

Dampening of IL-2 function in infants with severe respiratory syncytial virus disease

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Summary

A reduced ability to produce IL-2 and a limited response to this cytokine might affect the function of CD4⁺ T cells in RSV-infected infants.

Footnotes

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ABSTRACT

Background: FOXP3⁺ regulatory T cells (Tregs) restraint the destructive potential of the immune system. We have previously reported a pronounced reduction of circulating Tregs in infants with severe respiratory syncytial virus (RSV) disease. Because IL-2 is critical for Treg growth, survival and activity, we here analyzed IL-2 production and function in RSV-infected infants.

Methods: Phenotype, proliferation, IL-2 production and IL-2 signaling in CD4⁺ T cells were analyzed by flow cytometry. Serum CD25 levels were quantified by ELISA.

Results: CD4⁺ T cells from RSV-infected infants produced lower amounts of IL-2 and showed a reduced proliferative response compared with healthy infants. IL-2 increased CD4⁺ T cell proliferation and FOXP3 expression in both, healthy and RSV-infected infants. However, despite IL-2 induced a similar pattern of STAT5 phosphorylation, the proliferative response of CD4⁺ T cells and the expression of FOXP3⁺ remained significantly lower in RSV-infected infants. Interestingly, we found a negative correlation between disease severity and both, the production of IL-2 by CD4⁺ T cells and the ability of exogenous IL-2 to restore the pool of FOXP3⁺CD4⁺ T cells.

Conclusions: A reduced ability to produce IL-2 and a limited response to this cytokine might affect the function of CD4+ T cells in RSV-infected infants.

Key words: *Respiratory syncytial virus, infants, severe bronchiolitis, CD4+ T cells, FOXP3, IL-2, sCD25, CD25.*

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INTRODUCTION

Respiratory syncytial virus (RSV) infection is the commonest cause of bronchiolitis and hospitalization in infants accounting for about 100 000–200 000 deaths annually [1]. Pathogenesis of RSV infection involves both, cytopathic effects induced by the virus and an exacerbated inflammatory response which explain, at least partially, the airway damage [2, 3]. FOXP3+ regulatory T cells (Tregs) not only prevent autoimmune diseases and maintain immune homeostasis, but also regulate the immune response in the course of infectious diseases controlling tissue injury [4, 5]. In fact, experimental models of RSV infection have shown that depletion of Tregs leads to a more severe disease [6-8]. The role of Tregs during human RSV infection has not been clarified yet. However, the Treg cell compartment undergoes important changes in the course of severe RSV infection. In this regard, we have previously reported that severe RSV infection in infants is associated with a marked reduction in the frequency of peripheral blood Tregs [9].

Most studies analyzing the role of Tregs have focused on chronic conditions, such as autoimmunity, cancer, and persistent infectious diseases [10-13]. By contrast, very little is known about the function of Tregs in acute viral infections. Our previous findings showing the depletion of Tregs in RSV infected infants contrast with the increased frequencies of Tregs found in other acute viral diseases such as dengue [14] and influenza A virus (IAV) [15], suggesting a particular signature of RSV infection. Because Treg survival and function are dependent on IL-2 receptor signaling [16], we speculated that severe RSV disease could be associated with a dysregulation of the IL-2/IL-2R system. This hypothesis might also explain another key feature of RSV

infection, its inability to induce long-lasting immunity, a response strongly dependent on IL-2 function [17].

Here, we found that the production and function of IL-2 are compromised in infants with severe RSV infection. CD4⁺ T cells from RSV infected patients produced lower amounts of IL-2 and showed a limited proliferative response compared with cells from healthy donors. We also found that the addition of IL-2 failed to fully restore IL-2-dependent functions such as FOXP3 expression and CD4⁺ T cell proliferation. Interestingly, a negative correlation was observed between disease severity and both, IL-2 production by CD4⁺ T cells and IL-2 ability to increase the pool of FOXP3⁺CD4⁺ T cells.

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MATERIALS AND METHODS

Ethics Statement

Our study was approved by the Ethics Committee of the “Hospital de Pediatría Pedro de Elizalde”, Buenos Aires, Argentina, in accordance with the Declaration of Helsinki (Fortaleza 2013). Written informed consent was obtained from all donors or legal guardians.

Study population

We recruited 85 infants < 18 months old hospitalized at the “Hospital de Pediatría Pedro de Elizalde” (Buenos Aires, Argentina), with a severe episode of RSV bronchiolitis during the 2016-2017 respiratory seasons. We excluded children with history of prematurity, immunodeficiency, congenital heart disease, and chronic conditions. All of these infants required hospitalization. RSV infection was confirmed by direct immunofluorescence of nasopharyngeal aspirates. Disease severity was assessed by applying a clinical disease severity score (CDSS) based on the modified Tal score, which classified patients as having mild (0–4), moderate (5–8), or severe (9–12) RSV bronchiolitis at the time of sampling [18, 19]. Control group consisted in 40 infants admitted to the hospital for scheduled surgery (healthy donors, HD). They had no identifiable airway infections for a 4-week period before the study. Characteristics of the patients are shown in **Table 1**.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were obtained from blood samples (0.3 to 0.4 ml) from RSV-infected infants or HD, by Ficoll-Hypaque gradient centrifugation (GE Healthcare Life Sciences, Uppsala, Sweden). PBMCs were washed twice, and

suspended in complete culture medium: RPMI 1640 (Hyclone, Logan, Utah, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Natocor, Córdoba, Argentina), 200 mM L-glutamine and 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA).

Flow cytometry

Labeled mAb directed to CD3, CD4, CD8, CD14, CD19, CD25, CD39, FOXP3, IL-2, STAT5, and Ki-67 were all from BD Biosciences (San José, CA, USA). In all cases, isotype-matched mAb were used as controls. For intracellular IL-2 detection, cells were stimulated with 50 ng/ml PMA and 1 µg/ml ionomycin (Sigma-Aldrich) for 5 h in the presence of monensin (Golgi-Stop, BD Biosciences), and stained with anti-CD4 and anti-IL-2 mAbs, after cell fixation and permeabilization (BD Biosciences). To analyze STAT5 phosphorylation in CD4⁺ T cells, PBMCs were treated, or not, with IL-2 (20 ng/ml, Peprotech, Rocky Hill, NJ, USA) for 30 min at 37°C. Cells were then stained with a mAb directed to phosphorylated STAT5, after cell fixation and permeabilization. Data were acquired using a FACS Canto (Becton Dickinson) and analyzed with FlowJo software. Statistical analyses were based on at least 100,000 events gated on the population of interest.

Culture of PBMCs in the presence of IL-2

To test the proliferative response of CD4⁺ T cells, PBMCs from RSV or HD (1x10⁶ cells/ml) were activated with PHA (4 µg/ml, Sigma-Aldrich) and cultured in the absence or presence of IL-2 (20 ng/ml) during 3 d. Then, the expression of the proliferation marker Ki-67 was assessed by flow cytometry. The concentration of IL-2 used in these assays was selected on the basis of preliminary experiments performed

with PBMCs from healthy children. These experiments showed that the proliferation of PHA-activated CD4⁺ T cells reached similar values by using either 20 or 100 ng/ml of IL-2. To test the ability of IL-2 to increase the expression of FOXP3, resting PBMCs (1x10⁶ cells/ml) were cultured without or with IL-2 (20 ng/ml) for 24 h. In some experiments, resting PBMCs were cultured with IL-2 alone or IL-2 plus recombinant soluble CD25 (sCD25, 200 ng/ml, Peprotech), for 30 min or 24 h, and phosphorylation of STAT5 and FOXP3 expression was evaluated.

Culture of PBMCs in the presence of serum from RSV-infected or healthy children

Serum was obtained from HD and RSV-infected children, while PBMCs were obtained from unrelated healthy adult donors. Briefly, PBMCs (1x10⁶/ml) were incubated for 24 h in complete culture medium supplemented with heat-inactivated serum from healthy or RSV-infected children (final dilution 1:10), in the absence or presence of IL-2 (20 ng/ml). Then, the frequency of FOXP3⁺CD4⁺ T cells was analyzed. Flow cytometry crossmatch was used to detect the presence or lack of IgG antibodies on the surface of CD4⁺ lymphocytes, after incubation of PBMCs with serum samples. In no case, we detected the presence of cell-associated IgG antibodies.

Cytometric Bead Array (CBA)

PHA (4 µg/ml)-activated PBMCs (1x 10⁶/ml) were cultured during 3 d and the supernatants obtained were frozen until use. Measurement of IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ was assessed according to the manufacturer's protocol (BD Biosciences). Data were acquired using a FACS Canto.

ELISA

Serum levels of sCD25 were determined by ELISA (Thermo Fisher, Fredrick, MD, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Data normality was evaluated by Shapiro Wilk test. Groups were compared using the Chi-Square test or Wilcoxon signed rank test and/or Mann-Whitney test as appropriate. Correlations were assessed using Spearman correlation test. A p value <0.05 was considered statistically significant.

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RESULTS

Clinical characteristics of study population

The characteristics of RSV-infected and healthy infants enrolled in this study are summarized in Table 1. The CDSS of all admitted patients was equal or higher than seven. All admitted patients needed O₂ requirement. Those admitted to intensive care unit (n=3, 3.6%) required invasive mechanical ventilation for a median of 4.5 d (range, 3.6–5.9 d) and received broad-spectrum antibiotic therapy. RSV-infected children showed a higher frequency of B cells (CD19+ cells) and a lower frequency of CD8+ T cells compared with HD. The frequency of CD4+ T cells was similar in both groups.

CD4+ T cells from RSV- infected infants produce low amounts of IL-2 and show a decreased proliferative response

We first analyzed whether the production of IL-2, which is mainly mediated by conventional CD4+ T cells [20], might be reduced during severe RSV infection. PBMCs from RSV-infected and HD were stimulated for 5 h with PMA/ionomycin in the presence of monensin, and IL-2 production was analyzed by intracellular staining and flow cytometry in gated CD4+ T cells. We observed a similar frequency of IL-2+CD4+ T cells in RSV-infected and HD, however, the mean fluorescence intensity (MFI) of IL-2 staining was significantly lower in infected patients (**Fig. 1A**). Interestingly, an inverse correlation was found between disease severity (CDSS) and the MFI of IL-2 staining in activated CD4+ T cells from RSV-infected patients. Moreover, because young age represents one of the most important risk factors for the development of severe RSV disease [1], we re-analyzed our data by dividing patients into two groups; ≤6 months and >7 months. Both groups of patients showed a lower production of IL-2 compared with HD (**Fig. 1A, right panel**). To confirm that CD4+ T

cells from RSV-infected children actually produced low amounts of IL-2, we analyzed IL-2 production in the supernatants of PBMCs stimulated with PHA for 3 d, using a cytometric bead array (CBA). IL-2 levels were markedly lower in RSV-infected children compared with HD. Moreover, we found that cells from RSV-infected children produced lower amounts of IL-10 and IL-17. No significant differences were observed regarding the production of IL-4, IL-6, TNF- α , and IFN- γ (**Fig. 1B**)

Consistent with our observations indicating a deficient production of IL-2 in RSV-infected patients, we found that the proliferative response of CD4⁺ T cell induced by PHA, assessed by detecting Ki-67 antigen expression, was substantially lower in RSV-infected infants compared with HD. The addition of IL-2 significantly increased the proliferative response of CD4⁺ T cells, however, even in the presence of exogenous IL-2, the proliferative response of CD4⁺ T cells from RSV-infected infants was markedly lower compared with HD (**Fig. 1C**). This suggests that factors other than IL-2 production might also compromise the expansion of T cells in RSV-infected infants. Because the activation of STAT5 is one of the earliest events in IL-2 signaling through the high affinity IL-2 receptor [21], we analyzed STAT5 phosphorylation in response to IL-2 stimulation. We found a similar pattern of phosphorylation in CD4⁺ T cells from both RSV-infected infants and HD (**Fig. 1D**), suggesting that the IL-2-STAT5 pathway is preserved during infection.

IL-2 increases the frequency of FOXP3⁺ Tregs in PBMCs from RSV-infected infants

We have previously reported that severe RSV infection in infants is associated with a pronounced reduction in the frequency of circulating Tregs [9]. To explore whether exogenous IL-2 was able to restore the pool of Tregs, PBMCs were cultured with or

without IL-2 during 24 h, and the frequency of Tregs was then analyzed. IL-2 significantly increased the frequency of FOXP3+CD4+ T cells in both HD and RSV-infected infants. However, even after IL-2 treatment, the frequency of FOXP3+CD4+ T cells was shown to be significantly lower in RSV-infected infants compared with HD (**Fig. 2A**). Reanalysis of the data by dividing patients according to their age into two groups (≤ 6 months and > 7 months), revealed that IL-2 failed to normalize the frequency of FOXP3+CD4+ T cells in both groups of patients (**Fig. 2A, right panel**). Moreover, as shown in **Fig. 2B**, IL-2-treatment also increased Treg expression of the ectonucleotidase CD39, which hydrolyzes ATP into the immunosuppressive agent adenosine [22]. This suggests that the increased expression of FOXP3 in CD4+ T cells from RSV-infected infants induced by IL-2 was actually associated with a regulatory signature. Interestingly, we found a negative correlation between CDSS values and the frequency of FOXP3+CD4+ T cells in IL-2-treated PBMCs, suggesting that a more severe disease is associated with a limited response to IL-2 (**Fig. 2C**). Further confirming the compromise in the compartment of FOXP3+CD4+ T cells in RSV-infected infants, we found that the proliferative response of this cell subset in response to PHA stimulation, assessed either in the absence or presence of IL-2, was severely reduced (**Fig. 2D**).

Soluble CD25 limit IL-2 function during severe RSV infection

Elevated concentrations of sCD25 are found in autoimmunity, cancer and inflammatory conditions [20, 23]. Looking for factors that might affect the function of IL-2 in the course of RSV infection, we evaluated serum levels of sCD25 (IL-2 receptor α chain soluble form). It has been reported that serum from RSV-infected infants has increased amounts of sCD25 [24, 25]. In agreement with this observation, we found higher

amounts of sCD25 in the serum from RSV-infected infants compared with HD (**Fig. 3A**). However, no correlation was found between disease severity and sCD25 levels (not shown). Because the ability of sCD25 to inhibit IL-2 activity remains controversial [20, 26, 27], we evaluated the effect of recombinant sCD25 on the function of IL-2 in CD4⁺T cells from RSV-infected infants. We found that both, IL-2 dependant STAT5 phosphorylation and FOXP3 expression were significantly inhibited by the addition of recombinant sCD25 (**Fig. 3B and C**). Consistent with this observation, we found that serum from RSV-infected infants, but not from HD, diminished the frequency of FOXP3⁺CD4⁺ T cells in PBMCs isolated from healthy donors, cultured with or without IL-2 (**Fig. 3D**). Moreover, we found a significant positive correlation between the ability of serum from RSV-infected children to reduce the frequency of FOXP3⁺CD4⁺ T cells and their content in sCD25 (**Fig. 3D, right panel**).

Activated conventional CD4⁺ T cells and Tregs are the main sources of sCD25 [20]. Since severe RSV infection is associated with a marked depletion of Tregs [9] we speculated that conventional CD4⁺ T cells might be the primary source of sCD25. In fact, we found that FOXP3⁻ CD4⁺ T cells from RSV infected infants, express not only a higher frequency of CD25⁺ cells compared with HD, but also a great expression of CD25 measured as MFI (**Fig. 4A**). A very low or negligible percentage of CD25⁺ cells was detected in CD8⁺ T cells, B cells and monocytes (**Fig. 4B**). Interestingly, we observed an inverse correlation between the frequency of peripheral blood FOXP3⁺ Tregs and the levels of serum sCD25 when HD and RSV-infected infants were analyzed collectively (**Fig. 4C**), suggesting that sCD25 might actually act as a decoy receptor for IL-2 during severe RSV infection.

DISCUSSION

Our results suggest that both, the production and function of IL-2 is compromised in the course of severe RSV infection in infants. We found that conventional CD4⁺ T cells from RSV infected infants produce low amounts of IL-2. Moreover, we observed that exogenous IL-2 was unable to fully restore IL-2 dependent functions such as the proliferative response of CD4⁺ T cells and the expansion of Tregs. These observations suggest that different mechanisms contribute to limit the function of IL-2 in the scenario of RSV disease. Interestingly, this phenomenon might contribute to explain not only the depletion of circulating Tregs, but also an essential feature of RSV infection; its inability to promote a robust memory T cell response [2, 3, 28, 29]. In this regard, it should be mentioned that IL-2 is required for the effective generation of effector and memory CD8⁺ T cells [30]. Moreover, it has been reported that the impaired effector and memory function of CD8⁺ T cells as well as the antibody response observed in RSV-infected animals were markedly improved by administration of IL-2, being these effects associated to a reduced weight loss and illness in challenged mice [31].

A reduced capacity of conventional CD4⁺ T cells to produce IL-2 has been previously described in either autoimmunity or infectious diseases. Autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus are associated to a defective ability of CD4⁺ T cells to secrete IL-2 [32-35]. This defect appears to explain the decrease of Tregs, favoring the expansion of autoreactive T cells [32]. On the other hand, observations made in experimental models of infections induced by *T.gondi*, *L. monocytogenes* and *vaccinia virus*, have shown that the acute infection phase is associated to a limited production of IL-2, resulting in a diminished Treg

frequency [36, 37]. Regarding RSV infection, previous studies suggested that the production of IL-2 by CD4⁺ T cells might be limited. Using PBMCs from adults who have been naturally and recurrently exposed to RSV and *influenza A virus* (IAV), Fleming and coworkers have reported that *in vitro* exposure to RSV results in a reduced production of IL-2 and a low lymphocyte proliferative response, compared to IAV-stimulated cells, despite that PBMCs from adult donors expressed a similar frequency of specific T lymphocytes for both viruses [38]. Moreover, we have reported that activated CD4⁺ T cells are permissive to RSV infection, and also that infection promotes a marked inhibition of IL-2 production [39].

IL-2 availability during RSV infection might be limited, not only by a decreased production, but also by the high systemic levels of sCD25. Consistent with previous studies [24, 25], we found high amounts of sCD25 in the serum from RSV infected infants. Elevated serum concentrations of sCD25 have been reported in inflammatory conditions [20, 23]. Activated conventional T cells, Tregs and dendritic cells can release sCD25 by proteolytic cleavage of surface CD25 [20]. Our observations show a great expression of CD25 on conventional CD4⁺ T cells from RSV infected infants, suggesting that they might be the main source of sCD25. Regarding the biological significance of sCD25, it appears to compete with activated T cells for IL-2 binding, thereby reducing T cell proliferation [20, 26]. However, it has also been described that sCD25 might enhance the biological activity of IL-2. In fact, by forming a complex with IL-2, sCD25 has shown to enhance IL-2-mediated phosphorylation of STAT5 in CD4⁺ T cells, promoting their differentiation into inducible FOXP3⁺Tregs [27]. These observations suggest that sCD25 might either decrease or enhance the biological activity of IL-2, depending on the experimental setting. Our observations showing that

sCD25 partially inhibits the ability of IL-2 to induce both, STAT5 phosphorylation and FOXP3 expression in CD4⁺ T cells from RSV infected infants, suggest that sCD25 acts as a decoy receptor for IL-2 during severe RSV disease.

We found that IL-2 significantly increased the proliferative response and the expression of FOXP3 in CD4⁺ T cells from RSV-infected infants. However, even in the presence of IL-2, both the proliferation rate of CD4⁺ T cells and the expression of FOXP3 remained substantially lower in CD4⁺ T cells from RSV infected infants compared with HD. This suggests that factors other than IL-2 availability might compromise the activity of IL-2 during severe RSV infection. Contrasting with other acute viral infections such as those produced by measles virus and cytomegalovirus, RSV infection is not associated with a generalized immunologic hyporesponsiveness [2, 40]. On the other hand, our results showing that IL-2 induced a similar pattern of STAT5 phosphorylation in CD4⁺ T cells from RSV infected infants and HD, suggest that signaling through the IL-2 receptor is preserved. Several factors might account for the limited activity of IL-2 in the course of RSV infection. Mediators such as IL-4, TGF- β , type I interferons and Prostaglandin E2 are produced at high levels in the context of RSV infection, being all of them capable of damping the function of IL-2 [41-46].

All patients recruited in our study required hospitalization and supplemental oxygen. We analyzed the impact of disease severity on IL-2 production and IL-2 ability to restore the pool of FOXP3⁺CD4⁺ T cells. This analysis revealed a significant negative correlation for both parameters, suggesting that patients with a more severe disease produce lower levels of IL-2 and show a limited response to IL-2. It should be noted that although IL-2 failed to fully restore *in vitro* the function of CD4⁺ T cells from

RSV-infected infants, it significantly improved both, the proliferative response of CD4+ T cells and the frequency of FOXP3+CD4+ T cells. Interestingly, observations made in experimental models of RSV infection have shown that IL-2 administration improves disease outcome in RSV challenged mice [31]. Proof-of-concept clinical trials have shown that at low doses, IL-2 improves autoimmune and inflammatory conditions [32]. Further studies are needed to define whether IL-2 might represent a useful therapeutic tool in severe RSV infection.

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Table 1. Baseline Characteristics of Children Infected with Respiratory**Syncytial Virus**

	RSV patients N = 85	Healthy children N = 40
Demographic characteristics		
Age, mo, mean \pm SD	7.5 \pm 7.1	12.6 \pm 3.8
Male sex, n (%)	57 (67%)	22 (55%)
Disease severity		
CDSS, n (%)*		
	0-6	0 (0%)
	7-8	48 (57%)
	9-12	37 (43%)
O ₂ req, n (%)	85 (100%)	N/A
Hospital stay, days, mean \pm SD	7.3 \pm 0.2	N/A
PICU admission, n (%)	3 (3.6%)	N/A
Laboratory parameters		
WBC, counts/mm ³ , mean \pm SD	10,559 \pm 4,021	8,952 \pm 1,250
Lymphocytes, %, mean \pm SD	37.4 \pm 15.4	53.1 \pm 4.0
CD4+, %, mean \pm SD#	28.0 \pm 11.0	32.3 \pm 5.7
CD8+, %, mean \pm SD#	12.4 \pm 6.8	20.0 \pm 7.5
CD19+, %, mean \pm SD#	36.4 \pm 10.2	16.5 \pm 7.2

Mo: months

*CDSS was calculated using the modified-Tal score

PICU: pediatric intensive care unit

Percentages of cell subsets in the gate of lymphocytes (n=12 in each group)

FIGURE LEGENDS

Figure 1. CD4⁺ T cells from RSV-infected infants produce low amounts of IL-2 and show a limited proliferative response. (A) PBMCs from HD (n=8) and RSV-infected infants (n=20) were stimulated with PMA/Ionomycin in the presence of monensin for 5 h. Percentage of IL-2⁺CD4⁺ T cells and the MFI of IL-2 staining in the gate of IL-2⁺CD4⁺ T cells were analyzed by flow cytometry. The correlation between disease severity (CDSS) and the MFI of IL-2 staining is also shown (Spearman rank correlation test). Right panel shows the MFI of IL-2 staining in PBMCs from HD (n=8) and RSV-infected infants >7 months (n=6) and ≤6 months (n=13). (B) Levels of different cytokines in the supernatant of PHA-activated PBMCs after 3d of culture, quantified by a cytometric bead assay (CBA; n= 12 in each group). (C) PHA-activated PBMCs were cultured in the absence or presence of IL-2 (20 ng/ml) for 3 d (n=12 for each group). Frequency of Ki-67⁺CD4⁺ T cells in the gate of CD4⁺ T cells was then evaluated by flow cytometry. (D) PBMCs were treated, or not, with IL-2 (20 ng/ml) for 30 min at 37°C (n=10 for each group). Frequency of pSTAT5⁺CD4⁺ T cells was then analyzed by flow cytometry. Representative experiments are shown in A (middle panel), C (right panel) and D (right panel). Mean ± SEM of n donors are shown in A, B, C and D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. MFI (mean fluorescence intensity).

Figure 2. IL-2 increases the frequency of FOXP3⁺CD4⁺ T cells in PBMCs from RSV-infected infants. (A) PBMCs from HD (n=8) and RSV-infected infants (n=41) were stimulated, or not, with IL-2 (20 ng/ml) for 24 h. Frequency of FOXP3⁺CD4⁺ T cells was analyzed by flow cytometry. Left panel shows the analysis of all patients

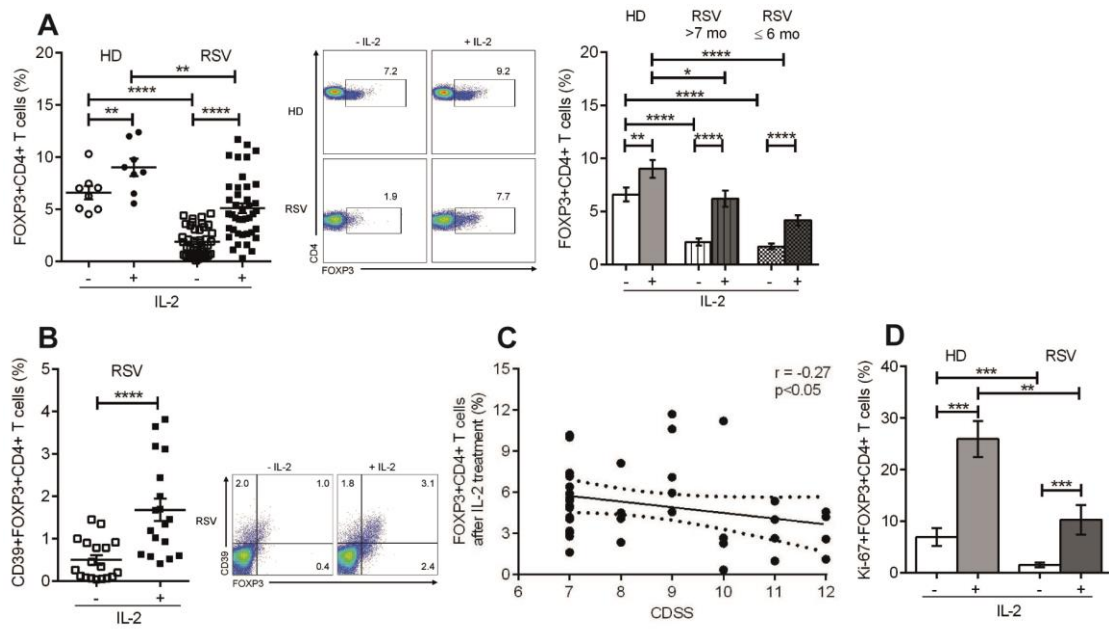
while the right panel shows the analysis of patients divided into two groups: >7 months (n=19) and ≤ 6 months (n=22). **(B)** Frequency of CD39+FOXP3+CD4+ T cells in PBMCs from RSV-infected children cultured for 24 in the absence or presence of IL-2 (20 ng/ml, n=18). **(C)** Correlation between the frequency of FOXP3+CD4+ T cells in PBMCs from RSV-infected infants cultured for 24 with IL-2 (20 ng/ml) and CDSS values, analyzed by using the Spearman rank correlation test. **(D)** PBMCs from HD and RSV-infected infants were activated with PHA (4 μ g/ml) and cultured in the absence or presence of IL-2 (20 ng/ml) for 3 d (n=12 for each group). Frequency of Ki-67+FOXP3+CD4+ T cells was evaluated by flow cytometry. Representative experiments are shown in A (middle panel) and B (right panel). Mean \pm SEM of n donors are shown in A (left and right panel), B (left panel), and D. **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 3. Soluble CD25 interferes with IL-2 function in RSV-infected children. **(A)** Levels of sCD25 in the serum from RSV-infected (n=24) and HD (n=21) were quantified by ELISA. **(B-C)** PBMCs from RSV-infected infants were cultured with IL-2 (20 ng/ml) or IL-2 plus sCD25 (200 ng/ml) for 30 min **(B)** or 24 h **(C)**. Then, the frequencies of pSTAT5+CD4+ T cells (n=12) or FOXP3+CD4+ T cells (n=7) were analyzed by flow cytometry (B and C, respectively). **(D)** PBMCs from healthy donors were incubated for 24 h with or without serum from healthy (n=9) or RSV-infected children (final dilution 1:10; n=9), in the absence or presence of IL-2 (20 ng/ml). Then, the frequency of FOXP3+CD4+ T cells was analyzed by flow cytometry (left and middle panel). Right panel shows the correlation between the ability of serum from RSV-infected infants to reduce the frequency of FOXP3+CD4+ T cells and serum levels of sCD25 (Spearman rank correlation test). Fold decrease of FOXP3+CD4+ T

cell frequency was calculated as the ratio between the frequencies of FOXP3+CD4+ T cells cultured in the absence and presence of serum from RSV-infected children. Representative experiments are shown in B (right panel) and D (middle panel). Mean \pm SEM of n donors are shown in A, B (left panel), C, and D (left panels). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 4. High expression of CD25 in conventional CD4+ T cells from RSV-infected infants. (A) Frequency and MFI of CD25 on gated FOXP3- CD4+ T cells from HD (n=9) and RSV-infected infants (n=21), evaluated by flow cytometry. (B) Percentage of CD25 on gated CD4+, CD8+, CD19+ and CD14+ cells from RSV-infected infants (n= 15). (C) Correlation between the frequency of FOXP3+CD4+ T cells and the serum levels of sCD25 in the group of healthy and RSV-infected children analyzed together, by using the Spearman rank correlation coefficient test. Representative experiments are shown in A (right panel). Mean \pm SEM of n donors are shown in A (left and middle panel) and B. ** $p < 0.01$ and **** $p < 0.0001$.

Figure 2



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Figure 3

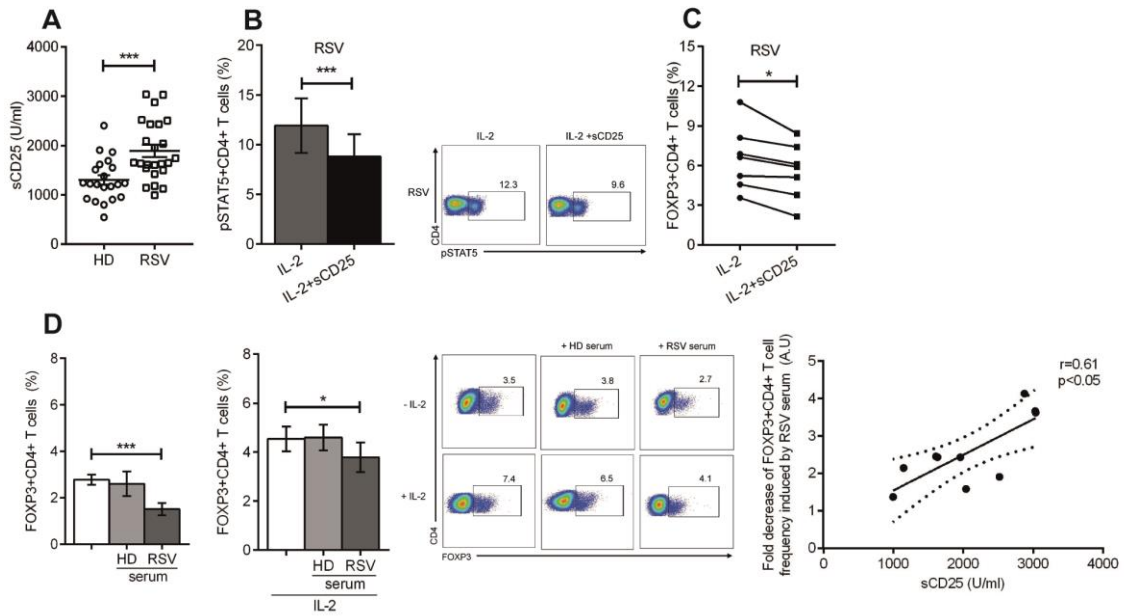
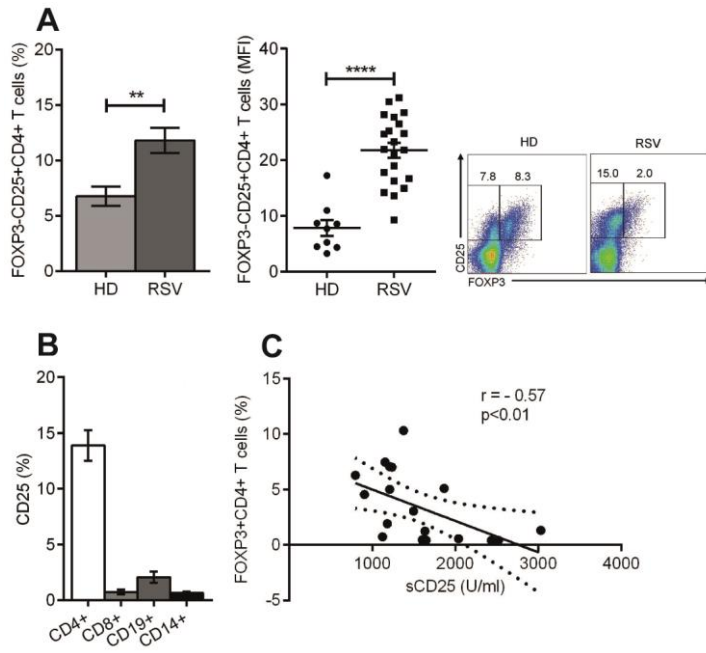


Figure 4



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