Chapter 2

Moderate local and systemic respiratory syncytial virus-specific T cell responses upon mild or subclinical infection.

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Abstract

Human respiratory syncytial virus (hRSV) infections are a major cause of severe respiratory disease in infants. It has been shown that there is an increased frequency of childhood wheezing in ex-bronchiolitic preteen children. This was postulated to be mediated by a vigorous virus-specific Th2 response influencing the further development of the immune system. Little is known about the possible role of the immune response to clinically mild hRSV infections in this respect. We have studied the hRSV-specific cellular immune response in infants with a laboratory-confirmed hRSV upper respiratory tract infection (URTI; *n*=13, mean age 12 months, range 2-22 months) in comparison with infants with non-hRSV mediated URTI (*n*=9, mean age 9.3 months, range 4-18 months) or infants with severe hRSV bronchiolitis (*n*=11, mean age 2.3 months, range 1-6 months). hRSV-specific cytokine producing cells were enumerated using the ELISPOT method in peripheral blood mononuclear cells and nasal brush T cells, collected during the acute and convalescent phase of the infection. Mixed Th1 (IFN-γ) and Th2 (IL-4 and IL-13) responses were detected in all three groups. Frequencies of hRSV-specific T cells were lower in both URTI groups than in the hRSV bronchiolitis group, and not significantly different between the hRSV URTI and the non-hRSV URTI group. The absence of vigorous virus-specific Th2 responses upon mild hRSV infection does not support the hypothesis that these infections influence the development of the immune system and that they predispose for the development of atopic disease.

Introduction

Human respiratory syncytial virus (hRSV) infections are the most important cause of severe respiratory disease in infants below 6 months of age. In particular, preterm infants [18] and infants with underlying cardiac and respiratory disease [19,20] have an increased risk to develop severe lower respiratory tract disease upon hRSV infection. Although most often associated with relatively mild upper respiratory tract infections (URTI) in immunocompetent adults, the virus may also cause severe respiratory disease in immunocompromised individuals and the elderly [17]. In moderate climate zones, hRSV causes yearly epidemics during the winter season, and at the age of three all infants have been infected at least once; reinfections continue to occur throughout life [17].

At present, no licensed hRSV vaccine is available, although several approaches are being exploited [118]. hRSV vaccine development remains hampered severely by vaccine trials in the 1960s when a formalin-inactivated whole virus preparation (FI-hRSV) was found to predispose infants for enhanced clinical disease upon subsequent natural hRSV infection [85,122,186,187]. Although the exact mechanism of this apparently immunopathological phenomenon remains unclear, studies in rodent models of hRSV infection have demonstrated that priming for a Th2 cellular immune

response is a key factor in this hypersensitivity response [86,188-190]. Studies in rodent models have also illustrated that not only FI-hRSV but also priming with the G protein of hRSV alone resulted in similar hypersensitivity responses upon subsequent hRSV infection, again associated with predominant Th2 responses [126,191-193].

Although several studies have suggested that primary hRSV infections in infants resulting in severe hRSV bronchiolitis are also associated with Th2 cellular immune responses [87,89], others have been unable to confirm this observation. Brandenburg *et al.* [88] found a predominant Th1 cellular immune response regardless of clinical severity.

Severe hRSV infection at a young age has been associated with episodes of wheezing and the development or exacerbations of asthma at the age of seven years, especially in infants with a family history of atopy [98]. In addition, Stein *et al.* [99] showed a relationship between severe hRSV infection in infancy and wheezing in later childhood, but this relationship was lost by the age of 13 years. Whether this relationship is causal or whether these phenomena result from a common physiological predisposition continues to be a subject of debate [194,195]. Because hRSV bronchiolitis and atopic disease have both been associated with Th2 cellular immune responses and rises in specific IgE, it has been hypothesized that the putative relationship between hRSV and asthma or allergy has an immunopathological basis [101-106]. Studies in a mouse model of airway hyperresponsiveness have shown that hRSV challenge in OVA-sensitized mice enhances and prolongs airway inflammation and airway hyperresponsiveness [107,196]. Recurrent hRSV infections in allergen-sensitized mice shift the immune response even further toward Th2 immune responses, airway inflammation and airway hyperresponsiveness [108].

A key factor in these processes of wheezing and airway hyperresponsiveness seems to be the production of the Th2 cytokine IL-13. Studies in rodent models of airway hyperresponsiveness have shown that IL-13 is the main mediator of the exacerbation of allergic response during hRSV infection. Moreover, IL-13 is induced preferentially during hRSV infection in the airway, and promotes airway hyperresponsiveness and damage [110-113,197]. In humans the Th2 cytokine IL-4 has been described to be involved in episodes of wheezing after hRSV-induced bronchiolitis [103]. Whether IL-13 also plays an important role in the induction of airway remodelling, wheezing and airway hyperresponsiveness in humans, as seen in rodent models of airway hyperresponsiveness after hRSV infection, remains to be proven.

Despite many studies investigating the role of hRSV bronchiolitis in the development of asthma or allergy, the putative role of hRSV-specific cellular immune responses in infants with relatively mild hRSV-induced URTI (RSV URTI) remains unclear. We selected infants with mild hRSV URTI, non-hRSV URTI or severe hRSV bronchiolitis, and characterized both the systemic and local hRSV-specific cellular immune responses. To this end, Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokine producing cells were

enumerated in peripheral blood mononuclear cells and T cell lines obtained from nasal brush samples following stimulation with autologous hRSV-infected B-lymphoblastic cell lines.

Materials & Methods

Patients and study design

Samples were collected in the framework of two different projects, both approved by the medical ethical committee of the Erasmus MC in Rotterdam. Informed consent was obtained from the parents. The first project was a prospective birth cohort study (Vigall) in which 129 healthy infants with (*n*=89) or without (*n*=40) FHA were followed until 2 years of age. Clinical specimens were collected at 6, 12, 18 and 24 months of age (routine visits), and during the acute and convalescent (approximately 14 days later) phases of each URTI, as defined by rhinorrhoea and at least one of the following criteria: fever, general malaise or loss of appetite. From this cohort 13 infants were selected with a laboratory-confirmed hRSV URTI (mean age 12 months, range 2-22 months) and 9 infants with a non-hRSV URTI (mean age 9.3 months, range 4-18 months). Patients with a non-hRSV URTI were selected based on the inclusion criteria mentioned above and a negative diagnosis for hRSV. In the second project (SSWO), clinical specimens were collected during the acute and convalescent (approximately 28 days later) phase from 11 infants hospitalized with severe hRSV-related bronchiolitis (mean age 2.3 months, range 1-6 months). Clinical samples included a heparinized peripheral blood sample and a nasal brush sample (Cytobrush Plus; Medscand Medical AB, Tomelilla, Sweden). Patient characteristics and viral diagnostics are mentioned in table I.

Plasma was isolated and stored at -70°C. Peripheral blood mononuclear cells (PBMC) were collected by density gradient centrifugation and stored at -135°C. Nasal brush cells (NBC) were harvested within 4 hr after sample collection in RPMI1640 medium (BioWhittaker, Verviers, Belgium) containing gentamycin by scrubbing the brush along the side of the tube and centrifuging for 5 min at 400 x *g*.

Laboratory diagnosis of hRSV infections

hRSV infections were diagnosed by combining the results of direct immunofluorescence (DIF) using fluorescein isothiocyanate (FITC)-labeled hRSV-specific monoclonal antibodies (DAKO, Glostrup, Denmark) on nasal brush cells [52] and virus isolation from nasal brush supernatant on HEp-2 cells.

Cell lines

Autologous B-lymphoblastic cell lines (BLCL) were established by transformation of PBMC with Epstein-Barr virus as described previously [198]. For the generation of target and stimulation cells, these BLCL were infected with hRSV A2 (multiplicity of infection [moi]: 100; ATCC-

VR1322) resulting in persistently hRSV-infected BLCL as described previously [199]. The percentage of hRSV-positive cells was checked by fluorescence activated cell scanner (FACScan) analysis with FITC-labeled hRSV-specific monoclonal antibody (DAKO). All BLCL were maintained in

Table I: Patient characteristics

Patient	Gender (M/F)	FHA (Y/N)	Age at onset (mo)	Month	Infectious agent	Symbol in figures 2-4	Fill
Group 1: hRSV URTI							
1	F	Y	20	Nov	hRSV	□	Gray
2	M	Y	16	Feb	hRSV	○	Gray
3	M	Y	12	Nov	hRSV	△	Gray
4	M	Y	22	Apr	hRSV	▽	Gray
5	F	Y	6	Jan	hRSV	◇	Gray
6	F	Y	10	Apr	hRSV	⬡	Gray
7	M	Y	8	Feb	hRSV	□	None
8	F	Y	7	Dec	hRSV	○	None
9	F	Y	15	Nov	hRSV	△	None
10	M	Y	18	Dec	hRSV	▽	None
11	F	Y	2	Nov	hRSV	◇	None
12	M	N	16	Jan	hRSV	⬡	None
13	F	N	4	Jan	hRSV	⊕	None
Group 2: non-hRSV URTI							
1	M	Y	5	Jan	ND	□	Gray
2	M	Y	6	Jan	rhino/corona	○	Gray
3	M	Y	7	Dec	ND	△	Gray
4	M	Y	18	Apr	corona	▽	Gray
5	F	N	4	Dec	rhino	◇	Gray
6	F	Y	18	Apr	ND	⬡	Gray
7	F	Y	11	Apr	ND	□	None
8	M	Y	11	Mar	ND	○	None
9	F	N	4	Nov	rhino	△	None
Group 3: hRSV bronchiolitis							
1	F	N	1	Jan	hRSV	□	Gray
2	F	Y	3	Jan	hRSV	○	Gray
3	M	N	1	Feb	hRSV	△	Gray
4	M	Y	1	Mar	hRSV	▽	Gray
5	F	N	3	Dec	hRSV	◇	Gray
6	F	N	1	Feb	hRSV	⬡	Gray
7	M	Y	3	Dec	hRSV	□	None
8	F	N	2	Dec	hRSV	○	None
9	M	N	2	Dec	hRSV	△	None
10	M	N	6	Feb	hRSV	▽	None
11	F	Y	2	Feb	hRSV	◇	None

M, male; F, female; FHA, family history of atopy; mo, months; ND, not determined

RPMI1640 medium (BioWhittaker) containing penicillin (100 U/ml; BioWhittaker), streptomycin (100 µg/ml; BioWhittaker), L-glutamin (2mM; BioWhittaker), and β-mercapto-ethanol (10^{-5}M; Merck KGaA, Darmstadt, Germany), further referred to as culture medium (CM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Greiner, Frickenhausen, Germany).
Within 24 hr after sample collection, nasal brush T cells (NBTC) were stimulated with phytohaemagglutinin (PHA) and gamma-irradiated allogeneic feeder cells and expanded with recombinant human IL-2 (rhIL2; Eurocetus, Amsterdam, the Netherlands) as described previously [200] in CM supplemented with 10% heat-inactivated human pooled serum (R10H). After 2-4 weeks of expansion, these NBTC were stored at -135°C.

ELISPOT assay

Two weeks before the ELISPOT assay, PBMC and NBTC were thawed, stimulated with PHA and gamma-irradiated allogeneic feeder cells, and expanded for 2 weeks in R10H supplemented with rhIL-2.

From the expanded PBMC, $CD8^+$ cells were isolated by positive selection with magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions, whereas remaining PBMC were tested as $CD8^-$ (mainly $CD4^+$) cells. NBTC were tested without prior separation in $CD8^+$ and $CD8^-$ cells. Cells were stimulated first with autologous hRSV-infected BLCL (effector-to-target ratio [E:T] of 2), autologous uninfected BLCL (E:T of 2) or R10H for 1.5 hr at 37°C in 96-wells V-bottom plates (Greiner). For IFN-γ 10,000-15,000 effector cells were used per well, whereas 100,000 effector cells were used for IL-4 and IL-13. Subsequently, the cells were transferred to ELISPOT plates with either a nylon bottom (for IFN-γ; Nalge Nunc, Rochester, NY, USA) or with a PVDF bottom (for IL-4 and IL-13; Millipore, Molsheim, France), which had been coated overnight at 4°C with a monoclonal antibody against IFN-γ (7.5 µg/ml; Mabtech AB, Stockholm, Sweden), IL-4 (15 µg/ml; Mabtech AB) or IL-13 (15 µg/ml; Mabtech AB) and blocked with R10H for 2 hr at 37°C. After 3-4 hours of incubation at 37°C, plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T; Merck KGaA, Darmstadt, Germany) and incubated overnight at 4°C with a secondary biotinylated antibody against IFN-γ (0.5 µg/ml; Mabtech AB), IL-4 (1 µg/ml; Mabtech AB) or IL-13 (1 µg/ml; Mabtech AB). Spots were visualized using streptavidine alkaline phosphatase (1:1,000; Mabtech AB) for 2 hr at room temperature, washing with PBS-T and adding NBT/BCIP substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) until spots appeared. Finally, washing the ELISPOT plates with distilled water stopped the color reaction. Spots were counted using an automated ELISPOT reader (Automated ELISA-Spot Assay Video Analysis Systems; A.EL.VIS GmbH, Hanover, Germany) for IFN-γ spots, or a stereomicroscope at 25-fold magnification for IL-4 and IL-13 spots. The number of hRSV-specific spots was determined by subtracting the number of spots detected after stimulation with uninfected BLCL from

the number of spots detected after stimulation with hRSV-infected BLCL (represented per 10^6 cells).

hRSV serology

An optimized "in-house" ELISA was used to determine hRSV-specific total IgG in plasma samples collected during routine sampling moments (at 6, 12, 18 or 24 months of age). In short, medium binding ELISA plates (Greiner) were coated overnight with 100 µl purified whole hRSV-A antigen at a concentration of 1 µg/ml in PBS at room temperature and washed three times with 300 µl PBS containing 0.5% Tween-20 (Merck KGaA). Plasma samples, diluted 1:100 in ELISA buffer (Meddens Diagnostics, Vorden, The Netherlands) supplemented with 5% normal goat serum (ICN, Irvine, CA, USA), were added and plates were incubated for 1 hr at 37°C. Next, plates were washed and 100 µl horseradish peroxidase-labeled goat anti-human IgG (Biosource, Camarillo, CA, USA) was added in ELISA buffer for 1 hr at 37°C. Finally, 100 µl TMB-substrate (Meddens Diagnostics) was added and staining reaction was stopped by addition of 2 M H_2SO_4. The extinction was measured at 450 nm with a reference filter at 620 nm.

Statistical analysis

Data were analyzed using nonparametrical tests. Differences between the two sampling moments (acute and convalescent) in each group were analyzed using the paired Wilcoxon test. Differences between the non-hRSV URTI and the hRSV URTI group during the acute or convalescent phase were analyzed using the Mann-Whitney test. Differences were considered statistically significant if the p-value < 0.05.

Results

Patient characteristics

Within the cohort of 129 infants followed up during their first two years of life (Vigall), 80 URTI episodes were reported, of which 13 were diagnosed as acute hRSV infections. An additional seven acute hRSV infections were diagnosed during routine sampling moments in the absence of clinical symptoms. Because a larger number of hRSV cases had been expected, prevalence of hRSV-specific IgG antibodies was measured in a number of plasma samples collected at the ages of 6 (n=54), 12 (n=49), 18 (n=21) and 24 months (n=26). Figure 1 shows the kinetics of plasma IgG antibodies to hRSV during this 2-year period. After a decline during the first year due to the loss of maternal antibodies, six of 21 samples (76%) were positive at 18 months and 22 of 26 samples (85%) were positive at 24 months of age. These data confirm that the majority of infants in the cohort had experienced at least one hRSV infection during their first 2 years of life. In most cases, these infections apparently did not result in URTI as defined in the Materials & Methods section, were not reported to the study doctor or were not diagnosed properly. Twenty laboratory-confirmed acute hRSV cases were identified and

13 were selected on basis of sample availability for characterization of hRSV-specific cellular immune responses. Of these 13 infants, eight were selected during the acute phase of an hRSV infection and five during a routine sampling moment. These were compared to responses in infants from the same cohort study with an URTI not related to an acute hRSV infection (*n*=9) and with responses in infants hospitalized with a severe hRSV-related bronchiolitis (*n*=11).

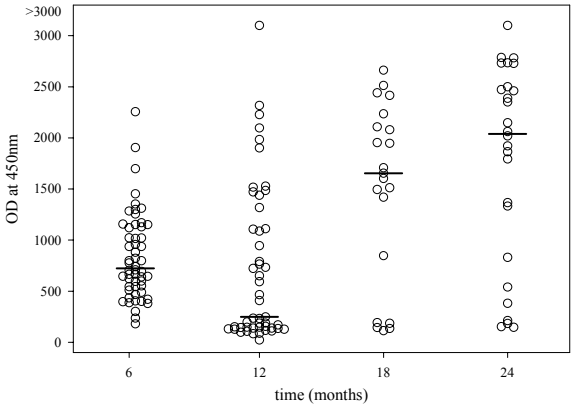

Figure 1: Detection of RSV-specific IgG during the different routine sampling moments. Results are shown for each individual infant and each routine sampling moment. The median of each group is represented by a line.

hRSV-specific cytokine producing T cells

ELISPOT assays were used to enumerate hRSV-specific Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4 and IL-13) producing T cells in the infants mentioned above. To compare systemic hRSV-specific T cell responses to those at the primary site of infection, NBTC were also tested. These NBTC were tested without separation of $CD8^+$ and $CD8^-$ T cells, whereas PBMC were tested after separation of $CD8^+$ and $CD8^-$ T cells. Furthermore, these hRSV-specific cellular immune responses were examined both during the acute and convalescent phase of infection.

Before and after expansion the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were measured by FACScan analysis. Mean percentages (± SD) of these cells in PBMC (p) or NBTC (n) in the acute (1) or convalescent (2) phase are shown in table II. Results show that the mean percentage of $CD4^+$ T cells did not change during expansion, whereas the percentages of $CD8^+$ T cells rose during this expansion. Note that for NBTC, these are the percentages after the first and second aspecific expansion cycle.

hRSV-specific T cell responses in severe hRSV-related bronchiolitis

In infants with severe hRSV-related bronchiolitis, IFN-γ producing T cells could be detected in the $CD8^+$ fraction of PBMC and in NBTC during both the acute and convalescent phase of infection. In the $CD8^-$ fraction of PBMC, IFN-γ producing T cells could only be detected during the convalescent phase of infection (figure 2). In contrast, in some infants Th2 cytokine (IL-4 and IL-13) producing T cells could be detected in the $CD8^-$

fraction of PBMC during both the acute and convalescent phase of infection, whereas in the $CD8^+$ fraction of PBMC and in NBTC, these cells could only be detected during the convalescent phase of infection (figure 3 and 4). Overall, the frequencies of hRSV-specific T cells, either Th1 or Th2, in infants with severe hRSV-related bronchiolitis were higher during the convalescent phase than the acute phase of infection.

These data confirm that our assays were of sufficiently sensitivity to detect hRSV-specific T cells producing Th1 and Th2 cytokines.

Table II: $CD3^+CD8^+$ and $CD3^+CD4^+$ cells in PBMC or NBTC

	before aspecific expansion		after aspecific expansion	
	$CD8^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	$CD4^+$ T cells
p1	15.2% (7.4)	50.9% (13.8)	36.8% (17.2)	51.2% (19.9)
p2	15.6% (6.4)	52.2% (14.1)	39.7% (14.7)	43.4% (14.2)
n1	50.6% (22.8)	18.8% (17.9)	64.1% (22.9)	18.3% (16.4)
n2	66.4% (21.3)	11.5% (11.9)	70.5% (20.6)	16.4% (15.3)

Mean % (SD) of cells collected during the acute (p1, n1) or convalescent (p2, n2) phase, measured before and after aspecific expansion. p, PBMC; n, NBTC.

RSV-specific T cell responses in hRSV vs. non-hRSV URTI

In infants with hRSV URTI as well as in infants with non-hRSV URTI, relatively low numbers of IFN-γ producing T cells (figure 2) and virtually no IL-4 and IL-13 producing T cells (figure 3 and 4) were detected in the $CD8^+$ or $CD8^-$ fraction of PBMC collected during the acute or convalescent phase of the infection. In NBTC from these infants, IFN-γ producing T cells could be detected during both the acute and convalescent phase of the infection (figure 2). Although a few individuals in the hRSV URTI group showed higher frequencies of hRSV-specific T cells, this did not result in a significant difference between the two groups. In NBTC from infants with non-hRSV URTI no IL-4- or IL-13 producing T cells could be detected, whereas in some infants with hRSV URTI these T cells were detected (figure 3 and 4).

Discussion

hRSV-specific T cell responses were studied in infants with mild hRSV URTI, non-hRSV URTI and severe hRSV bronchiolitis. Frequencies of hRSV-specific T cells were lower in both URTI groups as compared to the hRSV bronchiolitis group, but not significantly different between the hRSV or non-hRSV URTI groups. No substantial qualitative differences in the phenotype of the cellular immune response were found between the three groups.

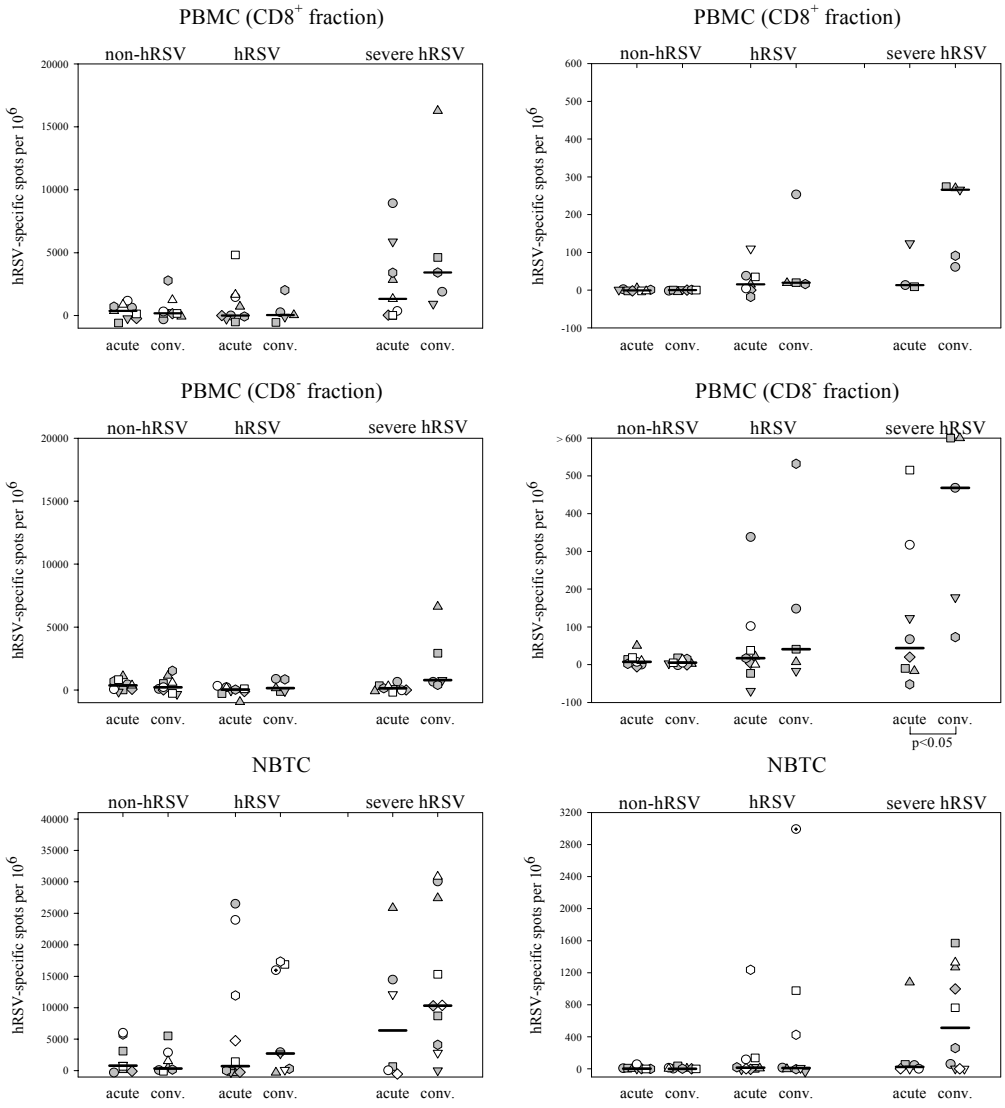

Figure 2: Detection of hRSV-specific IFN-γ producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IFN-γ producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

Figure 3: Detection of hRSV-specific IL-4 producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IL-4 producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

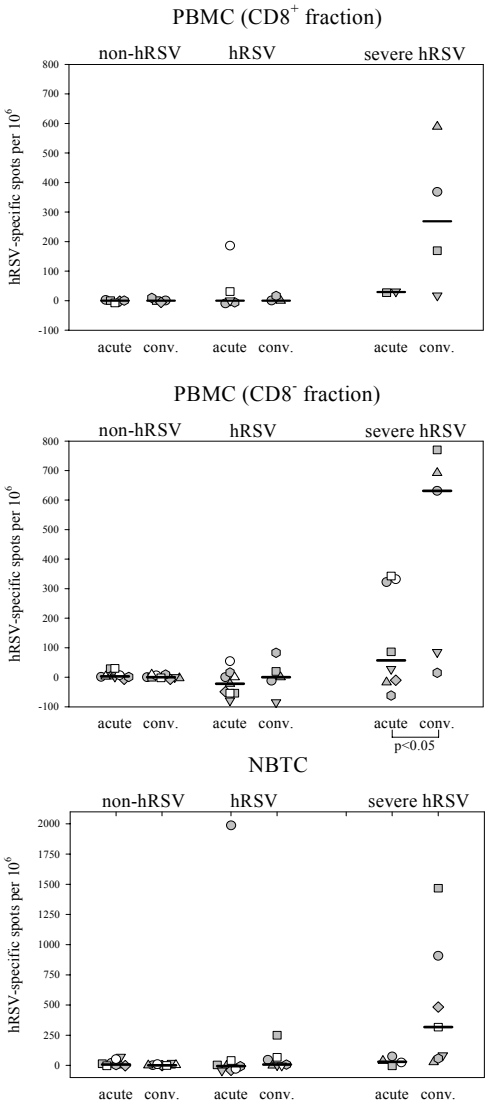

Figure 4: Detection of hRSV-specific IL-13 producing T cells per 10^6 within the $CD8^+$ fraction of PBMC (top graph), the $CD8^-$ fraction of PBMC (middle graph) and the NBTC (bottom graph) during the acute and convalescent phase of infection. Each graph represents the number of hRSV-specific IL-13 producing T cells for non-hRSV URTI (non-hRSV; left), hRSV URTI (hRSV; middle) and severe hRSV bronchiolitis (severe hRSV; right). The median of each group is represented by a line. Note the differences in the scales of the figures for the different populations tested in the ELISPOT assay.

We set out to test the hypothesis that infants with FHA would be predisposed to respond to hRSV infections during early childhood with a skewed virus-specific Th2 response. Because we detected only a limited number of acute hRSV infections in our prospective birth cohort study, however, which were found predominantly in the group of infants with FHA (table I), it was decided to limit our objectives to the characterization of the hRSV-specific T cell response in infants with mild or subclinical hRSV infections.

We identified no more than 20 laboratory-confirmed hRSV cases in 129 healthy infants followed during their first 2 years of life, of which 13 were associated with mild URTI and seven were found during routine sampling of clinically healthy infants. The prevalence of hRSV-specific serum IgG antibodies in infants' samples at the age of 18 or 24 months indicated that the majority of these infants had experienced at least one hRSV infection during their first 2 years of life, in accordance with other cohort studies [49,50]. Based on the literature, it was expected that few primary hRSV infections would be asymptomatic [1], most infections would be associated with URTI and 25-40% with hRSV bronchiolitis [201-203]. In our cohort study, two patients suffered from hRSV-induced bronchiolitis as described by others [202]. The limited number of URTI episodes reported (n=80 for 129 infants over a 2-year period) suggests underreporting in our study.

When comparing the three patient groups, the frequencies of hRSV-specific cytokine producing cells were significantly higher in hRSV

bronchiolitis patients than in URTI patients. The differences in age and timing of the convalescent phase sample in the URTI and the severe hRSV bronchiolitis groups, however, must be taken into consideration. Therefore, no firm conclusions may be drawn from these results, but it could be seen as a trend. Cells, both systemically and locally, producing the Th1 cytokine IFN-γ were measured at frequencies one to two log-values higher than cells producing the Th2 cytokines IL-4 or IL-13. Brandenburg *et al.* [88] previously studied hRSV-specific T cell responses in infants with hRSV infections of different clinical severity by specifically stimulating PBMC with autologous hRSV-infected BLCL, followed by expansion with rhIL-2, and subsequent characterization of the expanded cultures by measurement of cytokine production. In this study, it was found that responses were dominated by IFN-γ producing T cells, although IL-4 and IL-10 producing T cells could be detected at low frequencies. A difference was not detected in this respect between infants with severe or mild bronchiolitis. The results of our present study suggest that use of the ELISPOT assay on cells expanded with a nonspecific protocol (PHA + allogeneic feeder cells) is a more sensitive method for this purpose. We cannot rule out that the use of the expansion and cryopreservation steps introduces selection of certain subsets of T cells or changes in sensitivity/specificity. The data of both studies, however, suggest that, although low levels of Th2 cytokines or cytokine producing cells can be detected, the hRSV-specific T cell response is dominated by cells of the Th1 phenotype. The IFN-γ response detected in NBTC from infants with non-hRSV URTI can more easily be attributed to the presence of an hRSV-specific T cell response generated during an earlier hRSV infection.

Several studies have investigated the phenotype of the hRSV-specific T cell response in different patient groups. Aberle *et al.* [93] showed that infants suffering from severe hRSV infection had lower levels of IFN-γ mRNA expression in PBMC compared to infants with mild hRSV infection. Similar results were obtained by Bont *et al.* [204], who tested cytokine levels in nasal aspirates. They showed that ventilated infants had lower levels of IFN-γ in nasal aspirates than non-ventilated infants. Roman *et al.* [87] showed that infants suffering from hRSV-LRTI not only had decreased levels of IFN-γ in PHA-stimulated PBMC, but that the levels of IL-4 were decreased to a lesser extent, indicating a more Th2-like immune response. In contrast, our data suggest a more quantitative than qualitative difference in the hRSV-specific cellular response between patients with different disease severity. It is interesting to note that in our study IL-4 and IL-13 producing cells, rather than IFN-γ producing cells, were detected at higher levels in convalescent than in acute samples. This suggests a T cell phenotype switch over time as described previously for measles virus-specific T cells [205]. Our results are also in accordance with the data presented by Pala *et al.* [91], who showed that ex-bronchiolitic patients at the age of 7-8 had higher frequencies of hRSV-specific IL-4 producing T cells than control children. In this study, the frequencies of hRSV-specific IFN-γ producing T cells did not

differ between the two groups; however, the stimulation protocols were fundamentally different: Pala *et al.* used irradiated hRSV resulting in the stimulation of $CD4^+$ T cells only, whereas we used hRSV-infected autologous BLCL resulting in the stimulation of both $CD4^+$ and $CD8^+$ T cells.

When seeking a link between hRSV infections and the development or exacerbation of allergic asthma, IL-13 producing T cells may be of special interest, because this cytokine has been shown to play a central role in the pathogenesis of asthma and atopic disease [110,206]. IL-13 has been identified previously as a predominant cytokine produced by hRSV-specific T cells upon vaccination of macaques with FI-hRSV [128].

It has been suggested that in some cases, hRSV could establish a persistent infection in the host [207]. In addition, hRSV is known to cause recurrent infections throughout life, which could result in a chronic stimulation of hRSV-specific T cells. In a recent study, hRSV genome could be detected by RT-PCR in the lungs of three of seven adults who died of an asthma death and five of seven asthmatic patients who died of unrelated causes, as opposed to none of the seven control individuals [208]. We detected IL-13 producing cells predominantly in bronchiolitis patients during the convalescent phase. If a phenotype switch from Th1 to a mixed Th1/Th2 response indeed occurs after a primary hRSV infection, continuous re-stimulation could result in an increase of the frequency of hRSV-specific IL-13 producing cells. It could be hypothesized that boosting of IL-13 producing hRSV-specific T cells contributes to the pathogenesis of asthma. This hypothesis, however, could be tested best by prospectively comparing hRSV-specific cellular immune responses between infants with or without FHA and subsequently determining which of these children develop asthma or atopic disease.

In conclusion, our data suggest that asymptomatic primary hRSV infections are more common than considered previously. The hRSV-specific T cell response during mild or subclinical hRSV infections was similar to that observed in non-hRSV patients. On the other hand, the hRSV-specific T cell response in infants with hRSV bronchiolitis was higher quantitatively when compared to infants with hRSV URTI. It is unlikely that the immune response in infants with hRSV URTI could be a factor in driving the development of the immune system in the direction of skewed Th2 responses. The demonstration of hRSV-specific IL-13 producing T cells in hRSV bronchiolitis patients during the convalescent phase, however, suggests an alternative explanation for the observed link between bronchiolitis during early childhood and wheezing or development of atopic disease at a later age.

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