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ORIGINAL ARTICLE

IL-21 treatment recovers follicular helper T cells and neutralizing antibody production in respiratory syncytial virus infection

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Abstract

Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections in children under 1 year. RSV vaccines are currently unavailable, and children suffering from multiple reinfections by the same viral strain fail to develop protective responses. Although RSV-specific antibodies can be detected upon infection, these have limited neutralizing capacity. Follicular helper T (Tfh) cells are specialized in providing signals to B cells and help the production and affinity maturation of antibodies, mainly via interleukin (IL)-21 secretion. In this study, we evaluated whether RSV could inhibit Tfh responses. We observed that Tfh cells fail to upregulate IL-21 production upon RSV infection. In the lungs, RSV infection downregulated the expression of IL-21/interleukin-21 receptor (IL-21R) in Tfh cells and upregulated programmed death-ligand 1 (PD-L1) expression in dendritic cells (DCs) and B cells. PD-L1 blockade during infection recovered IL-21R expression in Tfh cells and increased the secretion of IL-21 in a DC-dependent manner. IL-21 treatment decreased RSV viral load and lung inflammation, inducing the formation of tertiary lymphoid organs in the lung. It also decreased regulatory follicular T cells, and increased Tfh cells, B cells, antibody avidity and neutralization capacity, leading to an overall improved anti-RSV humoral response in infected mice. Passive immunization with purified immunoglobulin G from IL-21-treated RSV-infected mice protected against RSV infection. Our results unveil a pathway by which RSV affects Tfh cells by increasing PD-L1 expression on antigen-presenting cells, highlighting the importance of an IL-21–PD-L1 axis for the generation of protective responses to RSV infection.

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infants, responsible for about 3 million hospitalizations and 74 500 deaths worldwide every year in children under 5 years of age.[1] There is no effective vaccine available against RSV, and passive immunization with monoclonal antibodies is used only in high-risk infants. Pregnant women and the elderly

are also target populations,[2] reinforcing the need for the development of an effective vaccine.

In humans, neutralizing RSV-specific antibodies are formed in the upper respiratory tract. However, reinfection with the same RSV strain is frequent in immunocompetent individuals, because antibody responses rapidly decline (approximately 3 months) to preinfection levels, which may facilitate recurring infections.[3,4] A correlation between nasal pre-existing RSV-specific humoral response and resistance to reinfection has been reported.[5] Other studies have correlated the presence of serum high-avidity RSV-specific immunoglobulin (Ig)G with protection,[6] although these antibodies have a short half-life in children[7] and adults.[8] RSV morbidity and mortality are increased in 2–4-month infants, in whom titers of maternal antibodies are reduced and have not yet been replaced by an endogenous antibody response.[9] The RSV fusion (F) protein is a glycoprotein on the envelope of the virion and the main target of antibodies leading to RSV neutralization; however, only a poor neutralizing antibody response is induced by the postfusion form of the RSV F protein. Antibodies that target the F prefusion form appear to be more effective as a neutralizing response.[10,11]

Effective B-cell responses require help from follicular helper T (Tfh) cells, which are found predominantly in germinal centers (GCs) of secondary lymphoid organs.[12–14] Tfh cells constitute the main source of interleukin (IL)-21.[15] IL-21 binds to interleukin-21 receptor (IL-21R) in naïve B cells and, along with other costimulatory signals, drives differentiation of either memory, GC B cells or plasma cells.[16] IL-21 is also crucial for affinity maturation and class switching of antibodies[17] that protect against invading pathogens.[18,19] Tfh cell activity can be negatively regulated by regulatory follicular T cells (Tfr cells), which are Foxp3-expressing follicular T cells with a regulatory phenotype.[20] Tfh cell regulation by Tfr cells involves different suppressor mechanisms, including downregulation of B-cell stimulation, delivery of direct negative signals and secretion of suppressive cytokines.[21]

In this study, we hypothesized that RSV targets humoral immune response efficiency by negatively modulating Tfh cells, thus preventing B-cell help. Our results showed that, upon RSV infection, Tfh cells failed to produce IL-21 and downregulated IL-21R. This correlated with a low-avidity RSV-specific humoral immune response, viral proliferation and lung tissue damage. GC response, which is stimulated by IL-21, is decreased by RSV infection, but can be recovered by treatment with recombinant IL-21. Treatment also induced the formation of tertiary lymphoid organs (TLOs) in the lung, reduced Tfr cells both in lung draining lymph nodes (LDLNs) and in lungs and restored high-affinity antibody production, decreasing viral replication and viral-induced lung inflammation. RSV infection also led to expression of programmed death-ligand 1 (PD-L1) in dendritic cells (DCs) and B cells, which correlates with decreased B- and Tfh-cell function. Blockade with anti-PD-L1 recovered IL-21R expression in Tfh cells and restored the secretion of IL-21 in a DC-dependent manner. Our results showed an intricate cooperation between the programmed cell death protein 1 (PD-1)/PD-L1 and the IL-21/IL-21R pathways in the generation of effective anti-RSV protective antibody responses.

RESULTS

RSV infection downregulates IL-21/IL-21R in Tfh cells via PD-L1 induction

PD-L1 is expressed on hematopoietic and nonhematopoietic cells; upon binding to PD-1 expressed in different cells, it delivers a signal with inhibitory effects for survival and function.[22,23] Tfh cells express PD-1 constitutively.[24] Previous studies demonstrated that RSV infection leads to PD-L1 upregulation in DCs.[25] PD-L1 upregulation in response to a helminth infection was reported to affect Tfh cells viability, leading to impairment of humoral responses.[26] In order to become fully differentiated Tfh cells, $CD4^+$ T cells need to receive first a signal from DCs in the T-cell zone and after that a signal from GC B cells in the GC.[24] We observed that not only DCs but also B cells in the lungs of RSV-infected mice upregulated PD-L1 (Figure 1a, b, Supplementary figure 2a).

IL-21 is one of the main signals delivered by Tfh cells to GC B cells to activate the production of high-affinity antibodies.[16,27] We next investigated whether RSV infection can modulate IL-21/IL-21R expression in Tfh cells. To test that, we infected mice with 1×10^7 plaque-forming units (PFUs) of RSV and after 5 days analyzed the intracellular expression of IL-21 or the surface expression of IL-21R in Tfh cells. RSV-infected mice did not induce IL-21 in Tfh cells (Figure 1c, d) and downregulated the expression of IL-21R in both Tfh cells (Figure 1e, f) and in B cells (Supplementary figure 2b). However, treatment with anti-PD-L1 (Supplementary figure 1a) increased IL-21 (Figure 1c, d) and restored IL-21R expression (Figure 1e, f) in lung Tfh cells, and increased IL-21R expression in B cells (Supplementary figure 2b). Together, these results suggested that PD-L1 induction by RSV was indeed necessary to block IL-21 production.

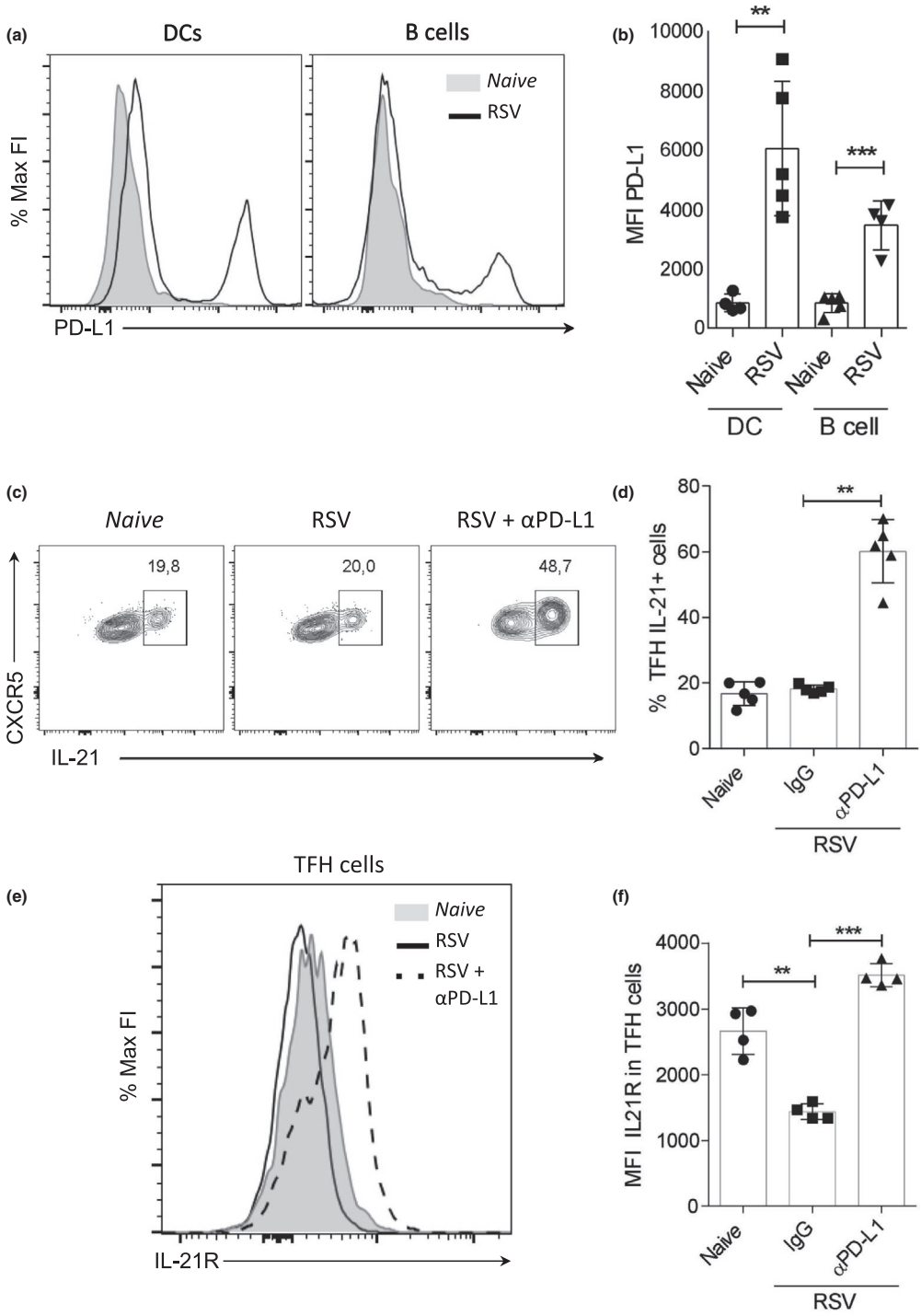

Figure 1. Programmed death-ligand 1 (PD-L1) blockade recovers interleukin (IL)-21/interleukin-21 receptor (IL-21R) expression in lung follicular helper T (Tfh) cells. BALB/c mice were intranasally infected with 1×10^7 plaque-forming units (PFUs) of respiratory syncytial virus (RSV) and treated intraperitoneally with two doses of 200 μg of anti-PD-L1 or isotype control on days 2 and 4 after infection. Five days after the infection, mice were killed and lungs were analyzed. (**a, b**) Mean fluorescence intensity (MFI) of PD-L1 in dendritic cells (DCs; $CD45^+CD11c^+CD19^-$) and B ($CD45^+CD11c^-CD19^+$) cells in RSV-infected or non-infected mice. (**c** and **d**) Gating and percentage of IL-21 expression or (**e** and **f**) IL-21R "/> in Tfh ($CD45^+CD4^+CXCR5^+PD1^+$) cells in naïve, RSV-infected or RSV-infected and treated with anti-PD-L1 mice. Results representative of three experiments, using five mice per experimental group. $^{**}P < 0.01$; $^{***}P < 0.001$.

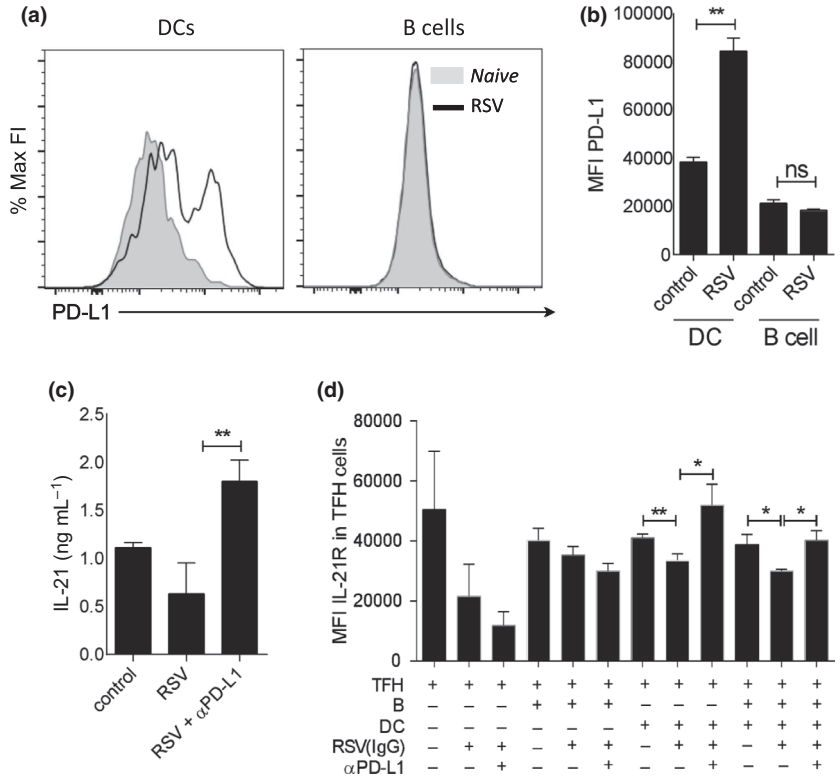

Figure 2. *In vitro programmed* death-ligand 1 (PD-L1) blockade recovers interleukin (IL)-21/interleukin-21 receptor (IL-21R) expression on follicular helper T (Tfh) cells in a dendritic cell (DC)-dependent manner. DCs ($CD11c^+CD19^-$), B cells ($CD11c^-CD19^+$) and Tfh cells ($CD4^+CXCR5^+PD1^+$) from BALB/c mice splenocytes were sorted, cocultured in different combinations and incubated with respiratory syncytial virus (RSV) and/or αPD-L1 for 4 days. (**a and b**) Mean fluorescence intensity (MFI) of PD-L1 in sorted DCs and B cells, in the presence or absence of RSV. (**c**) Supernatant IL-21 quantification from coculture of B cells, DCs and Tfh incubated with RSV and αPD-L1. (**d**) IL-21R MFI in Tfh cells from cocultures in different combinations of sorted B cells and DCs. Undetectable levels were considered zero for statistical purposes. The experiment was performed in triplicates. $*P < 0.05$; $**P < 0.01$. ns, not significant.

We next investigated whether PD-L1 upregulation in B cells and DCs was caused by a direct interaction of RSV with either of these cell populations. To answer this question, we sorted DCs and B cells and incubated them *in vitro* with RSV. Only DCs upregulated PD-L1 expression as a direct effect of RSV, expressing higher PD-L1 levels compared with B cells (Figure 2a, b). We hypothesized that, if DCs were the main direct RSV target for PD-L1 induction, blocking this signal would restore IL-21 and IL-21R levels in Tfh only if DCs were in the culture. We again sorted Tfh, DCs and B cells, and incubated them with RSV in different combinations. IL-21 production could only be detected upon PD-L1 blockade (Figure 2c). Interestingly, IL-21R expression was only recovered in Tfh cells upon PD-L1 blockade when cultured in the presence of DCs (Figure 2d) but not by Tfh cells when cultured exclusively with B cells. These results suggested that the direct induction of PD-L1 by RSV in DCs, but not B cells, could constitute a key mechanism of inhibition of the IL-21–IL-21R axis in Tfh cells, and this can be reversed by anti-PD-L1 treatment.

IL-21 treatment increases Tfh and reduces Tfr cell numbers in lungs upon RSV infection

Our results indicated that RSV interfered in the signaling between the IL-21–IL-21R and PD-1–PD-L1 pathways, inhibiting the development of an efficient humoral response against the virus. Although we had observed that PD-L1 blockade recovered IL-21 and IL-21R levels, a previous study had shown that this approach was not useful *in vivo*.[28] In that study, anti-PD-L1 treatment in RSV-infected mice induced proinflammatory cytokines, exacerbating pulmonary inflammation and host disease, leading to a moderate enhancement of Tfh cells numbers, and no improvement of antibody responses was observed up to 6 days following infection. Under the hypothesis that IL-21 modulation was connected to PD-L1 expression, we

decided to test an alternative approach, treating RSV-infected mice with recombinant, endotoxin-free IL-21 (Supplementary figure 1b). In the lungs, RSV infection decreased the percentages of Tfh cells (Figure 3a), contrary to what was observed in LDLNs. Furthermore, the absolute number of Tfh cells in the lung of naïve animals was low, and RSV infection was not able to increase this. IL-21 treatment increased the absolute numbers of Tfh cells in the lungs and LDLNs (Supplementary figure 3b) and partially restored Tfh cell percentages during RSV infection in the lungs (Figure 3a), with no effect in LDLNs.

In addition, we observed that Tfr cell percentages and absolute numbers were expanded in RSV-infected mice in both LDLNs and lungs (Figure 3b and Supplementary figure 3c). Tfr cells negatively regulate Tfh cell functions and are defined phenotypically as $CXCR5^+PD1^+CD4^+Foxp3^+$ T cells.[29,30] IL-21 treatment reversed this, decreasing Tfr cells numbers (Figure 3b and Supplementary figure 3c). Our data suggest that RSV can increase the relative percentages of Tfr/Tfh cells, and one of the mechanisms by which IL-21 treatment increases Tfh numbers in RSV infection *in vivo* might be the reduction of Tfr cells.

IL-21 treatment induces TLOs formation and neutralizing antibody responses, reducing severity of RSV infection

A logical prediction from these findings was that if IL-21 treatment could restore Tfh function *in vivo*, a direct effect would be observed over B cells and the anti-RSV antibody response. Accordingly, we observed that treatment with IL-21 in RSV-infected mice was able to induce the formation of TLOs resembling GCs inside of the lungs (Figure 4a). TLOs are ectopic lymphoid structures congregating mainly B and T cells, observed during acute inflammatory reactions, in infections, autoimmune disease and even cancer, and are associated with a protective response.[31] Moreover, IL-21 treatment increased proliferation capacity of total B cells in LDLNs and lungs (Supplementary figure 4a–c). It also resulted in increased titers of anti-F IgG (Figure 4b) and IgA (Figure 4c) in bronchoalveolar lavage starting at day 14 after infection. Finally, it increased anti-F IgG in serum (Figure 4d) and avidity of anti-RSV IgG (Figure 4e).

We then tested whether the antibodies produced upon IL-21 treatment would mediate reduction of severity in RSV infection. A hallmark of RSV infection is inflammatory pulmonary injury in peribronchial and perivascular areas (Figure 5a, b). Treatment with IL-21 reduced both peribronchial and perivascular inflammation (Figure 5a, b). IL-21 treatment also decreased numbers of RSV copies in lungs in RSV-infected mice (Figure 5c) and weight loss (Supplementary figure 1c) of infected animals, compared with untreated infected mice.

We next tested the neutralization capacity of the antibodies in the serum of RSV-infected mice *versus* serum of IL-21-treated mice, in Vero cells. Sera from IL-21-treated mice showed a significantly enhanced neutralization power (Figure 5d). To evaluate the protective potential of the generated antibodies, we pretreated naïve mice with purified IgG from serum of naïve mice or RSV-infected mice, either treated or not with IL-21, 2 days prior to RSV infection, and measured viral copies in lungs at day 5 after infection. The results indicated that a partial protection to RSV infection could be mediated by IgG alone, elicited by IL-21 treatment in infected mice (Figure 5e).

DISCUSSION

In this study, we unveil a pathway by which RSV can evade effector immune responses, impairing the generation of protective antibodies by inhibiting Tfh cell function, mainly the production of IL-21. Different studies report that targeting differentiation and/or function of immune cells constitutes a major virulence strategy for different viruses. Lymphocytic choriomeningitis virus negatively modulates antibody responses by killing Tfh cells via natural killer cell cytotoxicity.[32] Human immunodeficiency virus (HIV) infection is associated with a decrease in follicular regulatory T cell (Tfr cell) function, leading to Tfh cell proliferation, which is the major HIV-producing $CD4^+$ T-cell subpopulation, and consequently increasing viral replication.[33] Until now, modulation of Tfh cells by RSV had not been reported; and Tfh frequencies in humans during an RSV infection have not yet been described.

The production of IL-21 by Tfh cells coordinates the GC reaction and generation of high-affinity antibodies. The presence of RSV-specific high-affinity antibodies is associated with protection against RSV infection in infants under 3 months old.[6] Respiratory infections such as influenza are known to increase IL-21 production and Tfh cell responses.[34] However, the influence of IL-21 treatment during RSV infection was so far unexplored. Our studies demonstrated that IL-21 production by Tfh cells is inhibited specifically by RSV. In line with our findings, a cohort study that characterized the primary and secondary cytokine response to RSV infection did not detect IL-21 in swab nasal secretion samples from infants recruited during two consecutive winters.[35] Another study[36] demonstrated that IL-21 depletion during priming exacerbates immunopathology in mice

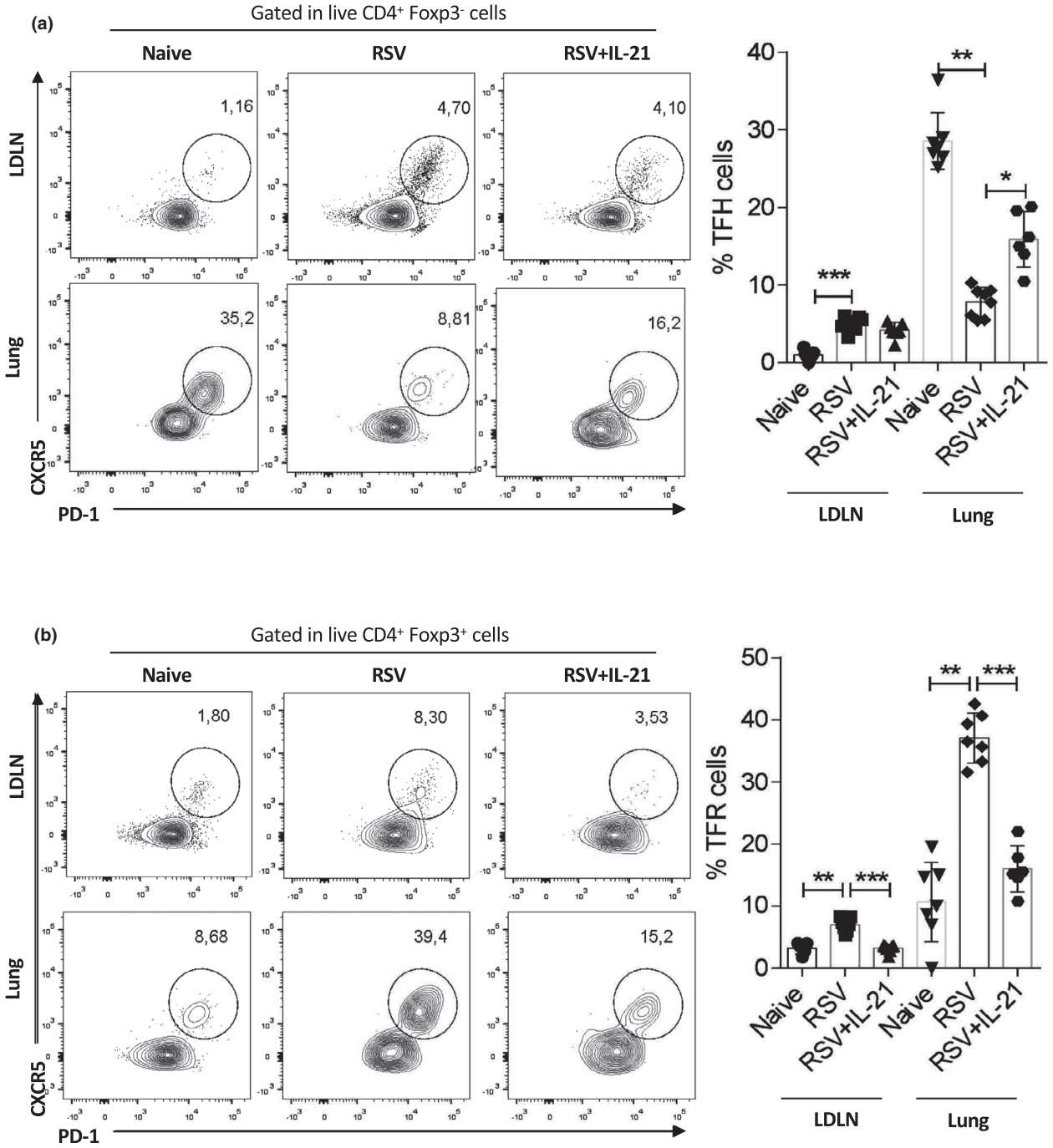

Figure 3. *In vivo* interleukin (IL)-21 treatment recovers the Tfr-to-Tfh ratio in lungs. BALB/c mice were intranasally infected with 1×10^7 plaque-forming units (PFUs) of respiratory syncytial virus (RSV) and treated subcutaneously with four doses of 0.5 μg of IL-21. (**a**) Representative gates of Tfh cells ($CD45^+CD4^+FOXP3^-CXCR5^+PD\text{-}1^+$) and quantification of Tfh cell percentages in lung draining lymph nodes (LDLNs) and lungs. (**b**) Representative gate of Tfr cells ($CD45^+CD4^+FOXP3^+CXCR5^+PD\text{-}1^+$) and Tfr cell percentages in LDLNs and lungs. Results show pooled data from two or three independent experiments, with n= 5-7 mice per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. PD-1, programmed cell death protein 1; Tfh, follicular helper T; Tfr, regulatory follicular T (cell).

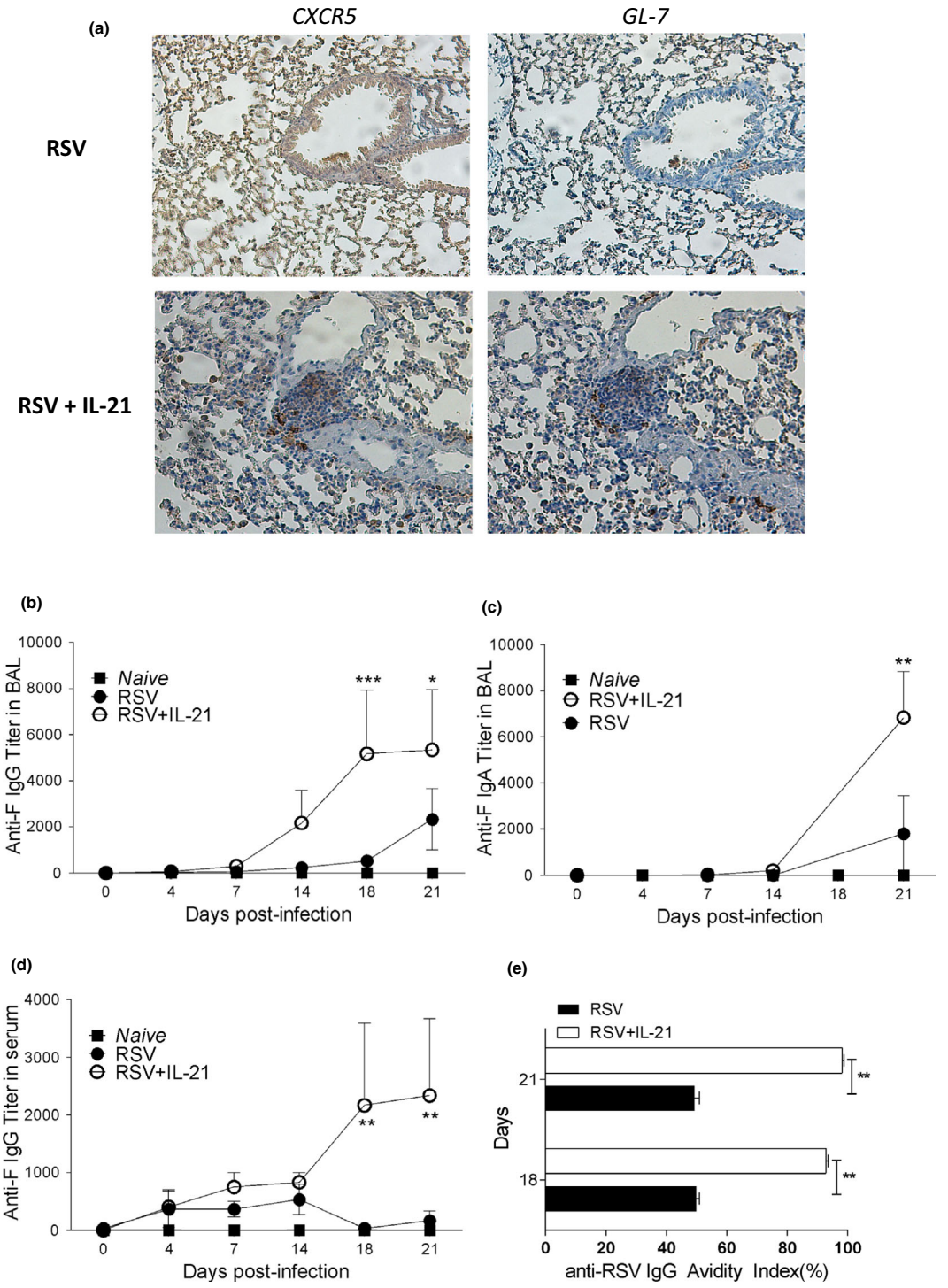

Figure 4. *In vivo* interleukin (IL)-21 treatment increases specific-antibody responses in respiratory syncytial virus (RSV)-infected mice. BALB/c mice were intranasally infected with 1×10^7 plaque-forming units (PFUs) of RSV and treated subcutaneously with four doses of 0.5 μg of IL-21. (**a**) Ectopic lymphoid formation in lung stained by CXCR5 and GL-7. (**b**) Anti-F IgG titers measured by ELISA in bronchoalveolar lavage (BAL). (**c**) Anti-F IgA titers measured by ELISA in BAL. (**d**) Anti-F IgG titers measured by ELISA in serum. (**e**) Total IgG avidity index on days 18 and 21 after infection. Results show pooled data from two or three independent experiments, with $n = 3$ or 5 mice per group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Ig, immunoglobulin.

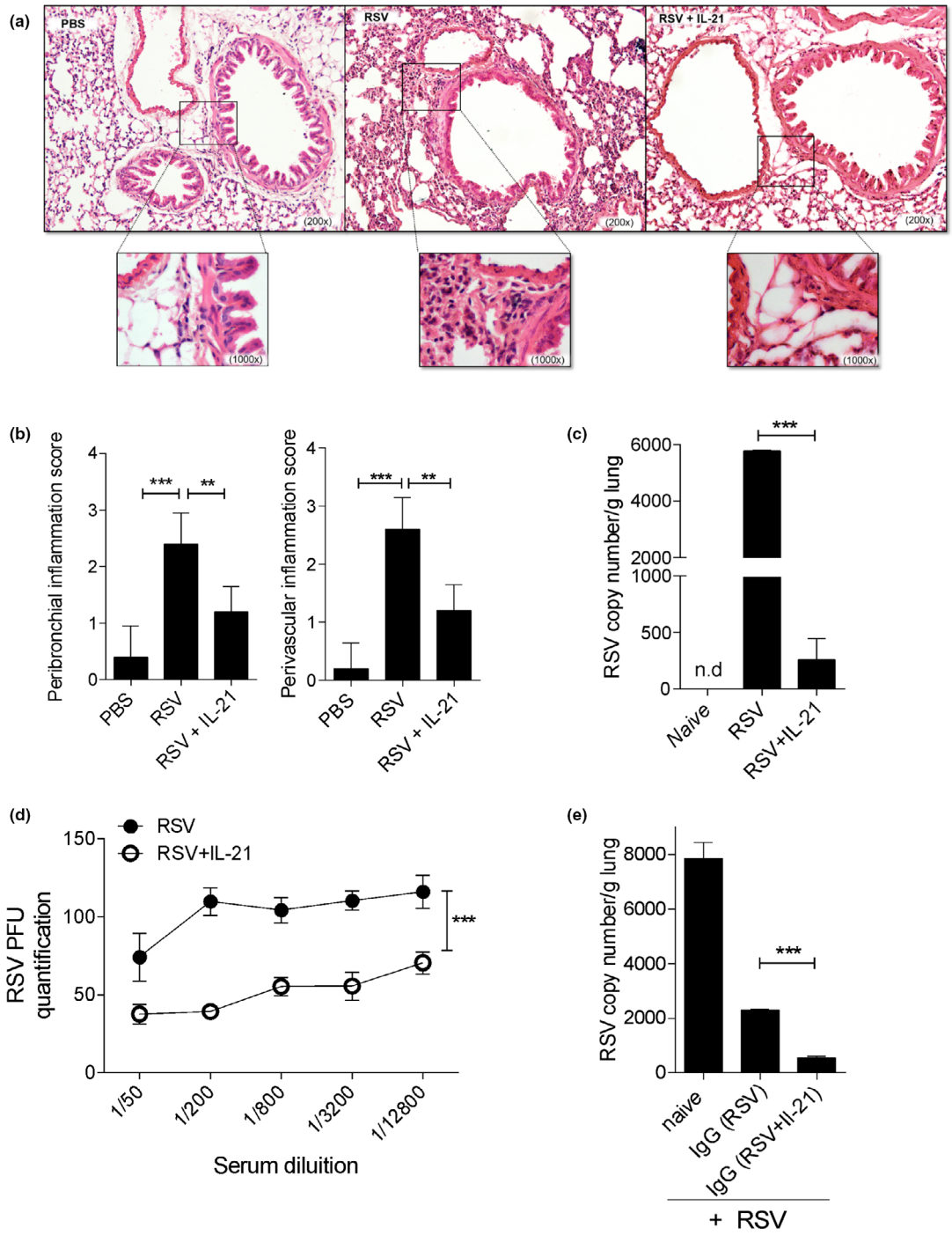

Figure 5. *In vivo* interleukin (IL)-21 treatment decreases respiratory syncytial virus (RSV) titers and lung inflammation. BALB/c mice were intranasally infected with 1×10^7 plaque-forming units (PFUs) of RSV and treated subcutaneously with four doses of 0.5 μg of IL-21. (**a**) Lung hematoxylin and eosin histology (200× and 1000× magnification), 21 days after infection. (**b**) Peribronchial and perivascular inflammation score. (**c**) Real-time (RT)-PCR quantification of RSV copies in the lungs, 21 days after infection. (**d**) *In vitro* neutralization capacity assay. (**e**) Passive immunization with purified IgG from RSV-infected mice or IL-21-treated RSV-infected mice for which RSV copies were measured in the lungs by RT-PCR. Results show pooled data from two or three independent experiments, with $n = 3$ or 5 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$. n.d., not detected; PBS, phosphate-buffered saline.

after RSV challenge, and reduces antibody production, evidencing a major role for IL-21 in RSV infection.

In fact, inhibition of IL-21 expression is a strategy employed by different pathogens to evade effector immune responses. In hepatitis C virus patients, serum IL-21 levels and the frequency of IL-21-producing Tfh cells in blood were found to be lower compared with healthy individuals.[37] Lower frequencies of IL-21-producing $CD4^+$ T cells were also associated with reduced proliferation and increased expression of the inhibitory receptors such as CTLA-4, Tim-3 and PD-1 on hepatitis C virus-specific $CD8^+$ T cells in a viral-persistence state.[38] In fact, IL-21 has potent and specific effects on mucosal antiviral responses, assisting viral clearance and regulating pulmonary T- and B-cell responses.[39] IL-21 treatment has been tested against several types of viral pathogens, such as hepatitis B virus,[40] hepatitis C virus[38] and HIV.[41,42] IL-21 knockout mice are profoundly impaired in B-cell responses, with reduced plasma cell formation and GC function, decreased B-cell proliferation, transition into memory B cells and antibody affinity maturation.[43] In addition, IL-21 induces IL-21R expression on activated B cells and Tfh cells.[44–46] All these studies underline the relevance of IL-21 for the generation of protective antibodies. In our study, RSV infection led to impairment of IL-21 expression in lung Tfh cells, whereas IL-21 treatment resulted in more efficient GC response, decreasing Tfr cells, increasing Tfh and B cells numbers as well as leading to the production of IgA and IgG antibodies that protected naïve animals that were challenged *in vivo* by RSV. Although our data do not show that the improved antibody response against RSV is dependent on the IL-21-recovered Tfh cell function, they demonstrate the strong correlation between the two phenomena.

When we investigated the lungs of RSV-infected mice at day 21 after infection, we did not observe the presence of TLOs. The RSV-infected mice that were treated with IL-21, however, showed TLOs mostly around the bronchi regions, with positive staining for CXCR5 and GL-7. Such tertiary structures are commonly observed in respiratory infections such as influenza.[47,48] In particular, mice lacking peripheral lymphoid organs and infected with high titers of influenza virus are able to organize B and T cells ectopically, in tissues, and thus clear the infection, highlighting the importance of TLOs as a local protective immune response.[49] The formation of TLOs is also observed in target organs of autoimmune disease, transplanted organs, solid tumors and chronic inflammation tissues (see the review by Luo *et al.* [50]). In human renal allograft rejection, TLO formation in grafts is dependent on IL-21.[51] One of the functional roles of TLOs is sustaining the generation of antibody responses. Our data support a role for IL-21 in inducing TLO formation and a protective antibody response against RSV infection.

We also observed that IL-21R was downregulated on lung Tfh cells in RSV-infected mice. This observation is consistent with the inhibition of IL-21 production by RSV. In humans, IL-21R defects cause severe primary immunodeficiency and reduced activity of natural killer, T and B cells, resulting in multiple infections.[52] Our results indicate that the downregulation of IL-21R is another important mechanism associated with the impairment of B-cell responses against RSV in mice, evidencing the relevance of the IL-21–IL-21R axis in the generation of protection to this virus.

Finally, we found that inhibition of IL-21 production during RSV infection depended on PD-L1 induction. Tfh cells naturally express PD-1.[12] PD-L1, one of the classic PD-1 ligands, is responsible for immune homeostasis, negatively modulating PD-1-expressing T cells[53] and B cells.[54] PD-1–PD-L1 interactions reduce Akt phosphorylation in PD-1-expressing cells, leading to decreases in function and cell survival.[55] PD-L1 expression in RSV-infected patients has not been described; however, PD-L1 has been previously reported to be increased in DCs[25,56] and B cells[28] of mice and also in bronchial epithelial human cells line[57] infected with RSV. PD-1 is usually studied with regard to its expression in T cells and used here as a marker for Tfh cells; however, different studies have reported that PD-1 can also be upregulated in DCs[58–60] and B cells,[61,62] where it can relay inhibitory signals for these cells as well. Our findings showed that RSV induced the expression of PD-L1 in lung DCs and B cells, and treatment with anti-PD-L1 restored the expression of IL-21/IL-21R in lung Tfh cells. In addition, our *in vitro* experiments showed that RSV could directly upregulate PD-L1 expression in DCs, but in not B cells. Similar to our findings regarding the role of PD-L1 and IL-21 in RSV infection, Cubas *et al.* demonstrated that HIV infection increased PD-L1 frequency in GC B cells leading to reduction of Tfh cell proliferation and IL-21 production.[63] Upregulation of PD-L1 in B cells mediated by RSV depended on DCs and T cells. It is possible that the mechanism by which PD-1–PD-L1 interactions inhibit Tfh cells function is by downregulation of c-Maf expression in these cells, a transcriptional factor involved in transactivation of both the promoter and enhancer of the IL-21 gene, and is inversely associated with PD-L1 expression.[24,64] We did not explore the capacity of RSV to induce PD-L1 expression in Tfh cells, which have been already shown to play a role in a colitis mice model.[65] PD-L1 upregulation in Tfh cells could be another important pathway to affect Tfh-cell function, decreasing IL-21 production. Several

studies have also suggested that blockade of PD-1 or PD-L1 may lead to a reversion of T-cell dysfunction in the context of chronic infection.[66] PD-L1 blockade in regulatory B cells can recover Th1 cell activity in visceral leishmaniasis in a canine model.[67] When we blocked PD-L1 *in vitro* during RSV infection, IL-21 secretion was also increased, supporting our *in vivo* data. We suggest that an early course of IL-21 therapy, rather than anti-PD-L1 or anti-PD-1, might constitute a better approach to circumvent Tfh-cell functional impairment resulting from RSV infection. Treatment with anti-PD-L1 has been previously reported to lead to high inflammatory responses in RSV infection[28]; more recently, in cancer patients with COVID-19 (coronavirus disease 2019), treatment with anti-PD-1 also resulted in exacerbated lung inflammation.[68] Before we can think of the use of anti-PD-1/PD-L1 antibodies as a viable approach for therapy in viral infections, more information is needed on the multiple signals these will affect in T, DC and B cells that may coexpress both markers in these situations.

In summary, our data underline the importance of IL-21 and highlight an intricate connection between the IL-21–IL-21R and the PD-1–PD-L1 axis in the regulation of Tfh- and Tfr-cell function for the development of a protective humoral response during RSV infection. Further investigation of this pathway, as well as clinical studies of IL-21 treatment in RSV-infected patients, will contribute to the development of new vaccine strategies, as well as immunotherapy, against respiratory infections.

METHODS

Viruses and cells

Vero cells (ATCC CCL81) were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA) and gentamicin (0.08 mg mL^{-1}; Novafarma, Brazil), maintained at 37°C with 5% of CO_2. These cells were used to propagate the RSV A2 strain. Viral PFUs were quantified using an RSV F protein-specific antibody (Millipore, Billerica, MA, USA). The virus produced was inactivated by UV light exposure for 30 min, at room temperature.

Animals

Female BALB/c mice aged 6–8 weeks were purchased from the Biological Center of Experimental Models (CEMBE) at Pontifícia Universidade Católica do Rio Grande do Sul. A well-established model to study RSV infection was previously developed in the BALB/c strain.[69] Mice were housed at the Biological Center of Experimental Models (CEMBE) facility and received water and food *ad libitum*. All animal procedures were performed in accordance with the guidelines of the Federation of Brazilian Societies for Experimental Biology and approved by the Pontifícia Universidade Católica do Rio Grande do Sul Ethics Committee for the Use of Animals (protocol number #13/00341).

Infection and treatment

For *in vitro* infection, a single-cell suspension of splenocytes was obtained, stained with specific antibodies for DCs ($CD11c^+CD19^-$), B cells ($CD19^+CD11c^-$) and Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) and sorted. Sorted cells were cocultured (5×10^3 DCs, 3×10^4 Tfh cells or 5×10^4 B cells per well) in different combinations, and later infected with 1×10^2 PFUs mL^{-1} of RSV for 4 days. In some experiments, 0.5 μg of anti-PD-L1 antibody (clone MHI5; eBioscience, USA) or IgG isotype control (Bio X Cell, USA) was added to the cultures, followed by analysis of IL-21R and PD-L1 expression.

For *in vivo* infection, mice were divided in four groups: two groups were infected intranasally with 1×10^7 PFUs of RSV, with one of them receiving subcutaneous over-the-shoulders treatment with 0.5 μg of recombinant IL-21 (eBioscience, USA) diluted in 100 μL of phosphate-buffered saline (PBS). The other two groups received PBS intranasally, with one of them subcutaneously administered recombinant IL-21. IL-21 was administered on days 2, 4, 8, 14 and 18 after infection. In some experiments, mice were treated on days 2 and 4 with 200 μg anti-PD-L1 antibody (clone MHI5, eBioscience, USA) or IgG isotype control (Bio X Cell, USA), both diluted in PBS. Bronchoalveolar lavage and blood collection occurred on days 0, 7, 14 and 21 after infection. Mice were killed at day 5 or 21 after infection; spleens, lungs and LDLNs were harvested for further analysis.

ELISA

The concentrations of murine IL-21 were determined by capture ELISA (R&D Systems, USA), according to the manufacturer's instructions. Undetectable levels were considered zero for statistical purposes. For quantification of IgG and IgA RSV-specific antibodies in mice serum and bronchoalveolar lavage, 96-well plates were sensitized overnight with RSV F protein (Sino Biological Inc, USA), washed and blocked for 1 h with blocking buffer (5% milk + 0.05% tween in PBS 1× buffer) and serum or bronchoalveolar lavage was added in dilutions of 1/10, 1/100, 1/500, 1/1000, 1/10 000 and incubated for 2 h at room temperature. Rabbit antimouse IgG or IgA antibody, both horseradish peroxidase conjugated (Invitrogen, USA), followed by 3, 3', 5, 5' - Tetramethylbenzidine (TMB) (Life Technologies, Brazil) were used to develop the assay. Plates were read at 450 nm using an EZ Read 400 Microplate reader (Biochrom).

An ELISA to measure the avidity of anti-RSV antibody was conducted as described above; however, plates were washed with 6 M urea–PBS. The results were expressed as an Avidity Index, which was calculated as previously described.[6] The avidity of RSV-specific total IgG was classified according to values that had been predetermined arbitrarily, defined as low (<50%), intermediate (50–70%) or high (>70%).

Neutralization assay

An assay to determine antibody neutralization capacity was performed as previously described.[70] Briefly, fourfold serial serum dilutions from 1:10 to 1:10 240 were prepared in virus diluent (Dulbecco's Modified Eagle Medium supplemented with 0% fetal calf serum and 1% gentamicin, 0.08 mg mL^{-1}; Novafarma). Serially diluted serum was challenged with an equal volume of the RSV-A2 strain, previously tittered to give approximately 150 PFUs per 50 μL of inoculum. The serum–virus mixtures were incubated at 37°C, 5% CO_2 for 1 h. Vero cells were seeded in 96-well plates, grown to a monolayer and then infected with 50 μL per well (in triplicates) of the serum–virus mixture. Plates were kept with 0.5% methyl cellulose, prepared in Dulbecco's Modified Eagle Medium with 2% fetal bovine serum and incubated at 37°C, 5% CO_2 for 3 days to allow plaque formation. To observe the syncytium formation, wells were incubated with primary anti-RSV antibodies (Millipore, USA) and secondary antibody (horseradish peroxidase rabbit antimouse IgG; Millipore, USA) for 1 h at 37°C. Nonspecific binding was blocked with a blocking buffer. The assay was developed with 4-chloro-1-naphthol with 0.01% H_2O_2 for 20 min in the dark. Syncytia were counted in an optical microscope and PFUs were calculated by the number of syncytia divided by the product of the virus dilution and the total volume in the well.

Flow cytometry

Cells were labeled with anti-CD3 (APC-C7, 17A2 clone), anti-CD27 (APC, LG.3A10 clone), anti-FOXP3 (PE, MF23 clone), anti-B220 (PerCP, RA3-6B2 clone), anti-CD8a (APC-H7 536.7 clone), anti-IL-21R (PE, 4A9 clone) and anti-CD19 (APC-Cy7, ID3 clone) from BD Biosciences; anti-CD4 (eFluor710-PerCP, clone RM4-5), anti-PD-1 (PE-Cyanine7, clone J43), anti-Bcl-6 (PE, mgl191E clone), anti-CXCR5 (APC, clone SPRCL5), anti-ICOS (FITC, clone 7E17G9), anti-CD45 (FITC, clone 30-F11) and anti-CD274 (PE, clone MIH5) from eBioscience; anti-CD25 (PE, PC61 clone), anti-GL7 (FITC, GL7 clone), anti-CD11c (PE/Cy7 N418 clone) and anti-Ki67 (PE or PerCP, clone 16A8) from BioLegend. Viability was determined with Fixable Viability Dye eFluor 780 or 450 from eBioscience. For intracellular staining, we used Foxp3 staining buffer set (eBioscience). Cells were analyzed using an FACSCanto II (BD Biosciences) with the FACSDiva software (BD Biosciences). Sorting was performed in an FASCAria (BD Biosciences).

Histology and immunohistochemistry

Lungs and spleens were fixed with 10% formalin, embedded in paraffin and cut into 5-μm sections (Olympus CUT 4060E). Hematoxylin and eosin staining was performed on sections to evaluate the cell infiltration and determine the inflammation scores. The score for peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as previously described.[71] Zero was attributed when no inflammation was detectable, 1 was attributed when occasional cuffing with inflammatory cells was observed, a value of 2 was given when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells and a value of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells.

For immunohistochemistry, sections were deparaffinized with xylol and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol. Antigen unmasking was performed by incubating the slides in 0.1 mol L^{-1} citrate buffer, pH 6, for 30 min at 95°C, followed by cooling at room temperature for 1 h. Sections were blocked in PBS with 4% bovine serum albumin and 5% mouse serum and later incubated with primary antibody anti-CXCR5 (clone 2G8, BD Biosciences) or anti-GL-7 (GL7 clone, Invitrogen) at a 1:500 dilution in PBS with 1% bovine serum albumin and 1.25% mouse serum. Biotinylated goat-derived secondary antibodies were detected by the avidin–biotin–horseradish peroxidase complex method (Dako Systems) using 3,3-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich) as a substrate.

Real-Time PCR

Total RNA was extracted from the lungs of infected animals using the Viral RNA/DNA Mini Kit (PureLink; Invitrogen, Canada) following the manufacturer's instructions. Complementary DNA was synthesized using random primers and the Sensiscript Reverse Transcription kit (QIAGEN, USA). The quality of complementary DNA for each sample was evaluated by amplification of the endogenous β-actin gene. Samples that did not amplify for β-actin were excluded. Real-time PCR was performed for the amplification of the RSV F protein gene using the primers and specific probes (forward-5′-AACAGATGTAAGCAGCTCCGTTATC-3′, reverse-5′-GATTTTTATTGGATGCTGTACATTT-3′ and probe 5′-FAM/TGCCATAGCATGACACAATGGCTCCT-TAMRA-3′). For the standard curve, ten-fold serial dilutions of 6×10^7 copies of a plasmid with the RSV F protein gene sequence were added to the same plate of quantitative PCR in duplicate. The results were measured by the StepOne Real-Time PCR System (Applied Biosystems) and used for further quantification of the samples viral load.

Passive immunization

Serum IgG from mice was purified using a Protein A-Sepharose column (Sigma, USA) following manufacturer's instructions. Mice were separated into three groups: the first group received IgG serum from RSV-infected mice, the second group received IgG serum from RSV-infected/IL-21-treated mice and the third group received serum from naïve mice. Each mouse received 300 μg of purified IgG intraperitoneally. After 2 days, animals were infected with 1×10^7 PFUs of RSV intranasally. Mice were killed at day 5 after infection and lungs were harvested for further analysis.

Statistical analysis

Data were tested for normal distribution using a Kolmogorov–Smirnoff test; differences between the groups were analyzed with one-way analysis of variance (ANOVA) followed by a

Bonferroni post-test, or paired *t*-tests using GraphPad Prism software (San Diego, CA, USA). Values demonstrated in graphs are the mean ± the standard deviation (s.d.). A level of significance of $P < 0.05$ was established for the analyses.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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