

Predominance of Th2 cytokines, CXC chemokines and innate immunity mediators at the mucosal level during severe respiratory syncytial virus infection in children

Jesus F. Bermejo-Martin¹, Maria C. Garcia-Arevalo¹, Raul Ortiz De Lejarazu², Julio Ardura³, Jose M. Eiros², Ana Alonso³, Vanesa Matías³, Maria Pino³, David Bernardo¹, Eduardo Arranz¹, Alfredo Blanco-Quiros¹

¹ Lab. de Inmunología de Mucosas, Dept. of Pediatrics and Immunology. Universidad de Valladolid, Facultad de Medicina, Ramón y Cajal 7, Valladolid 47005, Spain. Jesus F. Bermejo-Martin and Maria C. Garcia-Arevalo contributed equally to the development of this work.

² Hospital Clínico, Microbiología, Valladolid, Spain

³ Hospital Clínico, Pediatría, Valladolid, Spain

Correspondence : J.F. Bermejo-Martin
<bermejo@ped.uva.es>

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ABSTRACT. Profiling of immune mediators in both nasal and plasma samples is a common approach to the study of pathogenesis in respiratory viral infections. Nevertheless, mucosal immunity functions essentially independently from peripheral immunity. In our study, 27 immune mediators were profiled in parallel, in nasopharyngeal aspirates (NPAs) and plasma from 22 < 2 year-old children with a severe respiratory syncytial virus infection involving the lower respiratory tract, using a multiplex assay. NPAs from 22 children with innocent heart murmurs were used as controls. Differences in mediator concentrations between NPAs from patients and controls were assessed using the Mann-Whitney test. Ratios of innate/adaptive-immunity mediators, Th2/Th1-cytokines and CXC/CC-chemokines were calculated for NPAs and plasmas and differences were assessed using the Wilcoxon test. Associations mediators, severity and leukocyte counts were studied using the Spearman-Kärber test. **Results:** increased levels of Th1 cytokines (IL-1 β , IL-2, IL-12p70, IFN γ , TNF α), Th2 cytokines (IL-13, IL-4, IL-6, IL-10), chemokines (IP-10, IL-8, MIP1 α , MIP-1 β), growth factors (FGFb, PDGFbb, GCSF) and IL-1RA, IL-17 were observed in patient NPAs in comparison to controls. In the relative comparisons between patient NPAs and plasmas, a predominance of innate immunity mediators, Th2 cytokines and CXC chemokines was found at the mucosal level. No association between the level of each mediator in NPAs and plasma was found. In plasma, PDGFbb, VEGF, MIP-1 α , IL-8 correlated with severity; RANTES and IL-6 correlated with leukocyte counts. **Conclusions:** acute respiratory syncytial virus infection induces a relative predominance of innate-immunity mediators, Th2 cytokines and CXC chemokines in the mucosal compartment in infected children.

Keywords: cytokine, chemokine, infection, mucosa, plasma, RSV

Respiratory syncytial virus (RSV) is a major viral pathogen affecting both infants and adults. RSV infections usually last less than a week and tend to be more severe in children aged eight to 30 weeks. About 1 to 2% of all infants require hospitalization for bronchiolitis [1]. During the course of an RSV infection, inflammatory host cell recruitment to the lung is thought to play a central role in determining disease outcome. Chemokines mediate cell recruitment to sites of inflammation and are influenced by, and influence, the production of cytokines [2]. Cytokines and chemokines have been detected in nasopharyngeal aspirates (NPAs), serum and plasma during acute RSV disease in children [3-11]. The mucosal-associated immune tissues (MALT) represents a highly compartmentalized immunological system which functions essentially independently from the peripheral immune system appa-

ratus [12]. Consistent with this high degree of compartmentalization, the MALT is populated by phenotypically and functionally distinct B cells, T cells and accessory cell subpopulations as compared with systemic lymphoid tissues. At the same time, chemokines, growth factors and cytokines are differentially expressed among mucosal tissues [12]. As a consequence, when immune mediators are profiled in respiratory or plasma samples in the context of a viral infection affecting the respiratory tract, the compartmentalization of the immune system at these levels could influence the conclusions about the pathogenic events. The objective of our study was to study the particularities of the mucosal profiles of immune mediators in comparison to the plasma profiles, during a severe, respiratory syncytial virus infection in young children. For this, we profiled 27 immune mediators in parallel, in nasophary-

ryngeal aspirates and plasma from 22 < 2 year-old children with a severe respiratory syncytial virus infection involving the lower respiratory tract.

MATERIALS AND METHODS

Patients and samples

Children up to 24 months old showing clinical signs of lower respiratory tract infection of viral origin (tachypnea, prolonged expiratory time, wheezing, rales, chest retractions, dyspnoea of sudden appearance, fever) [13] needing hospitalisation in the Paediatric Department of the "Hospital Clínico Universitario" (HCU) in Valladolid (Spain) from December 1st, 2005 to March 31st, 2006. Evaluation of clinical severity was done at admission following the Wood's Clinical Asthma Score modified by Martinon-Torres *et al.* (M-WCAS) [13]. Twenty two children under 2 years of age, diagnosed with asymptomatic heart murmurs were recruited as controls. In all cases, an informed consent was requested from the parents or legal guardians prior to the inclusion of the child in the study. Approval of the Committee for Ethics in Clinical Research of our hospital was obtained prior to the beginning of recruitment. NPAs were harvested at admission from patients and controls by instillation of 2.5 mL of an isotonic saline solution into each nostril (NaCl 0.9%) as described elsewhere [3]. The samples were sent on ice to the virology laboratory for viral diagnosis, and the remainder of the sample was centrifuged and the supernatant stored at -70°C until cytokine, chemokine and growth factor evaluation. In parallel, an aliquot of 1 mL of blood was obtained only from those patients for whom the paediatricians had requested, at admission, analytical support for clinical reasons. Blood samples were taken in EDTA vacutainer tubes and plasma samples were obtained after proper centrifugation. Since blood extraction is an invasive technique, blood was not obtained from controls because of the ethical considerations.

Viral diagnosis

Patients were classified as having a suspected RSV infection using a rapid immuno-chromatographic test (NOW RSV test; Binax, Inc. Portland, ME, USA). Viral presence was subsequently confirmed in infected infants by direct immunofluorescent staining of viral cultures from NPAs (IMAGEN; DakoCytomation, Glostrup, Denmark), for RSV, adenovirus, parainfluenza 1, 2 and 3, influenza A and B viruses on MDCK, LLCMK2, A549 and Hep2 cells. All subjects included in the study were culture-positive for RSV, and culture-negative for all other viruses tested.

Immune mediator detection

Twenty-seven cytokines, chemokines and growth factors were profiled in both NPA supernatants and plasma samples using a multiplex assay (Biorad, Hercules, CA, USA) on a Luminex TM platform (Austin, TX, USA). Limits of detection (pg/mL) were as follows for NPAs and plasma respectively: eotaxin (2.95; 0.89), granulocyte macrophage colony-stimulating factor (GM-CSF) (6.76; 6.6), interleukin 1 receptor antagonist (IL-1RA), (2.17;

10.16), IL-5 (2.33; 2.3); IL-9: (2.21; 2.12); IL-13 (0.56; 0.43), IL-1 β (2.19; 2.2); monocyte chemo-attractant protein-1 (MCP-1) (1.28; 1.47); platelet-derived growth factor (PDGF-BB) (2.45; 2.39), vascular endothelial growth factor (VEGF) (2.79; 2.87), human fibroblast growth factor-basic (FGF-b) (2.81; 13.22), IL-2 (1.43; 1.08); IL-6 (2.46; 2.4), IL-10 (1.61; 1.62), IL-15 (1.92; 1.95), IL-8 (1.96; 1.86), macrophage inflammatory protein-1 α (MIP-1 α) (0.93; 0.95), regulated upon activation, normal T-cell expressed, and secreted (RANTES) (1.38; 1.35), granulocyte colony-stimulating factor (G-CSF) (2.01; 9.62), interferon γ (IFN- γ): (2.35; 10.42), IL-4 (0.26; 0.31), IL-7: (3.2; 3.17), IL-12p70 (2.76; 2.73), IL-17 (1.17; 1.21); interferon-inducible protein-10 (IP-10) (3.88; 3.18), macrophage inflammatory protein-1 β (MIP-1 β) (1.36; 1.24); and tumour necrosis α (TNF- α) (7.69; 30.96). Those values below the level of detection were reported as being equal to the level of detection. Levels of IL-9, IL-15 and GM-CSF in NPAs and IL-5 in both NPAs and plasma were consistently less than the limits of detection. Therefore, comparisons involving these mediators were not performed during the analysis of data.

White cell counts were performed on whole blood samples collected in EDTA-vacutainer tubes (BD Biosciences, San Jose, CA, USA) using a Sysmex XE-2100 analyzer (Roche, Basel, Switzerland).

Statistical analysis of data was performed using the Microsoft Excel and the SPSS 15.0 software for Windows. Differences in the levels of mediators between NPAs from patients and controls were assessed using the Mann-Whitney U test. Ratios between innate-immunity mediators (IL-6, TNF α , IL-1 β , IL-8) and adaptive immunity mediators (IL-4, IL-13, IL-2, IFN γ), between Th2 cytokines (IL-4, IL-10, IL-6, IL-13) and Th1 cytokines (IL-2, IL-12, TNF- α , IFN- γ) and between CXC chemokines [CXCL8 (IL8), CXCL10 (IP10)] and CC chemokines [CCL2 (MCP-1), CCL3 (MIP1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL11 (Eotaxin)] were calculated for each patient in both NPAs and plasma samples. Differences in these ratios between NPAs (n = 22) and plasma samples (n = 22) were assessed using the Wilcoxon signed ranks test with the Bonferroni's correction. Associations between mediators, severity parameters and blood counts were studied calculating the Spearman-Kärber correlation coefficients.

RESULTS

1. Clinical characteristics (data expressed as mean + SD). Recruited children were 8.3 + (5.6) months old. O₂ saturation at admission was 92.0 + (5.2), indicating a severe respiratory condition (normal O₂ saturation: > 96%). All children received treatment with oxygen and beta(2)-adrenoceptor agonists (salbutamol 0.15 mg/kg, every 4-8 hours). The mean time spent at hospital was 4.6 + (2.9) days, requiring oxygen for 2.3 + (2.7) days. A significant inverse association between O₂ saturation at admission and "days of hospitalization" was observed in our study (Spearman correlation coefficient: -0.42; *p* = 0.05). 2. Concentrations of mediators in NPAs and plasma samples. The concentration of cytokines, chemokines and growth factors in NPAs from patients and controls, along

with the concentrations of these mediators in plasma from patients is shown in *table 1*. The concentration of all mediators studied, with the exception of MCP-1, RANTES, IL-7, eotaxin and VEGF, were significantly higher in the NPAs from patients compared to those measured in NPAs from controls. Interestingly, while IL-9, IL-15 and GM-CSF were detected in plasma, they were not detected in NPAs.

3. Comparison of ratios between innate and adaptive immunity mediators in NPAs and plasma (*figure 1*). Ratios of IL-6/IL-4, IL-6/IL-13, IL-6/IL-2, IL-6/IFN γ , TNF α /IL-4, TNF α /IL-13, TNF α /IL-2, TNF α /IFN γ , IL-1 β /IL-4, IL-1 β /IL-13, IL-1 β /IL-2, IL-1 β /IFN γ , IL-8/IL-4, IL-8/IL-13, IL-8/IL-2, IL-8/IFN γ were significantly higher in patient NPAs ($p < 0.05$) than in patient plasma, indicating a relative predominance of innate-immunity mediators in the respiratory mucosa.

4. Comparison of ratios between Th2 and Th1 cytokines in NPAs and plasma (*figure 1*). Ratios of IL-13/IL-12, IL-4/IL-12, IL-10/IFN γ , IL-4/IL-2, IL-10/IL-2, IL-10/IL-12, IL-6/IL-2, IL-6/IL-12, IL-6/IFN γ and IL-13/IL-2 were significantly higher in patient NPAs ($p < 0.05$) than in patient plasma, indicating a relative predominance of Th2 cytokines in the respiratory mucosa.

5. Comparison of ratios between CXC and CC chemokines in NPAs and plasma (*figure 1*). Ratios of IL-8/MIP-1 β , IP-10/MIP-1 β , IL-8/MCP-1, IL-8/MIP-1 α , IL-8/RANTES, IL-8/eotaxin, IP-10/MCP-1; IP-10/MIP-1 α , IP-10/RANTES, IP-10/eotaxin were significantly higher in patient NPAs ($p < 0.05$) than in patient plasma, indicating a relative predominance of CXC-chemokines in the respiratory mucosa.

6. Study of the associations between the concentration of mediators in NPAs and plasma: interestingly, no significant association was found ($p > 0.05$) between the concentration of each mediator in NPA and plasma.

8. Association between mediators and severity: the following mediators showed a significant association between the concentration in plasma and severity; results expressed as [mediator, severity parameter (Spearman correlation coefficient; p)]: [PDGFbb, "days in hospital" (0.49; $p = 0.02$)], [VEGF, "days in hospital" (0.44; $p = 0.04$)], [MIP-1 α , M-WCAS (-0.48; $p = 0.025$)], [PDGFbb, "O₂ saturation" (-0.55; $p = 0.008$)], [IL-8, "O₂ saturation" (-0.54; $p = 0.001$)], [VEGF, "O₂ saturation" (-0.49; $p = 0.02$)]. In NPAs, the only significant association found was [IL-8, "days in hospital" (-0.43; $p = 0.044$)].

9. Association between mediators and white cell counts: we found the following significant associations between mediators in plasma and white cells counts in peripheral blood [mediator, cell type (Spearman correlation coefficient; p)]: [IL6, total leucocytes (0.48; $p = 0.02$)], [IL-6, neutrophils (0.48; $p = 0.02$)], [IL-6, monocytes (0.42; $p = 0.05$)], [RANTES, neutrophils (-0.5; $p = 0.01$)], [RANTES, monocytes (0.42; $p = 0.05$)]. Conversely, in NPAs, the only significant association found was [PDGFbb, monocytes (0.53; $p = 0.01$)].

DISCUSSION

In this work, we have simultaneously profiled 27 soluble immune mediators in both NPAs and plasma samples from children with a severe RSV infection. RSV induced an increase in 18 of the 27 mediators studied in the nasal mucosae, as assessed by the comparisons between NPAs from patients and controls. The increased mediators corresponded to Th1 cytokines (IL-1 β , IL-2, IL-12p70, IFN γ , TNF α), Th2 cytokines (IL-13, IL-4, IL-6, IL-10), chemokines (IP-10, IL-8, MIP1 α , MIP-1 β), growth factors (FGFb, PDEGFbb, GCSF) and also IL-1RA and IL-17. Theoretically,

Table 1
Concentration of mediators in NPAs and plasma. Results are expressed as median (pg/mL) + inter-quartile range

	[NPAs from patients] (n = 22)	[NPAs from controls] (n = 22)	[Plasma] (n = 22)
IL-1RA	8831.0 [16046.2]	3136.4 [4924.9]	2119.3 [807.8]
IL-13	3.7 [4.4]	1.4 [2.3]	19.8 [10.5]
IL-1 β	468.0 [1008.9]	55.4 [221.7]	17.5 [11.5]
FGF- β	7.7 [17.3]	2.8 [3.81]	141.0 [65.1]
IFN- γ	96.9 [55.4]	29.4 [65.4]	448.9 [134.3]
MCP-1	6.5 [19.4]	1.2 [6.0]	78.3 [49.9]
PDGF	17.4 [14.4]	4.8 [7.4]	4899.5 [8628.9]
IL-2	3.8 [4.4]	1.4 [0.3]	68.2 [60.7]
IL-6	237.7 [351.1]	8.6 [43.4]	61.5 [65.9]
IL-10	15.9 [23.3]	1.8 [3.7]	16.2 [13.7]
IP-10	13033.9 [19867.0]	3304.6 [4455.5]	1285.2 [888.0]
IL-8	7112.5 [6935.6]	799.8 [5156.9]	166.3 [452.6]
G-CSF	1370.3 [1707.3]	160.3 [455.2]	141.2 [65.9]
MIP-1 α	40.6 [76.2]	3.325 [29.8]	20.2 [9.4]
RANTES	72.9 [155.3]	63.1 [128.5]	5335.5 [3210.4]
IL-4	2.2 [1.9]	0.26 [0.8]	8.6 [3.8]
IL-7	9.4 [9.8]	8.6 [13.5]	10.0 [10.6]
IL-12P70	3.6 [2.3]	2.7 [0.0]	28.9 [22.6]
TNF- α	103.0 [999.9]	7.6 [0.0]	68.4 [132.9]
EOTAX.	35.7 [37.9]	29.0 [31.5]	161.1 [135.8]
IL-17	6.7 [11.5]	1.1 [4.3]	29.0 [23.4]
MIP-1 β	483.8 [1470.7]	38.5 [204.6]	92.6 [69.8]
VEGF	1230.7 [1683.3]	513.2 [1552.0]	75.4 [94.1]

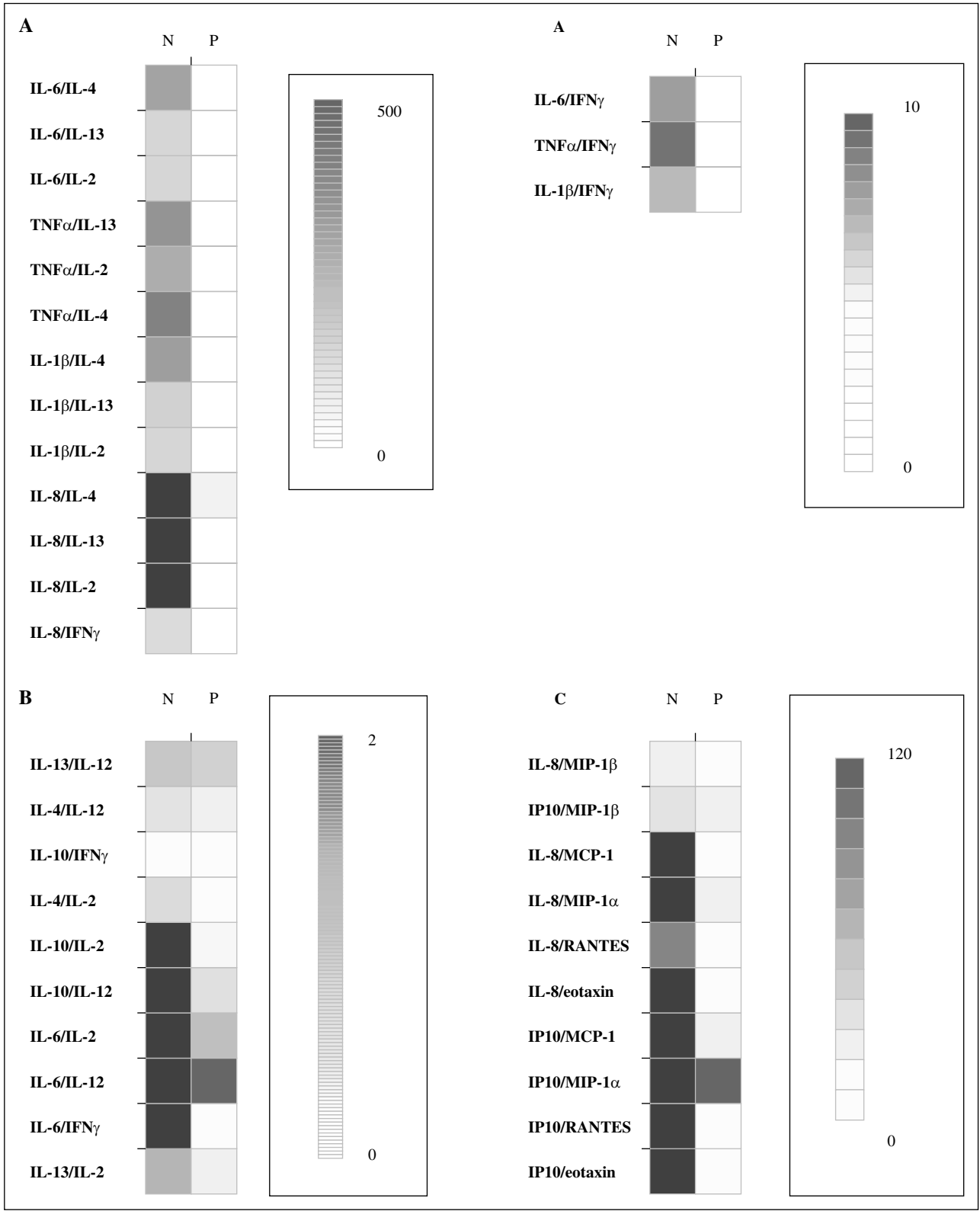


Figure 1
Ratios between innate and adaptive immunity mediators (A), between Th2 and Th1 cytokines (B) and finally between CXC and CC chemokines (C) in both NPAs (N) and plasma (P) are represented by a heat map using the JColorGrid software (© University of California San Francisco and University of California Berkeley, USA).

cally, IL-1 β , TNF α , IFN γ , IL-2, IL-12p70, IP10 (proinflammatory mediators) could promote inflammation in the respiratory tract and consequently contribute to the respiratory compromise showed by these children. Conversely, our findings showed that none of these mediators measured

in NPAs was associated with severity. IL-17 has been proposed to play a role in the increased mucus production in the bronchi of children with RSV infection [14]. IP10 is a lymphocyte-targeting CXC chemokine produced at high concentrations by activated bronchial epithelial cells in

response to infection [15]. A persistent increase of IP10 has been found in patients infected with the SARS-associated Coronavirus and a poor prognosis [16]. With regard to TNF α , the production of this cytokine during the inductive immune response to RSV seems to serve an important protective role, while exaggerated production of TNF α during the adaptive phase of the immune response seems to induce significant lung immunopathology [17]. Although not directly proinflammatory, bFGF synergistically potentiates inflammatory mediator-induced leukocyte recruitment, at least in part, by enhancing endothelial adhesion molecule up-regulation [18]. IL-13 is produced in large quantities by stimulated Th2 cells. IL-4 and IL-13 contribute as major effectors of Th2 inflammation and tissue remodelling [19, 20]. IL-10 is an anti-inflammatory cytokine produced by macrophages, T cells and B cells. It has been suggested that RSV may inhibit an effective immune response by inducing the production of this cytokine [21]. Alternatively to this point of view, the ability of IL10 to inhibit RSV replication has also been demonstrated in mice models [22]. IL-6 and IL-8 have been observed to occur in bronchial biopsy specimens of asthmatics [23]. The beta chemokine macrophage inflammatory protein-1 α (MIP1 α) and monocyte chemoattractant protein 1 (MCP-1) are also considered as possibly important in inciting the inflammatory response in asthma [24]. Since children suffering from severe RSV bronchiolitis seem to be predisposed to asthma, the elevation of these mediators could be related to the incidence of this disease afterwards. It is interesting that elevated levels of IL-6, IL-8, IFN- γ , and MIP-1 β , as well as of IL-10, may be protective against hypoxia in bronchiolitis [25]. Finally, GM-CSF may influence the function of polymorphonuclear cells attracted to the site of infection [26]. When comparisons of ratios between innate and adaptive immunity mediators in NPAs and plasma were performed, a predominance of innate-immune mediators was found in the mucosal compartment (NPAs). It could be a consequence of the early immune response to the virus, developed locally at the place where the virus replicates and initially comes into contact with the immune system, the respiratory mucosa [27]. The cells normally present in the nasal compartment could also contribute to the predominance of innate-immunity mediators at the mucosal level: these cells are basically epithelial cells of the nasal mucosa, and neutrophils [28]. The matched comparisons between several Th2/Th1 ratios in both types of sample (NPAs and plasma) indicated a relative predominance of Th2 cytokines in the mucosal compartment. Several factors could explain this situation. Under normal conditions, Th2 responses are typical of mucosal surfaces [29]. Additionally, during pregnancy, a range of soluble factors is produced by the placenta that switch maternal immune regulation towards a protective Th2 phenotype. These factors also influence the developing fetal immune system and all newborns initially have an immunological milieu skewed towards Th2 immunity [30]. Both the respiratory tract and the immune system undergo rapid maturation during the first year of life. Postnatal development seems to be affected by and affects responses against viral infections [31]. Viral factors could cause the Th2-predominant profile at the mucosal level. It has been reported that RSV evades the human adaptive immune response by skewing the Th1/Th2 cytokine balance toward increased levels of

Th2 cytokines [32]. Interference with the development of Th1 responses at the place of viral replication (the respiratory mucosa) could thus represent a mechanism of viral evasion. When the ratios between CXC and CC chemokines (or α and β -chemokines) were compared between both compartments, the mucosal compartment showed a relative predominance of CXCL8 (IL-8) and CXCL10 (IP-10). This finding agrees with previous reports [33], and indicates an active role of these chemokines in the respiratory system following infection with RSV. IP-10 recruits Th1 lymphocytes and monocytes to the lower respiratory tract [33]. IL-8 primarily mediates activation and migration of neutrophils from peripheral blood into tissue and is involved in the initiation and amplification of inflammatory processes [34]. These chemokines could promote virus clearance, but also participate in inflammatory pathogenic events following infection. Furthermore, we examined the relationships between the immune mediators of the plasma and mucosal compartments. The absence of a significant association between the concentration of each mediator in NPA and plasma indicates that both compartments maintain a certain degree of independence, since positive or negative increments in one compartment are not paralleled in the other. Finally, it is worth noting that that plasma mediators showed a higher number of significant associations with the parameters of clinical severity and with the leukocyte counts in peripheral blood than mediators in the mucosal compartment. This could occur as consequence of certain events taking place at the systemic level, such as leukocyte mobilization in response to the infection. Moreover, PDGF β and VEGF, two growth factors involved in angiogenesis [35], were inversely correlated with O $_2$ saturation and directly correlated with the duration of the hospitalization period. Since an inverse association between O $_2$ saturation and the parameter "days in hospital" was observed in the children of our study, PDGF and VEGF could be promoting angiogenesis in response to hypoxemia, as an attempt to ameliorate the uptake of O $_2$. VEGF has also been demonstrated to reduce *in vitro* RSV replication and inflammation [36].

CONCLUSION

Our study demonstrates that respiratory syncytial virus induces increases in Th1 and Th2 cytokines, chemokines and growth factors in the mucosal compartment. When ratios between mediators in the nasal mucosae and plasma were compared in parallel, a relative predominance of innate-immunity mediators, Th2 cytokines and CXC chemokines was observed in the mucosal compartment. This mucosal profile may contribute not only to viral clearance but also to the immuno-pathogenic events taking place in the respiratory tract.

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