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MAJOR ARTICLE

Respiratory Syncytial Virus (RSV) Infects CD4+ T Cells: Frequency of Circulating CD4+ RSV+ T Cells as a Marker of Disease Severity in Young Children

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Background. Although human airway epithelial cells are the main target of respiratory syncytial virus (RSV), it also infects immune cells, such as macrophages and B cells. Whether T cells are permissive to RSV infection is unknown. We sought to analyze the permissiveness of CD4+ T cells to RSV infection.

Methods. CD4+ and CD8+ T cells from cord blood, healthy young children, and adults were challenged by RSV or cocultured with infected HEp-2 cells. Infection, phenotype, and cytokine production by T cells were analyzed by flow cytometry or enzyme-linked immunosorbent assay. Expression of RSV antigens by circulating CD4+ T cells from infected children was analyzed by flow cytometry, and disease severity was defined by standard criteria.

Results. CD4+ and CD8+ T cells were productively infected by RSV. Infection decreased interleukin 2 and interferon γ production as well as the expression of CD25 and Ki-67 by activated CD4+ T cells. Respiratory syncytial virus antigens were detected in circulating CD4+ and CD8+ T cells during severe RSV infection of young children. Interestingly, the frequency of CD4+ RSV+ T cells positively correlated with disease severity.

Conclusions. Respiratory syncytial virus infects CD4+ and CD8+ T cells and compromises T-cell function. The frequency of circulating CD4+ RSV+ T cells might represent a novel marker of severe infection.

Keywords. respiratory syncytial virus; viral infection; infants; bronchiolitis; CD4+ T cells; interleukin 2; interferon γ; markers of disease severity.

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory disease in young infants [1–3]. The majority of children display a mild illness of the upper airways. However, 2%–5% will develop a severe bronchiolitis that requires hospitalization [4–7]. There are still relevant gaps in our understanding of pediatric RSV infection. Studies performed in mouse models have helped clarify the pathogenesis of infection. However, these experimental models do not reflect the course of human disease [8]. Hence, the immune mechanisms responsible for protection and pathogenesis remain poorly defined [9], and the characterization of risk factors is still incomplete [10]. In addition, although it is well known that the immune response against RSV infection is protective but fails to induce long-lasting immunity, the reasons underlying this defective response remain unknown [11, 12].

We have recently reported that severe RSV infection in young children results in the depletion of circulating CD4+ FOXP3+ regulatory T cells (Tregs) [13]. In the current study, using cord blood (CB) and peripheral blood from young children and adults, we have demonstrated that CD4+ T cells are permissive to RSV infection. Infection of CD4+ T cell affects neither cell viability nor the frequency of CD4+ FOXP3+ T cells, but decreased interleukin 2 (IL-2) and interferon γ (IFN-γ) production. Moreover, we found that RSV antigens are detected in circulating CD4+ and CD8+ T cells from infected young children. Interestingly, the frequency of circulating CD4+ RSV+ T cells positively correlated with disease severity.

MATERIALS AND METHODS

Ethics Statement

Our study was approved by the Ethics Committee at Hospital de Pediatría Pedro de Elizalde, Hospital de Pediatría S.A.M.I.C. Prof. Dr. Juan P. Garrahan, and Hospital de Clínicas José de San Martín, Buenos Aires, Argentina, in accordance with the Declaration of Helsinki. Written informed consent was obtained from all donors or legal guardians.

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Blood Samples

Blood samples from umbilical CB, healthy young children, and healthy adults were used. Cord blood samples were obtained from umbilical cord veins at birth. Peripheral blood samples were obtained from children aged <24 months admitted to the hospital for scheduled surgery and from adults aged 25–35 years. No donors had any hereditary disorder, hematologic abnormalities, or infectious complications. We also recruited 35 infants aged ≤18 months old who were hospitalized at Hospital de Pediatría Pedro de Elizalde with a severe episode of RSV bronchiolitis. We excluded children with history of prematurity, immunodeficiency, congenital heart disease, and chronic conditions. 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 [14, 15]. The CDSS of all admitted patients was always ≥7. Respiratory syncytial virus infection was confirmed by direct immunofluorescence of nasopharyngeal aspirates. Baseline characteristics of each patient are shown in Table 1.

Isolation of Cord Blood or Peripheral Blood Mononuclear Cells

Cord blood mononuclear cells and peripheral blood mononuclear cells (PBMCs) were obtained from blood samples by Ficoll-Hypaque gradient centrifugation (GE Healthcare Life Sciences). In all experiments we used freshly isolated PBMCs.

Isolation of $CD4^+$ and $CD8^+$ T Cells

$CD4^+$ and $CD8^+$ T cells from healthy CB mononuclear cells or adult PBMCs were purified by negative selection using a $CD4^+$ or $CD8^+$ T cell isolation kit (Miltenyi Biotec; purity >96%). Isolated cells were washed twice and suspended in culture medium: Roswell Park Memorial Institute 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Natocor), 200 mM L-glutamine, and 50 μg/mL gentamicin (Sigma-Aldrich).

Cell Lines

HEp-2 cells (human laryngeal carcinoma) and Jurkat cells (lymphoblastoid T-cell line) were maintained in culture medium following the recommendations from the American Type Culture Collection.

Respiratory Syncytial Virus Stocks

Unless otherwise stated, all experiments were performed with the subtype A, strain Long (gift of Elsa Baumeister, Malbrán Institute, Argentina). Virus was propagated and titrated on HEp-2 cells, as previously described [16, 17]. To inactivate RSV, the virus was exposed for 30 minutes to ultraviolet light, as previously described [18]. Studies by confocal microscopy were performed using a recombinant green fluorescent protein-expressing RSV (GFP-RSV; kindly supplied by Dr Mark E. Peeples and Dr Peter Collins [19], Research Institute at Nationwide Children's Hospital, Columbus, Ohio, and National Institutes of Health, Bethesda, MD, respectively).

Respiratory Syncytial Virus Infection Assays

Unless otherwise stated, PBMCs from healthy young children, purified $CD4^+$ or $CD8^+$ T cells from CB or adult blood samples (1×10^6 cells/mL), and Jurkat cells (0.5×10^6 cells/mL) were activated with phytohaemagglutinin (PHA, 4 μg/mL) for 24 hours. Then activated cells or monolayers of HEp-2 cells (40%/50% confluence) were incubated with RSV at multiplicity of infection (MOI) of 0.5 for 1 hour at 37°C and washed twice, and infection was revealed after 2–4 days by flow cytometry and/or real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR). For flow cytometry studies, cells were fixed, permeabilized (BD Biosciences), and labeled with a mouse anti-human RSV monoclonal antibody (mAb; EMD Millipore Corporation) and a Fluorescein isothiocyanate (FITC) goat antimouse immunoglobulin G (Dako). Respiratory syncytial virus infection was analyzed in the gate of live cells based on their forward and side scatter parameters. Susceptibility to RSV infection was also analyzed using an alternative approach based on the coculture of T cells or PBMCs with infected HEp-2 cells. Monolayers of HEp-2 cells (40%/50% confluence) were incubated with RSV using a MOI of 0.5 for 1 hour at 37°C. Then cells were washed and cocultured with isolated $CD4^+$ T cells or $CD8^+$ T cells (from CB or adult blood samples) or PBMCs (from young children) previously activated by PHA (4 μg/mL) for 24 hours (HEp-2: $CD4^+$/$CD8^+$ T-cell ratio = 1:10). Infection was analyzed after 2–4 days of culture by flow cytometry on gated $CD4^+$ or $CD8^+$ T cells. We also analyzed the permissiveness of resting $CD4^+$ T cells to RSV infection. To this aim, unstimulated $CD4^+$ T cells isolated from adult peripheral blood were incubated with RSV (MOI, 0.5) for 1 hour at 37°C, and infection was evaluated by flow cytometry as described above. When indicated, the inhibitory effect exerted by heparin and the antiviral drug ribavirin on RSV infection of HEp-2 cells, $CD4^+$ T cells, and Jurkat cells was assessed. Heparin (50 U/mL, from bovine intestinal mucosa; Sigma-Aldrich) was added to target cells together with RSV. After incubation for 1 hour at 37°C, the inoculum containing heparin was removed, and cells were washed and cultured for 3 days. Then infection was detected by staining with the anti-human RSV mAb and quantified by flow cytometry. Ribavirin (80 μM; Sigma-Aldrich) was added to cell cultures after viral adsorption. Briefly, target cells were incubated with RSV for 1 hour at 37°C. Cells were then washed and cultured for 3 days in the presence of Ribavirin. Later, infection was revealed by flow cytometry.

Real-time Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted using Trizol and subjected to reverse transcription using Improm-II Reverse Transcriptase

Table 1. Clinical Characteristics of Respiratory Syncytial Virus–Infected Children

Patient	Age, mo	Sex	CDSS	LOS at hospital, d	O_2 req	PICU admission	Length of symptoms at time of sampling, d	Associated pneumonia	Use of steroids	WBC, cell/mm^3	Lymphocytes, %	CD4$^+$ RSV$^+$, %
1	10	Male	12	10	Yes	Yes	3	Yes	Yes	11 100	26	20.00
2	2	Male	12	9	Yes	Yes	2	No	Yes	17 200	30	7.17
3	5	Male	9	7	Yes	No	3	Yes	Yes	4700	20	0.28
4	1	Male	10	8	Yes	No	2	No	Yes	11 300	40	0.13
5	3	Male	7	5	Yes	No	2	No	Yes	12 450	32	0.01
6	18	Male	8	5	Yes	No	2	No	Yes	8610	28	0.12
7	3	Female	7	5	Yes	No	2	No	Yes	10 100	30	0.18
8	2	Male	10	9	Yes	No	1	No	Yes	4700	32	0.66
9	3	Female	8	8	Yes	No	2	Yes	Yes	29 600	12	0.23
10	4	Male	7	6	Yes	No	3	Yes	Yes	10 200	34	0.10
11	15	Male	7	6	Yes	No	3	Yes	Yes	11 200	38	0.09
12	2	Female	8	5	Yes	No	3	No	Yes	7100	48	0.20
13	11	Female	11	10	Yes	Yes	1	Yes	Yes	5240	29	1.14
14	3	Female	7	5	Yes	No	2	No	Yes	12 800	55	0.28
15	4	Male	7	5	Yes	No	2	No	No	24 300	23	0.50
16	15	Female	8	5	Yes	No	2	No	No	13 900	28	0.11
17	2	Male	12	11	Yes	Yes	1	No	Yes	20 500	18	11.50
18	15	Male	7	5	Yes	No	2	No	No	10 100	34	0.08
19	4	Female	7	5	Yes	No	1	No	No	8920	35	0.16
20	18	Male	7	5	Yes	No	1	No	No	11 300	40	0.02
21	2	Female	11	11	Yes	Yes	1	Yes	Yes	25 000	40	0.07
22	16	Female	7	5	Yes	No	2	Yes	No	10 400	38	0.17
23	15	Male	7	7	Yes	No	3	Yes	Yes	7800	39	0.12
24	2	Female	8	7	Yes	No	2	No	Yes	6300	24	0.70
25	3	Female	8	7	Yes	No	1	No	No	8000	35	0.13
26	17	Male	7	7	Yes	No	2	Yes	Yes	11 500	34	0.17
27	2	Male	9	8	Yes	No	1	Yes	No	4610	45	0.56
28	18	Female	11	10	Yes	No	1	No	Yes	6450	25	0.66
29	16	Male	9	7	Yes	No	1	Yes	Yes	6210	42	0.17
30	17	Female	10	8	Yes	No	2	No	Yes	17 600	25	0.38
31	2	Male	8	7	Yes	No	2	Yes	Yes	8400	21	0.60
32	13	Female	10	8	Yes	No	2	Yes	Yes	19 500	22	0.41
33	7	Male	8	6	Yes	No	3	No	No	16 400	33	0.36
34	2	Male	12	12	Yes	Yes	1	No	Yes	24 600	11	18.20
35	2	Male	12	11	Yes	Yes	1	No	Yes	11 100	13	17.00

Clincial disease severity score was calculated using the Modified-Tal score.

Abbreviations: CDSS, clinical disease severity score; LOS, length of stay; PICU, pediatric intensive care unit; RSV, respiratory syncytial virus; WBC, white blood cell.

(Promega). Polymerase chain reaction analysis for RSV was performed with a real-time PCR detection system (Mx3000P, Stratagene) using SYBR Green as a fluorescent DNA-binding dye. The primer sets used for amplification were as follows: RSV-F5'-catccagcaaatacaccatcca-3' and RSV-R5'-ttctgcacatca taattaggagtatcaa-3'; GAPDH-F5'-cgaccactttgtcaagctca-3' and GAPDH-R5'-ttactccttggaggccatgt-3'. Primer sets yielded a single product of the correct size. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method using *GAPDH* as a reference gene [20].

Flow Cytometry

We used anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-FOXP3, anti–IL-2, anti–IFN-γ, anti–interleukin 5 (IL-5), anti–interleukin 17 (IL-17), and anti-Ki-67 mAbs, all from BD Biosciences. In all cases, isotype-matched mAbs were used as controls. Cell viability was evaluated using Annexin V and 7-AAD (BD Biosciences). For intracellular cytokine detection, cells were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin in the presence of monensin (Golgi-Stop, BD Biosciences) for 5 hours and then stained with anti-CD4, anti-CD8, anti–IL-2, anti–IFN-γ, anti–IL-5, or anti–IL-17 after cell fixation and permeabilization. The analysis of cytokine production was performed in the gate of live cells based on their forward and side scatter parameters. To analyze the proliferative response, cells were infected as described above. At day 1 after infection, cells were restimulated with anti-CD3 (1.2 μg/mL; Beckman Coulter) and anti-CD28 (1 μg/mL; BD Pharmingen) antibodies, and the expression of the proliferation marker Ki-67 was assessed after

3 days. Data were acquired using a FACSAria II (BD) and were analyzed with FlowJo software. Statistical analyses were based on at least 100 000 events gated on the population of interest.

Confocal Microscopy

Respiratory syncytial virus infection was also revealed by confocal microscopy using GFP-RSV. Monolayers of HEp-2 cells (40%/50% confluence), PHA-activated Jurkat cells, or PHA-activated CD4$^+$ T cells purified from adult blood samples were incubated with GFP-RSV (MOI, 1) for 2 hours at 37°C, washed twice, and cultured in medium supplemented with 2% FCS for 2 days. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus) and analyzed using the Fiji Image J software.

Quantitation of Interleukin 2 in Cell Supernatants

Quantification of IL-2 in cell supernatants was performed by enzyme-linked immunosorbent assay (BD Biosciences). Assays were performed in duplicates.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Data normality was evaluated by Shapiro-Wilk test. For comparisons between groups, Wilcoxon matched pair test, Friedman test, and Kruskal–Wallis test were used. Correlations were assessed using Spearman correlation test. $P < .05$ was considered statistically significant.

RESULTS

CD4$^+$ T Cells Are Permissive to Respiratory Syncytial Virus Infection

The permissiveness of T cells to RSV infection was analyzed using different T cell sources: the Jurkat T-cell line, CD4$^+$ and CD8$^+$ T cells isolated from CB and adult blood samples, and PBMCs from young children. Analysis by flow cytometry, confocal microscopy, and real-time quantitative RT-PCR showed that RSV successfully infects Jurkat cells. As expected, infection levels were lower in Jurkat cells compared with the epithelial cell line HEp-2 (Figure 1A). Activated cord blood CD4$^+$ T cells (left panel) as well as activated (left and middle panels) and resting (right panel) adult CD4$^+$ T cells, were also shown to be permissive to infection when challenged with RSV (Figure 1B**).** Coculture of CB, adult, or child CD4$^+$ T cells with HEp-2 infected cells also induced T-cell infection (Figure 1C). Respiratory syncytial virus was able to infect not only CD4$^+$ T cells but also CD8$^+$ T cells (Figure 1D).

We then analyzed whether infected CD4$^+$ T cells were capable of transmitting the infection to the epithelial cell line HEp-2. First, CB CD4$^+$ T cells were challenged with RSV. After 3 days of culture, both supernatants and cells were tested for their ability to transmit the infection to epithelial cells. In these experiments, HEp-2 cell monolayers (40%/50% confluence) were directly exposed to RSV or to supernatants from infected CD4$^+$ T cells, or cocultured with infected CD4$^+$ T cells (HEp-2: CD4$^+$ T-cell ratio of 1:10). HEp-2 infection was analyzed by flow cytometry at day 3 after challenge. We found that both supernatants and infected CD4$^+$ T cells transmitted the infection to HEp-2 cells (Figure 2A). As expected, we also found that heparin, RSV treatment with ultraviolet light, and the antiviral drug ribavirin markedly prevented RSV infection of HEp-2 cells (Figure 2B), CB CD4$^+$ T cells (Figure 2C), and Jurkat cells (Figure 2D).

Infection by Respiratory Syncytial Virus Compromises the Function of CD4$^+$ T Cells

We next determined whether infection of CD4$^+$ T cells resulted in a loss of cell viability. Results in Figure 3A show that the percentages of apoptotic and necrotic cells were similar in uninfected and infected CB CD4$^+$ T cells. Then we analyzed the impact of RSV infection on the production of cytokines by CD4$^+$ T cells. Data in Figure 3B and 3C show that RSV infection significantly impaired the production of IL-2 by CD4$^+$ T cells from CB, young children, and adults. No inhibition was observed using ultraviolet-treated RSV (Figure 3C). Respiratory syncytial virus infection also inhibited the production of IFN-γ by CD4$^+$ T cells from young children but not from adults, and a similar inhibition pattern was found in CD8$^+$ T cells (Figure 3D). No changes in the production of IL-5 and IL-17 were detected (not shown).

Considering the strong inhibition of IL-2 production induced by RSV infection, we then looked at the expression of CD25, the alpha chain of the high-affinity IL-2 receptor, expressed by activated T cells. We found that RSV infection significantly inhibited the expression of CD25 (Figure 4A). Moreover, according to the ability of RSV infection to suppress both the production of IL-2 and the expression of CD25, we found a lower expression of the cell proliferation marker Ki-67 in RSV-challenged CD4$^+$ T cells. It should be noted, however, that a significant inhibition of Ki-67 expression was observed in CD4$^+$ T cells from CB and young children samples but not in CD4$^+$ T cells from adult samples (Figure 4B). We have recently reported that RSV infection in young infants who required hospitalization induced a dramatic and prolonged reduction in the frequency of peripheral blood Tregs [13]. Hence, we analyzed whether in vitro infection by RSV resulted in the loss of this cell subset. We found that RSV infection did not induce any change in the frequency of Tregs (Figure 4C).

Circulating CD4$^+$ T Cells From Infected Infants Express Respiratory Syncytial Virus Antigens: Frequency of CD4$^+$ RSV$^+$ T Cells as a Possible Marker of Disease Severity

Having shown that CD4$^+$ and CD8$^+$ T cells are permissive to RSV infection, we wondered whether RSV antigens could be detected in peripheral blood T cells from infected infants. To this aim, we recruited 35 infants aged ≤18 months who were hospitalized at Hospital de Pediatría Pedro de Elizalde with

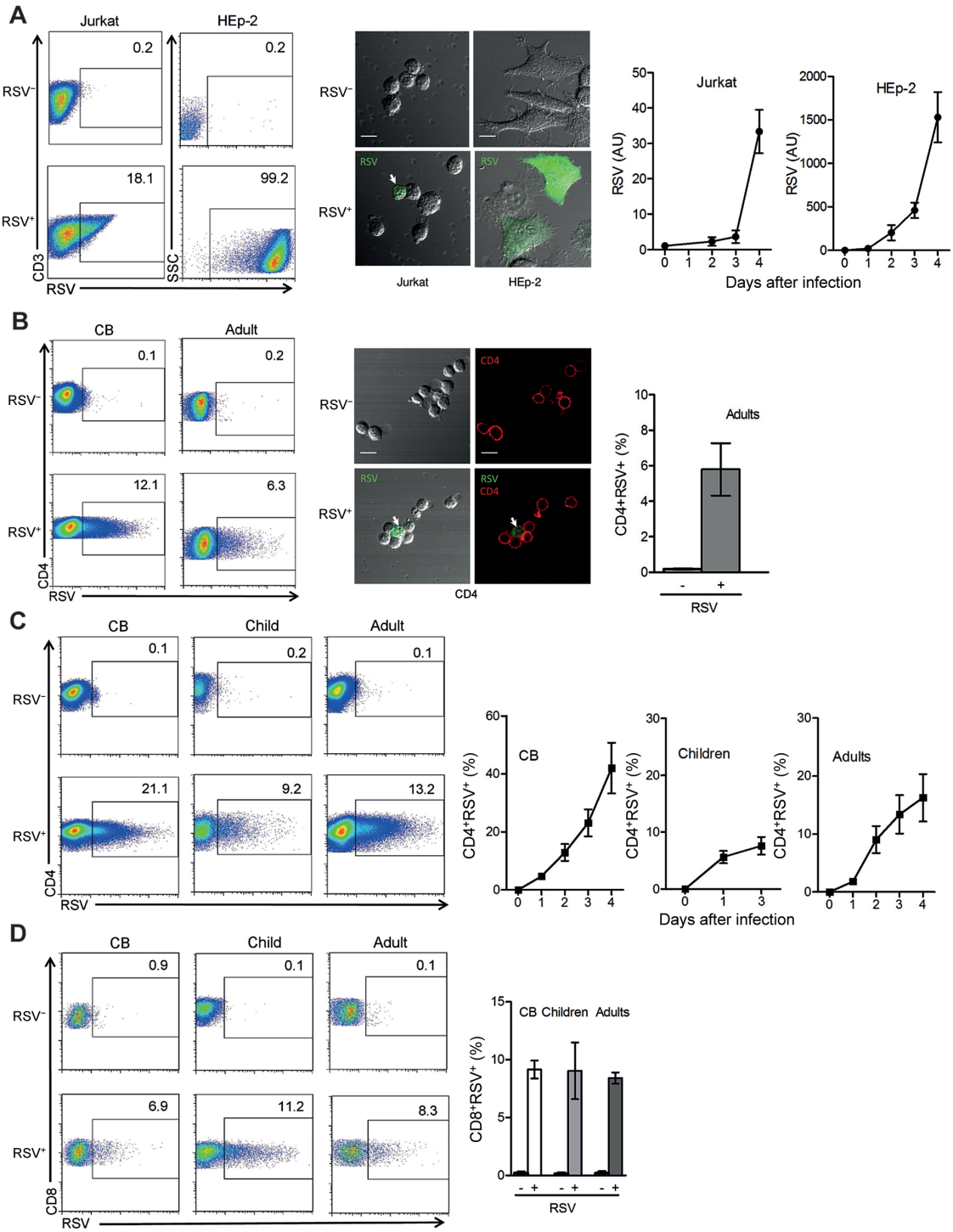

Figure 1. T cells are permissive to respiratory syncytial virus (RSV) infection. *A*, Jurkat cells (0.5×10^6/mL) or HEp-2 cell monolayers (40%/50% confluence) were challenged by RSV (subtype A; strain Long) at multiplicity of infection (MOI) of 0.5 for 1 h at 37°C. Cells were then washed, and infection was revealed by flow cytometry (left) or confocal microscopy (middle, green: RSV, bar: 10 μm) at day 4 after infection or by quantitative reverse-transcription polymerase chain reaction (RT-PCR) (right) at days 1, 2, 3, and 4 after infection. White arrows indicate green fluorescent protein-expressing RSV (GFP-RSV)–positive cells. *B*, Purified CD4+ T cells (1×10^6/mL) from cord blood (CB) or adult blood samples were activated by phytohaemagglutinin (PHA; 4 μg/mL) for 24 h, washed, and directly challenged by RSV (MOI, 0.5) for 1 h at 37°C. Cells were then washed, and infection was revealed at day 3 after infection by flow cytometry (left) or confocal microscopy (middle panel, green: RSV, red: CD4, bar: 10 μm). White arrows indicate GFP-RSV–positive cells. Right panel: Adult unstimulated CD4+ T cells were infected as described above, and infection was revealed by flow cytometry at day 3 after infection. *C* and *D*, Monolayers of HEp-2 cells (40%/50% confluence) were challenged, or not, by RSV (MOI, 0.5) for 1 h at 37°C. Then cells were washed and cocultured with isolated CD4+ T cells or CD8+ T cells (from CB or adult blood samples) or peripheral blood mononuclear cells (from young children) previously activated by PHA (4 μg/mL) for 24 h using a HEp-2/T-cell ratio of 1:10. Viral antigen expression was analyzed on gated CD4+ T cells at different times after infection (*C*) or on gated CD8+ T cells at day 3 after infection (*D*). Representative experiments are shown in *A* (left and middle), *B* (left and middle), *C* (left) and *D* (left). Mean ± SEM of independent experiments are shown in *A* (right, n = 4), *B* (right, n = 4), *C* (right, n = 5 for CB and adults, and n = 8 for children), and *D* (right, n = 5 for CB and adults, and n = 11 for children). Abbreviations: AU, arbitrary units; CB, cord blood; RSV, respiratory syncytial virus.

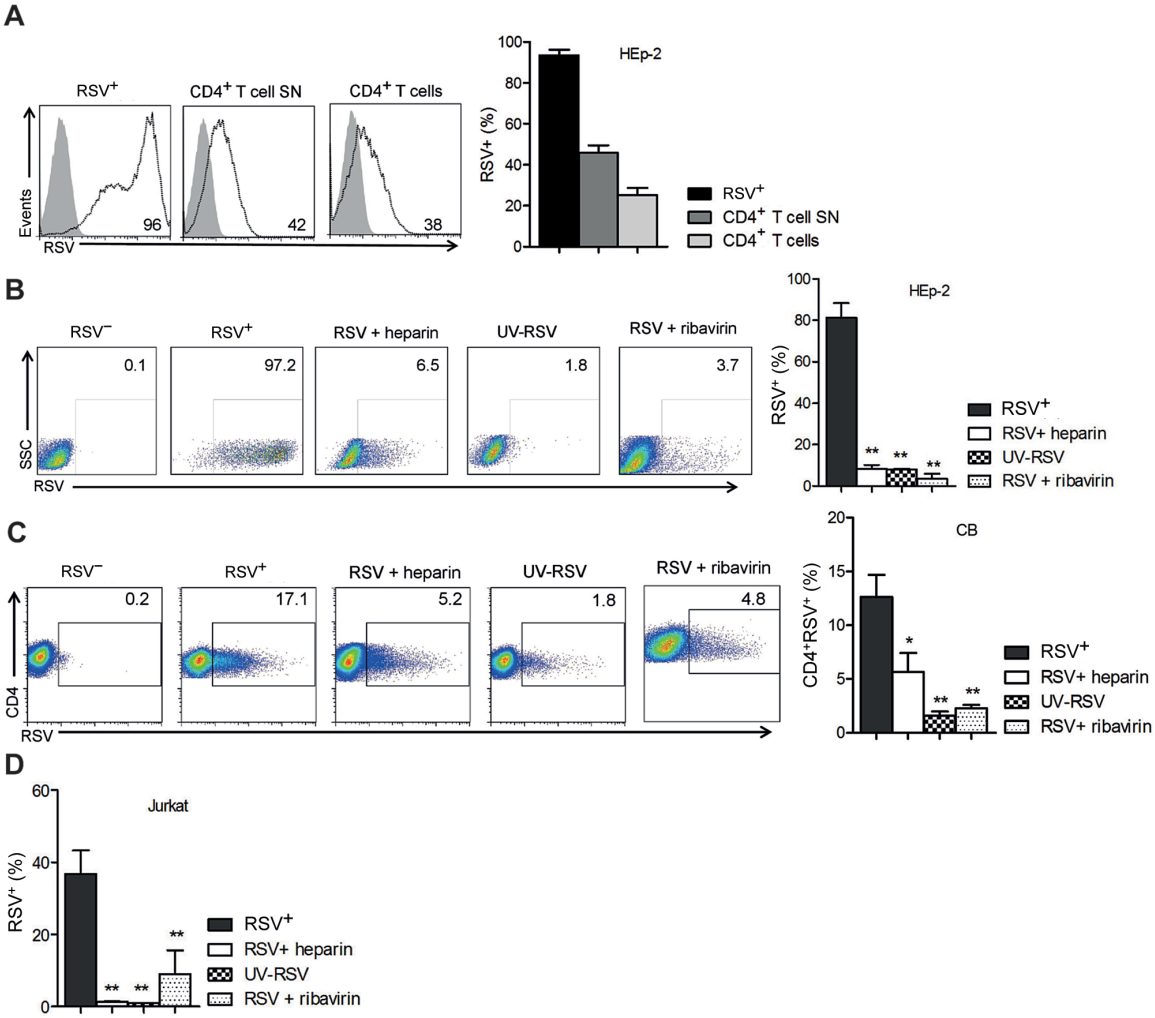

Figure 2. Infected CD4+ T cells transmit the infection to the epithelial cell line HEp-2. *A*, Purified CD4+ T cells (1×10^6/mL) from cord blood (CB) were activated by phytohaemagglutinin (PHA) for 24 h, washed, and challenged by respiratory syncytial virus (RSV) at multiplicity of infection (MOI) of 0.5, for 1 h at 37°C. Cells were then washed and cultured for 3 days. After this time, cell supernatants (SN) were harvested, and CD4+ T cells were washed again. Then HEp-2 cell monolayers (40%/50% confluence) were directly exposed to RSV or to supernatants from infected CD4+ T cells or cocultured with infected CD4+ T cells (HEp-2/CD4+ T-cell ratio of 1:10) for 3 days. Then HEp-2 infection was analyzed by flow cytometry. *B–D*, HEp-2 cell monolayers (*B*) as well as purified CB CD4+ T cells (*C*) or Jurkat cells (*D*) previously activated for 24 h with 4 µg/mL of PHA were challenged with RSV (MOI, 0.5) in the absence or presence of heparin (50 U/mL) or ribavirin (80 µM). When indicated, cells were challenged with ultraviolet-treated RSV. In all cases, infection was revealed after 3 days of culture by flow cytometry. Representative experiments are shown in *A* (left), *B* (left), and *C* (left), and the mean ± SEM of 5 experiments are shown in *A* (right), *B* (right), *C* (right), and *D*. **P* < .05, ***P* < .01 vs RSV+. Abbreviations: RSV, respiratory syncytial virus; SN, supernatant; UV, ultraviolet.

severe RSV bronchiolitis: (21 males, 14 females; age = 7.8 ± 6.6 months; white blood cells = 12263 ± 6437 counts/mm^3; lymphocytes = 30.8 ± 10.1% [mean ± SD]). Disease severity was analyzed using standard criteria, as described in the Materials and Methods. All admitted patients showed a CDSS ≥7. As a control group, we included 20 uninfected, aged-matched infants admitted for scheduled surgery: (11 male, 9 female; age = 12.6 ± 3.6 months; white blood cell = 8952 ± 1250 counts/mm^3; lymphocytes = 53.1 ± 10.2% [mean ± SD]). Baseline characteristics of each RSV-infected patient are shown in Table 1.

As expected, no expression of RSV antigens was detected in CD4+ or CD8+ T cells from uninfected infants. Interestingly, RSV antigens were detected in both circulating CD4+ and CD8+ T cells from RSV-infected infants (Figure 5A and 5B). Moreover, because the frequency of RSV+ cells in CD4+ and CD8+ T-cell populations showed a heterogeneous distribution among RSV-infected patients, ranging 0%–20%, we then analyzed whether the frequency of CD4+ RSV+ T cells was associated with disease severity. Figure 5C shows a strong positive correlation between CDSS and the frequency of CD4+ RSV+ T cells. This correlation appears to be mainly driven by the 6 patients showing percentages of CD4+ RSV+ T cells >1%. Of note, 5 of these 6 patients required admission to the pediatric intensive care unit. This observation strengthened the association between disease severity and percentages of circulating CD4+ RSV+ T cells >1%. To gain a better understanding of this

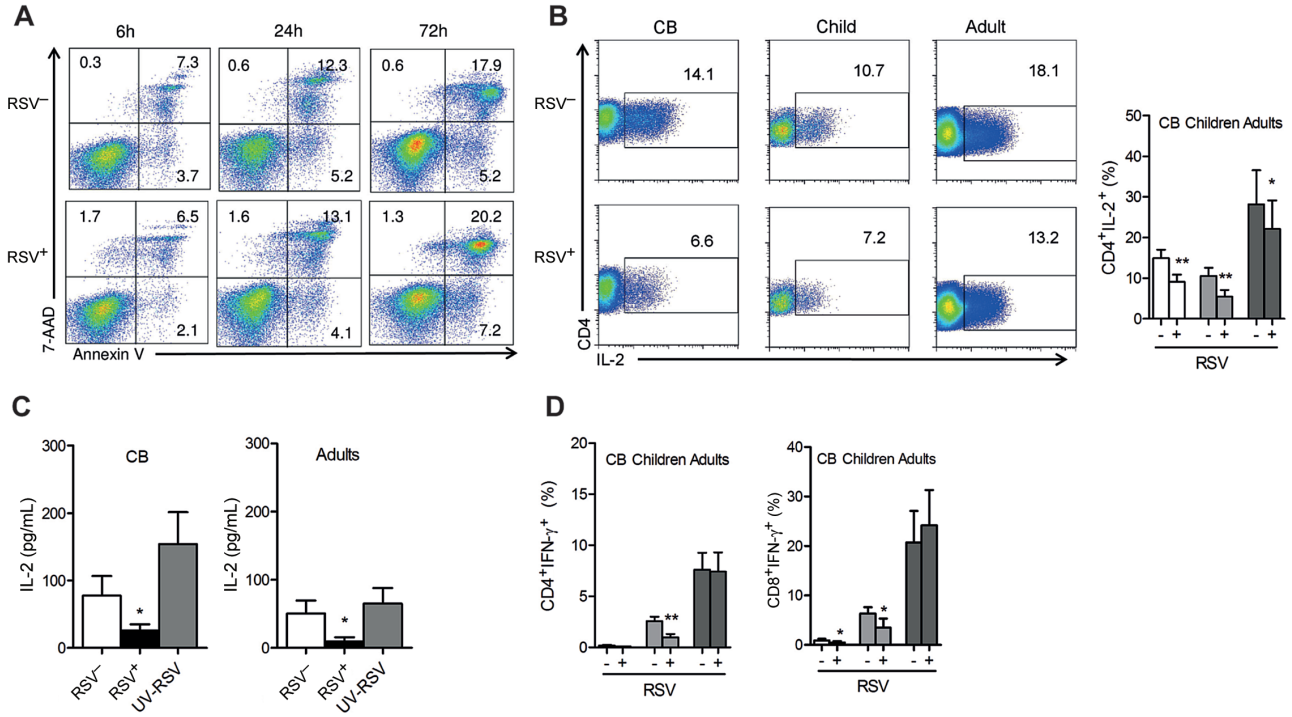

Figure 3. Respiratory syncytial virus (RSV) suppresses the production of interleukin 2 (IL-2) and interferon γ (IFN-γ) by CD4⁺ T cells. *A–D,* HEp-2 cells were challenged, or not, by RSV at multiplicity of infection (MOI) of 0.5 for 1 h at 37°C. Then cells were washed and cocultured with isolated CD4⁺ T cells or CD8⁺ T cells (from cord blood [CB] or adult blood samples) or peripheral blood mononuclear cells (from young children) previously activated by phytohaemagglutinin (PHA; 4 μg/mL) for 24 h. The HEp-2/T-cell ratio used was 1:10. After 3 days of culture, the frequency of Annexin-V/7-AAD–positive cells on gated CB CD4⁺ T cells was analyzed by flow cytometry (*A*). The production of IL-2 (*B* and *C*) and IFN-γ (*D*) was analyzed by flow cytometry on gated CD4⁺ (*B* and *D*, left) or CD8⁺ T cells (*D*, right) or by enzyme-linked immunosorbent assay in CD4⁺ T-cell supernatants (*C*). Representative experiments are shown in *A* and *B* (left). Mean ± SEM of different experiments are shown in *B* (right), *C*, and *D* (n = 7–8 for each group). **P* < .01 and ***P* < .05 vs RSV⁻. Abbreviations: CB, cord blood; IL-2, interleukin 2; IFN-γ, interferon γ; RSV, respiratory syncytial virus; UV, ultraviolet.

association, we also analyzed the correlation between CDSS and the frequency of CD4⁺ RSV⁺ T cells in our patient cohort, excluding those patients showing percentages of CD4⁺ RSV⁺ T cells >1% (patients 1, 2, 13, 17, 34, and 35, as depicted in Table 1). Even under these conditions, a significant positive correlation was found ($r = 0.42$; $P < .05$; n = 29).

Previous reports have shown that CD4⁺ T cells counts discriminate disease severity in children with RSV infection, with lower CD4⁺ T cell counts associated with more severe infection [21]. In agreement with this observation, we found not only a negative correlation between CDSS and the frequency of circulating CD4⁺ T cells but also a negative correlation between frequencies of CD4⁺ T cells and CD4⁺ RSV⁺ T cells (Figure 5D). There was no correlation between CD8⁺ RSV⁺ T cells and CDSS (data not shown). Taking together, our results suggest that the expression of RSV antigens by circulating CD4⁺ T cells might represent a novel marker of disease severity.

DISCUSSION

Although the human airway epithelium is the main target of RSV, immune cells are also susceptible to RSV infection. Respiratory syncytial virus productively infects human mast cells, stimulating the production of type I interferons, CXCL10 and CCL4 [22]. Alveolar macrophages and dendritic cells are also permissive to RSV infection [23–26]. In addition, previous reports have examined whether lymphoid cells were susceptible to RSV infection. Studies performed in murine models have shown that RSV infects B lymphocytes, but not T lymphocytes [27]. Also, it was described that RSV suppresses the proliferative response of lymphocytes through an interferon α–dependent mechanism [28]. On the other hand, Thurau and coworkers have reported that RSV infection of PBMCs induces the expression of RSV antigens in monocytes, B cells, and CD8⁺ T cells, but not in CD4⁺ T cells [18]. By contrast, our study shows that CD4⁺ T cells from CB, young children, and adults were permissive to RSV infection, as revealed by the intracellular staining of RSV antigens and flow cytometry. The reasons for the contrasting results are unclear. They could be explained by differences in the antibodies used to analyze the expression of RSV antigens. Whereas Thurau and colleagues used a homemade rabbit serum raised against purified RSV, we used a commercially available mouse mAb usually used in the diagnosis of RSV infection.

Our results demonstrate not only that CD4⁺ and CD8⁺ T cells are permissive to RSV infection but also that RSV infection compromises T-cell function. CD4⁺ T cells challenged by RSV showed a reduced ability to produce IL-2. A similar inhibition

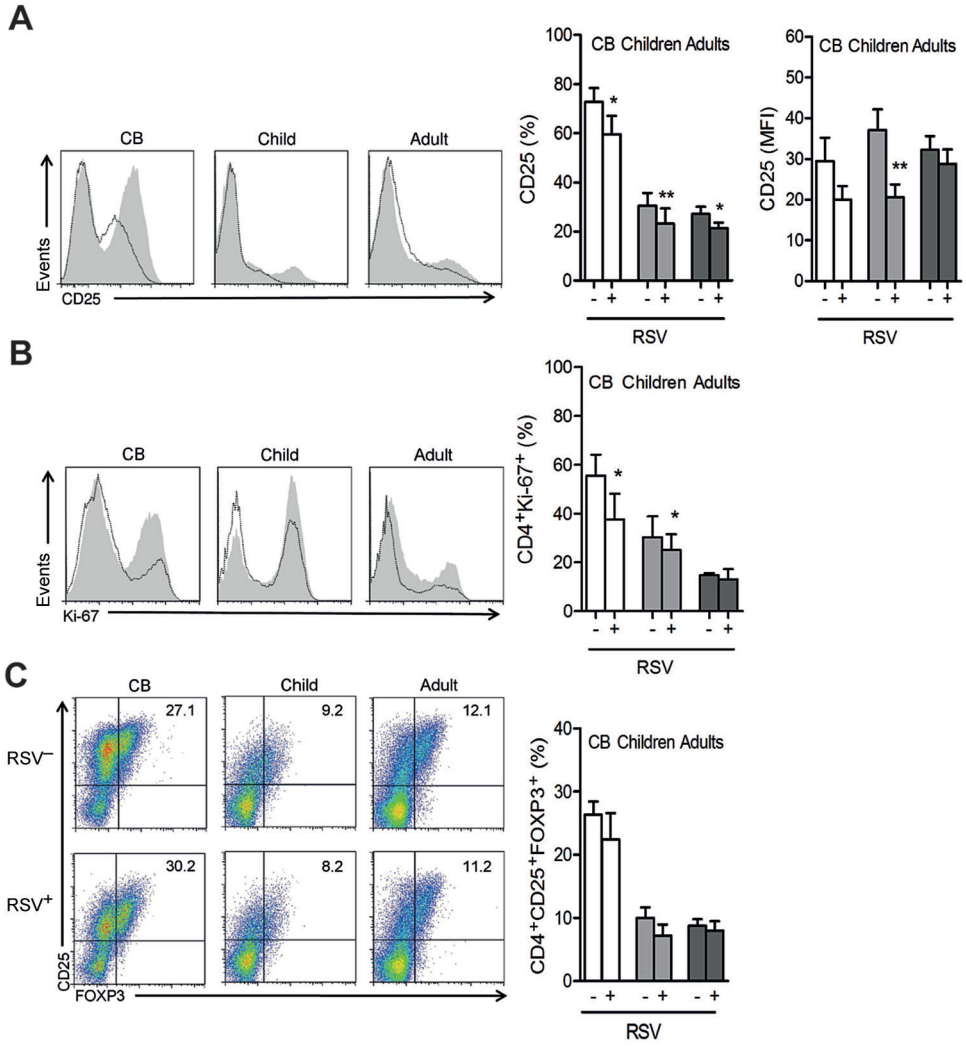

Figure 4. Respiratory syncytial virus (RSV) infection inhibits the expression of CD25 and Ki-67 by $CD4^+$ T cells without affecting the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells. *A–C*, HEp-2 cells were exposed, or not, to RSV at multiplicity of infection (MOI) of 0.5 for 1 h at 37°C. Afterward, cells were washed and cocultured with phytohaemagglutinin (PHA)–activated $CD4^+$ T cells (from cord blood [CB] or adult blood samples) or peripheral blood mononuclear cells (from young children) for 3 days using an HEp-2/$CD4^+$ T-cell ratio of 1:10. The expression of CD25 (*A* and *C*), Ki-67 (*B*), and FOXP3 (*C*) was analyzed by flow cytometry. Representative experiments are shown in *A* (left), *B* (left), and *C* (left), and the mean ± SEM of different experiments are shown in *A* (middle and right), *B* (right), and *C* (right) (n = 6 for CB and adults, and n = 10 for children). $^*P < .05$, $^{**}P < .01$ vs RSV^-. Abbreviations: CB, cord blood; MFI, mean fluorescent intensity; RSV, respiratory syncytial virus.

pattern was observed in $CD4^+$ T cells from CB and peripheral blood from young children and adults. Respiratory syncytial virus infection also inhibited the expression of CD25 and Ki-67 antigen in activated $CD4^+$ T cells. Moreover, a reduced production of IFN-γ by $CD4^+$ and $CD8^+$ T cells was observed in T cells from young children, but not in T cells from adult donors. This observation is particularly relevant because early age is one of the most important risk factors for developing a severe disease [29]. Together our observations suggest that by acting on either $CD4^+$ or $CD8^+$ T cells from young children, RSV might impair the development and/or the activity of type 1 T helper cells (Th1). These results are consistent with previous studies showing a decreased IFN-γ response in the course of RSV infection. Analysis of cytokine levels in nasal lavage fluid and PBMCs from infants with acute bronchiolitis due to RSV infection showed a type 2 T helper (Th2)-biased response [30–32]. Of note, alterations in the patterns of Th1/Th2 cytokine appear to be related to the pathogenesis of bronchiolitis due to RSV infection as well as the development of recurrent wheezing and asthma during infancy [31, 33]. Also consistent with our results, it has been shown that a defective production of IFN-γ in the course of RSV infection involves not only $CD4^+$ T cells but also $CD8^+$ T cells. In fact, $CD8^+$ T cells infiltrating the lung of RSV-challenged mice were shown to be defective in the production of IFN-γ [34].

Activated T cells represent the unique source of IL-2 [35]. Our study shows that RSV markedly inhibited IL-2 production by $CD4^+$ T cells. This cytokine plays a critical role not only in

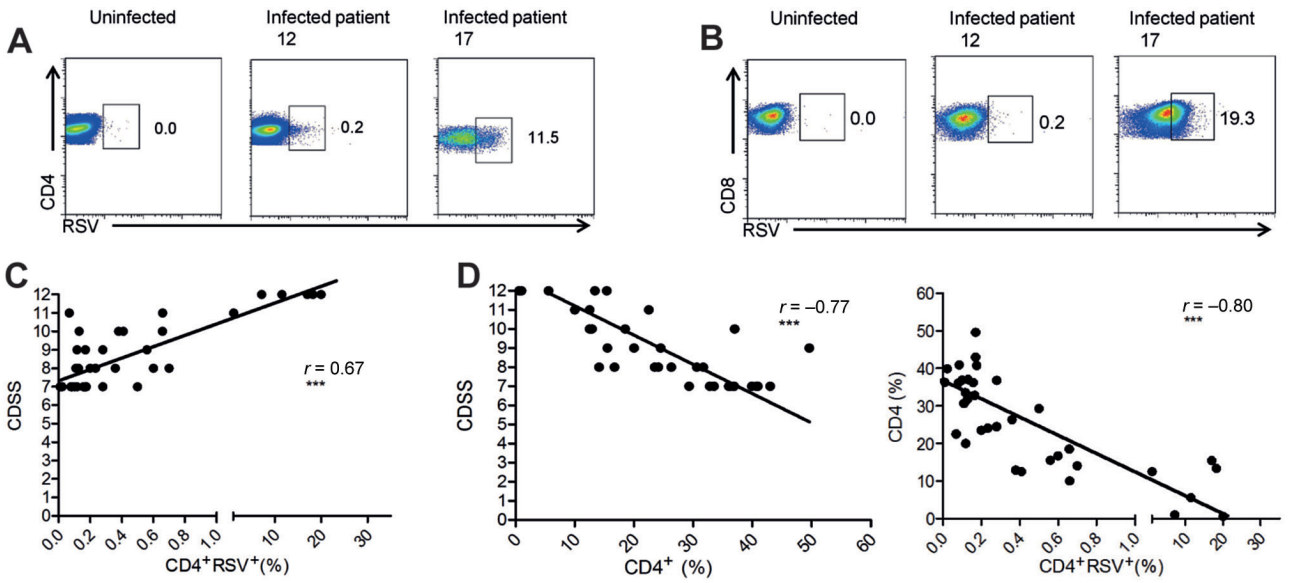

Figure 5. Frequency of circulating CD4+ respiratory syncytial virus (RSV)+ T cells as a possible marker of disease severity. *A* and *B*, Representative dot-plots of age-matched uninfected and RSV-infected young children, showing the frequency of CD4+ RSV+ T cells (*A*) and CD8+ RSV+ T cells (*B*) on gated CD4+ and CD8+ T cells, respectively. *C*, Correlation between clinical disease severity score (CDSS) and the percentage of CD4+ RSV+ T cells on gated CD4+ T cells from infected children (n = 35). *D*, Correlation between CDSS and the percentage of CD4+ T cells in infected children (left). Correlation between the percentages of CD4+ T cells and CD4+ RSV+ T cells in infected children (right). The CDSS was determined using the modified Tal Score. Correlations were evaluated by using Spearman rank correlation coefficient test. ***$P < .001$. Abbreviations: CDSS, clinical disease severity score; RSV, respiratory syncytial virus.

the expansion of Th1 cells but also in the generation of memory CD4+ and CD8+ T cells [36, 37]. Hence, a defective production of IL-2 in the course of RSV infection might explain why RSV, unlike many other viruses, does not induce durable protective immunity. In support of this hypothesis, previous studies have shown that the administration of a replication-defective adenovirus expressing IL-2 to mice challenged by RSV increases both the generation of memory CD8+ T cells and the antibody response against RSV [38]. Moreover, it was reported that vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin strains expressing RSV antigens induced protective immunity against viral infection, this effect being associated with the induction of memory CD4+ T cells producing IL-2 and IFN-γ [39].

Recognized risk factors for severe RSV infection include prematurity, young age, congenital heart disease, Down syndrome, and chronic lung diseases [4, 6]. Moreover, because the pathogenesis of RSV infection involves a strong inflammatory response, the relationship between the production of proinflammatory chemokines and cytokines and disease severity has been the focus of a number of published studies [43–45]. A significant finding of our study was the discovery that circulating CD4+ and CD8+ T cells from RSV-infected infants with severe illness express RSV antigens. Because the frequency of CD4+ RSV+ T cells showed a heterogeneous distribution among infected patients, we analyzed whether the increased frequencies in CD4+ RSV+ T cells were associated with disease severity. We found a positive correlation between CDSS and the frequency of CD4+ RSV+ T cells. This correlation appears to be mainly driven by the 6 patients with a higher percentage of CD4+ RSV+ T cells (ie, >1%), who all had high CDSS scores ranging from 10 to 12 (Table 1). However, the fact that a significant correlation was still observed when these 6 patients were excluded from the analysis suggests that the frequency of circulating CD4+ RSV+ T cells might represent a useful marker of disease severity, including but not limited to patients with a more aggressive disease. Further studies involving children with mild illness are required to test this hypothesis.

Notes

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