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STAT6 Regulates the Development of Eosinophilic versus Neutrophilic Asthma in Response to *Alternaria alternata*

Andrea C. Valladao,^{*,†} Charles W. Frevert,[‡] Lisa K. Koch,[†] Daniel J. Campbell,^{*,†} and Steven F. Ziegler^{*,†}

Human asthma is a heterogeneous disease characterized by the expression of both Th2 and Th17 cytokines. In vitro and in vivo studies have shown a reciprocal regulation between Th2 and Th17 pathways, suggesting a potential induction of neutrophil-promoting Th17 inflammation in the absence of a Th2 response. *Alternaria alternata* is a clinically relevant allergen that is associated with severe and fatal asthma exacerbations. Exposure to *A. alternata* is characterized by a predominant Th2 response, but can also induce the production of factors associated with Th17 responses (e.g., CXCL8) from epithelial cells. Using a mouse model, we found that wild-type mice develop an eosinophilic Th2 airway disease in response to *A. alternata* exposure, whereas IL-4^{-/-}, IL-13^{-/-}, and STAT6-deficient mice exhibit a primarily neutrophilic response. Neutrophilic asthma in STAT6^{-/-} mice was accompanied by elevated lung levels of TNF- α , CXCL1, CXCL2, and CXCL5, and was steroid resistant. Neutralization of Th17 signaling only partially reduced neutrophil numbers and total airway inflammation. Airway neutrophilia developed in RAG-deficient and CD4-depleted BALB/c mice, suggesting that the suppression of neutrophil responses is dependent on Th2 cytokine production by T cells and that airway neutrophilia is primarily an innate response to allergen. These results highlight the importance of combination therapies for treatment of asthma and establish a role for factors other than IL-17 as targets for neutrophilic asthma. *The Journal of Immunology*, 2016, 197: 4541–4551.

Asthma is a chronic inflammatory disease of the airways that can develop in response to a multitude of allergens. Fungi constitute one of the most important immunogens, with >80% of asthmatics in the United States having sensitization to one or more fungi; of these individuals, 75% have reactivity to *Alternaria alternata* (1). Large epidemiological studies have shown a strong correlation between *A. alternata* sensitization and an increased risk for severe and fatal asthma (2, 3). Mice exposed to *A. alternata* develop an allergic airway response characterized by increases in lung expression of the type 2 cytokines IL-4 and IL-13, eosinophil infiltration, and high levels of serum IgE (4–6).

IL-4 and IL-13 are critical to the development of allergic airway responses and are highly elevated in the bronchoalveolar lavage (BAL) fluid and sputum of asthmatic patients (7, 8). Recognition of these cytokines by a shared receptor unit, IL-4R α , results in the phosphorylation of the STAT6 (9, 10). STAT6 activation leads to cytokine-specific responses with IL-4 driving the differentiation of Th2 cells

and recruitment of eosinophils to the airways (11), and IL-13 inducing goblet cell hyperplasia and airway hyperresponsiveness (AHR) (12). Given their importance in the pathogenesis of asthma, IL-4 and IL-13 have become targets for the development of mAbs to modulate asthma. Clinical trials targeting IL-4 and IL-13 have had mixed results and overall only a minimal effect in disease burden (13).

In addition to the Th2 pathway, IL-17 and the Th17 pathway have been shown to play a key role in severe asthma pathology (14, 15). IL-17A mediates the development of neutrophilic airway inflammation via upregulation of CXCL chemokines (16, 17), AHR (18, 19), and corticosteroid resistance (18, 20). Moreover, Th17 cytokines can contribute to airway inflammation by collaborating with other cytokines, such as TNF- α , to upregulate the expression of neutrophil-attracting factors (21, 22).

Human asthma presents as a heterogeneous disease with various distinct phenotypes, including differential expressions of Th2 and Th17 signatures (23–25). IL-17A expression and neutrophils are present at high levels in patients with severe, persistent asthma (26, 27). Interestingly, IL-4 negatively regulates the differentiation of Th17 cells both in vitro (28, 29) and in vivo (30, 31), suggesting a possible correlation between blockade of the Th2 pathway and a potential increase in Th17-dependent neutrophilic airway inflammation (23, 31, 32). The cross-talk between Th2 and Th17 pathways and the development of different asthma phenotypes is still not completely understood. In this study, we used STAT6-deficient mice to show that: 1) IL-4 and IL-13 are necessary for the development of airway eosinophilia in response to *A. alternata*, and 2) blockade of STAT6 signaling promotes the development of nonatopic, neutrophilic asthma. Our findings suggest that patients with sensitization to *A. alternata* will benefit from therapies that target both eosinophilic and neutrophilic inflammatory responses.

Materials and Methods

Mice

Six- to eight-wk-old male and female BALB/c, Rag1^{-/-}, IL-4^{-/-}, and STAT6^{-/-} mice were obtained from Jackson Laboratories. IL-13^{-/-} mice

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; HDM, house dust mite; ILC, innate lymphoid cell; i.n., intranasal; KO, knockout; MCh, methacholine; PAS, periodic acid–Schiff; WT, wild-type.

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were a gift from Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). All animals were bred and housed in specific pathogen-free conditions at the Benaroya Research Institute animal facility, and all experiments were performed as approved by the Benaroya Research Institute Institutional Animal Care Committee.

Animal model

Mice received intranasal (i.n.) administration of 50 μ g of *A. alternata* extract (lots 188817 and 252218; Greer, Lenoir, NC) in a total volume of 50 μ l of PBS on days 0 and 1, rested, and then were challenged with 25 μ g of *A. alternata* extract on days 17, 18, and 19. Control mice were given PBS only. Mice were sacrificed 24 h after the final challenge, and inflammation was assessed as described later. For in vivo depletion of CD4⁺ cells, 200 μ g of anti-CD4 Ab (clone GK1.5; University of California, San Francisco Monoclonal Antibody Core) in a total volume of 200 μ l of PBS was injected i.p. on days -7, 0, 7, and 14. For in vivo depletion of innate lymphoid cells (ILCs), RAG knockout (KO) mice were given i.p. injections of 200 μ g of anti-Thy1.2 (clone 30H12; BioXCell) in a total volume of 200 μ l of PBS every 3 d for the length of the experiment. For IL-17RA and IFN- γ blockade, STAT6^{-/-} mice were injected i.n. on days 0, 1, 17, 18, and 19 with 200 μ g of anti-IL-17RA Ab (clone M751; Amgen) or anti-IFN- γ (clone XMG1.2; BioXCell) in a total volume of 50 μ l of PBS. Administration of the anti-IL-17RA and -IFN- γ Abs was followed 2 h later by i.n. administration of *A. alternata* extract as described earlier. For all experiments, control mice were given 200 μ g of isotype-matched rIgG in PBS. For steroid-sensitivity experiments, on days 17, 18, and 19, 2 h before each *A. alternata* challenge dose, mice were given i.n. doses of 40 μ g of dexamethasone (Bimeda-MTC Animal Health) in a total volume of 50 μ l of PBS or PBS only for controls.

Assessment of airway inflammation

At 24 h after the final challenge, mice were euthanized by i.p. lethal injection of 1 ml of 2.5% Avertin in PBS. BAL fluid was collected by intratracheal insertion of a catheter and four lavages of 1 ml of PBS. Lungs were perfused with 5 ml of PBS; the right upper lobe was removed and frozen in RNAlater (Thermo Fisher Scientific) for RNA isolation. The left lung was excised, digested with Liberase TM (Roche), and single-cell suspensions obtained. The remaining lung tissue was removed and placed in 10% neutral-buffered formalin (Leica Biosystems) for histological analysis. BAL TNF- α was detected by ELISA (R&D Systems). Lung tissue and BAL cellular differential counts were determined by flow cytometry analysis. For quantitative RT-PCR, RNA was isolated with the NucleoSpin RNA kit (Clontech). cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara), and mRNA expression levels were assessed using the SYBR Premix Ex Taq II (Takara) according to the manufacturer's instructions. The level of mRNA was normalized to GAPDH expression, and the results were analyzed by the 2^{- $\Delta\Delta C_t$} method.

Histology

Formalin-fixed lungs were embedded in paraffin, sectioned, and stained with H&E and periodic acid-Schiff (PAS) stains. Histological scores were determined by a blinded pathologist as follows: perivascular eosinophils (0–2), alveolar eosinophils (0–2), perivascular mononuclear cells (0–2), alveolar mononuclear cells (0–2), epithelial desquamation (normal = 0; elongation/distortion = 1; elongation, infolding, and narrowing = 2; loss of cells with broken airways = 3), area involved (<10% = 0, 10–75% = 1, >75% = 3), and goblet cell metaplasia by PAS stain (<5% = 0, 5–50% = 1, >50% = 2).

Abs and flow cytometry

The following Abs were purchased from BD Biosciences: purified CD16/CD32 (Fc block), anti-SiglecF PE (E50-2440), and anti-IFN- γ FITC (XMG1.2). The following Abs were purchased from BioLegend: anti-Ly6G FITC (1A8), anti-CD19 allophycocyanin (6D5), anti-CD45.2 PB (104), anti-CD4 BV650 (RM4-5), anti-B220 FITC (RA3-6B2), anti-CD19 FITC (6D5), anti-CD90.2 BV605 (53-2.1), anti-NKp46 PerCP-Cy5.5 (29A1.2), anti-CD25 BV421 (PC61), anti-ROR γ t PE (AFKJS-9), and anti-TCR β (H57-597). The following Abs were purchased from eBioscience: anti-CD8 PE-Cy5 (53-6.7), anti-CD11c PE-Cy7 (N418), anti-CD11c FITC (eBioN418), anti-CD45.2 PerCP-Cy5.5 (104), anti-CD3 eFluor 450 (17A2), anti-TCR $\gamma\delta$ FITC (eBioGL3), anti-MHC class II Alexa Fluor 700 (M5/114.15.2), anti-CD11b allophycocyanin-eFluor 780 and FITC (M1/70), anti-IL-17A PerCP-Cy5.5 (17B7), anti-TCR β Alexa Fluor 700 (IM7), anti-IL-13 PE (eBio13A), and anti-IL-4 PE-Cy7 (BVD6-24G2). Biotin anti-ST2 (DJ8) was purchased from MD Bioproducts. The following gating strategies were used: Eosinophils: CD45⁺CD11c⁺SiglecF⁺;

neutrophils: CD45⁺CD11c⁺CD11b⁺Ly6G⁺; T cells: CD45⁺CD4⁺TCR β ⁺; ILC stain: CD45.2⁺Lin⁺(CD11c, CD11b, TCR β , TCR $\gamma\delta$, B220, CD19)CD90.2⁺; ILC2: ST2⁺CD25⁺; ILC1: ST2⁺NKp46⁺ROR γ t⁺; ILC3: ST2⁺NKp46⁺ROR γ t⁺.

Single-cell suspensions were stained with Abs and analyzed on a BD LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo (Tree Star). For T cell and ILC intracellular staining, lung cells were cultured with PMA (1:2000; Sigma) and ionomycin (1:500; Sigma) in the presence of GolgiStop (BD Biosciences) for 3 h. Cells were then stained for surface markers and intracellular cytokines (Cytofix/Cytoperm; BD Pharmingen).

Measurement of AHR

Airway responsiveness was measured 24 h after the last challenge dose by invasive pulmonary function testing using the flexiVent system (Scireq, Montreal, QC, Canada). In brief, mice were anesthetized and paralyzed with i.p. injections of 50 mg/kg pentobarbital (Sigma-Aldrich) and 0.6 mg/kg vecuronium bromide, followed by tracheostomy and intubation to the flexiVent ventilator. After baseline measurement, mice were challenged for 10 s with saline aerosol, and at 4.5-min intervals with methacholine (MCh) at increasing concentrations (3.125, 12.5, and 50 mg/ml). For each MCh dose, the peak response was calculated as the mean of the six maximal values and used for calculation of airway resistance (cm H₂O/s/ml) and airway elastance (cm H₂O/ml).

Bone marrow chimeras

BALB/c CD45.1 and STAT6^{-/-} CD45.2 mice were used for reciprocal chimeras. Donor bone marrow was isolated from tibias and fibulas. Recipient mice were irradiated twice (4 h apart) with 450R, followed by tail vein injection of 1 $\times$ 10⁶ donor cells. Mice were allowed to reconstitute for 8 wk before the initial *A. alternata* dose.

Statistical analyses

A Student unpaired *t* test was used to determine statistical significance between two groups. Multivariable analysis was determined using one- or two-way ANOVA with Bonferroni's posttest. Data are presented as means $\pm$ SEM. Differences between groups were considered significant at the following values: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. All statistical analyses were performed using GraphPad Prism 6 software.

Results

Deficiency in Th2 cytokine signaling during *A. alternata* experimental asthma results in airway neutrophilia

Exposure to *A. alternata* induces airway inflammation characterized by increases in Th2 cytokines and infiltration of eosinophils (4–6). Recent reports of a house dust mite (HDM) murine model of asthma and an OVA-atopic march model have shown the development of a Th17 allergic phenotype after blockade of Th2 cytokines (23, 31). Considering the heterogeneity of Th2 and Th17 signatures in human asthma (23–25) and the in vitro regulation of IL-4 and IL-13 in IL-17 production (28, 29), we sought to determine the effects of IL-4 and IL-13 blockade in *A. alternata*-induced asthma. Wild-type (WT), IL-4^{-/-}, IL-13^{-/-}, and STAT6^{-/-} mice were intranasally sensitized with two consecutive doses of *A. alternata* extract, rested for 2 wk, and challenged three times. Disease development was assessed 24 h after the last challenge dose (Fig. 1A). Total cell infiltration in the BAL and lungs of all three KO strains was comparable with WT mice (Fig. 1B). However, whereas WT mice developed a predominant Th2 inflammation characterized by >50% of cells in the BAL consisting of eosinophils, IL-4^{-/-} and IL-13^{-/-} mice developed a mostly neutrophilic phenotype with eosinophils consisting of only a small percentage of total cell infiltrate. STAT6^{-/-} mice developed a primarily neutrophil (~40% of BAL cells) response with no detectable eosinophils (Fig. 1C, 1D). The change in lung inflammation between WT and KO strains was accompanied by decreases in lung expression of IL-4, IL-5, and IL-13 in IL-4^{-/-} and STAT6^{-/-} mice, as well as increases in IL-17A and IFN- γ in IL-4^{-/-} and STAT6^{-/-} mice, respectively (Fig. 1E). IL-13^{-/-}

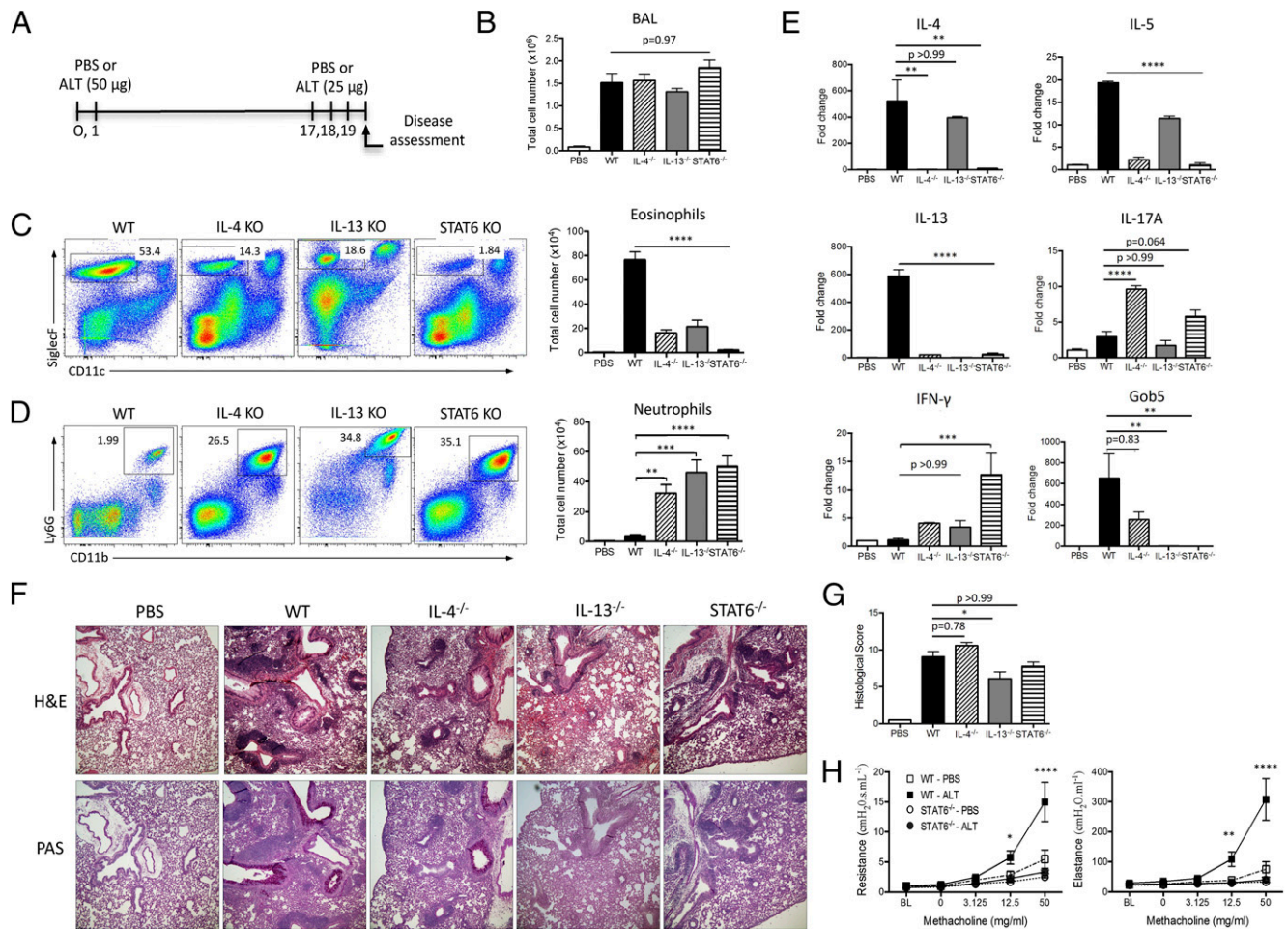


FIGURE 1. Deficiency in Th2 cytokine signaling during *A. alternata* experimental asthma results in airway neutrophilia. Model of *A. alternata* airway sensitization and challenge (**A**). Total cell numbers in BAL of WT, IL-4^{-/-}, IL-13^{-/-}, and STAT6^{-/-} mice (**B**). Percent BAL eosinophils (**C**) and neutrophils (**D**) by FACS (left) and total numbers (right). Lung mRNA expressed as fold increase over GAPDH (**E**). Representative histology images of H&E- and PAS-stained lung sections (original magnification ×40) (**F**) and inflammatory score (**G**). Lung resistance and elastance measured at baseline (BL) and increasing concentrations of MCh (**H**). Graphs show representative data of three independent experiments ($n = 3$ –5 mice per group) (**A**–**F**) and combined data from two independent experiments ($n = 4$ mice per group) (**H**). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

mice had comparable levels of IL-4 and IL-5 as that observed in WT mice, suggesting that IL-4 and IL-5 alone are not sufficient to drive airway eosinophilia.

Histologically, all mice displayed marked peribronchiolar and perivascular inflammatory infiltrates, as seen by H&E staining (Fig. 1F). PAS staining revealed decreased mucous production by IL-4^{-/-} mice and complete deficiency in IL-13^{-/-} and STAT6^{-/-} mice (Fig. 1F). These results were consistent with Gob5 expression by the different strains (Fig. 1E). Total lung inflammation, as assessed by histological score, was similar between WT and KO strains (Fig. 1G).

Excessive constriction of the airways after challenge with an allergen is a critical feature of asthma. Given that airway neutrophilia is associated with severe bronchoconstriction and persistent asthma (33), WT and STAT6^{-/-} mice were challenged with MCh to assess whether STAT6^{-/-} mice develop increased AHR. Airway reactivity was determined as a measure of lung resistance and elastance (Fig. 1H). *A. alternata* induced significant AHR in WT mice both at medium and at high doses of MCh (12.5 and 50 mg/ml). In contrast, STAT6^{-/-} mice did not display reactivity to MCh at any of the doses tested (Fig. 1H), showing that neutrophilia in response to *A. alternata* is not accompanied by AHR. Taken together, these results suggest that STAT6 signaling is

necessary for airway eosinophilia, and in the absence of this signaling pathway, *A. alternata* induces a neutrophilic airway response.

T cells are required for persistent airway eosinophilia but not neutrophilia in response to A. alternata exposure

To identify the cell types involved in the response to *A. alternata*, we performed bone marrow chimera studies. Host mice were irradiated and reconstituted with donor cells to create the following chimeric mice: WT $\rightarrow$ WT, WT $\rightarrow$ STAT6^{-/-}, and STAT6^{-/-} $\rightarrow$ WT. Eight weeks after irradiation, mice were challenged with *A. alternata* as previously described. In agreement with prior findings (5), STAT6 signaling deficiency in hematopoietic cells resulted in an inhibition of eosinophil infiltration into the BAL. Interestingly, these mice exhibited a neutrophil phenotype similar to that observed in full STAT6^{-/-} mice (Fig. 2A), suggesting that the regulation of airway eosinophilia versus neutrophilia by STAT6 occurs in hematopoietic cells.

Examination of various BAL cell types revealed no major differences in percent and total cell numbers between WT and KO mice (Supplemental Fig. 1A). However, given that Th2 cells have long been recognized as critical mediators of allergic asthma (34), we sought to determine the role of T cells in *A. alternata* airway

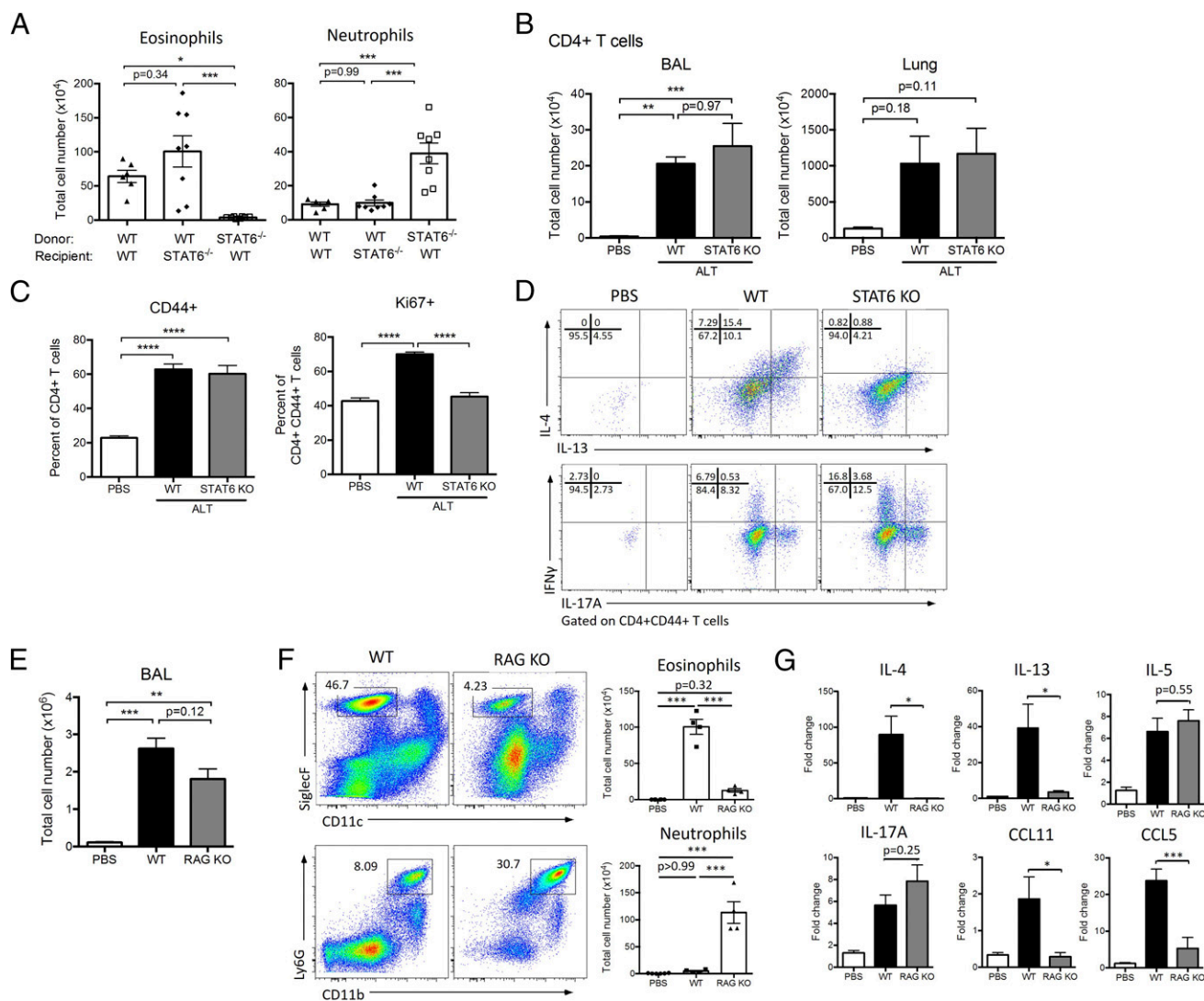


FIGURE 2. T cells are required for persistent airway eosinophilia but not neutrophilia in response to *A. alternata* exposure. Total number of eosinophils and neutrophils in BAL of WT and STAT6^{-/-} chimeric mice 24 h after last challenge (A). T cell profile in WT and STAT6^{-/-} mice after *A. alternata* challenge: total cell number in BAL and lung (B); activation and proliferation of lung CD4⁺ TCR β ⁺ cells (C); and intracellular cytokine staining of lung CD4⁺ T cells (D). WT and RAG^{-/-} mice airway response after *A. alternata* challenge. Total cell number in BAL (E). Percent BAL eosinophils and neutrophils by FACS (left) and total numbers (right) (F). Lung mRNA expressed as fold increase over GAPDH (G). Graphs show combined data from two independent experiments (A) and representative data from three independent experiments ($n = 3$ –5 mice per group) (B–G). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

responses. *A. alternata* exposure induced significant infiltration of CD4⁺ T cells in the BAL and lungs, with comparable numbers observed in WT and STAT6^{-/-} mice (Fig. 2B). Although both strains had similar amounts of activated T cells, as seen by CD44⁺ staining, STAT6^{-/-} mice had reduced numbers of proliferating CD4⁺Ki67⁺ T cells (Fig. 2C). In addition, STAT6^{-/-} mice had a higher percentage of IFN- γ ⁺ and IL-17A⁺ lung CD4⁺ T cells and a decrease in IL-4⁺IL-13⁺ double producers (Fig. 2D). To further determine the contribution of the adaptive immune system, disease development in response to *A. alternata* was assessed in RAG^{-/-} mice. Although not statistically significant, there was a trend toward reduced cell infiltrates in the BAL of RAG^{-/-} mice compared with WT mice (Fig. 2E). Interestingly, RAG^{-/-} mice developed a primarily neutrophilic response similar to that observed in STAT6-deficient mice (Fig. 2F). Lung mRNA expression showed significant decreases in IL-4 and IL-13, with little change in IL-17A (Fig. 2G). Although levels of IL-5 were unchanged in RAG KO mice compared with WT, the expression levels of CCL11 and CCL5 were significantly decreased (Fig. 2G) suggesting a need for these

chemokines in the persistence of lung eosinophilia. Similar results were obtained in WT mice depleted of CD4⁺ T cells (data not shown), suggesting that CD4⁺ T cells are necessary for the maintenance of airway eosinophilia in response to *A. alternata*. In contrast, CD4⁺ T cell depletion in STAT6^{-/-} mice only marginally affected total BAL cellularity and neutrophil numbers (Supplemental Fig. 1B, 1C), suggesting that the neutrophil phenotype is primarily an innate immune response.

ILCs do not drive lung neutrophilia in response to *A. alternata*

ILCs are critical components of the innate immune response to allergens. Activation of type 2 ILCs by IL-33 leads to the production of IL-5 and IL-13. The importance of this early response in the initiation of allergic airway responses has been shown in a model of *A. alternata* where absence of ILCs in the lung resulted in a complete ablation of the early eosinophil response to allergen (4). To determine the involvement of ILCs in lung neutrophilia after *A. alternata* exposure, we assessed the phenotype and cytokine production of ILCs in the lung of WT and STAT6^{-/-} mice

24 h after the last challenge dose. There were no major differences in the total number and percent of lung ILC1, ILC2, and ILC3 between WT and STAT6^{-/-} mice (Fig. 3A, Supplemental Fig. 2). Intracellular cytokine staining after ex vivo PMA-ionomycin stimulation showed no major differences in IFN- γ production by ILC1, but significant decreases in IL-5 and IL-13 by ILC2 in STAT6^{-/-} mice (Fig. 3B). Surprisingly, ILC3 from STAT6^{-/-} mice produced significantly less IL-17A compared with ILC3 from WT mice, suggesting that ILCs are not key contributors to airway neutrophilia. To confirm that ILCs were not involved in lung neutrophil infiltration after *A. alternata* exposure, we treated RAG KO mice with anti-CD90.2 Ab every third day during the length of the experiment to deplete ILCs. Control mice were treated with isotype IgG, and depletion efficiency was assessed by flow cytometry and total cell counts (Fig. 3D). Complete ablation of lung ILCs did not have any effect on the total cell number in BAL of RAG KO mice (Fig. 3C) or total amount of neutrophils infiltrating the lung (Fig. 3E), further demonstrating that ILCs are not driving airway neutrophilia.

Differential gene expression profile in lungs of WT and STAT6^{-/-} mice after *A. alternata* challenge

To further determine the factors contributing to the airway responses to *A. alternata*, we examined the gene expression of various critical lung cytokines and chemokines in WT and STAT6^{-/-} mice. Preliminary kinetic studies showed no differences in gene expression between WT and STAT6^{-/-} mice during the sensitization phase, but significant differences were observed

24 h after the first challenge dose (Supplemental Fig. 3A, 3B). Thus, an extended gene expression profile was further assessed at this time point. Compared with WT mice, STAT6^{-/-} mice had increased lung expression of the neutrophil-attracting chemokines CXCL1, CXCL2, and CXCL5, as well as increased expression of the IFN- γ -driven chemokines CXCL9 and CXCL10 (Fig. 4A). Furthermore, expression of the Th2-driven gene CCL11 and the alternative activating macrophage factors Arg1, Chi3l3 (Ym1), and Fizz1 was significantly downregulated in STAT6^{-/-} mice (Fig. 4B). No significant differences were observed in GM-CSF, IL-1 α , and IL-18 expression at this time point (Supplemental Fig. 3C). Interestingly, STAT6-deficient mice had increased lung expression of the macrophage-inducible C-type lectin (Mincle) and higher levels of TNF- α in BAL fluid (Fig. 4C, 4D), possibly implicating other innate cells, such as macrophages and DCs, as drivers of the neutrophil infiltration observed in the absence of STAT6 signaling.

Neutrophilic asthma in STAT6-deficient mice is partially dependent on IL-17A

IL-17A has been implicated as a key driver of neutrophilic asthma in both mice and humans (16, 21, 35). In addition, *A. alternata* exposure induces significant increases in lung tissue expression of IL-17A in both WT and STAT6^{-/-} mice and an increase in the number of CD4⁺IL-17A⁺ cells in the lungs of STAT6^{-/-} mice. Thus, to assess the role of IL-17A in the neutrophilic response, we immunized STAT6^{-/-} mice with anti-IL-17RA Ab 1 h before each challenge dose. Compared with control animals receiving

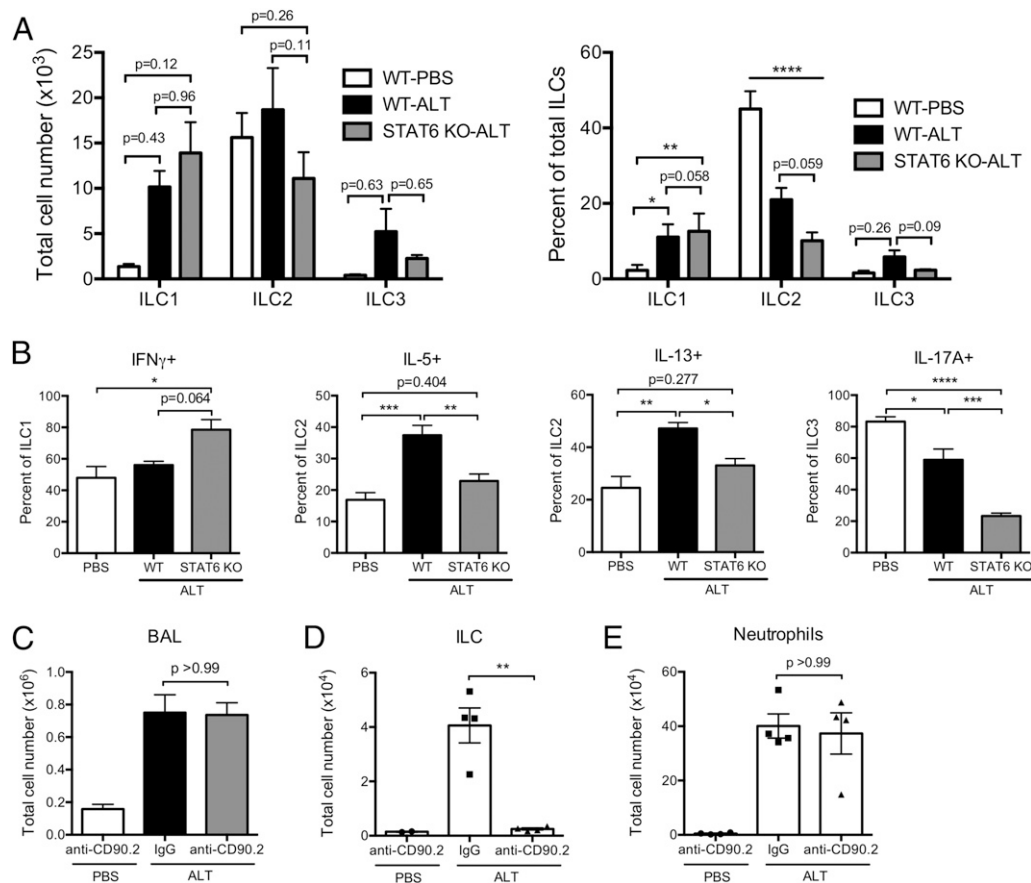


FIGURE 3. ILCs do not drive lung neutrophilia in response to *A. alternata*. Phenotype of lung ILCs in WT and STAT6^{-/-} mice. Total cell number and percent of ILC subsets (A). Percent of ILC subsets positive for intracellular cytokines (B). Airway inflammation in RAG^{-/-} mice treated with anti-CD90.2 Ab. Total cell infiltration in BAL (C). Total lung ILCs (D). Total number of neutrophils in lung (E). Graphs show combined data from three independent experiments ($n = 4$ mice per group). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

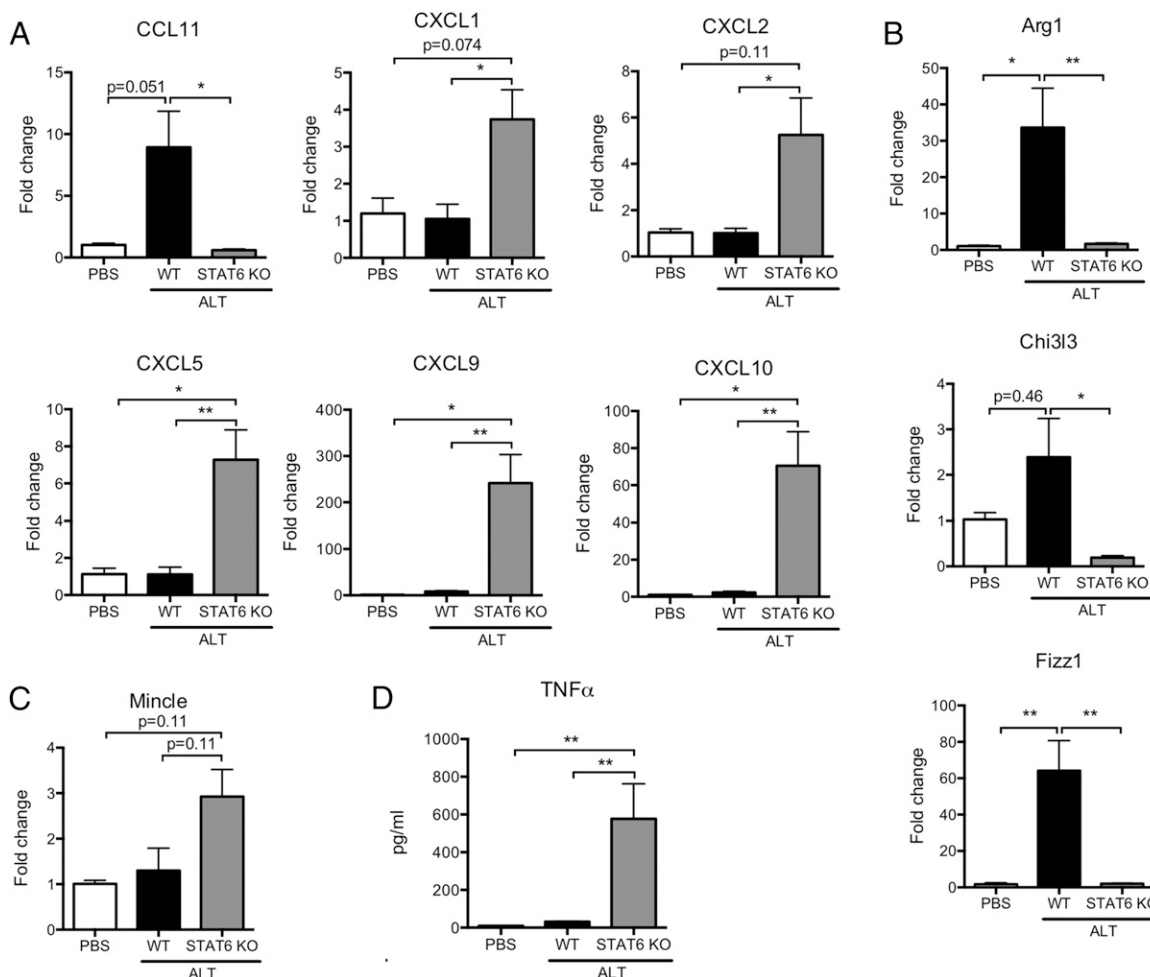


FIGURE 4. Differential gene expression profile in lungs of WT and STAT6 KO mice after *A. alternata* challenge. Lung mRNA expressed as fold increase over GAPDH 24 h after one *A. alternata* challenge: chemokines (**A**), macrophage-activating genes (**B**), and Mincle (**C**). Concentration of TNF- α protein in BAL fluid 24 h after one *A. alternata* challenge (**D**). Graphs show combined data from two independent experiments ($n = 4$ mice per group). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$).

anti-IgG Ab, treatment with the anti-IL-17RA Ab achieved a 50% reduction in total cell numbers in the BAL (Fig. 5A) and a decrease in the percent and total cell number of neutrophils and lymphocytes (Fig. 5B, 5C). The number of CD4⁺ IFN- γ ⁺ and CD4⁺IL-17A⁺ lung cells was slightly increased after anti-IL-17RA treatment (Fig. 5D). Histological analysis revealed diminished peribronchial cellular infiltrates and decreased epithelial thickening after blockade of the IL-17RA (Fig. 5E, 5F). These results suggest that neutrophil inflammation in response to *A. alternata* is partially dependent on IL-17A.

Airway neutrophilia in response to *A. alternata* is steroid resistant

To date, corticosteroids are the mainstream treatment for asthma due to their potent anti-inflammatory activity (36). However, severe, persistent asthma with neutrophil infiltrates is commonly steroid resistant (37). To assess the effect of steroids on *A. alternata*-induced asthma, we treated WT and STAT6^{-/-} mice with dexamethasone 1 h before each challenge dose. Dexamethasone significantly reduced the total number of cell infiltrates, specifically eosinophils and CD4⁺ T cells, in the BAL of WT mice compared with controls (Fig. 6A, 6B, 6D). In contrast, STAT6^{-/-} mice treated with dexamethasone had comparable levels of total BAL cellularity, percent, and number of neutrophils as that observed in control mice (Fig. 6A, 6C). Although not statistically

significant, there was a trend toward decreased IL-4, CCL11, and Gob5 expression in lungs of WT mice treated with dexamethasone (Fig. 6E). No differences were observed in expression levels of IL-5 and IL-13. In STAT6^{-/-} mice, dexamethasone treatment resulted in decreased lung expression of IFN- γ , but no changes in IL-17A, CXCL1, or CXCL5 (Fig. 6F). Histological examination in WT mice revealed diminished epithelial reaction and decreased cellular infiltrates after dexamethasone treatment compared with control (Fig. 6G, 6I). No changes were observed in PAS staining (Fig. 6G). STAT6^{-/-} mice did not display any changes in epithelial thickening or cell infiltrate as seen by H&E stain (Fig. 6H, 6I). These results demonstrate that eosinophil inflammation in response to *A. alternata* is steroid sensitive, whereas the neutrophilic response is steroid resistant.

Discussion

A. alternata is a clinically relevant allergen that has been associated with severe and fatal cases of asthma (1–3, 38). Studies into the pathogenesis of *A. alternata* have largely focused on the innate immune response during sensitization and the role of the epithelial-derived cytokines IL-33 and thymic stromal lymphopoietin (4–6, 39). In this study, we focus on the role of the adaptive immune system and the STAT6 signaling pathway in driving the persistence of pulmonary inflammation after *A. alternata* challenge, including the development of a previously unidentified airway neutrophilic response.

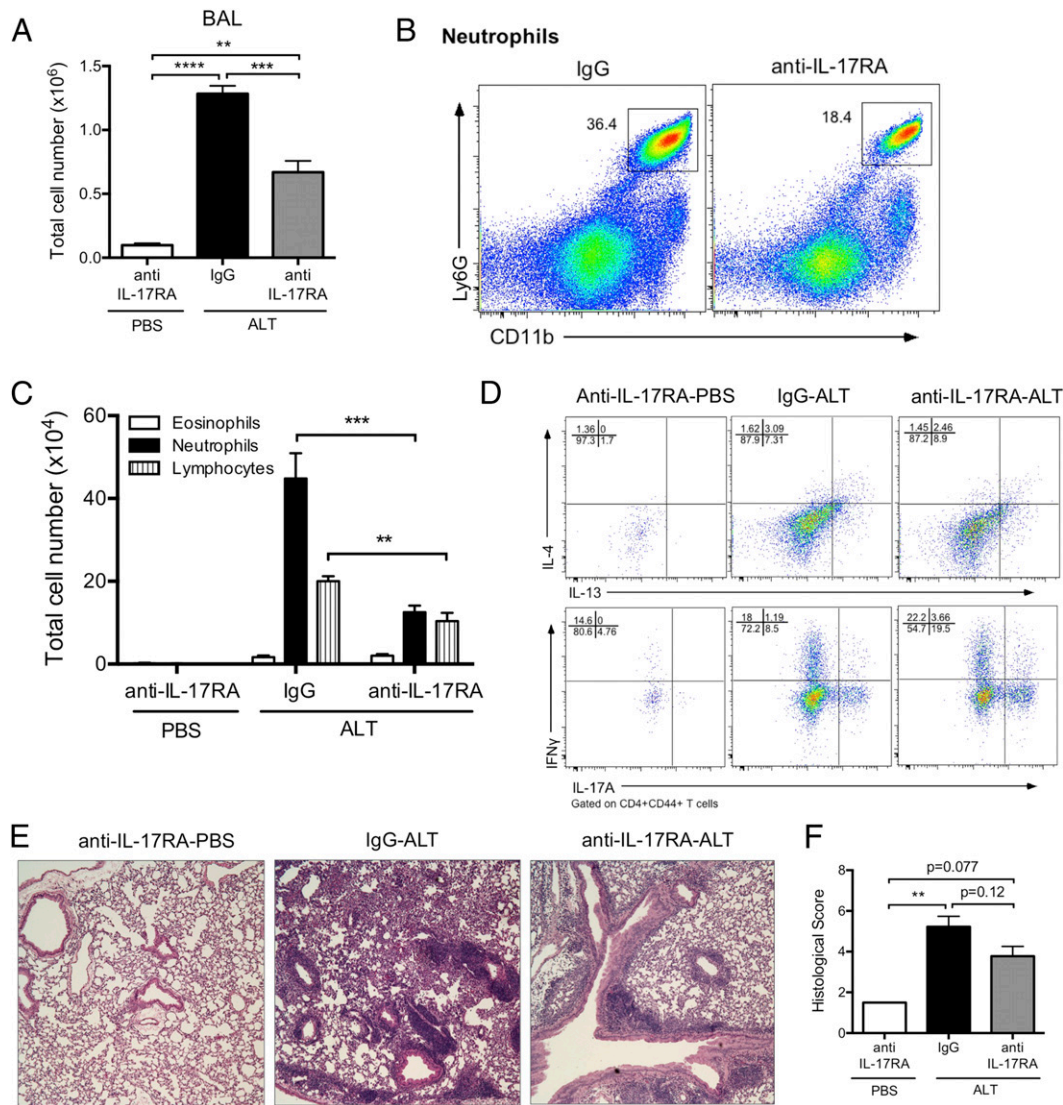


FIGURE 5. Neutrophilic asthma in STAT6 KO mice is partially dependent on IL-17A. Airway responses of STAT6 $^{-/-}$ mice treated with anti-IL-17RA or IgG control Ab. Total cell number in BAL (**A**). Percent BAL neutrophils by FACS (**B**). Total numbers of eosinophils, neutrophils, and lymphocytes in BAL (**C**). Intracellular cytokine staining in lung CD4 $^+$ T cells (**D**). Representative histology images of H&E-stained lung sections (original magnification $\times 40$) (**E**) and histological score (**F**). Graphs show combined data from two independent experiments ($n = 3$ –5 mice per group). All values are expressed as the means $\pm$ SEM (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The transcription factor STAT6 is required for IL-4 and IL-13 signaling, and it is essential for the induction of Th2-dependent airway responses (40, 41), as well as the late effector phases of asthma (10, 42). We and others (4–6) have shown that upon *A. alternata* exposure, mice exhibit increased lung expression of IL-4, IL-13, and IL-5 leading to the expansion of Th2 cells, airway eosinophilia, mucous production, and AHR. As expected, deficiency in the STAT6 signaling pathway resulted in a marked reduction in lung Th2 cytokine expression, eosinophilia, and mucous production. Airway eosinophilia was dependent on T cells, because WT mice depleted of CD4 $^+$ T cells failed to develop lung eosinophilia and Th2 airway inflammation. Our data demonstrate that *A. alternata*-induced production of IL-4 and IL-13 in the airways and activation of Th2 effector cells drives persistent airway eosinophilia and lung allergic inflammation.

Interestingly, even in the absence of a Th2-like eosinophilic response, STAT6-deficient mice challenged with *A. alternata* developed substantial airway disease. This response was characterized by significant neutrophil infiltration in BAL and lungs with

a higher percentage of IFN- γ $^+$ and IL-17A-producing CD4 $^+$ T cells. Given that eosinophilia in response to *A. alternata* is dependent on IL-33 (4) (Supplemental Fig. 1D, 1E), we sought to determine whether IL-33 also contributed to airway neutrophilia. Although IL-33-deficient mice had a significant increase in the percent and total cell number of neutrophils in the BAL compared with WT mice (Supplemental Fig. 1F), those numbers were substantially lower than those observed in STAT6-deficient mice (Fig. 1D) (average 5% and 5×10^4 neutrophils in IL-33 KO mice versus 35% and 50×10^4 in STAT6-deficient mice), indicating that there are other factors besides IL-33 regulating the eosinophil versus neutrophil response after *A. alternata* exposure. Unlike STAT6-deficient mice, IL-33R KO mice still have significant Th2 cytokine expression in the lung (Supplemental Fig. 1G). The presence of Th2 cytokines, albeit lower than that seen in WT mice, are likely sufficient to induce a Th2 eosinophilic response at the expense of a persistent neutrophil phenotype. Our results in STAT6-deficient mice are similar to models of HDM-induced asthma and OVA epicutaneous sensitization, and challenge where Choy et al. (23) and He et al. (31), respectively, showed that

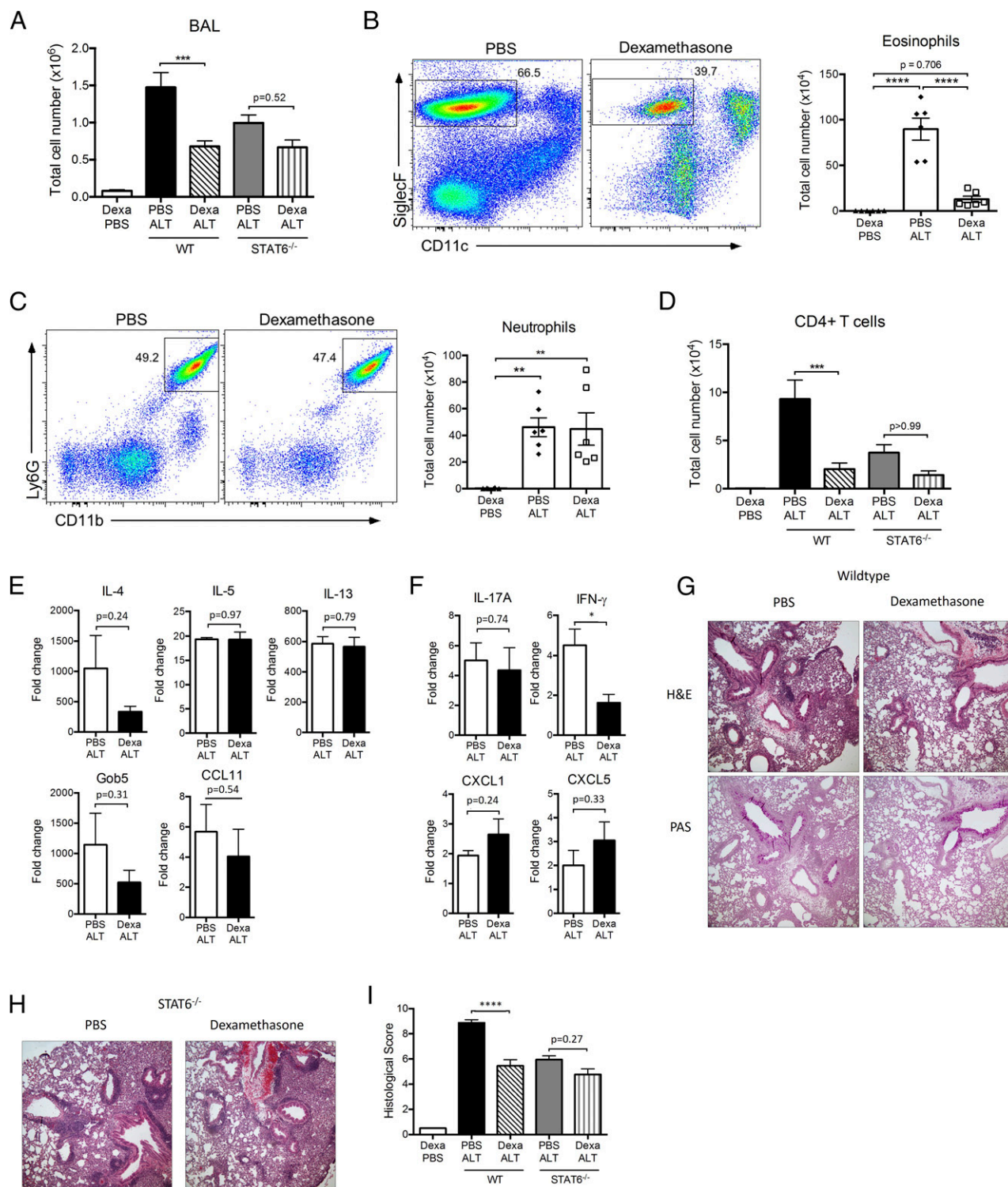


FIGURE 6. Airway neutrophilia in response to *A. alternata* is steroid resistant. Airway responses in WT and STAT6^{-/-} mice treated with dexamethasone before *A. alternata* challenge. Total cell number in BAL (A). Percent of BAL eosinophils in WT mice (B) and neutrophils in STAT6^{-/-} mice (C) by FACS (left) and total number (right). CD4⁺ T cell numbers in BAL of WT and STAT6^{-/-} mice (D). Lung mRNA expression of cytokines and chemokines in WT (E) and STAT6^{-/-} (F) mice. Representative H&E and PAS stains in WT lungs (G) and PAS stains in STAT6^{-/-} lungs (H). (G and H) Original magnification $\times 40$. Histological score determined by analysis of H&E stains (I). Graphs are representative of four independent experiments ($n = 3-6$ mice per group). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

blockade of IL-4 and/or IL-13 results in a switch from eosinophilic to neutrophilic inflammation in the airways. Taken together, these observations suggest a critical interplay between the STAT6 signaling pathway and the development of either eosinophilic or neutrophilic asthma in response to various allergens.

Along with previous studies (16, 17, 43), these reports have highlighted the role of the Th17 pathway as a key driver of neutrophilic asthma pathophysiology, further supporting reports implicating IL-4 and IL-13 in the inhibition of IL-17A protein expression both in vitro (28, 29) and in vivo (30, 31). Although Choy

et al. (23) demonstrated that reciprocal regulation of Th2 and Th17 responses in the airways leads to mutually exclusive phenotypes in asthmatic patients, they found no relationship between total counts of neutrophils in blood, sputum, or lamina propria and a Th17 molecular phenotype. In this article, we show that although there was a higher percentage of lung IL-17A-producing CD4⁺ T cells in STAT6^{-/-} mice compared with WT, there were no significant differences in the total lung IL-17A expression between both strains, suggesting that exposure to *A. alternata* induces IL-17A expression independent of the resulting inflammatory phenotype. In addition, blockade of the IL-17RA in STAT6^{-/-} mice failed to completely protect against neutrophil infiltration in the airways in response to *A. alternata* challenge. Moreover, neutrophil airway inflammation was observed in RAG-deficient mice, WT mice depleted of CD4⁺ T cells, and RAG-deficient mice depleted of ILCs, suggesting that IL-17A-producing cells contribute but are not required for the development of airway neutrophilia. Taken together, our results indicate that blockade of the Th2 pathway creates an environment where Th17 inflammation exacerbates the persistence of neutrophilia resulting from innate immune activation.

Higher expression of IFN- γ has been observed in patients with severe asthma and increased numbers of sputum neutrophils (36, 44, 45). Although, we found higher lung levels of IFN- γ expression and the IFN- γ -driven chemokines CXCL9 and CXCL10 in IL-4- and STAT6-deficient mice, acute neutralization of IFN- γ failed to protect against neutrophil accumulation in the lungs of STAT6 KO mice (Supplemental Fig. 4A–D). These results suggest that the Th1 pathway is not a key component in the development of airway neutrophilia and not a therapeutic candidate for neutrophilic asthma.

To better understand what other factors may be mediating neutrophil inflammation in response to *A. alternata*, we measured cytokine and chemokine expression in the lungs and BAL of STAT6^{-/-} mice after challenge with *A. alternata*. As expected, we found significant increased expression of the neutrophil-attracting chemokines CXCL1, CXCL2, and CXCL5, consistent with previous findings highlighting the role of these chemokines in neutrophilic asthma (21, 46, 47). This was accompanied by a complete decrease in Arg1, Fizz1, and Ch313, markers of alternatively activated macrophages (M2) and Th2 lung inflammation. Reduction of these markers might indicate the presence of classically activated macrophages (M1) in the lungs. We also observed increased levels of TNF- α in STAT6-deficient mice, which is consistent with various reports demonstrating a key role of TNF- α in neutrophil-mediated inflammation. Fei et al. (21) showed that TNF- α production by lung inflammatory DCs inhibited lung IL-5 expression, induced expression of CXCL1 and MIP-2, and promoted the development of airway neutrophilia at the expense of a Th2 eosinophilic response. IL-4 can also induce TNF- α mRNA destabilization leading to a reduction in neutrophil chemotaxis (48), providing further support to the role of STAT6 signaling in the inhibition of airway neutrophilia. We also observed, in lungs of STAT6^{-/-} mice, higher expression of Mincle, a macrophage-associated receptor that senses cell death and induces infiltration of neutrophils into damaged tissue (49).

Taken together, our studies show that *A. alternata* induces a potent early innate immune response characterized by production of neutrophil-attracting chemokines and cytokines. Further exposure to *A. alternata* results in the development of persistent Th2 eosinophilic lung inflammation that, when blocked, allows the early innate response to take over and present as neutrophilic asthma. Our model further supports the importance of IL-17A in airway neutrophilia, but adds to previous studies by showing that neutrophil inflammation in the lungs is driven by a variety of

factors that are differentially regulated in the presence or absence of the STAT6 signaling pathway.

Our findings are clinically relevant because we describe the pathology of *A. alternata*-induced asthma and the effects that different treatment options would have on disease persistence. We show that, whereas WT mice develop significant AHR, STAT6^{-/-} mice are protected from AHR and mucous production. Given that IL-13 and STAT6 signaling drive AHR and goblet cells metaplasia, it was expected that STAT6 KO mice would be protected from reactivity to MCh. However, *A. alternata* induces a severe lung disease with significant inflammatory cell infiltration in STAT6 KO mice that shares similar traits to neutrophilic asthma in humans (33). The apparent discrepancy between lung function and inflammation has been addressed in various studies where the relationship between neutrophils and IL-17A in the regulation and induction of AHR was assessed. Manni et al. (50, 51) found that in patients with neutrophil-dominant severe asthma, decreased forced expiratory volume in 1 s could be associated with both positive and negative changes in lung compliance, suggesting that there is a disconnect among airway inflammation, AHR, and lung compliance. Thus, lung stiffening may be mechanistically distinct from MCh responsiveness. In our model, we do not see AHR in STAT6 KO mice after challenge with *A. alternata*; however, lung histology analysis and overall cellular infiltrate point to strong airway inflammation.

Consistent with other asthma models (18, 52), we find that lung eosinophilia in WT mice is reduced upon dexamethasone treatment, whereas lung neutrophilia in STAT6^{-/-} mice is steroid resistant. This finding is important given that over the last decade, some clinical trials have explored the use of Abs against IL-4, IL-13, and/or IL-5 (13, 53, 54) as alternative treatments for both steroid-sensitive and steroid-resistant asthma. Unlike the success observed in clinical trials targeting IL-5 (54, 55), therapies targeting IL-4 and IL-13 have shown mixed results when reducing airway disease to allergens. Dupilumab, a humanized mAb against IL-4R α , was associated with fewer asthma exacerbations in patients with moderate-to-severe asthma and high type 2 phenotype (56). However, studies targeting patients with chronic asthma without first subsetting based on specific asthma phenotypes showed minimal benefits in reducing asthma exacerbations (57). The results obtained from these clinical trials highlight the complexity between different asthma phenotypes and suggest that treatments that are beneficial to one group might not have the same results on patients with different asthma signatures. Our studies suggest that some of the patients treated with Abs targeting Th2 cytokines might be suffering from a change in asthma phenotype toward a neutrophilic airway inflammation. Thus, treatment with corticosteroids would be insufficient, whereas alternative treatments such as IL-4 and IL-13 blockade would also be ineffective. Patients with sensitization to *A. alternata*, as well as other allergens such as HDM, would benefit from therapies that address both the Th2 eosinophilic response and the innate neutrophilic inflammation. Future studies will be aimed at determining the efficacy of various combination therapies including TNF- α and CXCR2 antagonists, in controlling airway eosinophilic and neutrophilic responses.

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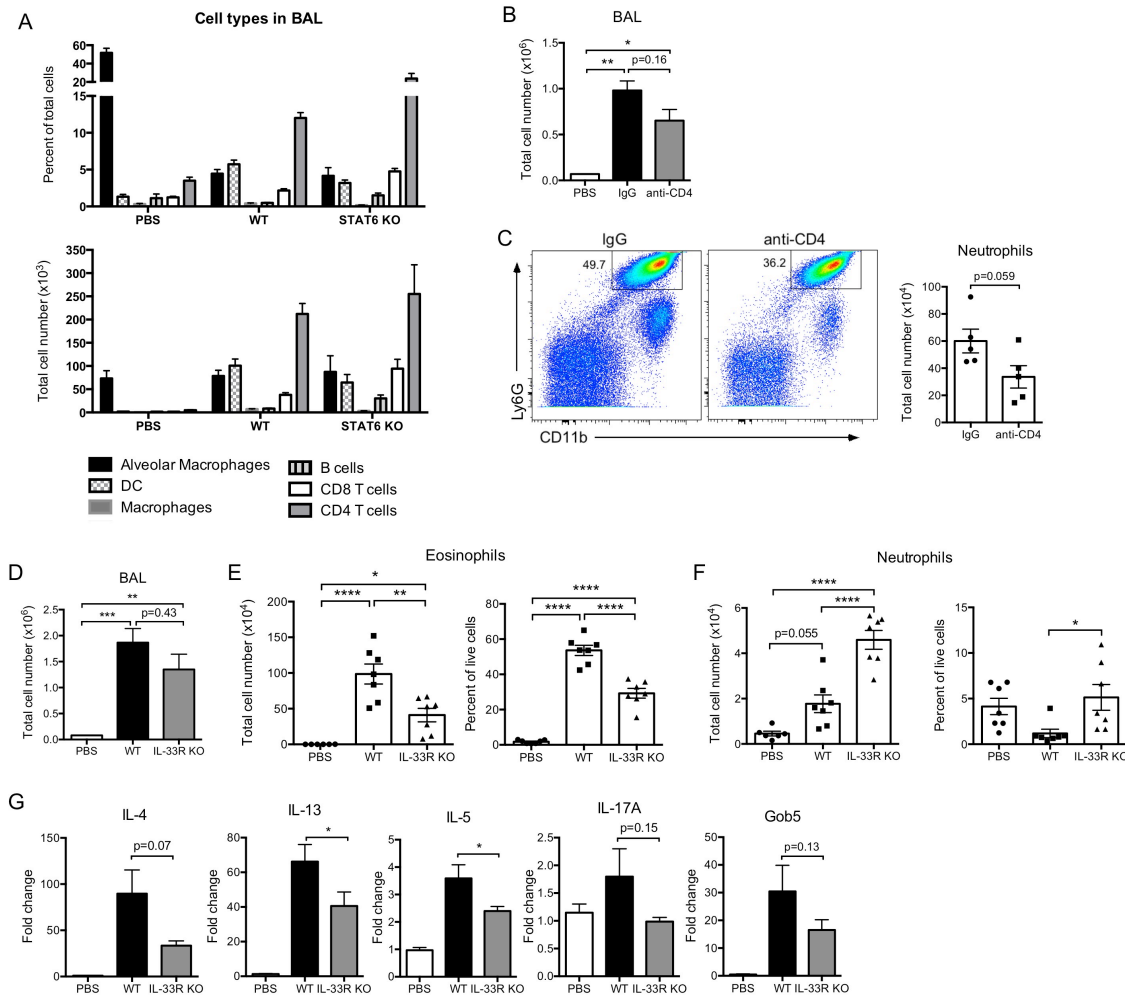
Disclosures

The authors have no financial conflicts of interest.

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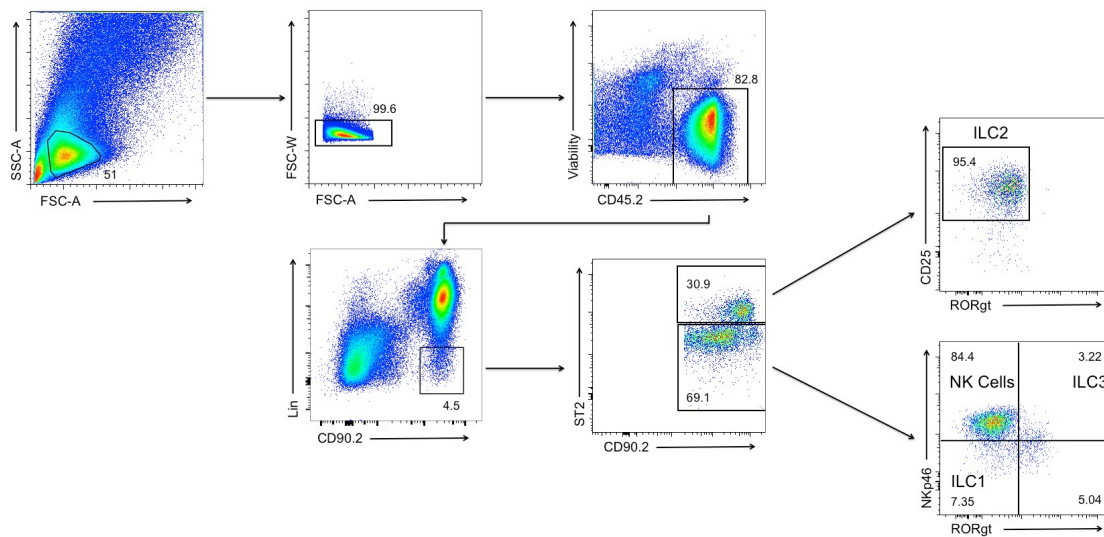
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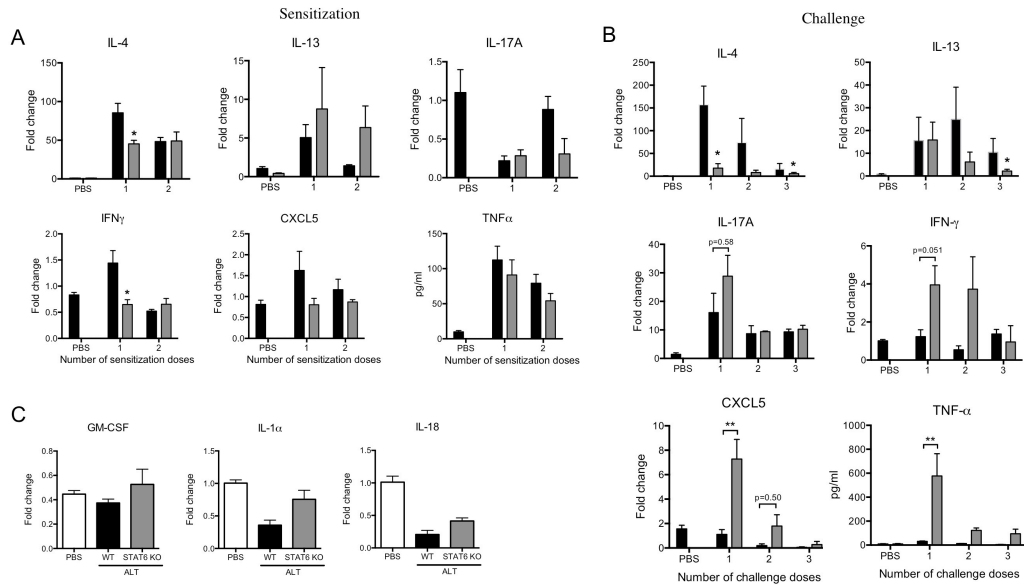


Supplemental Figure S1: Examination of cellular infiltrate in response to *A. alternata*.

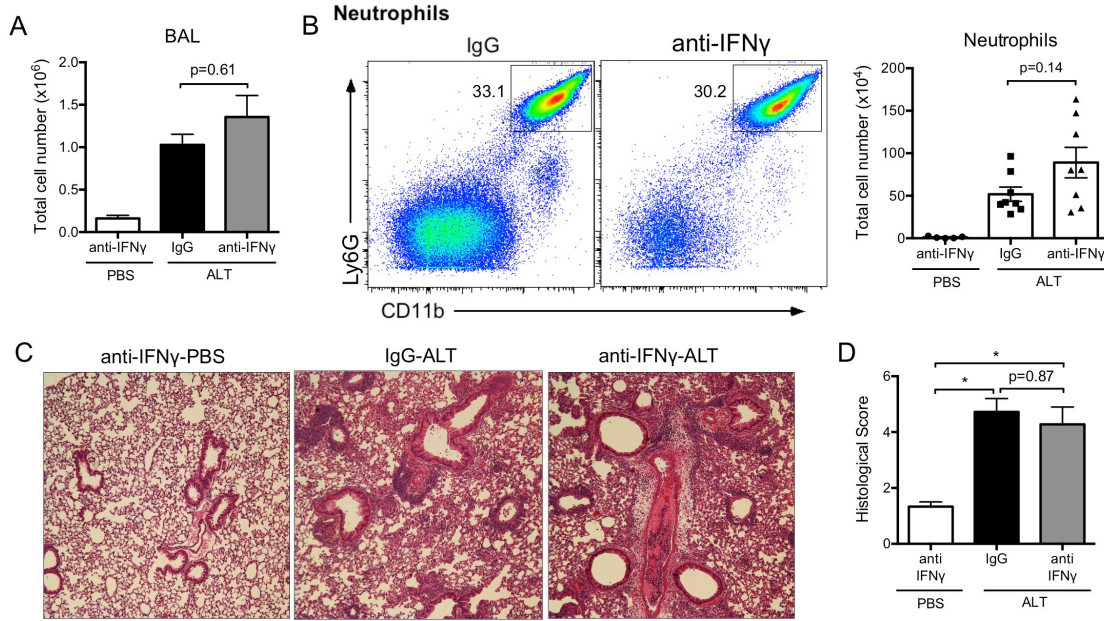
Cell types in BAL of WT and STAT6^{-/-} mice after ALT challenge (A). Airway responses of STAT6^{-/-} mice treated with anti-CD4 or IgG control antibody. Total cell number in BAL (B). Percent BAL neutrophils by FACS (left) and total numbers (right) (C). Airway responses of WT and IL-33R^{-/-} mice treated ALT. Total cell number in BAL (D). Total cell number and percent of BAL eosinophils (E) and neutrophils (F). Lung mRNA expressed as fold increase over gapdh (G). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs are representative of three independent experiments (n=3-6 mice/group) (A-C) and combined data from two independent experiments (D-G).



Supplemental Figure S2: Gating strategy for lung ILC phenotyping. Representative flow cytometry plots showing the strategy for identifying lung ILC populations by flow cytometry.



Supplemental Figure S3: Lung mRNA expression in response to *A. alternata* exposure. WT and STAT6^{-/-} lung mRNA (IL-4, IL-13, IL-17A, IFN γ , CXCL5) expressed as fold increase over gapdh and BAL TNF- α protein concentration 24 hours after one and two ALT sensitization doses (A) and one, two, and three ALT challenge doses (B). Lung mRNA (GM-CSF, IL-1 α , and IL-18) expressed as fold increase over gapdh 24 hours after the first ALT challenge dose (C). All values are expressed as the means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs show combined data from two independent experiments (n=4 mice/group).



Supplemental Figure S4. Neutrophilic asthma in STAT6 KO mice is not dependent on IFN γ . Airway responses of STAT6^{-/-} mice treated with anti-IFN γ or IgG control antibody. Total cell number in BAL (A). Percent BAL neutrophils by FACS (left) and total cell number (right) (B). Representative histology images of H&E stained lung sections (C) and histologic score (D). All values are expressed as the means $\pm$ SEM (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Graphs show combined data from two independent experiments (n=3-5 mice/group).