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Depletion of Circulating Regulatory T Cells during Severe Respiratory Syncytial Virus Infection in Young Children

To the Editor:

Respiratory syncytial virus (RSV) is the main cause of viral lower respiratory tract illness in infancy and early childhood. Each year, RSV is estimated to cause 34 million cases of lung infection and the deaths of up to 199,000 children less than 5 years of age worldwide. Children are usually infected by RSV during the first year of life, and virtually all by 3 years of age. Although in most cases the infection induces mild illness of the upper airways, 2 to 5% experience a severe bronchiolitis that requires hospitalization and respiratory support in an intensive

care unit. These patients show later a high susceptibility to develop recurrent wheeze and asthma (1, 2).

Our current understanding of the host response to RSV in humans remains rudimentary, because most observations have been performed in animal models that do not adequately reflect the course of human infection (3, 4). There is compelling evidence, however, that the host immune response has a prominent role in the pathogenesis of severe RSV infection (3, 4). $FOXP3^+CD4^+$ regulatory T cells (Tregs) have emerged as the most important cells able to prevent potentially harmful immune responses (5). Observations made in animal models clearly demonstrated that Tregs play a critical role in controlling lung inflammation in the course of RSV infection (6–8). The presence and function of Tregs during human RSV infection have not been yet analyzed. We here show that severe RSV infection of young children induces the selective depletion of peripheral blood Tregs.

Methods

Study population. We recruited 36 infants who were aged less than 18 months and admitted with severe RSV bronchiolitis to the "Pedro de Elizalde" Children's Hospital in Buenos Aires, Argentina. All of these infants required hospitalization for treatment of bronchiolitis. RSV bronchiolitis was confirmed by direct immunofluorescence of nasopharyngeal aspirates. The control group consisted of 10 uninfected healthy infants admitted to the hospital for scheduled surgery. They had no identifiable airway infections for a 4-week period before the study. Characteristics of the patients are shown in Table 1. The parents of the participants enrolled in this study gave informed consent, and the study was approved by the Ethics Committee of the "Pedro de Elizalde" Hospital.

Flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from 1 ml of blood collected in ethylenediaminetetraacetic acid tubes through a Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Freshly isolated PBMCs were stained with anti-CD4 (APC) and anti-CD45RA (PE-Cy7) antibodies (BD Biosciences, San Jose, CA), and FOXP3 were detected in permeabilized cells with anti-FOXP3 (Alexa Fluor 488, BD Biosciences). Monocytes were excluded from the analysis based on their forward and side scatter parameters and low CD4 expression. In all cases, isotype antibodies were used as negative controls. Data were acquired using a FACSAria II (Becton Dickinson, San Jose, CA) and analyzed with FlowJo software.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software. Groups were compared using the two-tailed unpaired *t* test for unpaired samples. Paired samples were compared using the paired *t* test. A *P* value less than 0.05 was considered statistically significant.

Table 1: Baseline Characteristics of Children Infected with Severe Respiratory Syncytial Virus

	Healthy (*n = 10*)	Patients (*n = 36*)
Age, mo	6.5 ± 0.9	4.5 ± 0.5
Sex, % male	60	69
Blood leukocytes, counts/mm^3	9,456 ± 513	10,490 ± 804
Treated with corticosteroids, %	—	47*

Data are presented as mean ± SEM.
*Blood samples were collected before the administration of corticosteroids.

Results and Discussion

Human $FOXP3^+CD4^+$ T cells comprise three phenotypically and functionally distinct populations: suppressive resting Treg cells (rTregs; $CD45RA^+FOXP3^{lo}$), suppressive activated Treg cells (aTregs; $CD45RA^+FOXP3^{hi}$), and nonsuppressive T cells ($CD45RA^-FOXP3^{lo}$) (9). By using a combination of anti-CD4, anti-CD45RA, and anti-FOXP3 antibodies, we analyzed the three different subsets of $FOXP3^+CD4^+$ T cells and the two different subsets of $FOXP3^-CD4^+$ T cells (naive and memory) in PBMCs from healthy children and children with severe RSV infection. Representative dot plots and the frequency of each population of $CD4^+$ T cells are shown in Figures 1A and 1B, respectively. Children with RSV infection showed a marked reduction in the frequency of Tregs compared with healthy children. rTregs decreased from 6.5 to 1.0%, and aTregs decreased from 0.4 to 0.1%. A less dramatic reduction in the frequency of $CD45RA^-FOXP3^{lo}$ nonsuppressive T cells from 2.3 to 1.3% was also observed. No changes were observed in the frequency of naive and memory $FOXP3^-CD4^+$ T cells. We conclude that the two subsets of $FOXP3^+CD4^+$ Tregs are markedly and selectively depleted during the course of severe RSV infection. Then, we wonder about the rate of recovery of Tregs after the episode of acute infection. To this aim, 21 to 26 days after hospital discharge we studied again the frequency of blood $FOXP3^+CD4^+$ Tregs in 6 of the 36 patients previously analyzed. Figure 1C shows that the frequency of rTregs and aTregs remained largely reduced for at least 3 weeks after the clinical resolution of RSV infection. As expected, there was not significant variation in circulating Tregs from the control children when measured 24 to 28 days after obtaining the first blood sample (Figure 1C).

Our observations reveal a dramatic and prolonged reduction in the frequency of the two populations of peripheral blood $FOXP3^+CD4^+$ Tregs in infants with severe RSV infection. These results are consistent with previous observations showing lower levels of FOXP3 mRNA in whole blood from children with RSV infection compared with healthy children (10). Very little information is available about human Tregs in the course of acute viral infection. However, increased frequencies of Tregs during human infection by dengue and influenza A virus (H1N1) have been reported (11, 12). In addition, observations made in murine models of RSV infection showed not only that depletion of Tregs resulted in enhanced disease, suggesting that Tregs suppress pathological immune responses, but also that infection induced the recruitment of Tregs in the lung and mediastinal lymph nodes (6, 13). Taking these observations into account, we speculate that the depletion of circulating Tregs in infants with RSV infection could reflect a massive recruitment of Tregs from the bloodstream to the infected lungs or the lung-draining lymph nodes. Alternatively, it could reflect

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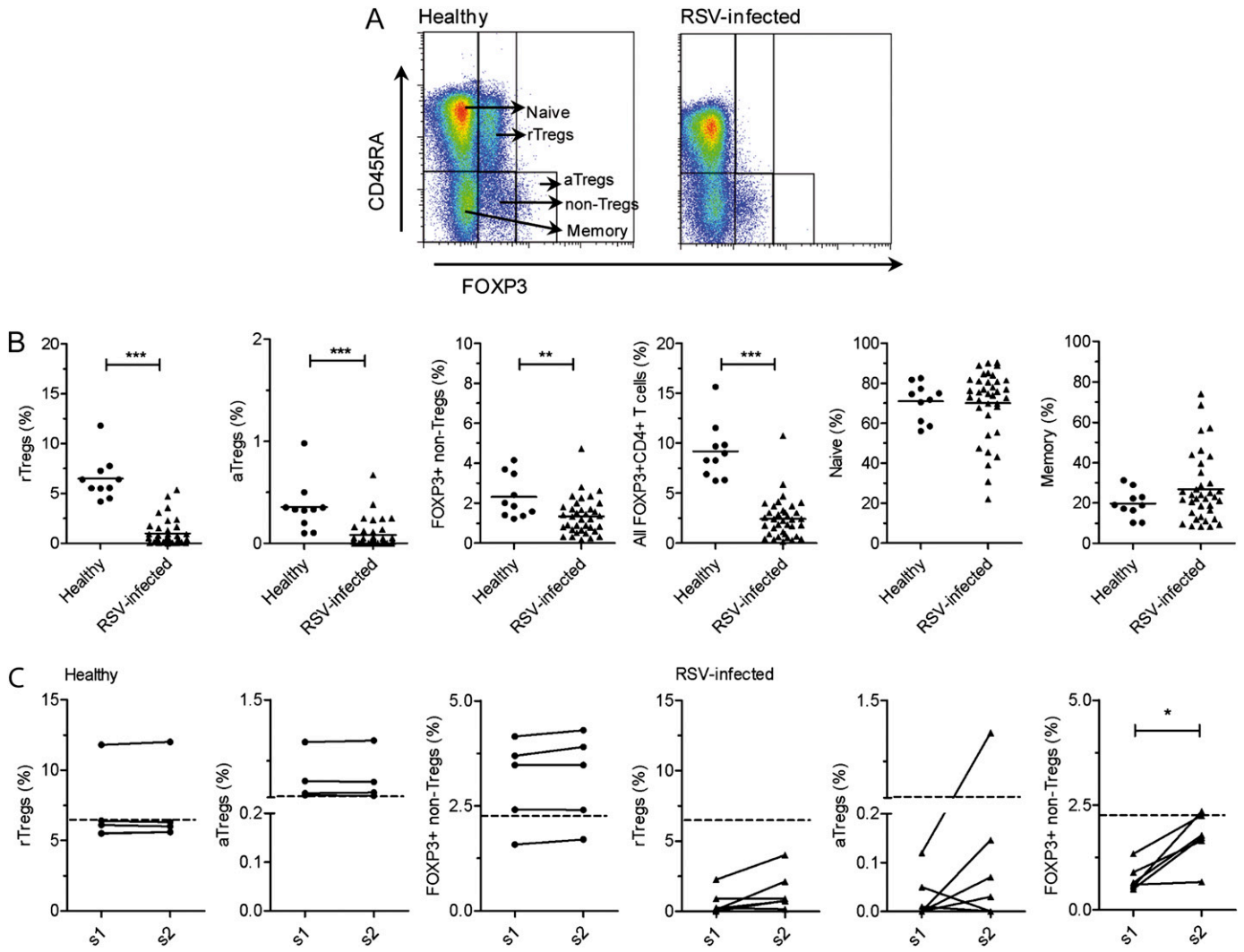

Figure 1. Frequencies of resting regulatory T cells (rTregs), activated Tregs (aTregs), and FOXP3$^+$ non-Tregs in the peripheral blood of healthy young children and young children with severe respiratory syncytial virus (RSV) infection. (*A*) Representative dot plots of CD45RA and FOXP3 expression in CD4$^+$ T cells are shown. (*B*) The frequency of each subset of FOXP3$^+$CD4$^+$ T cells (rTregs, aTregs, and FOXP3$^+$ non-Tregs), all FOXP3$^+$CD4$^+$ T cells, and FOXP3$^-$CD4$^+$ T cells (naive and memory) in children with RSV infection (n = 36) and healthy children (n = 10) is shown. (*C*) A second blood sample was taken from five healthy children (24–28 d after the collection of the first sample) and from six patients (21–26 d after the resolution of clinical infection), and the frequencies of the three subsets of FOXP3$^+$CD4$^+$ T cells was compared with those determined at the time of hospital admission. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

a high susceptibility to apoptosis of infant Tregs during the course of RSV infection as well as the possibility that in the setting of inflammation associated with severe RSV infection some Tregs might become unstable, losing FOXP3 expression and acquiring an effector-like phenotype (14). Further observations are needed to test these hypotheses. ■

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Obstructive Sleep Apnea and Preclinical Cardiac Damage: Need for U.S. Representative Study Sample

To the Editor:

I read the article by Querejeta Roca and colleagues with keen interest (1). These researchers analyzed data of 1,645 individuals free of cardiovascular disease at baseline. Certain laboratory variables, such as high-sensitivity troponin T (hs-TnT) and N-terminal brain natriuretic peptide, were measured in study participants. Obstructive sleep apnea (OSA) was diagnosed in 745 study subjects. Study participants were followed for a median 12.4 years. It was demonstrated that OSA severity was positively associated with greater levels of hs-TnT even after adjusting for potential confounders such as age, body mass index, sex, smoking, alcohol intake, hypertension, diabetes mellitus, renal function, pulmonary function tests, and lipid parameters. Furthermore, patients with elevated hs-TnT were noted to be at increased risk for incident heart failure, coronary heart disease, and death. The importance of this study is that it raises the question whether hs-TnT can be useful in detecting patients with OSA who are at increased risk for cardiovascular disease.

We congratulate the authors on this important study, which further supports the notion that OSA should be approached as a strong cardiovascular risk factor. Nevertheless, it is essential to note that 99% of the study participants were white (1). Therefore, these study findings may not apply to other groups living in the United States, such as Hispanics and African Americans, who compose a significant proportion of its population (2). In addition to that, it is known that patients of African American and Hispanic descent commonly have a greater burden of cardiovascular disease and metabolic disease (3). Therefore, it will be particularly important to evaluate whether OSA in those populations will translate into increased cardiovascular risk.

In conclusion, future studies with samples representative of the U.S. population should investigate whether detecting elevated hs-TnT in patients with OSA free of cardiovascular disease and resultant active therapeutic intervention will translate into decreased incidence of cardiovascular disease and mortality. Furthermore, it is important to study whether successful OSA treatment will decrease hs-TnT levels. ■

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