovirus infections at any time point. Numbers of macrophages were not significantly affected by the FHA.

The number of T lymphocytes in nasal brushes of these children also revealed age-related changes (Figure 5.1B). In contrast to the macrophage response, a comparable increase in the number of T lymphocytes with the advancing age of the child was observed in children during baseline (p=0.01) and during URTI (p=0.01). The median percentage of T lymphocytes increased from 0.5% at 6 months of age to 4% at 22 months at baseline. During URTI, an influx of T lymphocytes into the nose was observed at all ages (p=0.03), which was comparable between rhinovirus and RSV infections. At the age of 6 months, the median numbers of T lymphocytes increased from 0.5% at baseline to 1% in children with URTI, while in 22-month-old children these numbers increased from 4% to 8%. This represents a 3.8 fold increase per 12 months of age during URTI (Table 5.1). Numbers of T lymphocytes were not significantly affected by the FHA.

Age-related cytokine responses

The age-related increase in the influx of macrophages and T lymphocytes during URTI may result from the maturation of leucocyte-activating factors as Th1 cytokines. We speculated that changes at baseline in Th1-driving (IL-12), Th2-driving (IL-4) and regulatory cytokine (IL-10) responses with age could be responsible for the observed age-related increase in the effector cell responses.

Figure 5.2A shows a significant age-dependent decrease in the number of Th2-driving cytokine IL-4 positive cells (p=0.01), which decreased from median numbers of 20% at 6 months to 10% at 24 months (factor 0.6 (40%) per 12 months of age)(Table 5.1). For the regulatory cytokine IL-10, a trend was observed towards an age-related decrease in numbers of cells (p=0.09; Table 5.1). The median numbers of IL-10 positive cells were reduced from 41% at 6 months to 29% at 24 months of age (Figure 5.2B). The median numbers of the Th1-driving IL-12 cytokine positive cells remained unchanged (median 6%) with age (Figure 5.2C). No significant differences were found in nasal cytokine responses between children with a positive or negative FHA.

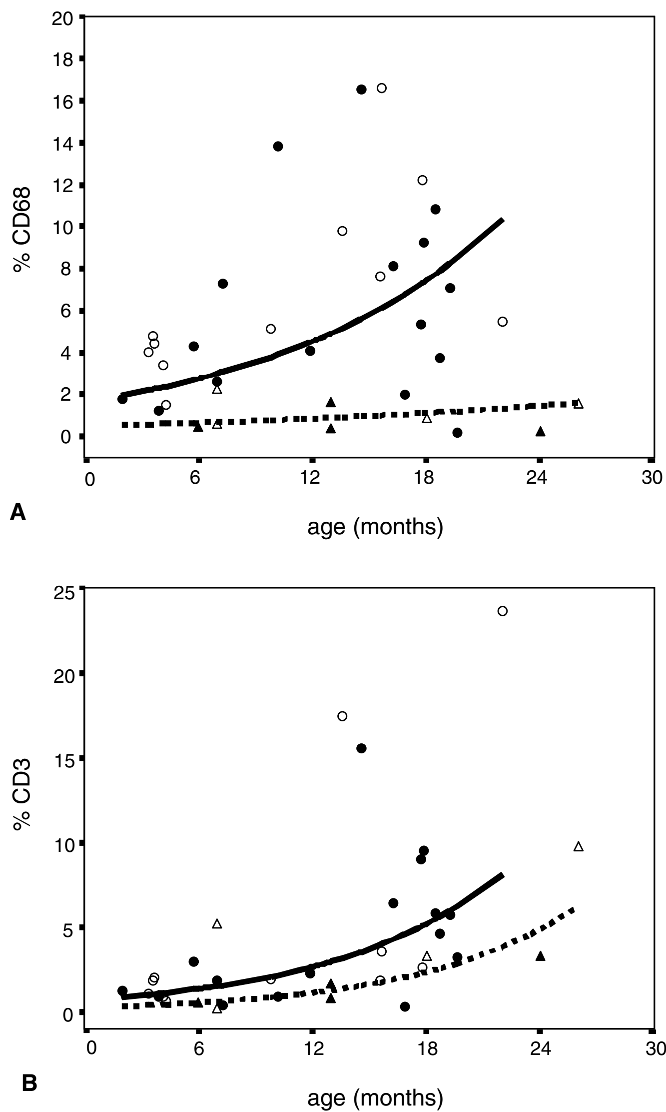

Figure 5.1: The age-related increase in percentages of A. nasal macrophages (CD68 positive) and B. T lymphocytes (CD3 positive) in children during URTI (circles, solid line; 27 samples in 24 children) and baseline (triangles, dashed line; 11 samples in 11 children) with a positive (closed symbols) or a negative FHA (open symbols). Curves correspond to linear regression lines after logarithmic transformation of the vertical axis.

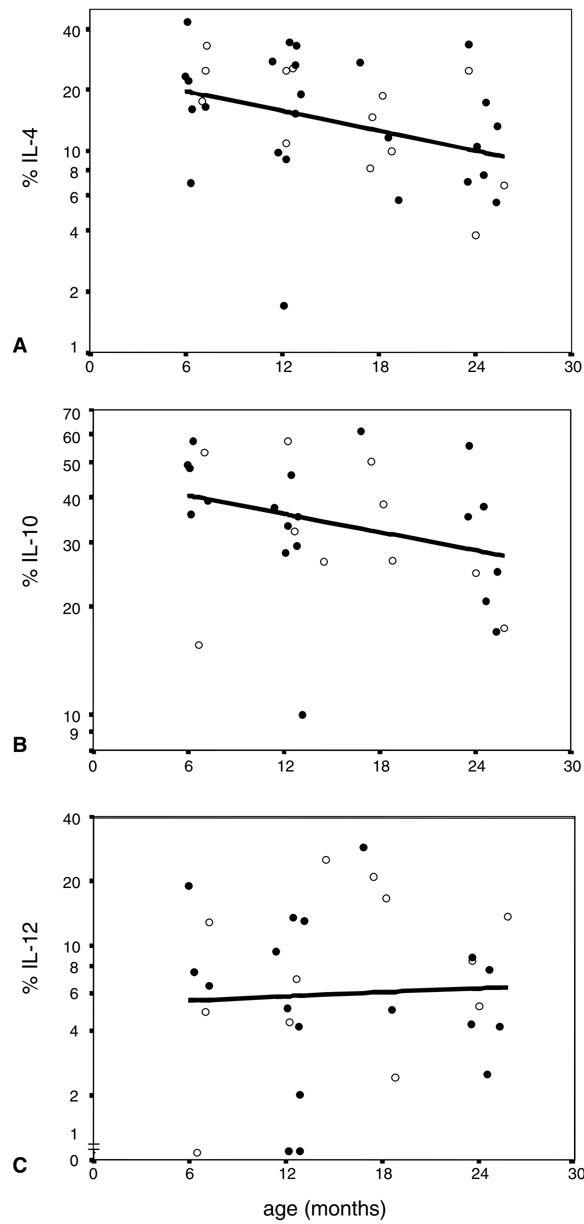

Figure 5.2: Age-related decrease in percentages of A. nasal IL-4 positive cells (38 samples in 33 children), and B. IL-10 positive cells (29 samples in 25 children), and C. equal percentages of IL-12 positive cells (30 samples in 26 children) in children during baseline with a positive (closed symbols) or a negative FHA (open symbols).

Age-related or infection-related immune maturation in children?

Several age-related factors could account for these changes in numbers of nasal macrophages, T lymphocytes, and IL-4 and IL-10 positive cells. Although these age-related differences could be a mere reflection of the natural maturation of a child's immune system, this could also result from recurrent stimulation by respiratory infections [222].

The cumulative incidence increased from $\sim$2 episodes at 6 months to $\sim$3 at 12 months, $\sim$5 at 18 months and $\sim$8 episodes at 24 months of age ($p< 0.001$). Three children suffered continuously from respiratory infections. Since the cumulative incidence of respiratory infections was positively related to age, we would expect to find that nasal immune responses would also be related to the number of respiratory infections. A positive relation was indeed found between numbers of respiratory infections and numbers of macrophages or T lymphocytes (p=0.02 and p=0.04 respectively, N=20). Numbers of macrophages increased 1.7 fold and T lymphocytes 1.5 fold per doubling of numbers of respiratory infections (Table 5.1). Additionally, during baseline, there was a trend towards a decrease in IL-4 positive cells (p=0.08, N=32) (Table 5.1) while no relation was found between IL-12 (N=30) or IL-10 (N=26) and the numbers of respiratory infections.

To discriminate between age-related or infection-related maturation, we performed a regression analysis for repeated measurements that included both variables. However, because of the high correlation between the age of the child and the number of respiratory infections, the effect of both variables on the IL-10 response proved difficult to separate. The strongest predictor for the T lymphocyte response during URTI and the IL-4 response in children during baseline turned out to be the age of the child. The age of the child remained related to both responses, even after adjusting for the numbers of respiratory infections (p=0.05 for T lymphocytes, p=0.09 for IL-4; Table 5.1). This is supported by the observation that, when the relation between the numbers of respiratory infections and the IL-4 and T lymphocyte responses was adjusted for age, this relation was not statistically significant (p=0.77 and p=0.85 respectively). However, the strongest predictor for the macrophage response during URTI turned out to be the number of respiratory infections as the relation remained statistically significant, even after adjusting for the age of the child (p=0.05; Table 5.1).

5.5 Discussion

This study is the first to show an age-related nasal immune maturation in 0-2 year old children. During baseline, numbers of Th2-driving (IL-4) and regulatory (IL-10) cytokine positive cells decreased with age. Interestingly, the percentages of nasal macrophages and T lymphocytes attracted to the nose during URTI were low until the age of 6 months, but increased rapidly with the age of the child. In contrast

Table 5.1: Relation between numbers of inflammatory cells or cytokine positive cells and the age of the child or the number of respiratory infections in children during URTI or during baseline.

	Age	**p-value**	**Adjusted p-value** [a]	**Respiratory infections**	**p-value**	**Adjusted p-value**[b]
URTI						
Macrophages	2.7 (2.2-3.4)	0.02	NS	1.7 (1.1-2.5)	0.02	0.05
T lymphocytes	3.8 (1.9-7.6)	0.01	0.05	1.5 (1.1-2.2)	0.04	NS
Baseline						
IL-4	0.6 (0.5-0.9)	0.01	0.09	0.8 (0.7-1.0)	0.08	NS
IL-10	0.8 (0.6-1.0)	0.09	NS	0.9 (0.8-1.1)	NS	NS
IL-12	1.1 (0.6-2.0)	NS	NS	1.1 (0.8-1.5)	NS	NS

Data given are the factors (95% confidence interval) at which the levels increase per 12 months of age or per doubling of the numbers of respiratory infections. p-values showing a statistically significant relation ($p \leq 0.05$) and p-values showing a trend ($p \leq 0.10$) are represented. When no statistical relation was found, this is indicated with NS (not significant).

URTI: Upper respiratory tract infection

[a] p-value for the relation with age adjusted for numbers of respiratory infections

[b] p-value for the relation with numbers of respiratory infections adjusted for age

to macrophages, the numbers of nasal T lymphocytes at baseline also increased. In 2 year-old children, the numbers of macrophages and T lymphocytes during baseline and recruited during URTI were comparable to those in adult patients with a common cold, as reported in our previous study [235]. This indicates that recruitment of inflammatory cells to the nose is mature at the age of 2 years. An assessment of whether the age-related increase in numbers of T lymphocytes in nasal brush samples is due to an increase of a particular type of T lymphocytes was not possible, but memory T lymphocytes are likely candidates. While children show high percentages of naive T lymphocytes ($\sim$82%) and only few memory T lymphocytes ($\sim$16%) in peripheral blood, adult T lymphocytes show the opposite phenotype ($\sim$48% naive, $\sim$49% memory) [16].

Th1-related cytokines, like IFNγ, TNFα, TNFβ, and IL-2, and IL-12 are effective in activating macrophages and cytotoxic T cell responses in the nose upon infection [125]. It has been suggested that there is a change in the natural balance between Th1 and Th2 cytokine production with the age of the child from Th2-skewed at birth to Th1-skewed in adulthood [56]. This could explain the age-related increase in effector cell responses we observed during URTI. Indeed, in our study, an age-related decrease in Th2-driving cytokine positive cells (IL-4) was observed in the noses of children during baseline. It is difficult to relate our *in vivo* data to known *in vitro* data obtained from cord blood and peripheral blood samples. In the *in vitro* studies, possibly due to different stimulation protocols and cytokine measurement techniques, slightly different outcomes have been observed, although all studies suggest immature Th1 responses in young children. Prescott and colleagues found an age-related decrease in the expression of Th2 cytokine IL-4 mRNA in allergen-stimulated cord blood and peripheral-blood mononuclear cells (PBMC) in children aged between 0 and 18 months [179]. However, others found an age-related increase in Th2 cytokine responses during the first year of life when peripheral blood cells were activated polyclonally or with allergen [243, 35].

In the present study also numbers of nasal IL-10 positive cells decreased with age. These high IL-10 responses in the nose of infants are in line with studies showing a high expression of IL-10 by peripheral blood mononuclear cells of infants compared to adults [182]. The role of IL-10 in immune maturation is complex. IL-10 can inhibit the production of Th1-driving cytokine IL-12 by dendritic cells [214], and therefore a decrease in the numbers of nasal IL-10 positive cells with age would allow an age-related increase in nasal IL-12 responses. This in turn would stimulate Th1-related cytokine production as IL-12 is a key factor in the stimulation of Th1 differentiation [136]. Recently it has become clear that IL-10 can inhibit many more cytokines (TNFα, IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-18) and therefore IL-10 is nowadays regarded as an important regulatory cytokine [147]. In this quality high expression of IL-10 in infancy may induce tolerance in early life and may regulate a balanced Th1 and Th2 maturation. This regulatory role of IL-10 in immune maturation has already been suggested by Yazdanbakhs

and colleagues, who found that high production of IL-10 during helminth infection was inversely related to the development of Th2-mediated allergic disease in children [236].

The reduction in the number of IL-10 and IL-4 positive cells is not paralleled by an increase in numbers of Th1-driving IL-12 positive cells. Although this does not preclude increase in protein production. Lower production of IL-12 protein by LPS-stimulated neonatal DCs has been observed by comparison with adult DCs [126]. Recently Upham and colleagues showed that protein production of IL-12p70 increased gradually from infancy to adulthood after stimulating peripheral and cordblood cells [233]. Unfortunately, our approach did not allow us to determine whether levels of IL-12 protein expression rather than cell numbers are upregulated. We were able to show decreased nasal Th2-driving (IL-4) and regulatory (IL-10) responses. Potentially, this can lead to the enhanced expression of several Th1-related cytokines and this could explain the age-related increase in nasal macrophages and T lymphocytes we observed during URTI.

Children with a positive or negative FHA did not differ in terms of nasal cytokine responses (IL-4, IL-10, IL-12). This concurs with findings from *in vitro* studies where polyclonally stimulated cord blood T lymphocytes from both groups of children did not differ in the production of Th1 and Th2 cytokines [91]. Differences in cytokine production have been observed between children that do and do not develop allergic disease. Van der Velden and colleagues showed that protein levels of Th2 cytokines produced by polyclonally stimulated blood lymphocytes increased from birth to 12 months of age in children with a high genetic risk for allergy who developed allergic disease at 2 years of age, whereas cytokine levels did not change in children who had a positive FHA but remained healthy [238]. In our cohort, no differences in nasal cell and cytokine responses were found between children with, and children without, elevated levels of total and allergen-specific IgE, which are risk factors for the development of allergic disease (data not shown). Nor were any differences found between children with, and children without, recurrent wheezing (data not shown). However, as none of our children had developed atopic dermatitis at the age of 2 and a proper diagnosis of asthma and allergic rhinitis cannot be made in these young children, we were not able to verify our expectations in the present study. A follow-up questionnaire on the development of allergic disease at the age of 6 will help us to address this question.

The hygiene hypothesis suggests that repeated respiratory infections influence immune maturation in children towards stronger Th1 responses and limit Th2 responses, resulting in a diminished chance of developing Th2-mediated allergic disease [222]. This is partly supported by the present study as macrophage responses during URTI were more strongly related to the increasing numbers of respiratory tract infections than to the age of the child. However, our data on the development of nasal IL-4 and T lymphocyte responses show that these responses were more strongly related to the age of the child. At present, we are not able to distinguish

between a strict age-related or infection-related nasal immune maturation and it may well be that both factors are important for a correct maturation. Furthermore, there were no differences in children with a high and a low genetic risk of developing allergic disease in terms of either the incidence of respiratory infections or of nasal macrophage and T lymphocyte response upon URTI, indicating that both groups of children are equally susceptible to respiratory infections. This is in agreement with studies showing that a parental history of allergic disease is a risk factor for lower respiratory tract infections, but not for upper respiratory tract infections, which comprise the majority of respiratory infections [115].

In conclusion, children sampled during baseline showed an age-related decrease in numbers of Th2-driving (IL-4) and regulatory (IL-10) cytokine positive cells in the nose. At 2 years, the numbers were about half of those detected at 6 months of age. This would allow Th1 responses to mature with the age of the child and may explain the infection- and age-related increase in numbers of nasal macrophages and T lymphocytes in children with rhinovirus and RSV-induced URTI. Whether this maturation is a reflection of the natural age-related maturation, the degree of exposure to respiratory infections, or possibly both, could not be resolved and needs further study.

5.6 Acknowledgement

We would like to thank all parents and children for their participation in the VIGALL study and Novartis for kindly providing the αIL-4 antibody. This study was supported by the Netherlands Asthma Foundation, the Netherlands Organisation for Health Research and Development and the Foundation 'Vereniging Trustfonds Erasmus Universiteit Rotterdam' in the Netherlands.

Chapter 6

RSV-induced bronchiolitis but not upper respiratory tract infection is accompanied by an increased nasal IL-18 response

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6.1 Abstract

The aim of this study was to investigate potential differences in the local nasal immune response between bronchiolitis and upper respiratory tract infection (URTI) induced by respiratory syncytial virus (RSV).

Nasal brush samples were obtained from 14 infants with RSV bronchiolitis and 8 infants with RSV URTI during infection (acute phase) and 2-4 weeks later (convalescent phase). Cytospin preparations were stained immunohistochemically for T cells, macrophages, eosinophils. Staining also took place for ICAM-1, Th1-like (IL-12, IFNγ), Th2-like (IL-4, IL-10) and pro-inflammatory cytokines (IL-6, IL-8, IL-18).

During both RSV-induced bronchiolitis and URTI, a cellular inflammation was observed. This was characterised by an increase in the numbers of nasal macrophages, which tended to be higher in bronchiolitis than URTI. Numbers of T lymphocytes and ICAM-1 positive cells increased during both bronchiolitis and URTI. There we no differences between the numbers in the groups. Interestingly, a distinct nasal pro-inflammatory cytokine response was observed in RSV-induced bronchiolitis. This is characterised by an increase in the number of IL-18 positive cells. This increase is specific for bronchiolitis, as a similar increase could not be detected in RSV-induced URTI. Numbers of IL-12 positive cells were higher in bronchiolitis, and numbers of IL-6 positive cells were higher in both bronchiolitis and URTI, while there were no differences between the groups. By contrast, the number of IL-8, IFNγ, IL-4 and IL-10 positive cells remained constant.

In conclusion, clear differences were found in nasal immune responses of children with RSV-induced URTI or bronchiolitis. The induction of a strong IL-18 response was typical for bronchiolitis, as this could not be observed in RSV-induced URTI, and could explain the eosinophilia that is frequently observed during bronchiolitis.

6.2 Introduction

Respiratory syncytial virus (RSV) infection in young children can either be restricted to the upper respiratory tract (URTI), leading to a simple cold, or include the lower airways and result in, for example, bronchiolitis [65]. Although RSV-induced bronchiolitis is nearly always preceded by symptoms of URTI, it is not clear why an RSV infection becomes more severe in some cases. An interesting question is whether there could be differences between the nasal immune responses of RSV-induced bronchiolitis and RSV-induced URTI.

The immunological effects of RSV infections are quite diverse and seem to encompass Th2, Th1, and pro-inflammatory responses. Increased levels of typical Th2-markers like eosinophil cationic protein (ECP) and RSV-specific IgE have been found in nasal lavage samples during bronchiolitis in infants [72, 252]. In line with the increased levels of ECP, higher peripheral blood eosinophil counts have also been observed [60, 72]. In addition, stimulated peripheral blood mononuclear cells revealed enhanced Th2 cytokine responses in infants during RSV bronchiolitis compared to controls [190]. Pro-inflammatory and Th1 responses have also been found in infants with bronchiolitis. For instance, the cytokines IFNγ, IL-2, IL-6, and IL-8 and chemokines MIP-1α, RANTES, were found in nasal lavage and blood samples during RSV bronchiolitis [1, 240, 73, 225, 30, 231]. During URTI in infants, elevated levels of pro-inflammatory and Th1 cytokines IL-1β, IL-6, IL-8 and TNFα have been observed in nasal samples [156]. Hardly any data is available comparing RSV-induced bronchiolitis and RSV-induced URTI.

This study investigated whether differences could be observed on the cellular level in the nasal immune response of infants with RSV-induced bronchiolitis and RSV-induced URTI. For this purpose, the numbers of nasal inflammatory cells, the numbers of Th1- and Th2-like cytokine positive cells, and the numbers of pro-inflammatory cytokine positive cells were determined in both groups of children. The results show distinct nasal immunological effects during RSV-induced bronchiolitis versus RSV-induced URTI.

6.3 Methods

Patients and study design

During the winters of 1998 and 1999, 14 infants (median age 9 weeks) seen at the Sophia's Children Hospital in Rotterdam (The Netherlands) with RSV-induced lower respiratory tract infection (bronchiolitis) were selected for inclusion in the study. The diagnosis of bronchiolitis was based on the diagnosis of an RSV infection in the presence of clinical symptoms and radiological findings characteristic for lower respiratory tract disease. In nearly all cases, the associated respiratory problems required hospitalisation of the affected children. Eight infants (median

age 26 weeks) were selected for comparison. They had participated in a prospective birth cohort study and presented with mild URTI symptoms caused by RSV without bronchiolitis. None of the children with URTI went to hospital. Physical examination was performed and nasal brush samples were taken from the URTI group within the first few days after the onset of infection (median 3 days). This was done within 24 hours after arrival in the hospital for the bronchiolitis group. To obtain baseline measurements, nasal brush samples were taken from all infants with URTI and from 11 of the 14 infants with bronchiolitis during the convalescent phase of the disease (2 to 4 weeks later). The study was approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam. Written informed consent was given by all parents for participation of their child in this study.

Nasal brushes and viral diagnostics

Cells were harvested from the nose with a cytobrush (Medscand Medical, Sweden) and processed as described elsewhere [82]. Cells were washed in RPMI 1640 medium (Life Technologies) and cytospin preparations were made on 10% (w/v) poly-L-lysine (Sigma) coated microscope slides. RSV infection was confirmed by direct immunofluorescent staining of nasal brush cells with antiviral antibodies [193] and/or by virus isolation from nasal brush supernatant.

Immunohistochemical staining of CD3, CD68, MBP, ICAM-1 and IL-18

Slides were fixed in acetone and placed in a semi-automatic stainer (Sequenza, Shandon, Amsterdam, The Netherlands). Immunohistochemical staining was performed as described elsewhere [82]. In brief, slides were pre-incubated with 10% (v/v) normal goat serum (CLB, The Netherlands) (10 minutes) and subsequently for 60 minutes with mouse anti-human monoclonal antibodies directed against CD3, CD68, major basic protein (MBP), ICAM-1, or IL-18 diluted in PBS supplemented with 1% (w/v) blocking reagent (Boehringer Mannheim, Germany) (Table 6.1). After incubation for 30 minutes with biotinylated goat anti-mouse Ig serum, slides were incubated for 30 minutes with either streptavidin alkaline phosphatase for CD3, CD68, and MBP or with polyclonal goat anti-biotin antibody for ICAM-1, and IL-18. After New Fuchsin (Chroma, Germany) staining, sections were counterstained with Gill's haematoxilin and mounted in glycerin-gelatin. Isotypic control antibody was used for control staining.

Table 6.1: Monoclonal antibodies

Antibody	Specificity	Cell type/ cytokine	Conc μ gml	Source
T3-4B5	CD3	T lymphocytes	4.2	DAKO, Denmark
EBM11	CD68	Macrophages	4.6	DAKO, Denmark
BMK-13	MBP	Eosinophils	0.2	Sanbio, The Netherlands
MEM-112	CD54	ICAM-1	144	Sanbio, The Netherlands
1-41-1	IL-4	IL-4	12	Novartis, Switserland
B-E8	IL-6	IL-6	10	Bender Medsystems, Austria
NAP II	IL-8	IL-8	1	Bender Medsystems, Austria
IC25-471	IL-10	IL-10	10	Instruchemie, The Netherlands
24945.11	IL-12p70	IL-12	5	R&D systems, United Kingdom
500-M87	IL-18	IL-18	20	Peprotech, United Kingdom
MD-1	IFNγ	IFNγ	2.5	Innogenetics, Belgium

MBP: Major basic protein

Tyramide signal amplification (TSA) staining for IL-4, IL-8, IL-10, IL-12 and IFNγ

A sensitive protocol based on the alkaline phosphatase method described in the previous section was used. Slides were incubated with mouse anti-human monoclonal antibodies directed against IL-4, IL-8, IL-10, IL-12, IFNγ, or an isotypic control antibody for 60 minutes (Table 6.1). After incubation with biotinylated goat anti-mouse Ig serum, endogenous peroxidase was blocked using 0.2% (w/v) azide, 0.02% (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Slides were next incubated with streptavidin conjugated peroxidase (30 minutes) (NEN Inc., USA), biotinyl tyramide in Tris/HCl buffer (10 minutes) for amplification of the signal, with alkaline-phophatase conjugated goat-anti-biotin and New Fuchsin substrate (30 minutes).

Immunohistochemical staining of IL-6

Sections were stained for IL-6 (Table 6.1) using the *poly*MICA immunohistochemical staining system of The Binding Site Ltd (Birmingham, UK) in accordance with the manufacturer's instructions.

Light microscope evaluation

A thousand cells stained with a purple-blue nucleus were counted in every nasal brush sample. All slides were blinded and counted by two independent investigators in order to ensure an objective analysis. The number of positively stained cells was calculated as a percentage of 1000 nasal brush cells. CD3 and ICAM-1 posi-

Table 6.2: Patient characteristics

	bronchiolitis	**URTI**
Number of patients	14	8
Age (weeks)[a]	9 (3-25)	26 (8-52)
Gender (male)	36%	63%
Smoking parent	36%	13%
ICU	50%	0%
Birth weight (grams) [a]	3150 (2295-4300)	3880 (2300-4950)
Duration pregnancy (weeks)[a]	38 (36-42)	40 (38-42)

URTI: Upper respiratory tract infection, ICU: Intensive care unit
[a] median (range)

tively stained cells had a red cell membrane. Red cytoplasmic staining was found for CD68, MBP, IL-4, IL-8, IL-10, IL-12, IL-18 and IFNγ. IL-6 positive cells had dark brown cytoplasmic staining. On the basis of morphology, both inflammatory and ciliated epithelial cells were found to stain positive for cytokines.

Statistical analysis

SPSS was used for the statistical analysis of cell numbers. Percentages of positive cells were log-transformed to obtain a normal distribution among all data. The paired sample T test was used to analyse differences between the two sampling moments. Differences in cytokine positive cells between patients with bronchiolitis and URTI and between different patient characteristics were analysed with the independent sample T test. Correlations between percentages of cells and the age of the child were tested using Spearman's correlation coefficient. Differences between patient groups and sampling moments were considered statistically significant when the p value ≤ 0.05.

6.4 Results

Patient characteristics

All but one of the 14 patients with RSV bronchiolitis needed hospital admission. Six infants were admitted to the medium care unit and seven to the intensive care unit (ICU). Three of the infants admitted to intensive care needed mechanical ventilation. All bronchiolitis infants suffered from a runny nose and cough, and three infants had wheezing symptoms. None of the eight infants with mild RSV URTI symptoms were admitted to the hospital. Patient characteristics are summarised in table 6.2.

Inflammatory cellular responses in RSV-induced URTI and bronchiolitis

During the acute phase of bronchiolitis as well as during URTI, there was a marked increase in the numbers of macrophages (CD68 positive cells) in nasal brush samples compared to convalescent samples (Figure 6.1A). The median numbers increased from 0.9% to 4.4% in URTI ($p=0.009$) and from 1.4% to 9.6% in bronchiolitis ($p=0.001$). Although the median number of macrophages in the acute phase was higher during bronchiolitis than during URTI, this was not statistically significant. The influx of macrophages was paralleled by a similar influx of T lymphocytes (CD3 positive cells). Statistically significant increases in numbers of T lymphocytes were observed in URTI (from median 1.7% to 2.3%; $p=0.02$), and a trend was also found towards increased numbers of T lymphocytes in bronchiolitis (from median 1.3% to 2.3%; $p=0.06$) (Figure 6.1B). No differences were found between the two groups.

The recruitment of inflammatory cells to the site of infection is often accompanied by an increased local expression of adhesion molecules that facilitates the migration of these cells [249]. This also seems to be the case in this study. An increase was observed in the number of ICAM-1 positive cells during both URTI (from median 11.7% to 18.4%) and bronchiolitis (from median 12.3% to 22.7%). However, compared to convalescence, significantly elevated numbers of ICAM-1 positive cells were only found during the acute phase of bronchiolitis ($p=0.04$)(Figure 6.1C).

Small numbers of eosinophils (BMK-13 positive cells; median 0%, range 0-0.4%, Figure 6.1D) were also found in nasal brush samples. No differences were observed in the number of eosinophils, either between acute and convalescent samples or between bronchiolitis and URTI in the acute phase (Figure 6.1D). However, during the convalescent phase in bronchiolitis, a small but significantly higher number of eosinophils was detected compared to the convalescent phase of patients presenting with URTI (median 0% versus 0.01%; $p=0.05$).

Th1- and Th2-like cytokine positive cells

Nasal brush samples were also stained for Th1-like cytokines IL-12 and IFNγ, and for Th2-like cytokines IL-4 and IL-10 (Figure 6.2). In bronchiolitis, median numbers of IL-12 positive cells increased from 3.2% at baseline (convalescence) to 6.6% during the acute phase of infection ($p=0.04$). There were no differences between bronchiolitis and URTI in terms of IL-12 positive cells. By contrast, no differences were found for IL-4, IL-10 and IFNγ positive cells between the acute and convalescent phase or between URTI and bronchiolitis. Similarly, no differences were found in the Th2/Th1 ratios (IL-4/IFNγ or IL-10/IL-12) for patients with RSV bronchiolitis and URTI at either sampling time point. However, there

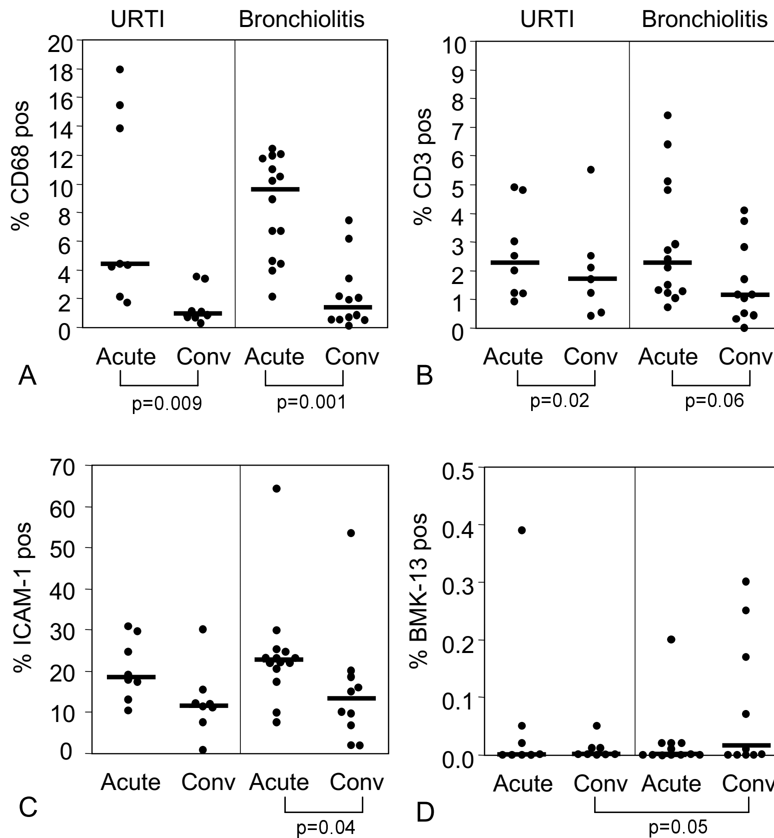

Figure 6.1: Percentages of (A) macrophages (CD68 positive), (B) T lymphocytes (CD3 positive), (C) ICAM-1 positive cells, and (D) eosinophils (MBP positive) during the acute and convalescent phase (conv) of RSV-induced upper respiratory tract infection (URTI) and bronchiolitis. Bars represent median percentages.

was a fall in the IL-10/IL-12 ratio during the acute phase of bronchiolitis compared to the convalescent phase (p=0.04).

Pro-inflammatory cytokines: IL-6, IL-8 and IL-18

By contrast with the Th1- and Th2-like cytokine positive cells, an increase in numbers of pro-inflammatory IL-18 positive cells was found during bronchiolitis compared to convalescence (from median 27.0% to 68.5%; p=0.01; Figure 6.3A). Interestingly, this increase was only observed during bronchiolitis and not during URTI. During the acute phase of infection, numbers of IL-18 positive cells were higher during bronchiolitis than URTI (p=0.001).

Although children with bronchiolitis and with URTI differed in age at the moment of sampling, the increase in numbers of IL-18 positive cells during bronchi-

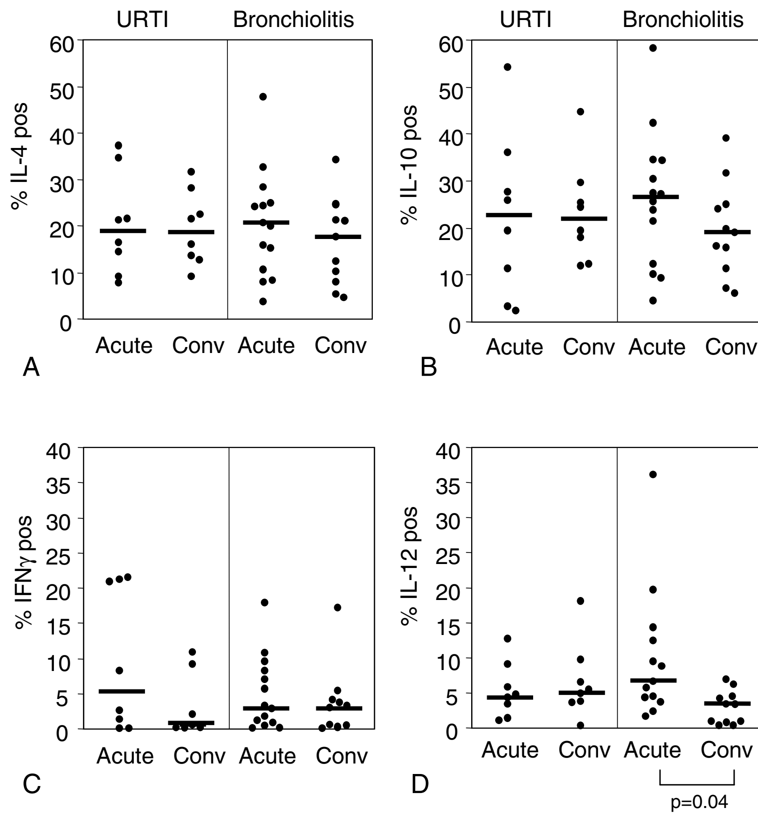

Figure 6.2: Percentages of IL-4 (A), IL-10 (B), IFNγ (C), and IL-12 (D) positive cells during the acute and convalescent phase (conv) of RSV-induced upper respiratory tract infection (URTI) and bronchiolitis. Bars represent median percentages.

olitis is not a consequence of the differences in age between both groups. As shown in figure 6.4, numbers of IL-18 positive cells did not relate to the age of the child during infection in either children with bronchiolitis or children with URTI. During baseline (convalescence) no differences in numbers were observed between bronchiolitis and URTI. Moreover, no age-dependent maturation was observed either. Among infants with bronchiolitis, numbers of IL-18 positive cells were not related to the severity of infection, as determined by the need for mechanical ventilation or admittance to the ICU.

In contrast to IL-18, the numbers of IL-6 positive cells increased for both bronchiolitis (from median 9.9% to 18.5%) and URTI (from median 19.0% to 30.9%) (Figure 6.3B). However, this increase was only statistically significant for bronchiolitis (p=0.03) but not for URTI. No differences were observed in terms of IL-6 positive cells between bronchiolitis and URTI during either the acute or conva-

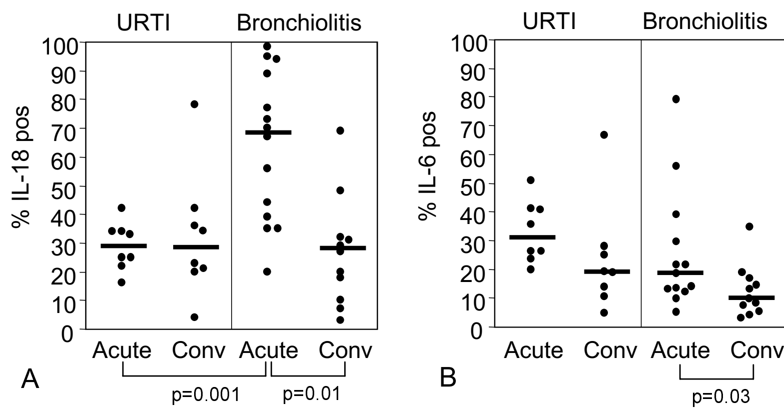

Figure 6.3: Percentages of IL-18 (A) and IL-6 (B) positive cells during the acute phase and convalescent phase (conv) of RSV-induced upper respiratory tract infection (URTI) and bronchiolitis. Bars represent median percentages.

lescent phase. Median numbers of IL-8 positive cells ranged between 0.5% and 45.1% and no differences were found between acute and convalescent sampling or between bronchiolitis and URTI (data not shown).

6.5 Discussion

During both RSV-induced URTI and bronchiolitis, a general nasal inflammation was observed. This was characterised by increased numbers of macrophages, T lymphocytes, and ICAM-1 positive cells, a finding that is in line with previous observations [85, 251]. Most importantly, this study showed a striking difference in cytokine responses between both types of infection, namely an increase in the number of IL-18 positive cells in nasal brush samples during bronchiolitis. This increase was not evident during URTI. The strong IL-18 response was accompanied by an increase in pro-inflammatory cytokine IL-6 and Th1-like cytokine IL-12 responses during bronchiolitis, but these responses were not different from patients with URTI.

IL-18 is a pro-inflammatory cytokine and its effect is closely related to that of IL-1, namely the induction of TNFα, IL-1, IL-6, IL-8, GM-CSF, ICAM-1, Fas ligand and several chemokines and the inhibition of IL-10 and IgE production [250, 153]. IL-18 is also involved in antiviral mechanisms and is, in combination with IL-12, a powerful inducer of IFNγ production from T lymphocytes and natural killer cells [55]. IL-18 can therefore stimulate Th1 responses. In these nasal brush samples, an increase was observed in IL-18 and IL-12 responses during bronchiolitis, but this was not accompanied by a corresponding increase in the numbers of IFNγ positive cells. Garofalo and colleagues did find higher levels of

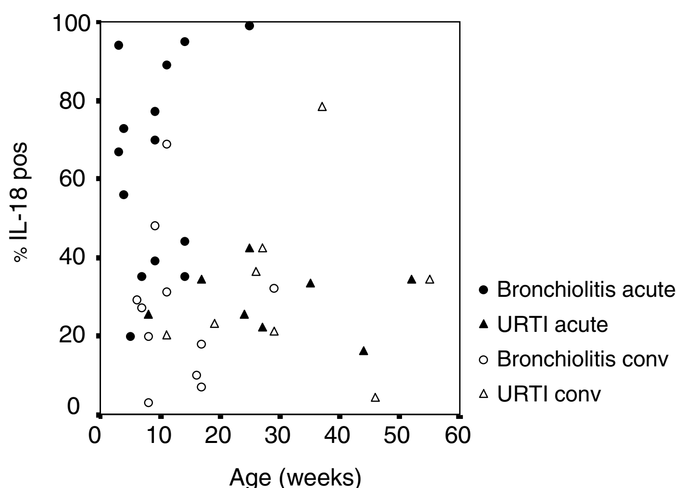

Figure 6.4: Percentages of IL-18 positive cells related to the age of the child during the acute and convalescent phase (conv) of RSV-induced bronchiolitis or URTI.

IFNγ protein in nasopharyncheal samples of infants with RSV bronchiolitis compared to RSV-induced URTI [73]. It is possible that the increase in the expression of IFNγ per cell was too small to be detected in this study due to the immaturity of the child's immune system. This is supported by the observation that when mononuclear cells or T lymphocytes from cord blood are stimulated with mitogen, lower levels of IFNγ protein are produced compared to cells isolated from adult blood [171, 40]. However, the approach in the present study did not allow to determine whether levels of IFNγ expression rather than cell numbers could have been upregulated. Despite this uncertainty, the observation of increasing numbers of IL-18 and IL-12 positive cells during RSV-induced bronchiolitis could explain the underlying mechanism of increased IFNγ production observed during general bronchiolitis in children [240].

With respect to the other pro-inflammatory cytokines IL-6 and IL-8, there was no similar distinction between bronchiolitis and URTI. Although IL-6 responses are increased during the acute phase of an RSV bronchiolitis compared to convalescence, this increase has also been observed for URTI. There were no differences in the number of IL-8 positive cells. However, others have found increased levels of IL-8 protein in nasal samples during bronchiolitis [1]. Noah and colleagues found elevated protein levels of both cytokines in nasal pharyngeal samples of infants with URTI [156]. Furthermore, levels of IL-6 and IL-8 proteins in plasma were higher in infants with severe compared to mild RSV infection [26, 30]. This discrepancy with the findings in this study is probably a consequence of the differences in the methods used to detect changes in the immune response. Where

this study determined changes in cell numbers, the other studies examined protein levels.

Nasal brushes are easy to carry out in infants and can adequately document cellular immune responses in the nose. In these brushes, it was observed on the basis of morphology that cytokines are not only produced by inflammatory cells but also by ciliated epithelial cells. This is in line with other studies indicating that cytokines are produced by epithelial cells after infection with RSV [9, 69]. This explains the higher percentages of cytokine positive cells observed in cytospins in the present study, as would be expected purely on the basis of the number of inflammatory cells present in such samples. A possible caveat of the study is the age difference between infants in both groups, which could have skewed the results. Although cytokine responses could depend on the age of the child [35], no evidence was found that the IL-18 data were affected. As shown in the figure 6.4, no age-related maturation of IL-18 responses was observed, either in infants with bronchiolitis or in infants with URTI. Moreover, at baseline (convalescence), numbers of IL-18 positive cells did not differ between the two groups and there was no age-related maturation. This shows that the differences in IL-18 response between both groups are not due to differences in age, but rather that this response represents an increase in IL-18 positive cells in children with severe RSV-induced infection. Similarly, possible confounders such as gender, birth weight, duration of pregnancy, cigarette smoking by the parents, and allergic disease in the family of the children did not differ significantly between both groups and did not affect the results (data not shown).

It remains unclear whether the distinct nasal immune response for IL-18 in RSV-induced bronchiolitis is functionally relevant. It would be interesting to determine whether this nasal increase in IL-18 could be a direct reflection of the immune response in the lower airway during RSV bronchiolitis or the severity of infection. A potential link between IL-18 expression and severity of disease has been postulated before. During tuberculosis, for instance, higher levels of IL-18 were found in patients with high-grade fever compared to patients without fever [257]. An additional example of the link between IL-18 and severity of disease was observed in asthmatic patients. In patients with asthma, not only were IL-18 levels significantly higher in serum during an acute exacerbation than on remission days, but IL-18 levels were also related inversely to peak expiratory flow [226]. Although patients were excluded who were positive for some of the respiratory viruses (RSV, parainfluenzavirus, influenzavirus), the study did not look for the most prevalent inducers of respiratory infection and asthma exacerbations, namely rhinovirus and coronavirus [137, 154]. It is therefore possible that the increase in levels of IL-18 during exacerbations is a direct reflection of a respiratory viral infection.

The increased IL-18 response during bronchiolitis could also be linked to the eosinophilia in nasal and peripheral blood samples. This has often described as

accompanying bronchiolitis [60, 72]. Until recently, the increase in numbers of eosinophils was thought to result from enhanced Th2 responses during infection [190]. The present study could not confirm this hypothesis as no Th2-skewed responses during bronchiolitis were observed. A previous study in our group showed that RSV-stimulated peripheral blood T lymphocytes taken during bronchiolitis produced predominantly Th1, and some Th2 cytokines, whatever the clinical severity of the underlying disease [30]. Similar results were recently published by Tripp and colleagues who found an increase in both RSV-specific Th1 and Th2 cytokine positive T lymphocytes during bronchiolitis compared to controls [231]. On the other hand, the present data do suggest an alternative explanation for the observed eosinophil influx. IL-18 has been shown to induce the expression of eosinophil chemoattractants RANTES and eotaxin [4, 38]. In combination with the increase in levels of ICAM-1 expression by IL-18 [262], this cytokine could be responsible for the eosinophilia observed during and after bronchiolitis.

In conclusion, despite the limitations of our study, clear differences were found in the local nasal immune response of children presenting with either RSV-induced URTI or bronchiolitis. RSV bronchiolitis was characterised by a strong increase in cells positive for the pro-inflammatory cytokine IL-18. The increase in IL-18 positive cells is specific for bronchiolitis, as a similar increase could not be detected in RSV-induced URTI. Although IL-6 and IL-12 responses also increased during bronchiolitis, these were not different from patients with URTI. The strong IL-18 responses during bronchiolitis could be a direct reflection of the severity of infection and could explain the eosinophilia which is frequently observed during bronchiolitis.

6.6 Acknowledgement

We would like to thank all children and parents who participated in this study, Afke Brandenburg for collecting nasal brush and blood samples of infants with bronchiolitis and Frank Kalthoff (Novartis) for kindly providing the αIL-4 antibody. This study was supported by the Netherlands Asthma Foundation, the Netherlands Organisation for Health Research and Development and the Foundation 'Vereniging Trustfonds Erasmus Universiteit Rotterdam' in the Netherlands.

Chapter 7

Prolonged nasal eosinophilia in allergic patients following common cold

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7.1 Abstract

Viral respiratory tract infections can cause both harmless common colds and severe asthma exacerbations and probably depend on the allergic status of the patient. To determine whether altered immunological mechanisms underlie these differences, we investigated nasal inflammation during naturally acquired common cold.

In a group of 16 patients (8 allergic), nasal brush samples were taken and nasal symptoms were recorded during common cold, 2 weeks later (convalescence) and at baseline (4 or more weeks without nasal symptoms). Nasal brush cells were stained immunohistochemically for Langerhans cells, T cells, monocytes, neutrophils, B cells, macrophages, NK cells, mast cells, eosinophils, eotaxin and RANTES.

Four rhinovirus, 4 coronavirus, 3 RSV, one Mycoplasma pneumoniae and one influenza A/enterovirus double infection were confirmed. Increased numbers of T cells, monocytes, macrophages, NK cells, eosinophils, RANTES and eotaxin positive cells, but not neutrophils, were observed during common cold in allergic and non-allergic patients, and increased numbers of mast cells in allergic patients. Compared to non-allergic patients, in allergic patients eosinophil influx persisted into convalescence.

In conclusion, prolonged nasal eosinophil influx was observed in allergic patients following common cold. What immunological factors can induce prolonged eosinophil influx and whether this may increase the risk of subsequent allergen induced hypersensitivity reactions have to be studied further.

7.2 Introduction

Common viral pathogens can cause both relatively harmless common cold symptoms and severe exacerbations of asthma. Differences in disease expression during common cold probably depend on the allergic status of the patient [78, 88, 89]. Although adult allergic patients do not appear to have an increased annual frequency of common colds [95], increased nasal [58] and pulmonary responses have been found during and after common cold [104]in allergic rhinitis and asthmatic patients. One can therefore ask what altered immunological mechanisms may render an allergic individual more susceptible to severe nasal and bronchial immune pathology upon viral encounter than non-allergic individuals.

Viral involvement in upper and lower airway pathology during common cold has been extensively tested and confirmed in adult volunteers. A wide variety of respiratory viruses can induce common cold symptoms [137]. Several studies have used experimentally induced human infection models to study the immune pathology of common cold [78, 88]. It has been shown that both mildly asthmatic and allergic rhinitis patients experimentally infected with rhinovirus type 16 have increased airway responsiveness during and after infection compared to healthy volunteers [78, 89].

Nasal and bronchial sampling studies during viral infection generally show increased numbers of lymphocytes, eosinophils and neutrophils [130, 173, 256, 230, 227]. Elevated levels of several cytokines and chemokines such as eotaxin, TNFα, IL-1β, IL-5, IL-6, IL-8 and IFNβ have also been detected during common cold in nasal and bronchial tissue [192, 66]. When immunological responses in asthmatic and nonasthmatic patients were compared during common cold, an enhanced eosinophilic response was observed in asthmatic patients during infection, which tended to be prolonged until convalescence [173, 66, 68]. With the exception of increased neutrophil and fibrinogen levels in atopics during common cold [173], no other immunological differences, such as differences in T helper 1 and 2 cytokine balances, have been observed so far between atopic and nonatopic patients.

Only few studies investigated immunological mechanisms of naturally acquired common cold [230, 192]. To clarify the immunological mechanisms underlying virally-induced immune pathology in allergic patients, we examined differences in both inflammatory cell types and eosinophil-specific chemokines in allergic and non-allergic patients in nasal brush specimens [82] during naturally acquired common cold.

7.3 Methods

Subjects

During the winters of 1998 and 1999, eight allergic patients (mean age 28 years) and eight non-allergic patients (mean age 31 years) with clinical symptoms of common cold participated in this study (Table 7.1). Within 4 days of the onset of a naturally acquired common cold, patients recorded the severity of nasal symptoms on a visual analogue scale (VAS) ranging from mild or absent (0) to severe (100). The total nasal symptom score was calculated as the sum of 5 individual nasal symptom scores recorded (runny nose, nasal blockage, sneezing, nasal itching and eye watering and irritation; ranging together from 0 to 500). Patients were considered to have a common cold and were included in the study when the total nasal symptom score was higher than 100 and when patients presented with at least symptoms of a runny nose and nasal blockage. Allergic sensitivity was confirmed by a positive skin prick-test reaction with a wheeldiameter of at least 2+ (Vivodiagnost; ALK Benelux BV, Groningen, the Netherlands) or detection of specific serum IgE (Phadiatop, Pharmacia CAP system, Upsala, Sweden) for house dust mite, grass pollen, birchpollen or cat and dog allergens. All sensitized patients had mild rhinitis symptoms without asthma. None of the patients had used topical and systemic corticosteroids for the previous 4 weeks. Each patient gave informed consent and the Rotterdam University medical ethics committee approved this nasal brush study.

Study design

Nasal brush samples were taken during the acute phase of the common colds and during convalescence (2-3 weeks later). Baseline samples were taken between 1 and 12 months after the common cold and when no nasal symptoms had been present for at least 4 weeks. At each sampling moment, patients recorded the severity of several nasal symptoms reported previously and general malaise on a VAS. All samples were taken outside the pollen season.

Nasal brushes

Cells harvested from nasal brush samples were collected as previously described by Godthelp et al. [82]. Cells were washed in 7 ml of RPMI 1640 medium (Life Technologies). The supernatant was collected and stored at $-80\,^{\circ}$C until viral and Mycoplasma RNA was isolated for PCR amplification. Cells were pelleted, placed in in Tissue-tek II OCT compound (Miles, Inc Diagnostics DIV., Terrytown, N.Y.) and snapfrozen in liquid nitrogen. Frozen sections (6 μm) were transferred to 10% poly-L-lysine (Sigma) coated microscope slides and stored at $-80\,^{\circ}$C until use.

Table 7.1: Patient characteristics

	Patient	**age (years)**	**Type of infection**	**Sensitization**
Non-allergic	1	32	Coronavirus OC43	-
	2	26	Coronavirus 229E	-
	3	33	Influenza A virus/enterovirus	-
	4	24	Rhinovirus	-
	5	39	RSV	-
	6	35	Rhinovirus	-
	7	29	ND	-
	8	26	ND	-
Allergic	1	31	Coronavirus OC43	HDM, grass and birch pollen
	2	23	Coronavirus OC43	HDM, grass pollen, cat
	3	26	*Mycoplasma pneumoniae*	HDM, cat
	4	37	Rhinovirus	HDM
	5	29	Rhinovirus	HDM
	6	26	RSV	Grass pollen
	7	30	RSV	Cat, dog
	8	24	ND	HDM, grass and birch pollen, cat

RSV: respiratory syncytial virus, HDM: house dust mite, ND: not detectable

Virus detection

The type of infection was confirmed in nasal brush samples within the first few days of common cold. Respiratory syncytial virus (RSV), adenovirus, influenza-virus types A and B, enterovirus and parainfluenzavirus type 1 and 2 infections were detected by immunofluorescent staining of nasal brush cells with antiviral antibodies or by viral isolation from 4 ml nasal brush supernatant. Rhinovirus, coronavirus and *Mycoplasma pneumoniae* infections were detected using amplification of viral RNA from 0.5 ml nasal brush supernatant by RT-PCR followed by hybridisation with either rhinovirus, coronavirus or *Mycoplasma pneumoniae* specific radiolabeled probes [6].

Immunohistochemical staining procedures

Slides with frozen nasal brush cells were defrosted and fixed in acetone for 10 minutes, rinsed in phosphate-buffered saline (PBS, pH 7.4) and placed in a semi-automatic stainer (Sequenza, Shandon, Amsterdam, The Netherlands). To block non-specific antibody binding, slides were incubated for 10 minutes at room temperature with a 10% normal goat serum (CLB) diluted in PBS supplemented with 3% bovine serum albumin (Sigma) and 0.1% azide (PBS/BSA). Subsequently, the slides were incubated for 60 minutes with mouse anti-human monoclonal antibodies for the cell markers and chemokines mentioned in table 7.2 (diluted in PBS/BSA). The slides were rinsed in PBS and incubated for 30 minutes with biotinylated goat anti-mouse Ig serum (1:50 in PBS/BSA + 10% human serum), rinsed in PBS and incubated either with streptavidin alkaline phosphatase (1:50 in PBS/BSA + 10% human serum; Biogenex, Klinipath, Duiven, The Netherlands) for cell type staining or with polyclonal goat anti-biotin antibody (1:50 in PBS/BSA + 10% human serum; Sigma) for chemokine staining for 30 minutes at room temperature. Sections were rinsed with distilled water and TRIS buffer (pH 8.5) and incubated for 30 minutes with New Fuchsin substrate (Chroma, Kongen, Germany). Finally, sections were counterstained with Gill's haematoxilin and mounted in glycerin-gelatin. Control staining was performed by the substitution of primary monoclonal antibody by an isotypic control antibody.

Light microscope evaluation

For every nasal brush sample, at least 2000 cells stained with a purple-blue nucleus were counted. The number of positively stained cells was calculated as a percentage of 2000 cells present. Positively stained cells had a bright red-stained cell membrane, red-stained cytoplasm, or both, depending on the cell type or chemokine evaluated. Cells were counted at a magnification of x400.

Table 7.2: Monoclonal antibodies used for immunohistochemical staining

Antibody	Specificity	Cell type/chemokine	Titer	Source
OKT6	CD1a	Langerhans Cells	1:25	Dept. Immunology, EMCR, Netherlands
T3-4B5	CD3	Total T cell pool	1:100	DAKO, Denmark
Leu2a	CD8	Cytotoxic T cells	1:100	B&D, Dorset, United Kingdom
Mon/1	CD14	Monocytes	1:600	CLB,Amsterdam, Netherlands
80H5	CD15	Neutrophils	1:25	Immunotech, Marseille, France
IOB4a	CD19	B cells	1:200	Immunotech, Marseille, France
EBM11	CD68	Macrophages	1:300	DAKO, Denmark
HP-3B1	CD94	Natural killer (NK) cells	1:50	Coulter, Netherlands
G3	tryptase	Mast cells	1:100	Chemicon , United Kingdom
BMK-13	MBP	Eosinophils	1:100	Sanbio, Uden, Netherlands
αEotaxin	Eotaxin	Chemokine	1:15	R&D systems, United Kingdom
αRANTES	RANTES	Chemokine	1:50	Chemicon, United Kingdom

MBP: Major basic protein

Statistical analysis

The statistical analysis of cell numbers was performed with SPSS. Differences in cell counts between the three sampling moments were analysed with the nonparametric Friedman test for related samples. Differences between sampling moments were considered when the p value was ≤ 0.05. Subsequently, differences between two sampling moments were analysed using the Wilcoxon signed rank test for paired samples. Differences between allergic and non-allergic patients at each sampling moment were measured with the Mann-Whitney U-test. Correlations between numbers of cell types, chemokines and clinical parameters were tested using Spearman's correlation coefficient. Differences between groups and sampling moments were considered to be statistically significant when p ≤ 0.05.

7.4 Results

Nasal symptoms and patient characteristics

All the patients investigated had a significantly elevated total nasal symptom score (Figure 7.1) and increased symptoms of general malaise during the acute phase of common cold as compared to convalescence and baseline. In 13 patients (81%), a virus or *Mycoplasma pneumonia* infection was confirmed (Table 7.1). During the acute and convalescent phase, no differences in systemic and local nasal symptoms were observed between allergic and non-allergic patients. At baseline, allergic patients reported slightly higher total nasal symptom scores (p=0.05) compared to non-allergic patients.

Inflammatory cell influx

During common cold, significantly increased numbers of CD3, CD14, CD68 and CD94 positive cells were observed as compared to convalescence and baseline samples (Figure 7.2). During baseline and convalescence generally, fewer than 5% monocytes, macrophages and Natural Killer cells (NK cells) were detected. This figure increased sharply during common cold to levels of up to 20% of cells present in the nasal brush samples. Baseline levels of CD3 positive T cells varied considerably between 0 and 6%. This increased to a maximum of 13% of the cells present during common cold. Somewhat fewer CD8 positive T cells than CD3 positive T cells were detected in nasal brush samples (range 0-6.6%) but there was a slight trend towards increased numbers during common cold (p=0.1 Friedman test). High numbers of neutrophils (CD15 positive cells) were observed (range 0.7-62.7%), but no significant differences were observed between the three sampling moments. Langerhans cells (CD1a positive) (median; range: 0;0-0.3% positive) and B cells (CD19 positive) (median;range: 0;0-0.4% positive) were de-

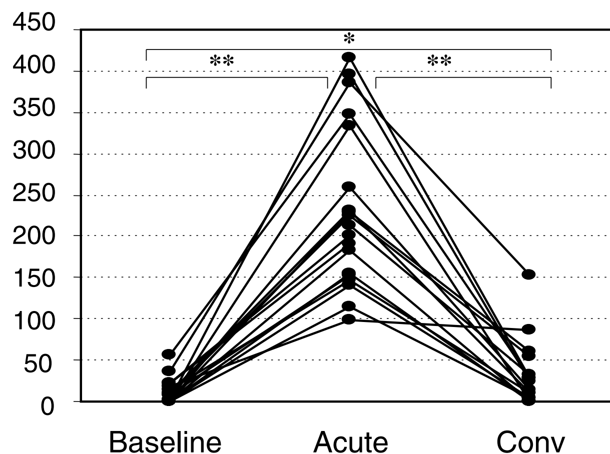

Figure 7.1: Total nasal symptom score during baseline, acute phase (acute) and convalescent phase (conv) of common cold (* $p < 0.05$, ** $p < 0.01$).

tected only in a few nasal brush samples. No differences in the cell types mentioned were observed between allergic and non-allergic patients during the acute and convalescent phases of common cold. At baseline, we observed significantly more macrophages in non-allergic than in allergic patients (p = 0.02).

Eosinophils, mast cells and eotaxin and RANTES positive cells

During the acute phase of common cold in allergic and non-allergic patients, significantly increased numbers of eosinophils (MBP positive cells) were detected as compared to baseline. No differences between allergic and non-allergic patients were observed during common cold and at baseline. However, during convalescence, allergic patients had significantly higher eosinophil levels than non-allergic patients (p=0.03) (figure 7.3A).

In allergic patients during the acute phase of common cold, increased numbers of mast cells (tryptase positive cells) were found as compared to convalescence and baseline (figure 7.3B), while in non-allergic patients no differences were found between the three sampling moments. The numbers of mast cells during common cold were significantly higher in allergic than non-allergic patients (p=0.04).

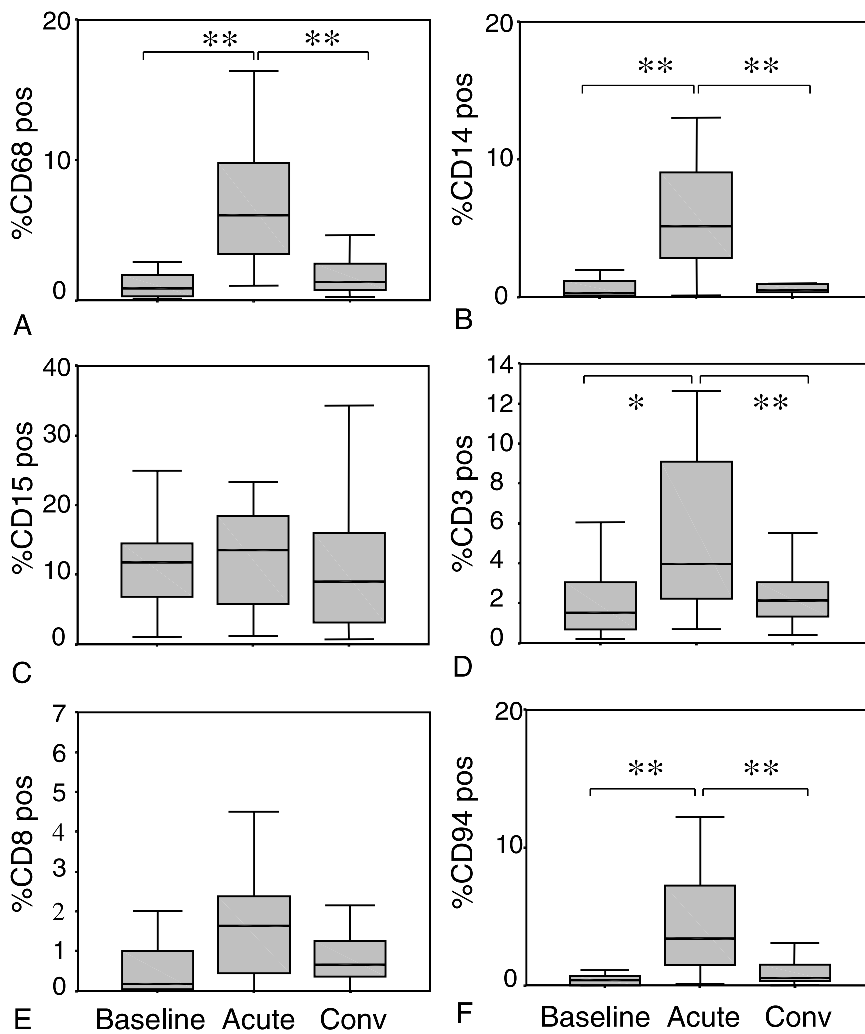

Figure 7.2: Percentage of A. macrophages (CD68), B. monocytes (CD14), C. neutrophils (CD15), D. CD3 positive T cells, E. CD8 positive T cells and F. natural killer cells (CD94) during baseline, acute phase (acute) and convalescent phase (conv) of common cold (* $p < 0.05$, ** $p < 0.01$).

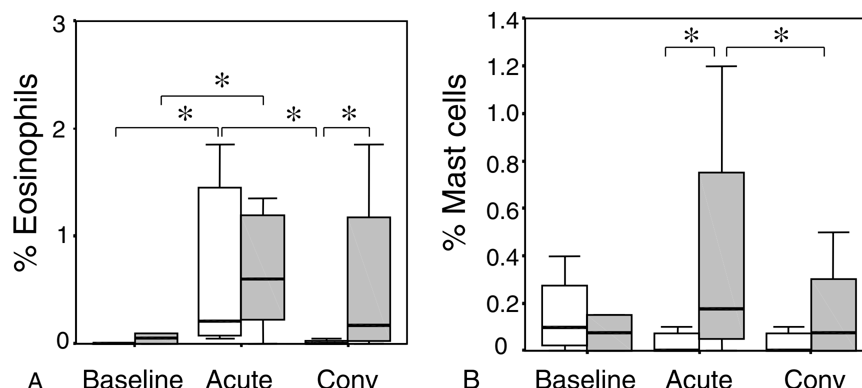

Figure 7.3: Percentage of A. eosinophils (MBP) and B. mast cells (tryptase) during baseline, acute phase (acute) and convalescent phase (conv) of common cold in allergic (gray bars) and non-allergic patients (white bars) (* $p < 0.05$).

Numbers of RANTES and eotaxin positive cells increased during common cold as compared to baseline in allergic and non-allergic patients (figure 7.4). Although numbers of eotaxin positive cells decreased after common cold, still higher numbers were detected during convalescence compared to baseline. Also numbers of RANTES positive cells remained elevated after common cold. No differences between allergic and non-allergic patients in numbers of eotaxin and RANTES positive cells were observed at all three sampling moments.

Correlation between cells, cytokines and nasal symptom severity

During the acute phase of common cold, several statistically significant positive correlations were found between different cell markers, chemokines and clinical parameters. The numbers of macrophages, monocytes, T lymphocytes and NK cells all correlated well to each other (data not shown). The number of eotaxin positive cells correlated well with T lymphocytes and NK cells (r=0.7; p=0.003 and r=0,8; p=0.001 respectively). There were also positive correlations between numbers of eotaxin positive cells and both upper respiratory symptoms and their feeling of general malaise (r=0.6; p=0.15 and r=0.6; p=0.02 respectively).

7.5 Discussion

To clarify the immunological mechanisms underlying immunopathology during common cold in allergic patients, we examined differences in inflammatory cell types and eosinophil-specific chemokines between allergic and non-allergic patients in nasal brush specimens during naturally acquired common cold. Both allergic and non-allergic patients showed an initial response of macrophages, mono-

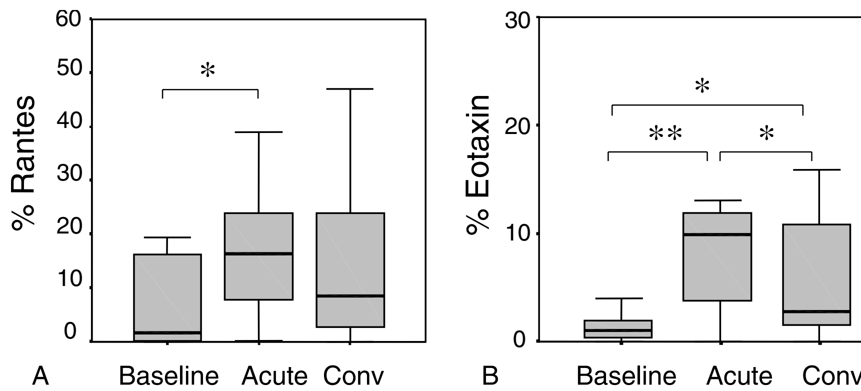

Figure 7.4: Percentage RANTES (A) and eotaxin (B) positive cells present in nasal brushes during baseline, acute phase (acute) and convalescent phase (conv) of common cold (* $p < 0.05$, ** $p < 0.01$).

cytes, T cells, NK cells and eosinophils in the nasal epithelium. However, a mast cell response was observed during the acute phase only in allergic patients. In addition, the increase in numbers of eosinophils during the acute phase only persisted into convalescence in allergic patients. These different immunological responses may underlie different disease expression during common cold in allergic and non-allergic patients.

A wide variety of respiratory viruses can induce common cold symptoms [137, 10] and can induce airway hyperresponsiveness in allergic patients [78, 89]. However, until now, almost all common cold studies used experimentally-induced infection models with rhinovirus type 16 in humans [88, 36]. Allergen provocation studies [67] have shown that immunological data derived from experimental studies can be very different from data acquired in naturally occurring disease, stressing the importance of studying natural disease. Little is known about the role of viruses other than rhinovirus in inducing inflammatory responses related to common cold.

The influx of inflammatory cells such as T cells, monocytes, macrophages and NK cells found in this study is comparable to what has been found in common cold studies in allergic patients and in controls [256, 230, 227]. In contrast to these studies, we did not observe a nasal influx of neutrophils [130, 173]. Since high numbers of neutrophils are found in the nose during all sampling moments but no differences were observed between allergic and non-allergic patients, it would not seem very likely that neutrophils play a role in explaining the difference between both patient groups. However, functional differences like the cytokine production may explain differences in airway hyperreactivity.

The mast cell response in this study, and studies showing increased levels of histamine in lavage samples during common cold in allergic subjects [100], indi-

cate a role for mast cells in airway hyperresponsiveness. In addition, after allergen challenge mast cells have been shown to produce mediators and cytokines which attract and activate eosinophils, leading to priming of subjects and induction of airway hyperresponsiveness. However, mediators such as histamine production alone do not result in priming phenomena. Therefore this is not a mast cell product which is very likely to explain airway hyperresponsiveness after common cold in allergic disease.

The most likely candidates for inducing immunopathology seem to be the eosinophils. Although increased numbers of eosinophils were detected in the nose of allergic and non-allergic patients during common cold, it was only in allergic patients that this eosinophilia persisted into convalescence. In asthmatic patients also, prolonged eosinophilia has been found after induced common cold in bronchial [173, 68]and nasal samples [66]. To our knowledge, these observations are the first which show persistent nasal eosinophilic responses in relatively mild allergic subjects after naturally acquired common cold. There have been reports of increased nasal and bronchial hypperreactivity in subjects with allergic rhinitis [78]. Influx of mast cells, release of mast cell products and persistence of eosinophils in the airway mucosa may cause an increase in airway hyperreactivity in allergic patients compared to non-allergic controls.

Contrary to what we expected on the basis of the findings above, no differences were found between allergic and non-allergic patients in eosinophil-specific chemokine positive cells. An increase in RANTES and eotaxin positive cells was observed during and after common cold in both groups. However, eosinophils may display enhanced susceptibility in allergic individuals for the chemokines mentioned, for example through altered chemokine receptor expression [259, 261]. Other mechanisms like increased expression of ICAM-1 in inflammatory and epithelial cells [230, 244, 43, 19], or decreased apoptosis of eosinophils may account for the prolonged eosinophilia in the nose after common cold [212]. The next step will be to measure actual quantities of chemokine produced by nasal brush cells in both allergic and non-allergic patients.

In conclusion, increased numbers of mast cells during infection and enhanced nasal eosinophilia after common cold in allergic patients may explain nasal and bronchial hyperreactivity and asthma exacerbations after viral infection. Further studies are needed to clarify why eosinophils tend to persist longer in the noses of allergic patients than in those of non-allergic patients after common cold.

7.6 Acknowledgement

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Chapter 8

Discussion

This thesis documents the first in vivo data on the nasal immune response elicited by viral upper respiratory tract infections (URTI) and the maturation of the nasal immune system in children during the first two years of life. In the VIGALL birth cohort study, that forms the central component of this thesis, we determined (i) the type and prevalence of respiratory viruses found during URTI, (ii) provided a detailed analysis of the immune response in the nose elicited by rhinovirus and respiratory syncytial virus (RSV) that together are responsible for ∼60% of all viral URTI episodes in infants, (iii) showed how this immune response changes over time and what molecular mechanism may underlie the maturation of the nasal immune response, and (iv) provide a framework of data that allows us to determine if and to what extend viral URTIs contribute to the maturation of the immune system and to the prevention of the development of allergic disease.

8.1 Epidemiology of respiratory infections

In order to study the potential role of viral upper respiratory tract infections during childhood on the maturation of the immune response and their effect on the development of allergic disease, it is necessary to first determine the types and prevalence of these viruses. Until recently attention was mainly focussed on respiratory syncytial virus (RSV), as this virus is the single most important pathogen during lower respiratory tract infection in infants. At the age of two years virtually all infants have been infected with RSV at least once [211]. However, whether these observations could be extrapolated to upper respiratory tract infections is questionable. Studies in adults show a higher prevalence of rhinovirus (∼50%) than of RSV during symptoms of URTI [137]. This suggests that rhinovirus might also be a relevant pathogen during upper respiratory tract infections in infants. Indeed we found that the incidence of rhinovirus (∼40%) during URTI was higher than for RSV (∼20%), where URTI is defined as runny nose, with at least one of the symptoms fever, general malaise, sleeping difficulties, and loss of appetite. Also during episodes of rhinitis (i.e. runny nose *without* fever, general malaise, sleeping difficulties and loss of appetite), rhinovirus was by far the most prevalent virus (∼40%), while RSV was only rarely detected. These observations in our birth cohort study are in line with the other available study in infants [242] and largely resembles the prevalences seen in adults [137]. As rhinovirus is the most prevalent viral respiratory pathogen in infants it is likely that this virus can contribute substantially to the development of the immune system. However, this contribution probably further depends on several host- and infection related factors as the type of host-immune response, the severity and timing of infection. Rhinovirus was also detected in ∼20% of asymptomatic infants. About 70% of the asymptomatic rhinovirus infected children did show symptoms of URTI or rhinitis in the week prior or after the sample moment, probably reflecting those children sampled just

after or prior to a clinical rhinovirus infection. It is unclear whether these subclinical rhinovirus infections may also affect immune maturation in infants.

Our study reveals that viruses as a group are predominantly responsible for URTI episodes ($\sim$70%) in children. Possible effects of URTI episodes on immune maturation and the development of allergic disease should therefore be mainly contributed to the viral origin of respiratory infections. We infer from our data that the remaining $\sim$30% of all URTI episodes originate from infection with bacteria or viruses for which we have not screened. However, a bacterial origin of URTI is rarely observed [137]. A likely candidate is the newly discovered human metapneumovirus (hMPV) [237]. This virus may cause mild respiratory problems but is more commonly associated with lower respiratory tract disease [62]. At the age of 5 years virtually all children had serum antibodies specific for hMPV [237]. The prevalence of this virus during influenza-like illnesses ranges between 2-16% depending on the study population [161, 220, 185]. The prevalence of hMPV during URTI and rhinitis in infants of the VIGALL study has still to be examined.

The potential role of viruses on the immune maturation and the development of allergic disease would be more complex to unravel when children that are prone to develop allergic disease, due to their genetic makeup, would be more or less susceptible to viral infections. This does not seem to be the case for URTI episodes. In our birth cohort study we were able to show that there was no difference in the number of respiratory infections, mainly consisting of URTI episodes, between a group of children with a positive family history of atopy (FHA) and those with a negative FHA. Confirming our observations, Koopman and co-workers also showed an absence of correlation between FHA and the number of URTI episodes [115]. This is in contrast with the increased risk for lower respiratory tract infections of children with a positive FHA [115]. These considerations are still valid when at the age of six we will determine which of the children from our birth cohort study display symptoms or signs of allergic disease.

8.2 Host immune responses during respiratory infection in infancy

The nasal immune responses during a rhinovirus or RSV infection revealed four important observations that provide new insights into possible immunological mechanisms during respiratory infections in infants. These observations may help us to elucidate the role of viral URTI in stimulating maturation of the immune system. Firstly, we found that nasal brushes of infants contain a remarkable high number of cells expressing the regulatory cytokine IL-10 and that these numbers are strongly reduced during a rhinovirus or RSV induced URTI. Secondly, we observed that infants even during the first two years of life are able to mount a nasal Th1 response against both viral pathogens. However this Th1 response is

different from that of adults as it is restricted to an increase in the level of TNFα and is not accompanied by an increase in IL-12 and IFNγ as is normally seen in adults. Thirdly, the immune response changes over time, with increasing numbers of macrophages recruited to the nasal mucosa allowing a more efficient eradication of pathogens with increasing age of the child. This issue will be discussed in the next section. And fourthly, we showed a difference in the nasal immune response between infections restricted to the upper respiratory tract or those that include the lower respiratory tract, with an increase IL-18 producing cells observed during RSV-induced bronchiolitis and not during RSV-induced URTI.

The interleukin 10 response during respiratory infection

We observed a high number of nasal brush cells positive for the regulatory cytokine IL-10 during periods when the child was free of respiratory infections. These numbers are reduced during a rhinovirus or RSV infection. This is in contrast with observations from studies in adults, where IL-10 responses are relatively low during baseline and increase during respiratory infection [46]. Discrepancies in the IL-10 immune responses between infants and adults suggest a different role for IL-10 between both groups. What exactly could be the role of IL-10 in infancy and how could the IL-10 response be regulated?

IL-10 has originally been described as a Th2 cytokine as it is produced by Th2 lymphocytes and is able to suppress the proliferation of Th1 lymphocytes [215]. Furthermore, IL-10 is produced by other cells associated with the Th2 response like B lymphocytes, mast cells, and eosinophils [147]. IL-10 may act as a growth factor for mast cells and as a growth and differentiation factor for B cells [147]. Moreover, IL-10 can inhibit the production of the Th1-driving cytokine IL-12 by dendritic cells and thereby favour the activation of Th2 lymphocytes [214]. In this quality IL-10 was thought to contribute to the pathogenesis of Th2 mediated allergic disease. As peripheral blood immune responses in infants have been described to be Th2-skewed at birth, these high IL-10 levels, we observed in nose brush samples, could therefore indicate that nasal immune responses are Th2-skewed as well. However, recent data suggest that IL-10 should also be considered as a regulatory or anti-inflammatory cytokine. This is since IL-10 is not only produced by Th2 associated cells, but also by Th1 and regulatory T lymphocytes, and in addition by monocytes, macrophages, and epithelial cells [147, 25]. Furthermore, IL-10 inhibits not only the production of various Th1-related or pro-inflammatory cytokines (TNFα, IFNγ, IL-2, IL-12, IL-18), but also of Th2 cytokines (IL-4, IL-5) [180, 160, 207, 139, 199]. Then IL-10 may also directly act on immature resting DCs resulting in DCs that stimulate T lymphocytes to become unresponsive [105]. Based on these anti-inflammatory properties, in adults, IL-10 has been linked to suppression of ongoing inflammatory responses following infection [3].

The role of IL-10 in the immune response may well vary depending on tem-

poral or spatial expression patterns. In adults, the production of IL-10 is known to occur relatively late in the immune response to pathogens reflecting its role in the downregulation of an active immune response [148]. However, in infants nasal IL-10 is expressed at a high level in the absence of an active immune response. The high level of IL-10 in infants could reflect induction of tolerance during the first few years of life, preventing excessive immune responses to the variety of more or less harmless antigens that the infant will encounter for the very first time in life. Additionally IL-10 induced tolerance is important to prevent an undesired immune response between mother and foetus, with reduced levels of IL-10 associated with increasing levels of abortion [175]. Furthermore the role of IL-10 may well depend on what kind of cells express IL-10. Expression of IL-10 by regulatory T lymphocytes may well induce local tolerance, whereas IL-10 expressed nearby resting immature DCs in the nasal mucosa may induce DC that stimulate peripheral T cell tolerance. Furthermore, IL-10 expressed by T lymphocytes interacting with B cells may well stimulate the Th2 response by enhancing B cell-mediated IgE production.

To understand the exact role of high IL-10 expression in the nose of infants, it is necessary to know what cells in the nose are the main producers of IL-10. In our nasal brush samples the most likely source of IL-10 is the epithelium. Approximately 40% of cells positive for IL-10 are ciliated and both our unpublished immunohistochemical observations and those of others have shown that epithelial cells can produce IL-10 [25]. These cells are likely to represent the majority of IL-10 positive cells. Inflammatory cells, in particular T lymphocytes and macrophages, can also produce IL-10 [147]. However, numbers of T lymphocytes and macrophages in nasal brushes of infants are low (less than 5% at baseline) and the increase in T lymphocytes and macrophages we have observed during URTI does not correlate with the decrease in IL-10 positive cells we have detected during URTI. Thus these cells are unlikely to represent many IL-10 positive cells. Furthermore, based on data from studies on adult nasal brushes, samples comprise $\sim$10% neutrophils, but these cells do not seem to produce IL-10 [31]. DCs, B lymphocytes, and mast cells can produce IL-10 but these cells are only found in relatively low numbers in nasal brush samples. In conclusion, the respiratory epithelium seems to be the most important source of IL-10 in the infant nose. Hopefully, immunohistochemical double staining procedures will become available for nasal brush samples in the near future to confirm these observations.

Epithelial cells are the first line of defence, suggesting that locally produced IL-10 may regulate immunity in infants. As IL-10 can suppress the immune response by inhibiting the production of various pro-inflammatory cytokines, it is likely that during URTI in infants the IL-10 production is actively reduced. This would allow the initiation of an effective host immune response consisting of Th1 and pro-inflammatory cytokine production. How is this reduction in numbers of nasal IL-10 positive cells during URTI established? Respiratory viruses can infect

respiratory epithelium, macrophages, and other leukocytes [106, 241, 79] and have been shown to directly regulate cytokine production by these cells [219]. Therefore, the epithelium may use infection by the viral pathogen as a trigger for the active downregulation of IL-10. Interesting in this respect is that some viruses like Epstein-Barr virus (EBV) encode an IL-10 homologue [218, 186]. Expression of virally encoded IL-10 may prevent the normal derepression of the immune response. Downregulation of IL-10 could also be indirect through other inflammatory cytokines. Type I interferons (IFNα and β) have been described to suppress IL-10 production by *Staphylococcus aureus* stimulated monocytes [63]. As these interferons are produced by epithelium, monocytes, and macrophages after infection by respiratory viruses [191, 196], they are a likely source of IL-10 suppression during respiratory infection in infants. Finally, also TGFβ1 and IL-18 may diminish the production of IL-10 [224, 234]. However, it is unlikely that production of IL-18 underlies the reduced IL-10 responses as in our study the number of IL-18 positive cells was not upregulated during URTI.

Fortuitously, we discovered that not only IL-10 is downregulated during a rhinovirus or RSV infection, but that this downregulation even persists upto two weeks after infection. In chapter 4 we observed the above-mentioned downregulation when comparing a healthy control group with a group of children during an URTI episode. In chapter 6 however, we could not detect a difference in the IL-10 levels during an URTI episode and a sample taken two weeks later. This convalescence sample we originally thought to represent the baseline expression level of IL-10. Comparing the percentages of cells positive for IL-10 in those three different sample moments, reveals that even two weeks after infection the level of IL-10 is still reduced compared to baseline. Such a long-lasting change in cytokine response following infection has also been observed in adults. In the present thesis it was shown that the numbers of nasal brush cells positive for chemokines RANTES and eotaxin remained relatively high until at least two weeks after common cold when compared to baseline (at least four weeks following infection) [235]. Possibly this prolonged inflammatory response plays a role in the induction of humoral and/or cellular memory responses against various pathogens [209]. Furthermore, various autoimmune diseases start after viral infection which could be a consequence of the this prolonged downregulation of IL-10 leading to activation of auto-reactive T lymphocytes [98].

The T helper 1 and 2 response during respiratory infection

In adults an effective host immune response upon infection consists of the induction of Th1-related and pro-inflammatory cytokines as IFNγ, IL-6, IL-8, IL-12, IL-18 and TNFα [46, 106, 11]. These cytokines play a central role in the attraction of immune cells to the site of infection and activation of these cells to clear the pathogen from the body. The question raises whether infants are capable of induc-

ing comparable Th1 and pro-inflammatory immune responses during respiratory infections and whether or not this immune response would change over time. This would validate the concept of a shift in the immune response from Th2-skewed at birth towards Th1 in adulthood due to repeated respiratory tract infections. To address this question we set out to determine the effect of rhinovirus and RSV upper respiratory tract infections on Th1- and Th2-related cytokine responses. These responses were determined in a combination of an immunohistochemical approach determining cell numbers staining positive for these cytokines (IL-4, IL-12, IL-18) and a biochemical approach determining cytokine responses at the protein level (IFNγ, TNFα).

In the experiments described in this thesis we observed a prominent Th1-like response evidenced by an increase in TNFα levels during rhinovirus- and RSV-induced URTI. In contrast to what has been reported for adults, this increase in TNFα is not accompanied by an increase of other pro-inflammatory and Th1-related cytokines like IFNγ, IL-12, and IL-18. The absence of an infant IFNγ or IL-12 response *in vivo* is in direct agreement with *in vitro* studies showing immaturity of IFNγ [40, 118] and of IL-12 responses [83]. Some *in vitro* experiments suggest that also the TNFα response in newborns is lower than in adults. However, we did not determine whether or not the extend of the TNFα response observed in the nasal brush samples of newborns was directly comparable to that of adults.

In adults, TNFα is characterized as a pro-inflammatory cytokine with Th1 stimulating properties. The biological effect of TNFα is the stimulation of functional activities of cytotoxic T lymphocytes, NK cells, and macrophages [229] and the recruitment of inflammatory cells to the site of infection [202]. Together with IL-12 and IL-18, TNFα can promote the development of Th1 lymphocytes [206]. Furthermore TNFα can stimulate production of macrophage-derived cytokines (for example IL-1, IL-6, IFNγ, IL-18) by various inflammatory cells and the epithelium [229, 55]. In this constitution, TNFα is a key cytokine during host-immune responses upon infection. In infants, although we have not observed an IL-12 response upon infection there is a basal level of IL-12 allowing a cooperative effect of TNFα and IL-12. Along these lines also IL-18 will contribute to the differentiation of T lymphocytes during URTI. In bronchiolitis this effect may even be more pronounced since we observed upregulation of IL-18. The effect of TNFα on the expression of the macrophage-derived cytokines could not be corroborated, as we did not observe an upregulation of IL-12, IL-18, or IFNγ during rhinovirus or RSV-induced URTI.

How could the production of TNFα be regulated *in vivo* during URTI in infants? The increased TNFα production during rhinovirus and RSV induced URTI might well be a consequence of the reduction in numbers of IL-10 positive cells. The IL-10 cytokine displays strong anti-inflammatory properties and it suppresses production of various cytokines, including TNFα [207]. Therefore a reduction in

expression of IL-10 during URTI may alleviate this suppressive effect and may enable the increase in production of TNFα. Alternatively, the production of TNFα could be stimulated directly by infection of epithelial and inflammatory cells with viral pathogens [17, 143]. Furthermore, production of TNFα could be stimulated indirectly by other cytokines as IL-12 [152] and IL-18 [181]. However, this mechanism of indirect stimulation is not very likely as no upregulation of IFNγ, IL-12, and IL-18 responses were observed during URTI in infants.

Although IL-18 responses are not found to be upregulated during rhinovirus and RSV-induced URTI, this is different for RSV-induced bronchiolitis. During bronchiolitis, the number of IL-18 positive cells is strongly upregulated, implicitly showing that the production of this cytokine in newborns is not intrinsically defect, as *in vitro* experiments have suggested [169]. What could be the role of the IL-18 response during bronchiolitis? IL-18 is mainly produced by monocytes and macrophages [55] but also by epithelial cells [37]. As virtually all nasal brush cells were positive for IL-18 during infection, this implicates that probably epithelial cells, neutrophils, and macrophages, the major components of nasal brushes, are all sources of IL-18 in infants. IL-18 activates various inflammatory cells including T lymphocytes, NK cells and macrophages to produce TNFα, IL-1, IL-6, IL-8, GM-CSF, and chemokines, while IL-10 and IgE production is inhibited [55, 153]. In addition, IL-18 enhances T and NK cell cytotoxicity and activates neutrophils [49, 129]. Furthermore as discussed above, IL-18 is in combination with IL-12, a powerful inducer of IFNγ production from T lymphocytes, NK cells and macrophages and thereby stimulates Th1 development [55]. In this constitution, IL-18 is thus an important cytokine in the antiviral host immune response and actively involved in the eradication of infection from the body. What could have induced this high nasal IL-18 response during bronchiolitis and why is no such response observed during RSV-induced URTI? In adults, various mechanisms are known by which the production of IL-18 could be stimulated. What mechanism underlies the IL-18 induction in infants during bronchiolitis is difficult to answer. The production of IL-18 can be induced directly by viral pathogens [195]. Whether differences in viral load between RSV-induced URTI and bronchiolitis could explain the differential expression of IL-18 seems unlikely. IL-18 can be stimulated also indirectly by various cytokines such as IL-1, TNFα, and IL-6 [55]. During bronchiolitis we did observe a small increase in numbers of IL-6 positive cells. However, this response did not differ between infants with RSV induced bronchiolitis and URTI. Upregulation of IL-6 is thus unlikely to account for the different IL-18 response between both groups of infants. Whether differences in IL-1 and TNFα responses are observed during RSV URTI and bronchiolitis remains to be studied. However, if both groups differ in the level TNFα production this is still unlikely to underlie the high IL-18 response during bronchiolitis. This is since numbers of IL-18 positive cells during URTI did not increase although elevated levels of TNFα were found.

Some studies have suggested that the high IL-18 response could result from the severity of infection. For instance, during tuberculosis, higher levels of IL-18 were found in patients with high-grade fever compared to patients without fever [257]. However, it is unlikely that the high IL-18 response in infants with bronchiolitis compared to the low response in infants with URTI results from the severity of infection. This is since we did not observe a different IL-18 response between infants with rhinovirus-induced URTI compared to infants with milder symptoms of rhinitis. Finally, the high IL-18 response during bronchiolitis could have resulted from differences in the site of infection. The fact that the lower respiratory tract is involved during bronchiolitis is an interesting possibility that could point towards a possible molecular interaction between upper and lower airways. Whether the high IL-18 response in the nose triggers disease expression in the lower respiratory tract or whether infection of the lower respiratory tract induces high IL-18 responses in the nose (and lungs) remains to be studied.

Our data suggest a possible explanation for the increased wheezing observed during and after RSV-induced bronchiolitis. Until recently it was thought that children had an increased risk to develop Th2-mediated allergic disease following bronchiolitis [210, 128]. This was concluded from the observation that wheezing symptoms following bronchiolitis resemble symptoms of asthma. However, it has become clear that although these transient wheezing episodes may persevere following bronchiolitis, they are not linked to increased chance of developing asthma in later life [217]. IL-18 has been linked to enhanced expression of matrix metalloproteinases that play an important role in airway remodelling [158, 103]. In infants, this remodelling could easily lead to long-lasting airway narrowing and symptoms of wheeze, with no predictive value for the later development of allergic disease.

In contrast to what we have described above, i.e. the downregulation of the regulatory cytokine IL-10 and concomitant upregulation of the Th1-related cytokine TNFα, we could not detect any effect of viral upper respiratory tract infections on the level of the Th2 cytokine IL-4. The old dogma would predict that an increase in the Th1 response would be accompanied by a decrease in the Th2 response. In the adult system this is mainly regulated through IL-12 and IFNγ [189]. As newborns lack an IL-12 and IFNγ response upon infection this could explain why they are not able to downregulate the Th2 response. Although there is no downregulation of the IL-4 response, the upregulation of the Th1 response still results in a shift in the Th1/Th2 balance towards Th1.

8.3 Maturation of local nasal immune responses

Data we have discussed above showed that in infants a nasal Th1 immune response is induced during rhinovirus and RSV-induced URTI, which consisted of an in-

crease in response of TNFα and a reduction in the regulatory cytokine response (IL-10). Based on this type of immune response, repeated URTIs are thus in theory capable of stimulating maturation of Th1 cytokine production as proposed by the hygiene hypothesis [222]. Prior to this thesis, only maturation of peripheral blood cytokine responses were studied. Our data allowed us to examined 1) whether immune maturation was observed in the respiratory mucosa of infants, i.e. a relative increase in Th1 and a decrease in Th2 cytokine production, and 2) whether these changes in cytokine response were related to the numbers of respiratory tract infections as suggested by the hygiene hypothesis or related to the age of the child as part of a natural occurring immune maturation.

Macrophages and T lymphocytes

This is the first study showing maturation of the immune response in the nasal mucosa during infection. Increasing numbers of macrophages and T lymphocytes are recruited to the nose during infection, as the infant grows older. Numbers of macrophages and T lymphocytes attracted to the nose during URTI were small in infants until the age of 6 months and thereafter rapidly increasing. At the age of two years numbers of macrophages and T lymphocytes during URTI were comparable with numbers in adults during common cold (data not shown).

Our data also revealed that, in contrast to the baseline numbers of macrophages that remained constant, the number of CD3 positive cells (T lymphocytes) gradually increased over time during baseline. Currently we do not know what subsets of T lymphocytes are responsible for the age-related increase in numbers of T lymphocytes during URTI or at baseline. The increase at baseline may well be a result of increasing numbers of memory T lymphocytes. For example, in peripheral blood it was found that percentages of memory T lymphocytes increased with age from 16% in infants to 49% in adults, while percentages of naive T lymphocytes decreased from 82% to 48% [16]. This gradual establishment of memory responses is also likely to occur in the nose as a result of the repeated encounter of childhood infections. Thus an increase in memory T lymphocytes is probably associated with the age-related increase in total pool of T lymphocytes. The increase observed during infection is likely to be a consequence of an influx of Th1 lymphocytes responding to the local invasion of the pathogen.

Recruitment of macrophages and T lymphocytes results from local production of cytokines that activate these cells and attract them to the site of infection. Th1-related and pro-inflammatory cytokines as IL-2, IL-12, IL-18, IFNγ and TNFα may act as leucocyte-activating factors [125]. As the production of Th1 and pro-inflammatory cytokines is opposed by Th2 and anti-inflammatory or regulatory cytokines [189, 160], age-related changes in these cytokines at baseline and/or during infection could underlie the increased recruitment of macrophages and T lymphocytes.

Th1, Th2 and regulatory cytokine responses

The observation of the maturation of the cellular immune response (macrophages and T lymphocytes) is supported by our observation of a concomitant maturation of the cytokine response. Our data present the first evidence for an age-related change in cytokine responses in the nose of infants. We observed a clear decrease in numbers of cells positive for the regulatory cytokine IL-10 and the Th2 cytokine IL-4 as the child grows older. The decrease in IL-4 with age is in line with an *in vitro* study by the Holt's group in Australia, who showed that IL-4 mRNA expression by allergen stimulated PBMCs slowly decreased from birth to two years of age in healthy children [179]. However, although several studies have pointed towards high production of IL-10 in infants compared to adults [182], this is the first study that shows an age-related decrease in IL-10 responses in infants.

As the regulatory cytokine IL-10 can downregulate the production of IL-12 [214], we had expected that an age-related decrease in numbers of IL-10 positive cells would enable an age-related increase in numbers of nasal brush cells positive for Th1-driving cytokine IL-12. This in turn would explain the increase in numbers of macrophages and T lymphocytes with age during URTI. However, no such increase in IL-12 response was observed in the nose of infants at baseline. Others did show maturation of IL-12 in peripheral blood samples. A recent cross-sectional study of Upham and colleagues showed that blood mononuclear cells activated with LPS or heat-killed *Staphylococcus aureus* had an impaired IL-12 production in neonates [233]. This IL-12 production gradually increased with age but even in 5- and 12-year old children levels had not reached adult levels. Also the production of other Th1 cytokines (IFNγ, TNFα, IL-2) has been shown to increase with age [243, 112, 35, 61]. In general, these protein levels did not reach adult levels until the age of 17 years. Several explanations could be given for the differences in results between the study presented in this thesis and studies mentioned above. First we have measured numbers of cells positive for IL-12 in the nose. Therefore we could have missed an increase in production of IL-12 protein with age. Furthermore, we measured cytokine maturation in infants until 2 years of age. The age-related increase in numbers of IL-12 positive cells in this age-interval could have been too small to be detected. Therefore, follow up of the children is necessary to study IL-12 responses in their noses at later age. Finally, maturation of other Th1 and pro-inflammatory cytokines as IFNγ and TNFα will further need to be studied in the nose of infants to clarify the increase in numbers of macrophages and T lymphocytes with age.

8.4 Age- versus infection-related maturation of the immune response

One of the goals set for this thesis is to try and determine whether repeated infections may contribute toward the maturation of the immune system. As discussed above we have shown that, as the child grows older, we could indeed detect maturation of the local nasal immune system both on cellular and on cytokine level. The question now raises whether this is a consequence of an intrinsic maturation of the immune system or whether it is this guided by recurrent infections that, as we have also shown, are able to elicit Th1 responses? Answering this question is complicated by the high degree of correlation between the age of a child and the number of infection a child has experienced. Furthermore, there might well be a different level of contribution of age- and infection-related maturation depending on what aspect of the immune maturation is considered. Statistical analysis of our data on the immune maturation in chapter 5 sheds some light on this complicated question. In this chapter it was shown that changes in IL-4 and T lymphocytes are stronger correlated with age than with the number of infections, whereas for macrophages the reverse was true. Although we are not able to fully resolve this complicated issue we are convinced that the chosen approach could resolve this important question in a larger birth cohort with a longer follow up period.

8.5 Regulation of immune maturation: a model

We observed that regulatory (IL-10) and Th2 (IL-4) responses in the nose decreased and Th1 responses (macrophages and T lymphocytes) increased with age. The next question is in what sequence these immune responses develop in infants. This is indeed critically dependent on the timing of the response and the function of the cytokines. Based on its regulatory effect during URTI, we suggest that IL-10 is the central player in the modulation of this immune maturation. Two reasons could be given for this. First, IL-10 is highly expressed in infants and thereby exerts a dominant immunosuppressive effect on the majority of Th1, Th2 and pro-inflammatory responses cytokines. Secondly, numbers of IL-10 are reduced during respiratory infection, which shows that this cytokine is subject to regulation. A central role for TNFα in the immune maturation is less likely. Induction of TNFα during respiratory infection seems a consequence of the reduction in IL-10, whereas TNFα has not been reported to be able to downregulate IL-10. Although the number of IL-4 positive cells decline as the child grows older, the number of IL-4 positive cells do not change during URTI. As this cytokine is not directly affected by respiratory infections we consider it less likely to contribute to the regulation of the maturation of the immune system. We would thus propose the

following model for the immune maturation, in which IL-10 is the central player (Figure 8.1):

1. Infants are born with high natural epithelial expression of the regulatory cytokine IL-10. This high level of IL-10 creates a local suppressive immune environment.

2. Viral respiratory tract infections directly downregulate the level of IL-10 allowing a TNFα-centred immune response and over time an reduction in IL-10 responses.

3. As a consequence of the relief of the anti-inflammatory effect of IL-10, subsequently the initially low Th1 responses may develop with age. This would allow the age-related increase in numbers of macrophages and T lymphocytes during URTI.

8.6 Immune maturation and the development of allergic disease

In this thesis we have shown that two of the most common respiratory viruses are able to induce a Th1 response in infants and that repeated infections, at least in part, contribute to the maturation of the immune system. The design of the VIGALL study allows us to determine whether the maturation of the immune system is different for those children who will or those who will not develop allergic disease. In addition we will be able to determine the relative contribution of viral upper respiratory tract infection to the expression of allergic disease, as has been suggested by the hygiene hypothesis.

In order to address this question we need to define which of the children will develop allergic phenotypes. To increase the chance to find a considerable number of children with allergic disease in later life, we included in the VIGALL study a high number of children (67%) with a positive family history of atopy. At the conclusion of this part of the study the children were two years old, which is still too young to allow a robust determination of their allergic status. In the total group of children median IgE levels rose from 7 IU/ml at 12 months to 18 IU/ml at 24 months, which conforms to the normal rule of thumb annual increase of 10 IU/ml. Children with specific IgE (20/79 at 12 months and 17/50 at 24 months) were nearly exclusively sensitised for cow's milk (17/20 at 12 months and 13/17 at 24 months), with a low percentage of children having a combination of cow's milk with IgE directed against egg, cat and/or HDM (3/20 at 12 months and 2/17 at 24 months). At 24 months only two children had an isolated house dust mite (HDM) sensitisation. Such a distribution of sensitisation with a high prevalence of IgE

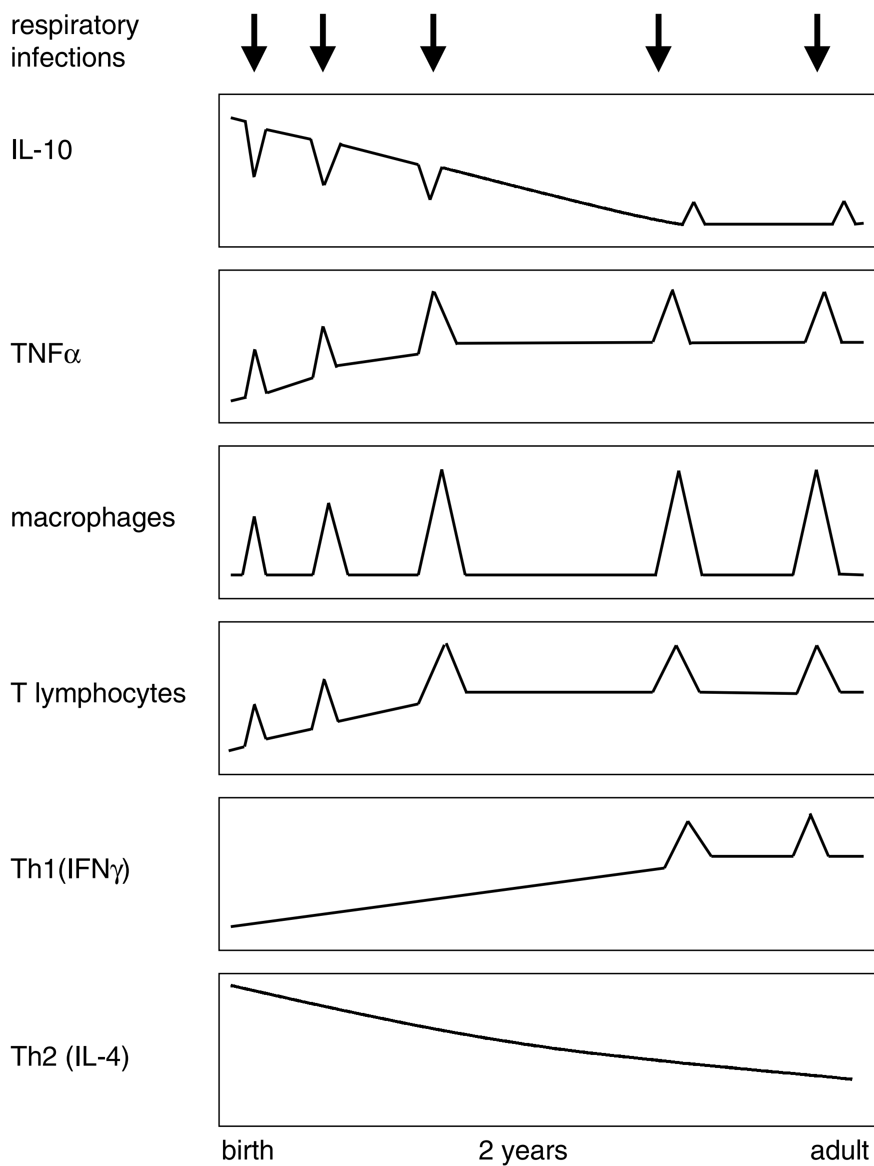

Figure 8.1: Model for sequence of events of local and peripheral immune maturation (see text for explanation). The depicted number of respiratory infections is an underestimation of the true numbers of respiratory infections.

directed against food allergen and a low prevalence of IgE directed against aeroallergens has been described before [122].

At the age of two, we found that 13% (11/82) of children had developed atopic dermatitis (AD), with the highest dermatitis score observed in the child with the multiple sensitisations of cow's milk, egg, cat, and HDM. Although total number of children is low, this is a clear suggestion that HDM sensitisation is a risk factor for the development of AD in our cohort. Two out of 5 children with IgE directed against HDM developed AD, whereas this was only the case for 1 out of 44 children without HDM sensitisation. Similar to other studies [247, 21, 57, 20] in the VIGALL birth cohort we also observed a trend for a higher risk of developing AD, allergen sensitisation, and elevated levels of total IgE (10 IU/ml or more at 12 months and 20 IU/ml or more at 24 months) in children with a positive compared to negative FHA (data not shown). The limited number of children in this study did not allow us to examine differences in host immune responses during (upper) respiratory tract infection and immune maturation with age as a function of allergic disease expression (AD, allergen sensitisation, or elevated levels of total IgE). As also a positive family history of atopy is linked to an increased chance for the later development of allergic disease, we used family history of atopy as a substitute for clinically diagnosed allergic disease. During respiratory infection we did not find differences in the nasal immune responses between children with a positive and negative family history of atopy. Also no differences were found in the pattern of immune maturation with age between both groups of children.

To get a clear insight whether immune responses during upper respiratory tract infection and maturation of these responses with age differ between children who will and will not develop allergic disease, prolonged analysis is needed. Therefore children will be studied at the age of 6 years, when a better diagnosis of allergic phenotypes is possible. This information is important to determine whether and to what extent differences in the immune maturation contribute to development of allergic disease and whether this is influenced by repeated viral respiratory tract infections during childhood.

8.7 Conclusion

In the current thesis the first *in vivo* data were documented on nasal immune responses elicited by viral upper respiratory tract infections and the maturation of the nasal immune system in children during the first two years of life. We found that infants show high responses of the regulatory cytokine IL-10 in the nose. This cytokine is probably involved in regulating local and peripheral tolerance against the various new antigens the baby encounters after birth and suppressing harmful inflammatory responses. This immuno-suppressive IL-10 milieu in the nose is downregulated during respiratory tract infection allowing an effective anti-

viral host immune response which in infants consist of high TNFα production, and which results in a gradual decrease in expression of IL-10 at baseline with age. This could subsequently lead to the development of Th1 and suppression of Th2 immunity with age. As allergic disease is characterized by overproduction of Th2 cytokines, a deregulation in immune maturation may underlie the increase in prevalence of allergic disease past decades. As proposed in the hygiene hypothesis, impaired immune maturation and enhanced risk of allergic disease could result from a relative lack of immune stimulation by respiratory tract infections due to an increased hygiene and health status. In the current thesis we provided evidence that respiratory tract infections could indeed stimulate immune maturation. Further prospective follow up studies with larger numbers of children and a longer follow up period are needed to elucidate whether respiratory infections protect children from developing allergic disease.

Summary

The human body has an extensive defence mechanism (immune system) for coping with pathogens. It is regulated by signalling molecules called cytokines. Cytokines are produced by various cells of the immune system such as leucocytes (e.g. T-cells and macrophages) but also by nasal and pulmonary epithelial tissue. There are several different types of cytokines. Th1 cytokines are involved in the eradication of bacterial and viral pathogens, while Th2 cytokines are involved in the defence against parasites. The production of Th1 cytokines is suppressed by Th2 cytokines and vice-versa so that the production of both cytokines is kept in balance. An overproduction of Th1 cytokines is found in auto-immune disorders, while allergic disease is frequently accompanied by high Th2 cytokine production. Furthermore, pro-inflammatory cytokines can induce general inflammatory reactions, while anti-inflammatory and regulatory cytokines may downregulate these responses.

Immune responses in newborns are immature. This is seen in relatively high levels of Th2 and regulatory cytokines and low levels of Th1 cytokines compared to adults. The infant immune system matures with age. This maturation process consists of a relative increase in the production of Th1 cytokines compared to Th2 cytokines. Viral respiratory infections in infants may stimulate immune maturation by their repeated Th1 stimulating effect, and thereby reduce the risk of a child developing Th2-mediated allergic disease. This hypothesis was first proposed by Professor Strachan in 1989 and is known as the 'hygiene hypothesis'. In the VIGALL study (VIGALL is the Dutch abbreviation for virally-mediated allergy), we examined whether respiratory infections predominantly induced by viruses may affect the maturation of the immune system and the development of allergic disease. We therefore looked to see which respiratory viruses are most prevalent in infants and what types of immune response are induced in the noses of these children during infection and when healthy. We then examined whether the number of respiratory infections and the maturation of the immune system were related.

Chapter 1 is a general introduction to the thesis. **Chapter 2** describes the aim and design of the study. One hundred and twenty-six infants participated in the VIGALL birth cohort study. Children were examined at the ages of 6, 12, 18 and 24 months and during a viral respiratory infection once in the first year and once in the second year of life. Each visit included an interview, a physical examination, and the collection of nasal brush and blood samples. Furthermore, various genetic and environmental characteristics of the child, general symptoms of disease, and symptoms suggestive of allergic disease were recorded using questionnaires and weekly symptom cards. Immune maturation may differ between children who will and will not develop allergic disease. To examine this issue, a considerable number of children in this study need to develop allergic disease. To increase the probability of this happening, we included a high number of children (67%) in the study whose parents suffer from allergic disease.

In our study of the potential role of viral respiratory tract infections during childhood on immune maturation, it was necessary to determine first the types

and prevalences of respiratory viruses in infants. Until recently, respiratory syncytial virus (RSV) was thought to be the most important inducer of respiratory infections in children as this virus was frequently found in during lower respiratory tract infections. In **chapter 3**, we show that rhinovirus is the most prevalent viral pathogen (40%), during both upper respiratory tract infection (URTI) and milder symptoms of rhinitis in 0-2 year old children. Remarkably, rhinovirus is even found in 20% of infants without any nasal symptoms. RSV (18%) and coronavirus infections (10%) were only observed during URTI. The high prevalence of rhinovirus in young children merits a study of the type of immune response induced by this virus and the possible impact of rhinovirus infections on the child's developing immune system.

In **chapter 4**, we look at the types of immune response induced in the noses of infants during URTI induced by rhinovirus or RSV. Is the response in infants predominantly characterised by Th2 cytokine production, which is frequently observed during RSV-induced lower respiratory tract infections, or are infants capable of inducing Th1 or pro-inflammatory cytokine response, which is the most effective response in adults to combat respiratory infections? We found high numbers of cells expressing the regulatory and anti-inflammatory cytokine IL-10 at baseline in infants. During URTI, the nasal immune system reduces these numbers considerably, allowing an induction of the Th1 and pro-inflammatory cytokine TNFα. No differences were observed in Th2 responses. This type of response in infants differs from that of adults since, in addition to TNFα, other Th1 and pro-inflammatory cytokines - IL-12, IL-18 and IFNγ - are also induced in adults during respiratory infection. Moreover, at baseline, adults usually produce only low levels of IL-10 and, during the late phase of infection, they tend to have more of an increase in IL-10 response, dampening the initial Th1 and pro-inflammatory response. The relatively high baseline expression of IL-10 in infants may prevent excessive harmful immune responses to harmless antigens which the babies encounter for the first time.

Several investigators have examined immune maturation *in vitro* in peripheral blood. In **chapter 5** we look at whether immune maturation has been observed *in vivo* in the noses of infants. This represents a more accurate reflection of the immune system. Furthermore, we look at whether maturation is natural and age-related or whether, as suggested by the hygiene hypothesis, it is affected by the numbers of respiratory tract infections. Numbers of macrophages and T lymphocytes attracted to the nose during URTI are low in infants until the age of 6 months and increase rapidly thereafter. At the age of two years, numbers of macrophages and T lymphocytes during URTI were comparable to numbers in adults during a common cold. This age-related increase in numbers of macrophages and T lymphocytes could be a result of the decrease we observed with age at baseline in numbers of cells positive for the regulatory cytokine IL-10 and the Th2 cytokine IL-4. The reason is that IL-10 and IL-4 could suppress the production of Th1

cytokines, which are necessary for the activation and attraction of T lymphocytes and macrophages. A decrease in IL-10 and IL-4 with age could therefore result in an increase in Th1 cytokines and a more effective attraction of T lymphocytes and macrophages with age during URTI. Whether this immune maturation results from an intrinsic maturation of the immune system or whether it is guided by recurrent respiratory infections is a complicated question since a high degree of correspondence is observed between the age of a child and the number of infections a child has experienced. We showed that changes in IL-4 and T lymphocytes were more strongly correlated with age than with the number of infections, whereas the reverse was true for macrophages. This suggests both an intrinsic maturation as well as an effect of recurrent respiratory infections.

Chapter 6 turns to nasal immune responses during RSV-induced lower respiratory tract infection (bronchiolitis) and compares them to immune responses during RSV-induced URTI. Symptoms of wheezing are often observed during and after bronchiolitis. As these symptoms are also found in patients with asthma, it is suggested that wheezing during and after bronchiolitis increases the risk of developing allergic disease. As allergic disease is characterised by the enhanced production of Th2 cytokines, it is suggested that bronchiolitis is also dominated by Th2 cytokine responses instead of the Th1 responses normally observed during URTI. In this chapter, however, we show that there is no enhanced Th2 (IL-4) or regulatory (IL-10) response during RSV bronchiolitis, but rather an increased pro-inflammatory or Th1 cytokine response (IL-18) compared to RSV-induced URTI. No major differences in percentages of other Th1 responses (IFNγ, IL-12) were found between the two groups. Further study is required to determine whether the production of Th1 cytokine TNFα is upregulated during bronchiolitis. These data therefore suggest that wheezing during and after bronchiolitis is not related to the development of Th2-mediated allergic disease, but may result from aspecific bronchoconstriction induced by enhanced pro-inflammatory cytokine (IL-18) production.

Chapter 7 looks at nasal immune responses in adult allergic patients during common cold. Patients with established allergic and asthmatic disease seem to have an increased risk of developing asthma exacerbations or enhanced reactions upon allergen encounter during and after respiratory viral infection. This chapter examines underlying mechanisms by comparing nasal inflammation in allergic and non-allergic adults during common cold. Increased numbers of inflammatory cells (T cells, monocytes, macrophages, NK cells), cells characteristic for allergic disease (eosinophils), and cells positive for RANTES and eotaxin (signalling molecules which attract inflammatory cells, including eosinophils) were observed during common cold in allergic and non-allergic adults. An increase in numbers of mast cells during common cold, which is also characteristic for allergic disease, was only observed in allergic adults. Compared to non-allergic adults, eosinophil influx in allergic adults persisted until two weeks after infection. This prolonged

nasal eosinophil influx may indicate that allergic patients are primed for enhanced allergic reactions upon allergen encounter following common cold.

In conclusion (chapter 8), this is the first study showing maturation of the immune response in the nose of infants. We found that nasal Th1 immune responses (characterized by macrophage and T lymphocyte responses) are low in infants and gradually increase with age, while regulatory IL-10 and Th2-type IL-4 responses were high and decreased with the age of the child. This thesis provides immunological evidence that this maturation could at least in part be regulated by repeated respiratory infections. Based on the high expression of IL-10 at baseline and its important role during URTI in infants, we would propose the following model for immune maturation in which IL-10 is the central player:

1. Infants are born with a high natural expression in the nose of the regulatory cytokine IL-10. This high level of IL-10 creates a local suppressive immune environment.

2. During viral respiratory tract infections, this immune suppression is relieved by the direct downregulation of IL-10 responses, allowing a TNFα-centred immune response during infection, which is necessary for a proper host immune response. Furthermore, suppressive baseline IL-10 responses will be reduced over time.

3. As a consequence of the relief of the suppressive effect of IL-10 with age, the initially low Th1 responses may now increase with age. More effective host immune responses as children grow older can therefore be explained by an increase in numbers of macrophages and T lymphocytes during URTI.

Finally, the question is whether the immune system matures differently in children who develop allergic disease and whether repeated viral respiratory infections protect the child from developing allergic disease. At the moment, the children in the VIGALL study are too young to give a proper diagnosis of allergic disease. Soon, the children will be 6 years old and will be examined for the development of allergic disease. This will allow us to determine the relative contribution of viral respiratory tract infections to the development of allergic disease.

Samenvatting

Het menselijk lichaam bezit een uitgebreid beschermingsmechanisme (afweersysteem) tegen ziekteverwekkers. Verschillende cellen zoals witte bloedcellen (bijvoorbeeld T cellen en macrofagen) maar ook cellen die de binnenkant van neus en longen bekleden (epitheelcellen) produceren signaalstoffen, ook wel cytokines genaamd, die het afweersysteem regelen. Er bestaan verschillende soorten cytokines. Th1 cytokines activeren specifiek het afweersysteem bij bacterile en virale infecties, terwijl Th2 cytokines betrokken zijn bij de afweer tegen parasitaire infecties. Th1 cytokines remmen de productie van Th2 cytokines en vice versa zodat de productie van beide type cytokines in balans wordt gehouden. Een overproductie van Th1 cytokines wordt wel gevonden bij auto-immuunziektes, terwijl allergie gepaard gaat met een hoge Th2 cytokine productie. Verder zorgen pro-inflammatoire cytokines voor een algemene ontstekingsreactie, terwijl anti-inflammatoire en regulatoire cytokines deze ontstekingsreactie remmen.

Kinderen worden geboren met een onrijp afweersysteem. Dit wordt gekenmerkt door een relatief hoge productie van Th2 en regulatoire cytokines en een lage productie van Th1 cytokines in vergelijking met volwassenen. Dit afweersysteem zal zich ontwikkelen, waarbij de productie van Th1 ten opzichte van Th2 cytokines wordt versterkt. Virale infecties in jonge kinderen kunnen mogelijk, door herhaalde activatie van de Th1 respons, deze ontwikkeling van het afweersysteem stimuleren en daarmee de kans op Th2 gekenmerkte allergische aandoeningen verminderen. Deze hypothese werd voor het eerst in 1989 door professor Strachan naar voren gebracht en wordt wel de 'hygiene hypothese' genoemd. In de VIGALL-studie (afkorting van Virus gemedieerde allergie) hebben we gekeken of luchtweginfecties, meestal veroorzaakt door een virus, een rol spelen in de ontwikkeling van het afweersysteem en allergie in jonge kinderen. Hiervoor hebben we allereerst onderzocht welke typen luchtwegvirussen in jonge kinderen het meest voorkomen en hoe het afweersysteem in de neus zich gedraagt tijdens een luchtweginfectie (verkoudheid) en wanneer het kind gezond is. Vervolgens hebben we gekeken of een relatie bestaat tussen het aantal verkoudheden en de ontwikkeling van het afweersysteem.

Het **eerste hoofdstuk** van dit proefschrift geeft een algemene introductie tot dit proefschrift. In **hoofdstuk 2** wordt het doel en de opzet van de studie beschreven. Honderdzesentwintig kinderen hebben deelgenomen aan de VIGALL-studie. De kinderen zijn onderzocht op de leeftijd van 6, 12, 18 en 24 maanden en tijdens een bovenste luchtweginfectie, één keer in het eerste levensjaar en één keer in tweede levensjaar. Tijdens deze onderzoeken is lichamelijk onderzoek verricht, is een anamnese afgenomen en zijn een beetje bloed en neusslijmvlies verzameld. Gedurende de hele duur van de studie is informatie verzameld over verschillende eigenschappen van de familie, de omgeving en over de gezondheid en ontwikkeling van allergie van het kind door middel van vragenlijsten en weekkaarten. Mogelijk verschilt de ontwikkeling van het afweersysteem tussen kinderen die wel en niet allergisch worden. Om dit te kunnen bestuderen moeten een redelijk aantal

kinderen in de studie allergie ontwikkelen. Om deze kans zo groot mogelijk te maken hebben we veel kinderen geïncludeerd waarvan bekend is dat de ouders allergisch zijn (67% van de kinderen).

Wanneer we willen weten of virale luchtweginfecties een effect hebben op de ontwikkeling van het afweersysteem, is het noodzakelijk eerst een goed overzicht te krijgen over welke typen virussen het meest voorkomen tijdens bovenste luchtweginfecties in deze jonge kinderen. Tot nu toe werd het respiratoir syncytieel virus (RSV) gezien als belangrijkste verwekker van luchtweginfecties bij kinderen omdat veel ernstige lagere luchtweg infecties in kinderen door dit virus worden veroorzaakt. In **hoofdstuk 3** staat echter beschreven dat het rhinovirus de meest voorkomende verwekker is van bovenste luchtweginfecties in 0-2 jarige kinderen (40%), zowel tijdens een milde als een ernstiger infectie. Rhinovirusinfecties werden zelfs gevonden in 20% van de kinderen die helemaal geen symptomen van een bovenste luchtweginfectie vertoonden. RSV (18%) en coronavirus (10%) kwam alleen voor tijdens een ernstiger bovenste luchtweginfectie. Nu we weten dat in jonge kinderen een groot deel van de bovenste luchtweginfecties door rhinovirus wordt veroorzaakt, is het interessant om te onderzoeken welk type afweer opgewekt wordt tijdens infectie met rhinovirus en of dit de ontwikkeling van het afweersysteem kan beïnvloeden.

In **hoofdstuk 4** hebben we onderzocht welk type afweerreactie jonge kinderen opwekken in de neus tijdens een bovenste luchtweginfectie, veroorzaakt door rhinovirus of RSV. Bestaat deze reactie voornamelijk uit een Th2 cytokine productie, zoals vaak wordt gezien bij een lagere luchtweginfectie door RSV, of zijn kinderen in staat een Th1 en pro-inflammatoire cytokine respons op te wekken, wat in volwassenen een effecieve respons is gebleken ter bestrijding van de luchtweginfectie? We vonden dat jonge kinderen normaal veel cellen met het regulatoire en afweeronderdrukkende cytokine IL-10 in het neusslijm hebben. Tijdens een bovenste luchtweginfectie reageerde het afweersysteem door dit hoge aantal cellen met IL-10 te verlagen, zodat een afweerreactie van het Th1 en pro-inflammatoire TNFTNFα mogelijk werd. Er was geen verandering te zien in de productie van Th2 cytokines. Deze afweerreactie in jonge kinderen is anders dan in volwassenen, aangezien volwassenen tijdens een infectie behalve TNFα ook veel van de Th1- en pro-inflammatoire cytokines IL-12, IL-18 en IFNγ maken. Bovendien hebben volwassenen normaal weinig IL-10 en is in de late fase van infectie juist een stijging van IL-10 zien om de initiële Th1- en pro-inflammatoire respons te remmen. De relatief hoge basale expressie van IL-10 in kinderen zorgt er mogelijk voor dat het afweersysteem van het kind niet op elke nieuwe, meestal onschadelijke, prikkel uit de omgeving reageert. Omdat rhinovirus en RSV infecties in jonge kinderen dus de productie van ten minste een deel van de Th1 cytokines kunnen opwekken, suggereert dit dat deze virusinfecties ook de ontwikkeling van het afweersysteem kunnen stimuleren.

De ontwikkeling van het afweersysteem is door verschillende onderzoekers *in vitro*(in een reageerbuis) op bloedcellen van kinderen onderzocht. In **hoofdstuk 5** hebben we onderzocht of ook een ontwikkeling plaatsvindt van het afweersysteem *in vivo* in het neusslijm van jonge kinderen. Dit is een meer natuurlijke weergave van het afweersysteem. Bovendien hebben we onderzocht of deze ontwikkeling komt door een natuurlijke ontwikkeling met de leeftijd of dat het aantal luchtweginfecties hier een effect op heeft, zoals is voorgesteld in de hygiene hypothese. Terwijl bij kinderen jonger dan 6 maanden het aantal afweercellen (macrofagen en T cellen) in de neus tijdens een bovenste luchtweginfectie nauwelijks toenam, werd de toename tijdens infectie steeds groter naarmate het kind ouder werd. Het aantal afweercellen tijdens infectie op de leeftijd van twee jaar was gelijk aan het aantal in volwassenen tijdens een verkoudheid. De toename in macrofagen en T cellen met de leeftijd kan het gevolg zijn van de daling die we zagen in het aantal cellen met het regulatoire cytokine IL-10 en het Th2 cytokine IL-4 in gezonde kinderen. IL-10 en IL-4 kunnen namelijk de productie van Th1 cytokines onderdrukken, die op hun beurt weer voor een aantrekking van macrofagen en T cellen kunnen zorgen. Een daling van IL-10 en IL-4 met de leeftijd kan dus zorgen voor een toename van Th1 cytokines en vervolgens meer macrofagen en T cellen tijdens infectie. Of de ontwikkeling van het afweersysteem in de neus het gevolg is van een natuurlijke ontwikkeling met de leeftijd of dat deze beïnvloed wordt door het aantal luchtweginfecties is moeilijk te beantwoorden. Naarmate het kind ouder wordt heeft het meestal ook meer infecties doorgemaakt waardoor het effect van beide variabelen moeilijk van elkaar te onderscheiden is. We zagen echter dat het aantal cellen met IL-4 en het aantal T cellen beter correleerden met de leeftijd dan met het aantal infecties, terwijl we het omgekeerde zagen voor het aantal macrofagen. Dit suggereert zowel een natuurlijk effect van de leeftijd als een effect van luchtweginfecties op de ontwikkeling van het afweersysteem in de neus van jonge kinderen.

In **hoofdstuk 6** is het type afweerreactie onderzocht in kinderen met een lagere luchtweginfectie (bronchiolitis) in vergelijking met kinderen met een bovenste luchtweginfectie veroorzaakt door RSV. Kinderen met een RSV bronchiolitis hebben namelijk een verhoogde kans op symptomen van piepen tijdens en na infectie, wat ook veel wordt gezien in patiënten met astma. Piepen tijdens en na een infectie wordt daarom vaak geassocieerd met de ontwikkeling van astma. Omdat astma wordt gekenmerkt door veel Th2 cytokine productie, hebben verschillende onderzoekers gesuggereerd dat tijdens een RSV bronchiolitis in jonge kinderen ook veel Th2 cytokines worden geproduceerd, in tegenstelling tot de Th1 cytokines die normaal tijdens bovenste luchtweginfectie worden aangemaakt. In dit hoofdstuk hebben we echter aangetoond dat kinderen tijdens een RSV bronchiolitis niet meer of minder cellen in het neusslijm hebben met Th2 (IL-4) of regulatoire (IL-10) cytokines dan kinderen met een bovenste luchtweginfectie, maar wel veel meer cellen met het Th1 of pro-inflammatoire cytokine IL-18. Kinderen met een bron-

chiolitis hadden ook een kleine toename van het Th1 cytokine IL-12, maar niet van IFNγ. Helaas hebben we de productie van TNFα niet gemeten in kinderen met bronchiolitis. Deze resultaten suggereren daarom dat piepen na bronchiolitis niet gerelateerd is aan het ontstaan van allergie of astma met Th2 cytokine productie als kenmerk, maar eerder resulteert uit een aspecifieke bronchoconstrictie als gevolg van een overvloedige pro-inflammatoire cytokine productie (IL-18) tijdens infectie. Onze resultaten verklaren dan ook waarom in andere studies geen verband gevonden wordt tussen een RSV bronchiolitis en de ontwikkeling van astma.

In **hoofdstuk 7** is het afweersysteem onderzocht tijdens een verkoudheid in volwassenen met een allergie. Volwassenen met allergie en/of astma lopen na een luchtweginfectie verhoogde kans om een astmaexacerbatie of allergische reactie te ontwikkelen. Om inzicht te krijgen in het onderliggend mechanisme van het afweersysteem, is in dit hoofdstuk het type afweerreactie van allergische en niet-allergische volwassenen tijdens een verkoudheid vergeleken. In beide groepen werd in de neus een verhoogd aantal algemene ontstekingscellen (T cellen, monocyten, macrofagen, NK cellen), cellen specifiek voor allergie (eosinofielen) en cellen met de chemokines RANTES en eotaxine (signaalstoffen met een aantrekkende kracht voor o.a. eosinofielen) aangetroffen tijdens verkoudheid. Het aantal mestcellen, eveneens kenmerkend voor allergie, was alleen verhoogd tijdens verkoudheid in allergische volwassenen. Bovendien hield de toename in aantal eosinofielen in allergische volwassenen tijdens verkoudheid langer aan, tot tenminste twee weken na infectie, dan in niet-allergische volwassenen. Deze relatief lange afweerreactie in allergische volwassenen na verkoudheid kan een verhoogde gevoeligheid geven waardoor makkelijk een allergische reactie kan ontstaan.

Concluderend (**hoofdstuk 8**), laat deze studie voor het eerst zien dat het afweersysteem in de neus zich ontwikkelt gedurende de eerste twee levensjaren. Th1 reacties (gekenmerkt door macrofagen en T cellen) zijn laag in jonge kinderen en nemen toe in de loop van de jaren, terwijl de regulatoire en Th2 cytokine productie hoog is en langzaam afneemt. Dit proefschrift levert immunologische bewijs dat deze ontwikkeling kan worden gestimuleerd door virale bovenste luchtweginfecties. Omdat het regulatoire cytokine IL-10 overvloedig wordt geproduceerd in jonge kinderen en bovendien een belangrijke rol speelt tijdens bovenste luchtweginfecties, stellen we voor de ontwikkeling van het afweersysteem het volgende model voor waarbij IL-10 centraal staat:

1. Jonge kinderen produceren veel van het regulatoire cytokine IL-10. Dit leidt tot een cytokine milieu in de neus waarbij grote delen van de overige cytokine productie worden onderdrukt.

2. Tijdens een bovenste luchtweginfectie reageert het afweersysteem hierop door het aantal cellen met IL-10 te verlagen waardoor een hogere productie van het Th1 cytokine TNFα mogelijk wordt, wat nodig is voor het opruimen

van de infectie. Bovendien zal zo met toenemende leeftijd de IL-10 productie afnemen.

3. Als gevolg van een afname van het onderdrukkende effect van IL-10 met de leeftijd kan nu de oorspronkelijk lage productie van Th1 cytokines toenemen naarmate het kind ouder wordt. Dit verklaart de verbeterde afweer naarmate het kind ouder wordt door toename in aantallen macrofagen en T cellen tijdens infectie met de leeftijd van de kinderen.

Tenslotte brengt dit ons bij de centrale vraag of de ontwikkeling van het afweersysteem verschilt tussen gezonde kinderen en kinderen met allergie en of virale infecties kinderen beschermen tegen de ontwikkeling van allergie en astma. Op het ogenblik zijn de kinderen uit het VIGALL onderzoek nog te jong om een nauwkeurige diagnose van astma te kunnen stellen. Binnenkort zijn alle kinderen 6 jaar en wordt onderzocht welke kinderen allergisch zijn. Deze informatie biedt ons de mogelijkheid om te onderzoeken of luchtweginfecties in jonge kinderen de kans op het ontwikkelen van allergie en astma beïnvloeden.

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Inesz

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1998-2003	Wetenschappelijk onderzoek naar 'virale respiratoire infecties en de ontwikkeling van de immuunrespons in de neus van jonge kinderen' op de afdeling Keel-, Neus- en Oorheelkunde, in samenwerking met de afdeling Kindergeneeskunde en het instituut voor Virologie (Erasmus Medisch Centrum in Rotterdam). De belangrijkste resultaten van dit onderzoek staan beschreven in dit proefschrift.
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List of Publications

- **I.J. van Benten**, A. KleinJan, H.J. Neijens, A.D.M.E. Osterhaus, W.J. Fokkens. Prolonged nasal eosinophilia in allergic patients following common cold. *Allergy 2001 Oct;56(10):949-56.*
- **I.J. van Benten**, C.M. van Drunen, L.P. Koopman, A. KleinJan, B.C. van Middekoop, L. de Waal, A.D.M.E. Osterhaus, H.J. Neijens, W.J. Fokkens. RSV-induced bronchiolitis but not upper respiratory infection is accompanied by an increased nasal IL-18 response. *J Med Virol. 2003 Oct;71(2):290-7.*
- **I.J. van Benten**, L.P. Koopman, H.G.M. Niesters, W.C.J. Hop, B.C. van Middelkoop, L. de Waal, C.M. van Drunen, A.D.M.E. Osterhaus, H.J. Neijens, W.J. Fokkens. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. *Pediatric Allergy and Immunology, 2003.*
- **I.J. van Benten**, C.M. van Drunen, L.P. Koopman, B.C. van Middelkoop, W.C.J. Hop, A.D.M.E. Osterhaus, H.J. Neijens, W.J. Fokkens. Age- and infection-related maturation of the nasal immune response in 0-2 year old atopic and non-atopic children. *Submitted for publication.*
- **I.J. van Benten**, C.M. van Drunen, J.L.M. Koevoet, L.P. Koopman, W.C.J. Hop, A.D.M.E. Osterhaus, H.J. Neijens, W.J. Fokkens. Reduced nasal IL-10 and enhanced TNFα responses during rhinovirus and RSV-induced upper respiratory tract infection in atopic and nono-atopic infants. *Submitted for publication.*
- L.P. Koopman, H. Savelkoul, **I.J. van Benten**, J. Gerritsen, B.J. Brunekeef, H.J. Neijens. Increased serum IL-10/12 ratio in wheezing infants. *Pediatr Allergy Immunol. 2003 Apr;14(2):112-119.*
- L. de Waal , L.P. Koopman, **I.J. van Benten**, A.H. Brandenburg, P.G. Mulder, R.L. De Swart, W.J. Fokkens, H.J. Neijens, A.D.M.E. Osterhaus. Moderate local and systemic respiratory syncytial virus-specific T-cell responses upon mild or subclinical RSV infection. *J Med Virol. 2003 Jun;70(2):309-18.*
- J. Tekstra, H. Beekhuizen, J.S. van De Gevel, **I.J. van Benten**, C.W. Tuk, R.H. Beelen. Infection of human endothelial cells with *Staphylococcus aureus* induces the production of monocyte chemotactic protein-1 (MCP-1) and monocyte chemotaxis. *Clin Exp Immunol. 1999, Sep; 117(3):489-95.*

- J.G. van Dijk, **I.J. van Benten**, C.G. Kramer, D.F. Stegeman. CMAP amplitude cartography of muscles innervated by the median, ulnar, peroneal, and tibial nerves. *Muscle Nerve 1999 Mar;22(3):378-89.*
- H. Beekhuizen, J.S. van de Gevel, B. Olsson, **I.J. van Benten**, R. van Furth. Infection of human vascular endothelial cells with *Staphylococcus aureus* induces hyperadhesiveness for human monocytes and granulocytes. *J Immunol. 1997; 158(2): 774-782.*

Abbreviations

AD	Atopic dermatitis
BCG	Bacillus Calmette-Guérin
CBMC	Cord blood mononuclear cells
CD	Cluster of differentiation
CMV	Cytomegalovirus
DC	Dendritic cell
FHA	Family history of atopy
HDM	House dust mite
hMPV	Human metapneumovirus
ICAM	Intracellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MBP	Major basic protein
MHC	Major histocompatibility class
NK	Natural killer cell
PIV	Parainfluenzavirus
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinine
PMA	Phorbol 12-myristate 13-acetate
RSV	Respiratory syncytial virus
TGF	Transforming growth factor
Th	T helper lymphocyte
TNF	Tumor necrosis factor
Tr	T regulatory lymphocyte
URTI	Upper respiratory tract infection
VAS	Visual analogue scale
VIGALL	Virus gemedieerde allergie

Ik trok een streep:
tot hier,
nooit ga ik verder dan tot hier.
Toen ik verder ging
trok ik een nieuwe streep,
en nog een streep.
De zon scheen
en overal zag ik mensen
haastig en ernstig,
en iedereen trok een streep,
iedereen ging verder.

Toon Tellegen