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Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection

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The inflammatory response to lung infections must be tightly regulated, enabling pathogen elimination while maintaining crucial gas exchange. Using recently described "depletion of regulatory T cell" (DEREG) mice, we found that selective depletion of regulatory T cells (Tregs) during acute respiratory syncytial virus (RSV) infection enhanced viral clearance but increased weight loss, local cytokine and chemokine release, and T-cell activation and cellular influx into the lungs. Conversely, inflammation was decreased when Treg numbers and activity were boosted using interleukin-2 immune complexes. Unexpectedly, lung (but not draining lymph node) Tregs from RSV-infected mice expressed granzyme B (GzmB), and bone marrow chimeric mice with selective loss of GzmB in the Treg compartment displayed markedly enhanced cellular infiltration into the lung after infection. A crucial role for GzmB-expressing Tregs has not hitherto been described in the lung or during acute infections, but may explain the inability of children with perforin/GzmB defects to regulate immune responses to infection. The effects of RSV infection in mice with defective immune regulation closely parallel the observed effects of RSV in children with bronchiolitis, suggesting that the pathogenesis of bronchiolitis may involve an inability to regulate virus-induced inflammation.

INTRODUCTION

Viral infections in the lower respiratory tract can be fatal. They not only cause cytopathic effects in infected cells within the airways, but also trigger cell infiltration into the lung tissue. This infiltration has to be tightly regulated in order to maintain gas exchange, suggesting that a delicate balance between an effective antiviral immune response and a life-threatening pathogenic reaction is essential to preserve the organ function while combating infection.

Human respiratory syncytial virus (RSV) is the major cause of serious lower respiratory tract infection in infants. Overexuberant and inappropriate immune responses have a major role in RSV disease[1] but the mechanisms leading to loss of immune regulation in the lungs of RSV-infected patients are not fully understood. Each year, RSV is estimated to cause 34 million cases of lung infection, about 3.4 million hospitalizations, and the deaths of 66,000–199,000 children under 5 years of age.[2] Despite the acute and long-term effects of RSV infection in infants and adults there is still no vaccine available.

Regulatory T cells (Treg) have a crucial role in controlling immune responses; most are $CD4^+$ and express the transcription factor Foxp3. In man, Treg deficiency causes dysregulated immunity with autoimmune disease affecting multiple organs.[3] Tregs also regulate immune responses in allergy[4,5] and chronic infections,[6] and are thought to limit the extent of an inflammatory response during viral infections. Studies of Friend virus infection demonstrate *in vitro* suppression of $CD8^+$ T-cell function by virus-induced Tregs.[7] In this infection, *in vivo* depletion of Tregs increases the antigen-specific $CD8^+$ T-cell responses and reduces viral burden.[8] In addition, Lund *et al.*[9] reported that Tregs contribute to the establishment of protective immunity by inducing early antiviral immune response at the site of infection. Lung Tregs have a crucial role in the maintenance of self-tolerance and in the regulation of the immune response

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Received 1 September 2011; accepted 23 November 2011; published online 11 January 2012. doi:10.1038/mi.2011.62

to pathogens and commensals. In addition, studies suggest that Tregs have a key role in contributing to the maintenance of immune homeostasis in the airways.[10] However, the role and suppressive mode of action of Tregs in acute viral lung infections is less clear.

To determine if pulmonary immune responses are regulated by Tregs during acute viral infections, we compared the effects of Treg depletion and Treg boosting during experimental RSV infection in mice. We found that depletion of Tregs before RSV challenge led to a decreased viral load but markedly increased disease, characterized by weight loss, delayed recovery, and increased influx of inflammatory cells into the lung and airways. Conversely, boosting Treg numbers and activity by injecting interleukin (IL)-2/anti-IL-2 immune complexes reduced pathology but did not prevent viral clearance. Notably, the ability of Tregs to regulate lung inflammation during RSV infection critically depended on their ability to produce granzyme B (GzmB), a cytotoxic mediator normally associated with antiviral $CD8^+$ T and natural killer (NK) cells. Thus, Tregs deploying a mechanism normally associated with elimination of virus-infected cells are key effectors responsible for immune regulation in the lung during acute viral infection.

RESULTS

Loss of Tregs increases T-cell-driven lung inflammation and RSV disease severity

Bacterial artificial chromosome-transgenic "depletion of regulatory T cell" (DEREG) mice express diphtheria toxin (DT) receptor and enhanced green fluorescent protein fusion protein under the control of the *foxp3* gene locus, allowing selective and efficient depletion of $Foxp3^+$ Treg cells by DT injection.[11] BALB/c DEREG mice were depleted of Tregs by injection of DT intraperitoneally (i.p.) on day −2, −1, 2, 5, and 8 post infection with human RSV A2. Flow cytometric analysis confirmed the depletion of $CD3^+CD4^+Foxp3^+GFP^+$ cells in the mediastinal lymph nodes, spleen, blood, and lung (**Supplementary Figure S1a** online). DT injections in the absence of infection of wild-type (WT) or DEREG mice did not cause neutrophil infiltration or any other detectable alterations in the lungs or airways (**Supplementary Figure S1b** and **c** online). As an index of disease severity, body weight was monitored daily in each individual mouse. BALB/c DEREG mice infected with RSV and depleted of Tregs showed increased and sustained weight loss and delayed recovery compared with control BALB/c mice (**Figure 1a**). Treg depletion during RSV infection led to an increase in total cell numbers in the lung and bronchoalveolar lavage fluid (BAL) on days 6 and 8 (**Figure 1c**) and day 14 (data not depicted) post RSV infection. In the absence of $Foxp3^+$ cells, a significant increase of $CD4^+Foxp3^-$ T cells was seen in the lung (data not depicted) and BAL on day 6 and 8 post RSV infection (**Figure 1d**), which was maintained until day 14 post infection (data not depicted). There was no difference in the expression of CD69 on $CD4^+Foxp3^-$ T cells in the lung or BAL between control BALB/c mice and Treg-depleted DEREG mice after RSV infection (data not shown). In the BAL, a significant increase of $CD8^+$ T cells was detected on day 6 and 8 (**Figure 1e** and **Supplementary Figure S3c** online) with similar results in the lung (data not depicted and **Supplementary Figure S3c** online). Antigen-specific $CD8^+$ T cells, detected using M2-specific pentamers, showed no increase on day 6 but increased at day 8 post RSV infection in Treg-depleted mice compared with control mice (**Figure 1e** and **Supplementary Figure S4a** and **b** online). In addition, M2 peptide restimulation of lung or BAL cells on day 8 post infection increased interferon (IFN)-γ production by $CD8^+$ T cells in Treg-depleted mice (**Figure 1e** and **Supplementary Figure S4b** online). Notably, Treg depletion led to an increase in total numbers of macrophages and neutrophils on day 6 and 8 post RSV infection (**Figure 1f**), while no differences were detected on day 4 (**Figure 1f**) or 14 (data not depicted). Unexpectedly, ablation of Tregs also led to an influx of eosinophils in the BAL on day 8 (**Figure 1f**), which are normally not detected after primary infection but have been found after RSV infection of mice vaccinated with formalin-inactivated RSV.[12] Depletion of Tregs caused no influx of neutrophils or eosinophils into the airways of uninfected DEREG mice, indicating that this response was specific to RSV infection (**Supplementary Figure S1b** online). In addition, a small increase of total numbers of dentritic cells in the lung ($CD11c^+$ cells; **Supplementary Figure S2a** online) on day 4 and 8 was detected. Alveolar macrophages were excluded by gating out autofluorescent cells. Expression levels of CD80 on $CD11c^+$ cells were elevated on day 6 and 8 (**Supplementary Figure S2b** online) while there was no change in the expression of CD86 (**Supplementary Figure S2b** online).

C57BL/6J (B6) mice can be infected with human A2 RSV, but are relatively resistant to viral growth and weight loss.[13] However, when Tregs were depleted in B6 DEREG mice, infection with RSV intranasally caused a striking weight loss of up to 15% by day 8 (**Supplementary Figure S3a** online). Furthermore, Treg depletion of B6 mice showed broadly similar effects to those shown in BALB/c mice (**Supplementary Figure S3b, c,** and **d** online). Furthermore, RSV infected, undepleted DEREG mice or B6 WT mice injected with DT did not show any differences from WT B6 mice infected with RSV (**Supplementary Figure S3** online).

In summary, Treg depletion during RSV infection manifests itself in markedly increased recruitment of inflammatory cells to the lung and increases disease severity in both BALB/c and B6 mice.

Absence of Tregs accelerates T-cell effector function

Treg depletion led to decreased viral load in the lung on day 4 post infection (**Figure 1b**), the peak of viral load in this infection model.[14] This suggests that in addition to dampening inflammation, Tregs inhibit early antiviral immunity. To investigate this further, we measured IFN-γ and GzmB by intracellular staining of lung T cells from RSV-infected mice, with or without Treg depletion. GzmB was detected directly *ex vivo* while IFN-γ was detected after 3-h phorbol 12-myristate 13-acetate/ionomycin restimulation. Treg depletion greatly increased IFN-γ and GzmB staining in $CD8^+$ and $CD4^+$ T cells in the lung on day 4 post infection (**Figure 2**). Therefore, we suggest that Tregs are

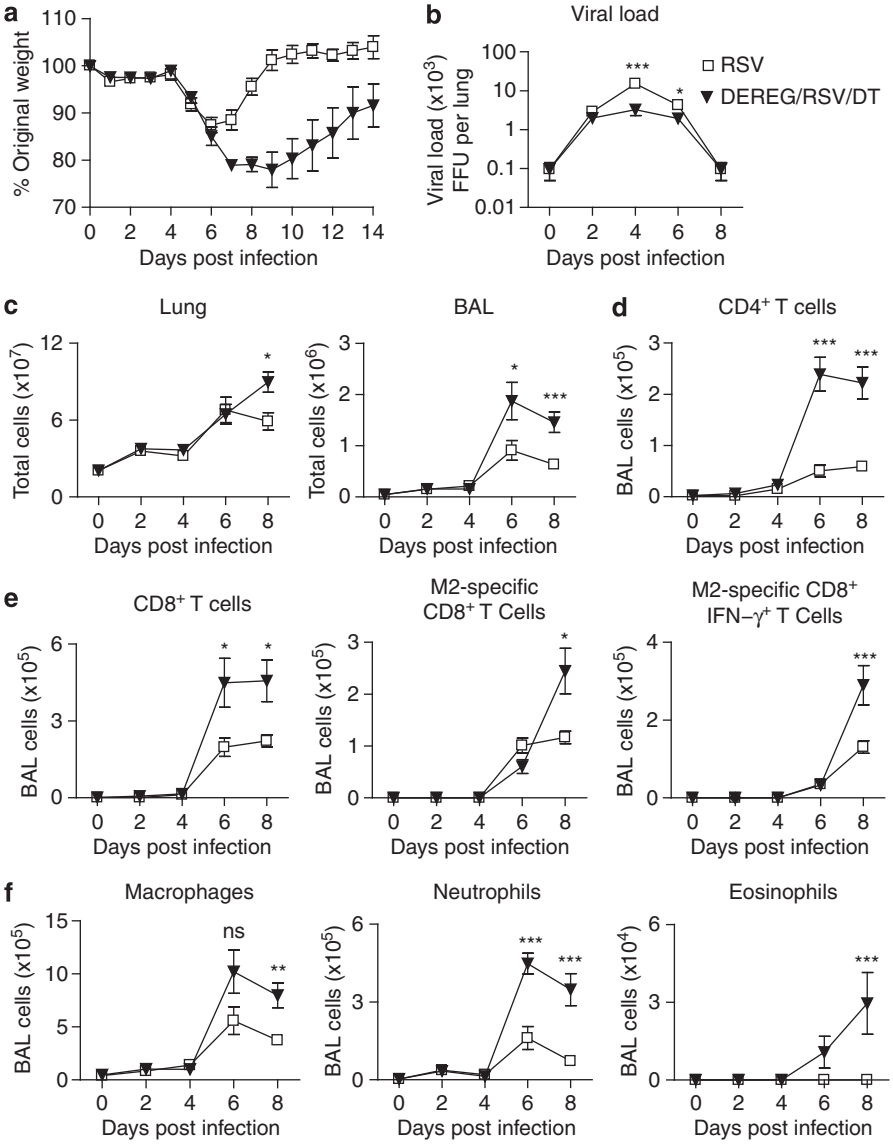

Figure 1 Depletion of Foxp3$^+$ cells decreases viral load, increases cellular influx into the lungs, and delays recovery during respiratory syncytial virus (RSV) infection. BALB/c mice or BALB/c depletion of regulatory T-cell (DEREG) mice were infected with 10^6 focus-forming units RSV intranasally (day 0). Where indicated, mice were injected intraperitoneally (days −2, −1, 2, 5, and 8) with 0.75 μg diphtheria toxin (DT). Lungs and bronchoalveolar lavage fluid (BAL) were obtained from naïve mice (day 0) or 2, 4, 6, or 8 post RSV infection. (**a**) Illness was monitored daily by weight for 14 days after RSV infection; percentage of original weight is shown. (**b**) Viral titer (FFU) was measured in lungs by immuno-plaque assay (detection limit at 1,000 FFU). (**c**) Total numbers of cells in the lung and BAL were enumerated in naïve or RSV-infected mice, with and without regulatory T-cell depletion. (**d**) Total numbers of CD3$^+$-gated CD4$^+$Foxp3$^-$ in the BAL were quantified using flow cytometry. (**e**) Total numbers of CD3$^+$-gated CD8$^+$ T cells, M2 pentamer$^+$ CD8$^+$ T cells, and M2 peptide-restimulated CD8$^+$IFN-γ$^+$ T cells in the BAL were quantified using flow cytometry. (**f**) Total numbers of macrophages, neutrophils, and eosinophils in the BAL were quantified using differential cell counting of hematoxylin and eosin-stained cytospins slides. Error bars indicate the s.e.m. Graphs are representative of at least two independent experiments with four mice per group in each. NS, not significant. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (*P<0.05, **P<0.01, ***P<0.001).

important for dampening the early activation of conventional T cells during RSV infection, thereby delaying viral clearance.

Lung Tregs express GzmB and can degranulate

Recent results using tumor models suggest that Tregs can mediate immune control in part through GzmB-dependent cytotoxicity.[15,16] Consistent with this possibility, we found that Tregs in the lungs (**Figure 3a** and **b**) and airways (data not depicted) of RSV-infected mice expressed GzmB by day 8 post infection. GzmB$^+$ Tregs were not found in the draining lymph nodes either on day 4 or 8 post RSV infection, indicating that Treg production of GzmB was restricted to the lung tissue (data not depicted).

To test whether Tregs from the lungs of RSV-infected mice can degranulate upon T-cell receptor activation, we captured transiently expressed CD107a on the cell surface with anti-CD107a antibodies following stimulation of lung-derived CD4$^+$Foxp3$^+$ T cells with anti-CD3/CD28-coated beads *ex vivo* (**Figure 3c**). Interestingly, after a 4-h restimulation, lung Tregs expressed as much surface CD107a$^+$ as CD8$^+$ cytotoxic T cells from the same tissue. Thus, Tregs requiring the expression of GzmB to

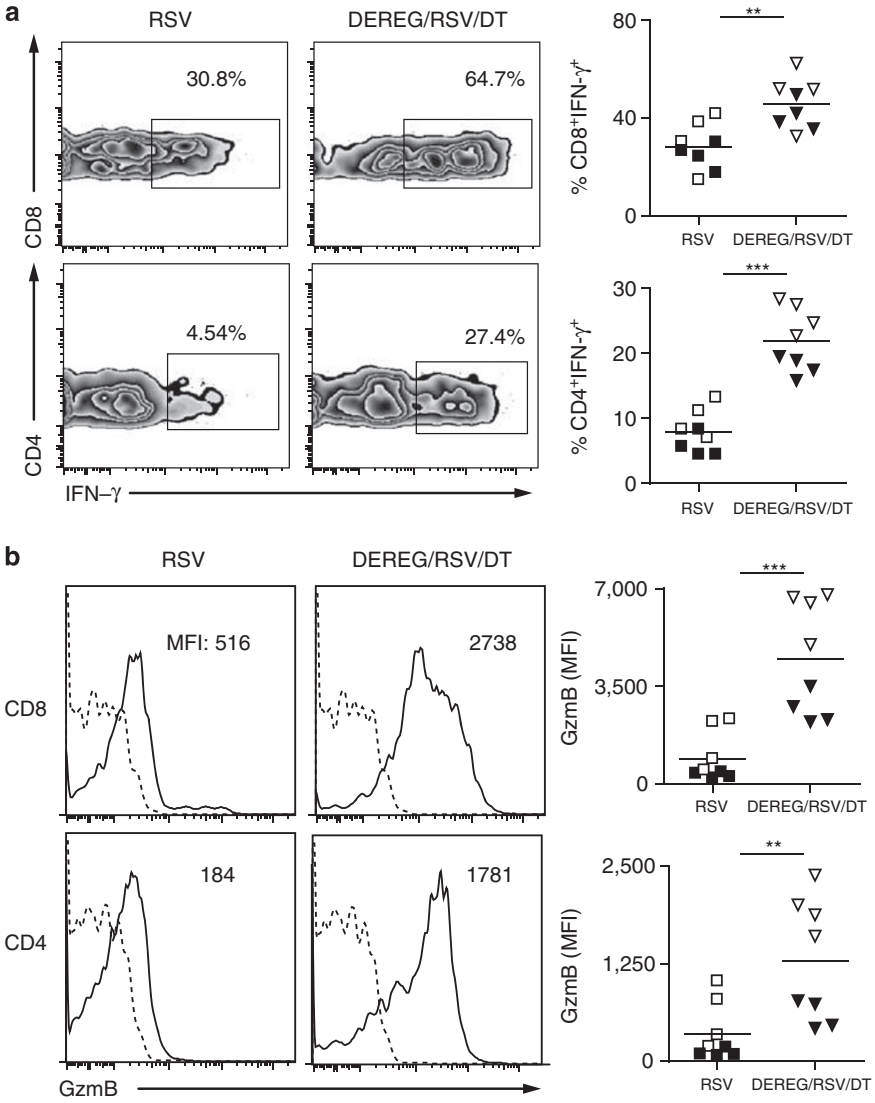

Figure 2 Foxp3$^+$ cells control early T-cell activation during respiratory syncytial virus (RSV) infection. CD3$^+$-gated, CD8$^+$ T cells and CD4$^+$Foxp3$^-$ T cells from lungs on day 4 of RSV infection (with or without regulatory T-cell depletion) were analyzed by flow cytometry (filled symbols, first study; open symbols, repeat). (**a**) Representative contour plots and percentage of intracellular interferon (IFN)-γ after *in vitro* phorbol 12-myristate 13-acetate/ionomycin restimulation. (**b**) Representative histograms and mean fluorescence intensity (MFI) of granzyme B (GzmB) (solid lines), and isotype control staining (dotted lines) directly *ex vivo*. Results from individual mice from two independent experiments are shown on the right. DEREG, depletion of regulatory T cell; DT, diphtheria toxin. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (**$P<0.01$, ***$P<0.001$).

perform their regulatory function are recruited to the lungs during acute RSV infection.

IL-2 complexes increase Treg frequencies and induce GzmB expression in lung Tregs

As the above experiments indicated that Tregs are crucial for the control of RSV infection, we tested the effect of enhancing their number and activity by injecting BALB/c mice i.p. with IL-2 immune complexes (IL-2 Cx)[17] for 3 days (days −2, −1, and 0). Examination of the spleen, mesenteric lymph nodes (mesLNs), and lung showed increased cellularity after IL-2 Cx injections (**Supplementary Figure S5** online), as well as increased frequencies of Tregs but not naïve CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ T cells (**Supplementary Figure S5** online and data not depicted). IL-2 Cx also expand NK cells[18] and the frequency of NK cells increased significantly in the lung but not in the spleen or mesLNs on day 1 (**Supplementary Figure S5d** online) and day 5 (data not depicted) after IL-2 Cx injections. In addition to increased frequency, Tregs in IL-2 Cx-injected mice displayed an activated phenotype, including elevated expression of CD25, glucocorticoid-induced TNFR (GITR), cytotoxic T-lymphocyte antigen 4, and inducible T-cell co-stimulator (ICOS) (**Supplementary Figure S5c** online). Less marked increases were also seen in intracellular Foxp3, programed death receptor ligand 1 (PDL-1), CD69, and intercellular adhesion molecule 1 (ICAM-1). In contrast, CD44 and folate receptor 4 (FR4) expression on lung Tregs were unaffected by IL-2 Cx injections. Interestingly, CD127 expression on Treg was strongly

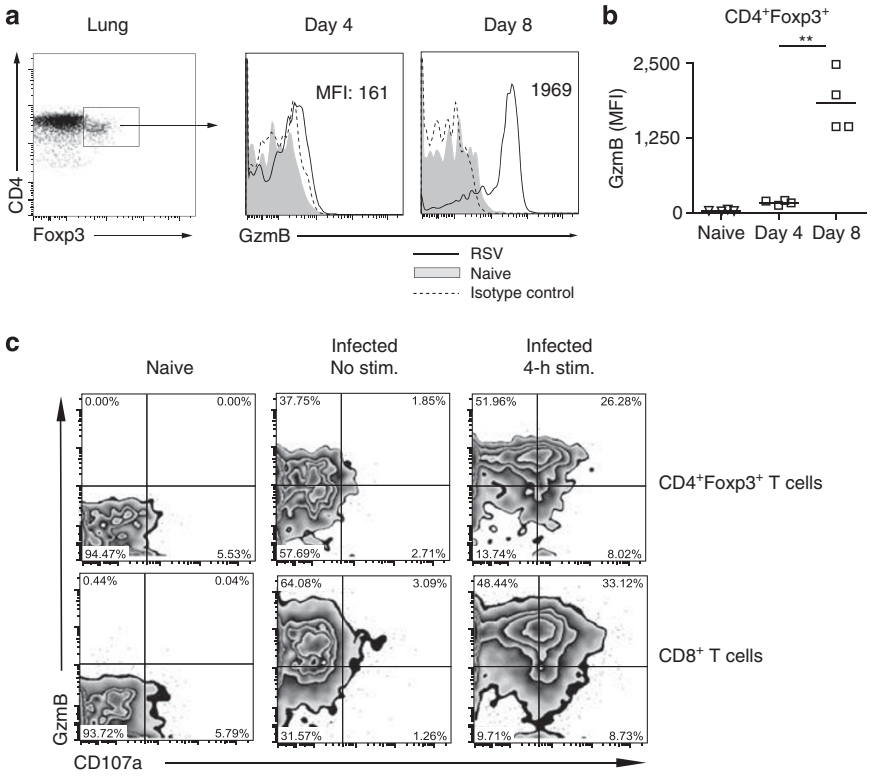

Figure 3 Foxp3$^+$ cells express granzyme B (GzmB) during respiratory syncytial virus (RSV) infection and degranulate upon T-cell receptor engagement. BALB/c mice were infected with RSV intranasally (day 0) and lungs were analyzed on day 4 and 8 post infection. (**a**) Representative plots of GzmB-expressing regulatory T cells (CD4$^+$Foxp3$^+$) from lungs of naïve and RSV-infected mice *ex vivo* on day 4 and 8 post infection. Cells from RSV-infected mice (solid black line), isotype control (dotted line) and stained cells from uninfected mice (gray). (**b**) Mean fluorescence intensity (MFI) of GzmB in CD4$^+$Foxp3$^+$ T cells in naive and RSV-infected mice in the lung on day 4 and 8 post RSV infection. (**c**) Representative contour plots of surface CD107a and intracellular GzmB in CD4$^+$Foxp3$^+$ T cells and CD8$^+$ T cells from the lung 8 days post RSV infection. Cells were stained *ex vivo* without ("no stim") or with restimulation for 4 h ("4 h stim") with anti-CD3/CD28-coated beads and compared with cells from naïve mice. Data are representative of two independent experiments. Stim., stimulation. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (**P<0.01).

downregulated by IL-2 Cx injections (**Supplementary Figure S5c** online). Notably, downregulation of CD127 expression has previously been shown in human Tregs.[19] The activation status of the Tregs returned to basal levels by day 5 post last injection (data not depicted).

Lung NK cells had high expression of GzmB after IL-2 Cx injections (**Supplementary Figure 5e** online). Surprisingly, IL-2 Cx injections also induced GzmB production by Tregs, which was detectable both in the lung and in the spleen (**Figure 4g** and data not depicted). RSV infection further increased GzmB production by lung Tregs in IL-2 Cx-injected mice (**Figure 4g**). Thus, IL-2 Cx injections increase the numbers and activation status of Tregs and NK cells, and importantly induce GzmB production in lung Tregs.

Boosting Tregs limits lung inflammation

Treg boosting by IL-2 Cx has previously been shown to inhibit allergic airway disease,[20] EAE, and allograft rejection.[21] Thus, we examined whether IL-2 Cx injections had beneficial effects in RSV lung disease. We gave IL-2 Cx injections i.p on day 1, 2, and 3 after RSV infection. As expected, weight loss in normal BALB/c mice infected with RSV peaked at day 6 and 7 with recovery starting at day 8. In contrast, mice injected with IL-2 Cx showed accelerated recovery (**Figure 4a**), even though the viral titers were similar to those found in untreated mice (**Figure 4b**). The total cell number in the BAL of IL-2 Cx-injected mice was significantly, although only slightly, decreased compared with phosphate-buffered saline (PBS)-injected mice after RSV infection (**Figure 4c**). Furthermore, while CD4$^+$Foxp3$^-$ T cells did not change in numbers between IL-2 Cx-injected and PBS-injected mice (**Figure 4d**), CD8$^+$ effector T-cell populations were decreased in the lung on day 8 post infection in the RSV/IL-2 Cx mice (**Figure 4e**). This decrease was even more remarkable for antigen-specific CD8$^+$ T cells detected by M2 pentamer staining or for IFN-γ-producing CD8$^+$ T cells after M2 peptide restimulation (**Figure 4e**). To address the role of IL-2 Cx on other cytokines, we measured IL-10 production from T cells after RSV infection of IL-2 Cx- or PBS-injected mice. Production of IL-10 from CD4$^+$ or CD8$^+$ T cells in the BAL did not change after IL-2 Cx injections at day 4 but were slightly lower on day 8 post RSV infection (**Supplementary Figure S6** online). To explore the activation status of effector T cells and NK cells in the airways after RSV infection and IL-2 Cx injections, their GzmB expression was analyzed. Surprisingly, NK cells, CD4$^+$ and CD8$^+$ T cells from the RSV/IL-2 Cx mice showed a drastic decrease in the expression of GzmB on

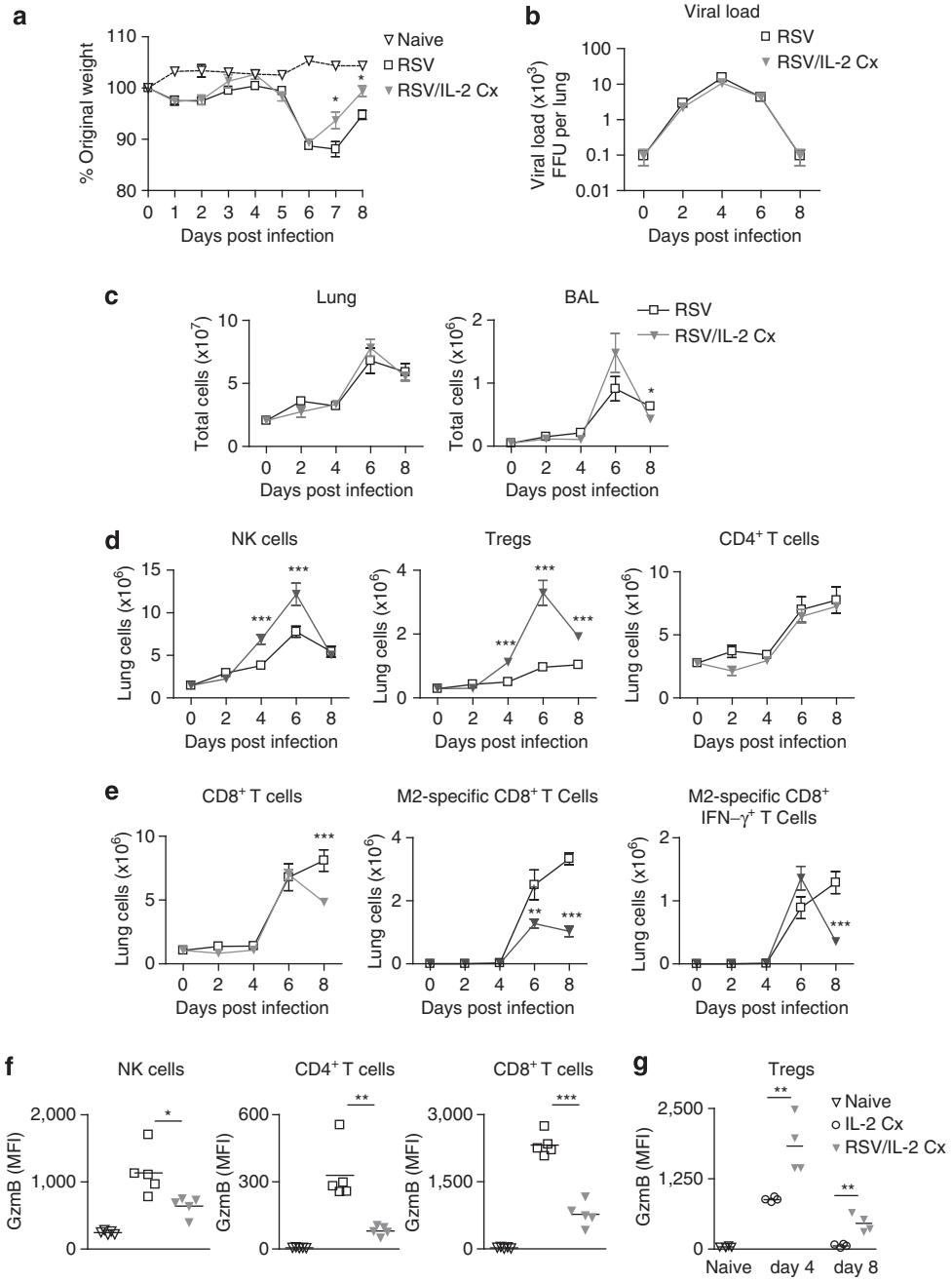

Figure 4 IL-2 immune complexes (IL-2 Cx) injections boost regulatory T cells (Tregs) and natural killer (NK) cells, and reduce cellular influx and T-cell activation in the lungs during respiratory syncytial virus (RSV) infection. BALB/c mice were infected with RSV intranasally and injected intraperitoneally up to three times (days 1, 2, and 3 post infection) with IL-2 Cx. Lungs and bronchoalveolar lavage fluid (BAL) were obtained from naïve mice (day 0) or 2, 4, 6, or 8 post RSV infection. (**a**) Illness was monitored by body weight after RSV infection. (**b**) Lung viral load measured by immuno-plaque assay (detection limit at 1,000 FFU). (**c**) Total numbers of cells in the lung and BAL. (**d**) Total numbers of NK cells ($CD3^-NKp46^+$), Tregs ($CD4^+Foxp3^+$), and $CD4^+Foxp3^-$ T cells in the lungs. (**e**) Total numbers of CD3-gated, $CD8^+$ T cells, M2 pentamer$^+$ $CD8^+$ T cells, and M2-restimulated $CD8^+$IFN-γ^+ T cells in the lung. (**f**) Quantification of the mean fluorescence intensity (MFI) of granzyme B (GzmB)-expressing NK cells, $CD4^+$ T cells, and $CD8^+$ T cells in the lung *ex vivo* on day 8 post infection. (**g**) Quantification of the MFI of GzmB-expressing $CD4^+Foxp3^+$ T cells in the lung of IL-2 Cx-injected or RSV-infected, and IL-2 Cx-injected mice on days 4 and 8 after RSV infection. Error bars indicate the s.e.m.; results are representative of at least two independent experiments (four mice per group). The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

day 8 post infection compared with RSV mice (**Figure 4f**). In addition, the number of neutrophils in the BAL was decreased in the RSV/IL-2 Cx mice at day 8 post infection (data not depicted).

In order to test whether the expanded NK cells or Tregs were responsible for the decrease in lung inflammation, RSV/IL-2 Cx mice were additionally injected with rabbit anti-mouse asialo GM1 (anti-aGM1) or anti-CD25 antibody (PC61). Depletion

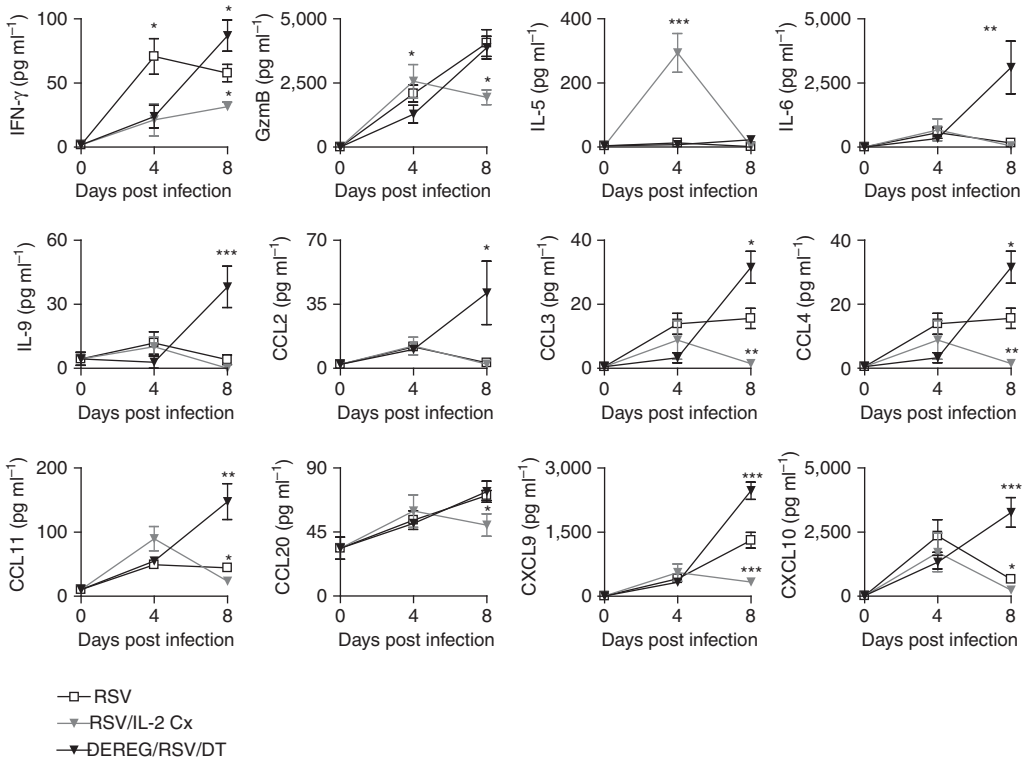

Figure 5 Foxp3$^+$ cells influence chemokine and cytokine production in the airways during respiratory syncytial virus (RSV) infection. Bronchoalveolar lavage fluid samples from BALB/c naïve mice, regulatory T cell (Treg)-depleted DEREG (depletion of regulatory T cell/diphtheria toxin (DT)), or IL-2 immune complexes-injected mice at different time points after RSV infection were analyzed for interferon (IFN)-γ, granzyme B (GzmB), interleukin (IL)-5, IL-6, IL-9, CCL2, CCL3, CCL4, CCL11, CCL20, CXCL9, and CXCL10. Error bars indicate the s.e.m. Graphs show pooled data from two independent experiments with four mice per group in each. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (**P*<0.05, ***P*<0.01, ****P*<0.001).

of NK cells with anti-aGM1 revealed that much of the GzmB in the BAL on day 4 post RSV infection was attributable to NK cells (**Supplementary Figure S7a** online). However, in IL-2 Cx-injected mice, NK cell depletion had little effect on BAL GzmB levels at day 4 post infection, suggesting that Tregs might be an additional source of GzmB as shown previously (**Supplementary Figure S7a** online and **Figure 3** and **4g**). In order to address the role of Tregs, RSV/IL-2 Cx mice were additionally injected with anti-CD25 antibody (PC61) on day −1 and 3 in order to eliminate Tregs. Analysis of CD4$^+$Foxp3$^+$ T cells in the lung on day 8 post infection confirmed depletion (**Supplementary Figure S7b** and **d** online). Notably, depletion of CD25$^+$ cells reversed the effects of the IL-2 Cx injections, increasing total cell numbers in the lymph node and BAL, and promoting infiltration of CD8$^+$ T cells into the lung (**Supplementary Figure S7c** and **d** online). This suggests that the effect of IL-2 Cx injections in our model is due to boosting of Tregs rather than NK cells, supporting the notion that *in vivo* boosting of Tregs limits lung inflammation and disease severity during RSV infection.

Tregs influence the chemokine and cytokine production in the airways

Chemokine and cytokine expression has an important role in the recruitment and activation of different cell types during RSV infection.[22] To elucidate the role of Tregs in this process, we analyzed the BAL samples from naïve mice, WT, Treg-depleted DEREG, or IL-2 Cx-injected mice at different time points after RSV infection (**Figure 5**). CXCL2, CCL5, IL-4, IL-10, IL-12p70, IL-13, IL-17, IL-21, IL-33, TNF-α, and TSLP could not be detected in any group at any time post RSV infection (data not depicted). In contrast, the levels of CCL11, CXCL10, CXCL9, CCL2, CCL3, IFN-γ, IL-5, IL-6, and IL-9 were significantly increased in the lungs of RSV-infected mice that had been depleted of Tregs (**Figure 5**). The converse was observed for CCL20, CXCL10, CXCL9, CCL3, CCL4, IFN-γ, GzmB, and IL-9 in mice receiving IL-2 Cx (**Figure 5**). Thus, removing Tregs was accompanied by an elevation of pro-inflammatory chemokine and cytokine levels in the airways. In contrast, boosting Tregs inhibited inflammation and decreased levels of these same chemokines and cytokines.

Tregs control acute RSV lung infection via GzmB

In order to test the possible role of GzmB in Treg suppression of lung inflammation, we attempted but failed to reconstitute Treg-depleted DEREG mice with Tregs from wt or GzmB$^{-/-}$ mice. Therefore, we used an alternative approach and constructed mixed bone marrow (BM) chimeric mice[23] using C57BL/6 hosts reconstituted with either 20% *Gzmb*$^{+/+}$ or *Gzmb*$^{-/-}$ BM, and 80% DEREG BM. In such chimeras, Tregs of DEREG origin can be depleted by DT injection, leaving only WT *Gzmb*$^{+/+}$ or knockout *Gzmb*$^{-/-}$ Tregs, respectively. If GzmB expression in Tregs has a fundamental role in regulating disease severity

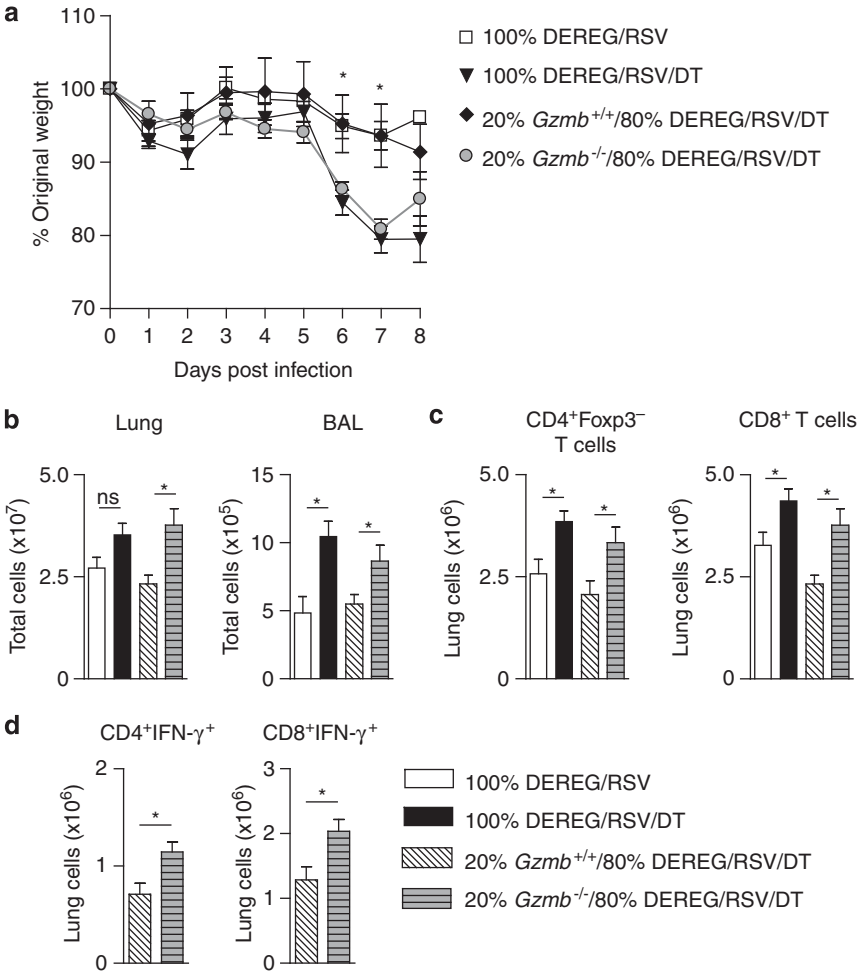

Figure 6 The regulatory function of Foxp3$^+$ cells during respiratory syncytial virus (RSV) infection is dependent on granzyme B (GzmB). C57BL/6J mixed BM chimeric mice were infected with RSV intranasally (day 0), with or without 0.75 μg diphtheria toxin (DT) intraperitoneally (days −2, −1, 2, and 5). (**a**) Illness was monitored by individual mouse weight measurement for 8 days after RSV infection, and is expressed as percentage of original weight. (**b**) Total numbers of cells were enumerated in the lung and bronchoalveolar lavage fluid (BAL) on day 8 post RSV infection. (**c**) Total numbers of CD4$^+$ Foxp3$^-$ T and CD8$^+$ T cells in the lung on day 8 post infection. (**d**) CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ T-cell expression of interferon (IFN)-γ after phorbol 12-myristate 13-acetate/ionomycin restimulation of lung cells was analyzed on day 8. Error bars indicate the s.e.m. Graphs are representative of two experiments with five mice per group in each. DEREG, depletion of regulatory T cell. NS, not significant. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t* test (*P<0.05).

during acute viral lung infection, infection-associated pathology in mice that only have *Gzmb*$^{-/-}$ Tregs should be more severe than in mice that contain *Gzmb*$^{+/+}$ Tregs.

Control chimeras with 100% DEREG BM with or without depletion of Tregs gave comparable results to those with non-chimeric mice. In these mice, DT injection led to a marked increase in weight loss, indicative of unabated pathological inflammation (**Figure 6** and **Supplementary Figure 3** online). This pathology was rescued in DT-treated mice that had been reconstituted with 80% DEREG and 20% *Gzmb*$^{+/+}$ BM, demonstrating that the 20% *Gzmb*$^{+/+}$ Tregs were sufficient to reconstitute regulation. Strikingly, mice reconstituted with 80% DEREG and 20% *Gzmb*$^{-/-}$ BM were not rescued and showed increased weight loss on day 6 and 7, similar to that seen in Treg-depleted mice that had been reconstituted with 100% DEREG BM (**Figure 6a**). Mice with GzmB-deficient Tregs displayed greater inflammatory cell influx into the lungs and airways, and elevated total T-cell numbers of CD4$^+$Foxp3$^-$ and CD8$^+$ T cells in the lung when compared with control chimeric mice (**Figure 6b** and **c**). In addition, total numbers of IFN-γ-producing T cells were increased in the lungs and BAL of *Gzmb*$^{-/-}$ Treg mice compared with *Gzmb*$^{+/+}$ Treg mice (**Figure 6d** and data not depicted). Thus, *Gzmb*$^{-/-}$ Tregs are unable to control lung pathology indicating that the regulatory effect of lung Tregs during acute RSV infection is dependent on their ability to produce GzmB.

DISCUSSION

The regulatory mechanism for controlling immune responses and preventing immunopathology during acute viral lung infection remains poorly understood. We used two complementary *in vivo* approaches to examine the contribution of Tregs in controlling virally induced lung inflammation: depletion of Tregs (DEREG mice) and boosting of Tregs (by IL-2 Cx injections). Depletion of Tregs before RSV infection caused increased

and sustained weight loss after infection, accompanied by an increase in inflammation and cell recruitment to the lung by day 6. Importantly, the general increase in cell recruitment was not reflected in recruitment of virus-specific $CD8^+$ T cells on day 4 or 6; only on day 8 was an enhanced virus-specific response evident in the lung and airway. Boosting Treg numbers and activity gave broadly opposite effect, diminishing inflammation but without affecting viral load. Together, these results underscore the key role of Tregs in determining the sequence of events and the final balance between effective antiviral immunity and controlling harmful immunopathology. Notably, after a systematic analysis of the Treg expression profile, we found that Tregs in the lungs of RSV-infected mice expressed GzmB and that this expression is essential for their function. This suggests that, Tregs might be able to co-opt a program normally employed by cytotoxic T cells and NK cells in order to regulate lung inflammation during acute viral infection.

We and others have recently shown that depletion of Tregs with anti-CD25 antibody enhances RSV-induced disease on day 8 and increases T-cell activation.[24–26] However, anti-CD25 also depletes activated T cells and does not deplete $Foxp3^+$ $CD25^-$ Tregs. In contrast, we now find that specific ablation of $Foxp3^+$ T cells during RSV infection in DEREG mice causes an increase in lung accumulation of effector T cells accompanied by a decrease in peak viral load. Our results are all the more striking as it is known that DT injection of DEREG mice does not fully deplete Tregs; some $FoxP3^+$ GFP^- cells remain and can subsequently expand.[27]

It has recently been shown that Tregs are essential for an early protective response in experimental herpes simplex virus infection of mice, enhancing effector cell recruitment into infected tissues, which promotes viral clearance.[9] In addition, Tregs can exert direct control over the proliferation of effector T cells,[28,29] which may account for the increase in effector T cells in Treg-depleted mice. This resembles the situation in acute Friend retrovirus infection, where Treg depletion decreases viral load while promoting elevated GzmB expression in $CD8^+$ T cells.[8] It is likely that increased GzmB expression by T cells is directly responsible for the reduced viral load through cytolytic attack on infected cells.[30]

Previous work has shown that, in addition to promoting viral clearance, $CD4^+$ and $CD8^+$ effector T cells are responsible for causing the weight loss and lung inflammation characteristic of RSV disease in mice.[30,31] Treg-depleted mice displayed an increase in weight loss and inflammatory cell infiltration into the lungs indicating that Tregs are essential for controlling the acute lung inflammation. Increased pro-inflammatory cytokine and chemokine levels were also indicative of greater lung and airway inflammation in Treg-depleted mice. We were surprised to find that depletion of $Foxp3^+$ T cells also caused an increase in the influx of neutrophils and eosinophils accompanied by elevated levels of inflammatory mediators IL-5, IL-9, CCL11, CXCL9, and CXCL10 in the airway on day 8 post RSV infection. This was not observed in the anti-CD25 depletion studies[24–26] or during primary RSV infection of normal mice.[31] However, the absence of Tregs also enhances eosinophil infiltration into the airways during allergic airway inflammation and increases levels of IL-4, IL-5, and IL-13,[5] suggesting that $Foxp3^+$ T cells may directly or indirectly regulate granulocyte influx into the airways and also suppress a Th2-biased immune response. Together, these findings support a major role for Tregs in regulating the immune response to lung infection both at the time of initiation of inflammation and at the peak of disease.

Boosting Tregs *in vivo* by IL-2 Cx injections after acute viral lung infection limited lung inflammation and disease severity during RSV infection, which was accompanied by reduced cytokine and chemokine production in the BAL. IL-2 Cx injections also caused a marked increase in lung NK cell numbers and activation. However, when Tregs were depleted with anti-CD25 mAb, IL-2 Cx injection no longer decreased RSV-associated lung pathology. This suggests that, even though NK cells can have an important role in viral clearance,[32,33] Tregs are the major mediators of the anti-inflammatory effect of IL-2 Cx during RSV infection.

Unexpectedly, we found that Tregs from the lung of RSV-infected mice express intracellular GzmB and degranulate after stimulation *in vitro*. GzmB expression by lung Tregs was also induced in mice injected with IL-2 Cx even in the absence of infection. Therefore, we speculate that the beneficial outcome of IL-2 Cx injections after RSV infection might be due to specific activation of GzmB-producing Tregs in the lung. To confirm that GzmB is essential for the regulatory function of Tregs in this model, we constructed mixed BM chimeric mice[23] using BM from DEREG mice (80%) and GzmB knockout mice (20%). Most cells (80%) in these chimeric mice are able to make GzmB but the Treg compartment is completely deficient in GzmB upon treatment with DT. As controls, we used chimeric mice with 80% DEREG and 20% WT BM. Chimeric mice with GzmB-deficient Tregs showed a very similar phenotype to fully Treg-depleted mice, exhibiting marked weight loss and increased lung inflammation.

These results reveal a crucial, cell-intrinsic, and unprecedented role for GzmB in the regulation of lung inflammation by Tregs during acute viral lung infection. In some tumor and transplantation models, Tregs have been suggested to kill dendritic cells or $CD8^+$ T cells via perforin, GzmB, or Fas-FasL-dependent mechanisms.[16,34–38] It is therefore possible that the requirement for GzmB in the Treg compartment during RSV infection reflects a cytolytic mode of regulation. Notably, there are also extracellular non-cytolytic functions of the serene protease GzmB.[39] GzmB cleaves aspartic acid residues (aspartase activity) and accumulates in extracellular fluids. Additionally, GmzB works together with perforin to kill cells,[40] but works without perforin to cleave the proteoglycan decorin in the extracellular space.[41] Whichever the mechanism, our unexpected finding of a requirement for $GzmB^+$ Tregs in the lung and airways during RSV infection is unlikely to be restricted to this model. As such, it may represent a general aspect of immunity during infections of the respiratory tract and other delicate barrier sites.

Interestingly, Bem *et al.*[42] have reported elevated GzmB expression in $CD4^+$ T cells in the BAL from children with severe RSV infection. In addition, many children with

haemophagocytic lymphohistiocytosis (a rare immune dysregulation syndrome) have genetic mutations in the perforin/granzyme pathway. Haemophagocytic lymphohistiocytosis is characterized by an uncontrolled immune response, especially to viral infections.[43] The results reported here raise the possibility that lack of GzmB production by Tregs in haemophagocytic lymphohistiocytosis leads to impaired immune regulation, and suggest that our findings could be directly relevant to human disease.

Our work raises a number of interesting issues for future studies. For example, what signals are regulating GzmB expression in Tregs in the lung and is the Treg role antigen specific? Nevertheless, even without knowing the answer to these questions we speculate that selectively enhancing GzmB-expressing Tregs could potentially be used as a therapeutic strategy in acute viral lung infections, dampening inflammation without elevating viral load. Our demonstration that Tregs have a pivotal role in controlling lung inflammation and the identification of a role for GzmB in the *in vivo* function of lung Tregs are important steps in elucidating the mechanisms by which the fine balance between clearing virus and controlling immune responses is achieved, so allowing the gas exchange function of the lung to be maintained in the face of viral attack.

METHODS

Mice, virus stocks, and infection. Six- to ten-week-old C57BL/6 or BALB/c mice (Harlan, Blackthorn, UK) were maintained in pathogen-free conditions under UK Home Office guidelines. DEREG mice on C57BL/6 or BALB/c background, and C57BL/6 *Gzmb*$^{-/-}$ (Jackson, Bar Harbor, ME[11,44]) were used in the study.

Plaque-purified human RSV (A2 strain from the ATCC, Teddington, UK) was grown in HEp-2 cells.[26] Age- and sex-matched mice were lightly anesthetized and infected intranasally with 10^6 focus-forming units (FFU) RSV in 100 µl.

Foxp3$^+$ cell depletion, IL-2 complex injections, NK cell and CD25 depletion. DEREG mice were injected with 0.75 µg of DT (Merck, Feltham, UK) in PBS i.p. on days −2 and −1, and on days 2, 5, and 8 after RSV infection to induce and maintain Foxp3$^+$ T-cell depletion. IL-2 Cx were obtained by mixing 1 µg rmIL-2 (Peprotech, Rocky Hill, NJ) and 5 µg anti-IL-2 (Clone JES6-1A12; eBioscience, San Diego, CA), and incubating at 37°C for 30 min. Age- and sex-matched BALB/c or C57BL/6 mice received daily i.p. injections of IL-2 Cx or PBS for three consecutive days. In some experiments, mice were additionally injected i.p. with 50 µl of rabbit anti-mouse asialo GM1 on days −2, 0, and 3 (Dako, Osaka, Japan), or with 200 µl of anti-CD25 antibody (PC61; 1 mg ml^{-1}) on days −1 and 3.

BM chimeras. C57BL/6 recipient mice were lethally irradiated (1000 rad γ irradiation). On the same day, 3×10^6 BM cells from C57BL/6 DEREG BM were injected intravenously into the irradiated recipients. Reconstitution determined as GFP$^+$ Tregs was 90–95% after 10–12 weeks. To obtain mixed BM chimeras in which the Tregs are GzmB deficient, we reconstituted irradiated C57BL/6 hosts with BM from *Gzmb*$^{-/-}$ (20%) and DEREG mice (80%). Thus, 80% of the Tregs can be depleted and the remaining Tregs are GzmB deficient, while 80% of the non-Treg cells are of WT origin. Control C57BL/6 hosts were reconstituted with WT BM (20%) and DEREG BM (80%).

Cell collection and preparation. Bronchoalevolar lavage (BAL) was carried out using 1 ml PBS containing 12 mM Lidocaine by flushing three times. In order to obtain single-cell suspensions, lungs were processed with the gentleMax dissociator (Miltenyi Biotech, Surrey, UK) using Collagenase D (50 µg ml^{-1}, Sigma, Dorset, UK). Total cell counts were determined by flow cytometry using Count Bright counting beads (Invitrogen, Paisley, UK), and dead cells were excluded by staining for 7-amino-actinomycin D (7-AAD, Sigma). For determination of cellular composition in the BAL, cells were transferred onto a microscope slide (Thermo Scientific, Braunschweig, Germany) using a cytospin centrifuge, and stained with hematoxylin and eosin (Reagena, Gamidor, Didcot, UK).

Flow cytometry. For flow analysis, the LIVE/DEAD Fixable Red Dead cell stain kit (Invitrogen) was used to exclude dead cells. Cells were incubated with FcγIII/II receptor antibody (BD Biosciences, Oxford, UK), and were labeled with the following antibodies (from BD Biosciences unless otherwise stated): V450-conjugated anti-mNKp46/NCR1, FITC-conjugated anti-FR4 (12A5, Biolegend, San Diego, CA), PE-Cy7-conjugated anti-CD3 (145-2C11), Pacific Blue or conjugated anti-CD4 (RM4-5), Alexa Fluor 700-conjugated anti-CD8α (53-6.7), PE-conjugated anti-CD25 (PC61), FITC-conjugated anti-CD44 (IM7), PE-conjugated anti-CD69 (H1.2 F3), FITC-conjugated anti-CTLA-4 (UC10-4F10-11), PE-conjugated anti-GITR (DTA-1), FITC-conjugated anti-CD127 (SB/199), PE-conjugated anti-ICOS (7E.17G9), FITC-conjugated anti-PDL-1 (MIH5), FITC-conjugated anti-CD19 (6D5), Alexa Fluor 700-conjugated anti-CD11c (HL3), PE-conjugated anti-CD86 (GL1), FITC-conjugated anti-CD80 (16-10A1), PE-conjugated MHC I Pentamer H-2Kd SYIGSINNI (M2; Proimmune, Oxford, UK), and PE-conjugated anti-ICAM-1 (3E2). Tregs were discriminated by staining for Pacific Blue-conjugated anti-CD4 (RM4-5) and Foxp3 through green fluorescent protein expression from DEREG mice. Intracellular staining for Foxp3, APC, or FITC-conjugated anti-Foxp3 (FJK-16 s, eBioscience), and APC-conjugated anti-human GzmB (GB12, Caltag, Buckingham, UK; Invitrogen) was performed using the Foxp3 staining kit (eBioscience) following the manufacturer's recommendations without restimulation. The anti-human GzmB antibody cross-reacts with mouse GzmB.[45] In order to detect intracellular IFN-γ production, cells were stimulated with 100 ng ml^{-1} phorbol 12-myristate 13-acetate and 1 µg ml^{-1} ionomycin or 5 µg ml^{-1} M2 peptide (82–90, SYIGSINNI, Proimmune) in complete RPMI. After 1 h incubation, monensin (Golgi Stop, BD Biosciences) was added. Following 2 additional hours of incubation, cell surface staining was followed by intracellular cytokine and Foxp3 staining with APC anti-IL-10 (JES5-16E3, eBioscience), Percp Cy 5.5 anti-IFN-γ, and FITC-conjugated anti-Foxp3 using the Foxp3 staining kit (eBioscience). The peptide restimulation was performed for 6 h and monensin was added for the last 4 h. Cells were acquired on a LSR II (BD Biosciences) with data analyzed using Flow Jo software (Ashland, OR). Cells were gated for live cells, singlets, and lymphocytes before analysis for indicated markers.

Degranulation assay. Lungs from RSV-infected mice were processed on day 8 post infection as previously described, and sorted for CD4$^+$ T cells with the CD4 negative selection kit (Miltenyi Biotech). T cells (5×10^5) were restimulated with anti-CD3/CD28-coated beads (Dynabeads, Invitrogen) for 4 h in the presence of anti-CD107a (1D4B, eBioscience) and GolgiStop (BD Biosciences) in 96-well, V-bottom plates. The LIVE/DEAD Fixable Red Dead cell stain kit was used to exclude dead cells. After restimulation, the cells were stained with anti-CD107a, anti-CD4, and anti-CD8.[38] Intracellular cytokine and Foxp3 staining with anti-human GzmB and anti-Foxp3 was performed as described above.

Chemokine and cytokine detection. Chemokines and cytokines were quantified by a 24-plex Luminex kit performed according to the manufacturer's instructions (Biolegend), and data acquired with a Luminex 100 (Applied Cytometry systems, Sheffield, UK). The concentration of cytokines in each sample was determined according to the standard curve using the software Starstation (Applied Cytometry Systems). In some experiments, GzmB levels in the BAL were measured by enzyme-linked immunosorbent assay following manufacturer's recommendations (R&D Systems, Abingdon, UK).

Immuno-plaque assay. RSV titer was assessed in lungs post RSV infection. Lung homogenate was titrated on HEp-2 cell monolayers in 96-well, flat-bottom plates. Twenty-four hours after infection, monolayers were washed, fixed with methanol, and incubated with biotin-conjugated goat anti-RSV antibody (Biogenesis, Poole, UK). Infected cells were detected using Streptavidin (Sigma) and DAB substrate (diaminobezindine tetrahydrochloride), and FFU enumerated by light microscopy.

Statistical analysis. Results are presented as means±s.e.m. The significance of results between the groups was analyzed by two-tailed, unpaired Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Values of $P < 0.05$ were considered significant (Prism software; Graph-Pad Software, La Jolla, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS

We thank C Reis e Sousa for critically reading the manuscript, and C Alyahya and PG Ashton-Rickardt for providing the *GzmB*$^{-/-}$ mice. This work was supported by the Centre of Respiratory Infections (CRI), the MRC & Asthma UK Centre in Allergic Mechanisms of Asthma, the Wellcome Trust (Program 087805/Z/08/Z; PO), and the MRC career development award (Grant G0800311; CJ). JL, CJ, FJC, and PJO designed the study; JL, HT, and LD performed the experiments; KEW and JS provided method protocols; TS provided DEREG mice; and JL, CJ, and PJO wrote the manuscript.

DISCLOSURE

The authors declared no conflict of interest.

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