



Research Article

Th2 cells promote eosinophil-independent pathology in a murine model of allergic bronchopulmonary aspergillosis

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Repeated inhalation of airborne conidia derived from the fungus *Aspergillus fumigatus* (Af) can lead to a severe eosinophil-dominated inflammatory condition of the lung termed allergic bronchopulmonary aspergillosis (ABPA). ABPA affects about 5 million individuals worldwide and the mechanisms regulating lung pathology in ABPA are poorly understood. Here, we used a mouse model of ABPA to investigate the role of eosinophils and T cell-derived IL-4/IL-13 for induction of allergic lung inflammation. Selective deletion of IL-4/IL-13 in T cells blunted the Af-induced lung eosinophilia and further resulted in lower expression of STAT6-regulated chemokines and effector proteins such as Arginase 1, Relm- α , Relm- β , and Muc5a/c. Eosinophil-deficient Δ dblGata mice showed lower IL-4 expression in the lung and the number of Th2 cells in the lung parenchyma was reduced. However, expression of the goblet cell markers Clca1 and Muc5a/c, abundance of mucin-positive cells, as well as weight gain of lungs were comparable between Af-challenged Δ dblGata and WT mice. Based on these results, we conclude that T cell-derived IL-4/IL-13 is essential for Af-induced lung eosinophilia and inflammation while eosinophils may play a more subtle immunomodulatory role and should not simply be regarded as pro-inflammatory effector cells in ABPA.

Keywords: *Aspergillus fumigatus* · allergy · eosinophils · IL-4/IL-13 · lung inflammation



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a chronic inflammatory condition affecting about 5 million people worldwide with a high incidence in asthma and cystic fibrosis patients [1,2]. ABPA is elicited by the omnipresent fungal opportunistic

pathogen *Aspergillus fumigatus* (Af), resembles the most common manifestation of allergic bronchopulmonary mycosis and is also considered as one of the severest forms of allergic eosinophilic asthma [3,4]. Treatment to date is largely limited to symptom-suppressive azole antimycotics and glucocorticoid therapy to reduce fungal burden and immunopathology, respectively [5].

Dependent on the host immune status Af can cause various diseases in humans. In contrast to invasive aspergillosis, which primarily affects immuno-compromised patients, ABPA is

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characterized by a type 2 immune response directed against inhaled fungal conidia [6–9]. This includes increased production of the cytokines IL-4, IL-5, and IL-13, elevated serum IgE levels, pronounced goblet cell hyperplasia with mucus deposition in airways and accumulation of Th2 cells and eosinophils in the lung [10–13].

IL-4 and IL-13 activate STAT6-regulated genes in various cell types and play a central role in mediating the effector phase of type 2 immune responses, including ABPA [14–18]. Both cytokines can be produced by many different cell types of the innate and adaptive immune system including Th2 cells, basophils, mast cells, type 2 innate lymphoid cells (ILC2s), and eosinophils [19–21]. It is therefore challenging to identify the predominant cellular source of IL-4/IL-13 in type 2 immune responses. In a model of OVA sensitization and challenge, we could previously show that T cell-derived IL-4/IL-13 is required for induction of allergic inflammation of the lung [22]. This might be different in ABPA since the abundantly recruited eosinophils could be a more important source of these cytokines as compared to Th2 cells. Furthermore, *Af* infection of the lung is also known to cause a Th17 response that can be cross-reactive to *Candida albicans* and may counteract a protective Th1 response [23,24]. In addition, repeated weekly intranasal exposure of mice to live *Af* conidia resulted in coevolution of a mixed Th1, Th2, and Th17 response where arterial remodeling in the lung was promoted by IL-4 and IL-10 from non-T cells [9].

Eosinophils are classically regarded as terminal effector cells in various allergic diseases [25–27]. This view is largely based on the fact that eosinophils store several cytotoxic proteins like eosinophil peroxidase and major basic protein in cytoplasmic granules that are released after activation and thereby inflict damage on surrounding tissue [28]. However, eosinophils may also have immunomodulatory functions and eosinophil-derived major basic protein has been shown to be dispensable for allergic airway inflammation [29–31].

In this study, we used a mouse model of ABPA with repetitive intranasal application of live *Af* conidia to immunocompetent mice to address the contribution of Th2 cells and eosinophils for allergic inflammation of the lung. We demonstrate that *Af* conidia induced massive recruitment of eosinophils to the lung while the number of neutrophils did not increase. T cell-derived IL-4/IL-13 appeared to be essential for lung eosinophilia, induction of chemokines associated with eosinophil recruitment, and goblet cell hyperplasia. Eosinophil-deficient Δ dblGata mice showed lower levels of IL-4, IL-17, and IL-23, and contained fewer Th2 cells in the lung parenchyma as compared to WT mice. Despite these effects, eosinophils appeared to be largely dispensable for goblet cell hyperplasia, mucus production, and lung pathology.

Results

Aspergillus fumigatus conidia elicit lung eosinophilia without neutrophil recruitment

Eosinophils and neutrophils are both known as potent innate effector cells associated with antifungal immune responses. However,

the kinetics and mechanisms of tissue accumulation of both cell types can vary depending on the organ, fungal burden, and the quality of the adaptive immune response. Here, we established a mouse model for ABPA by repetitive intranasal administration of 2×10^6 live conidia of *Af* and analyzed the lungs at different time points for eosinophil and neutrophil accumulation (Fig. 1A). We observed a selective increase of eosinophils but not neutrophils over time. On day 17, 3 days after the fifth *Af* conidia administration, eosinophils reached about 30% of total cells and a cell count of more than 5×10^6 in the lung while the number of neutrophils remained as low as in untreated mice (Fig. 1B–D, Supporting Information Figs. S1 and S2). Basophils and ILC2s showed only a small increase in total numbers (Supporting Information Fig. 3). We therefore conclude that this repetitive low-dose administration of *Af* conidia elicits a strong type 2 immune response with massive lung eosinophilia as it is also seen in human ABPA patients.

Af-induced lung eosinophilia requires T cell-derived IL-4/IL-13

The role of T cells and the Th2 cell-associated cytokines IL-4 and IL-13 in the context of allergy and more specifically in ABPA have been the subject of numerous studies [16–18]. Given the fact that IL-4 and IL-13 can be expressed by many different cell types including a variety of innate effector cells such as eosinophils, basophils, and ILC2s, it was unclear whether T cell-derived IL-4/IL-13 plays a crucial or rather redundant function in this ABPA model. To address this point, we compared WT mice with T cell-specific IL-4/IL-13-deficient (4–13Tko) mice, where IL-4 and IL-13 is specifically deleted only in the T cell lineage [32]. Surprisingly, without IL-4 and IL-13 from T cells, we observed completely abrogated eosinophilia (Fig. 2A–C). The *Af*-induced increase of mRNAs for IL-4 and IL-13 in the lung of WT mice was blunted in 4–13Tko mice (Fig. 2D). This indicates that Th2 cells are either the main source of these cytokines or play a critical role to recruit other IL-4/IL-13 producers to the lung. The impaired IL-4 and IL-13 expression was further accompanied by a lack of induction of IL-5, Ccl11 (eotaxin-1), and Ccl24 (eotaxin-2), critical molecules for eosinophil recruitment, and survival (Fig. 2D). In contrast to 4–13Tko mice, *Af*-challenged WT mice further upregulated the IL-4/IL-13-induced chemokine and ABPA marker Ccl17 (TARC) [33,34] as well as Cxcl1 and Ccl2 associated with neutrophil and monocyte recruitment, respectively (Fig. 2D). IFN- γ as an indicator for type 1 immunity was not upregulated upon *Af* administration in WT or 4–13Tko mice. Intriguingly, IL-17A was strongly induced by *Af* but comparable between both strains of mice, while IL-23A expression implicated in Th17 and Treg induction was generally very low and only induced in *Af*-challenged lungs of WT mice (Fig. 2D). Hence, repetitive intranasal administration of *Af* conidia in the absence of IL-4/IL-13-expressing Th2 cells appeared not to shift the immune response to a Th1- or Th17-dominated phenotype.

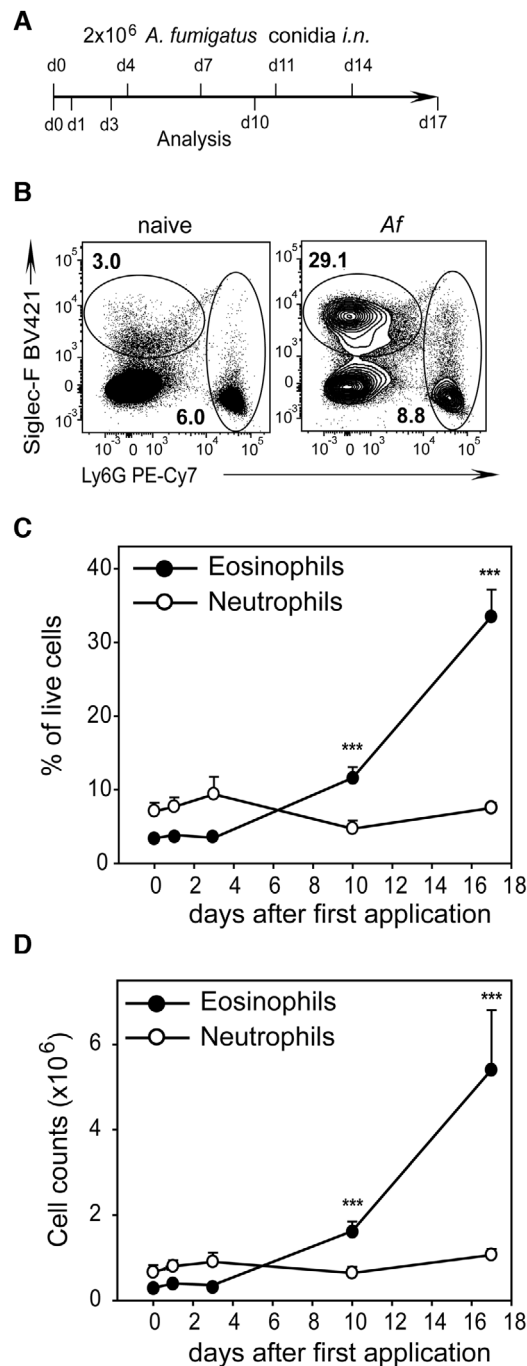


Figure 1. Recruitment kinetics of eosinophils and neutrophils to the lung during low-dose *Aspergillus fumigatus* (Af) conidia administration. (A) Schematic illustration of the experimental setup. (B) Flow cytometric analysis of single cell suspensions from the lung on day 0 (naïve) and day 17 (Af) after Af conidia administration. Gates indicate the frequencies of eosinophils (Siglec-F⁺Ly6G⁻) and neutrophils (Ly6G⁺) among pregated live CD11c⁻ cells (Supporting Information Fig. S1). Frequencies (C) and total numbers (D) of eosinophils and neutrophils in the lung at indicated days after Af conidia administration. Displayed are the mean + SEM from pooled data of two independent experiments except for d1 (only 1 experiment) with at least five mice per group and time point. Statistical significance was determined by Kruskal-Wallis test with Dunn's post hoc method against the control group (d0). *** $p < 0.001$.

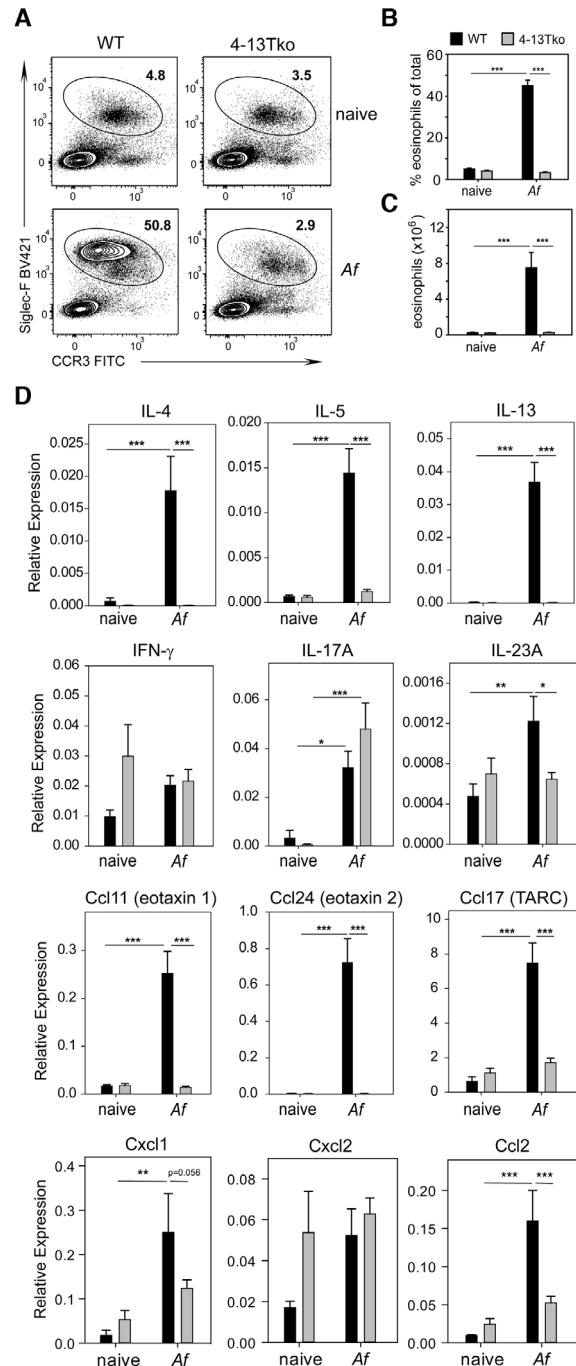


Figure 2. Af-induced lung eosinophilia requires T cell-derived IL-4/IL-13. (A) Flow cytometric analysis of single cell suspensions from the lung on day 0 (naïve) and day 17 (Af) after Af conidia administration in C57BL/6 (WT) and T cell-specific IL-4/IL-13-deficient (4-13Tko) mice. Gates indicate the frequency of eosinophils (Siglec-F⁺CCR3⁺) among pregated live Ly6G⁻CD11c⁻ cells. Frequencies (B) and total numbers (C) of lung eosinophils. Bars show the mean + SEM of pooled data from three experiments with a total of seven to nine mice per group. (D) Quantitative RT-PCR data from whole lung tissue of naïve and Af-challenged WT or 4-13Tko mice. Expression of indicated cytokines and chemokines was normalized to the expression level of the *Hprt* gene. Bars show the mean + SEM of pooled data from three experiments with a total of five to eight mice per group. Statistical significance was determined by two-way ANOVA with Holm-Sidak post hoc testing. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4–13Tko mice show normal *Af*-induced eosinophil development but reduced blood eosinophilia

Given the impaired *Af*-induced lung eosinophilia in 4–13Tko mice, we next addressed the question, whether eosinophil development or maturation in the BM is also affected in 4–13Tko mice. Therefore, we analyzed BM and blood from naïve and *Af*-challenged WT and 4–13Tko mice by flow cytometry. The frequency and total number of eosinophils (Siglec-F⁺SSC^{hi}) in the BM slightly increased after *Af* exposure in both WT and 4–13Tko mice (Fig. 3A and B). The upregulation of the eotaxin receptor CCR3 indicates an important maturation step for eosinophils in the BM [35]. Using this marker, we observed no significant differences in the number of mature eosinophils between WT and 4–13Tko mice (Fig. 3A and C). However, we found that the frequency of peripheral blood eosinophils was increased in *Af*-challenged WT mice but not in 4–13Tko mice (Fig. 3D and E). These results suggest that 4–13Tko mice are impaired in mobilizing eosinophils from the BM into the blood circulation and/or promoting survival of blood eosinophils while generation and maturation of eosinophils in the BM appear normal.

T cell-derived IL-4/IL-13 is required for *Af*-induced allergic lung inflammation

Having shown that T cell-derived IL-4/IL-13 is critical for *Af*-induced lung and blood eosinophilia, we further investigated whether this correlates with histopathological changes and expression of effector molecules associated with allergic lung inflammation. Hematoxylin- and eosin-stained lung sections of *Af*-challenged WT mice revealed massive eosinophil-dominated infiltrates that were not observed in 4–13Tko mice (Fig. 3F). Furthermore, periodic acid-Schiff (PAS) staining showed that only *Af*-challenged WT mice developed goblet cell hyperplasia in the lung (Fig. 3G). Quantitative RT-PCR analyses revealed that markers of IL-4/IL-13-activated goblet cells like Calcium-activated chloride channel regulator 1 (*Clca1*, also named *Gob5*) and Resistin-like-β (*Retnlb*) were only induced in *Af*-challenged WT mice (Fig. 3G). Similarly to mucus production, we could also only observe an increase in IgE levels in the serum of WT, but not 4–13Tko mice (Fig. 3H).

We further determined the frequency and total numbers of AAMs in the lung by flow cytometry using PD-L2 as a surface marker [36]. AAMs only increased in the lung of *Af*-challenged WT mice (Fig. 4A–C). The AAM-associated effector molecules Resistin-like-α (encoded by the *Retnlα* gene) and Arginase 1 (*Arg1*) [37, 38] were also strongly induced in *Af*-challenged WT mice, but not in 4–13Tko mice (Fig. 4D).

These findings demonstrate that the major effector pathways of *Af*-induced type 2 immunity-associated lung inflammation including eosinophilia, goblet cell hyperplasia, IgE production, and AAM accumulation are all critically dependent on T cell-derived IL-4/IL-13.

Eosinophils promote the IgE response and Th2 cell accumulation in the lung of *Af*-challenged mice

So far, we described the critical role of Th2 cells for lung eosinophilia and type 2 immunity in a model of ABPA. In addition to a variety of pro-inflammatory effector molecules, eosinophils can also produce significant amounts of IL-4 and IL-13 and may therefore promote Th2 accumulation and down-stream effector pathways in the lung. To determine the role of eosinophils for accumulation of Th2 cells in the lung of *Af*-challenged mice, we compared IL-4eGFP reporter mice (4get mice) with eosinophil-deficient 4get mice on day 17 after the first administration of *Af* conidia. Flow cytometric analysis demonstrated that the frequency of Th2 cells among all CD4 T cells in the lung increased from 2.7% in naïve to 25% in *Af*-challenged 4get mice and from 2.3% to 22% in 4get_ΔdblGata mice (Fig. 5A). Yet in total numbers, we observed about 1×10^5 more Th2 cells in the lung of *Af*-challenged 4get mice as compared to 4get_ΔdblGata mice (Fig. 5B). This difference was not apparent in the BAL where Th2 cell numbers were about fivefold lower as compared to total lung tissue in both strains of mice (Fig. 5B). The spleen did not show an increase of Th2 cells after *Af* challenge indicating that intranasal administration of *Af* conidia does not result in a systemic Th2 response (Fig. 5B). Further analysis revealed comparable AAM accumulation in the lung (Fig. 5C and D) but lower serum IgE levels in *Af*-challenged 4get_ΔdblGata as compared to 4get mice (Fig. 5E). These results demonstrate that eosinophils can enhance accumulation of Th2 cells in the lung and promote the IgE response in this model of ABPA.

Eosinophils promote expression of IL-4, IL-17, and IL-23 in the lung of *Af*-challenged mice

To further investigate eosinophil-dependent changes of cytokines and chemokines in the lung of *Af*-challenged mice, we performed quantitative RT-PCR analysis from whole lung tissue of 4get and 4get_ΔdblGata mice. IL-5 and IL-13 were strongly and IFN-γ was only moderately induced to similar levels in both strains of mice (Fig. 6A). In contrast, IL-4 upregulation was significantly stronger in *Af*-challenged 4get mice (Fig. 6A). This result could be confirmed at the protein level by ELISA (Fig. 6B). We also noticed increased expression of IL-17A and IL-23A in *Af*-challenged 4get mice that is consistent with a previous report demonstrating expression of both cytokines by eosinophils after acute high-dose *Af* conidia administration to the lung [39]. With regard to chemokines, we observed strong and comparable induction of Ccl11 and Ccl24 in both strains of mice, while Ccl17 and Cxcl2 were expressed at higher levels and Cxcl1 at lower levels in eosinophil-deficient 4get_ΔdblGata mice (Fig. 6A). This indicates that although eosinophils can promote IL-4 expression in the *Af*-challenged lung, this does not result in increased expression of IL-4/IL-13-induced chemokines. Furthermore, eosinophils seem to promote a mixed Th2/Th17 response in this ABPA model.

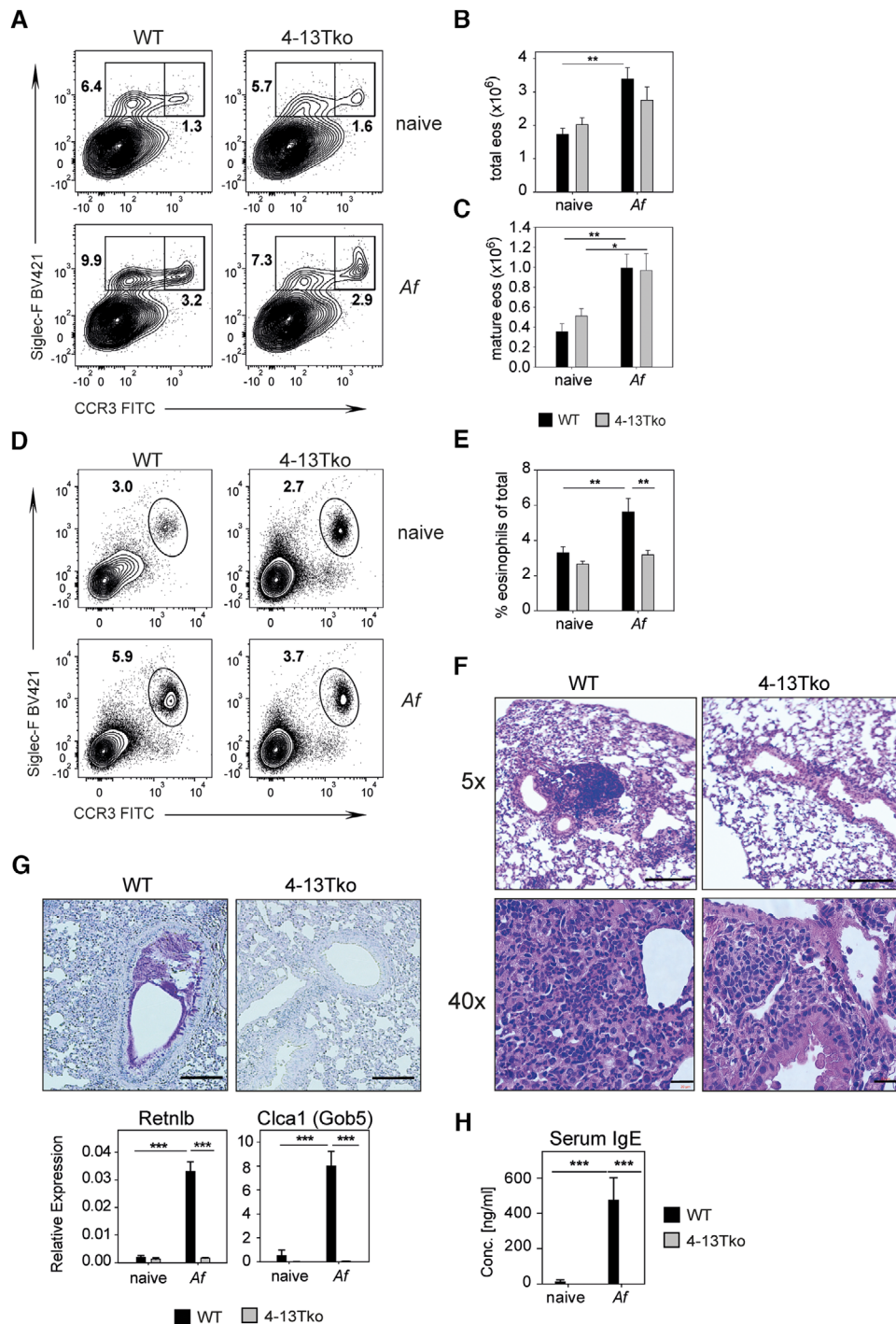


Figure 3. Th2 cells play a critical role for Af-induced blood eosinophilia, serum IgE and the type 2 immunity-driven mucus production in the lung. (A) Flow cytometric analysis of immature (Siglec-F⁺ CCR3⁻) and mature (Siglec-F⁺ CCR3⁺) eosinophils in the BM of naïve and Af-challenged WT and 4-13Tko mice. Total numbers of (B) total and (C) mature eosinophils in the BM. Bars show the mean + SEM of pooled data from three experiments with a total of seven to nine mice per group. (D) Flow cytometric analysis of peripheral blood eosinophils of naïve and Af-challenged WT and 4-13Tko mice. (E) Frequencies of eosinophils in peripheral blood. Bars show the mean + SEM of pooled data from two experiments with a total of four to eight mice per group. Statistical significance was determined by two-way ANOVA with Holm-Sidak post hoc testing. (F) Histological analysis of lung sections for eosinophilic cell infiltration from Af-challenged WT and 4-13Tko mice stained with H&E. (G) Assessment of goblet cell hyperplasia by histological Periodic acid-Schiff's reagent mucus staining and quantitative RT-PCR of goblet cell associated markers from whole lung tissue. (H) IgE ELISA from serum of naïve and Af-challenged WT and 4-13Tko mice. (F and G) Displayed are representative tissue sections of four to five mice per group from two experiments. Scale bars indicate 100 μ m for 5 $\times$ and 20 μ m for 40 $\times$ objective photographs. Expression was normalized to the *Hprt* gene. Bars show the mean + SEM of pooled data from three experiments with a total of five to eight mice per group. Statistical significance was determined by two-way ANOVA with Holm-Sidak post hoc testing. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

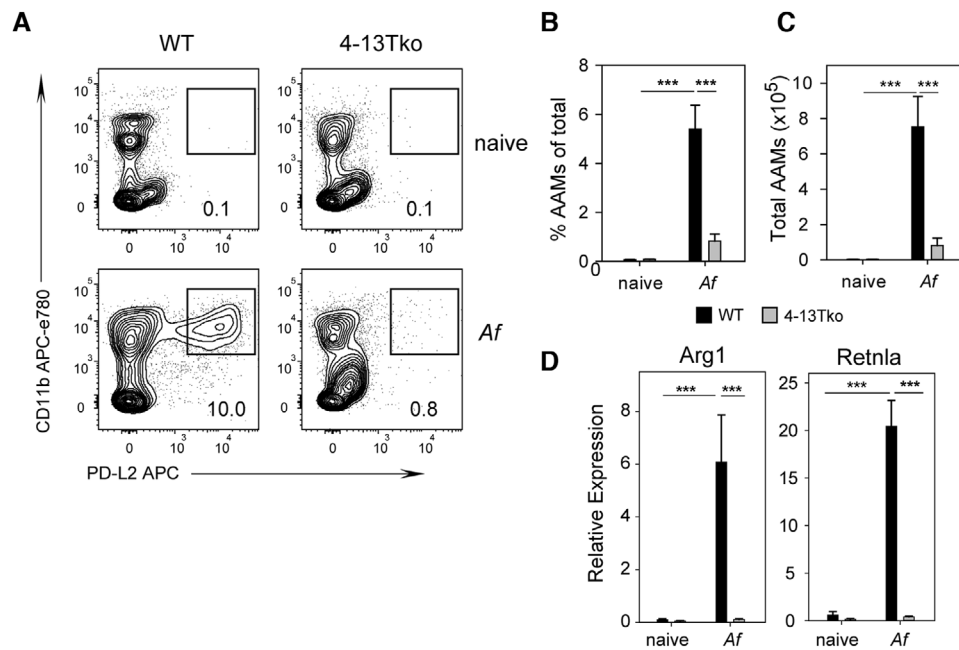


Figure 4. Th2 cells are required to elicit *Af*-induced alternative activation of lung macrophages. Flow cytometric analysis for alternatively activated macrophages (AAMs) in whole lung of naïve and *Af*-challenged B6 WT and 4–13Tko mice. (A) Representative plots for AAMs (CD11b⁺PD-L2⁺) among live cells with eosinophils gated out by Siglec-F/CD11c. (B) Percentage and (C) total numbers of AAMs in the lung of WT and 4–13Tko mice. (D) Quantitative RT-PCR data of AAM-associated markers from whole lung tissue. Expression was normalized to the *Hprt* gene. Bars show the mean + SEM of pooled data from three experiments with a total of —seven to nine (flow cytometry) or five to eight (qRT-PCR) mice per group. Statistical significance was determined by two-way ANOVA with Holm-Sidak post hoc testing. ****p* < 0.001.

Eosinophils are dispensable for ABPA-associated mucus production and lung edema

Eosinophils are generally believed to promote tissue damage and lung pathology in ABPA patients because they release a variety of pro-inflammatory proteins including major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. Therefore, we further investigated whether absence of eosinophils in 4get_ΔdblGata mice was associated with reduced goblet cell hyperplasia and less severe lung pathology. Unexpectedly, we observed strong and comparable induction of the goblet cell markers *Clca1* (*Gob5*) and *Muc5ac* in the lung of *Af*-challenged mice of both mouse strains (Fig. 7A). PAS staining further indicated similar percentages of PAS⁺ airways in 4get and 4get_ΔdblGata mice (Fig. 7B and C). As a further parameter of pathology, we determined the weight of the lung from naïve and *Af*-challenged mice. The weight of the lung increased from 0.2 g in naïve mice to 0.4 g in *Af*-challenged mice of both mouse strains (Fig. 7D). Based on these results, we conclude that eosinophils are largely dispensable for *Af*-induced lung edema in this established model of ABPA.

Discussion

In this study, we demonstrate that T cell-derived IL-4/IL-13 is an important signal to induce lung eosinophilia and ABPA-associated pathology in mice, when repetitively confronted with

low numbers of *Af* conidia to mimic chronic exposure to this fungal pathogen. Despite their well-known pro-inflammatory effector function, eosinophils appeared to be dispensable for lung pathology that developed with comparable severity in WT and eosinophil-deficient mice.

The lack of IL-4/IL-13 expression in T cells blunted the lung eosinophilia while eosinophil development in the BM was not affected. This supports the view that Th2 cells get locally activated in the lung and secrete IL-4/IL-13 to induce expression of the STAT6-regulated and eosinophil-recruiting chemokines *Ccl11*, *Ccl17*, and *Ccl24* in lung endothelial cells [40]. In addition, Th2-derived IL-5 may enhance the survival of recruited eosinophils in the lung.

We also observed that IL-4 expression and the number of Th2 cells in the lung parenchyma were reduced in the absence of eosinophils. This suggests that eosinophils can promote further recruitment or survival of Th2 cells in the *Af* conidia challenged lung consistent with previous reports using other models of allergic lung inflammation [41–43]. However, neither the frequency of Th2 cells among all CD4 T cells in the lung nor the total number of Th2 cells in the BAL was diminished in 4get_ΔdblGata mice. Interestingly, the expression of the Th2 recruiting chemokine *Ccl17*, which can be used as diagnostic marker in ABPA patients [34], was even higher in 4get_ΔdblGata as compared to control mice. This suggests that eosinophils promote the accumulation of CD4 T cells in the lung parenchyma rather than stimulating differentiation or selective recruitment of Th2 cells in this ABPA model.

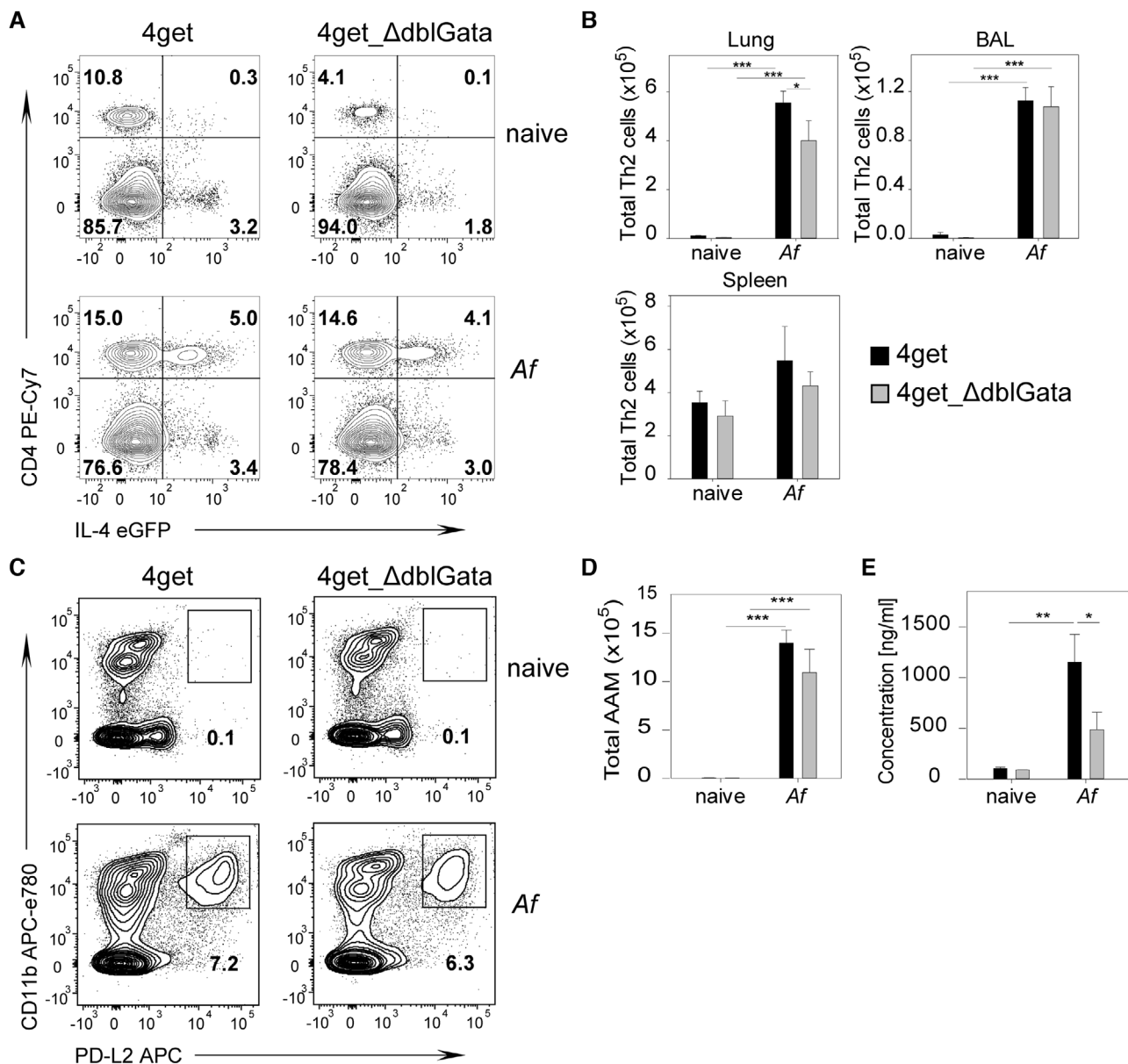


Figure 5. Eosinophils promote serum IgE and contribute to Th2 cell, but not AAM accumulation in the lung of Af-challenged mice. Flow cytometric analysis of single cell suspensions from the lung of naive and Af-challenged 4get and 4get_ΔdblGata mice. Plots show the frequencies of (A) Th2 cells (CD4⁺IL-4eGFP⁺) among pregated live Siglec-F[−]CD11c[−] cells to exclude eosinophils and autofluorescent cells. (B) Absolute numbers of total Th2 cells of whole lung, BAL, and spleen. (C) Representative plots of lung AAMs (CD11b⁺PD-L2⁺) gated from live cells with eosinophils gated out by CD11c/Siglec-F. (D) Absolute numbers of AAMs in the lung. (E) Serum IgE levels of naive and Af-infected 4get and 4get_ΔdblGata mice measured by ELISA. Bars show the mean + SEM of pooled data from three (lung, 6–9 mice/per group; IgE, 4–12 mice/per group) or four experiments (BAL and spleen, 8–11 mice/group). Statistical significance was determined by two-way ANOVA with Holm-Sidak post hoc testing. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Eosinophils can also express IL-13 and it has been demonstrated that IL-13-driven pathology can occur independently of IL-4 in other models of allergic airway inflammation [44–46]. Indeed, eosinophil-derived IL-13 can be sufficient for allergic lung inflammation as demonstrated in an adoptive transfer system [47]. However, we observed no reduction of total lung IL-13 in 4get_ΔdblGata mice indicating that eosinophils are not a major source of this cytokine in the ABPA model with repetitive low-dose

exposure to Af conidia. Interestingly, the induction of IL-17A and IL-23A expression was affected in 4get_ΔdblGata mice indicating that eosinophils can also promote a type 3 immune response in the lung that is characterized by increased levels of these cytokines. In fact, lung eosinophils have been demonstrated to express IL-17A and IL-23A in fungal respiratory disease [39,48].

The only minor difference of the type 2 inflammatory response in 4get_ΔdblGata mice was unexpected since several other reports

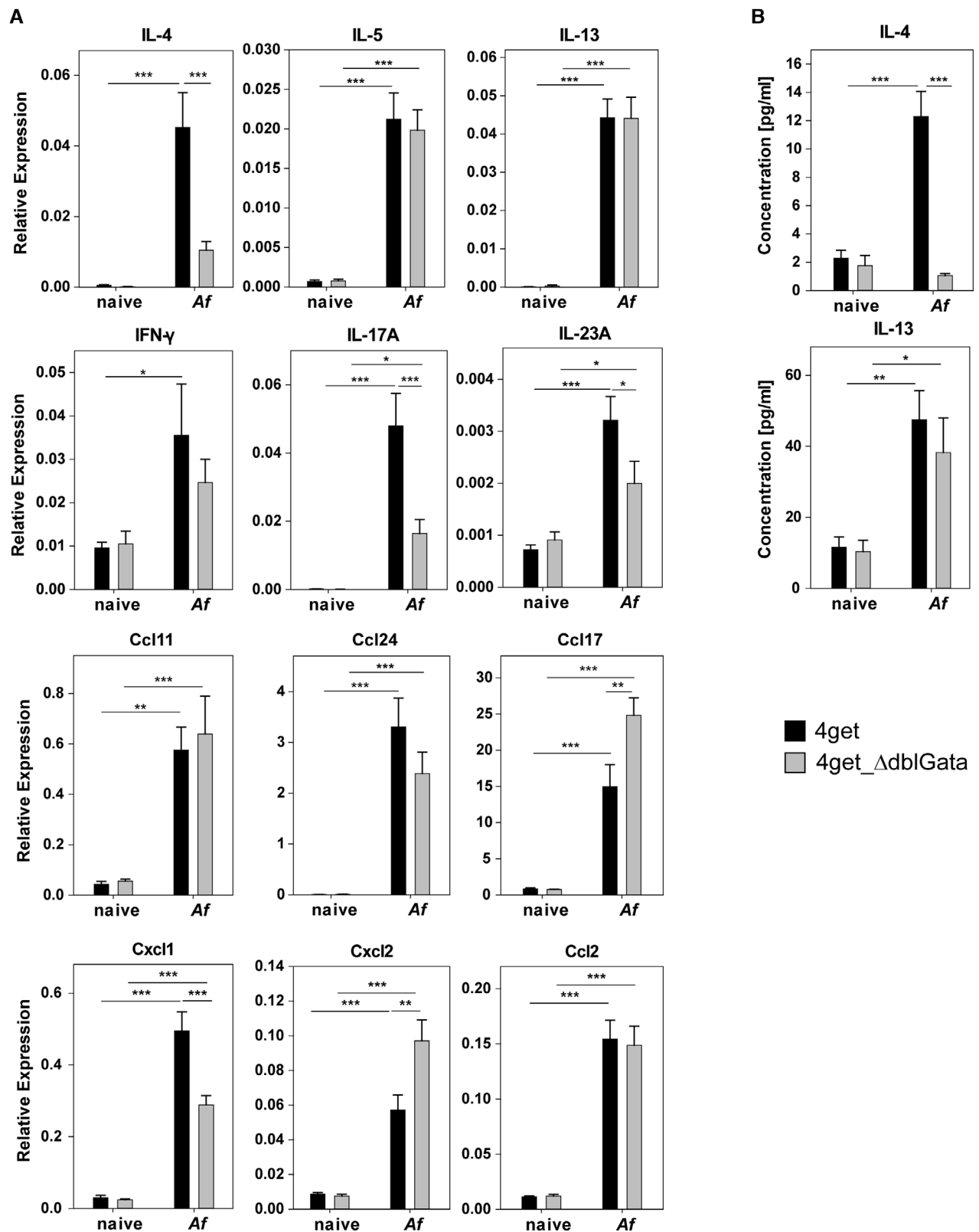


Figure 6. Eosinophils promote expression of IL-4, IL-17, and IL-23 in the lung of Af-challenged mice. (A) Quantitative RT-PCR data from whole lung tissue of naive and Af-challenged 4get or 4get_ΔdblGata mice. Expression of indicated cytokines and chemokines was normalized to the *Hprt* gene. Bars show the mean + SEM of pooled data from four experiments with a total of eight to 12 mice per group. (B) ELISA for IL-4 and IL-13 from BAL samples. Bars show the mean + SEM of pooled data from three experiments with a total of six to eight mice per group. Statistical significance was determined by Two-Way ANOVA with Holm-Sidak post hoc testing. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

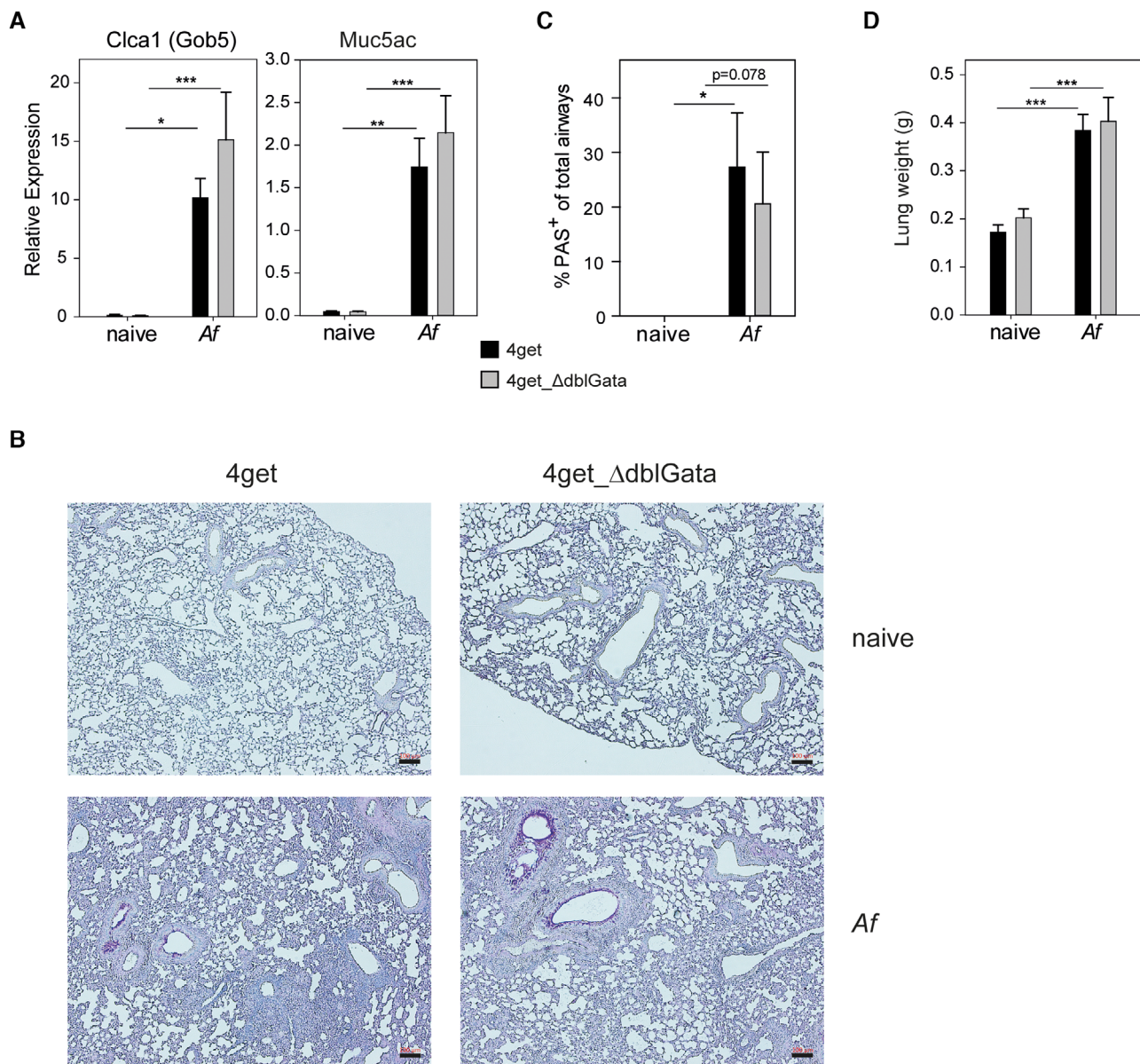


Figure 7. Eosinophils are dispensable for ABPA-associated mucus production and lung pathology. (A) Quantitative RT-PCR data from whole lung tissue of naïve and Af-challenged 4get or 4get_ΔdblGata mice for goblet cell associated markers of type 2 immunity-associated inflammation. Bars show the mean + SEM of pooled data from four experiments with a total of eight to 12 mice per group. Expression was normalized to the *Hprt* gene. (B) Histological analysis of lung sections from naïve and Af-challenged 4get or 4get_ΔdblGata mice stained with Periodic acid-Schiff's reagent. Displayed tissue sections are representative of —five to seven mice per group from two experiments. Scale bar indicates 100 μm. (C) Quantification of percentage of PAS⁺ airways of total airways. A total of 75–142 airways from five to seven mice per group were evaluated for presence of mucus. Bars show mean + SEM, resulting from independent countings of two experimenters. (D) Lung weight of naïve and Af-challenged 4get or 4get_ΔdblGata mice. Bars show the mean + SEM of pooled data from three experiments with a total of six to nine mice per group. Statistical significance was determined by Two-Way ANOVA with Holm-Sidak post hoc testing. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

had indicated that eosinophils are important effector cells that promote allergic inflammation in the lung. For example, eosinophils had been found to induce airway damage and bronchial hyper-reactivity in OVA-induced allergic lung inflammation in studies with IL-5-deficient mice [49] or anti-IL-5 treated mice [50]. Moreover, the Af cell wall component chitin elicits eosinophil recruitment and lung pathology [51]. Others have shown that allergic lung inflammation induced by repeated intranasal administration of

Af extracts is attenuated in ΔdblGata and CCR3-deficient mice on BALB/c background [27]. This result appears discrepant to our findings but might be explained by the fact that we used ΔdblGata mice on C57BL/6 background [42] and, more importantly, live Af conidia instead of Af extracts, which better resembles the natural situation.

Recently, human ABPA cases have been described, where application of the IL-5 receptor targeting antibody mepolizumab acted

beneficially in ameliorating pathology, implying a somehow pro-inflammatory role for eosinophils in this allergic disease [52–54]. However, although this treatment led to reduced eosinophilia, it should be taken into account that also other allergy-related cells, like basophils or antibody-producing B cells, are responsive to IL-5 and might be affected as well [55,56].

In conclusion, we found a dominant role for T cell-derived IL-4/IL-13 in establishing eosinophilia and allergic inflammation of the lung in a murine model of ABPA using live *Af* conidia. Eosinophils appeared to be largely dispensable for goblet cell hyperplasia, mucus production, and lung pathology. It remains to be investigated in further detail whether eosinophils are involved in fungal control and resolution of inflammation rather than exacerbation of ABPA.

Materials and methods

Mice

T cell-specific IL-4/IL-13-deficient (4-13Tko) [32] and eosinophil-deficient Δ dblGata [57] mice have been described. For this study, Δ dblGata mice had been crossed to 4get [58] mice in which expression of IL-4 mRNA is marked by the eGFP fluorescent protein. All mice were on C57BL/6 background, kept under SPF conditions and used at the age of 7–12 weeks. Animal experiments were performed with approval from the Local Government of Lower Franconia and according to the German animal protection law and guidelines of the European Union (Directive 2010/63/EU).

Intranasal administration of *Af* conidia

Instillation of freshly harvested fungal spores was performed as published before [59]. Briefly, mice were anaesthetized and received 2×10^6 live conidia of *A. fumigatus* ATCC 46645 (ref. PMID: 6160400) in 50 μ L PBS intranasally every 3–4 days for up to five times. Mice were analyzed on day 17 of the experiments unless indicated otherwise.

Organ sampling and generation of single cell suspensions

Spleens were carefully mashed through a 70 μ m cell strainer with a 5 mL syringe plunger. Femur and tibia bones were flushed out with FACS buffer (2% FCS, PBS) and single cell suspensions were generated by repetitively passing through a 20G syringe (BD Microlance, Franklin Lakes, NJ). Lungs were perfused with 8 mL of PBS through the right ventricle of the heart, placed into RPMI1640 (PAN-Biotech Inc., Aidenbach, Germany), cut into small pieces and digested with DNase DN25 (Sigma-Aldrich) and LiberaseTM (Roche, Basel, Switzerland) at 100 μ g/mL for 30 min at 37°C under continuous rotation. Debris was removed by gently mashing

through a 100 μ m cell strainer. Blood was collected in FACS blood buffer (2% FCS, 2000 U/mL Heparin, PBS) from the heart. Erythrocytes were lysed with ACK buffer (155 mM NH₄Cl, 9.99 mM KHCO₃, 0.10 mM EDTA) and cell suspensions filtered through a 70 μ m cell strainer afterward. BAL was flushed out with 2 mL PBS 0.1 mM EDTA intratracheally. To retrieve serum samples, blood was also collected from the heart of mice, transferred into SSTTM tubes (BD Microtainer, Franklin Lakes, NJ), allowed to clot for 30 min and centrifuged afterward according to manufacturer's protocol. Resulting supernatants were used for immunoglobulin ELISA measurements.

Flow cytometry

At first Fc-block (α -CD16/32, clone 2.4G2, BioXcell, West Lebanon, NH) was added to single cell suspensions to prevent unspecific staining. The staining was undertaken at 4°C in the dark for 20 min. For this, fluorophore-conjugated antibodies against the following targets were used: SIGLEC-F BV421 (E50-2440), PD-L2 APC (TY25), KLRG1 BV711 (2F1) from BD Biosciences (San Jose, CA), CD4 PEcy7 (RM4-5), CD11c PEcy7 (N418), CD49b AlexaFluor 488 (HM α 2), CD200R3 PE (Ba13), CCR3 FITC (J073E5), and Ly6G PEcy7 (1A8) all from BioLegend (San Diego, CA), ST2 PE (DJ8) from mdbiosciences (Egg b. Zürich, Switzerland), NKp46 PEVio770 from Miltenyi Biotec (Bergisch Gladbach, Germany), CD3e biotin (145-2C11), CD4 biotin (GK1.5), CD5 biotin (53-7.3), CD11b PEcy7 and APCeFluor780 (M1/70), CD11c PerCP-Cy5.5 (N418), CD45R biotin (RA3-6B2), Fc ϵ R1a PEcy7 (MAR-1), and NK1.1 PEcy7 (PK136), all from eBioscience/Thermo Fisher Scientific (Waltham, MA), as was the fixable ViaDye eFluor 506 and APC-conjugated Streptavidin. Data were acquired on BD FACS Canto II and BD LSRFortessa flow cytometry instruments and analyzed with FlowJo software (Version X, Treestar, Ashland, OR) according to the guidelines for the use of flow cytometry and cell sorting in immunological studies [60].

Quantitative RT-PCR

RNA from whole lung tissue was isolated with TriSure reagent (Bioline, Meridian Life Science Inc., Memphis, TN) and chloroform extraction. cDNA was generated with a High Capacity Reverse Transcription Kit from Applied Biosystems/Thermo Fisher Scientific (Waltham, MA). For the actual qRT-PCR, the SYBR Select Master Mix from Thermo Fisher Scientific (Waltham, MA) was used. Measurements were performed on a CFX-Connect instrument (Bio-Rad Laboratories, Inc., Hercules, CA) or a ViiA 7 Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific). Ct value results were normalized to *Hprt* as a reference gene. A list of primers used in this study is provided in Supporting Information Table S1. Primers are listed in Supporting Information Table S1.

Enzyme-linked immunosorbent assay

BAL supernatants and serum samples were analyzed via sandwich ELISA. For IL-4, an antibody pair of 11B11 (BioXcell, West Lebanon, NH) and BVD6-24G2-biotin (Biolegend), for IL-13 the murine IL-13 Standard ABTS ELISA Development Kit from Peprotech (Rocky Hill, NJ) were used. In both cases, alkaline phosphatase-conjugated Streptavidin (Southern Biotech Birmingham, AL) was applied to detect bound antibodies. IgE levels were assayed by pairing rat-anti-mouse IgE capture antibody (R35-72, BD Biosciences) and alkaline phosphatase-conjugated polyclonal goat-anti-mouse IgE detection antibody (Southern Biotech). Para-Nitrophenylphosphate (Southern Biotech) was used as chromogenic substrate and absorption was measured at a wavelength of 405 nm on a Multiskan FC 3.0 instrument (Thermo Fisher Scientific).

Histology

Lungs were fixed overnight in 4% para-formaldehyde (PFA) at 4°C, dehydrated, and embedded in paraffin. 3 µm sections were cut on a microtome and stained with H&E or Periodic-Acid-Schiff's reagent (PAS). Stained tissue sections were analyzed using a 5× objective for overview pictures or 20× and 40× objectives for selected representative bronchi and cellular infiltrates on a Zeiss Axio Vert.A1 microscope.

Statistical analysis

Statistical analysis of acquired data was performed with Sigmaplot (Version 12.3, Systat Software Inc., San Jose, CA). For the kinetic analysis (Fig. 1), criteria for a one-way ANOVA were not met and thus a Kruskal–Wallis test with the nominal variable of time was performed with Dunn's post hoc test against the control group of time point d0. In all experiments, comparing two different nominal variables (treatment and genotype), statistical significance was determined with two-way ANOVA and a Holm–Sidak post hoc test.

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Abbreviations: ABPA: Allergic bronchopulmonary aspergillosis · Af: *Aspergillus fumigatus* · BAL: bronchoalveolar lavage · FCS: fetal calf serum

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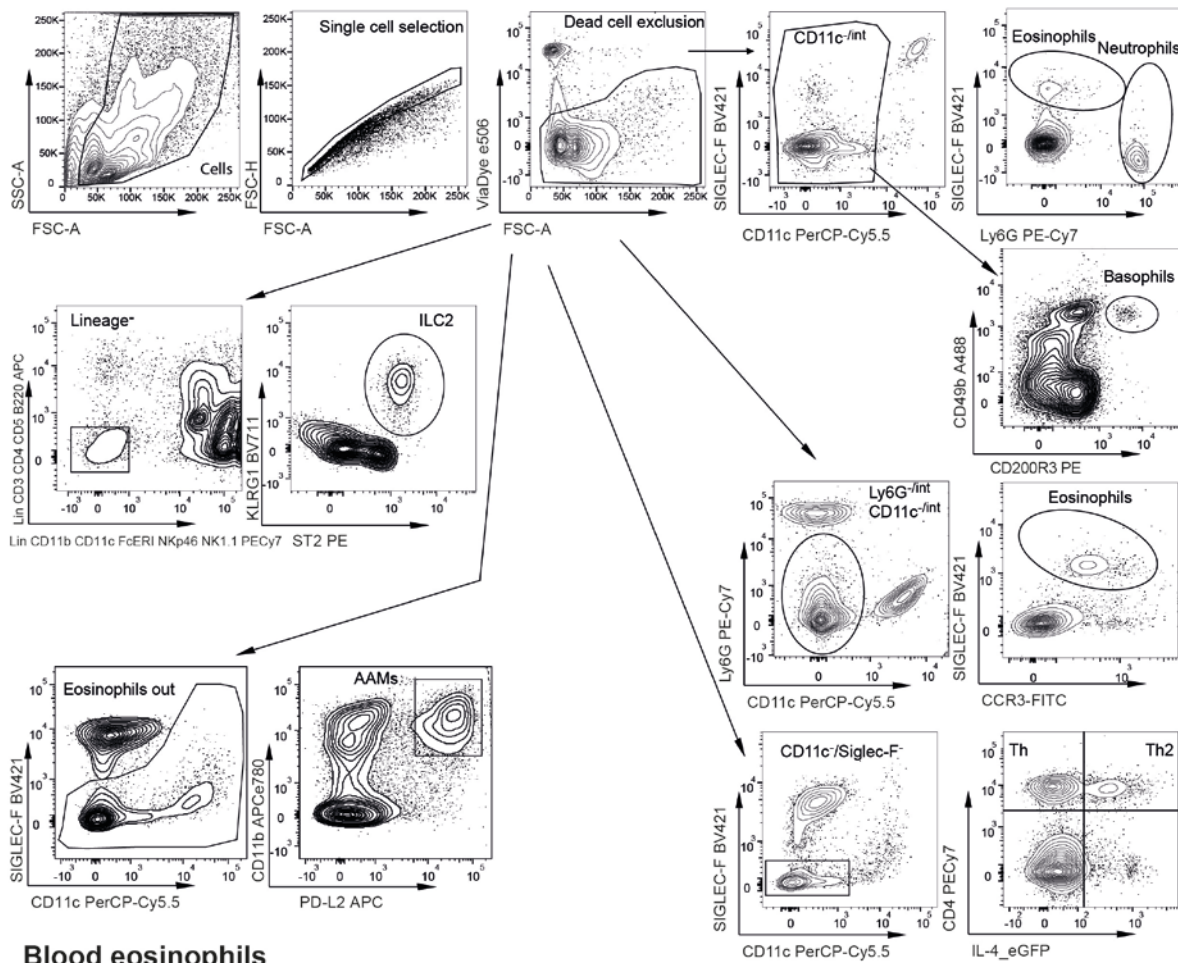
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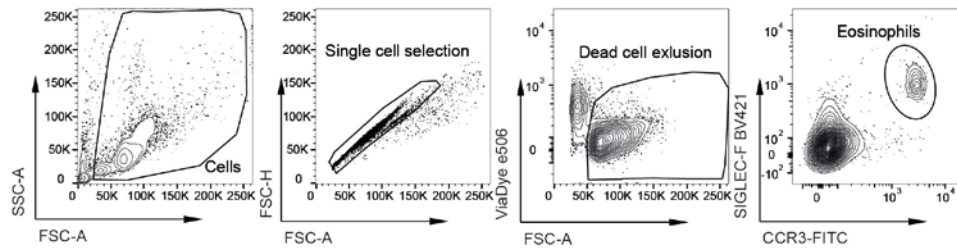
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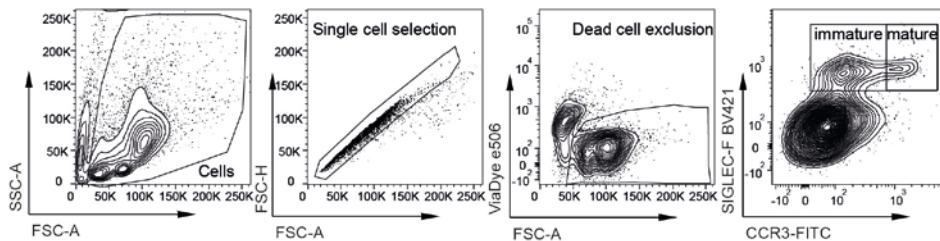
Figure S1
Lung



Blood eosinophils

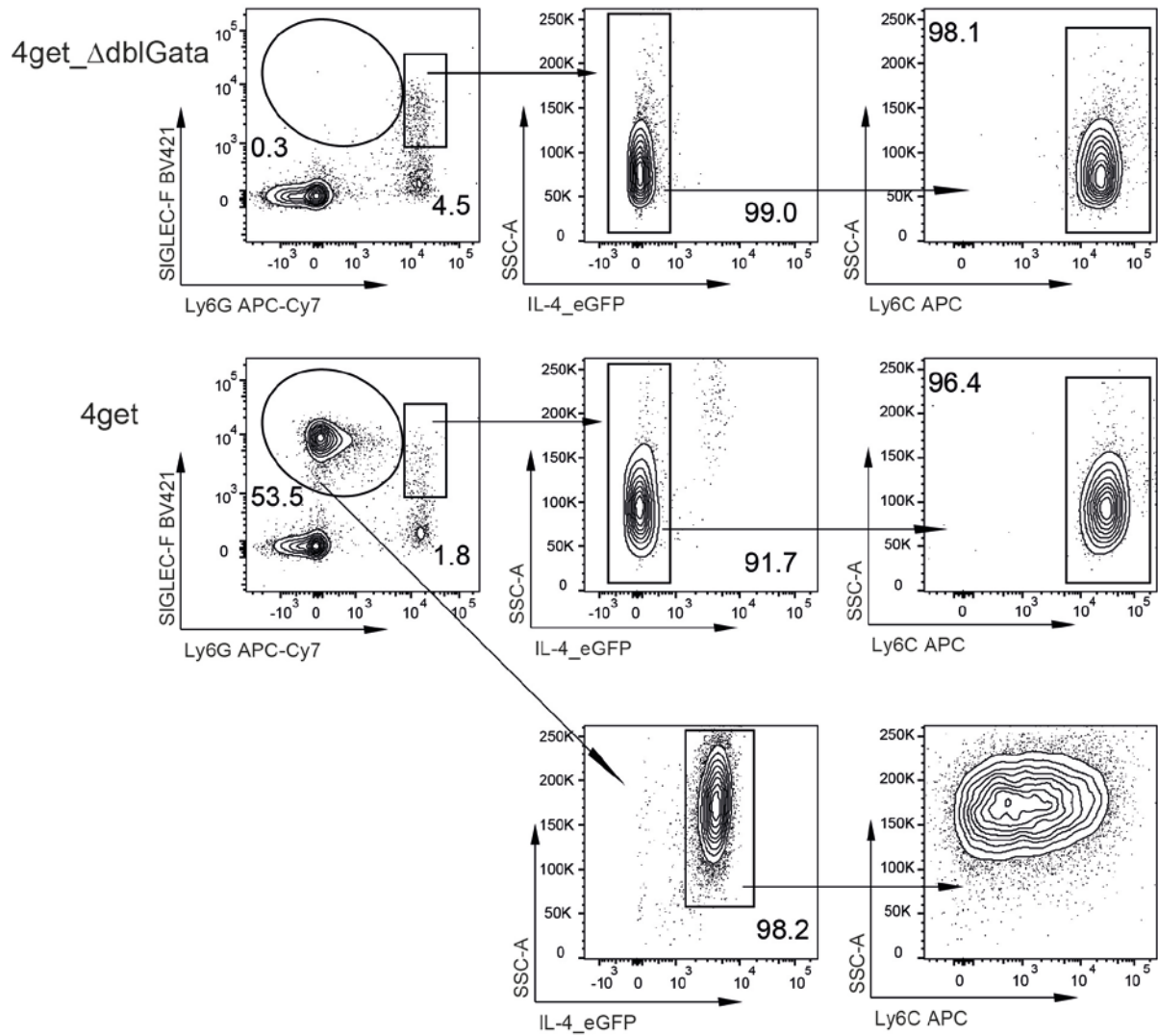


Bone marrow eosinophils



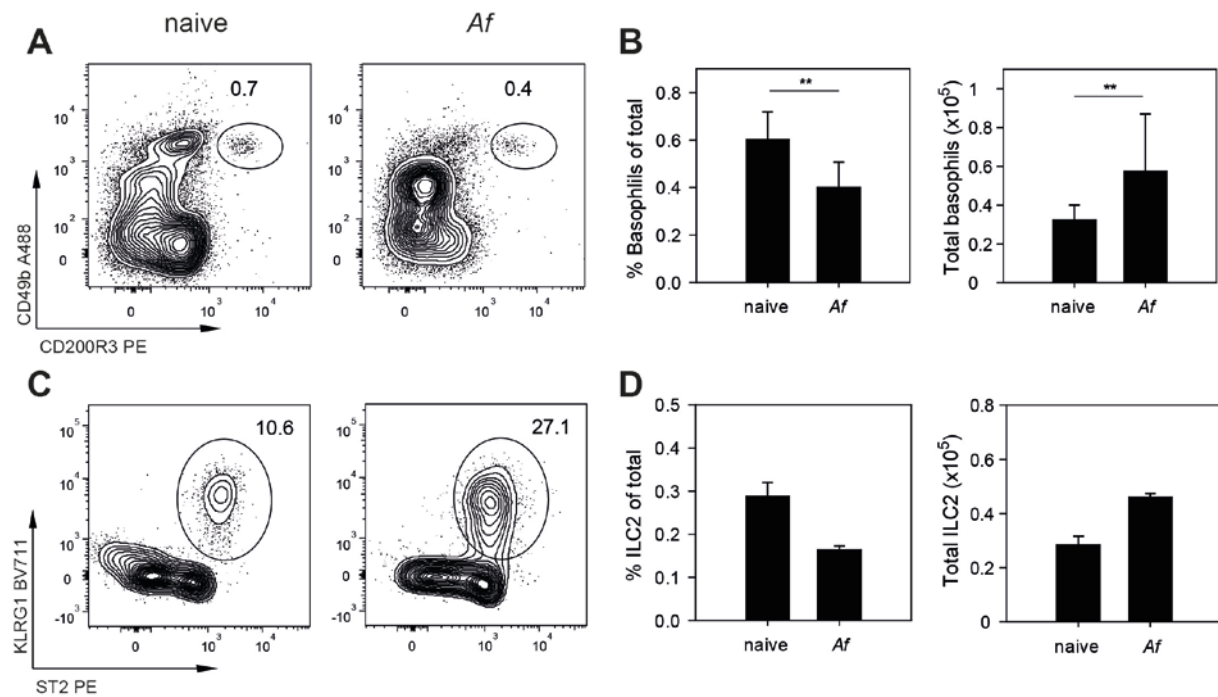
Supplemental Figure 1. Gating strategy to identify different cell types in tissues by flow cytometry. Single cell suspensions were stained with indicated antibodies and subjected to flow cytometric analysis. Hierarchical gating is shown from left to right with indicated gates within contour plots.

Figure S2



Supplemental Figure 2. Clarification of defining granulocyte gates. Comparison of SIGLEC-F/Ly6G double positive cells (IL-4_eGFP⁻/Ly6C⁺/SSC^{int}) being present in 4get and 4get_ΔdblGata, with bona fide eosinophils (SIGLEC-F⁺/IL-4_eGFP⁺/Ly6C^{variable}/Ly6G^{-/int}/SSC^{high}) being absent in 4get_ΔdblGata.

Figure S3



Supplemental Figure 3. Basophils and ILC2 numbers in repetitive low dose ABPA infection model. Representative flow cytometry plots of naïve and d17 Af-challenged lungs from Wt mice displaying **(A)** basophils and **(C)** ILC2. Relative and absolute quantification of **(B)** basophils and **(D)** ILC2. Bars show the mean+SD from one experiment with **(B)** 7 or **(D)** 2 mice per group. Statistical significance was determined by Student's t-test or Mann-Whitney U test in case of unequal variance.

Gene/Primer name	Sequence (5'-3')
Hprt_fw	GTTGGATACAGGCCAGACTTTGTT
Hprt_rv	GAGGGTAGGCTGGCCTATAGGCT
IL-4_fw	ACTTGAGAGAGATCATCGGCA
IL-4_rv	AGCTCCATGAGAACTAGAGTT
IL-5_fw	TCTGTACTCATCACACCAAG
IL-5_rv	AGGATGCTTCTGCACTTGAG
IL-13_fw	TGCTTTGTGTAGCTGAGCAG
IL-13_rv	GCAGTCCTGGCTCTTGCTTG
IFN γ _fw	TCGGATGAGCTCATTGAATG
IFN γ _rv	ACGCTACACACTGCATCTTG
IL-17A_fw	ACTACCTCAACCGTTCCACG
IL-17A_rv	CACACCCACCAGCATCTTCT
IL-23_fw	GCACCAGCGGGACATATGAATCTAC
IL-23_rv	GCAAGCAGAAGTGGCTGTTGTC
Ccl-11_fw	GAATCACCAACAACAGATGCAC
Ccl-11_rv	ATCCTGGACCCACTTCTTCTT
Ccl-17_fw	GAATGGCCCTTTGAAGTAA
Ccl-17_rv	TGCTTCTGGGACITTTCTG
Ccl-24_fw	AGGCAGTGAGAACCAAGT
Ccl-24_rv	GCGTCAATACCTATGTCCAA
RetnA_fw	CCATAGAGAGATTATCGTGGA
RetnA_rv	TGGTCGAGTCAACGAGTAAG
RetnB_fw	AAGCCTACACTGTGTTTCCTTTT
RetnB_rv	GCTTCCTTGATCCTTTGATCCAC
Gob5_fw	AAGCAGCTGTGCTTCAGCAG
Gob5_rv	CAGATTGGACTTATCCACAG
Arg1_fw	CTCGCAAGCCAATGTACACG
Arg1_rv	GTATGACGTGAGAGACCAGG
Muc5ac_fw	CTGTGACATTATCCCATAAGCCC
Muc5ac_rv	AAGGGGTATAGCTGGCCTGA
Cxcl1_fw	CCAAACCGAAGTCATAGCCACACTC
Cxcl1_rv	CCGTTACTTGGGGACACCTTTTAGC
Cxcl2_fw	GCTACAGGGGCTGTTGTGGC
Cxcl2_rv	GTCAGTTAGCCTTGCCTTTGTTCAG
Ccl2_fw	GCTCAGCCAGATGCAGTTAACG
Ccl2_rv	CTTGAGCTTGGTGACAAAACTACAGC

Table S1. The indicated primer sequences were used for RT-PCR analyses.