

Reduced Nasal IL-10 and Enhanced TNF α Responses During Rhinovirus and RSV-Induced Upper Respiratory Tract Infection in Atopic and Non-Atopic Infants

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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, nasal immune responses were examined 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 proinflammatory 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 TNF α . IL-10 responses were inversely related to TNF α responses. No enhanced responses were observed for IFN γ , 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 TNF α response. TNF α in turn could replace the immature production of IL-12, IL-18 and IFN γ during URTI to induce an effective clearance of

the viral infection and which could stimulate the maturation of Th1 cytokine production in infancy.

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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 2 years of life [Vesa et al., 2001]. In this study, nasal immune responses were examined

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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 [Brandenburg et al., 1997]. While a strong Th1-mediated response is required for the efficient eradication of respiratory pathogens [Papadopoulos et al., 2002], *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 [Lee et al., 1996; Sautois et al., 1997; Chalmers et al., 1998], but also that infants favour the production of Th2 cytokines over Th1 cytokines [Prescott et al., 1998; Ribeiro-do-Couto et al., 2001]. As a possible consequence, children in the first 2 years of life suffer on average from 6 to 8 respiratory infections a year. With the maturation of the immune system, this gradually declines to 3–4 a year in school-age children [Denny, 1995].

Rhinovirus is the most prevalent inducer of mild URTI in adults [Makela et al., 1998]. In infants and young children also, the majority of URTIs are induced by rhinovirus (manuscript *in press*) [Vesa et al., 2001]. 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 [Blomqvist et al., 2002]. 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 [Ukkonen et al., 1984].

There are very few studies examining immune responses in young children during URTI. Noah et al. [1995] found increased levels compared to baseline of Th1 and proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF α in nasal lavages of children aged 0–3.5 years with clinically defined URTI. 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 cytokine TNF α 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 [Sung et al., 2001]. We are not aware of any study comparing immune responses in children during URTI induced by rhinovirus or RSV.

In this study, 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 were examined in order to estimate the amount of stimulatory effect of these infections on immune maturation. To this end, Th1-related (IL-2, IL-12, IFN γ , TNF α), Th2-related (IL-4, IL-5), proinflammatory (IL-18) and regulatory (IL-10) cytokine responses were measured in nasal brush samples of infants (aged 2–26 months) with a defined rhinovirus or RSV infection.

METHODS

Participants Study I: RSV- and Rhinovirus-Induced URTI

To study nasal immune responses during URTI in infants, we selected infants (aged 2–26 months) from the VIGALL (Virus mediated allergy) birth cohort study [Koopman et al., 2000] 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 family history of allergy (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 [Lakwijk et al., 1998]. When both parents reported not having allergic disease, the children were classified as family allergy-negative.

As a control group, eight children were selected (matched for family history of allergy, 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 [Williams et al., 1995]. 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, only children infected by rhinovirus who suffered from URTI (N=20), from rhinitis (N=11), or who were asymptomatic during rhinovirus infection (N=7) were selected. Definitions of the symptom groups based on severity of rhinovirus infection are shown in Table I. 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

TABLE I. Definition of Symptom Groups

	Virus positive	Runny nose	General illness symptoms ^a
URT I	+	+	+
Rhinitis	+	+	–
Asymptomatic	+	–	–
Non-infected	–	–	–

^aGeneral malaise, fever, loss of appetite and/or sleeping difficulties.

or rhinitis, with one child having a non-specific cough. Immune 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 2 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 influenza virus, parainfluenza virus, RSV, adenovirus, cytomegalovirus, enterovirus and echovirus. For that purpose, nasal brush supernatant was cultured on Hep-2 cells and, after 1 week of culture, viruses were detected using immunofluorescence [Rothbarth et al., 1988]. Additionally, nasal brush cells from the pellet were stained with fluorescent-labelled antiviral antibodies to detect RSV, influenza virus, parainfluenza virus 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 [Pitkaranta et al., 1997].

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 described previously [Godthelp et al., 1996]. In brief, slides were pre-incubated with 10% v/v normal goat serum (CLB, The Netherlands) (10 min) and subsequently for 60 min with mouse anti-human monoclonal antibodies directed against IL-18 (20 µg/ml, clone 500-M87, Peprotech, London, UK) diluted in PBS supplemented with 1% blocking reagent (w/v) (Boehringer Mannheim, Germany). After incubation for 30 min with biotinylated goat anti-mouse Ig serum, slides were incubated with polyclonal goat anti-biotin antibody for 30 min. After incubation with New Fuchsin substrate (Chroma,

Kongen, Germany), sections were counterstained with Gill's haematoxylin 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 [Braunstaal et al., 2001] 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, clone 1-41-1, Novartis, Basel, Switzerland), IL-10 (10 µg/ml, clone IC25-471, Instruchemie, Delfzijl, The Netherlands), IL-12p70 (5 µg/ml, clone 24945.11, R&D systems, Abingdon, UK) or an isotypic control antibody for 60 min. 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 min) (NEN, Inc., Boston, MA), biotinyl tyramide in Tris-HCl buffer (10 min) for amplification of the staining signal, and with alkaline-phosphatase conjugated goat-anti-biotin (30 min). After incubation with New Fuchsin substrate (Chroma), sections were counterstained with Gill's haematoxylin 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, TNF α and IFN γ

Protein levels of IL-2, IL-4, IL-5, IL-10, TNF α and IFN γ were measured in a supernatant of nasal brush samples using the Cytometric Bead Array (CBA) system (BD Biosciences, San Diego, CA). 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 six 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 hr. 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 TNF α , and 7.1 pg/ml for IFN γ . Values lower than the sensitivity level of the assay were recorded as 0. For samples with concentration levels above the sensitivity level we normalized for sampling differences between patients using the

amount of protein recovered during the nasal brush. Protein concentrations were measured the Bradford method [Bradford, 1976] with Coomassie brilliant blue G-250 (Merck, Darmstadt, 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 inpatient 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 allergy 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 .

RESULTS

Symptomatology and Patient Characteristics

Infection-related symptoms and patient characteristics in each group of children are shown in Table II. Children with either rhinovirus- or RSV-induced URTI suffered by definition from a runny nose with additional infection-related symptoms (see "Methods"). 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 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 [Corne et al., 2001], 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 proinflammatory (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 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 (Fig. 1B), the Th1-related cytokine IL-12 (Fig. 1C), or the proinflammatory cytokine IL-18 (Fig. 1D) compared to non-infected children.

Increased Nasal Expression of TNF α 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 (TNF α , IFN γ , IL-2), Th2-related (IL-4, IL-5), and

TABLE II. Prevalence of Symptoms and Patient Characteristics in Infected and Non-Infected Children

	RSV URTI ^a [(N = 7), (%)]	Rhinovirus URTI ^a [(N = 20), (%)]	Rhinovirus rhinitis [(N = 11), (%)]	Rhinovirus asymptomatic [(N = 7), (%)]	Non-infected [(N = 8), (%)]
Runny nose	7 (100)	20 (100)	20 (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 ^b	5 (71)	11 (55)	5 (46)	5 (71)	4 (50)
Gender (boy)	2 (29)	6 (30)	6 (55)	3 (43)	5 (63)
Age (months) ^c	10 (2–18)	15 (3–22)	12 (4–23)	12 (6–18)	13 (6–26)

^aUpper respiratory tract infection.

^bFamily history of atopy.

^cMedian (range).

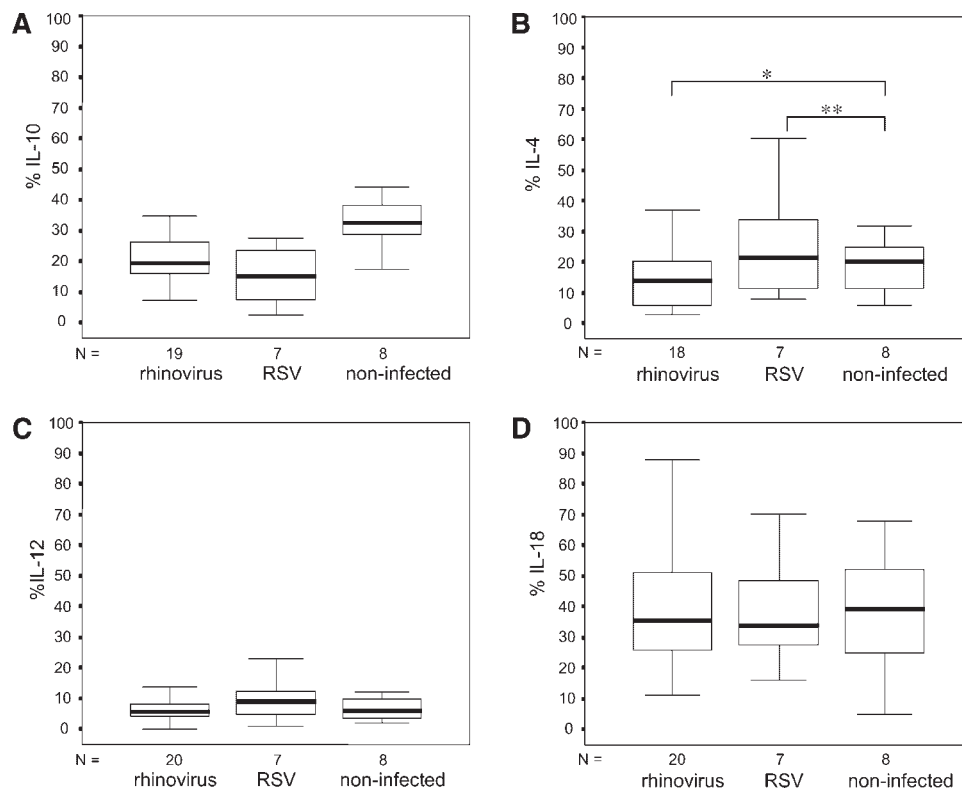


Fig. 1. Percentages of nasal brush cells positive for IL-10 (A), IL-4 (B), IL-12 (C), and IL-18 (D) in children with rhinovirus- or RSV-induced URTI compared to non-infected children. * $P < 0.05$, ** $P < 0.01$.

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). This precluded the possibility to link the relative expression levels based on cell numbers to the expression level based on the amount of proteins for these cytokines.

Only absolute levels of IFN γ and TNF α were high enough for analysis (IFN γ median: 59 pg/ml, range 0–279 pg/ml; TNF α median: 21 pg/ml, range: 0–794 pg/ml). Cytokine levels were expressed per mg total protein (median 138 μ g/ml, range 23–834 μ g/ml) measured in each individual sample. Children with rhinovirus-induced URTI produced significantly higher levels of TNF α protein (median 20 pg/mg total protein) than non-infected children (median 3.5 pg/mg total protein) (Fig. 2A) ($P < 0.01$). In addition, during RSV-induced URTI, an increase in levels of TNF α was observed (median 30 pg/mg total protein) ($P = 0.01$), which did not differ significantly from rhinovirus-induced URTI. In contrast to TNF α , no effect of viral infection could be detected on IFN γ responses (median rhinovirus 22 pg/mg total protein, RSV 49 pg/mg total protein, non-infected 39 pg/mg total protein) (Fig. 2B).

Nasal IL-10 and TNF α 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 I, II).

Figure 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%) (URT: $P = 0.03$; 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% vs. 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 TNF α responses upon rhinovirus infection (Fig. 3B). Children with rhinovirus-induced URTI and rhinitis had higher levels of TNF α (median 20 pg/mg total protein and 11 pg/mg total protein, respectively) than non-infected children (median 3.5 pg/mg total protein) (URT: $P < 0.01$; rhinitis: $P = 0.02$). Levels of TNF α did not differ

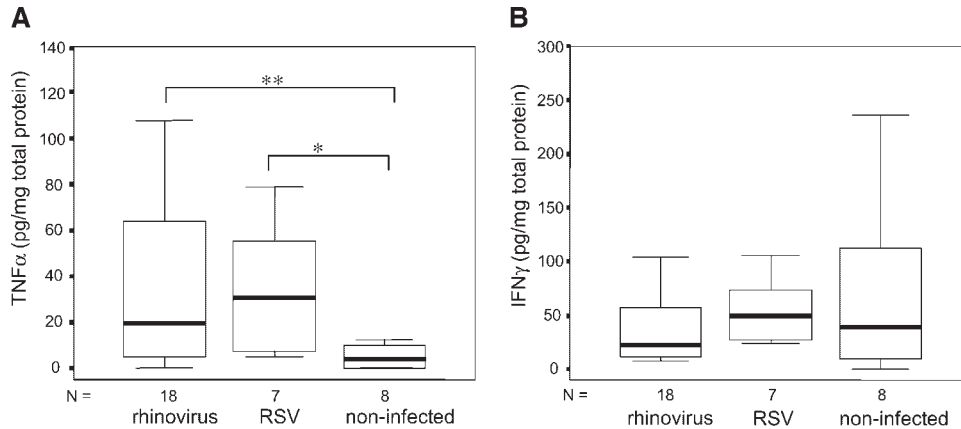


Fig. 2. Protein levels of TNF α (A) and IFN γ (B) in nasal brush samples of children with rhinovirus- or RSV-induced URTI compared to non-infected children. * $P < 0.05$, ** $P < 0.01$.

significantly between children with rhinovirus-induced URTI and rhinitis. Levels of TNF α 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 IFN γ 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.

High Correlation Between Nasal IL-10 and TNF α Responses

As IL-10 has been implicated in the down-regulation of various cytokines, including TNF α [Shin et al., 1999], we investigated a possible relationship between the decreasing numbers of IL-10 positive cells in nasal brush samples and the increasing protein levels of TNF α . To examine this relation, the individual values for both

variables at each sampling moment were plotted (Fig. 4). An inverse relation was observed between IL-10 and TNF α responses ($P = 0.04$).

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 the production of increased levels of TNF α protein. In contrast to adult responses during URTI [Corne et al., 2001], no response was observed in children involving the Th1-related cytokines IFN γ and IL-12, or proinflammatory cytokine IL-18. The immaturity of the in vivo IFN γ and IL-12 response is probably a reflection of in vitro data showing a low IFN γ and IL-12 response in neonatal dendritic cells and T lymphocytes after polyclonal stimulation [Chalmers et al., 1998; Langrish et al., 2002]. The data suggest that the Th1 response in infants, in the absence of normal adult-like IFN γ and IL-12 responses, largely depends on the increase in TNF α during URTI and that immune responses are only partially mature. The induction of a TNF α response

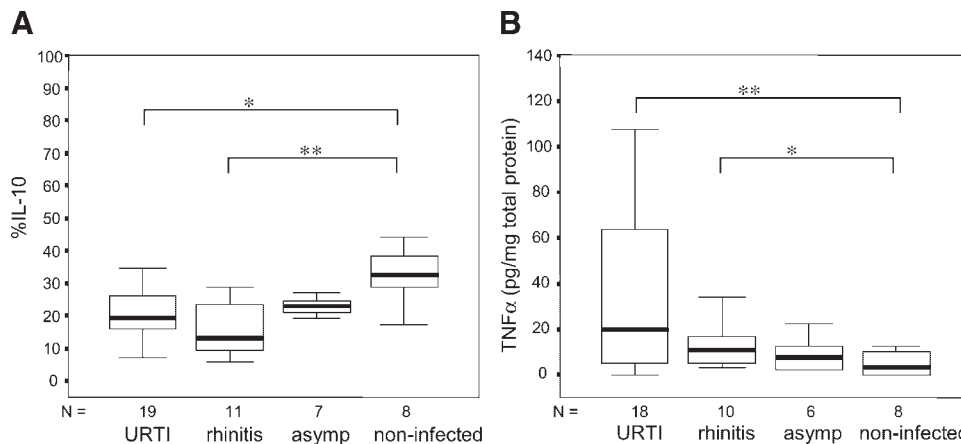


Fig. 3. Percentages of cells positive for IL-10 (A), and protein levels of TNF α (B) in nasal brush samples of rhinovirus-infected children with URTI or with rhinitis, and asymptomatic rhinovirus-infected children (asympt) compared to non-infected children. * $P < 0.05$, ** $P < 0.01$.

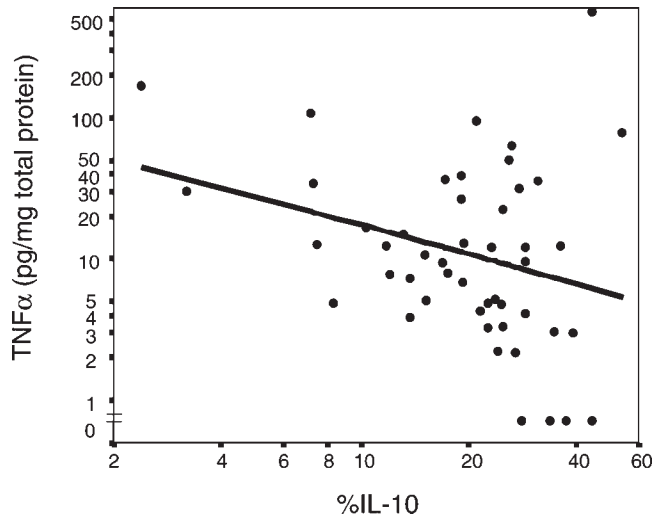


Fig. 4. Inverse relation between percentages of IL-10 positive cells and protein levels of TNF α in nasal brush samples.

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 TNF α responses suggested the alleviation of a suppressive effect of IL-10 on the expression of TNF α during URTI. Furthermore, we were able to show that the amount of IL-10 and TNF α 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 [Corne et al., 2001]. 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. What is similar between adults and children is that in both it is the epithelium cells of the nasal mucosa that produces IL-10. In immunohistochemical stainings no evidence was found for extensive IL-10 production by dendritic cells or T lymphocytes, with close all IL-10 positive staining cells being identified as epithelial cells. Furthermore, a substantial fraction of IL-10 positive cells in our cytopins carry cilia, identifying them uniquely as epithelial cells.

IL-10 plays a pivotal role in various immune responses and displays many distinct modes of action [Wakkach et al., 2000]. 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 inhibit directly the production of a wide range of cytokines, such as the Th1-related cytokines TNF α , IFN γ , IL-2, and IL-12 [Shin et al., 1999; Opal and DePalo, 2000], the proinflammatory cytokine IL-18 [Marshall et al., 1999], and the Th2-related cytokine IL-5 [Schandene et al.,

1994]. In this capacity, IL-10 can regulate immune responses by either preventing an inflammatory response or by limiting excessive ongoing inflammation [Akdis and Blaser, 2001]. 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 [Morrison et al., 2000].

In infancy, high numbers of IL-10 positive cells were observed in the nose 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 Reen [2002], which found that levels of IL-10 in newborns were higher than in adults as measured after stimulating cord or peripheral blood cells. These high levels of IL-10 during infancy could be a reflection of the IL-10 rich environment that prevails during pregnancy [Piccinni, 2002]. Suppression of the maternal and fetal immune responses by this cytokine seems to be necessary to maintain pregnancy and ensure survival of the fetal allograft. Additionally, an active role for IL-10 during infancy is suggested by the presence of IL-10 in breast milk [Garofalo et al., 1995]. 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 [Levings and Roncarolo, 2000; Groux, 2001].

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 [Yazdanbakhsh et al., 2002]. This idea was raised in a recent study by Van den Biggelaar et al. [2000], 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. 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 [Feng et al., 2002]. As increased IFN α production is common in adults with naturally acquired influenza virus infection [Kaiser et al., 2001] and in infants with lower respiratory tract infections [Moulin et al., 1996], up-regulation of this cytokine during URTI in children may explain the suppression of IL-10 responses. Alternatively, TGF β has also been shown to inhibit IL-10 mRNA synthesis in

monocytes [Suberville et al., 2001]. Although, in general, TGF β production is up-regulated during influenza virus infection in mice [Schultz-Cherry and Hinshaw, 1996], it is not known whether this is also the case in infants during respiratory infection and could therefore explain the down-regulation of IL-10 responses.

Bont et al. [2000] suggested that the high natural expression of IL-10 in infants may underlie their high susceptibility to respiratory infections and infection-related wheezing episodes. They observed that high levels of IL-10 produced by monocytes after bronchiolitis correlated with high numbers of wheezing episodes following infection. Our data also suggest a similar relation between IL-10 and wheezing. However, these observations should be considered cautiously as only a limited number of children are involved.

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 proinflammatory cytokines. This was indeed shown by an increase in levels of the Th1-stimulating cytokine TNF α during rhinovirus or RSV-induced URTI. This resembles the situation in adults, where IL-10 has been identified as a negative regulator of TNF α [Shin et al., 1999]. TNF α in turn may activate the anti-viral host immune response through the stimulation of functional activities of cytotoxic T lymphocytes, NK cells, and macrophages [Tracey and Cerami, 1993] and the recruitment of inflammatory cells to the site of infection [Sedgwick et al., 2000]. Furthermore, together with IL-12, TNF α can promote the development of Th1 lymphocytes [Shibuya et al., 1998]. Our data showing elevated levels of TNF α in infants are in line with previous studies showing a similar increase of TNF α in nasal lavage samples of children aged 0-3.5 years during URTI [Noah et al., 1995].

In adults, in addition to a TNF α response, there is also an increase in levels of Th1 cytokine IFN γ during URTI [Corne et al., 2001]. Other studies have found an increased production of Th1-related cytokines IL-12 and IFN γ protein when adult PBMCs were infected with rhinovirus [Papadopoulos et al., 2002], and an increased production of proinflammatory cytokine IL-18 by peripheral macrophages infected by influenza A virus [Sareneva et al., 1998]. Neonatal cord-blood dendritic cells are also capable of producing IL-12 after stimulation with influenza virus [Bartz et al., 2002]. 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 IFN γ protein

levels in nasal brush samples of rhinovirus- or RSV-infected children compared to non-infected children. Data from other studies suggest that the lack of IL-12, IL-18, and IFN γ responses during URTI could be a direct reflection of the immaturity of the child's immune system [Cohen et al., 1999]. LPS stimulated neonatal DCs fail to produce IL-12p70 or induce levels of IFN γ production by T lymphocytes comparable to those in adults [Langrish et al., 2002]. Interestingly, in the same experiment, levels of IL-10 and TNF α 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 IFN γ , while IL-10, IL-6, and TNF α was secreted spontaneously and enhanced after polyclonal stimulation [Zhao et al., 2002]. Our results are directly in agreement with these *in vitro* data that point to a largely immature immune system in infants that is able to mount correct IL-10 and TNF α responses upon infection, but fails to induce proper IL-12, IL-18 and IFN γ responses. It could therefore be the case that the mature production of TNF α during URTI replaces immature IL-12, IL-18 and IFN γ 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 [Strachan, 2000]. As we found that rhinovirus and RSV were capable of inducing Th1 host immune responses, characterised by increased TNF α 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 TNF α 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 proinflammatory cytokines IFN γ , IL-12 and IL-18. Further study is needed to determine whether URTI 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 TNF α . However, no enhanced responses were observed for the Th1-related and proinflammatory cytokines IL-12, IL-18 and IFN γ during URTI. This may indicate that immature responses of IL-12, IL-18 and IFN γ during URTI in infants are replaced by mature TNF α responses in order to induce a host immune response that can clear the viral infection effectively and can stimulate Th1 immune maturation.

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