

RelB-Deficient Dendritic Cells Promote the Development of Spontaneous Allergic Airway Inflammation

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Abstract

RelB is a member of the NF- κ B family, which is essential for dendritic cell (DC) function and maturation. However, the contribution of RelB to the development of allergic airway inflammation (AAI) is unknown. Here, we identify a pivotal role for RelB in the development of spontaneous AAI that is independent of exogenous allergen exposure. We assessed AAI in two strains of RelB-deficient (RelB^{-/-}) mice: one with a targeted deletion and one expressing a major histocompatibility complex transgene. To determine the importance of RelB in DCs, RelB-sufficient DCs (RelB^{+/+} or RelB^{+/-}) were adoptively transferred into RelB^{-/-} mice. Both strains had increased pulmonary inflammation compared with their respective wild-type (RelB^{+/+}) and heterozygous (RelB^{+/-}) controls. RelB^{-/-} mice also had increased inflammatory cell influx into the airways, levels of chemokines (CCL2/3/4/5/11/17 and CXCL9/10/13) and T-helper cell type 2-associated cytokines (IL-4/5) in lung tissues, serum IgE, and airway remodeling (mucus-secreting cell numbers, collagen deposition, and epithelial thickening). Transfer of RelB^{+/+} CD11c⁺ DCs into RelB^{-/-} mice decreased pulmonary

inflammation, with reductions in lung chemokines, T-helper cell type 2-associated cytokines (IL-4/5/13/25/33 and thymic stromal lymphopoietin), serum IgE, type 2 innate lymphoid cells, myeloid DCs, $\gamma\delta$ T cells, lung V β 13⁺ T cells, mucus-secreting cells, airway collagen deposition, and epithelial thickening. These data indicate that RelB deficiency may be a key pathway underlying AAI, and that DC-encoded RelB is sufficient to restore control of this inflammation.

Keywords: allergic airway disease; dendritic cells; RelB

Clinical Relevance

Our findings indicate that RelB deficiency may be a key factor underlying allergic airway inflammation, and that dendritic cell-encoded RelB may have important therapeutic applications because it is sufficient to restore control of allergic airway inflammation.

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RelB is a member of the NF- κ B family, which is involved in both canonical and noncanonical signaling pathways (1). It plays a crucial role in the development of lymphoid organs and in dendritic cell (DC) biology (2). RelB has also been implicated in regulating circadian rhythm, mitochondrial function (by regulating Sirtuin 3), and xenobiotic responses in the lung (3).

RelB is highly expressed in human and mouse DCs, and is critical for the function and maturation of these cells (4). It is also expressed at low levels in innate-immune cells, and certain stimuli are required to induce its expression (5). The effects of RelB activation on the immune response are cell-type specific. A lack of RelB prevents the differentiation of DCs and upregulation of costimulatory molecules and IL-12 (6). RelB-deficient (RelB^{-/-}) DCs are tolerogenic and stimulate the development of regulatory T cells (Tregs) (7). In contrast, Langerhans cell differentiation is not affected in the absence of RelB (8–10). Silencing of RelB in DCs has the potential to be an immunomodulatory therapy for autoimmune diseases (11).

In contrast, RelB also has the potential to act as a negative regulator of inflammatory responses (12–15). RelB^{-/-} mice develop multiorgan inflammation (16). RelB has been shown to regulate chemokine expression in mouse kidney fibroblasts and to have beneficial effects in human immunodeficiency virus-1-induced neuroinflammation by inhibiting the release of TNF- α from microglial cells (17, 18). Overexpression of RelB was shown to inhibit cigarette smoke-induced inflammation in the lungs, largely due to the suppression of cyclo-oxygenase-2, prostaglandin E2, and intracellular adhesion molecule-1 levels, which in turn reduced proinflammatory cytokine and chemokine production (15). We previously showed that RelB-sufficient DCs controlled memory T cells and established autoimmune disease in RelB^{-/-} mice in an indoleamine-2,3-dioxygenase (IDO)-dependent fashion (6).

RelB^{-/-} mice develop a T cell-dependent skin disease resembling human atopic dermatitis, associated with DC and mast cell infiltration, T helper type 2 (Th2) cytokine overexpression in the dermis, and elevated serum IgE (19, 20). Peribronchial and perivascular

inflammation has been observed in histological sections of lungs from RelB^{-/-} mice (19). Little is known about RelB's role in asthma and allergic airway inflammation (AAI); however, it is likely important, as a recent multi-ancestry genome-wide association study identified signals in regions of the genome relevant to both the control of RelB expression and RelB-mediated regulation of immune responses (21).

Asthma is predominantly an allergic airway disease (AAD) and has doubled in prevalence over the past three decades. Th2 cells are critical players in the pathogenesis of allergic asthma (22). An early-phase response occurs within minutes of exposure to allergens and causes the activation of mast cells after the cross-linking of IgE to high-affinity IgE receptors (Fc ϵ RI) (23). Mast cell activation causes the release of various mediators, such as histamine, type 2 cytokines (e.g., IL-4, IL-5, and IL-13), chemokines (e.g., eotaxin), prostaglandins, and leukotrienes. This response results in mucus hypersecretion and airway hyperresponsiveness (AHR) (24). A late-phase response then results in the activation and infiltration of leukocytes, consisting mainly of eosinophils, into the site of the allergic reaction (23). Together with memory Th2 cells, persistent IgE and cytokine production occurs, leading to the continuous recruitment and activation of mast cells and eosinophils (25). Type 2 innate lymphoid cells (ILC2s) were recently identified as mediators of type 2 immune responses in the lung. Upon exposure to allergens, the pulmonary epithelium produces IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which drive the expansion of ILC2s (26). This in turn leads to the production of type 2 cytokines, eosinophil influx, airway mucus hypersecretion, and remodeling (27). Other cells, including myeloid DCs (mDCs) and $\gamma\delta$ T cells, are implicated in type 2 immune responses (28, 29). Current mainstay treatments for asthma—inhaled corticosteroids and long-acting β -agonists—generally suppress the symptoms of disease but do not treat the underlying causes. Moreover, in many cases the allergen(s) underlying the generation of asthma is unknown. Understanding the underlying mechanisms of disease pathogenesis is necessary to identify potential therapeutic targets for asthma.

Therefore, in this study we investigated the mechanisms by which RelB^{-/-} mice develop spontaneous inflammation in the lung. We demonstrate that in the absence of RelB, features of AAI develop independently of exogenous allergen exposure, and that adoptive transfer of RelB-sufficient DCs can control memory T cells driving this pathology. Some of the results of these studies have been previously reported in the form of an abstract (30).

Methods

Ethics Statement

This study was performed in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of the University of Newcastle (A-2015-528) and University of Queensland (UQDI/220/13/NHMRC).

Mice

All mice were maintained in specific pathogen-free conditions. RelB total knockout mice were kindly provided by Dr. Falk Weih (Leibniz-Institute for Age Research, Fritz Lipmann Institute, Jena, Germany) (16). Mice homozygous for an insertional mutation in the RelB gene were generated as previously described (19). Age-matched wild-type (WT) mice from the same facility were used as controls. A mixture of male and female mice was used for experiments at 6–9 weeks of age.

Assessment of Airway and Parenchymal Inflammation

BAL was performed as previously described, and differential leukocyte counts were determined from a total of 250 cells (31, 32). Longitudinal sections were stained with hematoxylin and eosin (H&E), and parenchymal inflammation was assessed by enumerating the numbers of inflammatory cells in 20 randomized, high-powered fields (33).

Histopathology, Protein Isolation, ELISA, Serum Antibodies, Airway Remodeling, and Lung Function

Protein isolation, ELISA, serum antibodies, airway remodeling, and lung

function were assessed as previously described and/or in the MATERIALS AND METHODS section in the data supplement (33–39).

DC Transfer

RelB^{+/+} or RelB^{+/-} lymphatic and splenic CD11c⁺ DCs were purified by magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). Up to 2×10^6 magnetic-activated cell sorting-purified DCs (>90 CD11c^{hi}) were injected intravenously into each RelB knockout^{-/-} or RelB mutant^{-/-} recipient. The lungs of untreated and treated mice were analyzed 5 or 14 days after adoptive transfer, and the data for both time points were pooled.

Flow Cytometry

The numbers of ILC2s, mDCs, $\gamma\delta$ T cells, CD4⁺ T cells, CD8⁺ T cells, alveolar macrophages, and Tregs in lung homogenates were determined based on surface marker expression using flow cytometry (Table 1) (32, 34, 37). Cells were permeabilized and stained intracellularly for FoxP3 (eBioscience, San Diego, CA) according to the manufacturer's instructions. Flow-cytometric analysis was performed using a FACSAriaIII with FACSDiva software (BD Biosciences, North Ryde, Australia). Flow-cytometry antibodies were obtained from Biolegend (Karrinyup, Australia) or eBioscience (Table E1 in the data supplement).

Analysis of Thymic, Lung, and Spleen V β T Cells

Individual collagenase-treated, disaggregated thymi, lungs, and spleen were stained for CD4 (GK1.1), CD8, Foxp3 (FJK-16 s), and V β panel of antibodies (BD PharMingen).

Statistical Analysis

The data are presented as means $\pm$ SEM, with 6–10 mice in each group, and are representative of at least two independent experiments. Statistical significance for multiple comparisons was determined by nonparametric one-way ANOVA with Dunn's post test or by one-way ANOVA with Bonferroni's post test, where appropriate. A two-tailed Mann-Whitney test was used to determine differences between two groups. AHR was analyzed using two-way repeated-measures ANOVA with Tukey's post test. Analyses were performed using Prism V.6 software (GraphPad Software, San Diego, CA).

Results

Spontaneous Pulmonary Cellular Inflammation Develops in RelB^{-/-} Mice

To investigate the role of RelB in the spontaneous development of AAI, we used two different strains of RelB^{-/-} mice: a targeted-deletion total knockout (*open symbols* in the figures) and a RelB mutant expressing a major histocompatibility complex (MHC) class I transgene with a disruption in the *RelB* locus (*solid symbols* in the figures). RelB^{-/-} mice and their respective WT (^{+/+}) and heterozygous (^{+/-}) controls were killed at 6–8 weeks of age and pulmonary inflammation was assessed in BAL fluid (BALF) and lung tissue sections. RelB^{-/-} mice had increased numbers of total leukocytes, eosinophils, lymphocytes, macrophages, and neutrophils in BALF (Figures 1A–1E) compared with RelB^{+/+} and RelB^{+/-} controls. RelB^{-/-} mice also had increased total, airway, vascular, and parenchymal inflammatory scores (Figures 1F–1J).

Increased Chemokines and Th2-Associated Cytokines in the Lungs of RelB^{-/-} Mice

We next determined the levels of inflammatory chemokines and cytokines in the lungs that may have mediated the increased pulmonary inflammation in RelB^{-/-} mice. RelB^{-/-} mice had increased CCL2, CCL3, CCL4, CCL5, CCL11, and CCL17 (Figures E1A–E1F), and CXCL9, CXCL10, and CXCL13 (Figures E1G–E1I) protein in the lungs compared with RelB^{+/+} and RelB^{+/-} controls. Interestingly, RelB^{-/-} mice also had increased levels of IL-4 and IL-5 (Figures E2A and E2B), but not IFN- γ or IL-17A (Figures E2C and E2D). Total IgE was also increased in the serum of RelB^{-/-} mice (Figure E2E), as shown previously (20).

Spontaneous Airway Remodeling Develops in RelB^{-/-} Mice

Loss of control of inflammation can have damaging consequences for lung tissues. Thus, we next assessed features of airway remodeling in RelB^{-/-} mice. RelB^{-/-} mice had increased numbers of mucus-secreting cells (MSCs) around the airways, small airway epithelial cell area, and collagen deposition compared with RelB^{+/+} and RelB^{+/-} controls (Figures 2A–2C). Notably, both small airway epithelial cell area and collagen deposition were increased in RelB^{+/-} compared with RelB^{+/+} controls (Figures 2B and 2C). There were no differences in MSC numbers between RelB^{+/-} and RelB^{+/+} controls (Figure 2A). RelB^{+/-} mice had increased AHR, characterized by increased transpulmonary resistance in response to increasing doses of methacholine, compared with RelB^{+/+} and RelB^{-/-} mice (Figure 2D).

Adoptive Transfer of RelB-Sufficient DCs to RelB^{-/-} Reduces Inflammation in BALF and Lungs

RelB^{+/+} DCs were previously shown to reduce inflammation in RelB^{-/-} mice (6). We therefore assessed the importance of RelB expression in DCs for the control of spontaneous AAI. Purified lymphatic and splenic RelB-sufficient (RelB^{+/+} or RelB^{+/-}) CD11c⁺ DCs were adoptively transferred intravenously into RelB^{-/-}

Table 1. Characterization of Inflammatory Cells

Cell Type	Cell-Surface Antigens
ILC2s	CD45 ⁺ Lin ⁻ CD90.2 ⁺ CD2 ⁻ IL-7R α ⁺ CD25 ⁺ IL-33R α ⁺
mDCs	CD45 ⁺ CD11c ⁺ CD11b ⁺ F4/80 ⁻
$\gamma\delta$ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁻ CD8 ⁻ $\gamma\delta$ TCR ⁺
CD4 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻ $\gamma\delta$ TCR ⁻
CD8 ⁺ T cells	CD45 ⁺ CD3 ⁺ CD4 ⁻ CD8 ⁺ $\gamma\delta$ TCR ⁻
Regulatory T cells	CD45 ⁺ CD3 ⁺ CD4 ⁺ CD25 ⁺ FoxP3 ⁺
Alveolar macrophages	CD45 ⁺ F4/80 ⁺ CD11c ⁺ CD11b ⁻

Definition of abbreviations: ILC2s = type 2 innate lymphoid cells; mDCs = myeloid dendritic cells.

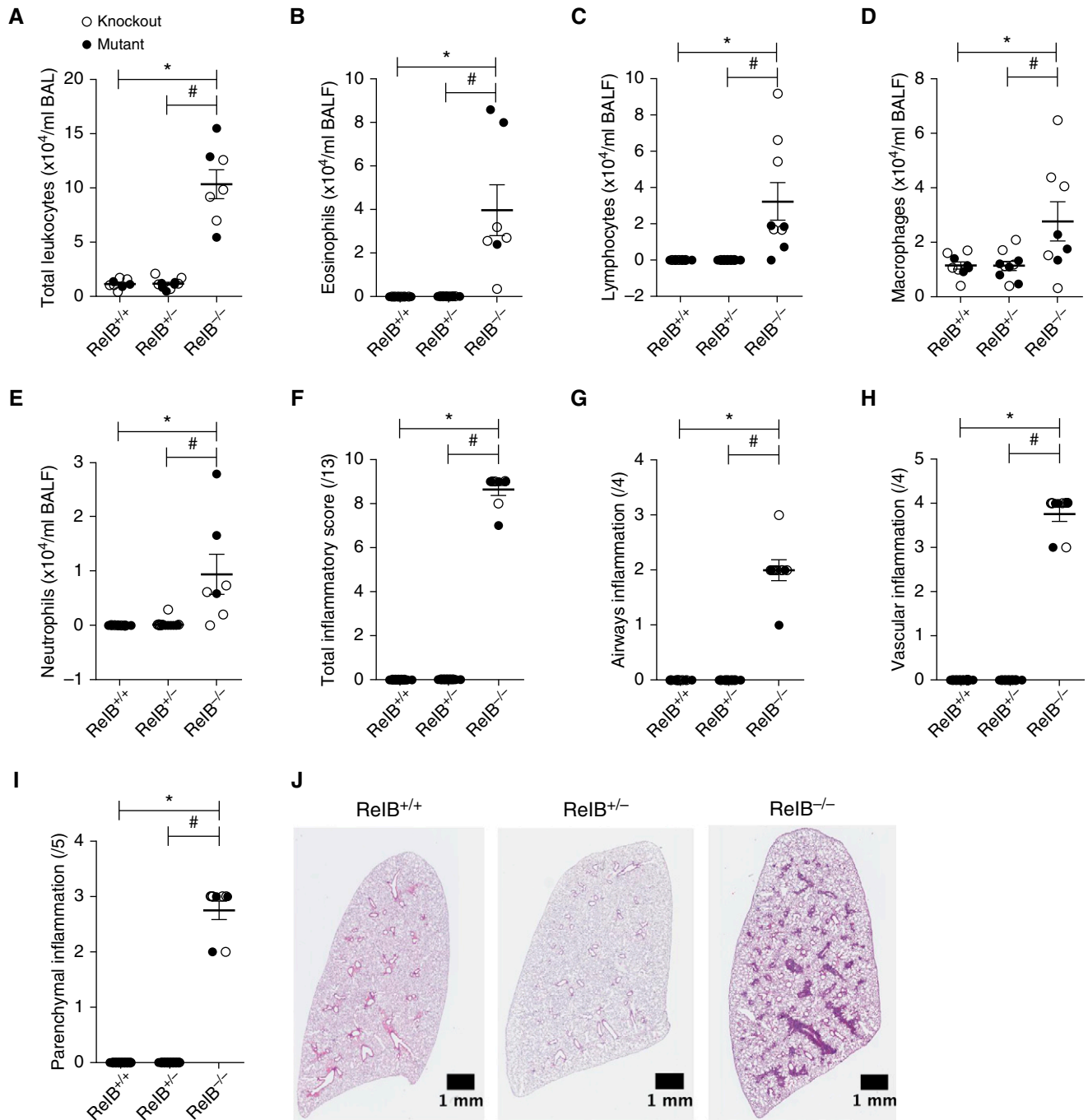


Figure 1. Spontaneous pulmonary inflammation in RelB-deficient mice. Wild-type ($+/+$), heterozygous ($+/-$), and RelB-deficient ($-/-$) knockout and mutant C57BL/6 mice were killed at 6–9 weeks of age and spontaneous pulmonary inflammation was assessed. (A) Total leukocytes, (B) eosinophils, (C) lymphocytes, (D) macrophages, and (E) neutrophils in BAL fluid (BALF). (F) Total, (G) airway, (H) vascular, and (I) parenchymal inflammation scores in hematoxylin and eosin (H&E)-stained lung sections. (J) Representative images of lung sections. Data ($n = 8$ –10) are presented as means $\pm$ SEM from two independent experiments. * $P < 0.05$ compared with RelB $^{+/+}$ mice, # $P < 0.05$ compared with RelB $^{+/-}$ mice. Open symbols represent RelB knockout mice; solid symbols represent RelB mutant mice. Scale bars: 1 mm.

mice. The lungs of untreated and treated mice were analyzed 5 or 14 days after adoptive transfer.

Adoptive transfer of RelB-sufficient DCs significantly reduced total

leukocytes and eosinophils in the BALF of RelB $^{-/-}$ mice, whereas neutrophils were increased (Figures 3A and 3B). Adoptive transfer of RelB-sufficient DCs

had no effect on the number of lymphocytes and macrophages. In the lungs of DC-treated RelB $^{-/-}$ mice, parenchymal (but not total, airway or vascular)

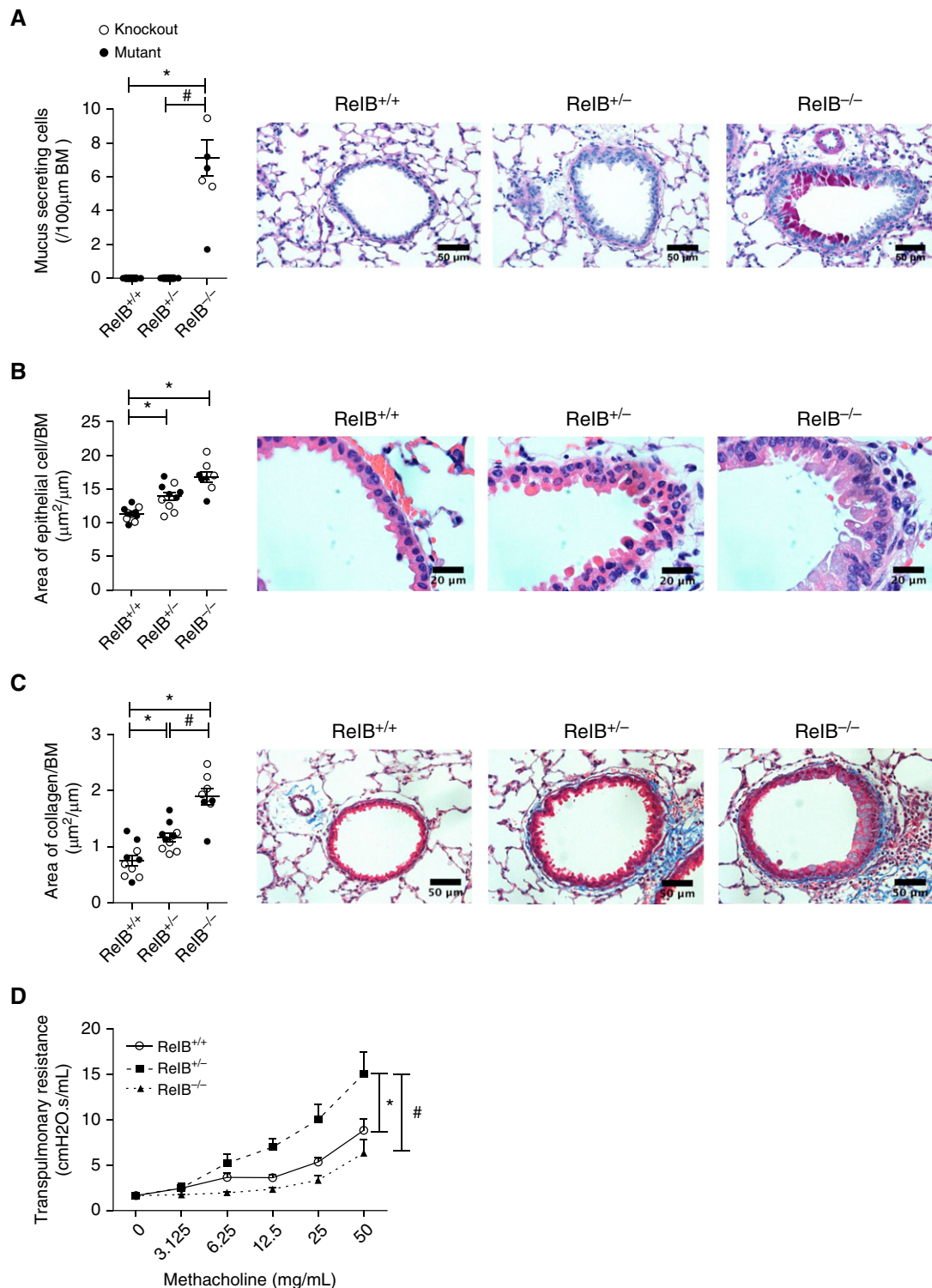


Figure 2. Spontaneous airway remodeling in RelB-deficient mice and increased transpulmonary resistance in RelB heterozygous mice. Wild-type (^{+/+}), heterozygous (^{+/-}), and RelB-deficient (^{-/-}) knockout and mutant C57BL/6 mice were killed at 6–9 weeks of age and airway remodeling was assessed. (A) Number of mucus-secreting cells around the airways in periodic acid Schiff-stained lung sections. (B) Small airway epithelial thickness in terms of epithelial cell area (μm²) per basement membrane (BM) perimeter in H&E-stained lung sections. (C) Area of collagen deposition (μm²) per BM perimeter in Masson's trichrome-stained lung sections. (D) Wild-type (^{+/+}), heterozygous (^{+/-}), and RelB-deficient (^{-/-}) mutant C57BL/6 mice were killed at 6–9 weeks of age and transpulmonary resistance was assessed. Data (n = 8–10) are presented as means ± SEM from two independent experiments. *P < 0.05 compared with RelB^{+/+} mice, #P < 0.05 compared with RelB^{+/-} mice. Open symbols represent RelB knockout mice; solid symbols represent RelB mutant mice. Scale bars: 50 μm (A and C) and 20 μm (B).

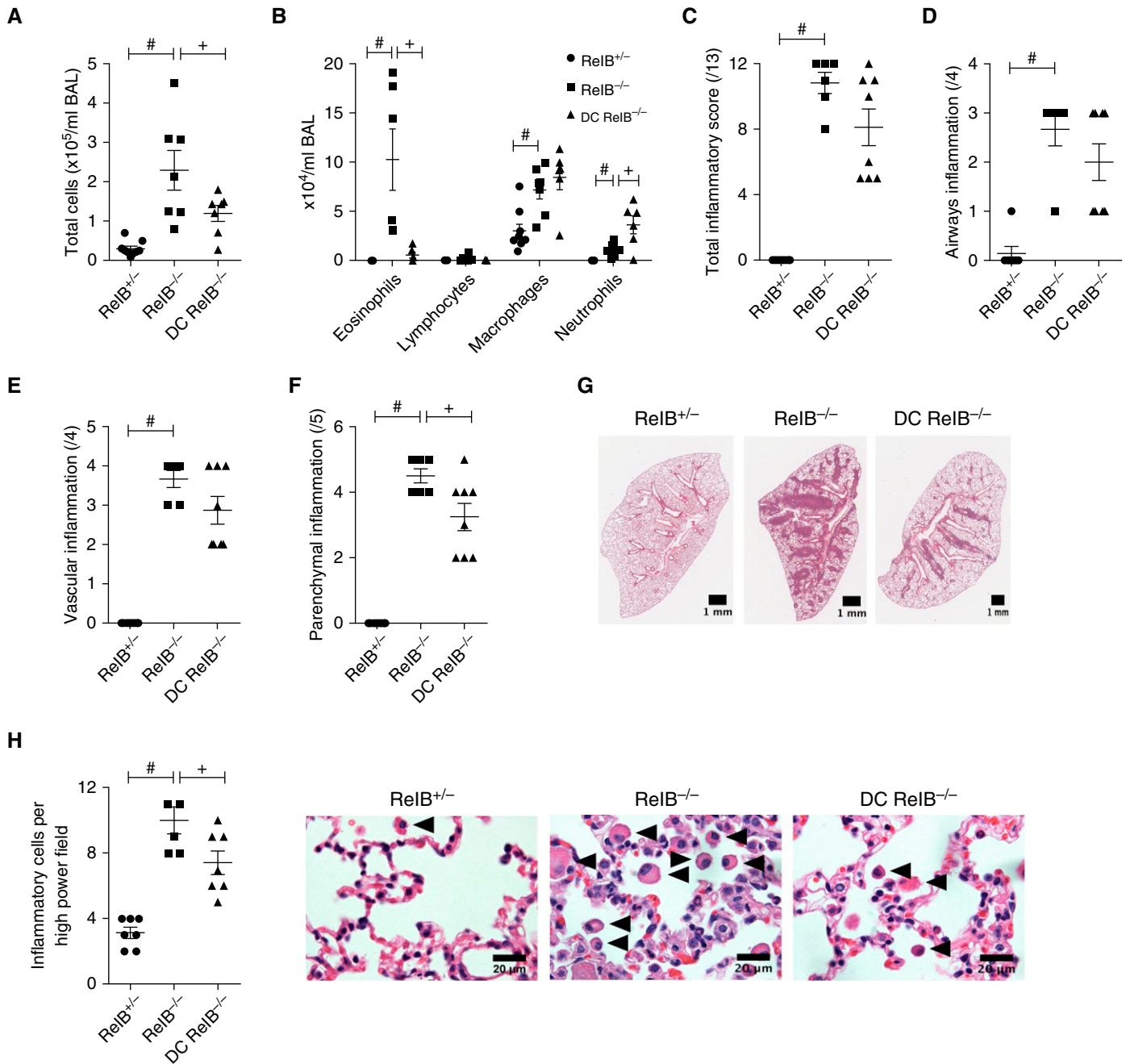


Figure 3. Adoptive transfer of RelB-sufficient dendritic cell (DCs) to RelB-deficient mice decreased pulmonary inflammation. Purified lymphatic and splenic CD11c⁺ DCs from RelB heterozygous (^{+/+}) mice were adoptively transferred intravenously into RelB-deficient (^{-/-}) mutant C57BL/6 mice and pulmonary inflammation was assessed 5 days after DC transfer. (A) Total leukocytes and (B) eosinophils, lymphocytes, macrophages, and neutrophils in BALF. (C) Total, (D) airway, (E) vascular, and (F) parenchymal inflammation scores in H&E-stained lung sections. (G) Representative images of lung sections. (H) The numbers of parenchymal inflammatory cells (arrowhead) were determined in H&E-stained lung sections. Data ($n = 6-9$) are presented as means $\pm$ SEM from three independent experiments. $^{\#}P < 0.05$ compared with RelB^{+/+} mice, $^{+}P < 0.05$ compared with RelB^{-/-} mice. Solid symbols represent RelB mutant mice. Scale bars: 1 mm (panel G) and 20 μm (panel H).

inflammatory scores were decreased (Figures 3C–3G), and there were fewer inflammatory cells per high-powered field in the parenchyma compared with untreated RelB^{-/-} mice (Figure 3H).

Adoptive Transfer of RelB-Sufficient DCs to RelB^{-/-} Mice Reduced the Numbers of ILC2s, $\gamma\delta$ T Cells, and mDCs in the Lung

We further explored the inflammatory leukocytes that infiltrated the lung in the

absence of RelB and their regulation by adoptive transfer of RelB-sufficient DCs. Consistent with the increases in the major inflammatory cell subtypes, RelB^{-/-} mice also had increased numbers of ILC2s, $\gamma\delta$ T cells, mDCs, CD4⁺ and

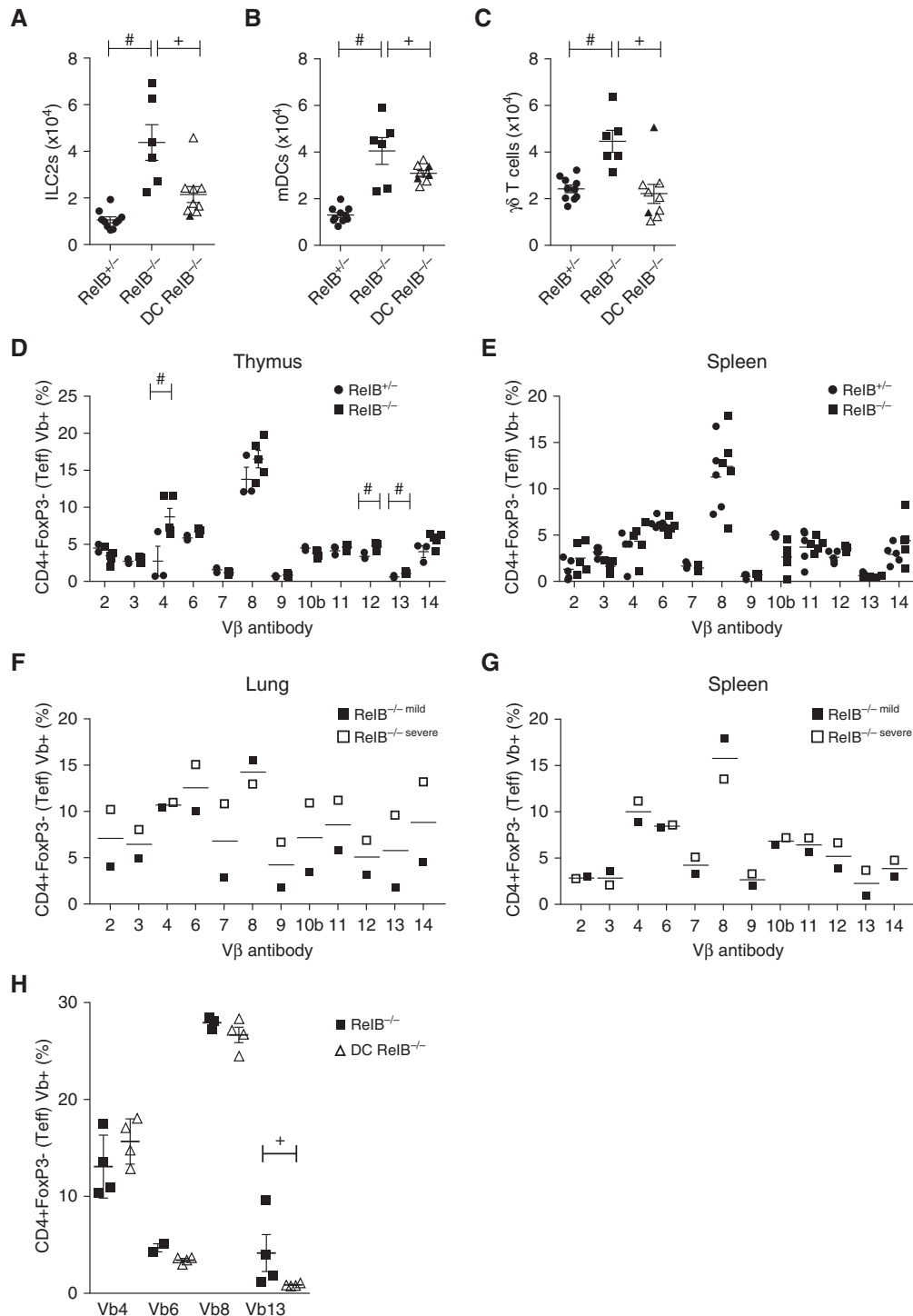


Figure 4. Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice decreased the numbers of type 2 innate lymphoid cells (ILC2s), myeloid DCs (mDCs), and $\gamma\delta$ T cells, and the proportion of V β 13⁺ CD4⁺FoxP3⁻ T cells in the lungs. Purified lymphatic and splenic CD11c⁺ DCs from RelB heterozygous (^{+/+}) or RelB wild-type (^{+/+}) mice were adoptively transferred intravenously into RelB-deficient (^{-/-}) mutant and knockout C57BL/6 mice, respectively, and the number of inflammatory cells in the lung was assessed by flow cytometry 14 days after DC transfer. (A) ILC2s were gated as CD45⁺ Lin⁻ CD90.2⁺ CD2⁻ IL7R α ⁺ CD25⁺ IL33R α ⁺, (B) mDCs were gated as CD45⁺ CD11c⁺ CD11b⁺ F4/80⁻, and (C) $\gamma\delta$ T cells were gated as CD45⁺ CD3⁺ CD4⁻ CD8⁻ $\gamma\delta$ TCR⁺. (D) Proportion of V β single-positive CD4⁺FoxP3⁻ T cells in the thymus, and (E) spleen of RelB^{+/+} and RelB^{+/-} mice. (F) Proportion of V β single-positive CD4⁺FoxP3⁻ T cells in the lungs and (G) spleen of RelB^{-/-} mice with moderate and severe disease. (H) Proportion of V β single-positive CD4⁺FoxP3⁻ T cells in the lungs of untreated RelB^{-/-} mice and RelB^{-/-} mice treated with RelB^{+/+} DCs. Data ($n = 2-8$) are presented as means $\pm$ SEM from two independent experiments. # $P < 0.05$ compared with RelB^{+/+} mice, + $P < 0.05$ compared with RelB^{-/-} mice. Open symbols represent RelB knockout mice; solid symbols represent RelB mutant mice.

CD8⁺ T cells, and CD4⁺CD25⁺FoxP3⁺ Tregs in the lung (Figures 4A–4C, E3, and E5). All but the CD4⁺ T cells and Treg subsets were reduced by adoptive transfer of RelB-sufficient DCs. Although we also observed a reduction in alveolar macrophages in RelB^{-/-} mice, adoptive transfer of RelB-sufficient DCs did not alter the number of these cells (Figure E3D).

Adoptive Transfer of RelB-Sufficient DCs to RelB^{-/-} Mice Reduced Lung Vβ13⁺ T Cells

We next assessed the identity of potentially expanded CD4⁺ effector T (Teff) cell clonotypes in the lungs of mice with moderate and severe disease. For this purpose, we determined Vβ expression of CD4⁺Foxp3⁻ T cells from the thymus, spleen, and lungs of RelB^{+/-} mice and RelB^{-/-} mice by flow cytometry (Figures 4D–4F). In the thymus, but not the spleen, of RelB^{-/-} mice, Vβ4, Vβ12, and Vβ13⁺ CD4⁺Foxp3⁻ T cells were expanded relative to RelB^{+/-} mice (Figures 4D and 4E). CD4⁺Foxp3⁻ T cells of multiple specificities infiltrated the lungs of untreated RelB^{-/-} mice in different proportions compared with their distribution in spleens (Figures 4F and 4G). As expected, few CD4⁺ T cells infiltrated the lungs of RelB^{+/-} mice (Figure E3A). RelB^{+/-} DC treatment of RelB^{-/-} mice specifically reduced the proportion of Vβ13⁺ CD4⁺ Foxp3⁻ T cells in the lungs (Figure 4H).

Adoptive Transfer of RelB-Sufficient DCs to RelB^{-/-} Mice Reduced Chemokine and Th2-Associated Cytokine Levels in the Lung

The reduction in pulmonary inflammatory cells after adoptive transfer of RelB-sufficient DCs suggested that inflammatory chemokines and cytokines might be altered. Adoptive transfer of RelB-sufficient DCs significantly reduced the levels of CCL2, CCL3, CCL4, CCL5, CCL11, and CCL17 (Figures 5A–5F), and CXCL9, CXCL10, and CXCL13 (Figures 5G–5I) in the lungs compared with untreated RelB^{-/-} controls. We again found that IL-4 and IL-5 were increased in the lungs of RelB^{-/-} mice (Figures 6A and 6B). We furthered this observation by detecting elevated IL-13, IL-25, IL-33, and TSLP levels (Figures 6C and 6E–6G). Adoptive transfer of RelB-sufficient DCs to RelB^{-/-} mice also

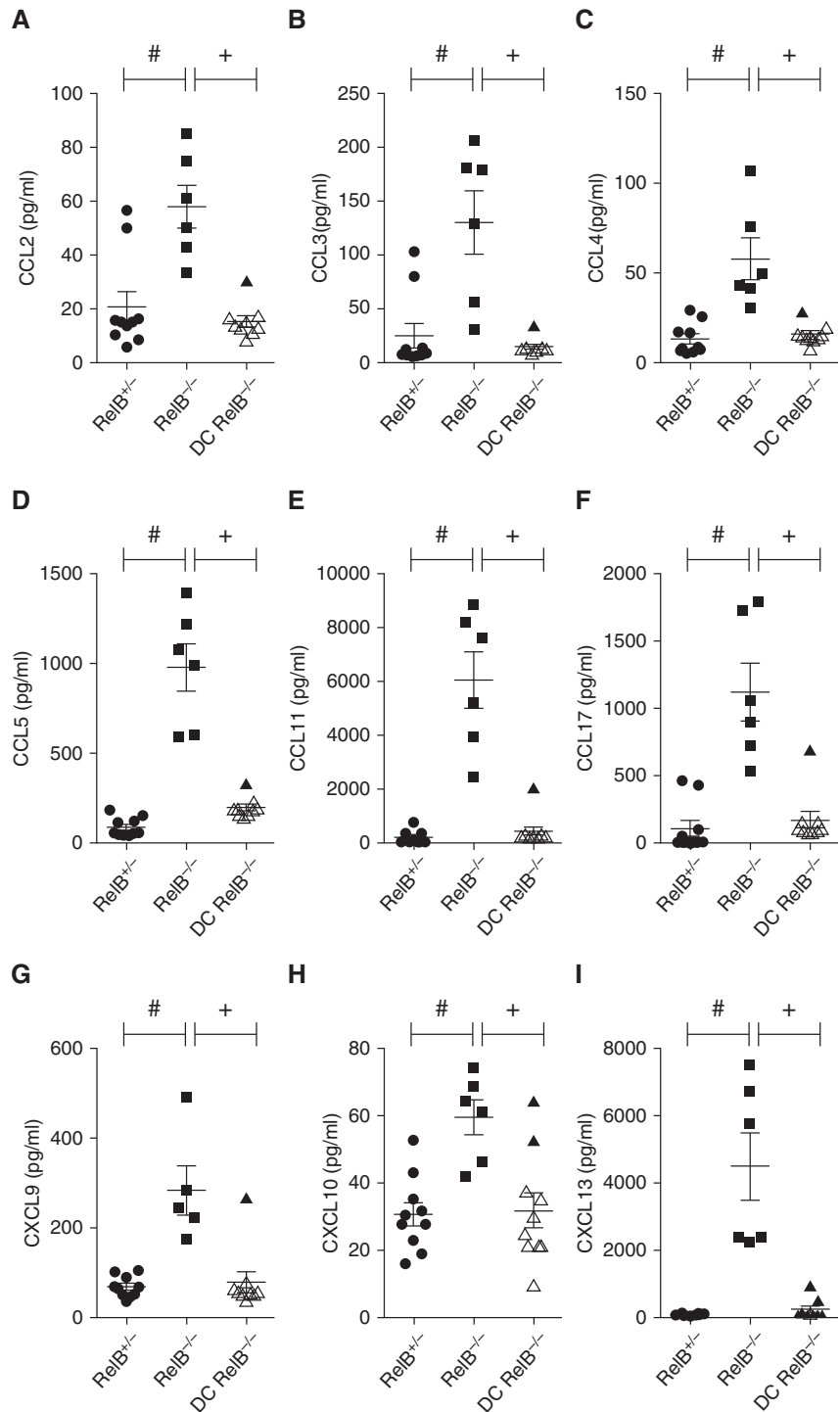


Figure 5. Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice decreased chemokine levels in the lung. Purified lymphatic and splenic CD11c⁺ DCs from RelB heterozygous (^{+/-}) or RelB wild-type (^{+/+}) mice were adoptively transferred intravenously into RelB-deficient (^{-/-}) mutant and knockout C57BL/6 mice, respectively. Protein was extracted from lung homogenates and chemokine levels were assessed by multiplex assay 14 days after DC transfer. (A) CCL2, (B) CCL3, (C) CCL4, (D) CCL5, (E) CCL11, (F) CCL17, (G) CXCL9, (H) CXCL10, and (I) CXCL13 protein levels in lung homogenates. Data ($n = 2-8$) are presented as means $\pm$ SEM from two independent experiments; # $P < 0.05$ compared with RelB^{+/+} mice, + $P < 0.05$ compared with RelB^{+/-} mice. Open symbols represent RelB knockout mice; solid symbols represent RelB mutant mice.

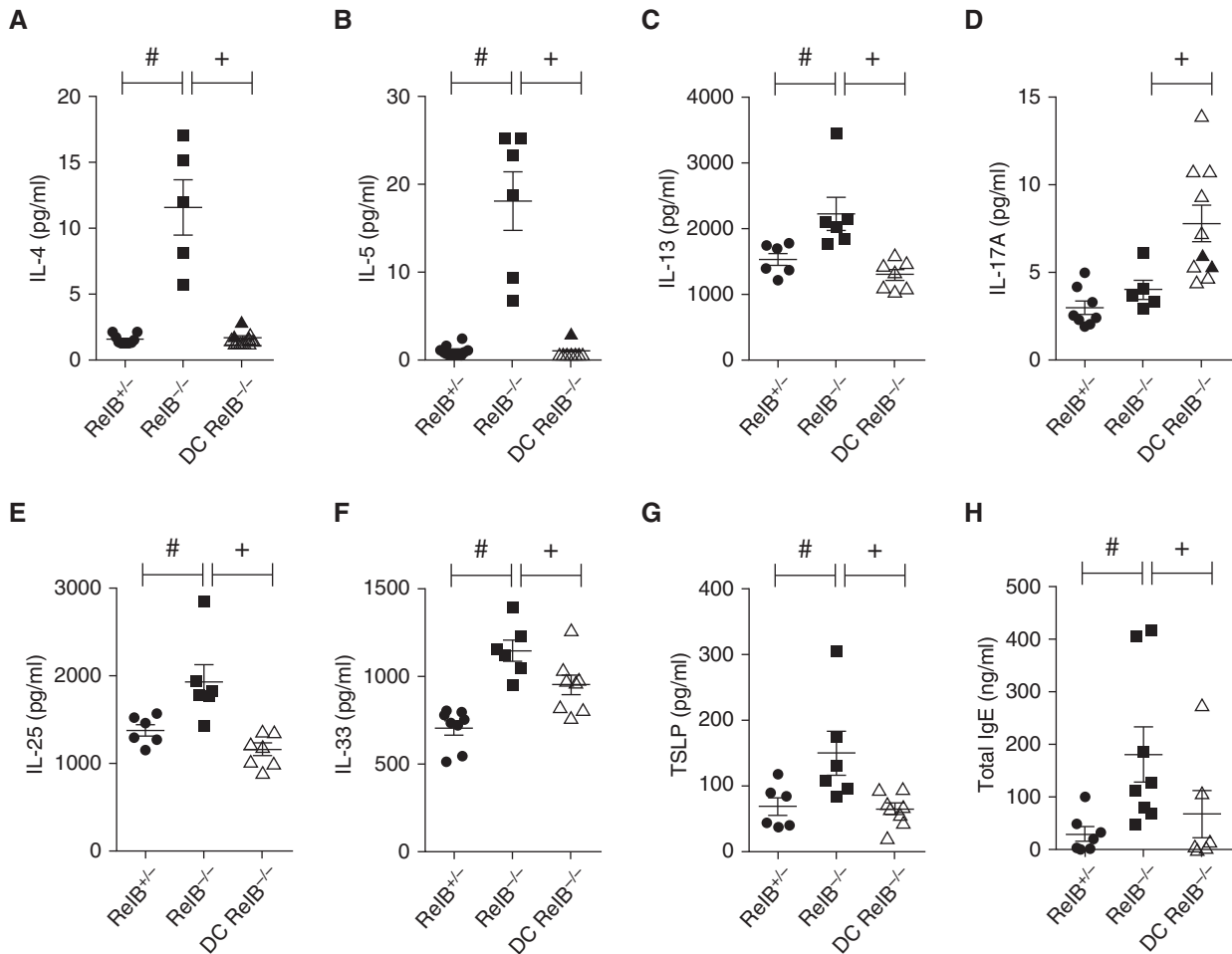


Figure 6. Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice decreased the levels of T-helper cell type 2-associated cytokines in the lung. Purified lymphatic and splenic CD11c⁺ DCs from RelB heterozygous (^{+/+}) or RelB wild-type (^{+/+}) mice were adoptively transferred intravenously into RelB-deficient (^{-/-}) mutant and knockout C57BL/6 mice, respectively. Protein was extracted from lung homogenates, and cytokine levels were assessed by multiplex assay or ELISA 14 days after DC transfer. Total IgE in serum was determined by ELISA 14 days after DC transfer. (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-17A, (E) IL-25, (F) IL-33, and (G) thymic stromal lymphopoietin (TSLP) in lung homogenates. (H) Total IgE in serum. Data ($n = 2-8$) are presented as means $\pm$ SEM from two independent experiments. # $P < 0.05$ compared with RelB^{+/+} mice, + $P < 0.05$ compared with RelB^{-/-} mice. Open symbols represent RelB knockout mice; solid symbols represent RelB mutant mice.

significantly reduced the levels of all of these cytokines (Figures 6A–6C and 6E–6G). Total serum IgE was also suppressed compared with that in untreated RelB^{-/-} controls (Figure 6H). In contrast to these Th2-associated cytokines, the levels of IL-17A were increased in the lung in response to adoptive transfer of RelB-sufficient DCs to RelB^{-/-} mice (Figure 6D). Levels of IFN- γ were reduced, but there was no difference in IL-10 levels (Figures E4A and E4B).

Adoptive Transfer of RelB-Sufficient DCs to RelB^{-/-} Mice Decreased Airway Remodeling

We then assessed the effect of DC transfer on spontaneous airway remodeling.

Adoptive transfer of RelB-sufficient DCs significantly reduced MSCs, small airway epithelial cell area, and collagen deposition around the small airways compared with RelB^{-/-} controls (Figures 7A–7C).

Discussion

In this study, we showed that RelB^{-/-} mice developed spontaneous AAI that was independent of exogenous allergen exposure. The mice developed spontaneous pulmonary inflammation characterized by increases in eosinophils, lymphocytes, macrophages, and neutrophils in BALF, and lung histopathology typical of AAI with airway remodeling. These events were

associated with increases in typical chemokines and Th2 cytokines in the lung. Transfer of RelB-sufficient DCs in the absence of exogenous allergen exposure to RelB^{-/-} mice suppressed AAI, chemokines, Th2 cytokines, and airway remodeling. Our data indicate that RelB deficiency in parenchymal and hemopoietic cells predisposes to AAI. Furthermore, clonally expanded V β 13⁺ CD4⁺ Teff cells may contribute to the development of AAI in RelB^{-/-} mice. Immunotherapy with DCs encoding RelB suppresses these Teff cells even in the absence of deliberate exogenous allergen exposure. Interestingly, the increased number of V β 13⁺ CD4⁺ Foxp3⁻ T cells in the RelB^{-/-} thymus suggests that autoreactive T cells may contribute to the

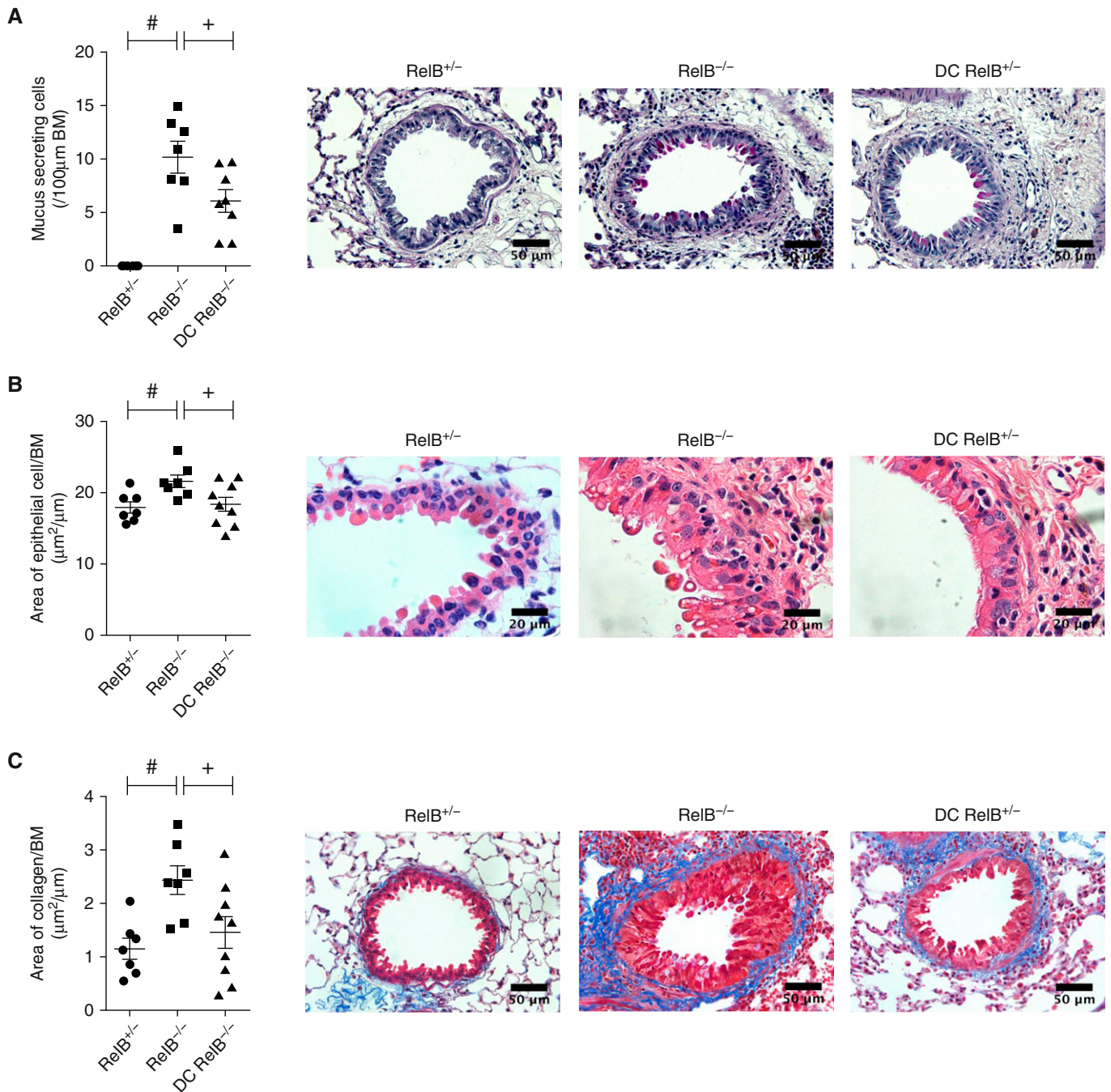


Figure 7. Adoptive transfer of RelB-sufficient DCs to RelB-deficient mice decreased airway remodeling. Purified lymphatic and splenic CD11c⁺ DCs from RelB heterozygous (^{+/-}) mice were adoptively transferred intravenously into RelB-deficient (^{-/-}) mutant C57BL/6 mice, and airway remodeling was assessed 5 days after DC transfer. (A) Number of mucus-secreting cells around the airways in periodic acid Schiff-stained lung sections. (B) Small airway epithelial thickness in terms of epithelial cell area (μm²) per BM perimeter in H&E-stained lung sections. (C) Area of collagen deposition (μm²) per BM perimeter in Masson's trichrome-stained lung sections. Data (*n* = 6–9) are presented as means ± SEM from three independent experiments. #*P* < 0.05 compared with RelB^{+/-} mice, +*P* < 0.05 compared with RelB^{-/-} mice. Solid symbols represent RelB mutant mice. Scale bars: 50 μm (A and C) and 20 μm (B).

development of AAI in a RelB-deficient setting. DCs encoding RelB also control features of lung inflammation, the Th2 inflammatory response, and airway remodeling. Together, these data may have

important implications for immunotherapy of allergic asthma.

In our study, we used two different strains of RelB^{-/-} mice: a targeted total knockout of the *RelB* gene (16) and a

mutant that was generated by chance integration of a class I MHC genomic clone as a transgene (8). Both strains were able to receive and respond to RelB-sufficient DCs from RelB^{+/-} littermates.

Other studies have shown that RelB^{-/-} mice have multiorgan inflammation, particularly in the lung and liver (16, 19). In the lungs of 10-day-old RelB^{-/-} mice, inflammation was mainly focused around the blood vessels, and by day 20 the perivascular infiltrate had extended into the parenchyma, leading to focal consolidation. Our analysis of adult RelB^{-/-} mice demonstrated similar inflammation around the airways and blood vessels, and in the lung parenchyma, as well as infiltration into the airway lumen.

Consistent with the conclusion that RelB deficiency leads to typical AAI, we observed that chemokines associated with allergic inflammation (CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CXCL9, CXCL10, and CXCL13) were increased in the lungs of RelB^{-/-} mice. The levels of CCL2, CCL3, CCL5, and CCL11 have also been shown to be elevated in the BALF of patients with allergic asthma (40–42). CCL2 enhances the secretion of IL-4, an important Th2 cytokine in asthma, by T cells (43, 44). CCL3, CCL5, and CCL11 are potent eosinophil chemoattractants (45–47). Although it was not specifically shown in asthmatic patients, CCL4 was increased in nasal secretions of patients with allergic rhinitis (48). CCL17 promotes the development of eosinophilia through the recruitment of Th2-type CD4⁺ T lymphocytes (49). The increase in these chemokines is consistent with the increase in eosinophils observed in the BALF of RelB^{-/-} mice. Increased levels of CXCL9 and CXCL10 have been observed in the plasma of asthmatic children with acute exacerbations, and elevated levels of CXCL13 have also been observed in BALF from patients with asthma and a murine model of ovalbumin-induced allergic inflammation (50, 51). RelB^{-/-} parenchymal cells are particularly prone to chemokine production due to the lack of transcriptional repression imposed by RelB. Upon stimulation by lipopolysaccharide, RelB^{-/-} kidney fibroblasts were found to produce high levels of chemokines (CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, and CXCL10) *in vitro*, and this was reduced by transfection with RelB cDNA (17). In support of a key role for RelB-deficient parenchyma in driving the development of AAI, transfer of RelB^{-/-} bone marrow into lethally irradiated WT controls does not induce AAI (6), even though stimulated DCs from these mice produce low levels of

IL-12p70 *in vitro* (52). However, it is important to note that RelB^{-/-} bone marrow chimeras also have no autoreactivity (6), which may remove the drive for proinflammatory chemokine production due to autoreactive effector T cell expansion in the lung.

The type of T cell-dependent immune response is determined by the production of specific cytokines. In addition to increased chemokines, we observed elevated levels of the Th2-associated cytokines IL-4, IL-5, and IL-13 in the lungs of RelB^{-/-} mice, but not the Th1-associated cytokine IFN- γ or the Th17-associated cytokine IL-17A. IL-4 plays a pivotal role in the differentiation of naive T cells into Th2 cells (53). It has been suggested that IL-5 plays a role in the etiology of AAI by regulating the growth, differentiation, and activation of eosinophils (54). Increased expression of IL-4 and IL-5 was associated with the accumulation of eosinophils in the airway lumen of RelB^{-/-} mice. These two cytokines promote Ig isotype switching to IgE (55, 56). The cross-linking of Fc ϵ R1 on eosinophils and mast cells stimulates degranulation with the release of histamine and inflammatory mediators that together elicit AAI. Our data suggest that typical type 2 responses and their inflammatory consequences dominate in the lungs of RelB^{-/-} mice.

Flow-cytometric analysis of lung homogenates from RelB^{-/-} mice identified increased numbers of ILC2s, mDCs, and $\gamma\delta$ T cells in the lung. ILC2s have recently emerged as a key player in type 2 immunity in the lungs of both mice and humans (57, 58). In allergy, mDCs and $\gamma\delta$ T cells skew T cell responses toward a Th2-mediated immune response (59). Consistent with the increase in ILC2s, the cytokines IL-25, IL-33, and TSLP, which activate and expand ILC2s to produce IL-4 and IL-13, were also increased in RelB^{-/-} lungs. IL-25 is secreted by eosinophils and epithelial cells (60), and promotes AHR, eosinophilia, and mucus hypersecretion in mice (61). IL-33 is released by the airway epithelium and stimulates the production of Th2 cytokines and serum IgE and IgA (62). TSLP is also secreted by the airway epithelium and plays a role in the initiation of allergic inflammation through the induction of OX40 ligand expression by DCs and subsequent differentiation of T cells into Th2 cells (63, 64). TSLP also

induces the secretion of CCL17 by mDCs, promoting the recruitment of Th2 cells to the airway (63). These epithelium-derived cytokines activate and expand ILC2s to increase production of IL-4, IL-5, and IL-13, which in turn promotes eosinophil recruitment and mucus hypersecretion. Determination of V β expression of CD4⁺FoxP3⁻ T cells infiltrating the lungs of RelB^{-/-} mice by flow cytometry identified CD4⁺FoxP3⁻ T cells of multiple specificities and in different proportions compared with thymic and splenic CD4⁺FoxP3⁻ T cells. This suggests that clonally expanded antigen-specific T cells may contribute to the development of AAI in RelB^{-/-} mice. Furthermore, increases in V β 13⁺ CD4⁺FoxP3⁻ T cells in the thymus of RelB^{-/-} mice suggest that autoreactive T cells may drive AAI, at least in part, in the RelB-deficient setting.

To rule out the possibility that an opportunistic infection in RelB^{-/-} mice drove the differences in phenotype, we tested for the presence of a range of different bacteria (*Helicobacter* spp.), endoparasites (*Aspicularis tetraptera* and *Syphacia obvelata*), and viruses (minute virus of mice, hepatitis, norovirus, parvovirus, rotavirus, and Theiler's encephalomyelitis virus) as part of normal monitoring in our mouse vivarium facilities. We found no evidence of these bacteria or viruses in RelB^{-/-} mice. We also performed a 16S analysis of fecal microbiomes and observed no significant difference in operational taxonomic units between RelB^{+/-} and RelB^{-/-} mice (data not shown). We attempted a 16S analysis of the BALF, but obtained no conclusive data.

RelB is highly expressed in DCs and is crucial for their maturation and DC-mediated immunity (4, 65). DCs play a critical role in priming T cells and controlling subsequent immune responses. RelB^{-/-} mice have defects in DC, but not Langerhans cell, development (66). In the current studies, adoptive transfer of RelB-sufficient DCs reduced pulmonary inflammation, chemokines, Th2 cytokines, and small airway remodeling.

Previously, we showed that transfer of RelB-sufficient DCs achieved long-term suppression of autoimmune disease and suppressed organ (liver, spleen, and pancreas) inflammation in RelB^{-/-} mice in an IDO-dependent fashion (6). IDO activity in RelB-sufficient DC-treated mice

was CD80/CD86 dependent, and promoted IFN- γ and suppressed IL-4 and IL-5 production by T cells (6). In our previous study we examined tissue inflammation, but not specifically a tissue with ongoing Th2 responses. Here, we showed that transfer of RelB-sufficient DCs suppressed the development of disease in AAD. Transfer of RelB-sufficient DCs to RelB^{-/-} mice significantly reduced the numbers of CD8⁺ T cells, V β 13⁺ CD4⁺ Teff cells, ILC2s, mDCs, and $\gamma\delta$ T cells; the Th2-associated cytokines IL-4, IL-5, IL-13, IL-25, IL-33, and TSLP; inflammatory chemokines; and serum IgE. We speculate that the specific reduction in V β 13⁺ CD4⁺ T cells in the lung may have resulted from the uptake of antigen by the transferred RelB^{+/+} DCs and presentation to CD4⁺ T cells. However, the generalized reduction in lung CD8⁺ and $\gamma\delta$ T cells likely resulted from reduced recruitment due to changes in cytokine and chemokine production. Our data also suggest that immunotherapy with DCs expressing RelB suppresses antigen-specific T cells even without deliberate exogenous exposure, although the underlying pathogenic antigen is unknown. We previously showed that RelB or CD80/86-sufficient DCs improved disease features in RelB^{-/-} mice. We also previously showed decreased levels of IL-4 and IL-5 in the serum of DC-treated mice (6). IL-25 and IL-33 also promote airway remodeling in allergic diseases (67, 68). The reduction in the levels of these cytokines in mice receiving DC treatment was associated with the suppression of airway remodeling with reductions in MSCs, collagen deposition, and small airway epithelial cell area.

Chronic airway inflammation promotes the induction of airway remodeling. The increases in MSCs within the airways, collagen deposition around the small airways, and airway epithelial cell area in RelB^{-/-} mice are consistent with previous observations (19). Surprisingly, despite the lack of inflammation in RelB^{+/+} mice, we observed an increase in small airway epithelial cell area and collagen deposition compared with RelB^{+/+} mice, although the change was smaller than that observed in RelB^{-/-} mice. We also found no significant difference between RelB^{+/+} and RelB^{-/-} mice in α smooth muscle actin area or density, as assessed by immunofluorescence staining of lung tissue

sections (data not shown). These data suggest that even heterozygous RelB deficiency may predispose to mild-to-moderate airway epithelial changes. In support of this observation, RelB^{+/-} mice have increased transpulmonary resistance in response to increasing doses of methacholine compared with RelB^{+/+} and RelB^{-/-} mice. We speculate that the epithelial alterations in RelB^{+/-} mice may improve the permeability for methacholine, thus increasing the response, and the massive inflammation and remodeling around the airways of RelB^{-/-} mice may reduce methacholine permeability. These changes differ from those observed in allergen-induced AAI models and are consistent with chronic exposure to lung-reactive CD4⁺ T cells, with consequent inflammatory effects in the airways of RelB^{-/-} mice from birth. We attempted to determine whether the allergic-like phenotype observed in RelB^{-/-} mice results from hypersensitivity to an allergen, such as house dust mite. However, RelB^{-/-} DCs pulsed with house dust mite failed to induce RelB^{-/-} T cell proliferation, suggesting that lung reactivity is self-antigen driven due to impaired negative selection and a RelB-deficient stromal environment, leading to a Th2 inflammatory response. The nature of the self-antigen is unknown, but most likely it is a lung self-antigen linked to thymic RelB deficiency, such as BPIFB1, an autoantigen associated with interstitial lung disease (69).

Recently, three patients with immune deficiency and inflammatory disease were identified in a consanguineous family with a homozygous C1191A mutation in the RelB gene that prevented RelB protein production (70). Similar to our observations in mice, T cells were skewed to effector memory cells. IL-2 and IFN- γ production was markedly reduced *in vitro*, and the TNF-induced p65 response was increased in patient fibroblasts. B cell maturation was defective, and T cell-dependent and -independent antibody responses were low to absent (71). Consistent with this immunodeficiency, each of these patients had persistent upper and lower respiratory tract infections with associated chronic cough and pneumonia. One patient had reactive airway disease from infancy, and the mother of two of the patients had psoriasis and eczema. Thus, homozygous

RelB deficiency in mice and humans is associated with severe immunodeficiency and inflammatory disease, the severity of which is exacerbated by pathogen exposure (20, 72). Heterozygous RelB deficiency may predispose to inflammatory autoimmune or atopic disease within the context of other genes and microbial exposure.

With respect to other contributory genes, a recent genome-wide association study and meta-analysis in 21,000 cases and 95,000 controls of multiple ancestry identified 10 new risk loci associated with atopic dermatitis, a disease clinically linked with asthma (21). Interestingly, some loci are associated with several key cells and factors that are altered in RelB^{-/-} mice, including Th2 and Treg cells (*IL13*, *IL2RA*, *IL7R*, and *IL2*), proinflammatory cytokine signaling (*IL6R*, *IL18R*, *STAT3*, *STAT6*, *IL22*, and *SOC3*), and antigen presentation/Langerhans cells (*HLA-DRB1* and *CD207*). Two loci (*TRAF3* and *PUS10*) highlight the potential role of the NF- κ B pathway. In myeloid cells, TRAF3 regulates the NF- κ B2 pathway and thus p52 (73), which partners with RelB in transcriptional regulation (74). The *PUS10* locus lies very close to *REL*, encoding the c-Rel subunit of NF- κ B. In Rel^{-/-} DCs, RelB transcription and DNA binding activity were shown to be severely depressed (1). In another study, using a systems biology approach, *NFKB1A* promoter polymorphisms were found to be associated with susceptibility to childhood inflammatory lung diseases (75). *NFKB1A* encodes for the I κ B α inhibitor of classical NF- κ B activity, and RelB regulates its stability in fibroblasts by mediating the phosphorylation steps involved in I κ B α degradation (76, 77). Together, these clinical studies, in the context of the current work and previous studies in RelB^{-/-} mice, strongly suggest that RelB deficiency is a key pathway underlying AAI, and that further translational insights will emerge from functional studies of RelB and its regulation in human inflammatory airway diseases and atopic eczema. Furthermore, DC-encoded RelB is sufficient to restore control of AAI through immune regulation of inflammation, which has important therapeutic applications. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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**RelB-deficient dendritic cells promote the development of spontaneous allergic
airway inflammation**

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ONLINE DATA SUPPLEMENT

Supplemental materials and methods

RelB-deficient dendritic cells promote the development of spontaneous allergic airway inflammation

Histopathology

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E (for histopathology) and periodic acid-Schiff (PAS-for mucus secreting cells). Histopathology was scored according to a set of custom-designed criteria and MSCs were enumerated in inflamed airways as previously described (E1).

Isolation of protein

Protein was extracted from whole lung. Briefly, tissues were homogenized in 500 µl of sterile Dulbecco's phosphate-buffered saline (Life Technologies, Mulgrave, Victoria, Australia) supplemented with PhoSTOP phosphatase and Complete ULTRA protease inhibitors cocktails (Roche Diagnostics, Mannheim, Germany) using a Tissue Tearor stick homogenizer (BioSpec Products, Bartlesville, Oklahoma, USA) on ice. Homogenates were then centrifuged at 8000xg for 10 minutes at 4 °C (E2). Supernatants were collected and stored at -20 °C for assessment by ELISA or multi-analyte flow assay kit.

Chemokine and cytokine concentration in lung

Protein concentration in lung homogenate were determined for CCL2, CCL3, CCL4, CCL5, CCL11 and CCL17, CXCL9, CXCL10, CXCL13, IL-4, IL-5 and IL-10 using a LEGENDplex multi-analyte flow assay kit (Biolegend, San Diego, California, USA) according to manufacturer's instructions. IL-13, IL-17A, IL-25, IL-33, TSLP and IFN-γ were

determined using mouse DuoSet ELISA kits (R&D systems, Minneapolis, USA) according to manufacturer's instructions.

Serum antibodies

Total IgE levels in serum was determined by ELISA (E3).

Airway remodeling

Lungs were formalin fixed, embedded and sectioned. Longitudinal sections were stained with H&E or Masson's Trichrome. Airway epithelial area (μm^2) and area of collagen deposition (μm^2) was assessed in a minimum of four small airways (basement membrane perimeter $<1000 \mu\text{m}$) per section (E2, E4-6). Data were normalized to basement membrane perimeter (μm) and quantified using ImageJ software (Version 1.49h, NIH, New York City, USA).

Lung function

Assessment of transpulmonary resistance was performed by whole body invasive plethysmography (Buxco electronics, Sharon, Connecticut, USA) as previously described (E3, E7).

Supplemental results

Figure E1. Increased chemokine levels in the lung of RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age. Protein was extracted from lung homogenates and chemokine levels assessed by multiplex assay. (A) CCL2, (B) CCL3, (C) CCL4, (D) CCL5, (E) CCL11, (F) CCL17, (G) CXCL9, (H) CXCL10 and (I) CXCL13 levels in lung homogenates. Data (n=8-10) presented as means $\pm$ s.e.m from two independent experiments * represents $P < 0.05$ compared to RelB $^{+/+}$ mice, # represents $P < 0.05$, compared to RelB $^{+/-}$ mice. Open symbols represent RelB knockout mice, closed symbols represent RelB mutant mice.

Figure E2. Increased IL-4, IL-5 and IgE levels in RelB-deficient mice. Wild-type ($^{+/+}$), heterozygous ($^{+/-}$) and RelB-deficient ($^{-/-}$) knockout and mutant C57BL/6 mice were sacrificed at 6-9 weeks of age. Protein was extracted from lung homogenates and cytokine levels assessed by multiplex assay. Total IgE in serum was determined by ELISA. (A) IL-4, (B) IL-5, (C) IFN- γ , and (D) IL-17A in lung homogenates. (E) Total IgE in serum. Data (n=8-10) presented as means $\pm$ s.e.m from two independent experiments * represents $P < 0.05$ compared to RelB $^{+/+}$ mice, # represents $P < 0.05$, compared to RelB $^{+/-}$ mice. Open symbols represent RelB knockout mice, closed symbols represent RelB mutant mice.

Figure E3. Number of CD4 $^{+}$, CD8 $^{+}$ T cells, regulatory T cells and alveolar macrophages in the lung. Purified lymphatic and splenic CD11c $^{+}$ DC from RelB heterozygous ($^{+/-}$) or RelB wild-type ($^{+/+}$) were adoptively transferred intravenously into RelB-deficient ($^{-/-}$) mutant and knockout C57BL/6 respectively and numbers of inflammatory cells in the lung were assessed by flow cytometry 14 days post DC transfer. (A) CD4 $^{+}$ T cells were gated as CD45 $^{+}$ CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$ $\gamma\delta$ TCR $^{-}$. (B) CD8 $^{+}$ T cells were gated as CD45 $^{+}$ CD3 $^{+}$ CD4 $^{-}$ CD8 $^{+}$ $\gamma\delta$ TCR $^{-}$. (C)

Regulatory T cells were gated as $CD45^{+} CD3^{+} CD4^{+} CD25^{+} FoxP3^{+}$. (D) Alveolar macrophages were gated as $CD45^{+} F4/80^{+} CD11c^{+} CD11b^{-}$. Data (n=2-8) presented as means $\pm$ s.e.m from 2 independent experiments. # represents $P < 0.05$ compared to $RelB^{+/-}$ mice, + represents $P < 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent $RelB$ knockout mice, closed symbols represent $RelB$ mutant mice.

Figure E4. Level of IFN- γ and IL-10 in the lung. Purified lymphatic and splenic $CD11c^{+}$ DC from $RelB$ heterozygous ($^{+/-}$) or $RelB$ wild-type ($^{+/+}$) were adoptively transferred intravenously into $RelB$ -deficient ($^{-/-}$) mutant and knockout C57BL/6 respectively. Protein was extracted from lung homogenates and levels of IFN- γ and IL-10 were assessed by multiplex assay 14 days post DC transfer. Data (n=2-8) presented as means $\pm$ s.e.m from 2 independent experiments. # represents $P < 0.05$ compared to $RelB^{+/-}$ mice, + represents $P < 0.05$, compared to $RelB^{-/-}$ mice. Open symbols represent $RelB$ knockout mice, closed symbols represent $RelB$ mutant mice.

Figure E5. Representative FACS plots for ILC2, mDCs and $\gamma\delta$ T cells. (A) Type 2 innate lymphoid cells (ILC2) were gated as $CD45^{+} Lin^{-} CD90.2^{+} CD2^{-} IL7R\alpha^{+} CD25^{+} IL33R\alpha^{+}$, (B) myeloid dendritic cells (mDCs) were gated as $CD45^{+} CD11c^{+} CD11b^{+} F4/80^{-}$, and (C) gamma delta ($\gamma\delta$) T cells were gated as $CD45^{+} CD3^{+} CD4^{-} CD8^{-} \gamma\delta TCR^{+}$.

Supplemental References

- E1. Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, Kaiko GE, Gibson PG, Foster PS, Hansbro PM. Neonatal chlamydial infection induces mixed T-cell responses that drive allergic airway disease. *Am J Respir Crit Care Med* 2007; 176: 556-564.
- E2. Haw TJ, Starkey MR, Nair PM, Pavlidis S, Liu G, Nguyen DH, Hsu AC, Hanish I, Kim RY, Collison AM, Inman MD, Wark PA, Foster PS, Knight DA, Mattes J, Yagita H, Adcock IM, Horvat JC, Hansbro PM. A pathogenic role for tumor necrosis factor-related apoptosis-inducing ligand in chronic obstructive pulmonary disease. *Mucosal Immunol* 2016; 9: 859-872.
- E3. Nair PM, Starkey MR, Haw TJ, Liu G, Horvat JC, Morris JC, Verrills NM, Clark AR, Ammit AJ, Hansbro PM. Targeting PP2A and proteasome activity ameliorates features of allergic airway disease in mice. *Allergy* 2017 (In press).
- E4. Beckett EL, Stevens RL, Jarnicki AG, Kim RY, Hanish I, Hansbro NG, Deane A, Keely S, Horvat JC, Yang M, Oliver BG, van Rooijen N, Inman MD, Adachi R, Soberman RJ, Hamadi S, Wark PA, Foster PS, Hansbro PM. A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. *J Allergy Clin Immunol* 2013; 131: 752-762.
- E5. Hansbro PM, Hamilton MJ, Fricker M, Gellatly SL, Jarnicki AG, Zheng D, Frei SM, Wong GW, Hamadi S, Zhou S, Foster PS, Krilis SA, Stevens RL. Importance of mast cell Prss31/transmembrane tryptase/tryptase-gamma in lung function and experimental chronic obstructive pulmonary disease and colitis. *J Biol Chem* 2014; 289: 18214-18227.
- E6. Liu G, Cooley MA, Jarnicki AG, Hsu AC, Nair PM, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, Tjin G, Wark PA, Walker MM, Horvat JC, Oliver BG, Argraves WS, Knight DA, Burgess JK, Hansbro PM. Fibulin-1 regulates the pathogenesis of tissue remodeling in respiratory diseases. *JCI insight* 2016; 1.
- E7. Starkey MR, Nguyen DH, Essilfie AT, Kim RY, Hatchwell LM, Collison AM, Yagita H, Foster PS, Horvat JC, Mattes J, Hansbro PM. Tumor necrosis factor-related apoptosis-inducing ligand translates neonatal respiratory infection into chronic lung disease. *Mucosal Immunol* 2014; 7: 478-488.

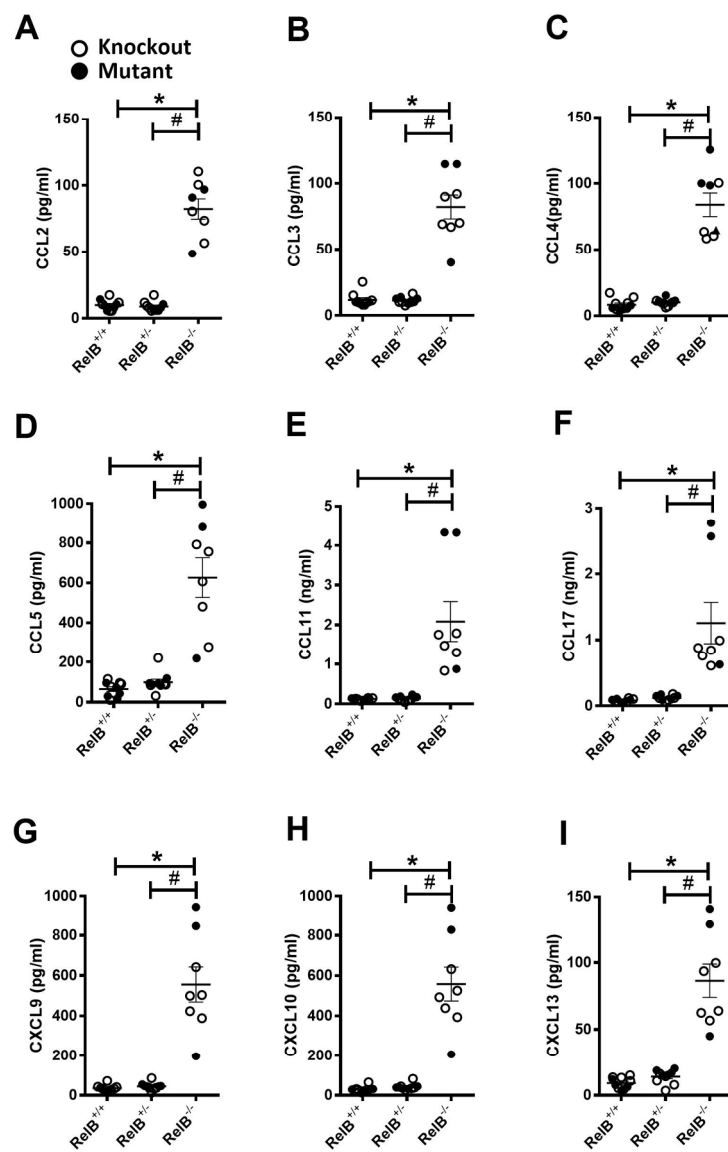


Figure E1

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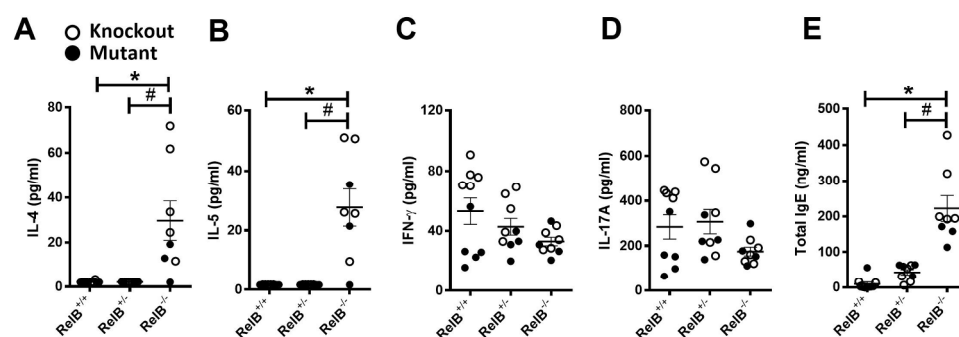


Figure E2

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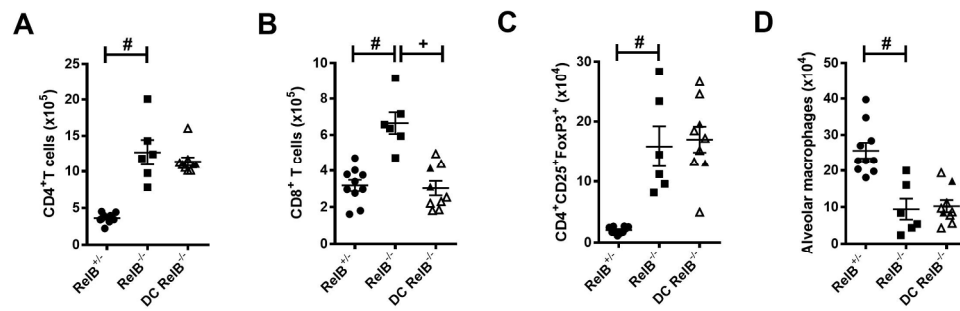


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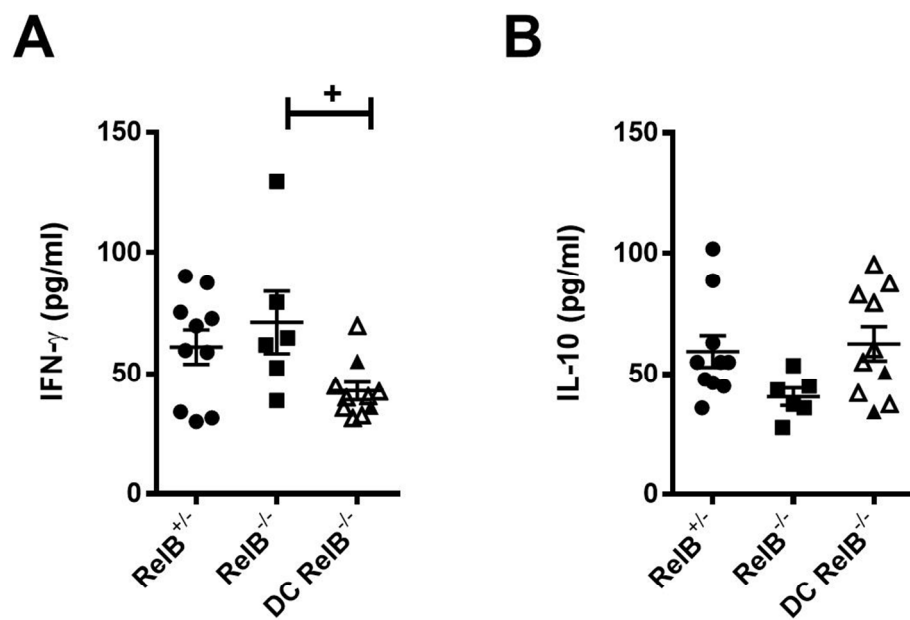


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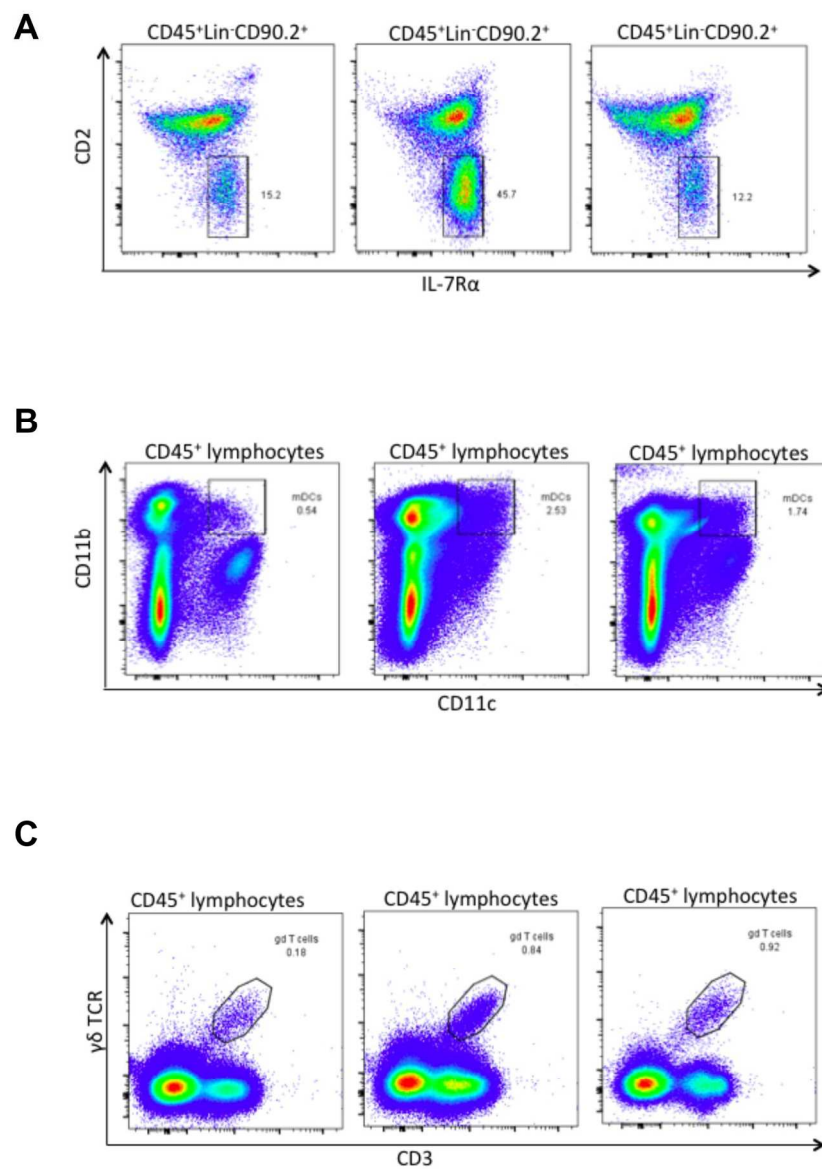


Figure E5

151x196mm (300 x 300 DPI)

Table E1. Antibodies used in flow cytometry analysis

Cell surface antigens	Clone	Fluorophore	Company
CD2	RM2-5	PE	Biolegend
CD25		FITC	Biolegend
Lineage (CD3/Ly-6G/CD11b/CD45R/Ter119)	17A2/RB6-8C5/MI/70/ RA3-6B2/Ter-119	AF700	Biolegend
CD90.2	30-H12	APC/Cy7	Biolegend
cKit	2B8	BV421	Biolegend
IL-33R α	DIH9	APC	Biolegend
CD45	30-F11	PerCPCy5.5	Biolegend
IL-7R α	A7R34	BV605	Biolegend
CD3	17A2	AF700	Biolegend
CD80	16-10 A1	PE	Biolegend
CD86	GL-1	FITC	Biolegend
CD45	30-F11	AF700	Biolegend
Gr1	RB6-8C5	APC-Cy7	Biolegend
CD11c	N418	BV510	Biolegend
F4/80	BM8	BV605	Biolegend
I-A/I-E	M5/114.15.2	APC	Biolegend
CD11b	M1/70	PerCPCy5.5	Biolegend
FoxP3	FJK-16s	PE	eBioscience
CD4	RM4-5	APC-Cy7	Biolegend
$\gamma\delta$ TCR	GL3	BV421	Biolegend
CD8a	53-6.7	BV510	Biolegend
CD3 ϵ	145-2C11	APC	Biolegend