

Toll-Interacting Protein, Tollip, Inhibits IL-13-Mediated Pulmonary Eosinophilic Inflammation in Mice

Yoko Ito^a Niccolette Schaefer^a Amelia Sanchez^a David Francisco^b
Rafeul Alam^a Richard J. Martin^a Julie G. Ledford^b Connor Stevenson^a
Di Jiang^a Liwu Li^c Monica Kraft^b Hong Wei Chu^a

^aDepartment of Medicine, National Jewish Health, Denver, CO, ^bDepartment of Medicine, University of Arizona College of Medicine, Tucson, AZ, and ^cDepartment of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Keywords

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Abstract

Toll-interacting protein (Tollip) is a key negative regulator of innate immunity by preventing excessive proinflammatory responses. Tollip genetic variation has been associated with airflow limitation in asthma subjects and Tollip expression. Whether Tollip regulates lung inflammation in a type 2 cytokine milieu (e.g., IL-13) is unclear. Our goal was to determine the *in vivo* role of Tollip in IL-13-mediated lung eosinophilic inflammation and the underlying mechanisms. Tollip-knockout (KO) and wild-type (WT) mice were inoculated intranasally with recombinant mouse IL-13 protein to examine lung inflammation. To determine how Tollip regulates inflammation, alveolar macrophages and bone marrow-derived macrophages from Tollip KO and WT mice were cultured with or without IL-13 and/or IL-33. IL-13-treated Tollip KO mice significantly increased lung eosinophilic inflammation and eotaxin-2 (CCL24) levels compared with the WT mice. IL-13-treated Tollip KO (vs. WT) macrophages, in the absence and

particularly in the presence of IL-33, increased expression of the IL-33 receptor ST2L and CCL24, which was in part dependent on enhanced activation of interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) and signal transducer and activator of transcription 6 (STAT6). Our results suggest that Tollip downregulates IL-13-mediated pulmonary eosinophilia in part through inhibiting the activity of the ST2L/IL-33/IRAK1 axis and STAT6.

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Introduction

Asthma, a chronic inflammatory disease of the airways, has increased in prevalence and severity in industrialized nations over the past 50 years [1]. To date, 2 major endotypes of asthma have been described: type 2 (T2) high and T2 low. Patients with T2-high asthma have increased eosinophils in sputum and airway tissue [2]. Eosinophils exert toxic effects on host tissue and exaggerate inflammation through the release of a variety of inflammatory mediators [3]. Asthmatics with a higher number of eosinophils in blood and/or sputum seem to have

more severe disease and more frequent exacerbations than those with lower eosinophil counts [4, 5].

Major regulators of asthma, such as interleukin-33 (IL-33), stimulate the innate or adaptive immune system to secrete T2 cytokines, such as IL-13. Because of the extensive use of mouse IL-13 treatment models in asthma research [2, 6–8], IL-13 has been considered as one of the key regulators in eosinophil recruitment and survival, and other pathological features of asthma, such as airway fibrosis. One of the major mechanisms by which IL-13 induces airway eosinophilia is through the production of eotaxins. Although several types of lung cells produce eotaxins, alveolar macrophages (AMs), particularly in the presence of T2 cytokine stimulation, are critical in eotaxin production. AMs are involved in asthma pathogenesis in both humans and mice [9–11]. IL-13 as well as IL-33 have been shown to increase the expression of eotaxin-2 (CCL24) and other chemokines, including CCL17, CCL18, and CCL22 [9]. However, the mechanisms regulating eotaxin-2 (CCL24) expression in macrophages exposed to T2 cytokines remain unclear.

Toll-interacting protein (Tollip) is a critical regulator of Toll-like receptor (TLR) and IL-1/IL-1 receptor (IL-1R) signaling by interacting with IL-1R-associated kinase 1 (IRAK1) and subsequently suppressing IRAK1 activity [12]. Many types of cells, including macrophages and epithelial cells, express Tollip [13–15]. Although Tollip is classically considered as a negative regulator in bacterial infection to prevent excessive inflammation and tissue damage, its role in noninfectious processes has not been well understood. Several Tollip single nucleotide polymorphisms have been implicated in sepsis [16], tuberculosis [13], idiopathic pulmonary fibrosis [17], and atopic dermatitis [18]. Recently, we reported that asthma subjects carrying the Tollip rs5743899 AG/GG genotypes demonstrate greater airflow limitation as indicated by a lower FEV₁/FVC ratio than those carrying the AA genotype [14]. Moreover, human airway epithelial cells with the AG/GG genotype express less Tollip protein, and produce more eotaxin-3 (CCL26) when stimulated with IL-13 [14]. However, the *in vivo* role of Tollip in regulating IL-13-mediated lung inflammation, especially eosinophilic inflammation, is not clear.

In the present study, we hypothesized that Tollip deficiency in an *in vivo* T2 cytokine (e.g., IL-13) milieu enhances eotaxin production and excessive pulmonary eosinophilic inflammation. To test this hypothesis, we used a Tollip-deficient mouse model of IL-13 treatment to determine the role of Tollip in eotaxin production and pulmonary eosinophilia. Additionally, we examined

the mechanisms by which Tollip downregulates eotaxin production by using cell culture models of AMs and bone marrow-derived macrophages (BMDMs) from Tollip-deficient mice.

Methods

Murine Model of IL-13-Induced Lung Inflammation

Tollip knockout (KO) and IRAK1 KO mice on the C57BL/6 background were obtained from Dr. Liwu Li at Virginia Polytechnic Institute and State University, and bred at National Jewish Health (NJH) Biological Research Center (BRC). ST2 KO mice on the BALB/c background were obtained from Dr. Rafeul Alam at NJH. Wild-type (WT) mice on the C57BL/6 and BALB/c background were purchased from the Jackson Laboratory and housed with the gene KO mice at NJH BRC under pathogen-free housing conditions. All of the experimental protocols were approved by the Institutional Animal Care and Use Committee at NJH. To establish an acute model of IL-13-induced airway disease, mice were intranasally instilled with 3 doses of IL-13 using modified methods as previously published [19, 20]. Mice (13–17 weeks old, both females and males) were administered intranasally with recombinant mouse IL-13 (rmIL-13; R&D Systems Inc., Minneapolis, MN, USA) at 500 ng/mouse prepared in 0.1% BSA (bovine serum albumin) or 0.1% BSA (control) once daily for 3 consecutive days. The mice were sacrificed 24 and 48 h after the last IL-13 treatment. Bronchoalveolar lavage (BAL) was performed with 1 mL of saline. BAL cells were used for the leukocyte count, and BAL fluid (BALF) was saved for ELISA.

Mouse Lung Histopathologic Analysis

The apical lobe of the mouse right lung was fixed in 10% formalin, embedded in paraffin, and cut at 5- μ m thickness for histopathologic analysis. Lung sections were stained with hematoxylin and eosin (HE), and evaluated in a blinded fashion under a light microscope using a histopathologic inflammatory scoring system as we described previously [21]. A final score per mouse on a scale of 0–26 (least to most severe) was obtained based on an assessment of the quantity and quality of peribronchiolar and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates, and parenchymal pneumonia.

Murine Macrophage Culture

Murine primary AMs were obtained by lavaging the lungs. AMs were isolated by adherence and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum) and penicillin/streptomycin. At 6–8 h after adherence in 96-well culture plates, AMs were stimulated with rmIL-13 (10 ng/mL) \pm rmIL-33 (10 ng/mL; Pepro Tech, Rocky Hill, NJ, USA). Cell culture supernatants were collected for CCL24 protein measurement. Cells were saved in RLT lysis buffer (Qiagen Inc., Germantown, MD, USA) at -80°C for subsequent total RNA extraction and RT-PCR.

Due to the limited number of AMs available for the mechanistic studies, BMDMs were used to study how Tollip regulates CCL24 production in the presence or absence of IL-13 and/or IL-33. Bone marrow cells from Tollip KO (C57BL/6 background), ST2 KO (BALB/c background), IRAK1 KO (C57BL/6 back-

ground), and WT (C57BL/6 and BALB/c) mice were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin and recombinant mouse macrophage colony-stimulating factor 25 ng/mL; R&D Systems Inc.) for 7 days to differentiate them into BMDMs. On day 7, BMDMs were harvested by trypsinization and then seeded at 2×10^5 cells/well onto 24-well cell culture plates. On day 8, cells were stimulated by rmIL-13 \pm rmIL-33, and in some conditions cells were preincubated with an anti-mouse ST2L/IL-33R antibody (R&D Systems Inc.) or the rat IgG2B isotype control (R&D Systems Inc.) for 2 h. At the indicated time points, culture medium and cells were collected for protein and mRNA determination.

ELISA

A murine CCL24 ELISA kit (R&D Systems Inc.) was used to quantify CCL24 in murine BALF and macrophage culture medium. Murine IL-33 ELISA (R&D Systems Inc.) was used to measure IL-33 in BALF. The detection range for CCL24 and IL-33 was from 15.6 to 1,000 pg/mL.

Quantitative Real-Time PCR

Quantitative real-time PCR was conducted using Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) to determine relative mRNA levels of ST2L and IL-33. Target gene expression was normalized by the housekeeping gene 18S rRNA. The comparative threshold cycle method ($\Delta\Delta C_t$) was applied to determine the relative levels of target genes.

Western Blot Analysis

Equal amounts of protein from samples with different treatments were separated on 10 or 15% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with a goat anti-Tollip antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA), a rabbit anti-IRAK1 antibody (Cell Signaling Technology Inc., Danvers, MA, USA), a rabbit anti-pIRAK1 antibody (Phospho-Thr209; Aviva Systems Biology, San Diego, CA, USA), a goat anti-ST2L antibody (R&D Systems Inc.), a rabbit anti-STAT6 or anti-pSTAT6 (Phospho-Tyr641, Cell Signaling Technology Inc.) antibody, or a mouse anti-GAPDH antibody (Santa Cruz Biotechnology Inc.).

Blots were then incubated with appropriate horseradish peroxidase-linked secondary antibodies and developed with ECL Western blotting substrate. Densitometry was performed using the NIH Image-J software. Activation of IRAK1 or STAT6 was measured by analyzing their phosphorylation levels as indicated by the ratio of phosphorylated versus nonphosphorylated IRAK1 or STAT6.

Statistical Analysis

If the data were normally distributed, 1-way analysis of variance (ANOVA) followed by a Tukey post hoc test was used for multiple comparisons. The Student *t* test was used when 2 groups were compared. For nonparametric data, the Kruskal-Wallis test for multiple comparisons or the Mann-Whitney test for 2 group comparisons were applied. Pearson correlation analysis was performed to determine the relationship of lung eosinophils and CCL24 levels. A *p* value <0.05 was considered significant.

Results

Tollip Inhibits Eosinophil Recruitment into the Lung of IL-13-Treated Mice

After 24 h of the last IL-13 treatment, the total leukocyte counts in BALF were increased in both WT and Tollip KO mice, and were higher in Tollip KO mice than WT mice, although a significant difference was not achieved ($p = 0.06$; Fig. 1a). Importantly, following IL-13 treatment, the percentage and number of eosinophils in BALF were significantly higher in Tollip KO mice than WT mice (Fig. 1b, c). Additionally, lymphocytes were also increased in Tollip KO mice as compared to the WT mice (Fig. 1d). IL-13 increased the number of neutrophils in both WT and Tollip KO mice, but there was no significant difference between the 2 groups (Fig. 1e). The number of macrophages in BALF did not change after IL-13 exposure (Fig. 1f). Examination of lung histopathology of IL-13-treated Tollip KO mice revealed more severe infiltrate of inflammatory cells including eosinophils around large airways and in the alveolar space (Fig. 2a–f). The histopathology score, an indication of overall inflammation levels, was significantly higher in IL-13-treated Tollip KO mice than the WT mice (Fig. 2g).

We also collected BALF at 48 h after the last IL-13 treatment. Tollip KO mice showed higher eosinophil and lymphocyte counts in BALF than WT mice, but the differences did not reach the statistically significant level (eosinophils – WT: $40,311 \pm 9,905$ cells/mL, KO: $110,574 \pm 26,881$ cells/mL, $p = 0.07$; lymphocytes – WT: $4,215 \pm 1,119$ cells/mL, KO: $16,712 \pm 5,172$ cells/mL, $p = 0.13$).

Tollip Reduces Lung Secretion of CCL24 into BALF

As the focus of our study was to investigate how Tollip regulates eosinophilic inflammation, our mechanistic studies were centered on the pathways related to eotaxin expression, particularly at the earlier time point (e.g., 24 h) to mimic acute inflammation related to asthma exacerbations. In line with the BAL eosinophil data, IL-13-treated Tollip KO mice showed higher levels of CCL24 protein in BALF than the WT mice at 24 h (Fig. 3a) and at 48 h (WT: 638 ± 256 pg/mL, KO: $1,654 \pm 397$ pg/mL, $p = 0.06$). Notably, CCL24 protein levels were positively correlated with BAL eosinophil counts in both Tollip KO and WT mice (Fig. 3b).

Tollip Suppresses IL-13-Induced CCL24 in Cultured Murine Macrophages

As CCL24 produced by macrophages is responsible for IL-13-mediated airway eosinophilia in a murine model

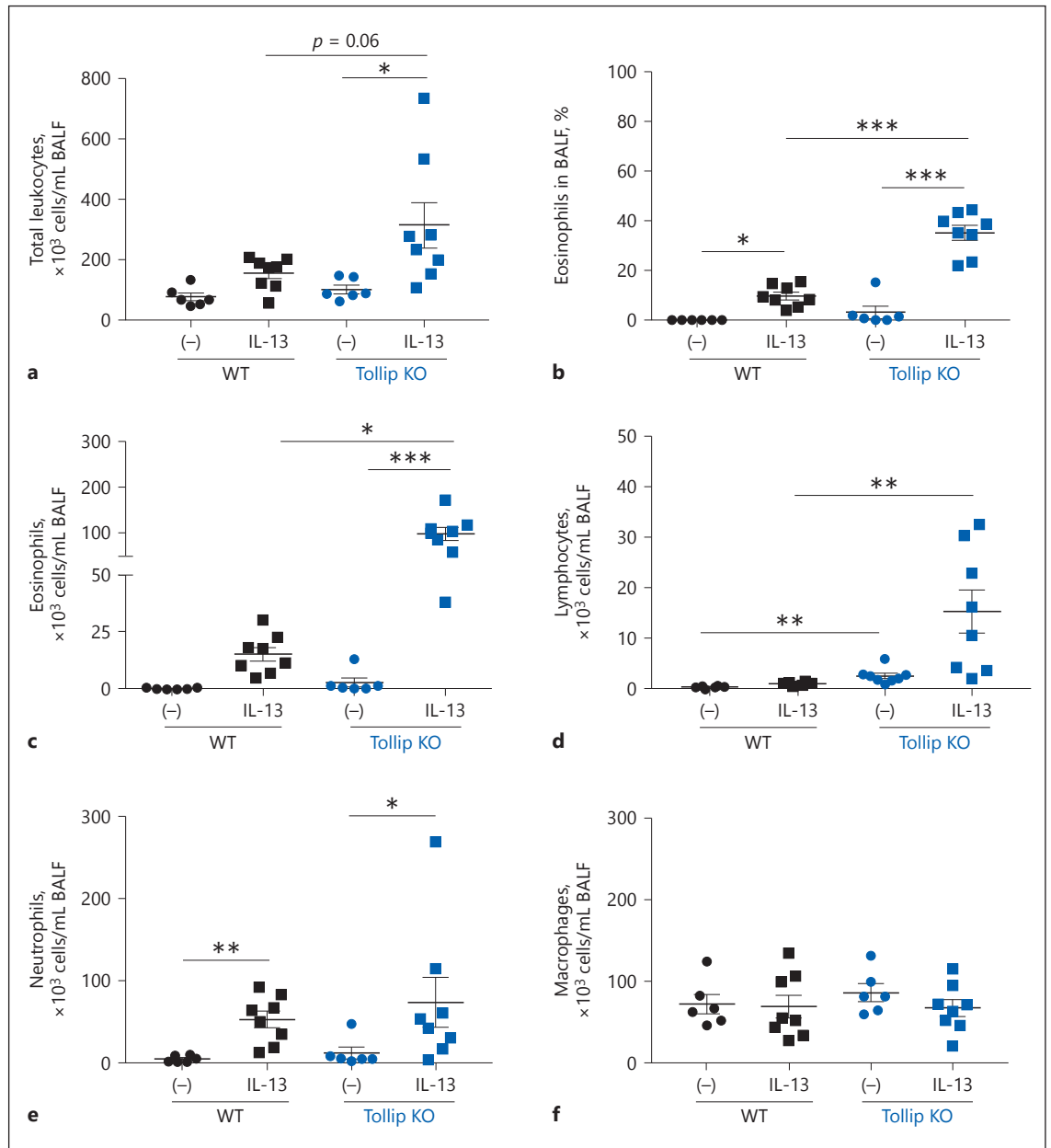


Fig. 1. Tollip KO mice treated with IL-13 show exaggerated eosinophil recruitment into the airways. Tollip KO and WT mice were treated with recombinant mouse IL-13 and sacrificed 24 h after the last IL-13 treatment. BALF was collected for counting the total numbers of leukocytes (a), eosinophils (b, c), lymphocytes (d), neutrophils (e), and macrophages (f). $n = 6-8$ mice/group. Data are expressed as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

[7], we determined the impact of Tollip expression on CCL24 secretion by macrophages. To test if IL-13-exposed AMs from Tollip KO mice produce more CCL24 than the WT cells, AMs were treated with rmIL-13. After IL-13 treatment, AMs from Tollip KO mice showed sig-

nificantly higher levels of CCL24 secretion than the WT mice (Fig. 3c).

As further mechanistic studies in murine AMs were difficult to pursue due to the limited number of cells, we utilized BMDMs from Tollip KO and WT mice. We con-

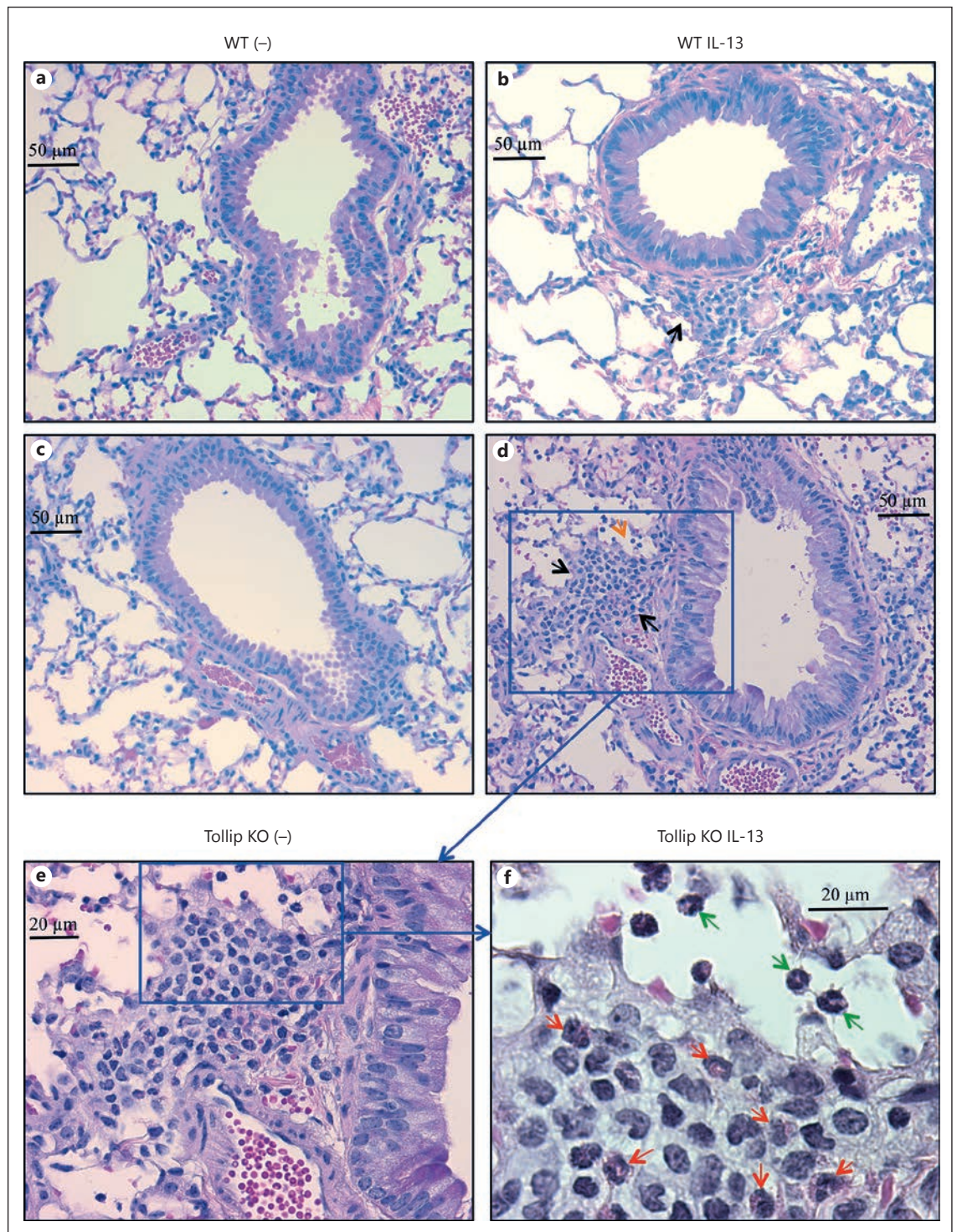
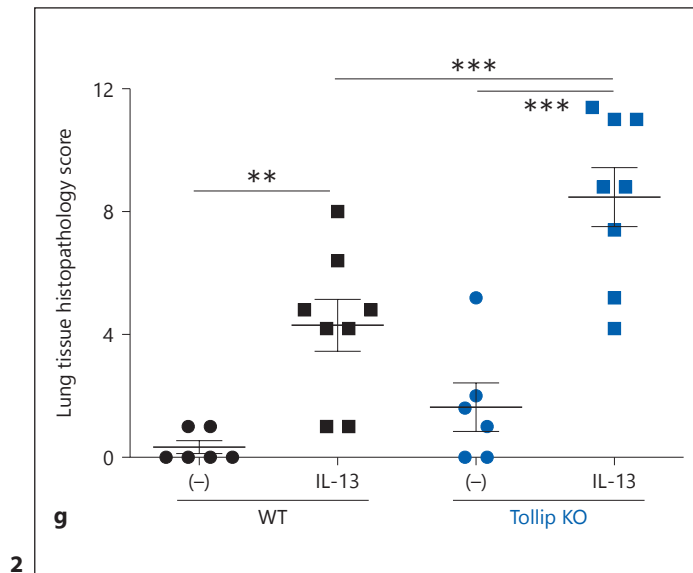


Fig. 2. Tollip KO mice treated with IL-13 show increased lung tissue inflammation. Tollip KO and WT mice were treated with recombinant mouse IL-13 and sacrificed 24 h after the last IL-13 treatment. Histopathologic images of HE-stained lung tissues are from WT (**a, b**) and Tollip KO (**c, d**) mice. Black and orange arrows indicate leukocyte infiltrate around an airway and in the alveolar space, respectively. To further identify inflammatory cell types in

panel **d** (original magnification, $\times 200$), higher magnification images of the framed inflammation area are shown in panel **e** ($\times 400$) and panel **f** ($\times 1,000$). The red arrows indicate eosinophils around the airway, while the green arrows indicate eosinophils in the alveolar space (**f**). **g** Histopathology score. $n = 6-8$ mice/group. Data are expressed as means \pm SEM. ** $p < 0.01$ and *** $p < 0.001$.

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firmly that IL-13-treated BMDMs from Tollip KO mice produced higher levels of CCL24 protein than those from the WT mice (Fig. 3d), which was consistent with the data in cultured AMs and BALF *in vivo*.

Tollip Decreases IL-13-Induced ST2L Expression and IL-13/IL-33-Mediated CCL24 Production in Murine BMDMs

How Tollip regulates CCL24 expression under IL-13 stimulation remains unclear. A previous study using the BMDM culture system demonstrated the synergistic effects of IL-13 in combination with IL-33, another key cytokine in the T2 inflammation, on eotaxin expression, which is largely dependent on the induction of the IL-33 receptor ST2L by IL-13 [22]. Importantly, the ST2L protein level was significantly higher in IL-13-treated BMDMs from the Tollip KO than the WT mice (Fig. 4a). As various cell types in the lung constitutively express IL-33, our data suggest that ST2L upregulation associated with Tollip deficiency may enhance the crosstalk between IL-13 and IL-33 pathways, and further increase eotaxin expression and eosinophilic inflammation. Therefore, we stimulated BMDMs with IL-13 and IL-33 simultaneously and measured CCL24 secretion. IL-13 and IL-33 stimulation in Tollip KO mouse BMDMs synergistically increased CCL24 secretion, which was markedly (about 6-fold) inhibited by a ST2L-neutralizing antibody (Fig. 4b). The contribution of ST2L to IL-13- and IL-33-mediated CCL24 expression was further revealed by the finding that ST2L KO BMDMs produced significantly less CCL24

than the WT cells (Fig. 4c). Moreover, as previous reports demonstrated that IL-33 amplifies IL-13-induced ST2L expression [22], we examined whether Tollip deficiency enhances ST2L induction by IL-13 and IL-33. As shown in Figure 4d, ST2L mRNA and protein were markedly induced by IL-13 and IL-33 in BMDMs from the Tollip KO mice.

Tollip Downregulates IL-13/IL-33-Induced CCL24 in Part by Inhibiting IRAK1 Activation

IL-33 is a member of the IL-1 family. Upon IL-33 stimulation, MyD88 (myeloid differentiation primary response protein 88), IRAK1, IRAK4, and TRAF6 (tumor necrosis factor receptor-associated factor 6) are all recruited to ST2L, which activates MAP kinase and nuclear factor (NF)- κ B [23]. Tollip may negatively regulate the IL-33 signaling pathway by inhibiting the activation of IRAK1 [24]. To determine if CCL24 induction by IL-13 and IL-33 is regulated through IRAK1 activation, we measured CCL24 production in IL-13- and IL-33-stimulated BMDMs of IRAK1 KO and WT mice. The dependence of CCL24 expression on IRAK1 was supported by the lack of CCL24 production in IL-13- and IL-33-stimulated BMDMs from IRAK1 KO mice as compared to the WT mice (Fig. 5a). Notably, Tollip KO BMDMs with IL-13 and IL-33 stimulation showed greater IRAK1 activation than WT cells (Fig. 5b), supporting Tollip's inhibitory effect on IRAK1 activation and subsequent CCL24 production. Having shown the inhibitory role of Tollip in IRAK1 activation, we determined if Tollip inhibited STAT6 activation, a pathway involved in IL-13-induced ST2L expression [25]. STAT6 activation was compared in BMDMs of WT and Tollip KO mice at 60 min after IL-13 and IL-33 costimulation to mimic the coexistence of these 2 cytokines in our IL-13-treatment mouse model. We found that STAT6 activation was moderately higher in BMDMs of Tollip KO mice than WT mice (Fig. 5c), suggesting an inhibitory role of Tollip in the early IL-13 signaling event.

IL-33 Protein Is Constitutively Secreted into the Mouse Airway, and Tollip Inhibits IL-33 mRNA Induction by IL-13 in AMs

As BMDMs from the Tollip KO mice increased CCL24 secretion after IL-13 and IL-33 costimulation, we sought to determine if IL-33 protein levels in BALF were different between WT and Tollip KO mice. Although IL-33 protein was detectable in the BALF of control mice, it was not further induced by IL-13 treatment (Fig. 6a) in WT or Tollip KO mice. As the murine macrophage is

one of the major IL-33 sources [26–30], we examined whether IL-13 alone induced IL-33 expression in AMs. IL-33 mRNA was significantly induced by IL-13 in Tollip KO AMs, but not in WT AMs. The induction of IL-33

mRNA by IL-13 tended to be greater in AMs from the Tollip KO mice than the WT mice (Fig. 6b). However, IL-33 protein in culture medium from IL-13-treated AMs was undetectable by ELISA. AMs were stimulated with

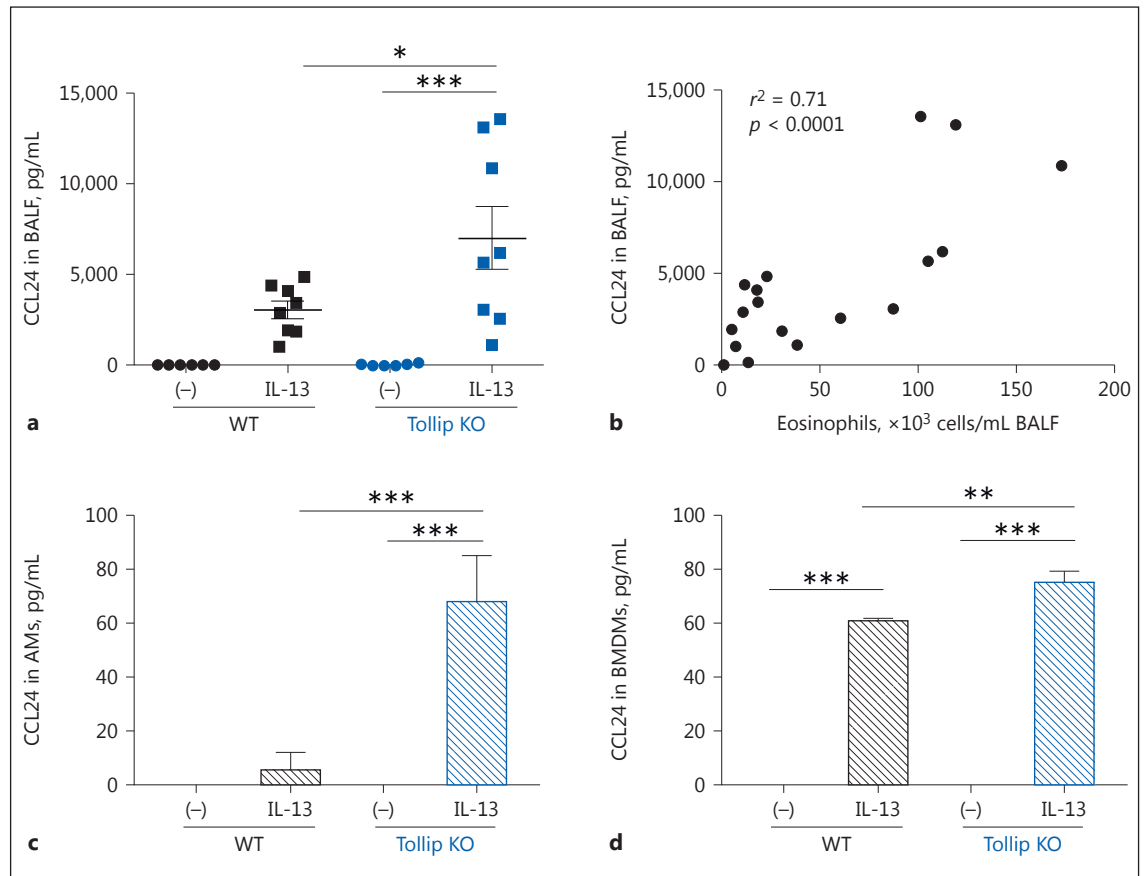


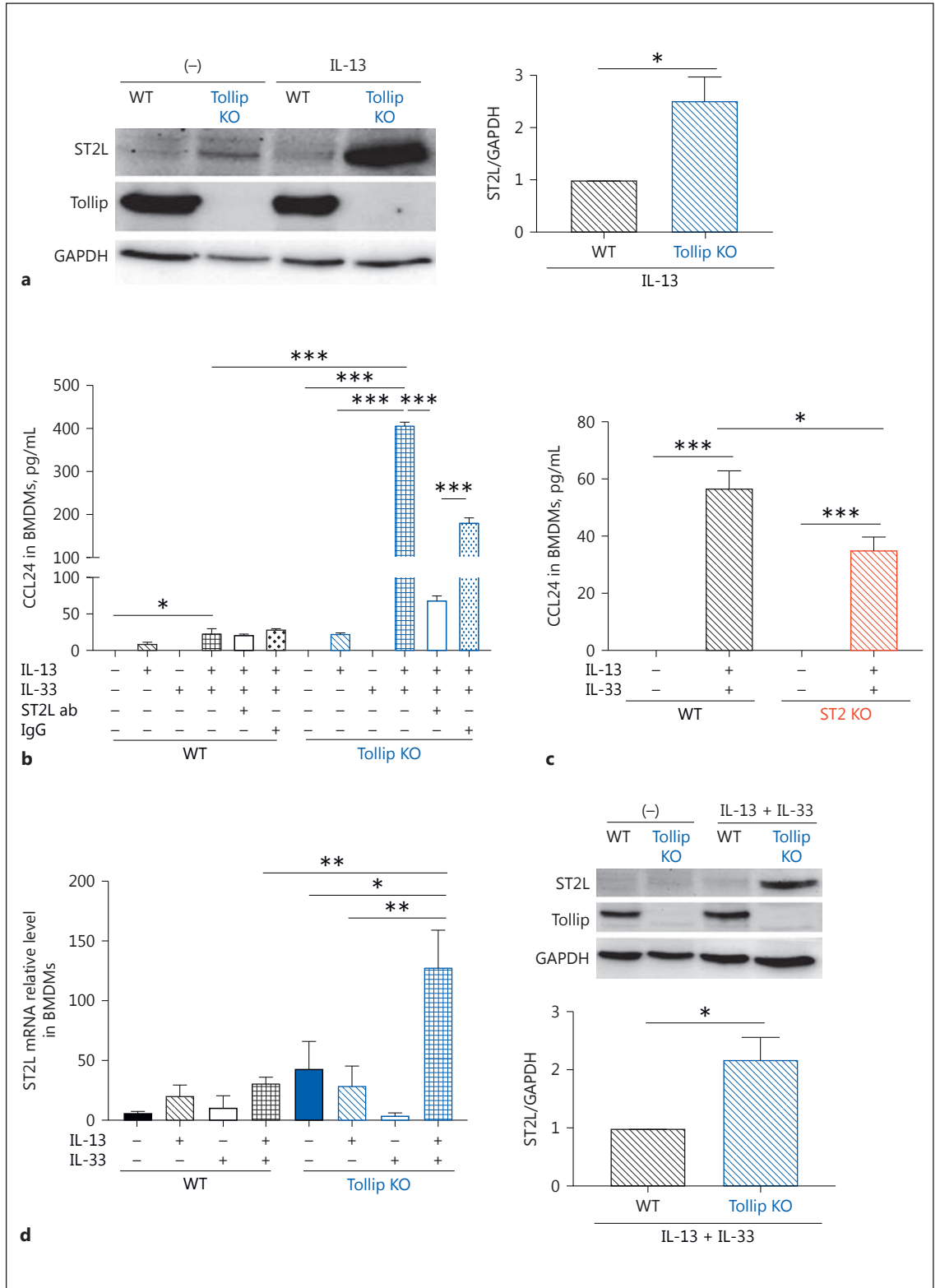
Fig. 3. Tollip deficiency enhances IL-13-induced CCL24 production in mouse BALF and macrophages. Tollip KO and WT mice were treated with IL-13 and sacrificed 24 h after the last IL-13 treatment. **a** CCL24 protein levels in BALF. $n = 6-8$ mice/group. **b** Scatterplot showing a positive correlation between the number of eosinophils and the level of CCL24 protein in BALF. **c** CCL24 protein

levels in culture medium of Tollip KO and WT mouse AMs stimulated with IL-13 for 48 h. $n = 6-8$ replicates from 2 independent experiments. **d** CCL24 protein measured in culture medium (\pm IL-13 for 24 h) of BMDMs from Tollip KO and WT mice. $n = 9$ replicates from 3 independent experiments. Data are expressed as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 4. Tollip deficiency increases IL-13-induced ST2L expression, and CCL24 production by IL-13 and IL-33 in murine BMDMs. **a** BMDMs from Tollip KO and WT mice were incubated with IL-13 for 24 h: a representative Western blot of IL-13-induced ST2L protein expression (left); densitometric analysis of ST2L protein (right). **b** CCL24 protein levels in culture medium of Tollip KO and WT mouse BMDMs stimulated with IL-13, IL-33, and a combination of IL-13 and IL-33 in the presence or absence of a ST2L neutralizing antibody (ST2L ab) or the IgG2B isotype control

(IgG) for 24 h. **c** CCL24 protein levels in culture medium of ST2L KO and WT mouse BMDMs stimulated with IL-13 and IL-33 for 48 h. **d** BMDMs from Tollip KO and WT mice were cultured with IL-13 and/or IL-33 for 24 h: ST2L mRNA relative levels (left); a representative Western blot of ST2L protein and densitometric analysis (right). Data are expressed as means \pm SEM. $n = 9$ replicates from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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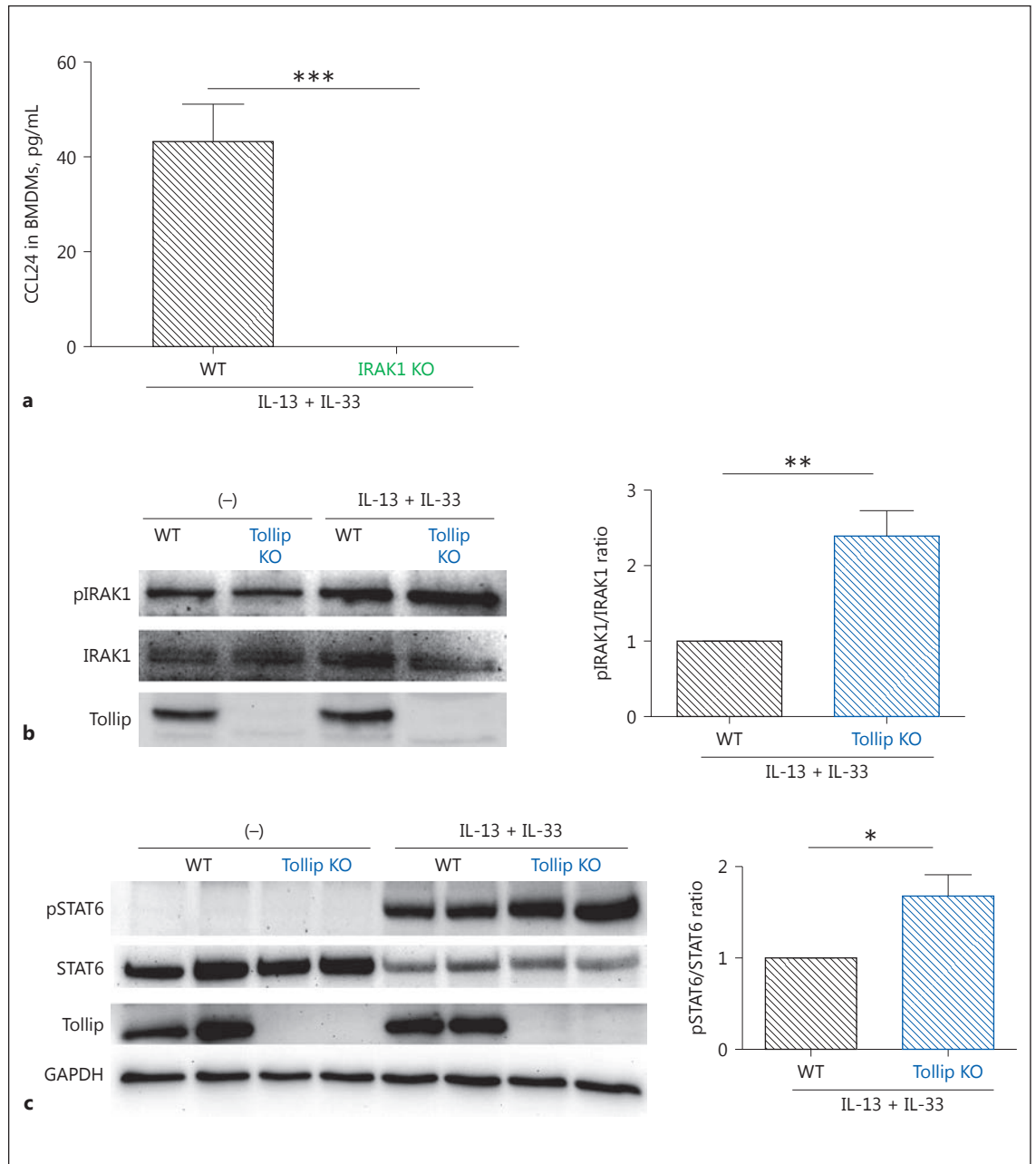


Fig. 5. Role of IRAK1 and STAT6 in CCL24 induction by IL-13 and IL-33 in murine BMDMs. **a** CCL24 protein levels in culture medium of IRAK1 KO and WT mouse BMDMs stimulated with IL-13 and IL-33 for 48 h. $n = 9$ replicates from 3 independent experiments. **b** BMDMs from Tollip KO and WT mice were incubated with IL-13 and IL-33 for 24 h: a representative Western blot of phosphorylated IRAK1 (pIRAK1) and total IRAK1 proteins (left); densitometric analysis of IRAK1 activation, as determined by the

ratio of pIRAK1 to total IRAK1 (right). $n = 9$ replicates from 3 independent experiments. **c** Representative Western blot of phosphorylated STAT6 (pSTAT6) and STAT6 in Tollip KO and WT mouse BMDMs stimulated with IL-13 and IL-33 for 60 min (left); densitometric analysis of STAT6 activation, as determined by the ratio of pSTAT6 to total STAT6 (right). $n = 3$ replicates from 2 independent experiments. Data are expressed as means \pm SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

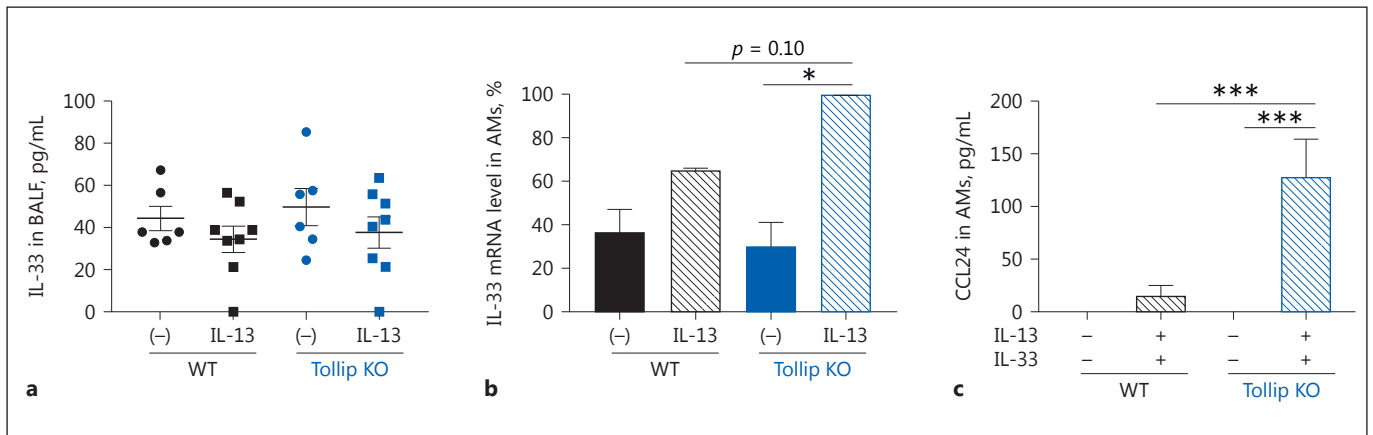
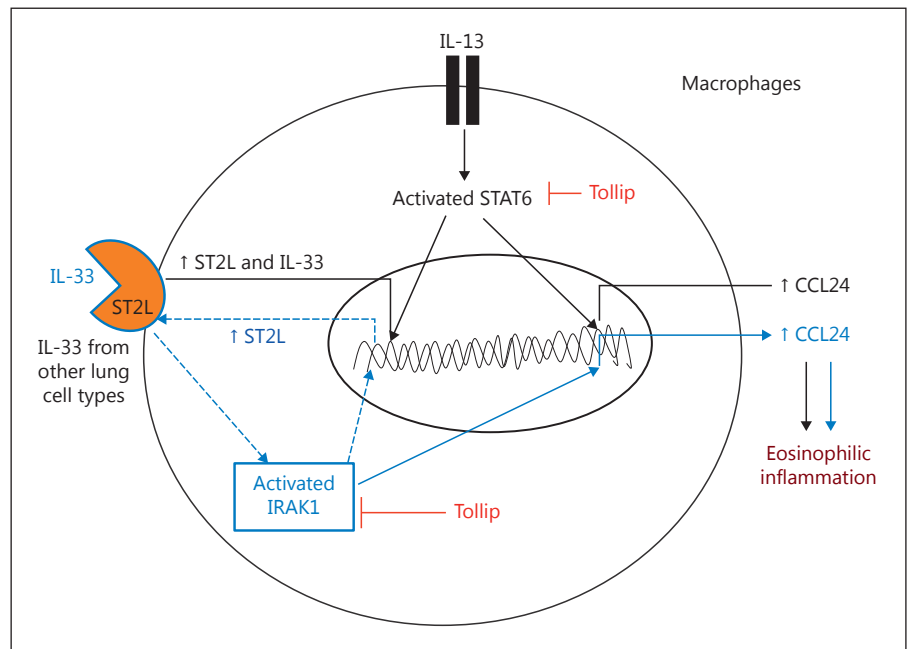


Fig. 6. IL-33 expression in mouse BALF and AMs. **a** Tollip KO and WT mice were treated with IL-13 and sacrificed 24 h after the last IL-13 treatment. IL-33 protein in BALF was measured by ELISA. $n = 6-8$ mice/group. **b** AMs from Tollip KO and WT mice were incubated with IL-13 for 48 h. IL-33 mRNA levels were expressed as the percentage of IL-13-treated AMs from the Tollip KO mice.

c CCL24 protein levels in culture medium of Tollip KO and WT mouse AMs with or without stimulation of IL-13 and IL-33 for 48 h. Data are expressed as means \pm SEM. $n = 6-9$ replicates from 2 or 3 independent experiments for culture of AMs. * $p < 0.05$ and *** $p < 0.001$.

Fig. 7. Proposed mechanisms whereby Tollip attenuates CCL24 production and pulmonary eosinophilic inflammation. In lung cells such as macrophages, IL-13 induces CCL24 expression in part through activating STAT6. Importantly, STAT6 activation will subsequently increase the expression of IL-33 receptor ST2L, which binds the preexisting (constitutive) IL-33 in the lung to further induce CCL24 expression and eosinophilic inflammation in part through activating IRAK1. Tollip inhibits CCL24 and eosinophilic inflammation by suppressing the activity of IRAK1 as well as STAT6.



both IL-13 and IL-33 to mimic the coexistence of these 2 cytokines in our IL-13-treatment mouse model. Indeed, AMs from the Tollip KO mice, as compared to the WT mice, increased the production of CCL24 (Fig. 6c), which was consistent with our data obtained from BMDMs (Fig. 4b).

Discussion

This is the first report demonstrating the regulation of in vivo lung eosinophilic inflammation by Tollip in the context of T2 cytokine treatment. Our results suggest that Tollip deficiency promotes pulmonary eosinophilia by enhancing CCL24 production. Major findings in this

study include: (1) IL-13-treated Tollip KO mice show exaggerated pulmonary eosinophilia and CCL24 production, and (2) Tollip KO murine macrophages stimulated with IL-13 alone or in combination with IL-33 are characterized by enhanced ST2L expression, IRAK1 activation, and CCL24 production.

In this report, we clearly demonstrated that Tollip deficiency enhances IL-13-induced pulmonary eosinophilia along with CCL24 secretion into the airway lumen. Although various mediators such as eotaxins and IL-5 are known to promote murine lung eosinophilic inflammation, how these mediators are regulated under a T2 cytokine milieu remains poorly understood. In mouse lungs, AMs, in response to IL-13, serve as a major source of CCL24 that contributes to sustained recruitment of eosinophils into the airway lumen [7]. Indeed, we found that CCL24 production is higher in murine macrophages (AMs and BMDMs) from the Tollip KO mice than the WT mice. Once IL-13 binds to IL-13R α 1, STAT6 is activated and translocated to the nucleus where CCL24 transcription is upregulated [6]. We determined whether Tollip modifies the activation of STAT6, and observed marginal enhancement of STAT6 activation by IL-13 in BMDMs from the Tollip KO mice, suggesting that Tollip may downregulate CCL24 in part through reducing STAT6 activation. While we do not know how Tollip exactly affects STAT6 activation, Tollip is known to modify several signaling pathways (e.g., IL-1 β R and TGF- β R) by promoting degradation of ubiquitin-conjugated proteins [31–33]. Therefore, we propose that Tollip regulates the IL-13-STAT6 signaling pathway possibly by interacting with STAT6, but this awaits further confirmation.

One innovative aspect of our current study is to unravel the role of Tollip in inhibiting IL-13-mediated upregulation of the IL-33 receptor ST2L. IL-33, a member of the IL-1 cytokine family, is involved in asthma airway inflammation [34]. The binding of IL-33 to ST2L leads to IL-1R accessory protein (IL-1Racp) recruitment and the formation of heterodimeric signaling complex that involves MyD88, IRAK1, IRAK4, and TRAF6, and the activation of mitogen-activated protein kinase and NF- κ B [35]. ST2L expression is higher in endobronchial biopsies of severe asthmatics than healthy controls and mild/moderate asthmatics, and associated with multiple T2-high asthma biomarkers, including blood eosinophils and eotaxin mRNA expression [36]. Notably, ST2L is induced by IL-13 in part through the STAT6 pathway [22, 25]. Therefore, IL-33 has been shown to amplify CCL24 production in IL-13-pretreated macrophages [22]. As Tollip is a negative regulator for TLR and IL-1R pathways by

inhibiting IRAK1 activation [24], we tested whether Tollip regulates the IL-13/IL-33/ST2L pathway, which was never evaluated before. Our data have extended previous reports [22] in that IL-13, particularly in the presence of IL-33, significantly increases ST2L expression and CCL24 production in murine macrophages. Our study demonstrates for the first time that Tollip deficiency leads to a greater induction of ST2L expression by IL-13 and/or IL-33 in macrophages. Moreover, we have found that Tollip deficiency results in greater induction of CCL24 that is dependent on ST2L as Tollip's inhibitory effect on CCL24 is partially abrogated by an ST2L-neutralizing antibody. Thus, greater ST2L expression on macrophages from Tollip KO mice renders the cells more sensitive to the stimulation of IL-33 that is constitutively expressed in the lung and can be further upregulated by IL-13 treatment [26, 37]. We are aware that, unlike the previous study [22], we did not observe a synergistic effect of IL-13 and IL-33 stimulation on ST2L expression in BMDMs from the WT mice. This may be explained by the use of a higher dose of IL-33 (20 ng/mL) in the previous study, and a lower dose of IL-33 (10 ng/mL) in our current study.

Having shown that IL-33/ST2L signaling is involved in CCL24 production, we further evaluated the role of the downstream signaling event of this pathway in eotaxin expression in the context of Tollip. IRAK1 is an integral component of the IL-33/ST2L signaling, but its contribution to IL-13/IL-33-mediated eotaxin production is unknown. Our data suggest that Tollip deficiency allows greater IRAK1 activation, and that IRAK1 deficiency significantly diminishes IL-13/IL-33-induced CCL24 expression. Together, our data indicate that Tollip acts on several checking points to downregulate CCL24 expression in the IL-13/IL-33 setting. The relative contribution of these checking points to Tollip's effects on eotaxin expression deserves further investigation.

Lung macrophages and epithelial cells produce IL-33 as an alarmin [26]. IL-33 is induced in AMs of ovalbumin-challenged mice [28, 38]. Intriguingly, we did not see increased IL-33 in BALF of IL-13-treated Tollip KO mice as compared to the WT mice. Notably, in cultured AMs from the Tollip KO mice but not the WT cells, there was significant induction of IL-33 mRNA by IL-13. However, IL-33 protein was not detectable in AM supernatants. Together, our findings suggest that Tollip has a minimal impact on IL-33 protein release during its regulation of lung eosinophilic inflammation. Instead, as described in Results, IL-33 is constitutively released into the lung (e.g., airway lumen). We propose that greater induction of ST2L expression in lung cells (e.g., macrophages) of

Tollip KO mice by IL-13 would increase the sensitivity of cells responding to endogenous/local IL-33, thereby promoting eosinophilic inflammation.

There are several limitations to the current study. First, because gene knockdown in human primary macrophages is very challenging, we were not able to knockdown Tollip in human primary AMs in order to translate our findings from mice to humans. Second, although lymphocyte recruitment is increased in Tollip KO versus WT mice, we have not assessed its contribution to eosinophilic inflammation. Recent studies have indicated that T2 innate lymphoid cells (ILC2s) contribute to the persistence of airway eosinophilia in patients with severe asthma through localized production of IL-13 [39]. Therefore, dissecting the role of Tollip in regulating ILC2s would further reveal how Tollip affects lung eosinophilic inflammation. Finally, our mechanistic study was focused on macrophages. In our pilot study, we examined if airway epithelial cells are involved in Tollip's function related to eosinophilic inflammation. As CCL24 levels in cultured mouse tracheal epithelial cells were not detectable, we did not pursue the epithelial model for the mechanistic studies. However, future studies are warranted to determine the role of Tollip in regulating eotaxin expression by primary human airway epithelial cells.

In Figure 7, we propose the novel mechanisms by which Tollip inhibits pulmonary eosinophilic inflam-

ation. Briefly, IL-13-stimulated macrophages increase ST2L in part through STAT6 activation, and then CCL24 expression in part through IRAK1 activation in the context of lung constitutive and perhaps inducible IL-33 secretion. Tollip attenuates the activity of the IL-13/STAT6/ST2L/IL-33/IRAK1/eotaxin axis in part by inhibiting STAT6 as well as IRAK1 activation. As our previous study demonstrated that asthmatics carrying the Tollip rs5743899 AG/GG genotype (vs. the AA genotype) express less Tollip protein and greater airflow limitation (less FEV₁/FVC), our current study likely offers an innovative and more precise approach to reducing airway inflammation in asthma patients. Enhancing Tollip expression or suppressing IRAK1 activation in asthmatics carrying the rs5743899 AG or GG genotype may effectively reduce airway inflammation and improve pulmonary function.

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Disclosure Statement

The authors declare no competing financial interests.

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