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Lack of IL-1 Receptor Signaling Reduces Spontaneous Airway Eosinophilia in Juvenile Mice with Muco-Obstructive Lung Disease

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Running title: Role of IL-1 receptor signaling in airway eosinophilia

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

ABSTRACT

Previous studies demonstrated spontaneous type 2 airway inflammation with eosinophilia juvenile *Scnn1b*-Tg mice with muco-obstructive lung disease. Interleukin-1 receptor (IL-1R) signaling has been implicated in allergen-driven airways disease, however, its role in eosinophilic inflammation in muco-obstructive lung disease remains unknown. In this study, we examined the role of IL-1R signaling in the development of airway eosinophilia and type 2 inflammation in juvenile *Scnn1b*-Tg mice. We determined effects of genetic deletion of *Il1r1* on eosinophil counts, transcript levels of key type 2 cytokines, markers of eosinophil activation and apoptosis, and tissue morphology in lungs of *Scnn1b*-Tg mice at different time points during neonatal development. Further, we measured endothelial surface expression of intercellular adhesion molecule 1 (ICAM-1), an integrin involved in eosinophil transendothelial migration, and determined effects of eosinophil depletion using an anti-IL-5 antibody on lung morphology. Lack of IL-1R reduced airway eosinophilia and structural lung damage, but did not reduce levels of type 2 cytokines and associated eosinophil activation in *Scnn1b*-Tg mice. Structural lung damage in *Scnn1b*-Tg mice was also reduced by eosinophil depletion. Lack of IL-1R was associated with reduced expression of ICAM-1 on lung endothelial cells and reduced eosinophil counts in lungs from *Scnn1b*-Tg mice. We conclude that IL-1R signaling is implicated in airway eosinophilia independent of type 2 cytokines in juvenile *Scnn1b*-Tg mice. Our data suggest that IL-1R signaling may be relevant in the pathogenesis of eosinophilic airway inflammation in muco-obstructive lung diseases, which may, in part, be mediated by ICAM-1 dependent transmigration of eosinophils into the lungs.

Keywords: Eosinophil, interleukin-1, migration, type 2 inflammation

INTRODUCTION

Airway eosinophilia is an important phenotype in muco-obstructive lung diseases including asthma and chronic obstructive pulmonary disease (COPD) (1, 2). Airway eosinophils are key effector cells in allergic asthma mediating airway hyperresponsiveness, inflammation and remodeling through the release of pro-inflammatory mediators (3). Additionally, airway eosinophilia is associated with more severe lung disease in patients with COPD (4, 5). *Scnn1b*-Tg mice present with spontaneous muco-obstructive lung disease caused by airway surface dehydration and reduced mucociliary clearance (MCC) leading to airway mucus obstruction, chronic inflammation and structural lung damage (6–9). Previous longitudinal studies of *Scnn1b*-Tg mice demonstrated that during the first weeks of life this muco-obstructive phenotype is associated with the development of spontaneous eosinophilic inflammation and type 2 inflammation (9–11). Recent studies suggest that this airway eosinophilia may be in part triggered by allergen retention (12), but the pathogenesis of eosinophilic inflammation and its relationship to type 2 inflammation remain incompletely understood.

Previous studies in mouse models of experimental asthma identified a role of interleukin-1 receptor (IL-1R) signaling in the pathogenesis of airway eosinophilia and type 2 inflammation in allergen-induced airways disease (13–15). In a house dust mite (HDM)-induced model of allergic airway disease it was shown that HDM exposure triggers release of interleukin-1 α (IL-1 α) from airway epithelial cells, which acts in an autocrine manner to induce the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-33 (IL-33) that in turn activate dendritic cells to drive adaptive type 2 immunity (16). In a previous study, we identified a role of IL-1R signaling in the pathogenesis of neutrophilic airway inflammation in *Scnn1b*-Tg mice (17). Specifically, we found that airway mucus obstruction triggers hypoxic necrosis of a subset of airway epithelial cells leading to the release of IL-1 α (17–20). Further, we showed that IL-1R signaling contributes to the development of neutrophilic airway inflammation, mucus plugging and structural lung damage in this model of muco-obstructive lung disease (17). However, the role of IL-1R signaling in the pathogenesis

of spontaneous airway eosinophilia and type 2 inflammation observed in juvenile *Scnn1b*-Tg mice remains unknown.

The aim of this study was, therefore, to examine the role IL-1R signaling in the development of spontaneous airway eosinophilia and type 2 inflammation present in juvenile *Scnn1b*-Tg mice. To achieve this goal, we determined effects of genetic deletion of *Il1r1* on eosinophil counts in bronchoalveolar lavage (BAL) and lung tissue, transcript levels of key type 2 cytokines and eosinophil chemoattractants (*Il13*, *Il5*, *Ccl11*, *Ccl24*), eosinophil activation and apoptosis, eosinophil granule protein levels and tissue morphology in lungs of wild-type (WT) and *Scnn1b*-Tg mice at different time points during neonatal development. Further, we measured endothelial surface expression of intercellular adhesion molecule 1 (ICAM-1), an integrin involved in eosinophil transmigration from the vasculature into the lungs. Finally, we treated *Scnn1b*-Tg mice and WT controls with anti-IL-5 antibody to deplete eosinophils from the lungs and evaluated effects on lung morphology.

MATERIALS AND METHODS

Experimental animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany or were in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Ethical Review Committee of Queen's University Belfast (Belfast, UK). *Scnn1b*-Tg mice were studied on a C57BL/6N background as previously described (21). *Scnn1b*-Tg mice were inter-crossed with *Il1r1*^{-/-} mice on the C57BL/6 background (Jackson Laboratories, Bar Harbor, ME, USA) to generate *Scnn1b*-Tg/*Il1r1*^{-/-}, *Scnn1b*-Tg, *Il1r1*^{-/-} and WT mice in the second generation and all analyses were performed on littermates. Offspring were genotyped as previously described (6, 22) and mice were studied at 3, 8, 10, 12 and 14 days of age. All animals were housed in specific pathogen free animal facilities and had free access to chow and water.

Eosinophil depletion

Mice were treated by subcutaneous injections of 100 µg of either anti-IL-5 antibody (TRFK5, BioXCell, NH, USA) or an isotype control antibody (TNP6A7, BioXCell, NH, USA) at the age of 3, 6, 9 and 12 days after birth. At the age of 14 days, mice were killed by overdose of pentobarbital, BAL fluid was collected and lungs were inflated and fixed in formalin for assessment of structural lung damage as described below.

Histology and airway morphology

Murine lung tissue was fixed in formalin and embedded in paraffin, sections were cut at 5 µm and stained with Alcian blue periodic acid-Schiff (AB-PAS) and with hematoxylin and eosin (H & E) as described previously (9). Airway mucus content was assessed in the left proximal main axial airway by determining the volume of AB-PAS positive material per surface area (nl/mm²). For assessment of structural lung damage, right lungs were inflated to 25 cm of fixative pressure and mean linear intercepts (MLI) as a measurement of distal airspace enlargement and the destructive index (DI) as a measurement of alveolar wall destruction were evaluated as previously described (6, 23).

Flow cytometry

Multiparameter flow cytometry was performed using an LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany). Single cell suspensions were obtained from whole lung and surface stained with fluorescent antibodies to delineate cell populations and surface expression of ICAM-1 and annexin V. Flow cytometry was performed immediately after cellular staining with exclusion of nonviable cells and doublets, and data were analyzed with FlowJo software (v10; TreeStar, Ashland, OR) as previously described (12). Additional details are provided in the online supplement.

Real-time RT-PCR

Semi-quantitative real time RT-PCR was performed on an Applied Biosystems 7500 Real Time PCR

System using TaqMan Universal PCR master mix and inventoried TaqMan gene expression assays for *Ilf5* (Mm00439646_m1), *Ilf13* (Mm00434204_m1), *Ccl11* (Mm00441238_m1), and *Ccl24* (Mm00444701_m1) (Applied Biosystems, Darmstadt, Germany). Relative fold change of target gene expression was determined by normalization to expression of the reference gene *Actb* (Mm00607939_s1).

BAL cell counts and eosinophil granule protein measurement

Mice were deeply anesthetized by means of intraperitoneal injection of 120 mg/kg ketamine and 16 mg/kg xylazine (Sigma-Aldrich, Taufkirchen, Germany) and exsanguinated. BAL and differential cell counts were performed as previously described (9) and detailed in the online supplement. Concentrations of the eosinophil granule proteins eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil peroxidase (EPO) and in BAL fluid were quantified by ELISA (Biomatik, Cambridge, Canada).

Statistics

All data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) and are reported as mean $\pm$ SEM. Statistical analyses were performed using Student's t-test, Mann-Whitney Rank Sum test, one-way ANOVA, Kruskal-Wallis test or Pearson correlation coefficient as appropriate and $P < 0.05$ was accepted to indicate statistical significance.

RESULTS

Lack of IL-1R reduces spontaneous eosinophilic airway inflammation in juvenile *Scnn1b*-Tg mice

To determine if IL-1R signaling contributes to the development of spontaneous airway inflammation in juvenile *Scnn1b*-Tg mice we compared inflammatory cell counts in BAL fluid collected from WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 8, 10, 12 and 14 days of age (Figure 1A-D). Consistent with previous studies (6, 9), juvenile *Scnn1b*-Tg mice presented with an increase in BAL total

inflammatory cell counts compared to WT littermates (Figure 1A) associated with spontaneous neutrophilic as well as eosinophilic airway inflammation (Figure 1C and D). Lack of IL-1R had no effect on the number of total cells or macrophages in the BAL fluid of juvenile *Scnn1b*-Tg mice (Figure A and B), but reduced airway neutrophils as previously shown in studies of neonatal and adult *Scnn1b*-Tg mice (17) (Figure 1C). In addition, lack of IL-1R largely reduced the development of airway eosinophilia in juvenile *Scnn1b*-Tg mice. Specifically, eosinophil counts in BAL were significantly reduced in *Scnn1b*-Tg/*Il1r1*^{-/-} mice compared to *Scnn1b*-Tg mice of the same age from 10 days of age onward (Figure 1D). These results demonstrate that IL-1R signaling contributes to the development of spontaneous eosinophilic airway inflammation in juvenile *Scnn1b*-Tg mice.

Lack of IL-1R delays, but does not prevent, the onset of type 2 airway inflammation in juvenile *Scnn1b*-Tg mice

Our previous studies demonstrated that spontaneous airway eosinophilia is associated with transient type 2 airway inflammation in juvenile *Scnn1b*-Tg mice (9, 24). To examine the role of IL-1R signaling in this age-dependent type 2 inflammation associated with muco-obstructive lung disease, we next performed longitudinal measurements of transcript levels of the key type 2 cytokines *Il13* and *Il5*, and eosinophil chemoattractants *Ccl11* and *Ccl24*, (Figure 2A-D) in lung tissues from neonatal to juvenile WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice (i.e. at 3, 8, 10, 12 and 14 days of age). Consistent with previous studies, we observed no difference in expression of these type 2 cytokines between *Scnn1b*-Tg mice and WT littermates of neonatal age (9). However, transcript levels were increased in juvenile *Scnn1b*-Tg mice peaking around day 8 to 10 before beginning to wane (Figure 2A-D). *Scnn1b*-Tg/*Il1r1*^{-/-} mice showed a significant reduction in the expression of *Il13* and *Il5* transcripts at day 8 compared to their *Scnn1b*-Tg littermates of the same age (Figure 2A and B). Similarly, a trend toward reduction in expression of the eosinophil chemoattractants *Ccl11* and *Ccl24* was observed at 8 days of age (Figure 2C and D). These results suggest that IL-1R may contribute to the initiation of type 2 inflammation in this model. However, at later time points, lack of IL-1R had no effect on transcript

levels of any of these key type 2 cytokines demonstrating that IL-1R signaling is not essential for the upregulation of type 2 cytokines in juvenile *Scnn1b*-Tg mice (Figure 2A-D). When viewed in combination with BAL eosinophil counts (Figure 1D) these data show that IL-1R is implicated in eosinophilic airway inflammation in juvenile *Scnn1b*-Tg mice independent of type 2 inflammation.

Lack of IL-1R does not prevent activation of airway eosinophils in juvenile *Scnn1b*-Tg mice

Previous studies showed a shift in the eosinophil phenotype during allergic inflammation to a Siglec $F^{\text{high}}/CD11c^{\text{low}}$ phenotype that is associated with enhanced recruitment of eosinophils to the airways (25). Because we observed a sustained decrease in airway eosinophil counts, but not type 2 cytokines in lungs from juvenile *Scnn1b*-Tg/*Il1r1*^{-/-} mice, we therefore examined the effects of IL-1R knockout on this eosinophil phenotype in lungs from WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice by flow cytometry (Figure 3A-C). In these studies, we compared eosinophil activation, as determined from the Siglec $F^{\text{high}}/CD11c^{\text{low}}$ phenotype, at 8 days of age when transcript levels of type 2 cytokines were still in the normal range observed in WT mice, and at 12 days of age when type 2 cytokine transcripts were increased to similar levels in *Scnn1b*-Tg/*Il1r1*^{-/-} compared to *Scnn1b*-Tg mice (Figure 2A-D). At both time points *Scnn1b*-Tg mice had a significantly higher proportion of Siglec $F^{\text{high}}/CD11c^{\text{low}}$ eosinophils compared to WT littermates (Figure 3B and C). In *Scnn1b*-Tg/*Il1r1*^{-/-} mice the proportion of Siglec $F^{\text{high}}/CD11c^{\text{low}}$ airway eosinophils was reduced compared to *Scnn1b*-Tg mice at 8 days, but increased to similar levels at 12 days of age. (Figure 3A-C). As an independent measure of eosinophil activation, we determined levels of secreted eosinophil granule proteins ECP, MBP and EPO in BAL fluid from 14 day old WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice. Consistent with low-grade airway eosinophilia in this age group (Figure 1D) (9, 11, 12), all three secreted eosinophil granule proteins were detected at low levels in juvenile WT mice (Figure E1). In *Scnn1b*-Tg mice, ECP and MBP remained unchanged, whereas EPO was significantly increased compared to WT controls (Figure E1). A similar pattern was observed in BAL fluid of *Scnn1b*-Tg/*Il1r1*^{-/-} mice, where reduced levels of eosinophils were associated with reduced levels of EPO compared to *Scnn1b*-Tg mice (Figure E1C). Taken together,

these results suggest that IL-1R signaling is not essential for eosinophil activation that is associated with type 2 airway inflammation in juvenile *Scnn1b*-Tg mice.

Lack of IL-1R reduces endothelial ICAM-1 expression and eosinophil recruitment to the lungs in juvenile mice

It was previously shown that IL-1R signaling activates endothelial cells and promotes leukocyte transmigration by inducing the expression of the surface integrin ICAM-1 (26, 27). To examine the potential role of IL-1R in recruitment of eosinophils from the circulation into the lung, we first determined eosinophil numbers by flow cytometry in whole lung tissue from WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 3, 8, 10 and 12 days of age (Figure 4A). Similar to BAL fluid, whole lung eosinophil counts were increased in *Scnn1b*-Tg mice compared to WT mice at 10 and 12 days of age. Lack of IL-1R reduced eosinophil numbers in lung tissue of both WT and *Scnn1b*-Tg mice with significant reductions at 8 and 10 days in WT and 10 and 12 days in *Scnn1b*-Tg mice (Figure 4A). Next, we examined expression levels of ICAM-1 on endothelial cells by flow cytometry in WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 14 days of age (Figure 4B). Endothelial ICAM-1 expression was unchanged in *Scnn1b*-Tg compared to WT mice. However, expression of ICAM-1 was significantly reduced in both *Il1r1*^{-/-} and *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 4B). To examine if differences in apoptosis may be involved in reduced eosinophil counts in *Scnn1b*-Tg/*Il1r1*^{-/-} compared to *Scnn1b*-Tg mice, we examined annexin V surface expression as a marker of apoptotic cell death on airway eosinophils from 14 day old WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 4C). The percentage of apoptotic cells was ~21% increased in *Scnn1b*-Tg compared to WT mice but remained unchanged in *Scnn1b*-Tg/*Il1r1*^{-/-} compared to *Scnn1b*-Tg mice demonstrating that eosinophil apoptosis was not affected by the lack of IL-1R (Figure 4C). Taken together, these data show that lack of IL-1R reduced endothelial ICAM-1 expression and total lung eosinophil counts, but did not affect apoptotic cell death, in WT and *Scnn1b*-Tg mice. These data suggest that IL-1R signaling is implicated in eosinophil migration from the blood into the lung tissue and that abrogation of this function contributes to the reduction of

eosinophilia observed in lung tissue and BAL from *Scnn1b*-Tg/*Il1r1*^{-/-} compared to *Scnn1b*-Tg mice.

Lack of IL-1R and eosinophil depletion reduces structural lung damage in juvenile *Scnn1b*-Tg mice

We previously demonstrated emphysema-like structural damage of distal airspaces in juvenile *Scnn1b*-Tg mice, however, the relationship between airway eosinophilia and early lung damage has not been studied (7, 9). We, therefore, compared MLI and DI in WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 14 days of age (Figure 5A-C). As expected from previous studies (9, 28), MLI and DI were increased in *Scnn1b*-Tg compared to WT mice. No difference was observed in MLI and DI between WT and *Il1r1*^{-/-} mice. However, in *Scnn1b*-Tg/*Il1r1*^{-/-} mice MLI and DI were significantly reduced compared to *Scnn1b*-Tg mice (Figure 5A-C). To determine the specific role of eosinophils in structural lung damage more directly, we next performed eosinophil depletion experiments. We treated neonatal *Scnn1b*-Tg mice and WT controls with anti-IL5 antibody or isotype control and examined effects on emphysema-like structural lung damage. Anti-IL5 treatment specifically depleted eosinophils without reducing neutrophil or macrophage counts in BAL fluid of juvenile *Scnn1b*-Tg mice (Figure 6A-D). Eosinophil depletion was associated with a significant decrease in MLI in *Scnn1b*-Tg mice (Figure 6F). In addition, we found a direct correlation between BAL eosinophil counts and MLI in *Scnn1b*-Tg mice (Figure 6G). These results support that activated airway eosinophils contribute to the development of structural lung damage in early muco-obstructive lung disease in juvenile *Scnn1b*-Tg mice.

DISCUSSION

This study demonstrates for the first time that impaired airway mucus clearance triggers spontaneous eosinophilic airway inflammation in juvenile mice that is, in part, mediated by IL-1R signaling independent of type 2 cytokine expression (Figure 1 and 2). We show that lack of IL-1R signaling had no effect on eosinophil activation or apoptosis in the airways, but is associated with reduced expression of the integrin ICAM-1 on endothelial cells and may thereby contribute to reduced migration of eosinophils from the circulation into the lung tissue (Figure 3 and 4). Finally, reduction of

airway eosinophilia in juvenile *Scnn1b*-Tg mice, either by genetic deletion of IL-1R or by eosinophil depletion, was associated with a decrease in emphysema-like distal airspace enlargement suggesting that activated eosinophils contribute to early structural lung damage (Figure 5 and 6). Collectively our results provide novel insights into the role of IL-1R signaling in the in vivo pathogenesis of airway eosinophilia in muco-obstructive lung disease.

Our results show that the development of spontaneous eosinophilic airway inflammation characteristic of juvenile *Scnn1b*-Tg mice (9–11) requires intact IL-1R signaling in addition to chemotactic factors released in type 2 airway inflammation (Figure 1). We speculate that the continued presence of type 2 inflammatory cytokines despite attenuated airway eosinophilia in juvenile *Scnn1b*-Tg/*Il1r1*^{-/-} mice may be driven by release of interleukin-13 (IL-13) from a spectrum of innate and adaptive immune cells that are activated by inhaled allergens and other irritants retained in mucostatic airways of juvenile *Scnn1b*-Tg mice. This hypothesis is supported by a recent study demonstrating that IL-13 levels were increased in airway epithelial cells, macrophages, type 2 innate lymphoid cells (ILC2), and T helper type 2 (TH2) cells in juvenile *Scnn1b*-Tg mice vs. WT mice at baseline, and that IL-13 production by these cell types is further increased upon allergen challenge (12). Whilst IL-1R was not essential for the upregulation of type 2 cytokines in *Scnn1b*-Tg mice, our longitudinal measurements in neonatal and juvenile mice indicate that IL-1R signaling may contribute to the initiation of this inflammatory response (Figure 2). This is in line with previously published data showing that IL-1R is implicated in the initiation of type 2 inflammation in a HDM-induced model of allergic lung disease (16).

Lack of IL-1R reduced eosinophils not only in the airways, but also in the whole lung (Fig. 4A) suggesting that trans-endothelial migration of eosinophils from the blood into the lung tissue may be a potential mechanism for the observed reduction in airway eosinophilia in juvenile *Scnn1b*-Tg/*Il1r1*^{-/-} mice. In this context, interleukin-1 (IL-1) is a well-known activator of the vascular endothelium modulating the adhesive properties of endothelial cells via adhesion molecules such as ICAM-1 that are essential for

the regulation of leukocyte migration into tissues during inflammation (29–31). We observed a small but significant downregulation of ICAM-1 on lung endothelial cells from WT (~22%) and *Scnn1b*-Tg (~12%) mice lacking IL-1R (Figure 4B). ICAM-1, a major endothelial adhesion molecule has previously been shown to be induced by IL-1 and contributed to eosinophil adhesion to the vascular endothelium (32, 33). Of note, previous studies have shown that even small changes in ICAM-1 expression can result in physiologically relevant changes in leukocyte adhesion (34). The downregulation of ICAM-1 in IL-1R deficient mice in our study may therefore lead to reduced transendothelial migration of eosinophils into the lung tissue, thereby reducing the pool of tissue eosinophils available to migrate into the airways of juvenile *Scnn1b*-Tg mice along the chemoattractant gradient set by type 2 cytokines and eosinophil chemoattractants. Of note, this notion is supported by a previous study showing that knockout of IL-1R on structural lung cells, but not inflammatory cells, was required in order to demonstrate a reduction in airway eosinophilia in mice with HDM-induced asthma (16). Collectively, these data suggest IL-1R and/or ICAM-1 as potential therapeutic targets to inhibit airway eosinophilia in allergen-induced asthma and other muco-obstructive lung diseases.

Finally, we show that lack of IL-1R reduced emphysema-like lung damage, as determined from measurements of MLI and DI in juvenile *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 5A-C). In previous studies we demonstrated that neutrophil elastase (NE), macrophage elastase (matrix metalloproteinase 12 [MMP-12]) and cathepsin S released from activated neutrophils and macrophages in mucostatic airways, are major contributors to structural lung damage in *Scnn1b*-Tg mice (28, 35, 36). While airway neutrophils were also reduced in *Scnn1b*-Tg/*Il1r1*^{-/-} mice (Figure 1C), it is important to note that *Scnn1b*-Tg/*NE*^{-/-} presented with residual emphysema suggesting that other factors may contribute to emphysema formation in this model. However, lack of IL-1R had no effect on macrophages (Figure 1B) constituting the primary source of MMP-12 in the airways making it unlikely that the striking reduction in emphysema observed in juvenile *Scnn1b*-Tg/*Il1r1*^{-/-} mice in this study was determined by alterations in macrophage function. We therefore depleted eosinophils to assess their specific role in structural lung damage in juvenile *Scnn1b*-Tg mice. Eosinophil depletion by treatment with anti-IL-5 antibody

had no effect on neutrophil or macrophage counts in BAL fluid, but resulted in a significant decrease in MLI and more preserved architecture of the distal airspaces in *Scnn1b*-Tg mice (Figure 6A-F). Of note, the effect of eosinophil depletion on elevated MLI was less complete than the effect of genetic deletion of IL-1R that resulted in a reduction of airway eosinophils and neutrophils, and normalized MLI almost completely in juvenile *Scnn1b*-Tg mice (Figure 1 and Figure 5). A role for eosinophils in the development of early structural lung damage in *Scnn1b*-Tg mice is also supported by the direct correlation between airway eosinophils and distal airspace enlargement in juvenile *Scnn1b*-Tg mice (Figure 6G). In this context, we found that EPO secreted by activated eosinophils was significantly increased in the airways of juvenile *Scnn1b*-Tg mice. As EPO was previously shown to be cytotoxic to a variety of cell types including the airway epithelium (37, 38) and to be negatively correlated with FEV₁ in asthma patients (39), we speculate this eosinophil granule protein may be a potential candidate for eosinophil-mediated structural lung damage in *Scnn1b*-Tg mice (Figure E1). Taken together, our results support that eosinophils, in addition to neutrophils, contribute to the development of early structural lung damage in this model of muco-obstructive lung disease.

In summary, we show that IL-1R signaling contributes to spontaneous airway eosinophilia in juvenile *Scnn1b*-Tg mice featuring muco-obstructive lung disease. This role of IL-1R signaling was independent of type 2 cytokine expression and possibly mediated via the surface integrin ICAM-1 on vascular endothelial cells and concomitant activation of eosinophil migration from the circulation into the lung tissue. Additionally, we demonstrate that inhibition of airway eosinophilia in juvenile *Scnn1b*-Tg mice by genetic deletion of IL-1R or eosinophil depletion was associated with reduced structural lung damage. Collectively, our results support an important role of IL-1R signaling in the in vivo pathogenesis of airway eosinophilia and neutrophilia and suggest IL-1R as a potential novel therapeutic target (40) to inhibit this hallmark phenotype in muco-obstructive lung diseases.

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FIGURE LEGENDS

Figure 1. Lack of IL-1R reduces spontaneous eosinophilic airway inflammation in juvenile *Scnn1b*-Tg mice. (A-D) Total cell (A), macrophage (B), neutrophil (C) and eosinophil (D) counts in bronchoalveolar lavage (BAL) fluid from juvenile wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg and double mutant *Scnn1b*-Tg/*Il1r1*^{-/-} mice at indicated time points during development (8 days, 10 days, 12 days and 14 days of age) as determined from May-Grünwald Giemsa stained cytopins. n=5-28 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with WT. [†]*P* < 0.05, [‡]*P* < 0.001 compared with *Scnn1b*-Tg.

Figure 2. Lack of IL-1R delays, but does not prevent the onset of type 2 airway inflammation in juvenile *Scnn1b*-Tg mice. (A-D) Longitudinal measurements of transcript levels of *Il13* (A), *Il5* (B), *Ccl11* (C) and *Ccl24* (D) in lungs from juvenile wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg and double mutant *Scnn1b*-Tg/*Il1r1*^{-/-} mice at indicated ages during postnatal development (3 days, 8 days, 10 days, 12 days and 14 days of age). n=4-8 mice per group. **P* < 0.05, ***P* < 0.01 compared with WT. [†]*P* < 0.05, [‡]*P* < 0.001 compared with *Scnn1b*-Tg.

Figure 3. Lack of IL-1R does not prevent activation of airway eosinophils in juvenile *Scnn1b*-Tg mice. (A) Example dot plots showing 20,000 events in lung tissues of *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 8 days and 12 days of age showing activated eosinophil phenotype (SiglecF^{high}/CD11c^{low}). (B-C) Percent of total activated lung eosinophils expressing the SiglecF^{high}/CD11c^{low} phenotype at 8 days (B) and 12 days (C) of age. n=5-18 mice per group. **P* < 0.01 ***P* < 0.001 compared with wild-type (WT). [†]*P* < 0.05 compared with *Scnn1b*-Tg.

Figure 4. Lack of IL-1R reduces endothelial ICAM-1 expression and eosinophil recruitment to the lungs of juvenile wild-type (WT) and *Scnn1b*-Tg mice. (A) Eosinophil counts in whole lung determined by flow cytometry in WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and double mutant *Scnn1b*-Tg/*Il1r1*^{-/-} mice between 3 and 12 days of age. (B) ICAM-1 expression on endothelial cells from WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 14 days of age determined by flow cytometry. (C) Percentage of apoptotic

eosinophils in BAL fluid from WT, *Il1r1*^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 14 days of age determined by annexin V expression. n=5-22 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to WT. [†]*P* < 0.05, [‡]*P* < 0.01 compared with *Scnn1b*-Tg.

Figure 5. Lack of IL-1R reduces structural lung damage in juvenile *Scnn1b*-Tg mice. (A) Representative lung sections from 14 day old wild-type (WT), *Il1r1*^{-/-}, *Scnn1b*-Tg and double mutant *Scnn1b*-Tg/*Il1r1*^{-/-} mice stained with hematoxylin and eosin (H & E). Scale bar = 100μm. (B) Mean linear intercept (MLI) and (C) destructive index (DI) was accessed from H & E stained lung sections. n=5-7 mice per group. **P* < 0.001 compared to WT. [†]*P* < 0.05 compared with *Scnn1b*-Tg.

Figure 6. Eosinophil depletion reduces structural lung damage in *Scnn1b*-Tg mice. Wild-type (WT) and *Scnn1b*-Tg mice were treated with anti-IL-5 antibody or isotype control at the age of 3, 6, 9 and 12 days after birth and endpoint studies were performed at the age of 14 days. (A-D) Total cell (A), macrophage (B), neutrophil (C) and eosinophil (D) counts in bronchoalveolar lavage (BAL). (E) Representative lung sections stained with hematoxylin and eosin (H & E). Scale bar = 100μm. (F) Mean linear intercept (MLI) was accessed from H & E stained lung sections. (G) Relationship between BAL eosinophil counts and MLI in *Scnn1b*-Tg mice. n=7-14 mice per group. **P* < 0.001, ***P* < 0.0001 compared to WT, [†]*P* < 0.05, [‡]*P* < 0.001 compared with *Scnn1b*-Tg.

Figure 1

148x110mm (600 x 600 DPI)

Figure 2

148x117mm (600 x 600 DPI)

Figure 3

110x161mm (600 x 600 DPI)

Figure 4

115x124mm (600 x 600 DPI)

Figure 5

121x140mm (300 x 300 DPI)

Figure 6

160x166mm (300 x 300 DPI)

Lack of IL-1 Receptor Signaling Reduces Spontaneous Airway Eosinophilia in Juvenile Mice with Muco-Obstructive Lung Disease

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Online Data Supplement

SUPPLEMENTAL METHODS

Flow cytometry

Multiparameter flow cytometry was performed using an LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany). In brief, mice were deeply anesthetized by means of intraperitoneal injection of 120 mg/kg ketamine and 16 mg/kg xylazine (Sigma-Aldrich, Taufkirchen, Germany) and exsanguinated. Following vascular perfusion with PBS to remove blood cells, lungs were excised and placed in 1 ml of PBS on ice for immediate processing. Each lung was removed from PBS, placed in a petri dish and minced to pieces no larger than 2-3 mm before being transferred to a 15 ml conical tube with 1 ml of PBS containing 0.2% collagenase 2 (Worthington Biochemical, Lakewood, NJ) and 30 µg/ml DNase I (Sigma-Aldrich, Taufkirchen, Germany) and incubated for 45 minutes at 37°C with gentle rocking. Digested lung solutions were passed through a 100 µm mesh to obtain single cell suspensions. Red blood cells were lysed with red blood cell lysis buffer (BD Pharm Lyse, BD Biosciences, Heidelberg, Germany), and single cells were finally resuspended in DMEM/+10% FCS medium. Cells for flow analysis of apoptotic cell death were obtained from BAL as described below. Cells were counted and up to 2×10^6 cells were loaded into 96 well plates to be stained. Before surface staining with specific monoclonal fluorochrome-conjugated antibodies, cells were incubated with Fc Block (BD Biosciences, Heidelberg, Germany). For detection of macrophages (CD45⁺CD11c⁺SiglecF⁺), eosinophils (CD45⁺CD11c^{low}SiglecF⁺), neutrophils (CD45⁺CD11b⁺Ly6G⁺), cells were stained with CD4- fluorescein isothiocyanate (FITC), SiglecF-phycoerythrin (PE), CD11c-PE-CF594, Ly6G-peridinin- chlorophyll-protein complex (PerCp)-Cy5.5, Ly6C-PE-Cy7, CD3-V500, CD11b-BV605, CD45- allophycocyanin (APC), CD8-Alexa Fluor (AF)700. For detection of endothelial cells (CD45⁺CD31⁺) and ICAM-1 staining, cells were stained with CD45-PE-Cy7, CD31-APC and ICAM1-PerCp-Cy5.5. For analysis of eosinophil apoptotic cell death, cells were stained with Annexin V-PE, CD45-AF700, Siglec F-PE, and CD11c-Brilliant violet (BV)421. Cell-surface staining was performed for 30

minutes at 4°C. Cell viability was assessed by staining with live/dead fixable eFluor780 for 30 minutes prior to sample analysis. All antibodies were obtained from BD Biosciences (Heidelberg, Germany), Biolegend (San Diego, CA) and eBiosciences (San Diego, CA). Flow cytometry was performed immediately after cellular staining with exclusion of nonviable cells and doublets, and data were analyzed with Flow Jo software (v10; TreeStar, Ashland, OR).

BAL cell counts

Mice were deeply anesthetized by means of intraperitoneal injection of 120 mg/kg ketamine and 16 mg/kg xylazine (Sigma-Aldrich, Taufkirchen, Germany) and exsanguinated, and the trachea was cannulated. Lungs were lavaged with PBS by using a weight-adjusted volume of 0.035 ml/g body weight. This weight-adjusted lavage volume approximates the vital capacity of the developing rodent lung and was used to account for differences in lung size of the age populations of mice included in our study (E1, E2). BAL was repeated twice, both fractions were pooled and centrifuged at 300g for 5 minutes at 4°C. BAL total cell counts were performed, and cell numbers were determined per milliliter of lavage fluid to improve comparability of results from developing mice with different lung sizes, as previously described (E3, E4). BAL differential cell counts were performed from cytopspins stained with May-Grünwald Giemsa, with a total of at least 400 cells counted per mouse.

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SUPPLEMENTAL FIGURES

Figure E1

Figure E1. Eosinophil granular protein concentrations in bronchoalveolar lavage (BAL) fluid.

(A-C) Concentrations of eosinophil cationic protein (ECP) (A), major basic protein (MBP) (B), and eosinophil peroxidase (EPO) (C) were determined in BAL fluid from juvenile wild-type (WT),

Il1r1^{-/-}, *Scnn1b*-Tg and *Scnn1b*-Tg/*Il1r1*^{-/-} mice at 14 days of age. n=4-12 mice per group. **P* < 0.05,

***P* < 0.0001 compared with WT. †*P* < 0.05 compared with *Scnn1b*-Tg.