11 Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one year follow-up study

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11.1 Abstract

Respiratory syncytial virus (RSV) bronchiolitis is associated with subsequent recurrent wheezing episodes. To determine whether cytokine responses during infection can be of predictive value for the development of recurrent wheezing, we performed a follow-up study in 50 hospitalized children with RSV bronchiolitis. Monocyte and lymphocyte cytokine responses *in vitro* were studied during the acute phase of disease, and again during the convalescent phase, 3-4 weeks later. Monocyte cytokine responses, including interleukin-10 (IL-10), were measured in whole blood cultures, stimulated with lipopolysaccharide and interferon-g (LPS+IFNγ). In addition, T cell cytokine responses, including IFNγ and IL-4 production, were measured in whole blood cultures, stimulated with phytohemaglutinin (PHA) or αCD2+αCD28. Cytokine responses were analysed in relation to the development of recurrent episodes of wheezing, documented by parents in a diary during a one year follow-up period. IL-10 responses during the acute phase of RSV bronchiolitis were comparable to healthy controls. During the convalescent phase, IL-10 responses were significantly increased in patients as compared to healthy controls ($p<0.001$). At follow-up, 27 children (58%) had recurrent episodes of wheezing. IL-10 levels, measured during the convalescent phase, were significantly higher in patients who developped recurrent wheezing during the year following RSV bronchiolitis than in patients without recurrent episodes of wheezing ($p=0.006$). Moreover, IL-10 responses during the convalescent phase correlated significantly with the number of wheezing episodes ($r=0.42$, $n=46$, $p=0.004$). Interestingly, no association was found between IFNγ responses, IL-4 responses or IFNγ/IL-4 ratios and recurrent wheezing. We conclude, that monocyte IL-10 responses *in vitro* upon stimulation with nonspecific stimuli may have predictive value for the development of recurrent wheezing following RSV bronchiolitis. Moreover, our results indicate, that not only allergen-driven Th2 cytokine responses can lead to asthmatic symptoms, but also virus-induced changes in cytokine responses may result in asthmatic symptoms.

11.2 Introduction

The occurrence of recurrent episodes of wheezing in early childhood following respiratory syncytial virus bronchiolitis has been well documented (1-5). Actually, RSV bronchiolitis is followed by recurrent wheezing in 20-80% of the cases (6-8). It is thought, that these wheezing episodes are triggered by viral upper respiratory tract infections and they appear to be independent of atopy (9). In addition, follow-up studies show bronchial hyperresponsiveness 4 to 8 years after hospitalization for RSV bronchiolitis (6,7,10).
Although RSV infection induces cytokine production by a number of cells in vivo and in vitro, the relation of these cytokine responses to recurrent wheezing is largely unknown. CD4+ T cells can be functionally divided into Th1 and Th2 cells (11). This division is based on the profile of cytokine production. Th1 cells selectively secrete IFNγ and promote cell-mediated immunity. In contrast, Th2 cells secrete IL-4, IL-5 and IL-13. These cytokines are involved in humoral immunity and are thought to contribute to allergic asthmatic inflammation. Recent studies suggest that a Th2 cytokine profile during RSV bronchiolitis is associated with wheezing during follow-up (12,13).
To our knowledge, the role of monocytes/macrophages in recurrent wheezing following RSV bronchiolitis is unknown. However, it is evident that alveolar macrophages participate in the immune response during RSV infections. Alveolar macrophages recovered from bronchoalveolar lavage (BAL) fluid from children with severe RSV bronchiolitis are quantitatively the most important cell type (14). *In vitro* data show that monocytes can readily be infected by RSV (15,16). They have the ability to produce a spectrum of cytokines, including IL-12 and IL-10. Interleukin-12 is required for the initiation of the antiviral immune response (17,18), whereas IL-10 has several properties, including down-regulation of cytokine production by Th1-like T cells and inhibition of antigen presentation by antigen presenting cells (APC) (19,20). Alveolar macrophages in BAL fluid from RSV bronchiolitis patients show increased expression of pro-inflammatory cytokines, including IL-1b and TNFα, as compared to healthy controls (21). In addition, *in vitro* data show induction of IL-6, IL-8 and IL-10 production by macrophages infected with RSV (22).
The aim of this study was to determine whether cytokine responses during the acute and convalescent phase of RSV bronchiolitis are associated with the subsequent development of recurrent wheezing. We therefore studied T cell and monocyte cytokine responses in hospitalized children with RSV bronchiolitis and related these cytokine responses to recurrent episodes of wheezing in a one year follow-up period.

11.3 Methods

11.3.1 Study population

Fifty children were included during one winter epidemic in 4 hospitals in the Netherlands (Wilhelmina Children's Hospital, Utrecht; Beatrix Children's Hospital, Groningen; St. Elisabeth Hospital, Tilburg; Rijnstate Hospital, Arnhem). Inclusion criteria were: hospital admission, lower respiratory tract symptoms, age < 13 months and immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Infants with wheezing illness prior to RSV bronchiolitis were not included. One investigator (L.B.) took the history of atopy of parents, grandparents and siblings (asthma, documented food allergy,

eczema, hay fever) and inquired whether either parent had smoked in the presence of the infant during the follow-up period. Twenty-seven control children aged < 13 months without evidence of atopy or infection were selected for this study during the same winter season. Included were infants prior to minor surgery, prior to cardiac surgery in the absence of hemodynamic compromise, healthy prematurely born infants, healthy infants screened for congenital disorders and infants with mild anemia. The study was approved by the Medical Ethical Committee in all participating centers. Parents of subjects and controls gave written informed consent.

11.3.2 Whole blood cultures

Heparinized venous or arterial blood was taken within 24 hours after admission, from subjects in the Wilhelmina Children's Hospital, Utrecht (n=24). Three to four weeks later, during the convalescent phase, heparinized blood was taken from all subjects (n=50). Freshly taken heparinized blood was diluted 1:10 in RPMI 1640 medium (Life Technologies, Grand Island, NY) and aliquoted (150 µl) into 96-well culture plates (Nunc International, Denmark).

The whole blood culture stimulated with LPS is a suitable *ex vivo* method to study monocyte cytokine production under conditions in which many of the physiologically relevant cellular interactions remain intact (23). To induce monocyte IL-10 and IL-12 production, lipopolysaccharide (LPS) (100ng/ml)+IFNg (20 ng/ml) was added and cultures were incubated for 48 hours at 37°C in 5% CO_2. It has been shown that maximal monocyte IL-10 production is observed after 48 hours, which is relatively late compared to that of monocyte pro-inflammatory cytokines (20). Also monocyte IL-12 production is (sub)optimally induced after 48 hours stimulation (24). Furthermore, it has been established that monocytes are the main producers of IL-10 and IL-12 in LPS stimulated whole blood cultures (25).

To induce lymphocyte cytokine production, phytohaemagglutinin (PHA) (50µg/ml) or anti-CD2,1 (1:12000)+anti-CD2,2 (1:12000)+anti-CD28 (1:3000) monoclonal antibodies (anti-CD2/28 Moabs, CLB, Amsterdam, The Netherlands) were added and cultures were incubated for 48 hours at 37°C in 5% CO_2.

All cultures were performed in quadruplicate. Pooled supernatants were kept at -70°C.

11.3.3 Cytokine assays

In supernatants of LPS+IFNγ stimulated blood cultures IL-12 and IL-10 were measured. In supernatants of PHA stimulated cultures IFNγ and IL-4 were measured. Concentrations of IL-10, IFNγ and IL-4 were determined using ELISA kits supplied by the Dutch Laboratory for Blood Transfusion (CLB, Amsterdam, the Netherlands). The detection limit for IL-10 was 2.5 pg/ml, for IFNγ 25 pg/ml and for IL-4 1.0 pg/ml. Concentrations of IL-12 were determined using ELISA kit from R&D (Oxon, United Kingdom), the detection limit was 7.8 pg/ml. This assay recognizes only the IL-12 heterodimer and not the individual subunits of the dimer. When cytokine values were not detectable, for statistical analysis the minimum detectable level was used. When IL-10 responses were above the maximum detectable level (300 pg/ml), this level was used.

11.3.4 Follow-up evaluations

Follow-up data were collected during one year following discharge. Follow-up was performed using diaries, which were developed for this study. One investigator instructed parents how to use the diary. Starting three weeks after discharge from the hospital, parents noted the presence of "coughing" and "wheezing" on a daily base. A disease episode was defined as the presence of symptoms for two or more consecutive days. At the end of the follow-up period, patients were classified as "recurrent wheezing" if more than one episode of wheezing was noted following discharge. In addition, at the end of the study period, one investigator contacted the general practitioners of the patients by telephone and inquired if "asthma" had been diagnosed.

11.3.5 Statistical analysis

Cytokine production and IFNγ/IL-4 ratios were analyzed after logarithmic transformation. Mean (geometric mean) and standard error of mean (SEM) of cytokine levels are calculated of logarithmically transformed values. Chi-square test was used to evaluate whether gender, the need for mechanical ventilation and the presence of a positive family history of atopy were associated with recurrent wheezing. Differences in age at onset of disease, IL-10 and IL-12 responses and IFNγ/IL-4 ratios between infants with and without recurrent wheezing and infants with and without a family history of atopy were analyzed with unpaired Student's t-test. The relation between cytokine response in the acute and convalescent phase were analyzed with paired Student's t-test. Pearson's correlation coefficient was used to analyze the relation between cytokine levels and the number of reported wheezing episodes. All tests of significance were two-sided.

11.4 Results

11.4.1 Subject characteristics

The investigated population consisted of 50 patients and 27 controls. Twenty-nine patients (58%) were boys, median age was 3 months. Eleven patients (22%), including 3 pairs of twins, were born prematurely (range: 29 4/7 - 36 5/7 weeks). In the control group, 17 children were boys (63%), the median age was 4 months and 2 controls were born prematurely. Thirty-six patients (72%) and 18 controls (67%) had a positive family history of atopy. Respiratory distress was present in all children. Three infants had had apnea prior to admission. Fourteen subjects (28%) needed mechanical ventilation. In one child chronic lung disease was diagnosed, none of the patients had cardiac disease or an immunodeficiency. None of the patients received ribavirin or systemic anti-inflammatory agents, including corticosteroids. Patients did not receive inhaled steroids during RSV bronchiolitis. All patients survived.

11.4.2 Cytokine responses

Cytokine responses in LPS+IFNγ stimulated whole blood cultures from patients during the acute phase (n=24) and convalescent phase (n=50) are shown in Figure 11.1 and 11.2. During the acute phase, IL-12 production was significantly lower in patients than in controls (geometric mean 28 vs 66 pg/ml, $p = 0.007$). During the convalescent phase, IL-12 responses in patients increased to levels that were not significantly different from controls (44 pg/ml). In contrast, the amount of IL-10 produced during the acute phase

was not significantly different from controls (26 vs 38 pg/ml). During the convalescent phase, however, IL-10 production (112 pg/ml) was significantly higher than in the acute phase ($p<0.001$) and higher compared to controls ($p < 0.001$).
In PHA-stimulated whole blood cultures of patients during the acute phase of disease, both IL-4 and IFNγ responses were low (n=24). Actually, in 11 patients, levels of either cytokine were below detection limits. During the acute phase of disease, IL-4 production was lower than in controls (2.9 vs 9.6 pg/ml, $p < 0.001$), as were IFNγ responses (89 vs 602 pg/ml, $p = 0.001$). In the convalescent phase both IL-4 and IFNγ responses returned to values that were not significantly different from controls (n=49). In one patient no supernatant of PHA-stimulated whole blood cultures was collected for technical reasons.
In cultures stimulated with αCD2+αCD28 the same response pattern of IL-4 and IFNγ were seen. During the acute phase, in comparison to controls, we found decreased production of IL-4 (4.2 vs 8.1 pg/ml, $p = 0.03$) and IFNγ (8.1 vs 186 pg/ml) ($p < 0.001$). In the convalescent phase IL-4 response (6.6 pg/ml) and IFNγ response (170 pg/ml) were comparable to controls.

11.4.3 Follow-up data

Diaries were returned of 46 (92%) patients in follow-up, whereas 4 patients were lost in follow-up. Twenty-seven children (59%) had 2 or more episodes of wheezing (range 2-11 wheezing episodes). In patients requiring mechanical ventilation, 43% had recurrent episodes of wheezing in the follow-up period, which was not significantly different from-non-ventilated infants. The number of episodes of wheezing correlated strongly with the number of episodes of coughing ($r = 0.76$, $p<0.001$).
One or more episodes of coughing in follow-up was documented in 43 patients (93%). In 4 patients (9%) one episode of coughing was noted, in 7 children (15%) 2 episodes of coughing were noted and in 32 patients (70%) 3 or more episode of coughing was noted. Asthma was diagnosed by a physician in 16 patients (35%). These patients had all more than one documented episode of wheezing.
A positive history of atopy was noted in 19 infants with recurrent wheezing (70%) and in 15 infants without recurrent wheezing (79%), which was not significantly different. Six infants were exposed to tobacco smoke by at least one parent during follow-up, three had recurrent episodes of wheezing. Age at onset of disease and gender were not associated with the occurrence of recurrent wheezing.
IL-10 responses during the convalescent phase were significantly higher in infants with recurrent wheezing than without recurrent wheezing ($p=0.006$) (Figure 11.3). The difference was found between IL-10 responses in the convalescent phase in infants with and without physician diagnosed asthma ($p=0.004$). Moreover, IL-10 levels during the convalescent phase correlated with the number of episodes of wheezing ($r=0.42$, $p=0.004$) (Figure 11.4).

Figure 11.1 *Ex vivo IL-12 production during the acute and convalescent phase of RSV bronchiolitis. Blood was obtained from RSV bronchiolitis patients within 24 hours after admission to the hospital (acute phase) and 3-4 weeks later (convalescent phase) and controls. Whole blood cultures were stimulated for 48 hours with LPS (100ng/ml) and IFNγ (20 ng/ml). Data represent individual values and mean ± SEM. Data were analyzed after log transformation.*

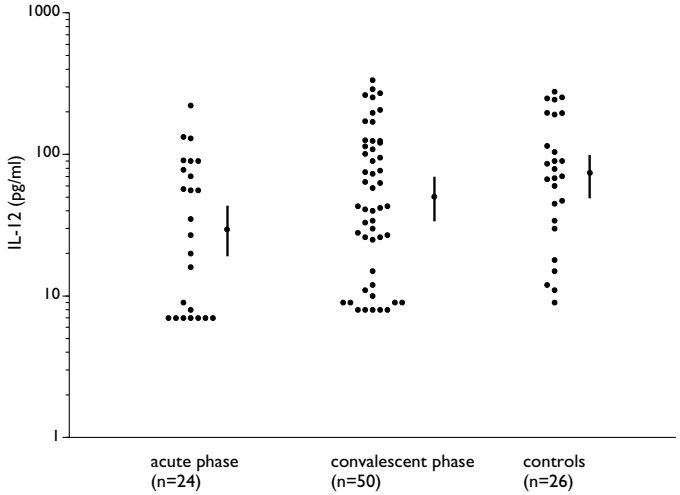


Figure 11.2 *Ex vivo IL-10 production during the acute and convalescent phase of RSV bronchiolitis. Samples and cultures as described in the legends to Figure 11.1. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.*

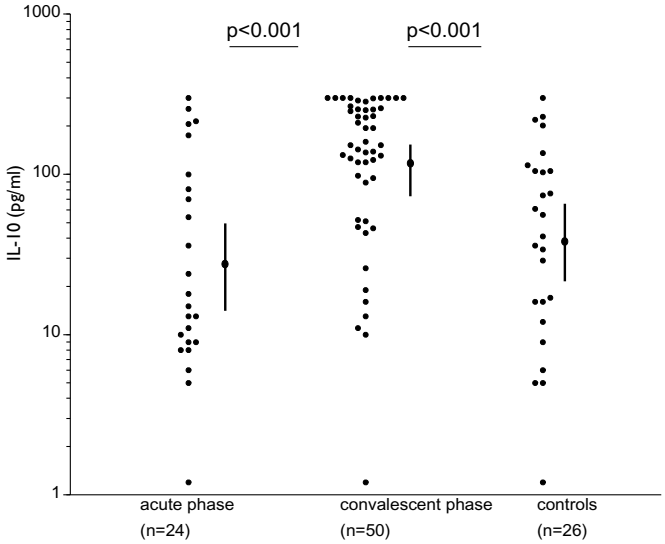
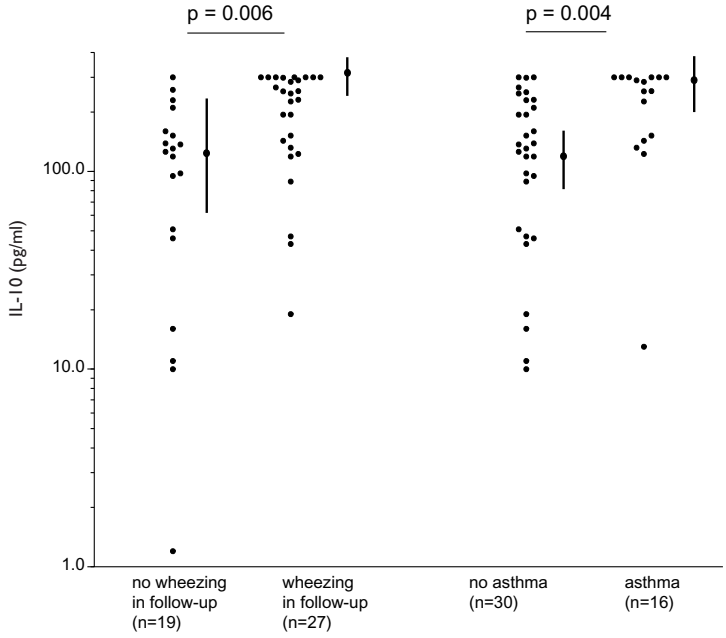


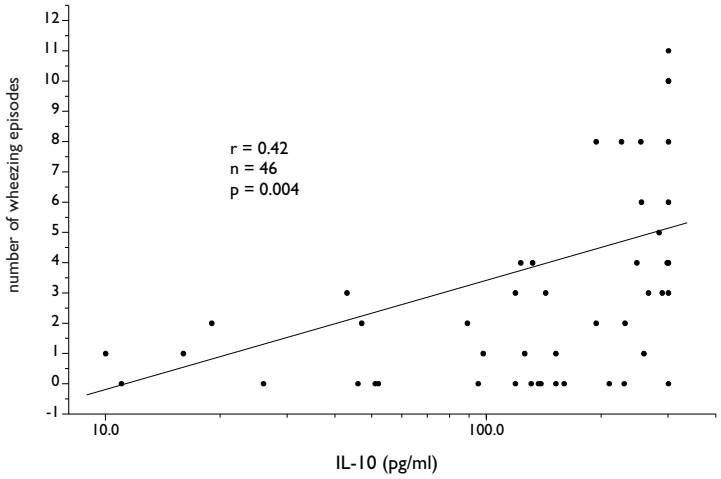
Figure 11.3 *Ex vivo IL-10 production in patients with and without subsequent recurrent wheezing and physician-diagnosed asthma.*
Blood was obtained from RSV bronchiolitis 3-4 weeks after admission (convalescent phase) and controls. Cultures as described in the legends to Figure 11.1. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



We considered the possibility that the association between IL-10 responses and recurrent wheezing could only be found during the first months of the follow-up period following RSV bronchiolitis. We therefore evaluated the association between IL-10 responses during the convalescent phase and wheezing during the last 3 months of the study period (the winter season). During this period 20 patients had ≥ 1 wheezing episode, and again, a difference was found in IL-10 responses was found between infants with and without a wheezing episode ($p = 0.02$).

Interleukin-12 responses were not associated with recurrent wheezing during follow-up. In addition, no differences in IL-4 and IFNγ responses in both PHA and αCD2+αCD28 stimulated blood cultures were found between wheezing and non-wheezing infants. Moreover, IFNγ/IL-4 ratios in PHA stimulated cultures (Figure 11.5a) during the convalescent phase were comparable for wheezing and non-wheezing infants. As expected, in infants with a positive family history of atopy, decreased IFNγ/IL-4 ratios were found in PHA stimulated cultures ($p<0.05$) (Figure 11.5b). In αCD2+αCD28 stimulated cultures the same association was seen, although this did not reach a significant level. Monocyte cytokine responses, including IL-10 responses during the convalescent phase, were not associated with family history of atopy.

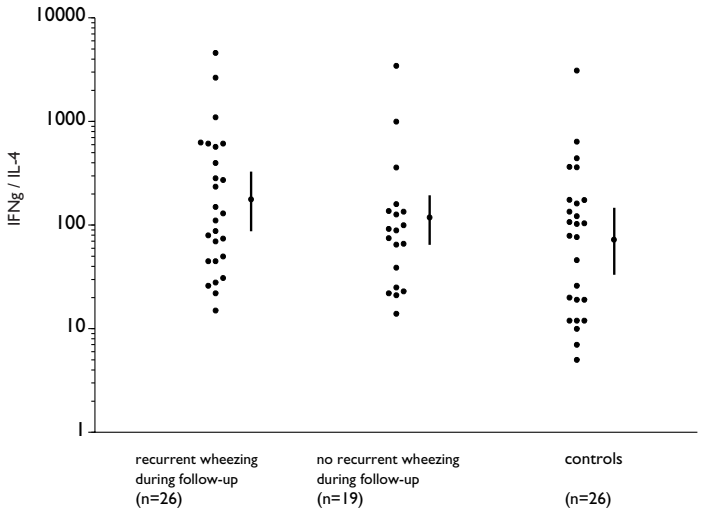
Figure 11.4 *Relation between ex vivo IL-10 production and number of wheezing episodes during a one year follow-up period.*
Blood was obtained from RSV bronchiolitis patients 3-4 weeks after admission (convalescent phase). Cultures as described in the legends to Figure 11.1. Data represent individual values. Linear regression line is drawn.



11.5 Discussion

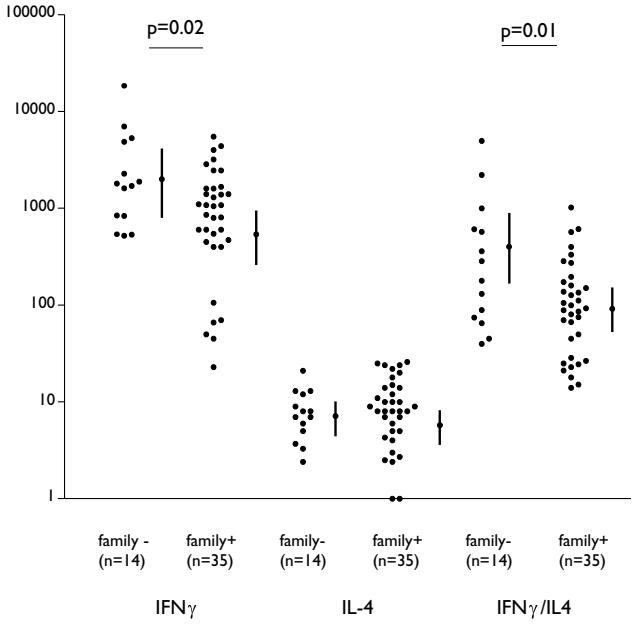
The results of this study demonstrate that increased monocyte IL-10 response *in vitro* upon stimulation with aspecific stimuli during the convalescent phase of RSV bronchiolitis is associated with the development of subsequent recurrent episodes of wheezing, during a one year follow-up period. Moreover, there was a highly significant correlation between IL-10 production in the convalescent phase and the number of wheezing episodes. The same association was found between IL-10 response and physician-diagnosed asthma. Recurrent wheezing during the year following clinical bronchiolitis was not associated with a family history of atopy. In agreement with the latter finding, T cell cytokine responses (IFNg and IL-4) in vitro upon stimulation with nonspecific stimuli were not associated with recurrent wheezing.
Clinical risk factors for the development of recurrent wheezing following RSV bronchiolitis were established by previous investigators (9,26-28). These risk factors include male gender, low age at onset of disease, and disease severity. In our study, boys were not more likely to develop recurrent wheezing than girls, consistent with findings of Sims et al., but not with findings of McConnochie and Roghmann, who found an increased risk for boys (6,29). Furthermore, we did not find a relation between either age at onset or disease severity during RSV bronchiolitis and subsequent recurrent wheezing, consistent with most other studies (3,6,7). Finally, some reports indicate an increased risk for post-bronchiolitis wheezing in children with a positive family history of atopy, but most recent reports, including ours do not support this suggestion (6,8,30).

Figure 11.5a *Relation between ex vivo IFNγ/IL-4 ratios in PHA stimulated whole blood cultures and the development of recurrent wheezing following RSV bronchiolitis.*
Blood was obtained from RSV bronchiolitis patients 3-4 weeks after admission to the hospital (convalescent phase) and from controls. Whole blood cultures were stimulated for 48 hours with PHA (50μg/ml). Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



Ex-vivo IL-10 production was significantly increased during the convalescent phase of RSV infection. Although IL-10 can be produced by different cell types, including monocytes, Th2 cells and B cells, it has been shown that IL-10 in LPS stimulated whole blood cultures, IL-10 is most likely monocyte derived (19,20,25). In the LPS+IFNg stimulated cultures in the present study, IL-8 was decreased significantly (data not shown), indicating that the increased IL-10 response is not explained by a general increase in monocyte cytokine responses.
Two mechanisms can explain changes in cytokine responses by monocytes in peripheral blood during RSV bronchiolitis. The presence of RSV ribonucleic acid (RNA) in the blood and the potential to cause productive infection *in vitro* in monocytes suggest that during RSV bronchiolitis, changes in monocyte function could result from direct infection (15,31). Another explanation is that changes in monocyte cytokine responses are the systemic consequence of local production of cytokines and other mediators by epithelial cells and macrophages in the respiratory tract during RSV infection. Finally, we note that the immune response in respiratory tract and changes in cytokine production by local macrophages are potentially different from what is found in circulating monocytes. More research is required to evaluate whether cytokine responses by circulating monocytes reflect cytokine responses by macrophages in the respiratory tract.

Figure 11.5b *Ex vivo IFNγ and IL-4 production and IFNγ/IL-4 ratios in PHA stimulated whole blood cultures in patients with a positive/negative family history of atopy.*
Samples and cultures as described in the legends to Figure 11.5a. Data represent individual values and mean ± SEM. Data were analyzed after log transformation.



Ex-vivo IL-12 production was significantly decreased during the acute phase of RSV bronchiolitis. Different viruses, including measles virus, have been shown to inhibit IL-12 production in vitro by monocytes/ macrophages (24,32). Although the effect of RSV on IL-12 production by monocytes/macrophages has not been investigated, it is conceivable that, RSV itself effectively inhibits IL-12 production. More study is needed to evaluate whether low IL-12 responses play a role in the pathogenesis of acute RSV bronchiolitis.
We propose two possible mechanisms by which increased production of monocyte IL-10 leads to recurrent wheezing. On the one hand, increased monocyte/macrophage IL-10 responses *in vivo* may result in suppression of Th1 cells and enhancement of Th2 cells by antagonizing IL-12 (33-35). As a result, this could then lead to allergic asthmatic airway inflammation. This latter possibility is not supported by our data, that show the absence of an association between IFNg/IL-4 ratios and recurrent wheezing. On the other hand, it is conceivable that in vivo increased IL-10 production leads to decreased antiviral immunity in the lower airways, as a result of suppression of antigen presentation by pulmonary macrophages. One could then speculate that viral infections in the upper respiratory tract more easily leads to infection and inflammation of the lower respiratory tract, leading to wheezing and bronchial hyperresponsiveness. This explanation is in line with the

clinical picture of wheezing following RSV bronchiolitis, usually associated with upper respiratory symptoms (8).

In this study we found decreased IFNγ and IL-4 responses in patients in the acute phase of RSV bronchiolitis. We recently described this finding and showed in addition, that depressed lymphocyte proliferative responses and T cell cytokine responses are markers of disease severity (36).

Overwhelming evidence is available that a Th2-like cytokine response pattern leads to allergic asthmatic airway inflammation. Therefore, one could hypothesize, that a Th2-like cytokine response pattern also plays a role in recurrent wheezing following RSV bronchiolitis. For example, CD4+ T-cells, specific for the RSV attachment protein (protein G) secrete IL-4 and IL-5, but little IL-2 (i.e. a Th2-like pattern) when stimulated with antigen (37). This hypothesis is supported by the study of Renzi et al. showing an association between a Th2 cytokine response after allergen (*Dermatophagoides farinae)* stimulation 5 months after hospitalization for bronchiolitis, and subsequent wheezing in 26 infants (13). In contrast to our study, in Renzi's study 43% of the patients were negative for RSV and patients requiring mechanical ventilation or with radiographic evidence for bacterial infection were excluded. Interestingly, in our study we did not find evidence for an association between IFNg/IL-4 ratios and the subsequent development of recurrent wheezing. In the present study, IFNγ/IL-4 ratios in both PHA and αCD2+αCD28 stimulated cultures were comparable for infants with and without recurrent wheezing in the follow-up period. We were capable of detecting a lower IFNγ/IL-4 ratio in infants with a family history of atopy, which resulted mainly from differences in IFNγ responses. These data demonstrate that our methods are suitable to detect biologically significant differences in IFNγ/IL-4 ratios. We note, however, that other Th2-like cytokines, including IL-5 and IL-13, have not been measured. When other Th2-like cytokines are used to assess the Th1-Th2 cytokine balance or when other *in vitro* stimuli are used, it is conceivable that other results can be found with respect to role of the Th1-Th2 cytokine balance in recurrent wheezing following RSV bronchiolitis.

We conclude that monocyte IL-10 production increases during the course of RSV bronchiolitis and that increased IL-10 production is associated with the development of recurrent wheezing and physician diagnosed asthma. We did not find support for a role of IFNγ/IL-4 balances in the development of recurrent wheezing following RSV bronchiolitis. This study indicates that not only allergen-driven Th2 cytokine responses can result in asthmatic symptoms, but also virus induced changes in monocyte cytokine responses can lead to asthmatic symptoms.

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