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IL-33 Exacerbates Eosinophil-Mediated Airway Inflammation

Bartosz Stolarski, Mariola Kurowska-Stolarska, Peter Kewin, Damo Xu, and Foo Y. Liew

IL-33 has emerged as an important mediator in the immunopathogenesis of allergy and asthma. However, the role of IL-33 in eosinophil-mediated inflammation has not been fully explored. In this article, we report that IL-33 directly stimulates eosinophil differentiation from CD117⁺ progenitors in an IL-5-dependent manner. Although resting eosinophils expressed moderate levels of the IL-33R α -chain (ST2L), eosinophils that accumulated in the airways of mice with OVA-induced asthma expressed increased amounts of ST2L. In vitro, IL-33 and GM-CSF are potent inducers of ST2L expression on eosinophils, and IL-33 induced the production of IL-13, CCL17, and TGF- β by eosinophils. In adoptive-transfer experiments, IL-33 exacerbated eosinophil-mediated airway inflammation by increasing the levels of eosinophils, macrophages, lymphocytes, IL-13, TGF- β , CCL3, CCL17, and CCL24 in the lungs. IL-33 also enhanced the eosinophil-mediated differentiation of airway macrophages toward the alternatively activated macrophage phenotype in an IL-13-dependent manner. Taken together, this study demonstrates that the IL-33/ST2 signaling pathway activates airway eosinophils that exacerbate airway inflammation in an autocrine and paracrine manner. *The Journal of Immunology*, 2010, 185: 000–000.

Eosinophils are multifunctional leukocytes that play an important role in the homeostasis of mammary gland development (1). Eosinophils are also generally regarded as the major effector cells in type 2 inflammatory diseases, including helminthic infection, asthma, and allergy (2, 3). The important role of eosinophils in the immunopathogenesis of asthma was shown in studies using eosinophil-deficient mice that develop markedly attenuated disease (4, 5). In asthma, eosinophils mediate the inflammatory process through the release of cytotoxic granules and lipid mediators that induce tissue damage and affect nerves, which, in turn, causes bronchoconstriction (2, 3, 6–8). In addition, eosinophils show numerous immune regulatory functions, including production of a range of cytokines and chemokines that leads to the exacerbation of inflammation, mucus secretion, and lung remodeling (2, 3, 7, 8). Eosinophils may be one of the primary sources of IL-4 during the initiation phase of type 2 immune responses (9), and they can recruit T cells to the lungs during development of asthma (10). There is also growing evidence that eosinophils can serve as APCs (11). The development of eosinophils is governed by several transcription factors, including GATA-1, PU.1, and C/EBP, as well as an array of cytokines, in particular GM-CSF, IL-3, IL-5, and IL-9 (2, 12). Although IL-3 and GM-CSF also support the development of other cells, IL-5 seems to be more specific and

efficient at promoting eosinophil lineage, as demonstrated in IL-5-deficient mice (13–15).

IL-33 is a new cytokine of the IL-1 family and is expressed by a variety of tissue stromal cells, predominantly in skin, lung, and the CNS, suggesting that it may play a physiopathological role in these organs (16, 17). Unlike IL-1 and IL-18, the protein-maturation process is not necessary for IL-33 bioactivity (18, 19). Intriguingly, pro-IL-33 also contains a DNA-binding domain that may regulate gene function in epithelial cells (17). Therefore, IL-33 may operate in an autocrine and paracrine fashion in complex immune responses. IL-33 signals via a heterogeneous receptor complex consisting of IL-33R α -chain (ST2L) and IL-1R accessory protein and triggers the activation of NF- κ B and MAP kinases (20–22).

IL-33 is expressed at substantial levels in the lung epithelial cells and macrophages of mice with OVA-induced airway inflammation (22). Importantly, patients with asthma show high levels of IL-33 in serum (23) and in the lungs, where this cytokine is principally located in epithelial cells (24) and smooth muscle cells (25). The pathogenic role of IL-33 in airway inflammation emerged from experiments with intranasal administration of IL-33 into naive mice that then exhibited eosinophilia and high concentrations of IL-5, IL-13, chemokines, IgE, and mucus production in the lungs, as well as airway hyperresponsiveness (16, 24, 26). Moreover, IL-33 was able to exacerbate airway inflammation induced by A_gs (22, 27, 28). Examination of the cell types involved in these processes revealed the contribution of IL-33-activated alternatively activated (M2) macrophages (24). Although IL-33 was reported to activate human eosinophils in vitro (29–31), its impact on eosinophil differentiation and the contribution of IL-33-activated eosinophils to airway inflammation in vivo remain obscure. In this article, we report that IL-33 can directly stimulate eosinophil differentiation from CD117⁺ hematopoietic progenitor cells. Importantly, IL-33 can exacerbate eosinophil-mediated airway inflammation by increasing the levels of eosinophils, macrophages, lymphocytes, IL-13, TGF- β , CCL3, CCL17, and CCL24 in the lungs. Thus, the IL-33/ST2 signaling pathway activates eosinophils that exacerbate airway inflammation in an autocrine and paracrine manner, suggesting novel pathways for therapeutic intervention in allergic inflammatory diseases.

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; eos, eosinophil; EPO, eosinophil peroxidase; i.n., intranasally; M2, alternatively activated; M Φ , macrophages; mac, macrophage; MR, mannose receptor; ST2L, IL-33R α -chain; WT, wild-type.

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Materials and Methods

rIL-33

Murine and human rIL-33 were produced in-house (18, 24) or were obtained from PeproTech (Rocky Hill, NJ), yielding similar results.

Mice

BALB/c mice were purchased from Harlan Olac (Bicester, U.K.). ST2^{-/-} mice on the BALB/c background were described previously (32, 33) and were kept at the Biological Services facilities of the University of Glasgow, in accordance with the U.K. Home Office guidelines. C57BL/6, IL-13^{-/-}, and IL-5^{-/-}/IL-9^{-/-}/IL-13^{-/-} triple knockout mice on the C57BL/6 background (34, 35) were provided by Dr. Andrew McKenzie (Laboratory of Molecular Biology, Medical Research Council, Cambridge, U.K.).

Eosinophil differentiation

CD117⁺ hematopoietic cells were purified from bone marrow by CD117 magnetic microbeads after the depletion of mature and committed cells using a Lineage Cell Depletion Kit (both from Miltenyi Biotec, Auburn, CA). The purity of CD117⁺ cells was evaluated by staining with PE-conjugated anti-CD117 (Miltenyi Biotec) and FITC-anti-IL-5R α Abs (BD Biosciences, San Jose, CA). Bone marrow or purified CD117⁺ hematopoietic cells (purity $\geq$ 95%) from BALB/c, ST2^{-/-}, IL-13^{-/-}, and IL-5^{-/-}/IL-9^{-/-}/IL-13^{-/-} mice were cultured in RPMI 1640 (supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.05 M 2-ME) and IL-5 (10 ng/ml) or IL-33 (2, 10, or 50 ng/ml) (all cytokines were from PeproTech) with or without anti-IL-5 neutralizing Ab or isotype control (10 μ g/ml; eBioscience). After 4–5 or 7–8 d of culture, cells were stained with PE-conjugated anti-Siglec-F (early eosinophil differentiation marker; BD Biosciences), allophycocyanin-conjugated anti-CCR3 (an eosinophil marker; R&D Systems, Minneapolis, MN), FITC-conjugated anti-ST2L (MD Biosciences, Zurich, Switzerland), PerCP-conjugated anti-Gr-1 (a granulocyte marker; BD Biosciences), PE-conjugated anti-Fc ϵ R1 (a basophil marker; BD Biosciences), FITC-conjugated anti-CD11c (a dendritic cell marker; BD Biosciences), and allophycocyanin-conjugated F4/80 (a macrophage marker) Abs or with the appropriate isotype control followed by FACS analysis. To analyze the effect of IL-33 on CCR3 expression, Siglec-F⁺ wild-type (WT) or ST2^{-/-} eosinophils from IL-5-driven cultures were separated using a FACS Aria cell sorter and then stimulated with IL-33 (10 ng/ml) in media containing a small amount of IL-5 (2 ng/ml). After 24 h, the cells were stained with allophycocyanin-conjugated anti-CCR3 Ab. Cytospins were prepared from some cultures and stained in the presence of potassium cyanide for eosinophil peroxidase (EPO; exclusive to eosinophils), according to a protocol published previously (36), or with H&E.

Eosinophil activation in vitro

Mouse eosinophils were obtained from in vitro cultures described above or from bone marrow of WT and ST2^{-/-} mice that were injected with IL-5 (2 μ g/mouse, i.p.) every second day for 7 d. Mature eosinophils were sorted by FACS Aria based on the expression of Siglec-F⁺CCR3^{high}Gr-1^{intermediate} into medium containing GM-CSF (1 ng/ml) or IL-5 (2 ng/ml), both eosinophil survival factors. Purified populations of eosinophils (purity $\geq$ 97%) were cultured in medium with IL-5 (2 ng/ml, control) supplemented with GM-CSF (1 or 10 ng/ml), IL-4 (10 ng/ml), IL-33 (10 or 50 ng/ml), or a combination of these cytokines. After 24 or 48 h, cells were stained with FITC-conjugated anti-ST2L and allophycocyanin- or PerCP-conjugated anti-CCR3, followed by FACS analysis. To assay for cytokine and chemokine production, purified eosinophils were incubated in medium with GM-CSF (1 ng/ml, survival and ST2L-inducing factor; control) with or without IL-33 (10 or 50 ng/ml). Culture supernatants were collected after 48 h. Cell viability was evaluated by counting in trypan blue and was $\geq$ 94%.

Macrophage and eosinophil cocultures

Bone marrow cells from ST2^{-/-} mice were cultured in the presence of M-CSF (20 ng/ml) for 6 d. The purity of bone marrow-derived macrophages was determined by FACS using a macrophage marker F4/80 (anti-F4/80 Ab, clone BM8; eBioscience); cell fractions containing $\geq$ 96% of F4/80⁺ macrophages were used for additional experiments. ST2^{-/-} macrophages (5×10^5 /well/2 ml) were cultured alone or with FACS Aria-sorted BALB/c eosinophils (3×10^6 /well/2 ml) and stimulated for 72 h with IL-5 (2 ng/ml) or IL-33 (10 ng/ml) with or without neutralizing anti-IL-13 Abs (R&D Systems) or isotype controls (both 10 μ g/ml). The expression of TLR2 (clone 6C2; eBioscience) and mannose receptor (MR) (Serotec, Oxford, U.K.) on macrophages was analyzed by FACS.

OVA-induced airway inflammation

WT and ST2^{-/-} mice were injected i.p. with 100 μ g OVA (Sigma-Aldrich, St. Louis, MO) in 2% alum (Aluminum Hydroxide Gel Adjuvant; Brenntag, Mulheim, Germany) and then were challenged intranasally (i.n.) on days 8, 9, and 10 with 10 μ g OVA or PBS, as described previously (37). Mice were sacrificed 48–120 h after the last challenge. Serum, bronchoalveolar lavage (BAL), and lungs were harvested and analyzed as described previously (38). In addition, BAL cells were stained with PE-conjugated anti-Siglec-F, allophycocyanin-conjugated anti-CCR3, and FITC-conjugated anti-ST2L Abs or their isotype control.

Adoptive transfer of eosinophils

Eosinophils were harvested from peritoneal wash of BALB/c mice injected with IL-33 (1 μ g/mouse for 7 consecutive days). The cells were then sorted by FACS Aria based on Siglec-F and CCR3 expression (purity $\geq$ 98%). The cells were then incubated for 2 d with GM-CSF (1 ng/ml), stained with CFSE (2 μ M), and inoculated i.n. into ST2^{-/-} recipients (10×10^6 /mouse). IL-33 (1 μ g/mouse) or PBS was administered i.n. on days 1, 2, and 3. Control recipient mice received PBS instead of cells, followed by IL-33 or PBS inoculations. All mice were sacrificed on day 4, and serum, BAL, and lungs were analyzed as described previously (38). BAL cells were stained with FITC-conjugated anti-F4/80 Abs, PE-conjugated anti-TLR2, and allophycocyanin-conjugated anti-MR or allophycocyanin-conjugated anti-CD4 or isotype controls.

ELISA

Murine cytokines IL-4, IL-13, IL-10, and TGF- β and murine and human chemokines CCL3, CCL11, CCL24, and CCL17 were analyzed by ELISA using paired Abs (BD Biosciences and R&D Systems). In some experiments, murine 20-plex Luminex (BioSource International, Camarillo, CA) was also used.

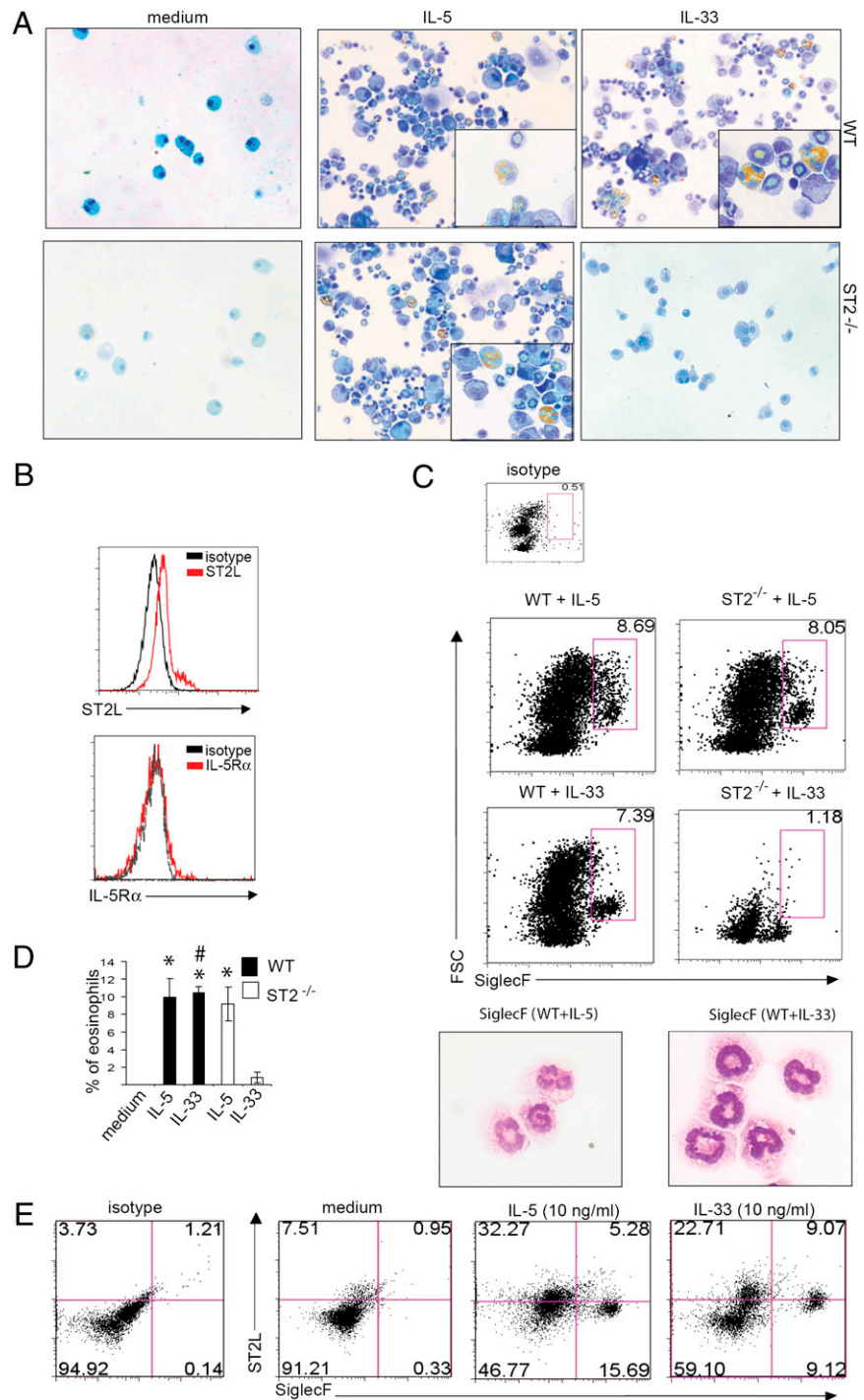
Statistical analysis

ANOVA followed by the Tukey test or the Student *t* test was applied to all in vitro data. Analysis between in vivo groups was examined by ANOVA followed by the Student *t* test. All data are expressed as mean $\pm$ SEM. A *p* value < 0.05 was considered statistically significant.

Results

IL-33 induces eosinophil differentiation from hematopoietic progenitor cells

IL-33 can induce eosinophilia in mice (16, 22, 26). However, it is not clear whether eosinophils differentiate as a direct effect of IL-33 on bone marrow cells or are induced indirectly via IL-5 produced by other cell types, such as Th2 cells activated by IL-33. To address this question, we used different methods and various eosinophil markers (39). Initially, we incubated whole bone marrow cells from WT or ST2^{-/-} mice with IL-33 or IL-5 for 5 or 8 d. IL-5 and IL-33 induced eosinophil differentiation from bone marrow precursors of WT mice as determined by the presence of EPO (eosinophil granule-associated marker; Fig. 1A). By contrast, IL-5, but not IL-33, induced eosinophil differentiation in ST2^{-/-} mice. No EPO⁺ cells were detected in the culture with medium alone in WT or ST2^{-/-} cultures. These data indicate that IL-33 can induce eosinophil development from bone marrow cells in an ST2-dependent manner. To determine whether IL-33 can act directly on hematopoietic progenitor cells, CD117⁺ (c-kit⁺) cells were purified from bone marrow of WT or ST2^{-/-} mice after depletion of committed or mature lineage⁺ cells, and cultured as above. Freshly isolated CD117⁺ progenitors expressed ST2L. The expression of IL-5R α on these cells was below the detection level of flow cytometry (Fig. 1B). Eosinophil differentiation was analyzed by FACS using the surface eosinophil-specific marker Siglec-F. IL-5 induced comparable numbers of Siglec-F⁺ cells that stained positively with eosin in WT and ST2^{-/-} CD117⁺ cell cultures ($10 \pm 2.5\%$ and $9.2 \pm 2.5\%$, respectively), whereas IL-33 induced Siglec-F⁺ eosin⁺ eosinophils only in WT cultures but not in ST2^{-/-} CD117⁺ cell cultures ($10.5 \pm 0.5\%$ and $1.2 \pm 1\%$, respectively) (Fig. 1C, 1D). There were

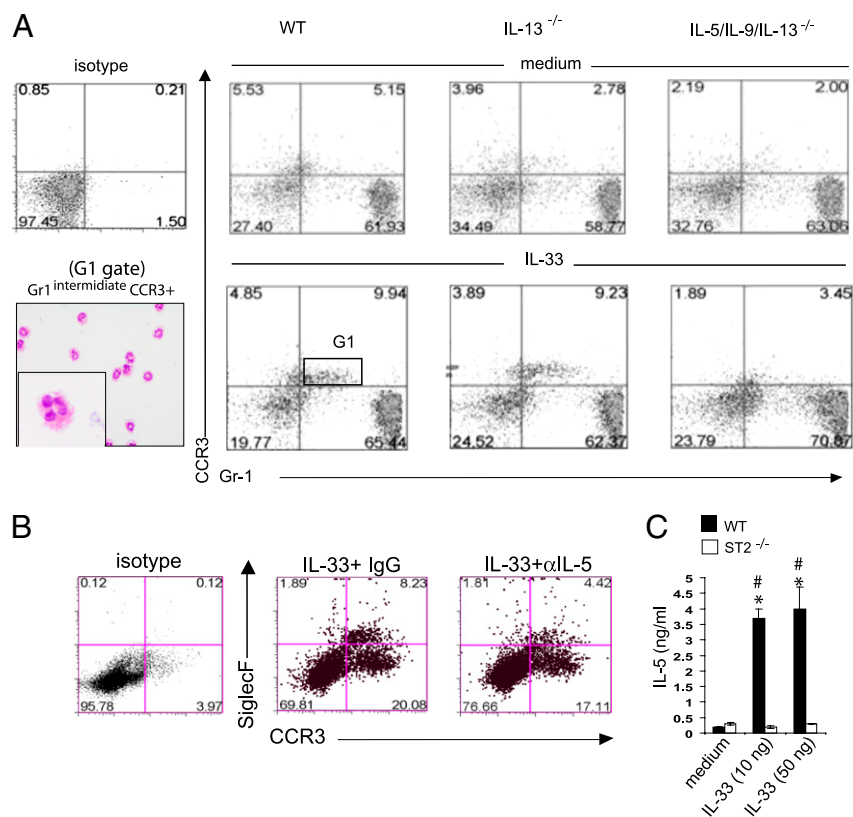


no Siglec-F⁺ cells detected in cultures with medium alone (data not shown). Similar data were obtained for 8-d cultures (data not shown). In addition, IL-33-driven Siglec-F⁺ eosinophils expressed ST2L (Fig. 1E). Therefore, these results indicate that IL-33 is able to act directly on hematopoietic progenitor cells and to stimulate their differentiation into mature eosinophils.

Given that IL-33 stimulates the production of cytokines associated with type 2 immunity and eosinophil development (2, 16, 22), we examined the contribution of IL-5, IL-9, and IL-13 to the IL-33-induced development of eosinophils from bone marrow precursors. Bone marrow cells from WT, IL-13^{-/-}, and IL-5/IL-9/IL-13^{-/-} triple knockout mice were cultured as above, and the mature eosinophil population characterized by the expression of

CCR3 was evaluated by FACS (CCR3⁺Gr-1^{intermediate}) and eosin⁺ staining. IL-33 induced comparable numbers of CCR3⁺Gr-1⁺ eosinophils from the precursors of IL-13^{-/-} and WT mice (9.5 ± 2 and $9 \pm 1.9\%$, respectively) but not from the precursor cells of triple knockout mice ($2.5 \pm 1.5\%$) (Fig. 2A). These results suggest that IL-5 and/or IL-9, but not IL-13, are required for the IL-33-induced eosinophil differentiation from precursor cells. Because IL-5 is known as a major eosinophil differentiation factor (13), we cultured CD117⁺ hematopoietic progenitor cells with IL-33 in the presence of an IL-5-neutralizing Ab. Anti-IL-5 treatment markedly reduced the IL-33-induced differentiation of eosinophils (Siglec-F⁺CCR3⁺) from CD117⁺ cells (Fig. 2B). Consistent with this finding, WT CD117⁺ cells stimulated with IL-33 produced

FIGURE 2. IL-33-induced eosinophil differentiation from hematopoietic progenitor cells is IL-5 dependent. Bone marrow (A, 2×10^6 /ml) or CD117⁺ hematopoietic cells (B, C, 0.5×10^6 /ml) from BALB/c, C57BL/6, ST2^{-/-} (BALB/c background), IL-13^{-/-} (C57BL/6 background), or IL-5^{-/-}/IL-9^{-/-}/IL-13^{-/-} triple knockout mice (C57BL/6 background) were cultured with IL-5 (10 ng/ml) or IL-33 (10 or 50 ng/ml) with or without IL-5-neutralizing Ab (or isotype control, 10 μ g/ml) for 5 d. A, Representative data for the expression of eosinophil markers CCR3^{high} and GR-1^{intermediate} and eosin-stained CCR3^{high} and GR-1^{intermediate}-sorted cells (gate G1). Original magnification $\times 20$; insets $\times 100$. B, Representative FACS data of mature eosinophil markers Siglec-F and CCR3. C, IL-5 levels in culture supernatants were determined by ELISA. Data are means $\pm$ SEM and are representative of at least three independent experiments. * $p < 0.05$, IL-33 versus medium; # $p < 0.05$, WT versus ST2^{-/-}.



substantial amounts of IL-5 compared with unstimulated cultures or IL-33-treated ST2^{-/-} CD117⁺ cells (Fig. 2C). Together, these data suggest that IL-33 is able to act locally in the bone marrow compartment and directly stimulates eosinophil differentiation from hematopoietic progenitor cells in an IL-5-dependent manner. Furthermore, an increase in Fc ϵ RI⁺ cells, likely basophils, was detected in cultures stimulated with IL-33, as reported previously (Supplemental Fig. 1) (40).

IL-33/ST2 is required for optimal expression of CCR3 on differentiating eosinophils

CCR3 is a major chemokine receptor responsible for the mobilization of mature eosinophils from bone marrow and their trafficking to sites of inflammation (2). To investigate whether IL-33 is involved in the regulation of CCR3 expression during eosinophil differentiation, FACSARIA-sorted Siglec-F⁺ eosinophils from IL-5-driven bone marrow cultures (7 d; Fig. 3A) were incubated with IL-5 (2 ng/ml) with or without IL-33 for 24 h, and CCR3 expression was determined by FACS. As expected, the majority of IL-5-driven Siglec-F⁺ eosinophils expressed CCR3. However, IL-33 further increased the surface density of Siglec-F on these eosinophils (Fig. 3B). We next investigated whether there was any difference in the basal expression of CCR3 between IL-5-driven eosinophils from WT and ST2^{-/-} mice. Eosinophils from ST2^{-/-} mice expressed significantly less CCR3 than did WT eosinophils (Fig. 3C). These results indicate that the endogenous IL-33/ST2 signaling pathway enhances the expression of CCR3 on eosinophils.

Airway eosinophils readily express ST2L

Next, we investigated the regulation of ST2L expression on mature eosinophils by cytokines released during the type 2 immune response. Bone marrow cells were differentiated in the presence of IL-5 and sorted by FACSARIA for CCR3^{high}Siglec-F⁺ cells (>98% pure). The cells were then cultured in medium containing IL-5 (2 ng/ml) in the presence of IL-33, GM-CSF, and IL-4 (all

10 ng/ml) or a combination of these cytokines for 48 h. Expression of ST2L on eosinophils incubated in medium with IL-5 alone was below the sensitivity of FACS (Fig. 4A) or was very low (Fig. 1E). IL-33 and GM-CSF, but not IL-4, increased ST2L expression on WT eosinophils (Fig. 4A). An additive effect of GM-CSF and IL-33 on ST2L expression was also observed. We then investigated ST2L expression on BAL eosinophils during mouse OVA-induced airway inflammation (see *Materials and Methods*). BAL cells were isolated 48–120 h after the last Ag challenge, and the expression of ST2L on CCR3⁺ eosinophils was determined. As expected (22, 24), eosinophils (CCR3⁺) accumulated in BAL as early as 24 h and peaked 120 h after the last challenge (data not shown). The BAL eosinophils readily expressed ST2L, peaking at 48 h (80% of eosinophils) and declining thereafter (Fig. 4B, data not shown). ST2L was not detected in the control eosinophils from ST2^{-/-} mice. Together, these results demonstrate that mature resting eosinophils express low levels of ST2L, which can be markedly increased by cytokines, including IL-33 and GM-CSF, and at sites of inflammation.

IL-33 enhances eosinophil functions

Because the lungs of asthma patients and mice with OVA-induced allergic inflammation abundantly express IL-33 (22, 24, 25), and eosinophils at sites of inflammation express high levels of ST2L, we investigated in detail the contribution of IL-33 to eosinophil functions. Eosinophils are a source of pleiotropic mediators, including cytokines, chemokines, and tissue-damaging granule proteins (2). To investigate whether IL-33 can modulate the production of these mediators, FACSARIA-sorted CCR3⁺Siglec-F⁺ WT and ST2^{-/-} eosinophils were incubated in medium containing suboptimal amounts of GM-CSF (1 ng/ml, eosinophil survival and ST2L-inducing factor) in the presence or absence of IL-33 (10 or 50 ng/ml) for 48 h. IL-33 triggered the production of IL-13 and IL-6 and strongly increased the production of CCL17 and TGF- β

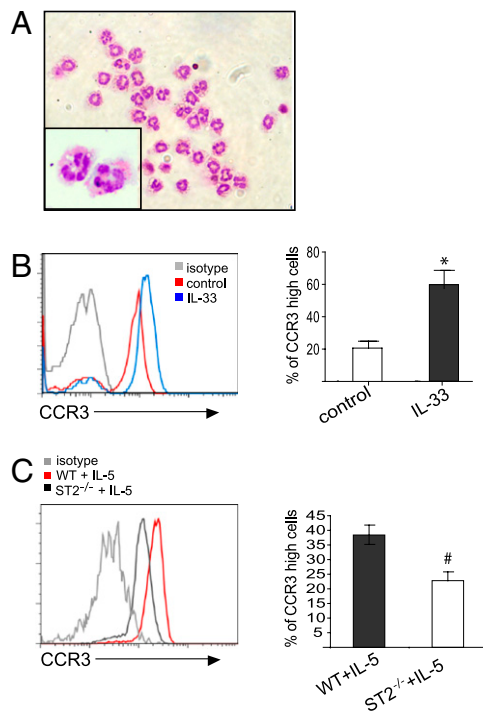


FIGURE 3. IL-33 increases eosinophil CCR3 expression. FACSARIA-sorted Siglec-F⁺ eosinophils from IL-5-driven (10 ng/ml) BALB/c (A–C) and ST2^{-/-} (C) bone marrow cells (cultured for 7 d with IL-5) were stimulated with IL-33 (10 ng/ml) (B) or IL-5 (10 ng/ml) (C) for 24 h, followed by FACS analysis for CCR3 expression. A, Purity of sorted eosinophils by the presence of eosin (pink cytoplasm) is shown (original magnification $\times 20$; inset $\times 100$). B and C, Expression of CCR3 by FACS. Data are means $\pm$ SEM and are representative of at least three independent experiments; * $p < 0.05$, IL-33 versus medium; [#] $p < 0.05$, WT versus ST2^{-/-}.

from WT eosinophils, but not ST2^{-/-} eosinophils, in a dose-dependent manner (Fig. 5A). IL-33 had little or no effect on the production of CCL11, CCL24, IL-4, CCL2, CXCL-10, and CCL3 (data not shown). IL-17, CXCL1, CXCL9, fibroblast growth factor, IL-1a, IL-2, IL-5, IL-10, IL-12, IFN- γ , IL-1 β , TNF- α , and vascular endothelial growth factor remained below the level of detection (data not shown). Together, these data demonstrate that IL-33 enhances multiple features of eosinophil functions; therefore, an IL-33-rich environment in asthma and allergy may contribute to the pathogenic activities of eosinophils.

IL-33-activated eosinophils induce airway inflammation in ST2^{-/-} recipients

We next investigated the contribution of IL-33 to eosinophil-mediated airway inflammation in vivo. FACSARIA-sorted WT eosinophils (CCR3^{high}Siglec-F⁺) were stained with CFSE and adoptively transferred i.n. into ST2^{-/-} mice. Recipients then received IL-33 or PBS i.n. for the subsequent 3 consecutive days. Mice were culled 24 h after the last IL-33 challenge, and BAL fluid and cells were analyzed. Differential cell counts confirmed the presence of eosinophils in mice that received cells and a complete lack of eosinophils in the control groups given IL-33 or PBS only (Fig. 6A). Mice that received eosinophils + IL-33 exhibited markedly higher total cell, eosinophil, macrophage, and lymphocyte counts than did those that received eosinophils + PBS. BAL from the recipients of eosinophils + IL-33 also contained significantly higher numbers of host eosinophils (CFSE⁻) than did the group given eosinophils + PBS (Fig. 6A). These data indicate that

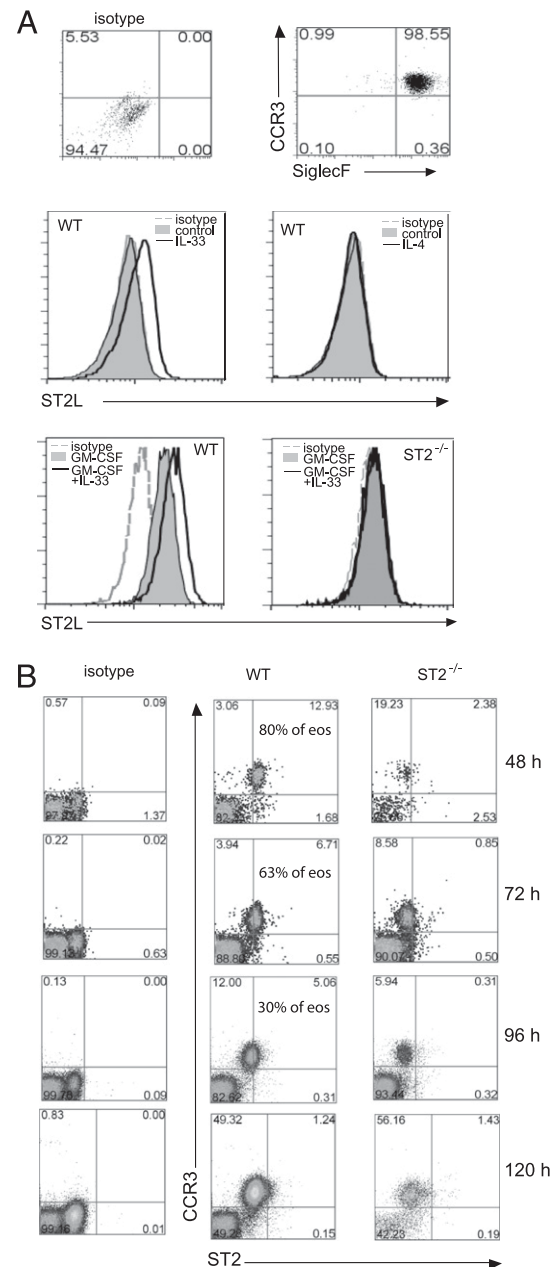


FIGURE 4. Airway eosinophils express ST2L. A, Mature murine FACSARIA-sorted BALB/c or ST2^{-/-} eosinophils (Siglec-F⁺CCR3^{high}) were cultured in medium with IL-5 (2 ng/ml, control) supplemented with GM-CSF (10 ng/ml), IL-4 (10 ng/ml), IL-33 (10 ng/ml), or a combination of these cytokines. Purified eosinophils (CCR3⁺Siglec-F⁺) expressed ST2L when cultured with IL-33 or GM-CSF but not with IL-4. IL-33 further increased the intensity of ST2L expression induced by GM-CSF. Data are representative of two independent experiments. B, WT and ST2^{-/-} mice were sensitized with OVA + alum and challenged with OVA. BAL cells were collected 48–120 h after the last OVA challenge and stained with anti-Siglec-F, anti-CCR3, and anti-ST2L Abs. At 48 h, up to 80% of BAL eosinophils (F4/80⁺CCR3⁺ cells) were ST2L⁺. Data are representative of 10 mice.

IL-33, given to IL-33-unresponsive (ST2^{-/-}) recipients, stimulated donor WT eosinophils to produce mediators that were likely responsible for attracting the recipient's inflammatory cells into the lungs. BAL fluid-analysis shows that, in contrast to the PBS and IL-33-alone groups, both recipient groups that received eosinophils showed the presence of CCL17, CCL3, CCL11, CCL24, IL-13, and TGF- β . However, mice that received eosinophils + IL-33

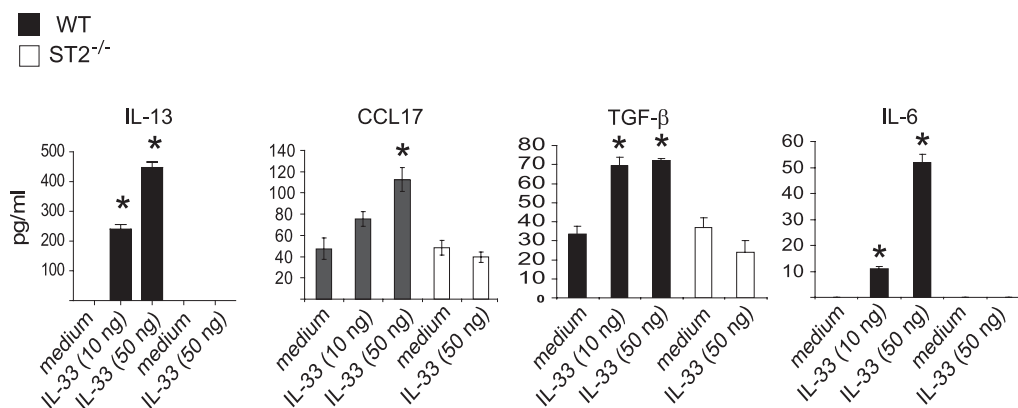


FIGURE 5. IL-33 enhances eosinophil functions in vitro. FACSARIA-sorted BALB/c or ST2^{-/-} eosinophils (Siglec-F⁺CCR3^{high}) were incubated for 48 h in medium containing GM-CSF (1 ng/ml, control medium) with or without IL-33. Cytokine and chemokine concentrations in culture supernatants were analyzed by ELISA. Data are means $\pm$ SEM and are representative of at least three independent experiments. * p < 0.05, IL-33 versus control medium.

produced significantly more CCL17, IL-13, TGF-β, CCL3, and CCL24 than did those given eosinophils with PBS (Fig. 6B).

Macrophages were the major cell type found in the BAL of the recipients given eosinophils + IL-33 (Fig. 6A). Because M2 macrophages play an important role in asthma by producing chemokines and mediating tissue fibrosis (20, 27, 35), we investigated the phe-

nototype of the recipients' macrophages. BAL cells were stained for F4/80, CCR3, MR (an M2 macrophage marker), and TLR2 (a marker of proinflammatory subset of macrophages, M1). Alveolar macrophages from control mice had low expression of MR that was consistent with their quiescent phenotype (24). The presence of adoptively transferred eosinophils in the airways increased the percentage

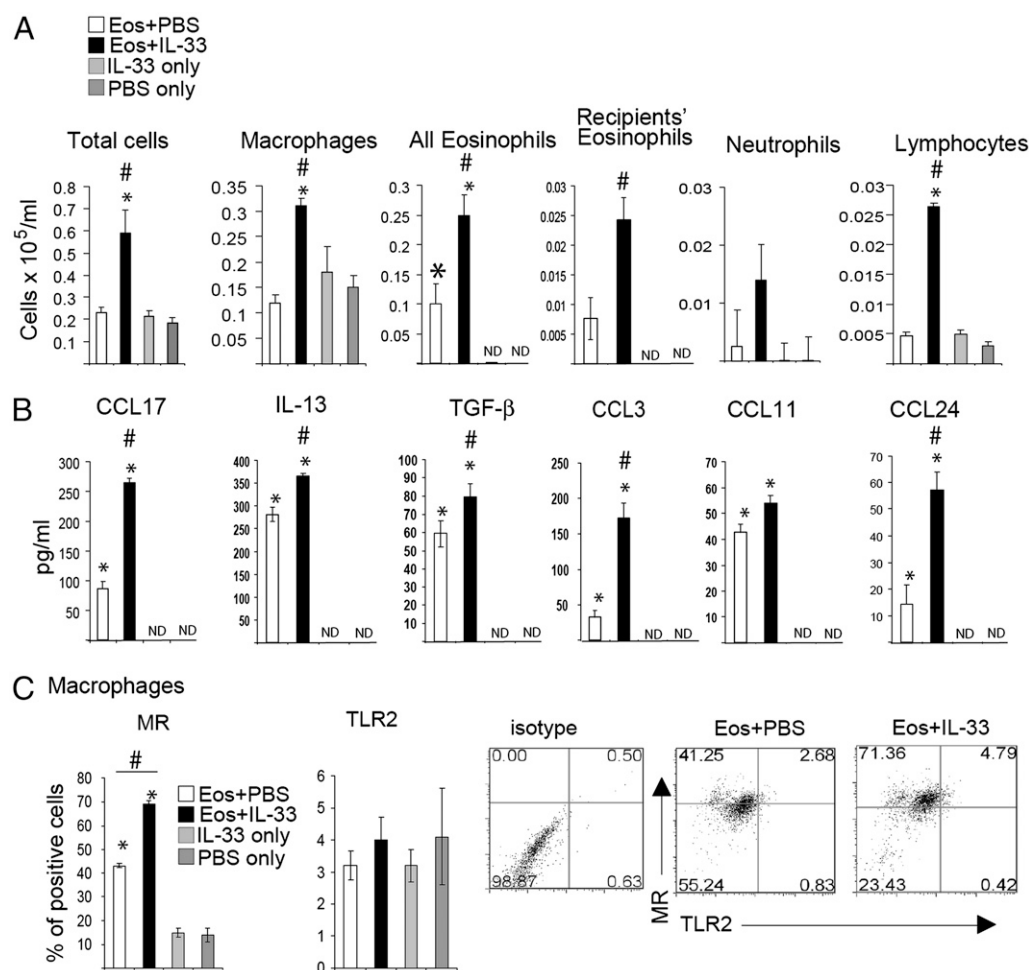


FIGURE 6. IL-33-activated eosinophils induce airway inflammation. FACSARIA-sorted WT BALB/c eosinophils (Siglec-F⁺CCR3^{high}) were stained with CFSE and adoptively transferred i.n. into ST2^{-/-} recipients that were subsequently administered IL-33 or PBS for 3 d i.n. Control groups received PBS instead of cells. Mice were culled 24 h after the last IL-33 administration. BAL differential cell counts (A), BAL cytokine and chemokine concentrations (B), and TLR2 and MR expression on BAL macrophages (F4/80⁺CCR3⁺) (C) were determined. Data are means $\pm$ SEM (n = 5 mice/group) and are representative of two independent experiments. * p < 0.05, eosinophils versus PBS or IL-33 alone; # p < 0.05, eosinophil + IL-33 versus eosinophil + PBS.

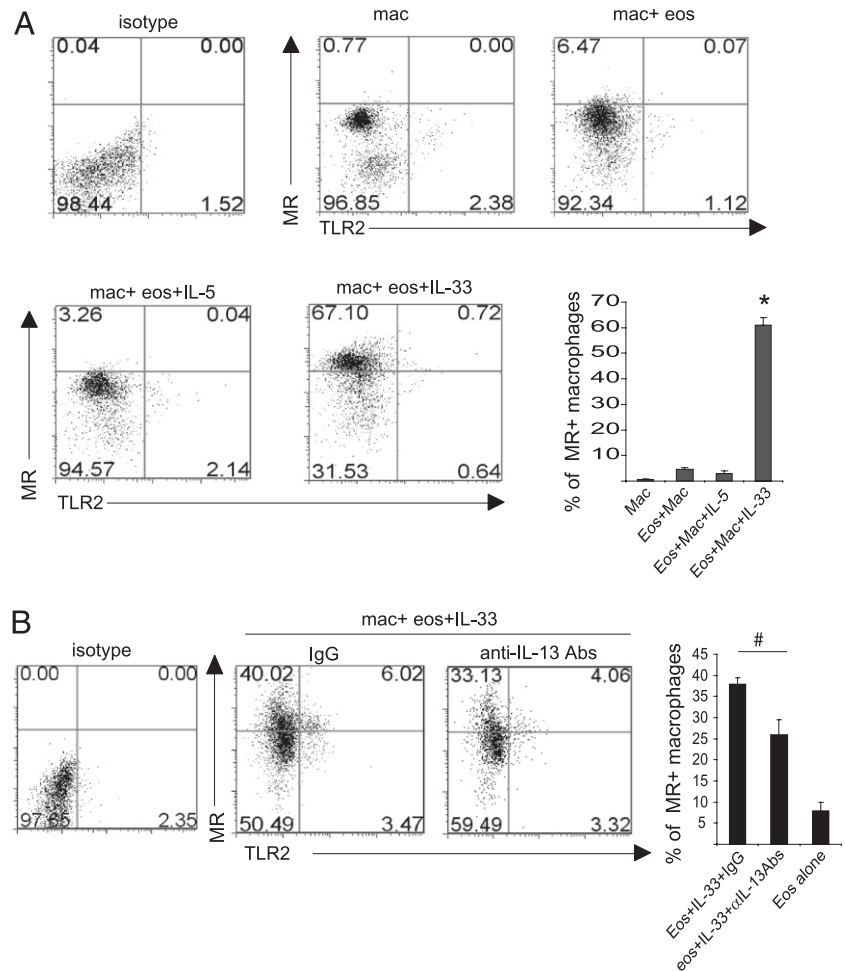


FIGURE 7. IL-33-activated eosinophils enhance macrophage switching to M2 phenotype. Macrophages from ST2^{-/-} mice were cultured alone or with FACSARIA-sorted WT BALB/c eosinophils (Siglec-F⁺CCR3^{high}) in the presence of IL-5 (5 ng/ml), IL-33 (10 ng/ml), or IL-33 + neutralizing anti-IL-13 Ab or isotype control (10 μ g/ml). After 48 h, macrophages were stained with anti-TLR2 and anti-MR Abs. **A**, IL-33-activated eosinophils increased the percentage of MR⁺ but not TLR2⁺ macrophages. **B**, The IL-33-induced increase in MR⁺ macrophages was significantly blocked by anti-IL-13 Ab. Data are means $\pm$ SEM and are representative of at least two independent experiments. * p < 0.05, eosinophils + macrophages + IL-33 versus other cultures; # p < 0.05, anti-IL-13 versus IgG. eos, eosinophil; mac, macrophage.

of MR⁺ macrophages. However, mice that received eosinophils + IL-33 had a significantly greater percentage of MR⁺ macrophages (F4/80⁺CCR3⁻) than did mice that received eosinophils + PBS (Fig. 6C). The percentage of TLR2⁺ macrophages was low, and there was no difference between the groups. Therefore, these data indicate that IL-33-activated eosinophils can increase the differentiation of alveolar macrophages toward the M2 phenotype.

To investigate the mechanism by which IL-33-activated eosinophils promoted M2 polarization, we cocultured ST2^{-/-} macrophages with WT eosinophils in vitro in the presence or absence of IL-33 or IL-5. Eosinophils alone modestly elevated the percentage of MR⁺ macrophages, which was markedly increased by the presence of IL-33 but not IL-5 (Fig. 7A). Because IL-13 is a well-defined M2-differentiation factor (41) and was found to be produced by eosinophils upon IL-33 stimulation (Fig. 5A), we tested whether IL-13 produced by IL-33-activated eosinophils may be responsible for the enhanced M2 polarization. The presence of anti-IL-13 neutralizing Abs partially, but significantly, reduced the number of MR⁺ macrophages polarized by IL-33-activated eosinophils (Fig. 7B), indicating that IL-33-activated eosinophils polarized macrophages to the proinflammatory M2 phenotype, at least in part, via IL-13. The proposed mechanism by which IL-33 enhances eosinophil-mediated inflammation is shown in Fig. 8.

Discussion

In this report, we demonstrate that IL-33 can directly induce eosinophil differentiation from bone marrow precursors. We also show that mature eosinophils acquire high levels of IL-33R (ST2L) at sites of airway inflammation. IL-33 stimulates eosinophils to

produce elevated levels of proinflammatory cytokines and chemokines and enhances eosinophil-mediated polarization of M2 macrophages in vitro and in vivo. Importantly, we provide evidence

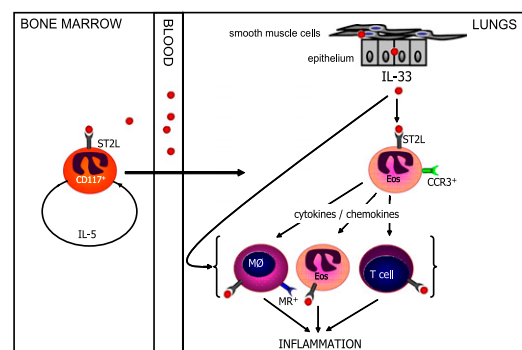


FIGURE 8. Schematic representation of the contribution of IL-33-activated eosinophils to airway inflammation. IL-33 is overexpressed by epithelial and smooth muscle cells in the lungs during experimental and clinical asthma (22, 24, 25) and is increased in the serum of asthma patients (23). IL-33 can directly induce differentiation of hematopoietic precursors into mature eosinophils in an IL-5-dependent manner. IL-33 activates M2 macrophages (24) and T cells (22) to produce chemokines and IL-5, respectively, which attract mature eosinophils to the lungs. Eosinophils that accumulate in the airways express higher amounts of ST2L and, hence, are more responsive to IL-33 stimulation. IL-33-activated eosinophils further exacerbate inflammation by producing cytokines and chemokines themselves as well as by a direct interaction with other airway cells. Eos, eosinophil; MΦ, macrophages.

that IL-33 can exacerbate eosinophil-induced airway inflammation in vivo (Fig. 8).

IL-33 induces sustained eosinophilia in vivo (16, 24, 26), and the enhanced eosinophil differentiation in the bone marrow is also a hallmark of clinical and experimental asthma (7, 42, 43). We showed in this study that IL-33 induces eosinophil differentiation from bone marrow CD117⁺ precursor cells in an IL-5–dependent and IL-13–independent manner. It can be concluded that IL-33 is capable of inducing the expansion of a subpopulation of CD117⁺ granulocyte/monocyte progenitors that were characterized by their dependency on IL-5 (44); however, it is not clear whether this is achieved in a direct or indirect fashion. Our finding is consistent with a recent study demonstrating that human hematopoietic CD34⁺ cells from asthma patients express ST2L and produce IL-5 in response to IL-33 (45). Thus, IL-33–driven eosinophilia is likely, at least in part, a consequence of IL-5 produced by IL-33–induced CD117⁺ hematopoietic precursors in the bone marrow compartment in an autocrine manner.

We also showed in this study that the IL-33/ST2 signaling pathway enhances the expression of CCR3, which plays an important role in facilitating mobilization of eosinophils from bone marrow to the peripheral blood and, ultimately, trafficking to the sites of inflammation (46). Thus, one of the key consequences of IL-33 activation of eosinophils is the differentiation, maturation, and acquisition of cellular migratory capability of eosinophils from the bone marrow to the inflammatory foci. The lack of an optimal expression of CCR3 on ST2^{−/−} eosinophils is likely responsible for fewer recruited eosinophils in BAL of ST2^{−/−} mice compared with WT mice in OVA-induced airway inflammation (22) (Fig. 4B).

Clinical and experimental studies established an important role for eosinophils in pulmonary diseases (7, 43). However, the precise mechanisms for the localized activation of eosinophils in the asthmatic lungs are not fully understood. This issue is of particular relevance in light of the recent finding that airway eosinophils are not responsive to IL-5 because these cells shed their surface IL-5R when they enter the airway lumen (47). Given the abundant expression of IL-33 in the stromal tissue of asthmatic lungs (24, 25, 48), it is likely that IL-33 triggers the activation of eosinophils that have been recruited to the airways. Consistent with this notion, we found that eosinophils that accumulated in the airways of mice with OVA-induced asthma expressed high levels of ST2L. Eosinophils activated via the IL-33/ST2 pathway may contribute to the exacerbation of airway inflammation by increasing the number of host macrophages, lymphocytes, and eosinophils in the lungs, probably through the elevated levels of cytokines and chemokines, including CCL17, IL-13, TGF- β , and CCL3. Purified eosinophils produced substantial amounts of CCL17, IL-13, and TGF- β upon IL-33 stimulation in vitro. A recent study suggests that pulmonary, eosinophil-dependent CCL17 release is required for the localized recruitment of effector T cells during experimental asthma (10). Thus, IL-33 could be an important driver for CCL17 production by eosinophils, leading to the accumulation of lymphocytes in the airways.

IL-33 seems to have no direct effect on CCL24 and CCL3 production by eosinophils. The elevated levels of these chemokines in the BAL of ST2^{−/−} recipients (that received WT eosinophils and IL-33) are likely due to an indirect effect of IL-33–stimulated eosinophils affecting other cell populations in the lungs, including alveolar macrophages. This is consistent with earlier reports that BAL M2 macrophages are major producers of CCL24 in experimental asthma (24, 49). In this study, we showed that IL-33–activated eosinophils in the airways could enhance the polarization of alveolar macrophages toward the M2 phenotype, which is known to be pathogenic in type 2 inflammation (24, 41). IL-13, a recognized M2 differentiation factor that is released by eosinophils upon IL-33

stimulation, can be responsible, at least in part, for the increased differentiation of M2 macrophages. During the preparation of this manuscript and consistent with our data, it was reported that IL-33 can trigger the production of IL-13 from eosinophils and that this was important in eosinophil- and macrophage-driven fibrosis (50).

Interestingly, IL-33 administered locally into the lungs helps to retain a significant proportion of adoptively transferred eosinophils, probably due to an induction of chemokines (e.g., CCL24 and CCL3) in the airways. The prolonged survival of eosinophils may also contribute to that process. Survival of adoptively transferred eosinophils could be enhanced as a result of an interaction with other cells or as a direct effect of IL-33. A direct antiapoptotic effect of IL-33 on human eosinophils in vitro was reported recently (29, 31, 51).

Taken together, the IL-33/ST2L signaling pathway regulates multiple features of eosinophil functions that can have a significant impact on allergic inflammation. Accumulating experimental and clinical evidence show that IL-33 is equally important in the development or activation of other cell types (such as dendritic cells, basophils, Th2 cells, mast cells, and macrophages) that are involved in the pathogenesis of allergy and asthma (16, 22, 24, 26, 28, 40, 48, 52, 53). In view of the fact that anti-IL-5 therapy in asthma patients has met with limited success (54, 55), targeting the IL-33/ST2L signaling pathway may represent a novel therapeutic approach to asthma management.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure 1

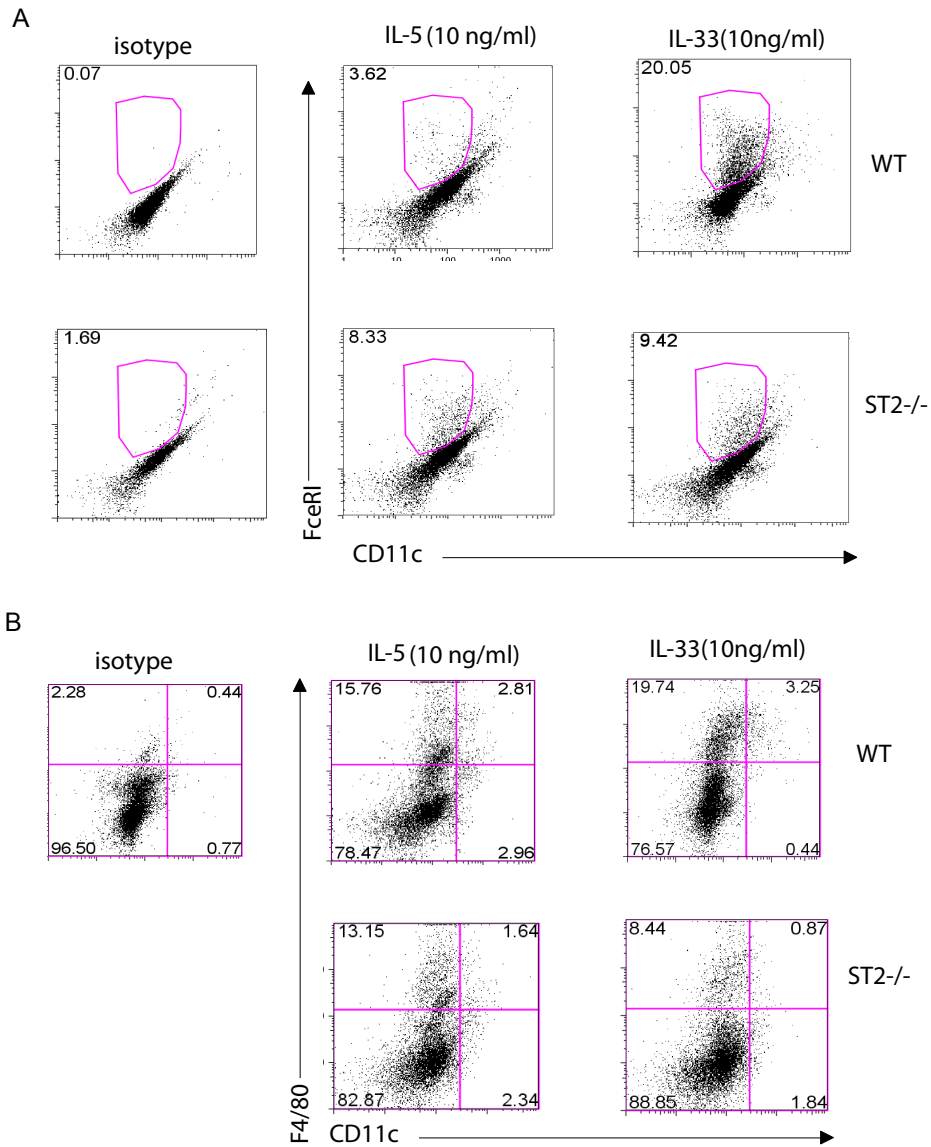


Figure 1. The effect of IL-33 on the development of basophils, dendritic cells and macrophages *in vitro*. A, FcεRI positive cells expand in cultures stimulated with IL-33. B, F4/80 positive cells expand in cultures stimulated with IL-33 or IL-5. CD117⁺ cells were purified from bone marrow of WT and ST2^{-/-} mice after depletion of committed or mature lineage-positive cells and cultured in the presence of IL-5 (10 ng/ml) or IL-33 (50 ng/ml) for 4 days. Cell phenotype was analyzed by FACS using FITC-anti-CD11c, PE-anti-FcεRI and APC-anti-F4/80 antibodies or isotype controls.