

Role of IL-18-transformed CD274-expressing eosinophils in promoting airway obstruction in experimental asthma

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Abstract

Background

IL-5-dependent residential and IL-18-transformed pathogenic eosinophils have been reported; however, the role of IL-18-transformed CD274-expressing pathogenic eosinophils compared to IL-5-generated eosinophils in promoting airway obstruction in asthma has not yet been examined.

Methods

Eosinophils are detected by tissue anti-MBP and anti-EPX immunostaining, CD274 expression by flow cytometry, and airway resistance using the Buxco FinePointe RC system.

Results

We show that *A. fumigatus*-challenged wild-type mice, and different gene-deficient mice including naïve CC10-IL-18-transgenic mice, accumulate mostly peribronchial and perivascular CD274-expressing eosinophils except naïve CD2-IL-5-transgenic mice. Additionally, we show that CD2-IL-5 transgenic mice following rIL-18 treatment accumulate high number of CD274-expressing perivascular and peribronchial eosinophils with induced collagen, goblet cell hyperplasia and airway resistance compared to saline-challenged CD2-IL5 transgenic mice. Furthermore, we also show that even *A. fumigatus*-challenged *IL-5*^{-/-} mice and rIL-18 given $\Delta db/GATA$ mice accumulate

CD274-expressing eosinophil-associated asthma pathogenesis including airway obstruction. Most importantly, we provide evidence that neutralization of CD274 and IL-18 in *A. fumigatus*-challenged mice ameliorate experimental asthma. Taken together, the data presented are clinically significant in establishing that anti-IL-18 neutralization is a novel immunotherapy to restrict asthma pathogenesis.

Conclusions

We demonstrate that IL-18 is critical for inducing asthma pathogenesis, and neutralization of CD274 is a potential immunotherapeutic strategy for asthma.

Abbreviations

ΔdblGATA

GATA1 deficiency

A. fumigatus

Aspergillus fumigatus

BALF

bronchoalveolar lavage fluid

EPX

eosinophil peroxidase

i.n

intranasal

IL

interleukin

***IL-18*^{-/-}**

IL-18 knockout

***IL-5*^{-/-}**

IL-5 knockout

MBP

major basic protein

MCh

methacholine

PAS

periodic acid schiff

PBS

phosphate-buffered saline

RI

airway resistance

Tg

transgenic mice

WT

wild type

1 INTRODUCTION

Asthma is a chronic inflammatory lung disease characterized by activation of inflammatory cells and mediators, variable obstruction, hyperresponsiveness, and remodeling of the airway.¹⁻³ In the United States, nearly 25 million people (8% of adults and 9% of children) suffer from asthma, and it is responsible for over 5000 deaths per year. Several studies have provided evidence that asthma is a Th2-type malady that is induced in response to food or environmental antigens.⁴⁻⁷ The lung inflammatory response in asthma is characterized by induced expression of multiple gene-encoding cytokines, chemokines, and adhesion molecules that are associated with the recruitment of eosinophils and Th2 lymphocytes.⁸ Asthma patients show evidence of food and aeroallergen hypersensitivity.⁹ Chronic asthma is also characterized by structural changes in the lung through a Th2-mediated eosinophilic inflammatory response known as airway remodeling.^{8, 10} Clinical studies and experimental modeling have established that Th2 cytokine (IL-4, IL-5, and IL-13) signaling is essential for disease pathogenesis.¹¹⁻¹³ These cytokines have a proven role in inducing eosinophilic inflammation in a number of tissues in several diseases.^{5, 14-16} Numerous studies in mice^{17, 18} and humans¹⁹ have supported an important role for eosinophilic inflammation in allergen-induced asthma. It is believed that asthma pathogenesis is prompted by the induction of IL-5-associated eosinophilia in response to several types of allergens. IL-5 is a well-established eosinophil differentiation and growth factor which is induced in several allergic diseases including asthma.^{20, 21} Of note, the intrinsic molecular pathway of IL-5-regulated eosinophilia is largely unknown and remains to be defined.⁹ Most recently, IL-5-responsive pathogenic eosinophil subsets have been reported in mice; however, they have not been validated in human allergic diseases.²² Anti-IL-5 therapy causes significant improvement in eosinophilia in asthma patients, but the eosinophilia returns to the tissue once therapy is withdrawn.^{15, 19, 23-25} Additionally, a clinical report demonstrated no optimal response to anti-IL-5 therapies in substantial prototype patients,²⁶ and functional tissue eosinophils were detected following post 750 mg mepolizumab treatment.²⁷ In addition, the effects of anti-IL-5 immunotherapy on several characteristics of asthma pathogenesis, like improved mucus production and airway resistance, have not been fully studied. It is possible that the long-term effects of anti-IL-5 may compromise innate immunity of the gastrointestinal tract, as eosinophils reside in the gastrointestinal tract and are involved in parasite exclusion. Recently, we presented our novel finding that IL-18 can generate IL-5-independent subsets of eosinophils and transform IL-5-responsive naive eosinophils into CD274-expressing pathogenic eosinophils.²² Several clinical reports show that IL-18 is induced in allergic patients, including asthmatics.²⁸⁻³³ CD274 (PDL1) is known to be expressed on T cells, B cells, macrophages, and dendritic cells (DCs).³⁴ Previous reports indicated that CD274 gene-deficient mice showed reduced levels of airway hyperreactivity and reduced asthmatic inflammation by enhancing production of IFN- γ by iNKT cells.³⁵ However, we previously reported that iNKT cells are not critical in promoting asthma pathogenesis, as *A. fumigatus*-challenged iNKT cell-deficient mice are not protected from eosinophilic inflammation.³⁶ Thus, the current study provides insight into the critical role of IL-18 and IL-18-responsive pathogenic CD274⁺ eosinophils in asthma pathogenesis, including promoting airway hyperresponsiveness in *A. fumigatus*- or cytokine-induced

asthma. Herein, we provide mechanistic evidence for the significance of IL-18-associated CD274 in experimentally induced asthma. Accordingly, we propose neutralization of IL-18 or CD274 as a potential target therapy for human asthma.

2 MATERIALS AND METHODS

2.1 Mice

Specific pathogen-free BALB/c, $\Delta db/GATA$ (GATA1 deficiency) BALB/c background mice, and C57BL6 and $IL-18^{-/-}$ C57BL6 background mice were obtained from the Jackson Laboratory. $IL-5^{-/-}$ and CD2-IL-5 transgenic (CD2-IL-5 Tg) BALB/c background mice were provided by Marc Rothenberg, MD, PhD (Cincinnati Children's Hospital Medical Center, Cincinnati OH).³⁷ CC10-IL-18 transgenic (CC10-IL-18 Tg), C57BL6 background mice were obtained from the laboratory of Dr. Jack Elias, MD, PhD, Yale University.³⁸ The mice were maintained in a pathogen-free barrier facility. All experiments were performed on age- and gender-matched 6- to 8-week-old mice. The Tulane Institutional Animal Care and Use Committee (IACUC) approved the animal protocols, which were employed in accordance with NIH guidelines. We used both C57BL6 and BALB/c strains of mice based on the availability of respective gene-deficient and transgenic mice. The CD2-IL-5 Tg, $IL-5^{-/-}$, and $\Delta db/GATA$ mice were BALB/c background and CC10-IL-18 Tg and $IL-18^{-/-}$ were C57BL6. The double-knockout $IL-5^{-/-}/IL-18^{-/-}$ mice were generated by mating individual $IL-5^{-/-}$ with $IL-18^{-/-}$ mice that provided $IL-5^{-/-}/IL-18^{-/-}$ double gene-deficient mice in F2 generation. Both $IL-5^{+/+}$ and $IL-18^{+/+}$ mice were used as littermate controls, as described previously.^{39, 40} Experimental asthma was induced using established methods.^{5, 41, 42}

2.2 Measurement of airway resistance (RI)

The trachea was surgically exposed in anesthetized mice and a cannula was inserted and tied with a suture to prevent air leakage.^{43, 44} Mice were placed in the Buxco Finepointe RC system chamber (Data Sciences International [DSI] St. Paul, MN) and connected to a ventilator. Mechanical ventilation was started with the appropriate respiratory rate and tidal/stroke volume. After two baseline measurements lasting 3 min, mice were exposed to phosphate-buffered saline (PBS) or methacholine (MCh) (3.125 to 50 mg/ml) and the airway resistance (RI) was measured as per the manufacturer's protocol.

Additional details regarding the method are provided in the [supporting information](#).

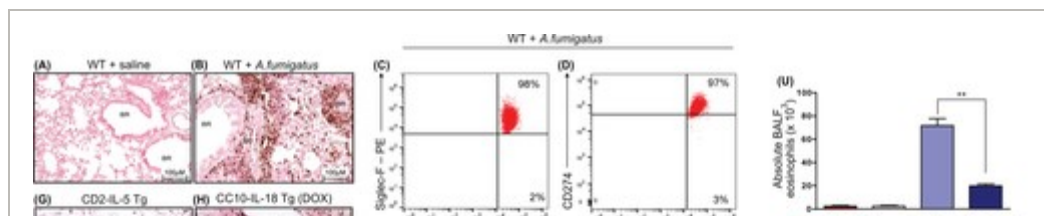
2.3 Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical significance comparing different groups of mice was determined using unpaired InStat GraphPad Prism5 Version 5.03 (San Diego, CA) software and the nonparametric one-way ANOVA Kruskal–Wallis test followed by Dunn's corrections for multiple comparisons. A p -value $< .05$ was considered statistically significant.

3 RESULTS

3.1 Analysis of eosinophil subtypes in the lungs of *A. fumigatus*-induced experimental asthma

Recently, we reported that IL-5 differentiated naïve eosinophils into the CD274-expressing eosinophils from bone marrow precursors in response to IL-18 and CD274-expressing and non-expressing eosinophils are present both in mice and humans.^{22, 33} Therefore, in this study, we examined the eosinophil subtypes in allergen-challenged experimental asthma. Experimental asthma was induced by challenging BALB/c mice with *A. fumigatus* extract as per established protocol.^{5, 42} Histopathological analysis of major binding protein (MBP) and morphometric quantification of lung sections showed that most of the eosinophils in *A. fumigatus*-challenged WT mice accumulated in the peribronchial and perivascular epithelium compared to very few eosinophils in the lung parenchyma of saline-challenged wild-type (WT) mice (Figure 1A,B). Our analysis detected ~97% CD274⁺CCR3⁺Seglec-F⁺ eosinophils in the BALF of *A. fumigatus*-challenged WT mice based on the isotype controls (Figure 1C–F). Furthermore, we showed that CD2-IL-5 Tg mice accumulate eosinophils in the lung parenchyma (Figure 1G) compared to the peribronchial and perivascular eosinophils accumulation in CC10-IL-18 Tg (DOX) mice (Figure 1H). Eosinophil accumulation in the peribronchial and perivascular regions is observed in all *A. fumigatus*-challenged *IL-5*^{−/−} (Figure 1L) and *IL-18*^{−/−} mice (Figure 1N), but in low numbers compared to *A. fumigatus*-challenged WT mice (Figure 1B,P). Saline-challenged mice do not show any eosinophils in the lungs (Figure 1I–K,M,O). BALF analysis of CCR3⁺Seglec-F⁺ eosinophils of *A. fumigatus*-challenged WT (Figure 1C,D) and *IL-5*^{−/−} (Figure 1Q,S) mice showed that more lung eosinophils express CD274 compared to the *A. fumigatus*-challenged *IL-18*^{−/−} mice (Figure 1R,T). The absolute number of BALF eosinophils (Figure 1U,V) along with CD274⁺ and CD274[−] eosinophils in *A. fumigatus*-challenged WT, *IL-5*^{−/−} (Figure 1Q,S), and *IL-18*^{−/−} mice is presented in Figure 1R,T. More BALF eosinophils in *IL-18*^{−/−} mice are CD274[−] eosinophils (Figure 1X) compared to *IL-5*^{−/−} mice (Figure 1W). Very few eosinophils show the expression of CD274 in *IL-18*^{−/−} mice. This may be caused by antibody cross-reactivity, as CD273 and CD274 have 70% amino acid homology,^{45, 46} or due to compensatory mechanism by which IL-1 family of some inflammatory cytokine bind to IL-18R and induce CD274 expression on cells.⁴⁷ The schematic representation of *A. fumigatus*-induced experimental asthma protocol is presented in Figure S1A with flow cytometry gating strategy for CCR3⁺ Siglec-F⁺ eosinophil analysis in Figure S1B. In addition, we show quantification of MBP⁺ tissue eosinophils in saline- or *A. fumigatus*-challenged WT, *IL-5*^{−/−} and *IL-18*^{−/−}, and CD2-IL-5 Tg and CC10-IL-18 Tg mice (Figure S1C,D,E).



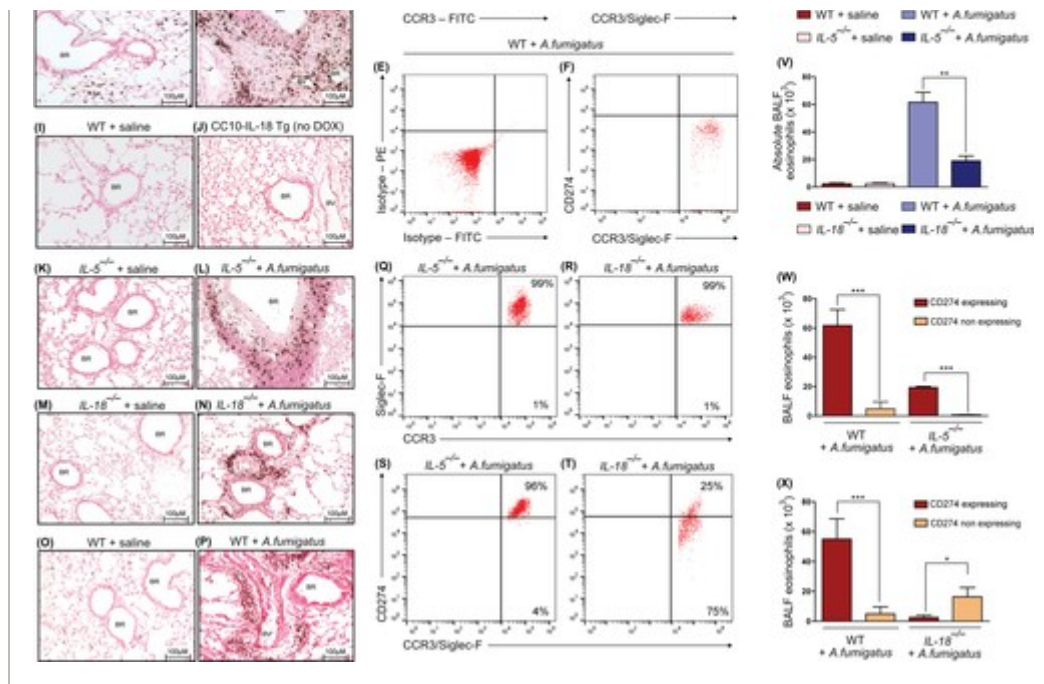


FIGURE 1

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Lung-accumulated eosinophils express CD274 following the induction of experimental asthma. A representative photomicrograph of anti-MBP-immunostained lung sections shows peribroncheal and perivascular eosinophil accumulation of MBP+ cells in an *A. fumigatus*-challenged WT (BALB/c) murine model of experimental asthma (A, B). A representative flow cytometry analysis of BALF live CCR3⁺Siglec-F⁺ eosinophils (C) and CD274⁺ eosinophils (D) based on isotype controls (E, F). A representative lung photomicrograph indicated most MBP⁺ eosinophils accumulate in the lung parenchyma of CD2-IL-5 Tg mice (G) compared to the peribroncheal eosinophil accumulation in DOX-inducible CC10-IL-18 Tg mice (N). Saline-challenged WT and CC10-IL-18 (no DOX) Tg mice show no baseline eosinophils (I, J). Similar peribroncheal eosinophils were observed in *A. fumigatus*-challenged *IL-5*^{-/-} (L) and *IL-18*^{-/-} (N) mice compared to no eosinophils in respective saline-challenged mice (K, M). Flow cytometry analysis detected CD274 expression in the BALF of all CCR3⁺Siglec-F⁺ eosinophils of *A. fumigatus*-challenged *IL-5*^{-/-} mice (Q, S) compared to mostly CD274⁻ eosinophils in *A. fumigatus*-challenged *IL-18*^{-/-} mice (R, T). BALF eosinophil quantitation shows a significantly increased number of eosinophils in *A. fumigatus*-challenged WT, *IL-5*^{-/-}, and *IL-18*^{-/-} mice compared to the respective saline-challenged WT (BALB/c, C57BL/6) mice (U, V). Significantly reduced CD274 expression in the BALF of *A. fumigatus*-challenged *IL-5*^{-/-} (W) mice compared to increased CD274 expression in *A. fumigatus*-challenged *IL-18*^{-/-} (X) with their respective *A. fumigatus*-challenged WT (BALB/c, C57BL/6) mice. Data are expressed as mean ± SD, *n* = 8–10 mice/group. **p* < .05; ***p* < .001; ****p* < .0001. Photomicrographs presented are 100X original magnification

3.2 Comparative analysis of IL-5, IL-13, and IL-18 in *A. fumigatus*-challenged WT and *IL-5*^{-/-} mice

BALF eosinophils and active cytokines IL-18, IL-5, and IL-13 were analyzed in *Aspergillus*-challenged WT and *IL-5*^{-/-} mice by performing ELISA analysis. The levels of IL-18 were highly elevated compared to IL-5 and IL-13 (Figure 2A–C). These data indicate that IL-5 may negatively regulate IL-18, which promotes eosinophil accumulation even in *IL-5*^{-/-} mice

following *A. fumigatus* challenge. The anti-NLRP3, anti-F4/80, and anti-IL-18 immunofluorescence staining shows that NLRP3 is induced in the F4/80⁺ lung-accumulated macrophages (Figure S2A, i-ii) and NLRP3-IL-18 colocalization further indicates that NLRP3 activation produces IL-18 in *A. fumigatus*-challenged mice (Figure S2B, i-ii). These data provide the evidence that lung-accumulated macrophages are the source of IL-18 in *A. fumigatus*-challenged mice. Additionally, eosinophil-induced airway resistance was analyzed in *A. fumigatus*-challenged WT and *IL-5*^{-/-} mice using the Buxco Finepointe RC system. This analysis indicated that both *A. fumigatus*-challenged WT and *IL-5*^{-/-} mice have increased airway resistance compared to respective saline-challenged mice (Figure 2D). Furthermore, goblet cell hyperplasia was detected in *A. fumigatus*-challenged *IL-5*^{-/-} mice and WT mice, but no goblet cells were observed in saline-challenged mice (Figure 2E). Quantitative analysis showed reduced goblet cells in *IL-5*^{-/-} mice compared to WT mice (Figure 2F). We also observed significantly reduced collagen accumulation in *A. fumigatus*-challenged *IL-5*^{-/-} mice compared to WT mice (Figure 2G). The reduced airway resistance in *A. fumigatus*-challenged *IL-5*^{-/-} mice compared to WT mice is consistent with reduced levels of IL-13 protein (Figure 2B), mRNA levels (Figure S1F), goblet cell hyperplasia, and collagen accumulation in the lungs of *A. fumigatus*-challenged *IL-5*^{-/-} mice compared to WT mice. Interestingly, we observed induced MUC1 and MUC5AC protein levels in both *A. fumigatus*-challenged WT and *IL-5*^{-/-} mice (Figure 2H). These data indicate that IL-5 is redundant for the generation of the CD274-expressing eosinophils that is responsible for promoting airway obstruction even in allergen-challenged *IL-5*^{-/-} mice.

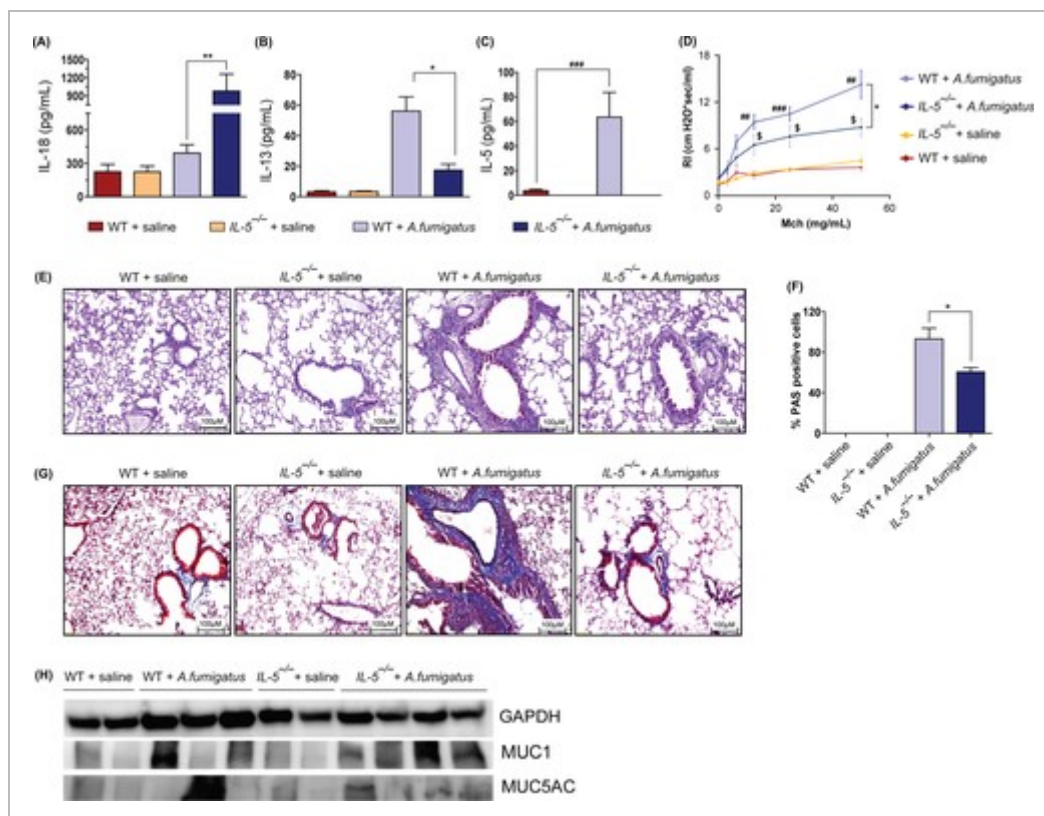


FIGURE 2

Induced IL-18 is associated with airway resistance and goblet cell hyperplasia in *A. fumigatus*-challenged WT and *IL-5*^{-/-} mice. ELISA analysis detected elevated levels of IL-18 (A), decreased levels of IL-13 (B), and no IL-5 (C) in the BALF of *A. fumigatus*-challenged *IL-5*^{-/-} mice compared to *A. fumigatus*-challenged WT (BALB/c) mice. Airway resistance (RI) in response to different concentrations of methacholine (Mch) is shown in *A. fumigatus*-challenged *IL-5*^{-/-} and WT mice (D). A representative photomicrograph of goblet cell hyperplasia (E) and quantitation of PAS-positive goblet cells in WT, *IL-5*^{-/-}, *A. fumigatus*-challenged WT, and *A. fumigatus*-challenged *IL-5*^{-/-} mice (F). Representative photomicrographs of lung sections with Masson's trichrome staining show perivascular and peribronchiolar collagen accumulation in the *A. fumigatus* and WT mice compared to *IL-5*^{-/-} and *A. fumigatus*-challenged *IL-5*^{-/-} mice (G). Immunoblot analysis of MUC1, MUC5AC, and GAPDH protein levels in *IL-5*^{-/-} and WT (BALB/c) *A. fumigatus*-challenged mice (H). Data are expressed as mean ± SD, *n* = 8–10 mice/group. **p* < .01, ***p* < .001; ****p* < .0001 (WT + *A. fumigatus* vs. *IL-5*^{-/-} + *A. fumigatus*); ##*p* < .001, ###*p* < .0001 (WT + *A. fumigatus* vs. WT + saline); and \$*p* < .04 (*IL-5*^{-/-} + *A. fumigatus* vs. *IL-5*^{-/-} + saline). Photomicrographs presented are 100X original magnification

3.3 Analysis of CD274⁺ eosinophils induced upon administration of rIL-18 in CD2-IL-5 transgenic mice

We previously showed that most of the eosinophils present in CD2-IL-5 transgenic mice do not express CD274.²² Therefore, we next examined whether rIL-18-challenged mice can transform all eosinophils into the CD274⁺ pathogenic eosinophils that exacerbate airway resistance and other characteristics of asthma pathogenesis. Accordingly, both naive WT mice and CD2-IL-5 transgenic mice were challenged with 10 µg of rIL-18 in 50 µl saline or saline alone via intranasal (i.n.) route as in the presented schematic protocol (Figure S2C). We used the i.n. route to examine local overexpression of IL-18-induced responses in eosinophilic asthma, which has been previously reported.^{38, 48, 49} CCR3⁺Siglec-F⁺ eosinophil samples in the BALF of saline and rIL-18-challenged WT mice and CD2-IL-5 transgenic mice were analyzed for CD274 expression using flow cytometry. BALF analysis indicated that rIL-18-challenged WT mice showed a larger number of CCR3⁺ and Siglec-F⁺ eosinophils compared to saline challenged (Figure 3A,B), which was further increased by ~3-fold in rIL-18- challenged CD2-IL-5 transgenic mice (Figure 3C,D). The rIL-18-treated CD2-IL-5 transgenic mice had a significantly higher number of CD274⁺ eosinophils compared to rIL-18-challenged WT mice (Figure 3F–H). The quantification of eosinophils in BALF showed that rIL-18-challenged CD2-IL-5 transgenic mice have ~3-fold higher number of induced CD274⁺ eosinophils compared to rIL-18-challenged WT mice (Figure 3I,J). rIL-18 treatment induced peribronchial and intraepithelial accumulation of eosinophils in the lungs of CD2-IL-5 transgenic mice compared to rIL-18-challenged WT mice. Saline-treated controls showed fewer baseline eosinophils (anti-MBP) in the lungs of compared to rIL-18-challenged WT and CD2-IL-5 transgenic mice (Figure 3K). Additionally, the anti-EPX staining revealed very high number of perivascular and peribronchial intact and degranulated eosinophils (extracellular eosinophilic granules) in rIL-18-challenged CD2-IL-5 transgenic mice compared to saline-challenged mice (Figure 3L). In comparison, anti-MBP staining revealed a low number of mostly intact eosinophils in rIL-18-challenged CD2-IL-5 transgenic mice (Figure 3M).

Furthermore, we observed increased PAS-stained goblet cell hyperplasia in rIL-18-treated CD2-IL-5 transgenic mice compared to WT mice, and very few to none were detected in saline-treated CD2-IL-5 transgenic mice and WT mice, respectively (Figure 3N,O). Furthermore, to establish that rIL-18-challenged CD274⁺ eosinophils have a critical role in promoting airway resistance, we examined airway resistance in rIL-18- challenged WT and CD2-IL-5 transgenic mice in response to different concentrations of methacholine. The rIL-18-challenged CD2-IL-5 transgenic mice showed significantly increased airway resistance compared to rIL-18-challenged WT mice (Figure 3P). Saline-challenged CD2-IL-5 transgenic mice showed similar airway resistance as rIL-18-challenged WT mice; this may be due to the presence of baseline endogenous IL-18 in CD2-IL-5 transgenic mice (Figure 3Q). Furthermore, a significant increase in the levels of eosinophil-active cytokines IL-5 and IL-13 was observed in rIL-18-challenged CD2-IL-5 transgenic mice and WT mice compared to saline-challenged mice (Figure S2D,E). We also observed induced collagen accumulation in the lungs of rIL-18-challenged CD2-IL-5 transgenic mice compared to WT mice (Figure S2F, i-iv). The morphometric quantitation of MBP⁺ and EPX⁺ cells in the lungs of saline- and rIL-18-challenged WT and CD2-IL-5 transgenic mice is presented in Figure S2G,H. These data establish that IL-18 is indeed critical in transforming IL-5-responsive eosinophils to CD274⁺ pathogenic eosinophils that promote asthma pathogenesis.

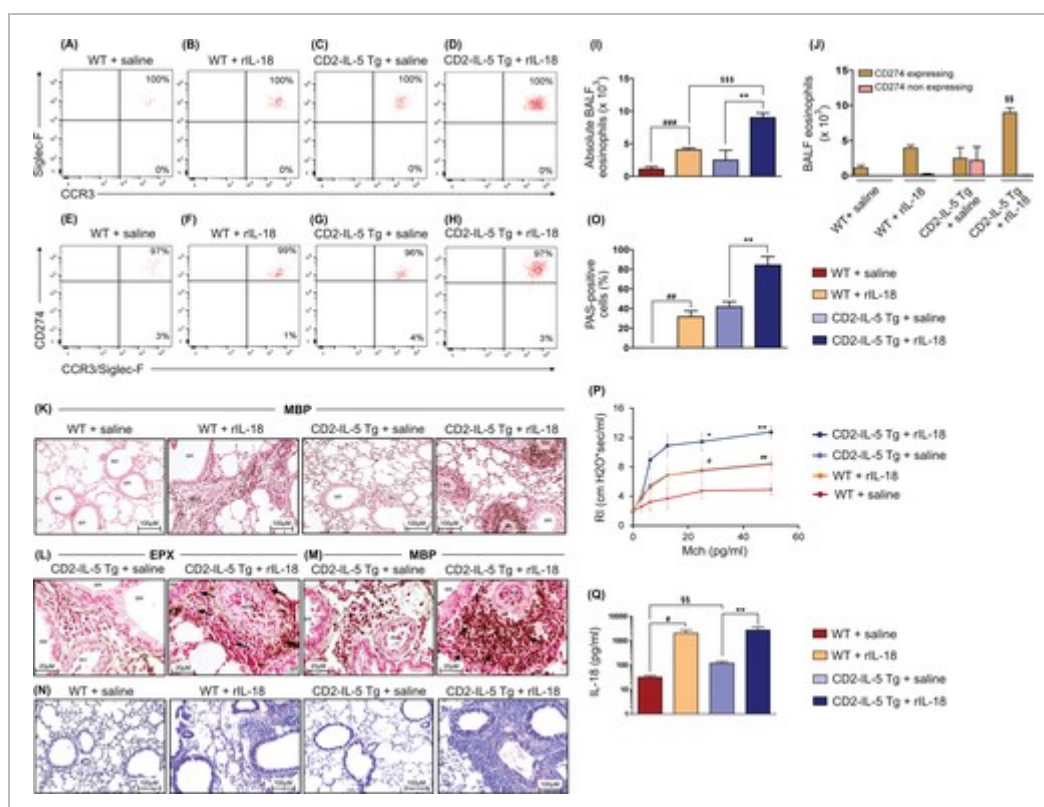


FIGURE 3

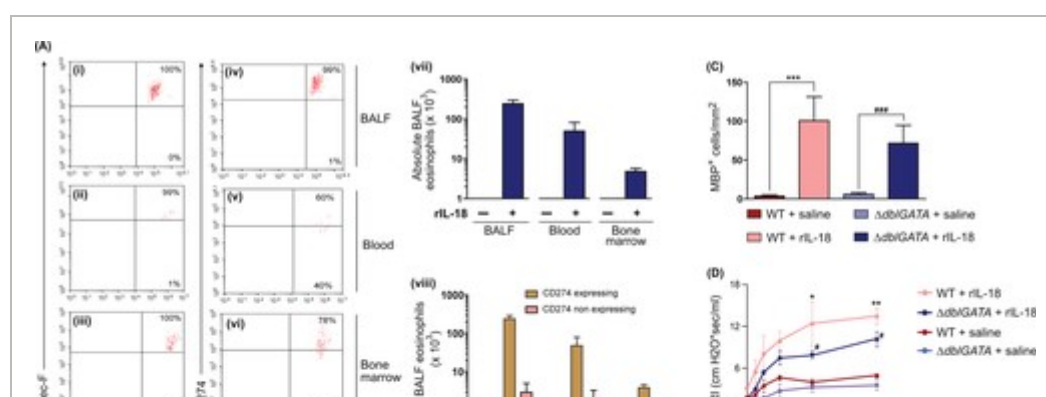
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Asthma pathogenesis in IL-18-induced CD274⁺ pathogenic eosinophils in CD2-IL-5 transgenic mice. Induced detection of CCR3⁺Siglec-F⁺ eosinophils (A–D) and CD274 expression (E–H) in the BALF of CD2-IL-5 Tg and WT (BALB/c) rIL-18-treated mice compared to respective controls. The quantification of CD274⁺ eosinophils in BALF of saline- and rIL-18-treated WT

mice and CD2-IL-5 Tg mice (I, J). A representative photomicrograph lung section of anti-MBP immunostained lung sections of saline- and rIL-18-treated WT and CD2-IL-5 Tg (K) detected high numbers of MBP⁺ eosinophils. The anti-EPX stained tissue sections show intact and degranulated (extracellular granules) eosinophils (indicated by black arrows) (L) compared to intact anti-MBP stained eosinophils (indicated by black arrows) in tissue sections of saline- and rIL-18-treated CD2-IL-5 Tg mice (M, original magnification 400X). A representative photomicrograph of PAS-stained goblet cell hyperplasia and quantitation of rIL-18-treated CD2-IL-5 Tg mice compared to WT mice (N, O). Airway resistance (RI) in response to methacholine was measured in rIL-18- or saline-treated CD2-IL-5 Tg and WT mice (P). ELISA analysis detected highly induced protein levels of IL-18 in the serum of rIL-18-treated CD2-IL-5 Tg mice compared to WT mice (Q). Data are expressed as mean $\pm$ SD, $n = 10$ – 12 mice/group. $*p < .02$, $**p < .001$; $***p < .0001$ (CD2-IL-5 Tg + saline vs. CD2-IL-5 Tg + rIL-18); $\#p < .02$, $##p < .001$ (WT + saline vs. WT + rIL-18); $$$$p < .001$ (WT + rIL-18 vs. CD2-IL-5 Tg + rIL-18); and $^{ss}p < .001$ (WT-saline vs. CD2-IL-5 Tg + saline). Photomicrographs presented are 100X (K, N) and 400X (L, M) original magnification, respectively

3.4 IL-18-induced eosinophilic asthma pathogenesis in $\Delta db/GATA$ mice

Next, to establish that IL-18 is indeed critical for the *in vivo* maturation of CD274⁺ eosinophils that promotes asthma pathogenesis, WT and $\Delta db/GATA$ mice were challenged intranasally with 10 μ g of rIL-18 in 50 μ l saline or saline alone. The $\Delta db/GATA$ mice are deficient in mature eosinophils but have bone marrow eosinophil precursors.⁵⁰ We present direct evidence that rIL-18 is critical for the *in vivo* generation of CD274⁺ pathogenic eosinophils. The rIL-18 challenge to $\Delta db/GATA$ mice showed induction of CD274⁺CCR3⁺SiglecF⁺ eosinophils in the BALF, blood, and bone marrow (Figure 4A, i-viii; Figure S3A, i-vi) and peribronchial accumulation of lung eosinophils compared to saline-challenged $\Delta db/GATA$ mice (Figure 4B). The morphometric quantitation of MBP⁺ cells showed a comparable number of tissue-accumulated eosinophils in the lungs of rIL-18-challenged WT and $\Delta db/GATA$ mice (Figure 4C). We also found that induction of IL-18-generated CD274⁺ eosinophils accumulation resulted in induced IL-13 protein with goblet cell hyperplasia, collagen accumulation, and airway obstruction in rIL-18-challenged $\Delta db/GATA$ mice compared to saline-challenged $\Delta db/GATA$ mice (Figure 4D–G). The morphometric quantitation of goblet cells in the lungs of saline- and rIL-18-challenged WT and $\Delta db/GATA$ mice is presented in Figure 4H.



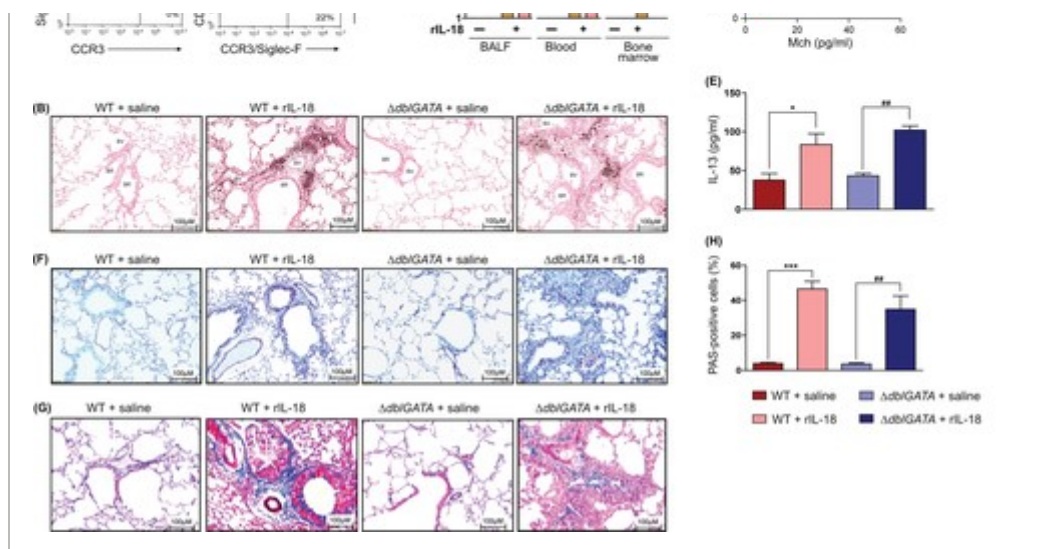


FIGURE 4

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IL-18-induced eosinophilic asthma pathogenesis in $\Delta db l G A T A$ (*GATA1 gene-deficient*) mice. Induced detection of CD274 expression in CCR3⁺Siglec-F⁺ eosinophils in the BALF (A, i–ii), blood (A, iii–iv), and bone marrow (A, v–vi) of rIL-18-challenged $\Delta db l G A T A$ mice. The quantification of CD274⁺ eosinophils in BALF, blood, and bone marrow of saline- and rIL-18-challenged $\Delta db l G A T A$ mice (A, vii–viii). A representative photomicrograph of anti-MBP immunostained lung sections with quantitation of eosinophils in saline- and rIL-18-challenged WT (BALB/c) and $\Delta db l G A T A$ mice (B, C). Airway resistance (RI) in response to methacholine was measured in $\Delta db l G A T A$ and WT (BALB/c) mice challenged with or without rIL-18 (D). ELISA analysis detected comparable induced IL-13 protein levels in the BALF of rIL-18-challenged WT and $\Delta db l G A T A$ mice compared to the saline-challenged mice (E). A representative photomicrograph of PAS-stained goblet cell hyperplasia with quantitation of rIL-18-challenged WT and $\Delta db l G A T A$ mice (F, H). Masson's trichrome-stained lung sections showed collagen accumulation in rIL-18-challenged $\Delta db l G A T A$ mice and WT (BALB/c) mice compared to saline-challenged mice (G). Data are expressed as mean $\pm$ SD, $n = 6$ –10 mice/group. * $p < .01$, ** $p < .001$, *** $p < .0001$ (WT + saline vs. WT + rIL-18); # $p < .01$, ## $p < .001$ ($\Delta db l G A T A$ + saline vs. $\Delta db l G A T A$ + rIL-18). Photomicrographs presented are 100X original magnification

3.5 Analysis of asthma pathogenesis in IL-5^{-/-}/IL-18^{-/-} mice

Since both IL-5 and IL-18 can generate, proliferate, and transform naive and pathogenic eosinophils, we further tested the hypothesis that IL-5 and IL-18 synergy is critical in promoting eosinophil-induced asthma pathogenesis. Accordingly, we generated endogenous IL-5 and IL-18-deficient (IL-5^{-/-}/IL-18^{-/-}) mice. Experimental asthma was induced in IL-5^{-/-}/IL-18^{-/-} mice along with littermate-matched control mice following the schematic protocol (Figure S3B).⁵ Anti-MBP immunohistochemical analysis detected no eosinophils in the lung sections of *A. fumigatus*-challenged IL-5^{-/-}/IL-18^{-/-} mice compared to high levels in *A. fumigatus*-challenged littermate-matched control mice. Saline-challenged mice did not show any lung eosinophilia (Figure 5A–D). Like tissue eosinophilia, very few baseline eosinophils (with no CD274⁺ eosinophils) were detected in the BALF of IL-5^{-/-}/IL-18^{-/-} mice by flow cytometry analysis compared to high levels in *A. fumigatus*-challenged littermate-matched

control mice (Figure 5E–H). The quantification of absolute eosinophils in BALF and accumulation in lung tissue showed they were highly induced in *A. fumigatus*-challenged littermate-matched control mice compared to almost none in *IL-5*^{−/−}/*IL-18*^{−/−} mice (Figure 5I,J). Additionally, we observed highly reduced baseline bone marrow eosinophils (Figure 5K) and *A. fumigatus* challenge-induced blood eosinophilia (Figure 5L) in the *IL-5*^{−/−}/*IL-18*^{−/−} mice compared to *IL-5*^{−/−} and *IL-18*^{−/−}, and littermate-matched control mice. Lastly, airway resistance analysis indicated that *A. fumigatus*-challenged *IL-5*^{−/−}/*IL-18*^{−/−} mice showed significantly improved airway resistance compared to *A. fumigatus*-challenged littermate-matched control mice (Figure 5M). Some airway resistance was still observed in *A. fumigatus*-challenged *IL-5*^{−/−}/*IL-18*^{−/−} mice that may be due to the *A. fumigatus*-induced IL-13 in *IL-5*^{−/−}/*IL-18*^{−/−} mice that is an established enhancer of mucus production. In addition, asthmatic *IL-5*^{−/−}/*IL-18*^{−/−} mice had significantly lower levels of eosinophil-active inflammatory cytokines than their *A. fumigatus*-challenged littermate-matched control mice; IL-5 and IL-13 levels were almost undetectable in saline- or *A. fumigatus*-challenged *IL-5*^{−/−}/*IL-18*^{−/−} mice (Figure S3C,D). The morphometric quantitation of MBP⁺ cells showed only a few baseline eosinophils in saline- and *A. fumigatus*-challenged lungs of *IL-5*^{−/−}/*IL-18*^{−/−} mice compared to high levels in littermate-matched control WT mice (Figure S3E). These data further confirm that the eosinophils observed in *IL-5*^{−/−} mice are due to the presence of endogenous IL-18, which is consistent with the report that *IL-5*^{−/−} mouse bone marrow eosinophil precursors develop into mature eosinophils in response to rIL-18.²²

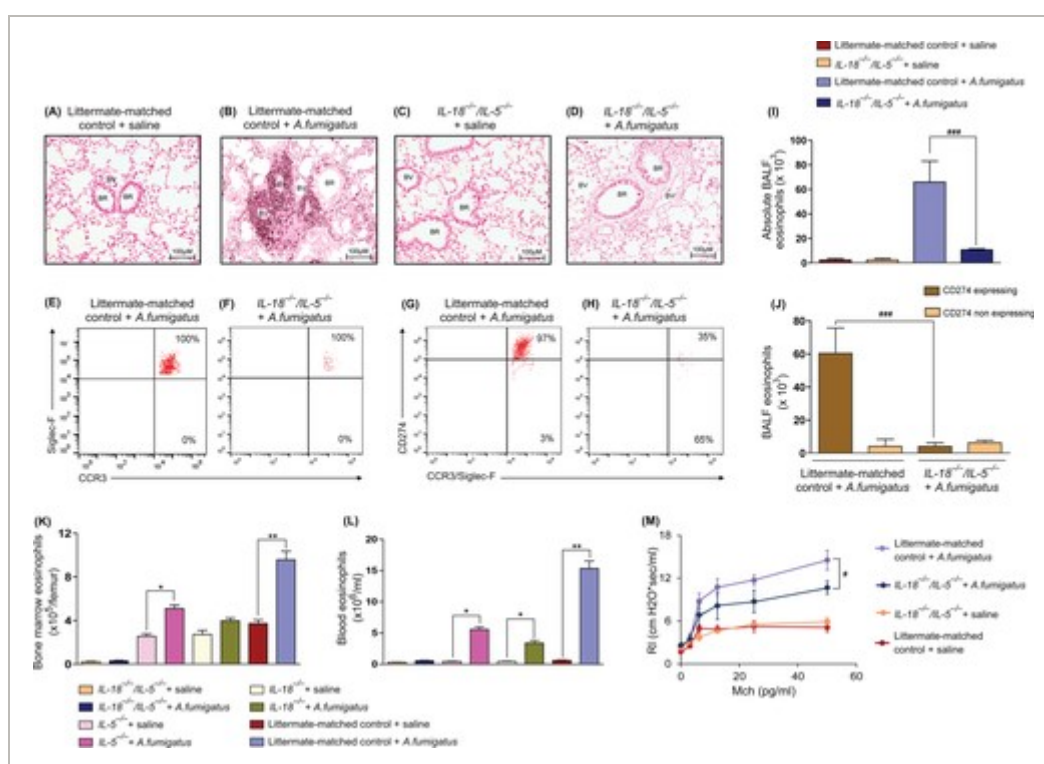


FIGURE 5

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IL-5^{−/−}/*IL-18*^{−/−} double gene-deficient mice are deficient in bone marrow, blood, and lung eosinophils following allergen-induced asthma. A representative photomicrograph of anti-MBP antibody immunostained lung sections shows no

accumulation of eosinophils in asthmatic *IL-5^{-/-}/IL-18^{-/-}* mice compared to *A. fumigatus*-challenged littermate-matched control mice (A–D). Representative flow cytometry analysis of CCR3⁺Siglec-F⁺ eosinophils (E, F) and CD274 expression (G, H) in the BALF of *A. fumigatus*-challenged littermate-matched control mice and *IL-5^{-/-}/IL-18^{-/-}* mice. Eosinophil quantitation of BALF in *A. fumigatus*-challenged littermate-matched control and *IL-5^{-/-}/IL-18^{-/-}* mice compared to saline-challenged littermate-matched control mice (I, J). Baseline bone marrow and *A. fumigatus*-induced blood eosinophils in *IL-5^{-/-}/IL-18^{-/-}* mice (K, L). Airway resistance (RI) in response to methacholine was measured in *A. fumigatus*-challenged littermate-matched control and *IL-5^{-/-}/IL-18^{-/-}* mice (M). Data are expressed as mean ± SD, *n* = 6–10 mice/group. **p* < .01, ***p* < .001 (control vs. *A. fumigatus*); #*p* < .01, ###*p* < .0001 (littermate-matched control + *A. fumigatus* vs. *IL-5^{-/-}/IL-18^{-/-}* + *A. fumigatus*). Photomicrographs presented are 100X original magnification

3.6 Neutralization of CD274 ameliorates *A. fumigatus*-induced experimental asthma

Next, we aimed to establish whether IL-18-induced CD274⁺ eosinophils are responsible for the induction of experimental asthma. Accordingly, we neutralized CD274⁺ cells *in vivo* in an experimental mouse model of asthma by intraperitoneal injection of neutralizing anti-CD274 antibody (1 mg two times per week for 3 weeks) as in the presented schematic protocol (Figure S4A). Neutralization of CD274 in *A. fumigatus*-challenged mice resulted in reduced airway eosinophils in BALF compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 6A). A quantification of CD274⁺ eosinophils in BALF by flow cytometry showed a ~3-fold reduction upon neutralization of CD274 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 6B). A similar reduction in perivascular and peribronchial tissue eosinophils was detected upon neutralization of CD274 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice; no eosinophils were detected in saline-challenged mice (Figure 6C). Furthermore, lung function tests (airway resistance analysis) indicated that *A. fumigatus*-challenged mice upon CD274 neutralization show significantly improved airway resistance compared to *A. fumigatus*-challenged isotype control-treated mice (Figure 6D). A significant reduction in PAS-stained mucus-producing goblet cells was detected upon CD274 neutralization in *Aspergillus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 6E), and a morphometric analysis indicated significantly reduced goblet cells upon neutralization of CD274 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 6F). Flow cytometry analysis of BALF cells showed reduced expression of CD274 in the CCR3⁺SiglecF⁺ eosinophils of *A. fumigatus*-challenged mice upon neutralization of CD274 compared to isotype control-treated *A. fumigatus*-challenged mice (Figure S4B,C). Reduced collagen accumulation was observed in allergen-challenged mice upon neutralization of CD274 compared to *A. fumigatus*-challenged isotype control-treated mice (Figure S4D, i-iv). ELISA analysis showed significantly reduced levels of IL-18 and IL-13 in *A. fumigatus*-challenged mice treated with anti-CD274 mice compared to isotype control-treated and *A. fumigatus*-challenged mice (Figure S4E,F). Morphometric quantitation of anti-MBP staining showed a statistically

significant reduction in lung tissue-accumulated eosinophils in *A. fumigatus*-challenged mice treated with anti-CD274 antibody compared to isotype control-treated and *A. fumigatus*-challenged mice (Figure S4G). The presented data also indicate that CD274 neutralization downregulates also T-cell function in *A. fumigatus*-challenged mice, suggesting that CD274⁺ eosinophil accumulation-induced responses are indeed responsible for promoting asthma pathogenesis.

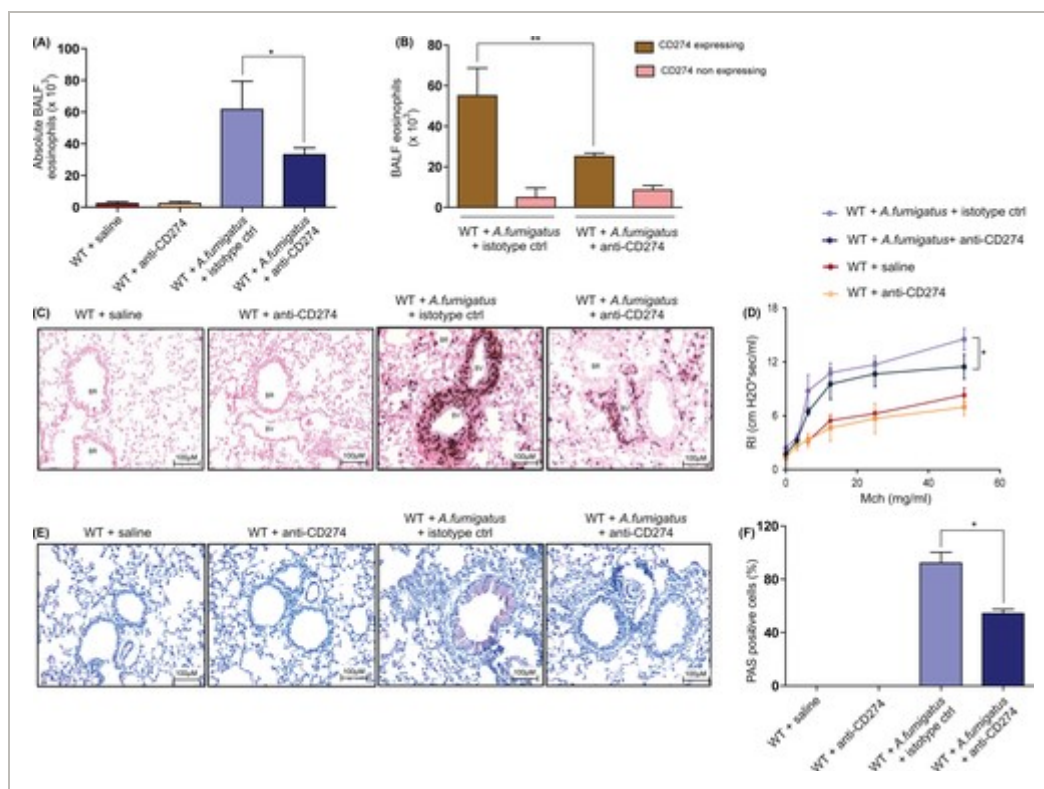


FIGURE 6

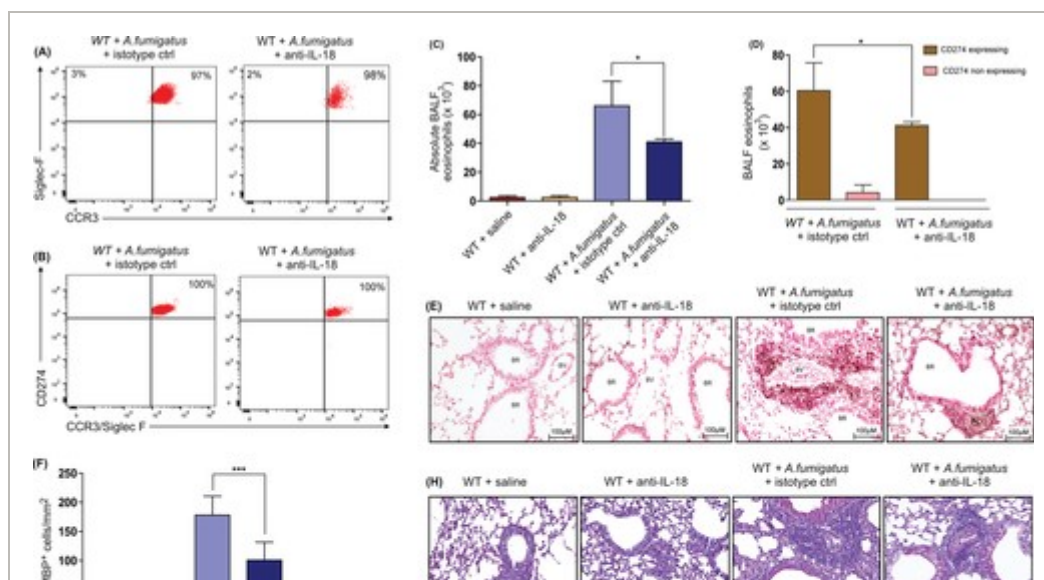
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CD274-expressed eosinophils are indeed responsible for asthma pathogenesis in mice, including airway obstruction. A significant difference was detected in CD274⁺ BALF eosinophil numbers in the *A. fumigatus*-challenged WT mice upon neutralization of CD274 and *A. fumigatus*-challenged WT mice treated with matching isotype control (A, B). A representative photomicrograph of anti-MBP antibody immunostained eosinophils in lung sections of *A. fumigatus*-challenged anti-CD274-treated and isotype control-treated WT mice (C). Airway resistance (RI) in saline- and *A. fumigatus*-challenged anti-CD274-treated and anti-isotype control-treated mice is shown (D). A representative photomicrograph of goblet cell hyperplasia (E) and respective cell numbers in *A. fumigatus*-challenged anti-CD274-treated and isotype control-treated mice (F). Data are expressed as mean $\pm$ SD, $n = 8-12$ mice/group. * $p < .01$, ** $p < .001$ (WT + *A. fumigatus* + anti-CD274 vs. WT + *A. fumigatus* + isotype control). Photomicrographs presented are 100X original magnification

3.7 Neutralization of IL-18 protects *Aspergillus*-induced CD274-expressing eosinophil-mediated induction of experimental asthma

We further examined the effectiveness of IL-18-neutralizing antibody in restricting the

generation and transformation of CD274⁺ pathogenic eosinophils that induce asthma in *A. fumigatus*-challenged mice. Accordingly, we intraperitoneally (IP) injected anti-IL-18-neutralizing antibody (200 µg two times per week for 3 weeks) and isotype control in WT mice following the schematic protocol presented in Figure S5A. Previous studies have shown that the IP injection route is pharmacologically relevant for slow absorption and long-term body retention of neutralizing antibodies.^{51, 52} BALF eosinophils (Figure 7A,C) and CD274⁺ eosinophils were analyzed by flow cytometry. The neutralization of IL-18 in *Aspergillus*-challenged mice showed significantly reduced numbers of CD274⁺ eosinophils compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 7B,D). Furthermore, a similar reduction in peribronchial accumulation of tissue eosinophils was detected by anti-MBP immunostaining (Figure 7E, i-iv), and morphometric analysis indicated that MBP⁺ cells are significantly reduced upon neutralization of IL-18 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 7F). Lung functional analysis indicated that *A. fumigatus*-challenged mice treated with anti-IL-18 show significantly improved airway resistance compared to *A. fumigatus*-challenged isotype control-treated mice (Figure 7G), which correlates with the reduced number of CD274⁺ eosinophils. We also detected goblet cell hyperplasia via PAS staining in *A. fumigatus*-challenged mice (Figure 7H) and morphometric analysis indicated that PAS-positive goblet cells are significantly reduced upon neutralization of IL-18 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure 7I). Furthermore, a similar reduction was also observed in collagen accumulation upon neutralization of IL-18 in *A. fumigatus*-challenged mice compared to isotype control-treated *A. fumigatus*-challenged mice (Figure S5B, i-iv). IL-18 neutralization does not give complete protection against disease pathogenesis, including CD274⁺ eosinophils, as these mice still show induced IL-18 in the blood and BALF (Figure S5C, D). These data establish that anti-IL-18-treated *A. fumigatus*-challenged mice have restricted differentiation and lung accumulation of CD274⁺ pathogenic eosinophils, resulting in improved asthmatic characteristics including mucus production and airway obstruction, and that anti-IL-18-neutralizing antibody may be used as a novel strategy to restrict asthma pathogenesis without compromising gastrointestinal innate immunity.



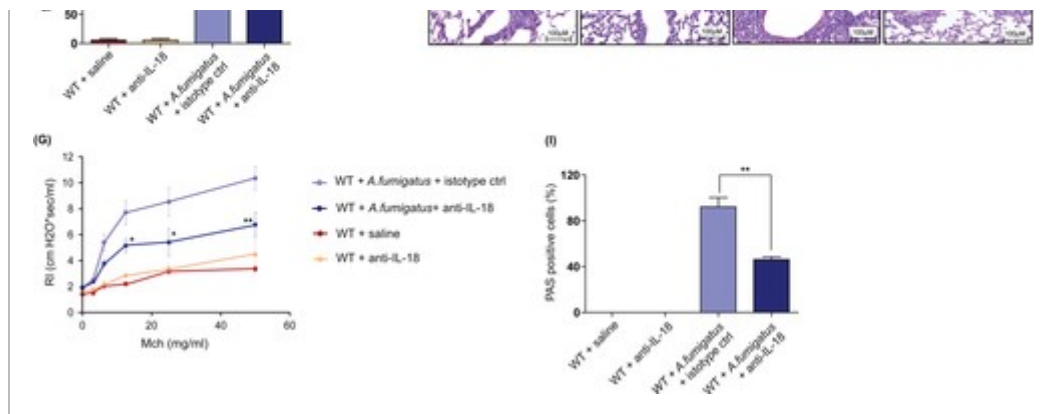


FIGURE 7

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Neutralization of IL-18 ameliorates *A. fumigatus*-induced experimental asthma. A flow cytometry analysis of CCR3⁺Siglec-F⁺ eosinophils and CD274⁺ eosinophils in the BALF of *A. fumigatus*-challenged anti-IL-18-treated and isotype control-treated mice (A, B). A quantification of flow cytometry analysis of CCR3⁺Siglec-F⁺ BALF eosinophils and CD274⁺ BALF eosinophil numbers in *A. fumigatus*-challenged anti-IL-18-treated and isotype control-treated mice (C, D). A representative photomicrograph of anti-MBP antibody immunostained lung sections of *A. fumigatus*-challenged anti-IL-18-treated and isotype control-treated mice (E). Morphometric quantification of MBP⁺ cells, expressed as cells/mm² (F). Airway resistance (RI) of all four groups of *A. fumigatus*- and saline-challenged anti-IL-18-treated and isotype control-treated mice was measured (G). A representative photomicrograph of goblet cell hyperplasia (H) with respective cell numbers in *A. fumigatus*-challenged anti-IL-18-treated and isotype control-treated mice are shown (I). Data are expressed as mean ± SD, *n* = 8–12 mice/group. **p* < .01, ***p* < .001, ****p* < .0001 (WT + *A. fumigatus* + anti-IL-18 vs. WT + *A. fumigatus* + isotype control). Photomicrographs presented are 100X original magnification

4 DISCUSSION

The burden of immune-related diseases, including asthma, is increasing globally, particularly in the Western world^{53, 54} Most asthma studies are focused largely on analyses of the cellular and molecular events induced by allergen exposure in sensitized animals and humans.^{55–59} Studies have identified elevated production of IgE and several inflammatory cytokines that lead to lung function abnormalities.^{58, 60–64} Previous findings from our group and others have indicated that eosinophil recruitment into inflammatory tissue is a complex process regulated by Th2 cytokines (IL-5, IL-13, and IL-15) and eosinophil-associated chemokines in asthma pathogenesis.^{12, 13, 65, 66} Asthma patients tend to have both food and aeroallergen hypersensitivity.^{11–13} It is believed that asthma pathogenesis is prompted by the induction of IL-5-associated eosinophilia in response to allergen exposure. IL-5 is a well-known eosinophil differentiating and growth factor that is induced in several allergic diseases including asthma,^{20, 21} and its effector functions and roles in various diseases are under renewed scrutiny.^{67, 68} The intrinsic molecular pathway of IL-5-regulated eosinophilia is largely unknown and remains to be defined.⁹ A recent report indicated that allergic lungs have two eosinophil subsets, CD101⁺ and CD101[−], and that CD101-expressing eosinophils

are IL-5-responsive inflammatory eosinophils.⁶⁹ However, we reported that IL-5 transgenic naive mice have no CD101⁺ eosinophils, which is inconsistent with a recent report showing CD101⁺ eosinophils as IL-5 dependent.⁶⁹ In addition, we found that CD2-IL-5 transgenic mice eosinophils become both CD101⁺ and CD274⁺ eosinophils kinetically only following *ex vivo* exposure to IL-18, and that eosinophils have two naive and pathogenic (CD274⁺) eosinophil subsets.²² Furthermore, we also showed that while healthy humans have blood eosinophils, high levels of CD101-expressing and CD274⁺ eosinophils are associated with allergic diseases. Notably, IL-18 is increased in a number of food and aeroallergen-induced allergic diseases,²⁸⁻³⁰ including asthma.^{31, 33, 70} Several clinical reports show that IL-18 is induced in allergic patients including asthmatics,²⁸⁻³³ and experimental modeling shows that IL-18 overexpression in the lung promotes the asthma phenotype in mice.^{71, 72} Several reports have recognized the role of IL-18 in asthma and allergic diseases; however, the significance and functional mechanism of IL-18 in eosinophil biology and in promoting allergic diseases have never been fully examined. Herein, we provide the first evidence that IL-18 is involved in differentiation of CD274⁺ eosinophils *in vivo*, which induces pathogenesis of the disease. Previous reports indicated that PD-1 and its ligands CD274 and CD273 play a role in allergic asthma by involving T-cell development, maintenance, function, and tolerance^{34, 35, 73, 74}; however, the factor that regulates CD274⁺ eosinophils was not known. The current study illustrates the significance of IL-18 in regulating CD274 expression on inflammatory cells in asthma. Additionally, CD274^{-/-} mice show clearing of adenovirus infection, *Leishmania mexicana*-mediated infection, and reduced airway hyperactivity.^{35, 75, 76} We also show that despite different genotypes, allergen-challenged mice have induced IL-18 levels and accumulate eosinophils in the epithelial layer around the airway, and BALF analysis indicates that these eosinophils all express CD274.²² Studies have shown increased IL-18 and IL-18R in lung tissues of patients with fatal asthma.^{77, 78} The role of IL-18 in IgE production and mast cell biology has previously been explored,⁷⁹ but the direct role of IL-18 and IL-18-differentiated eosinophil subsets in asthma pathogenesis has not been established. Thus, the precise mechanism of IL-18-induced pulmonary eosinophilic or non-eosinophilic inflammation is not clearly understood. Based on these reports, we hypothesize that the IL-18-differentiated CD274⁺ eosinophil subset is critical in promoting asthma pathogenesis, including mucus production and collagen deposition in the lung. In this study, we provide important evidence on the significance of IL-18-differentiated eosinophils in promoting asthma pathogenesis following rIL-18 delivery to IL-5 transgenic mice. The rIL-18-treated CD2-IL-5 transgenic mice showed that most of the characteristic features observed in human asthma, like peribronchial and perivascular eosinophilia-induced accumulation of collagen, goblet cell hyperplasia, and increased airway hyperactivity. This study is the first to provide direct evidence of the critical role of IL-18-differentiated pathogenic CD274⁺ eosinophil subsets in asthma pathogenesis. We used different background strain (BALB/c and C57BL6) mice because all gene-deficient and transgenic mice were not available from one background strain. Since BALB/c, C57BL6, and littermate-matched IL5^{-/-}IL18^{-/-} are crossbred mice that show similar baseline airway hyperactivity, using both mice in different experimental setups does not pose any risk of bias in our results.

Furthermore, our experiments provide evidence on the critical role of CD274 in promoting asthma pathogenesis. *A. fumigatus*-challenged anti-CD274-treated and anti-IL-18-treated mice show significantly improved asthma pathogenesis, including airway hyperreactivity. Additionally, we showed clinically relevant supportive data illustrating the effectiveness of aCD274 and IL-18 neutralization in protecting induction of CD274⁺ pathogenic eosinophils and mucus-producing goblet cell hyperplasia in asthmatic mice. We show that IL-13, goblet cells, and airway hyperactivity are reduced in *A. fumigatus*-challenged *IL-5*^{-/-} mice compare to WT mice. Of note, IL-13, goblet cells, and airway hyperactivity reduction are similar to the reduced number of IL-18-responsive CD274⁺ eosinophils in *IL-5*^{-/-} mice. Additionally, the anti-CD274-treated mice show reduced eosinophils, IL-13, and airway hyperactivity after *A. fumigatus* challenge. Most importantly, this study also shows that even rIL-18-treated $\Delta db/GATA$ mice show the accumulation of CD274⁺ eosinophils, induced airway obstruction, and IL-13-associated mucus-producing goblet cells. The $\Delta db/GATA$ mice are deficient in eosinophils but have eosinophil stem cell precursors,⁵⁰ and these precursors generate mature eosinophils upon rIL-18 challenge; this is consistent with our earlier report that IL-18 is also capable of generating and maturing eosinophils from bone marrow precursors.²² Of note, this is the first report that presents an *in vivo* study showing direct evidence of the generation of eosinophils in response to IL-18 from eosinophil stem cell precursors in eosinophil-deficient $\Delta db/GATA$ mice. Thus, the presented data strongly support our hypothesis that IL-18-responsive CD274⁺ eosinophils are responsible for eosinophilic asthma pathogenesis, including the induction of airway obstruction. These investigations also provide a strong preclinical rationale to consider regulation of IL-18 signaling and differentiation of naive eosinophils to CD274-expressing pathogenic eosinophils for therapeutic trials in asthmatic patients. In support of these presented data, and to establish the synergy of IL-18 and IL-5-induced eosinophilia in allergic asthma, we generated *IL-5-IL-18* double-knockout mice (*IL-18*^{-/-}/*IL-5*^{-/-}) that completely depleted both naive and pathogenic blood and tissue eosinophilia and significantly reduced airway hyperactivity following the induction of *A. fumigatus*-induced experimental asthma. In short, the presented data support the therapeutic effectiveness of CD274 and IL-18 neutralization for eosinophilic asthma. Taken together, the current study advances our knowledge of eosinophil biology and the existence of an eosinophil subset that is critical for promoting mucus, mucus cell hyperplasia, and airway resistance. We present for the first time strong novel data that establish the significance of induced IL-18 and its responsive CD274⁺ eosinophils in asthma pathogenesis and provide evidence for new targeted therapeutic and diagnostic interventions. First, we show allergen-challenged WT, *IL-5*^{-/-}, or *IL-18*^{-/-} mice develop CD274⁺ eosinophilic inflammation and airway obstruction in the lungs. Second, we show that rIL-18 treatment induces perivascular and peribronchial eosinophilia and increases the severity of asthma pathogenesis in CD2-IL-5 transgenic mice. Third, we show that rIL-18 treatment caused the development of lung eosinophilia-associated asthma pathogenesis, including airway obstruction, even in eosinophil-deficient $\Delta db/GATA$ mice. Fourth, we show that *IL-5*^{-/-}/*IL-18*^{-/-} double gene-deficient mice are protected from induction of CD274⁺ eosinophilic lung inflammation and airway hyperactivity. Lastly, we show that both IL-18 and CD274

neutralization protect mice from asthma pathogenesis. Based on our previous report of CD274⁺ eosinophil subsets and their presence in human allergic patients^{22, 80, 81} and current findings, we propose anti-CD274 and anti-IL-18 clinical immunotherapy trial (with proper control placebo) to improve quality of life for asthma patients. The anti-CD274 or anti-IL-18 immunotherapy will restrict only pathogenic eosinophils and will not disturb the IL-5-responsive eosinophil-induced innate immunity in patients suffering from allergic asthma or other diseases.

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CONFLICT OF INTEREST

All authors have no financial conflict of interest.

AUTHOR CONTRIBUTIONS

AM—conceptualization, supervision, funding acquisition, visualization, project administration, and writing—review and editing. DM, HKK, and CSY—methodology, review, and editing. SUV—partial funding acquisition, data curation, formal analysis, investigation, methodology, and writing original draft. All authors helped with reviewing and editing the manuscript.

Supporting Information



Filename	Description
all15180-sup-0001-FigS1.pdf PDF document, 182.8 KB	Fig S1
all15180-sup-0002-FigS2.pdf PDF document, 1.8 MB	Fig S2
all15180-sup-0003-FigS3.pdf PDF document, 408.5 KB	Fig S3
all15180-sup-0004-FigS4.pdf PDF document, 1.6 MB	Fig S4
all15180-sup-0005-FigS5.pdf PDF document, 1 MB	Fig S5
all15180-sup-0006-TableS1.pdf PDF document, 99.7 KB	Table S1

Filename	Description
all15180-sup-0007-Supinfo.pdf PDF document, 292.6 KB	Supplementary Material

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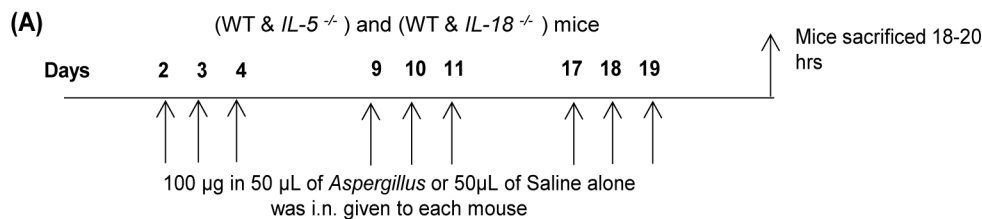
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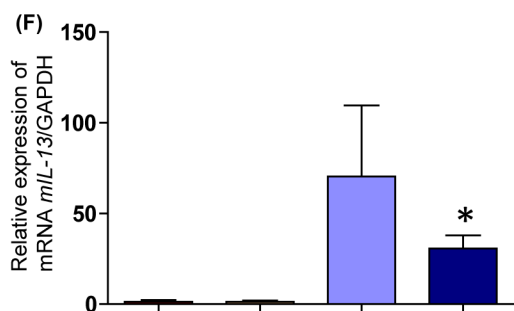
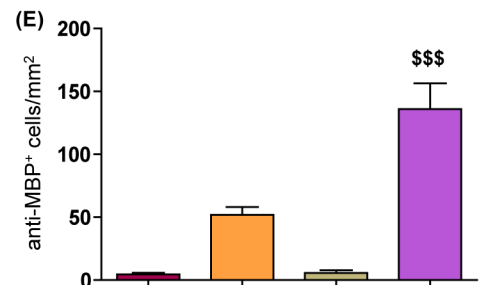
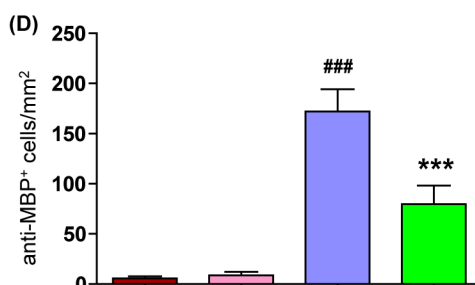
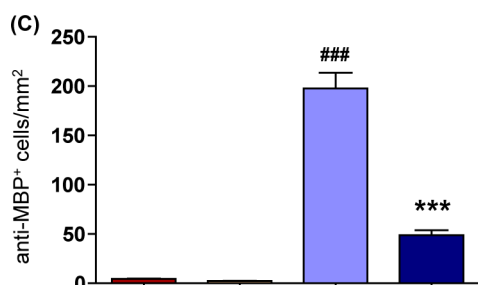
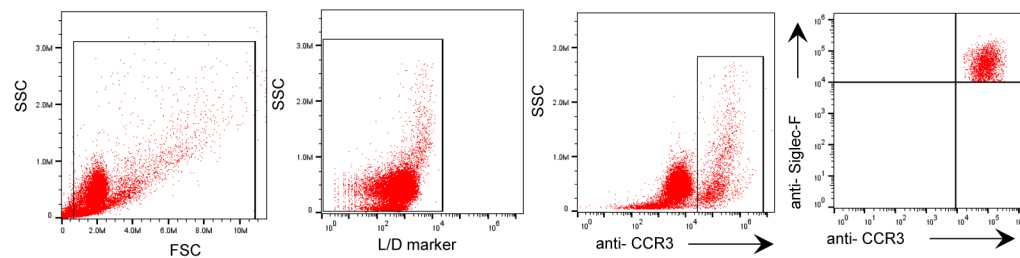
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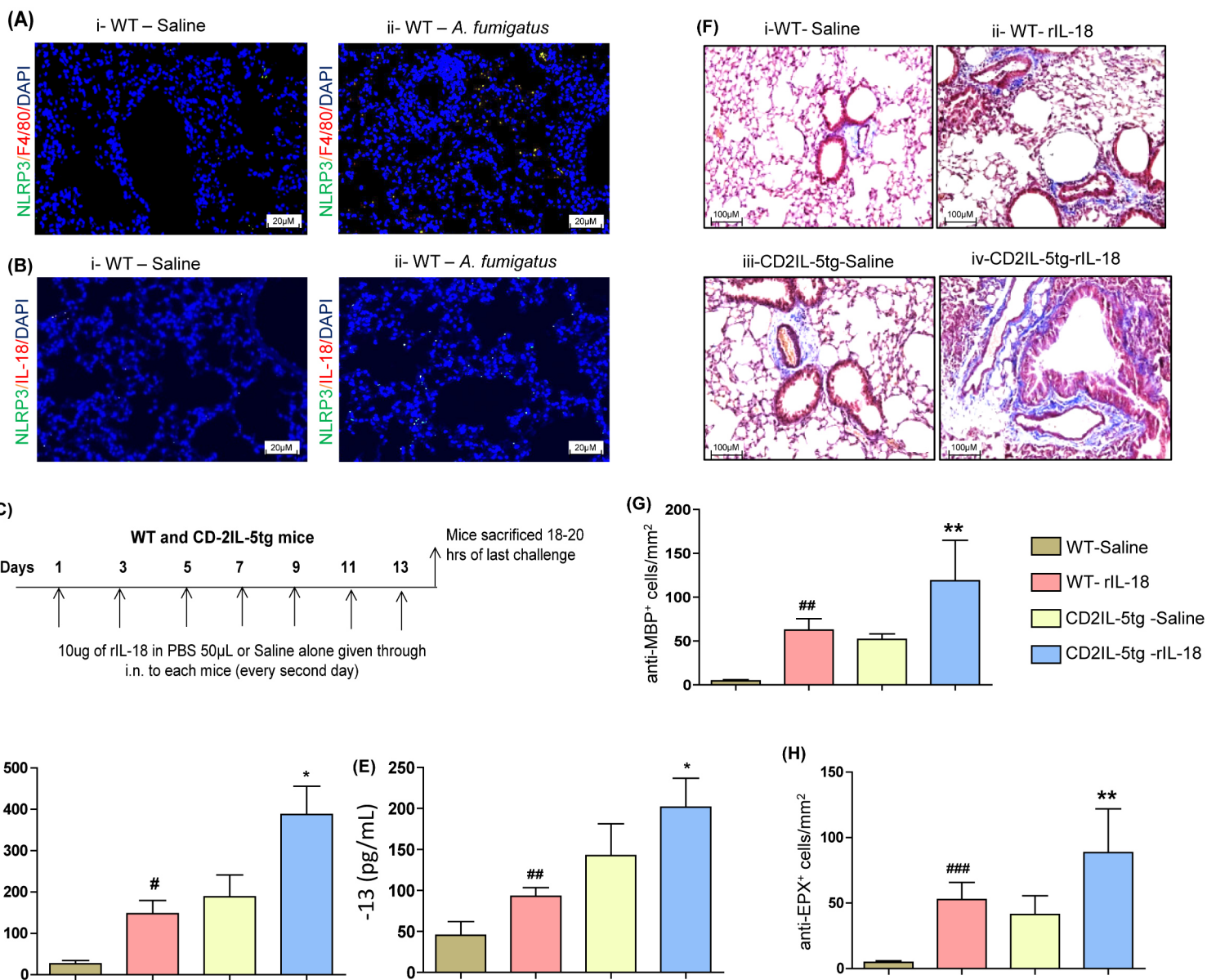
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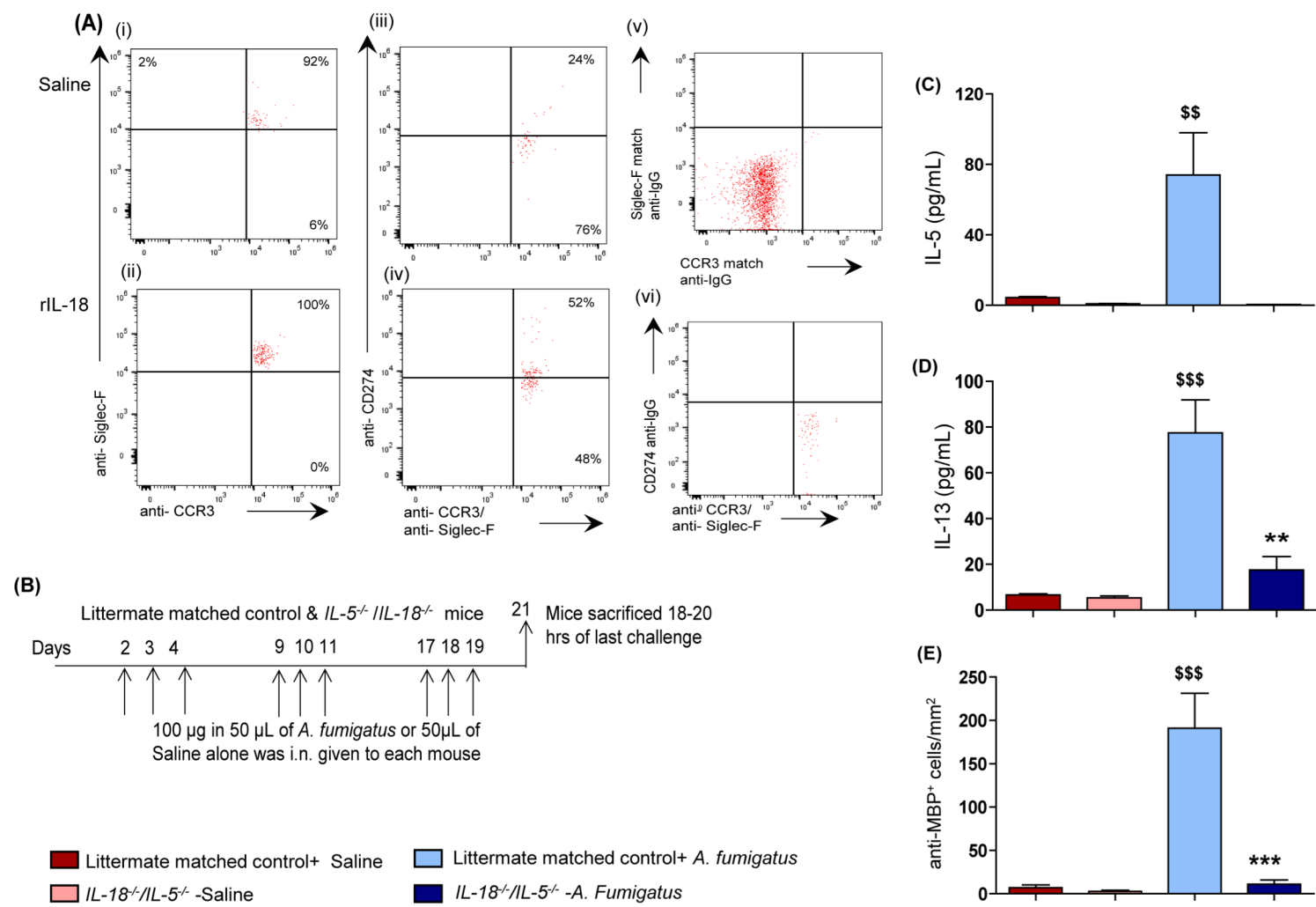
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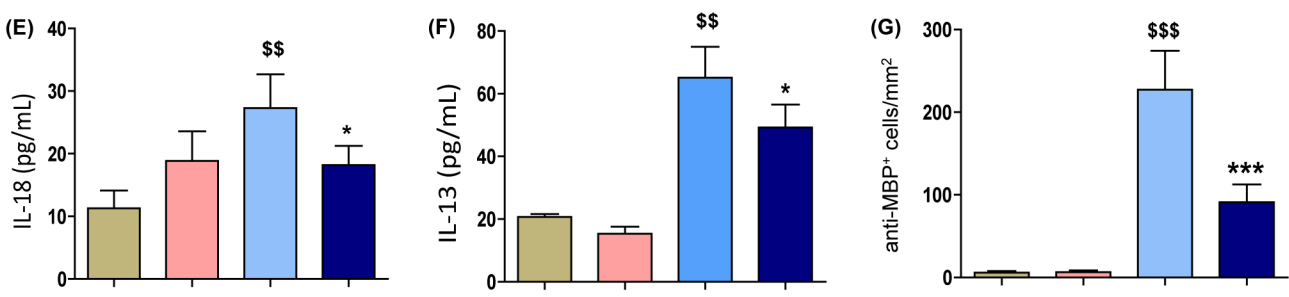
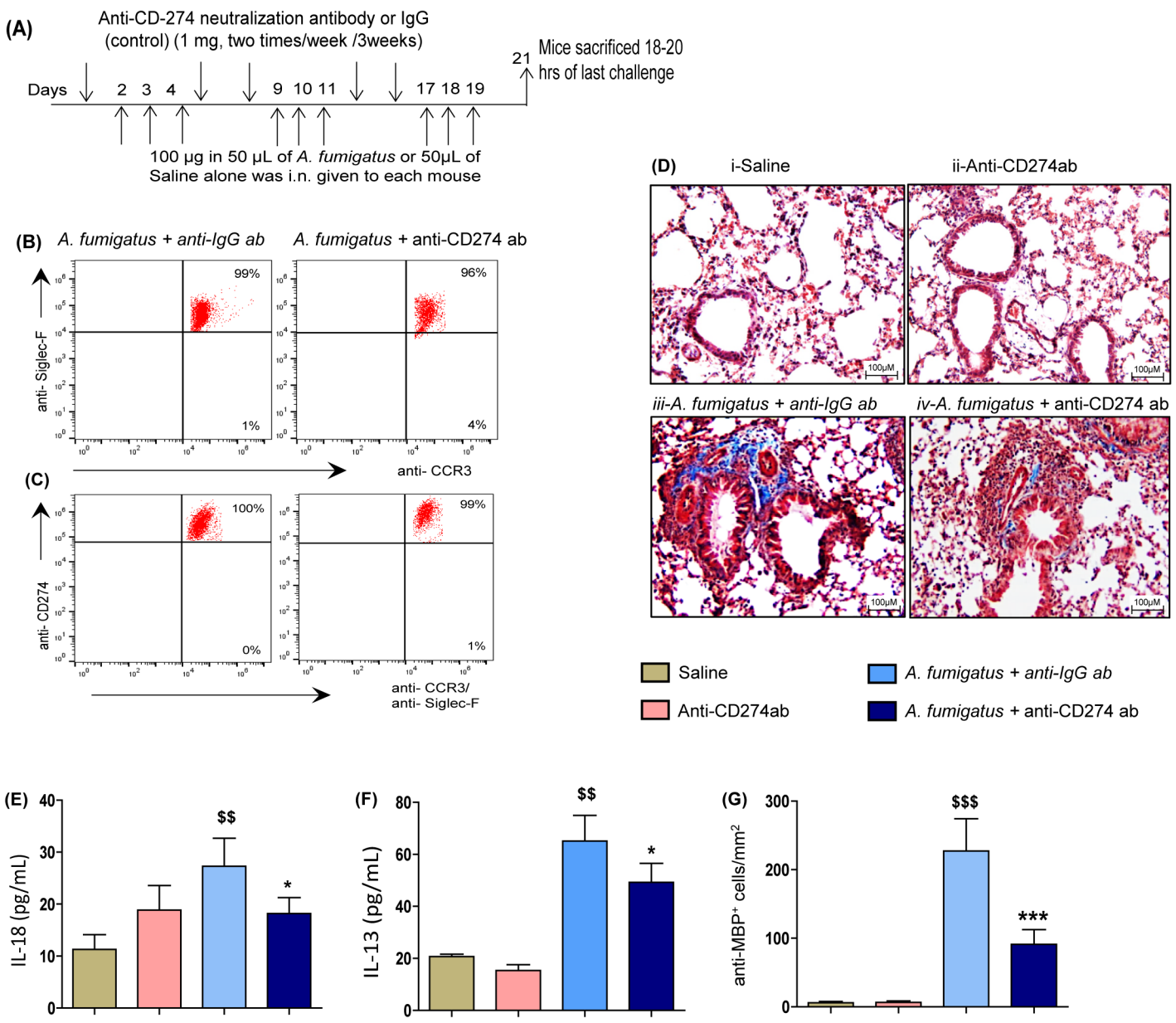


(B) Gating strategy

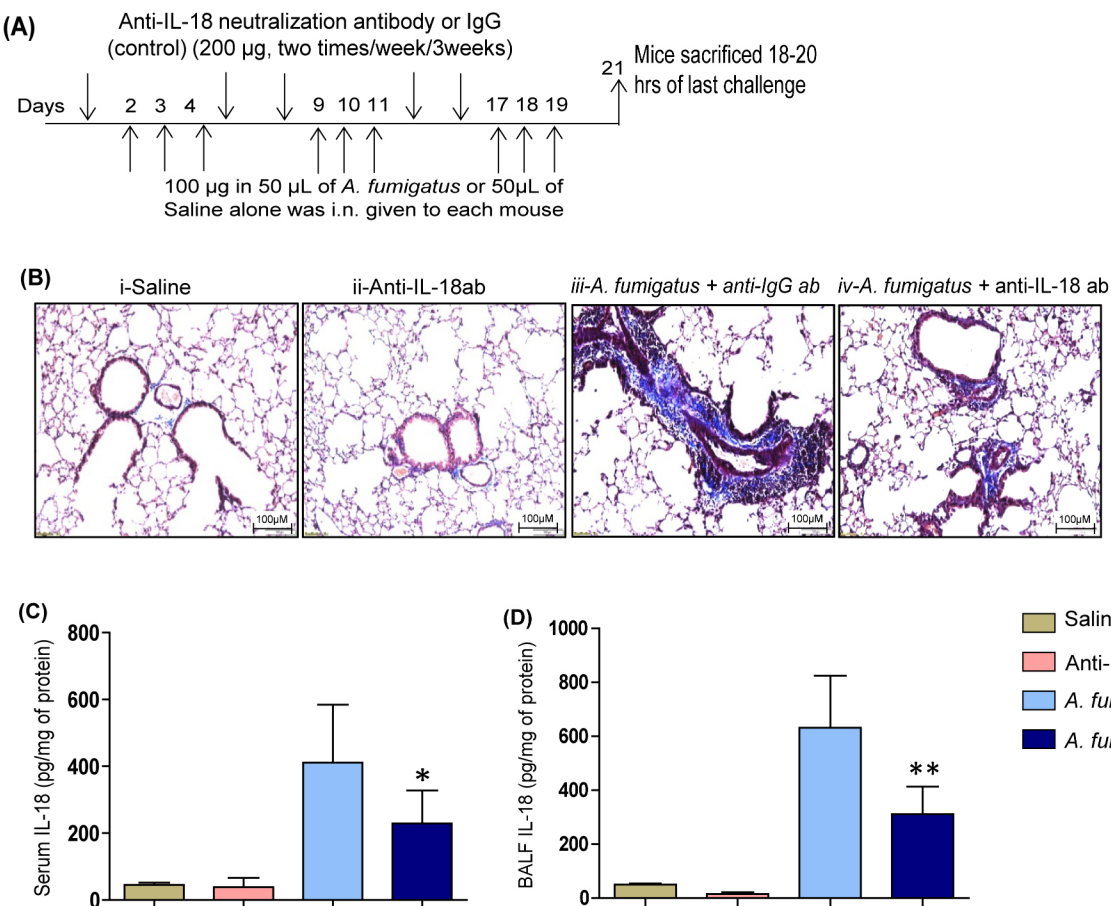








Suppl. Figure 4 _ Anil et al,



Supplemental Table S1 - A list of antibody sources

Antigen	Cat No	Source	Dilution
CD193 (CCR3) J073E5- FITC	144512	BioLegend	1:50
CD274 (B7-H1, PD-L1) 10F.9G2- PE/Cyanine7	124314	BioLegend	1:50
Siglec-F E50-2440- PE	552126	BD Biosciences	1:50
Anti-rat Anti-Eosinophil Major Basic Protein (MBP)	(MT-14.7)	Mayo Clinic	1:6000
Anti-mouse Eosinophil Peroxidase (EPX)	(MM82.2.1)	Mayo Clinic	
NLRP3 Rabbit mAb (D4D8T)	15101S	Cell Signaling Technology	1:100
F4/80 Rabbit mAb (D4CBV)	30325S	Cell Signaling	1:100
IL-18 mouse McAb	60070-1-Ig	Proteintech	1:100
Biotinylated Anti-Goat IgG	BA-9500	Vector Laboratories, Inc	1:200
Biotinylated goat anti-rat IgG	BA-9400	Vector Laboratories, Inc	1:250
Biotinylated goat anti-mouse IgG	BA-9200	Vector Laboratories, Inc	
FITC Donkey anti-rabbit IgG	406403	Biogen	1:200
PE Mouse anti-rabbit IgG	SC-3753	Santa Cruz Biotechnology	1:200
Streptavidin, Alexa Fluor™ 488	532354	Thermo Fisher Scientific	1:200
Streptavidin - Alexa Fluor™ 594	532356	Thermo Fisher Scientific	1:200
Mucin 1 (SM3) (MUC1)	sc-53381	Santa Cruz Biotechnology	1:500
Mucin 5AC (45M1)	sc-21701	Santa Cruz Biotechnology	1:500

Supporting information

**Role of IL-18 transformed CD274 expressing eosinophils in promoting
airway obstruction in experimental asthma**

Anil Mishra, PhD., Dewan Majid, MD., Hemanth Kumar Kandikattu, PhD., Sathisha
Upparahalli Venkateshaiah, PhD

METHODS

Experimental allergic airway murine model of asthma

Both C57BL/6 and BALB/c mice strain were used to examine the eosinophils subpopulation role in the mouse model of allergic asthma using established methods as described previously.^{1, 2} The mice were lightly anesthetized with isoflurane (Iso-Flo; Abbott Laboratories, North Chicago, IL), and 100 µg (50 µl normal saline) of *Aspergillus fumigatus* (*Aspergillus*) extract (Greer Laboratories, Lenoir, NC) or 50 µl of normal saline alone was given through intranasal using a micropipette with the mouse held in the supine position (**Suppl Fig. 1A**).

Anti-IL-18 and anti-CD274 neutralization antibody treatment

Anti-IL-18 Clone -YIGIF74-1G7 or Rat IgG2b, κ (control) (200 µg, two times per week for 3 weeks) (Bio X Cell, West Lebanon, NH) and anti-CD274 neutralization antibody Clone-10F.9G2 or Rat IgG2b, κ (control) (1 mg, two times per week for 3weeks) (Bio legend, San Diego, CA) was administered intraperitoneally to mice 30 min before challenging them with *Aspergillus* extract following the protocol described above (**Suppl Fig. 4A,5A**).

Bronchoalveolar lavage fluid (BALF)

The mice were euthanized by CO₂ inhalation. Immediately thereafter, a midline neck incision was made, and the trachea was cannulated. The lungs were lavaged two times with 1.0 ml PBS containing 1% FCS and 0.5 mM EDTA. The recovered BALF was centrifuged at 400-g for 5 minutes at 4°C and re-suspended in PBS containing

1% FCS and 0.5 mM EDTA. Total cell numbers were counted with a hemocytometer and BALF cells were used for measuring eosinophils levels using flow cytometer as an indication of lung eosinophilia.

Flow cytometric analysis

BALF eosinophils were stained with anti-mCCR3, anti-mSiglecF, and anti-mCD274 with their respective isotype controls antibodies as listed in **Table S1** (obtained from Thermo Fisher Scientific (eBioscience), San Diego, CA and Bio legend, San Diego, CA). A live/dead marker was used to exclude dead cells. Data were acquired with a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) and Novocyte (ACEA Biosciences, San Diego, CA) and analyzed with FlowJo software version 7.1 (Tree Star).^{3, 4} Positive cells were identified by comparison to the appropriately conjugated isotype controls (**Suppl Fig. 1B**).

Analysis of eosinophils

Paraffin embedded lung tissue sections (5µm) were immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP) as described previously.¹ In brief, endogenous peroxidase in the tissues was quenched with 0.3% hydrogen peroxide in methanol followed by non-specific protein blocking using 3% normal goat serum. Tissue sections were then incubated with rat anti-MBP antibody (1:6000 dilution) overnight at 4°C (Mayo Clinic, Scottsdale, AZ), followed by a 1:250 dilution of biotinylated goat anti-rat IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. Slides were further

developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate, and counterstained with nuclear fast red. Negative controls included replacing the primary antibody with normal rabbit serum to control for endogenous biotin and peroxidase activity. Lung sections were immunostained with a mouse monoclonal antibody specific for EPX as per protocol described previously.^{5, 6} Eosinophils were quantified by counting the anti-MBP or anti-EPX positive cells in the lung tissues. Eosinophil numbers and area of tissue section were measured and calculated with the assistance of digital morphometric analysis (Lumenera Corporation, Infinity Analyze 6.1.0). The tissue sections were taken from the same position in each set of mice, and at least four random sections per mouse were analyzed for eosinophil levels, expressed as eosinophils/mm² as described previously.^{1, 7}

Immunofluorescence analysis

Paraffin-embedded mouse lung tissue sections (5µm) were deparaffinized and dehydrated and staining was carried out using methods described previously.^{8, 9, 10} Antigen retrieval was carried out using the Antigen Unmasking solution citric acid-based (Vector labs) method, blocked with 5% normal goat serum to reduce nonspecific binding, and incubated with specific primary antibodies (F4/80 (D4CBV) Rabbit mAb, Cat-30325S; NLRP3 (D4D8T) Rabbit mAb, Cat- 15101S; IL-18 mouse McAb, Cat-60070-1-Ig) followed by secondary antibodies conjugated with FITC or PE antibodies as listed in **Table S1**. Immunostained sections were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The images

were captured using an Olympus BX43 microscope with appropriate filters, and photomicrographs are presented as original magnification 400X.

Analysis of goblet cell hyperplasia

Lung tissue samples were fixed with 10% paraformaldehyde in phosphate buffer (PBS) pH 7.4, embedded in paraffin, cut into 5 µm sections, and fixed to positively charge slides. Periodic Acid Schiff (PAS) reaction staining (Poly Scientific R&D Corp, Bay Shore, NY) was performed on the tissue sections according to the manufacturer's recommendations. Quantification of mucus-stained cells was assessed with morphometric analysis using the Metamorph Imaging System (Lumenera Corporation, Infinity Analyze 6.1.0) as described. Lung sections were taken from the same position in each set of mice and at least 4-5 random sections/mouse were analyzed. Using digital image capture, tissue regions associated with the entire perivascular/peribroncheal region were viewed and goblet cells were quantified as the percentage of total mucus-producing cells relative to the total number of airway epithelial cells. ¹

Masson's trichrome collagen staining

Tissue sections obtained from the different groups of mice were fixed with 4% formaldehyde and embedded in paraffin. Then, 5-µm sections were cut and placed on positively charged glass slides. Collagen staining was performed on tissue sections using Masson's trichrome staining (Poly Scientific R&D Corp, NY) method

for the detection of collagen fibers according to the manufacturer's recommendations.^{1, 11}

ELISA analysis

Cytokine levels in serum, BALF and lung homogenates were measured using DuoSet ELISA kits (IL-5, IL-13, and IL-18 from Thermo Fisher Scientific, San Diego, CA). The detection limits were 4 pg/ml for IL-5, IL-13, and IL-18 (19 pg/mL).^{1, 11, 12}

Immunoblot Analysis

Lung tissue samples were obtained from the different treatment groups were homogenized in lysis buffer, and homogenates were electrophoresed in 4–12% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. The membranes were then incubated with blocking buffer -5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Immunoblotting was performed using a specific primary antibody for MUC1 and MUC5AC (Santa Cruz Biotechnology), followed by an anti-rabbit secondary antibody conjugated with horseradish peroxides (Cell Signaling Technology) **Table S1**. The signal was developed using Clarity Max ECL Substrate (Bio-Rad) according to the manufacturer's instructions. Equal loading was further verified by immunoblotting the same membrane with GAPDH (Cell Signaling Technology) using a similar protocol following stripping with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific).^{1, 8}

RNA isolation and quantitative transcript analysis by polymerase chain reaction (qPCR)

RNA was isolated from the lung tissues by the TRIZOL method. RNA samples (500 ng) were subjected to reverse transcription using Bioscript reverse transcriptase (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. IL-13 mRNA levels were quantified using gene-specific primers and real-time PCR (IQ5, Bio-Rad). The primers used in the study were as follows: mL-13—F-5'-CATGGCGCTCTGGGTGACTG-3', R5'-CGGCCAGGTCCACACTCCATAC-3'; mGAPDH F 5'-ACCCAGAAGACTGTGGATGG-3', R 5'-CACATTGGGGGTAGGAACAC-3'. The gene expression data were normalized to GAPDH amplified from the same cDNA mix and expressed as relative gene expression as described earlier.¹

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Supplementary Figure legends.

Supplementary Figure 1. A diagrammatic representation of the protocol followed for induction of asthma in mice (WT (BALB/c) & *IL-5*^{-/-}) and (WT (C57BL/6) & *IL-18*^{-/-}) is shown (**A**). A representative flow cytometer dot blot analysis of BALF cells were analyzed by selecting live CCR3 positive leukocytes for the detection of CCR3⁺Siglec-F⁺ eosinophils (**B**). Morphometric quantification of MBP⁺ positive cells expressed as cells/mm² in WT (BALB/c), *IL-5*^{-/-}, (WT (C57BL/6) and *IL-18*^{-/-} mice is shown (**C,D**). Morphometric quantification of MBP⁺ positive cells expressed as cells/mm² in WT (BALB/c), CD-2IL-5tg, CC10-IL-18tg (NoDOX) and CC10-IL-18tg (DOX) mice is shown (**E**). RT-PCR analysis detected reduced mRNA *IL-13* levels in *Aspergillus*-challenged *IL-5*^{-/-} compared *Aspergillus*-challenged WT (BALB/c) mice lung tissues (**F**). Data are expressed as mean± SD, n=8-10 mice/group. *p<0.01, ***p<0.0001- WT (BALB/c)- *Aspergillus*-challenge vs *IL-5*^{-/-} *Aspergillus*-challenge; WT (C57BL/6)- *Aspergillus*-challenge vs *IL-18*^{-/-} *Aspergillus*-challenge; ###p<0.0001- WT (BALB/c)- *Aspergillus*-challenge vs saline; WT (C57BL/6)- *Aspergillus*-challenge vs saline; \$\$\$p<0.0001- CD2-IL-5tg vs CC10-IL-18tg (DOX).

Supplementary Figure 2. Immunofluorescence analysis detected induced macrophages (F4/80⁺) and NLRP3⁺ double positive cells and NLRP3 and IL-18 colocalized double positive cells were observed (**Aii-Bii**) in *Aspergillus* challenged lung sections compared to saline challenged mice (**Ai-Bi**). A diagrammatic representation of rIL-18 challenges in the CD2-IL-5tg and WT (BALB/c) mice protocol is shown (**C**). ELISA analysis detected a significant increase the levels of

eosinophil active cytokines IL-5 and IL-13 in rIL-18-challenged CD2-IL-5tg mice and WT (BALB/c) mice compared to mice challenged with saline (**D,E**). Masson's trichrome stained lung sections showed collagen accumulation in the rIL-18-challenged CD2-IL-5 and WT (BALB/c) mice. rIL-18- challenged CD2-IL-5tg mice showed induced accumulation of lung collagen compared to WT (BALB/c) mice (**F i-iv**). Morphometric quantification of MBP⁺ and EPX⁺ positive cells expressed as cells/mm² (**G,H**). Data are expressed as mean $\pm$ SD, n=10-12 mice/group, #p<0.02, ##p<0.001, ###p<0.0001- WT (BALB/c) -saline vs WT (BALB/c) -rIL-18; *p<0.02, **p<0.001- CD2-IL-5tg -saline vs CD2-IL-5tg-rIL-18. Representative photomicrographs presented as x100 of original magnification.

Supplementary Figure 3: A representative flow cytometry dot blot analysis showed induced detection of CCR3⁺Siglec-F⁺ eosinophils (**A i-ii**) and CD274 expression (**A iii-iv**) based on isotype-matched anti-IgG (**A v-vi**) in the blood of rIL-18 given WT (BALB/c) mice compared to saline given mice. A diagrammatic representation of *Aspergillus*-challenged *IL-5*^{-/-}/*IL-18*^{-/-} mice and littermate control protocol is shown (**B**). ELISA analysis showed no detection IL-5 levels (**C**) and detected decreased of IL-13 protein levels (**D**) in the BALF of *Aspergillus* -challenged *IL-5*^{-/-}/*IL-18*^{-/-} mice compared to the challenged littermate matched control mice. Morphometric quantification of MBP⁺ positive cells, expressed as cells/mm² (**E**). Data are expressed as the mean $\pm$ SD, n=6-10 mice/group, **p<0.001, ***p<0.0001- *Aspergillus*-challenged littermate matched control vs *IL-5*^{-/-}/*IL-18*^{-/-} double gene

deficient mice; $^{**}p<0.001$, $^{***}p<0.0001$ - *Aspergillus*-challenged littermate matched control vs saline challenged mice.

Supplementary Figure 4: A diagrammatic representation of anti-mouse rabbit polyclonal anti-CD274 neutralization antibody treatment and allergen-challenged protocol is shown (A). Flow cytometry dot blot analysis of CCR3⁺Siglec-F⁺ eosinophils in the BALF of *Aspergillus*-challenged anti-CD274 neutralized and anti-IgG non-neutralized mice are shown (B,C). Representative light microscopy photomicrographs of lung sections with Masson's trichrome staining show perivascular and peribronchiolar collagen accumulation after 3 weeks of *Aspergillus*-challenged anti-IgG non-neutralized compared to anti-CD274 neutralized mice (D, i-iv). ELISA analysis detected decreased IL-18 and IL-13 levels in the BALF of *Aspergillus*-challenged anti-IgG non-neutralized compared to anti-CD274 neutralized mice (E, F). Morphometric quantification of MBP⁺ positive cells, expressed as cells/mm² (G). Representative photomicrographs presented as 100x of original magnification. Data is expressed as mean $\pm$ SD, n=8-12 mice/group. $^{*}p<0.01$, $^{***}p<0.0001$ - anti-CD274 neutralized vs anti-IgG non-neutralized mice; $^{**}p<0.001$, $^{***}p<0.0001$ - *Aspergillus*-challenged anti-IgG non-neutralized vs saline mice.

Supplementary Figure 5: A diagrammatic representation of anti-mouse rabbit polyclonal anti-IL-18 neutralization antibody treatment and *Aspergillus*-challenged protocol is shown (A). Representative light-microscopy photomicrographs of lung

sections with Masson's trichrome staining show perivascular and peribronchiolar collagen accumulation after 3 weeks of *Aspergillus*-challenged anti-IgG non-neutralized compared to anti-IL-18 neutralized mice (**B, i-iv**). IL-18 ELISA in BALF and serum of *Aspergillus*-challenged anti-IgG non-neutralized and anti-IL-18 neutralized mice (**C,D**). Data is expressed as mean $\pm$ SD, n=8-12 mice/group. *p<0.01, **p<0.001- anti-IL-18 neutralized ab vs anti-IgG non-neutralized mice. Photomicrographs presented are 100X original magnification.