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IL-33 Amplifies the Polarization of Alternatively Activated Macrophages That Contribute to Airway Inflammation[1]

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Alternatively activated macrophages (AAM) play a crucial role in type 2 immunity. Mice deficient in ST2, a receptor for the latest member of the IL-1 family, IL-33, have impaired type 2 immune responses. We therefore reasoned that IL-33/ST2 signaling may be involved in the differentiation and activation of AAM during airway inflammation. We report herein that IL-33 changed the quiescent phenotype of alveolar macrophages toward an AAM phenotype that expressed mannose receptor, IL-4Rα, and produced high levels of CCL24 and CCL17 in an IL-13-dependent manner during IL-33-induced airway inflammation. Neutralization of AAM-derived CCL24 led to an amelioration of IL-33-induced eosinophilia in the lungs. Moreover, depletion of alveolar macrophages reduced IL-33-induced airway inflammation. Additionally, the attenuated OVA-induced airway inflammation in ST2$^{-/-}$ mice was associated with a decrease in AAM differentiation. In vitro, IL-33 amplified IL-13-induced polarization of alveolar- and bone marrow-derived macrophage toward an AAM phenotype by increasing the expression of arginase I, Ym1, as well as the production of CCL24 and CCL17. IL-13/IL-4Rα signaling was crucial for IL-33-driven AAM amplification by inducing the expression of ST2L. Finally, we showed that IL-33 was more abundantly expressed in the lung epithelial cells of asthma patients than those from healthy controls, suggesting that IL-33 may be involved in lung macrophage activation in clinical asthma. Taken together, we demonstrate here that IL-33/ST2 plays a significant role in the amplification of AAM polarization and chemokine production which contribute to innate and Ag-induced airway inflammation. ***The Journal of Immunology,*** **2009, 183: 0000–0000.**

Macrophages are specialized hematopoietic cells distributed throughout different tissues and organs where they play a central role in homeostasis, tissue remodeling, and host defense. Depending on the microenvironment, macrophages can be polarized to various distinct subsets and the heterogeneity of circulating monocytes may predefine their polarization fate once they arrive at tissues (1, 2). Polarized macrophages have been broadly classified as M1 or M2 macrophages. Classically activated M1 macrophages are differentiated by type 1 inflammatory cytokines and microbial products, and they are potent effector cells against microorganisms and tumor cells and are mainly associated with pathologic type 1 inflammation. M1 macrophages express most TLRs and opsonic receptors and secrete IL-12, TNF-α, IL-1β, IL-23, IL-6, CXCL10 (IP-10), and CCL5 (RANTES) and express inducible NO synthase (iNOS). M2 macrophages, in contrast, are characterized by their low secretion of IL-12 and can be further subdivided into three groups: M2a (alternatively activated macrophages, AAM[3]), induced by IL-4 or IL-13 or IL-21; M2b, induced by immune complexes and agonists of TLRs or IL-1 receptors; and M2c (immunosuppressive), induced by IL-10, TGF-β, or glucocorticoids (3, 4). M2 macrophages ameliorate type 1 inflammatory responses and adaptive immunity, and they promote and regulate type 2 immune responses, angiogenesis, and tissue repair (3). The M2 macrophage profile, and that of the AAM in particular, can be characterized by abundant expression of nonopsonic receptors such as the mannose receptor (MR, CD206) and CD163. Moreover, arginase I is up-regulated in AAM, resulting in generation of polyamines and proline contributing to wound healing and pathological fibrosis (3–6). Other important markers for AAM are chitinase-like lectin Ym1, resistin-like secreted protein FIZZ1, and acidic mammalian chitinase (7). Furthermore, a distinct chemokine profile, including CCL17 (TARC), CCL22 (MDC), and CCL24 (eotaxin-2), has been associated with AAM activation (5).

IL-33 was discovered in 2005 as a new member of the IL-1 family (8). Similar to IL-1β and chromatin-associated cytokine HMGB1, IL-33 may act as both a cytokine and a NF (9–20). As a cytokine it signals through its interaction with receptor complex consisting of membrane-bound ST2L (IL-1 receptor-like 1 molecule) and IL-1R accessory protein, leading to NF-κB and MAPK activation (8, 18, 21). Recently, single Ig IL-1R-related molecule

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[3] Abbreviations used in this paper: AAM, alternatively activated macrophage; BAL, bronchoalveolar lavage; BMM, bone marrow-derived macrophage; i.n., intranasal(ly); iNOS, inducible NO synthase; MR, mannose receptor; qPCR, quantitative PCR; ST2, IL-1 receptor-like 1 molecule; ST2L, membrane-bound ST2; sST2, soluble ST2; WT, wild type.

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(SIGIRR/TIR8) has been identified as a negative regulator of IL-33/ST2L signaling (22). In the present study we demonstrate that IL-33 is a hitherto unrecognized amplifier of the alternatively activated macrophages and that IL-33-activated AAM significantly contribute to type 2 immune responses.

Materials and Methods

Mice

BALB/c mice were purchased from Harlan Olac. $ST2^{-/-}$ mice (deficient for membrane-bound ST2L and soluble ST2) and $IL\text{-}4^{-/-}$ on BALB/c background were bred and kept at the Biological Services facilities of the University of Glasgow in accordance with the U.K. Home Office guidelines. $IL\text{-}4R\alpha^{-/-}$ mice were provided by Dr. J. Alexander (University of Strathclyde, Glasgow, U.K.) and $ST2^{-/-}$ mice were originally provided by Dr. A. McKenzie (Laboratory of Molecular Biology, Medical Research Council, Cambridge) (19).

Patients

Endobronchial biopsies were obtained from 10 atopic asthmatics (3 women; average age, 37 years (range, 20–60 years); forced expiratory volume in 1 s (FEV_1), 83% (range, 42–100%)) and 10 normal controls (5 of 10 atopic; 4 women; average age, 22 years (range, 19–26 years), FEV_1, 104.2% (range, 80–118%)) as described previously (23). Each subject provided informed, written consent. The patients were recruited in the Department of Asthma, Allergy and Respiratory Science, King's College, London, U.K. Asthmatics had a clear history of relevant symptoms, documented reversible airways obstruction (20% improvement in FEV_1 either spontaneously or after administration of inhaled β_2-agonist), and/or histamine PC_{20} <8 mg/ml measured within 2 wk before biopsy. None had ever smoked and there was no history of other respiratory disease. All subjects were clinically free of respiratory infection and had no systemic glucocorticoid therapy for at least 1 mo before the study. Atopy was defined as a positive skin prick test (wheal at 15 min >3 mm in diameter in the presence of positive histamine and negative diluent controls) to one or more extracts of common local aeroallergens. Normal control subjects were healthy, life-long nonsmoking volunteers who had no history of lung disease.

Macrophage culture

Bone marrow cells from BALB/c, $ST2^{-/-}$, $IL\text{-}4^{-/-}$, or $IL\text{-}4R\alpha^{-/-}$ or human peripheral blood monocytes ($CD14^+$ cells; purity, ≥97%) were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 M 2-ME, and M-CSF (20 ng/ml) for 6 days. Some cultures were conducted in FCS-free medium, X-Vivo-15 (BioWhittaker). Murine cells were evaluated by flow cytometry (FACS) for macrophage marker and cell preparations containing ≥96% of $F4/80^+$ macrophages (anti-F4/80 Ab; eBioscience, clone BM8) were used for additional experiments. Murine bone marrow-derived macrophages (BMM); FACSAria-sorted $F4/80^+CCR3^-$ (anti-CCR3 Ab; R&D Systems) alveolar macrophages or human macrophages were stimulated for 4, 24, and 48 h with IL-4, IL-13, IFN-γ (all 10 ng/ml; PeproTech), LPS (10 ng/ml; Sigma-Aldrich) and IL-33 (20 ng/ml; PeproTech), or in combination. In some cultures, macrophages were preincubated with IL-13 or IL-33 or medium alone. After 24 h cells were washed twice and recultured in fresh medium supplemented with either IL-33 or IL-13 for a further 48 h. Culture supernatants were analyzed for cytokines and chemokines by ELISA. The expression of TLR2 (eBioscience, clone 6C2), TLR4 (eBioscience, clone MTS510), ST2L (MD Biosciences, clone DJ8), and MR (Serotec) were analyzed by FACS. The expression of arginase I, iNOS, and Ym1 were analyzed by quantitative PCR (qPCR).

Recombinant IL-33

Murine and human IL-33 proteins were either produced in-house (18, 24) or obtained from PeproTech with similar results.

ELISA

Murine cytokines IL-4, IL-13, IL-10, TGFβ, and murine as well as human chemokines CCL11, CCL24, and CCL17 were analyzed by ELISA using paired Abs (BD Biosciences). In some experiments, murine 20-plex Luminex (BioSource International) was also used.

Flow cytometry and FACSAria sorting

Macrophages, bronchoalveolar lavage (BAL) cells, and lung cells were incubated with Fc blocking anti-mouse CD16/32 Abs followed by PE- or allophycocyanin-conjugated anti-F4/80, FITC-conjugated anti-TLR2 (all from eBioscience), allophycocyanin-conjugated anti-MR (Serotec), allophycocyanin- or PerCP-conjugated anti-CCR3 (R&D Systems), FITC-conjugated anti-ST2L (MD Biosciences), PE-conjugated anti-IL-4Rα (BD Pharmingen), and PE-conjugated anti-TLR4 (eBioscience) Abs or appropriate isotype controls in different combinations. Alveolar macrophages were sorted based on their expression of F4/80 and lack of CCR3 expression.

qPCR

qPCR was conducted as described previously (24, 25) with specific probe/primer or primer sets for arginase I (Mm01190441_g1; Applied Biosystems), r18S (Applied Biosystems), CCL-5 (Mm01302427_m1; Applied Biosystems), ST2L (18), Ym1 (forward, catgagcaagacttgcgtgac; reverse, ggtccaaacttccatcctcca; Invitrogen), and iNOS (forward, cagctgggctgtacaa acctt; reverse, cattggaagtgaagcgtttcg; Invitrogen).

OVA-induced airway inflammation

Wild-type (WT) and $ST2^{-/-}$ mice were sensitized i.p. with 100 μg of OVA (Sigma-Aldrich) in 2% alum (aluminum hydroxide gel adjuvant; Brenntag) on day 1 and then challenged intranasally (i.n.) on days 9, 10, and 11 with 10 μg of OVA or PBS (26). Mice were sacrificed 3 h after Ag challenge on days 9 and 10 as well as 24 h after last challenge (day 12). Serum, BAL cells, and lungs were harvested and analyzed as described previously (27).

IL-33-induced airway inflammation

IL-33 (4 μg/mouse) or PBS was administered i.n. for 3 or 6 consecutive days. To evaluate the contribution of CCL24 and IL-13 to IL-33-induced inflammation, mice were given neutralizing Abs (R&D Systems, clones 106521 and 38213, respectively) or appropriate isotype controls (20 μg/mouse) together with IL-33. To evaluate the contribution of alveolar macrophages to IL-33-induced airway inflammation, clodronate or control liposomes (40 μl/mouse) were administered i.n. (28) 72 and 24 h before 3 consecutive days of i.n. IL-33 inoculations. Clodronate was a gift of Roche Diagnostics. It was encapsulated in liposomes as described earlier (29). All mice were sacrificed on day 6 or 7, and serum, BAL cells, and lungs were analyzed as described previously (27). Some lungs were digested further by incubation with an enzyme cocktail containing collagenase V (0.85 mg/ml; Sigma-Aldrich), collagenase D (1.25 mg/ml; Roche), dispase (1 mg/ml; Invitrogen), and DNase (30 μg/ml; Invitrogen). After 1 h at 37°C, cell suspensions were filtered and analyzed. Tissue inflammation was scored using a semiquantitative scoring system assessing the degree of eosinophilic inflammation: 0, no eosinophils; 1, eosinophils make up <10% of total infiltrate or total infiltrate is <20 cells; 2, eosinophils make up 10–50% total infiltrate; and 3, eosinophils make up >50% total infiltrate.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections (6 μm) of bronchial biopsies by the alkaline-phosphatase anti-alkaline-phosphatase technique (23) using mAb against human IL-33 (Nessy-1; Alexis Biochemicals). Briefly, sections were incubated with primary mAb (1/100) for 4 h at room temperature. After washing, sections were incubated with secondary Ab (rabbit anti-mouse, 1/30; Dako) followed by mouse anti-alkaline phosphatase complex (1/30; Dako). Positive signals were detected using Fast Red (Sigma-Aldrich). The number of $IL\text{-}33^+$ cells was counted by a histologist blinded to the source of the samples. The results are expressed as the number of $IL\text{-}33^+$ cells per mm^2 of bronchial mucosa.

Statistical analysis

ANOVA followed by Tukey's test or Student's *t* test was applied to in vitro studies. Analysis between in vivo groups was examined by Mann-Whitney *U* test or ANOVA followed by Student's *t* test. All data are expressed as means ± SEM. Values of $p < 0.05$ were considered significant.

Results

IL-33 enhances the polarization of AAM

Given the well-established contribution of IL-33/ST2 to type 2 immune responses (8, 18) and the expression of IL-33R on macrophages (19) we reasoned that IL-33 may be involved in AAM macrophage activation. To characterize the effect of IL-33 on macrophage activation we stimulated WT and $ST2^{-/-}$ bone marrow-derived macrophages with IL-33, IFN-γ (a M1 activation stimulus), IL-4 or IL-13 (known AAM activation stimuli), or a

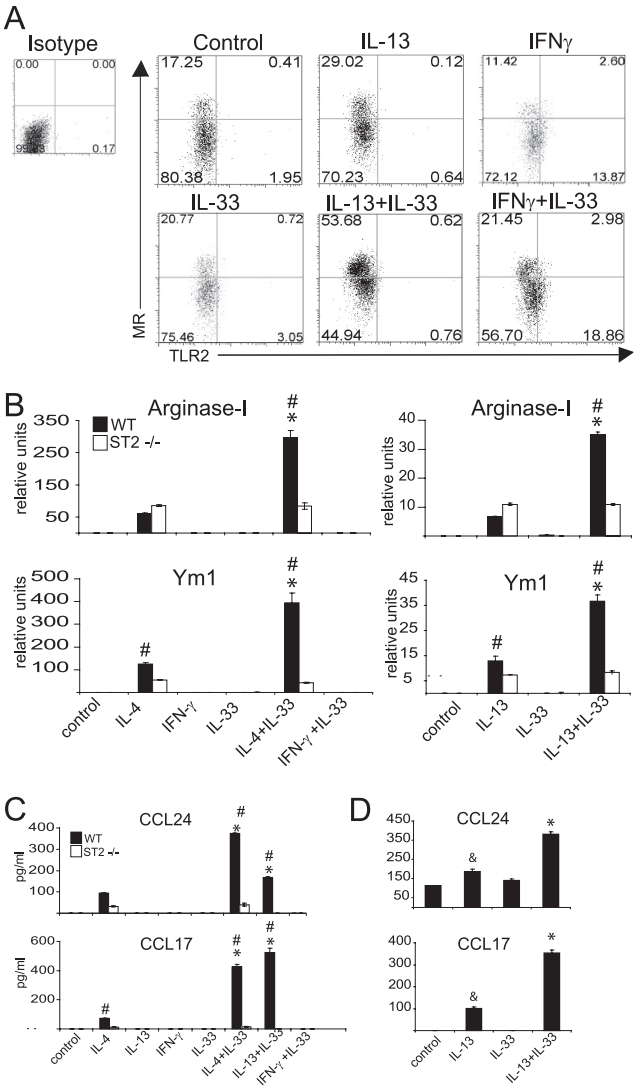

FIGURE 1. IL-33 enhances the polarization of AAM from bone marrow precursors. *A–C*, BM cells from BALB/c or ST2$^{-/-}$ mice were cultured in the presence of M-CSF (10 ng/ml). After 6 days, macrophages (F4/80^{+}) were stimulated for 48 h with IL-13 (10 ng/ml), IL-4 (10 ng/ml), IFN-γ (10 ng/ml), murine IL-33 (20 ng/ml), or combinations of these reagents. Surface markers (*A*) and mRNA expression of arginase I and Ym1 (*B*) were analyzed by FACS and qPCR, respectively. *C*, CCL24 and CCL17 production were analyzed by ELISA. *D*, Human monocyte-derived macrophages were stimulated with IL-13 (2 ng/ml), human IL-33 (20 ng/ml), or in the presence of both for 48 h. Data are means ± SEM and are representative of at least three independent experiments and three donors. *, $p < 0.05$, IL-33 treated vs other samples; #, $p < 0.05$, WT vs ST2$^{-/-}$ sample; &, $p < 0.05$, IL-13 vs control. Relative units indicates percentage of 18S expression.

combination of these cytokines for 48 h. IL-4, IL-13, and IFN-γ were used in suboptimal doses so as to reveal any synergistic/additive effect with other cytokines. Analysis of M1 and AAM cell markers revealed that macrophages cultured with medium alone contained a modest population of cells expressing MR or TLR2 (Fig. 1*A*). As expected, suboptimal doses of IL-13 and IFN-γ had a modest effect on MR and TLR2 expression. IL-13 increased, and IFN-γ decreased the percentage of MR^{+} cells while IFN-γ increased the percentage of TLR2^{+} cells. IL-33 alone had no apparent effect on the expression of MR and TLR2. In contrast, IL-33 strongly enhanced the expression of MR, but not TLR2, in the presence of IL-13 (Fig. 1*A*). A similar synergistic effect was also observed for IL-4 and IL-33 on MR expression (data not shown). Consistent with these findings, IL-33 strongly enhanced the mRNA expression of the AAM markers, arginase I and Ym1, in the presence of IL-13 or IL-4 in the BMM from WT but not from ST2$^{-/-}$ mice as determined by qPCR (Fig. 1*B*). Analysis of chemokine production revealed that macrophages cultured with medium, IL-13, IFN-γ, or IL-33 alone did not produce detectable CCL24 or CCL17. In the presence of IL-4, WT macrophages released a modest amount of CCL24 and CCL17. ST2$^{-/-}$ macrophages released a minimal amount of both chemokines. WT macrophages stimulated with IL-33, in the presence of IL-4, released 3.5-fold more CCL24 and 5-fold more CCL17 than did those stimulated with IL-4 alone (Fig. 1*C*). Although IL-13 and IL-33 on their own did not produce detectable levels of chemokines, these cytokines synergized to trigger a release of high concentrations of CCL17 and CCL24 (Fig. 1*C*). IFN-γ did not induce CCL17 or CCL24 production in combination with IL-33. Consistent with the lack of effect on TLR2 expression, IL-33 alone or in combination with IFN-γ had no influence on M1 cytokine and chemokine profile, including IL-12, TNF-α, and CXCL10 (data not shown). IL-10 and IL-5 were not detectable in these cultures. Similar results were obtained in FCS-free cultures. Taken together, these data demonstrate that IL-33 significantly augments the polarization of mouse alternatively activated macrophages induced by IL-13 and, to a lesser extent, by IL-4.

To investigate whether IL-33 also synergizes with IL-13 in human AAM activation, monocyte-derived macrophages were stimulated with IL-13, IL-33, or a combination of both cytokines. As shown in Fig. 1*D*, IL-13 alone stimulated the production of both CCL24 and CCL17. IL-33 alone had no influence on chemokine production; however, in the presence of IL-13, IL-33 strongly enhanced the production of both chemokines. This result demonstrates that IL-33 also augments CCL17 and CCL24 production by human macrophages.

IL-33 amplification of AAM polarization is IL-4Rα-dependent

Given the well-established involvement of IL-4, IL-13, and IL-4Rα (shared by IL-4 and IL-13) in AAM differentiation (3, 30, 31), we evaluated the contribution of these factors to IL-33-amplified AAM development. BMM from WT, IL-4$^{-/-}$, or IL-4Rα$^{-/-}$ mice were cultured as described above. IL-33 alone induced only a modest amount of CCL17 or CCL24 production. However, in the presence of IL-13 or IL-4, IL-33 strongly increased the chemokine productions in WT and IL-4$^{-/-}$ but not in IL-4Rα$^{-/-}$ macrophages (Fig. 2, *A* and *B*). The synergistic effect of IL-13/IL-4 with IL-33 on chemokine productions was markedly inhibited by the presence of IFN-γ. Taken together, these data demonstrate that synergistic effect of IL-33 and IL-13/IL-4 on AAM polarization is IL-4Rα-dependent.

IL-13 increases macrophage responsiveness to IL-33 by increasing ST2L expression

To further investigate the mechanism underlying the synergistic effect of IL-33/ST2 and IL-4Rα signaling on AAM differentiation, macrophages were preincubated with IL-13 or IL-33. After 24 h, the cells were washed and fresh medium was supplemented either with IL-33 or IL-13 and the cells were cultured for a further 48 h. Preincubation of macrophages with IL-13 followed by IL-33 stimulation dramatically increased the production of CCL17 and CCL24 compared with cells preincubated with IL-13 or stimulated with IL-33 alone (Fig. 2*C*). In contrast, preincubation of macrophages with IL-33 followed by IL-13 stimulation did not show any synergistic effects of these two cytokines on chemokine production. These data indicate that IL-13 increases the responsiveness of

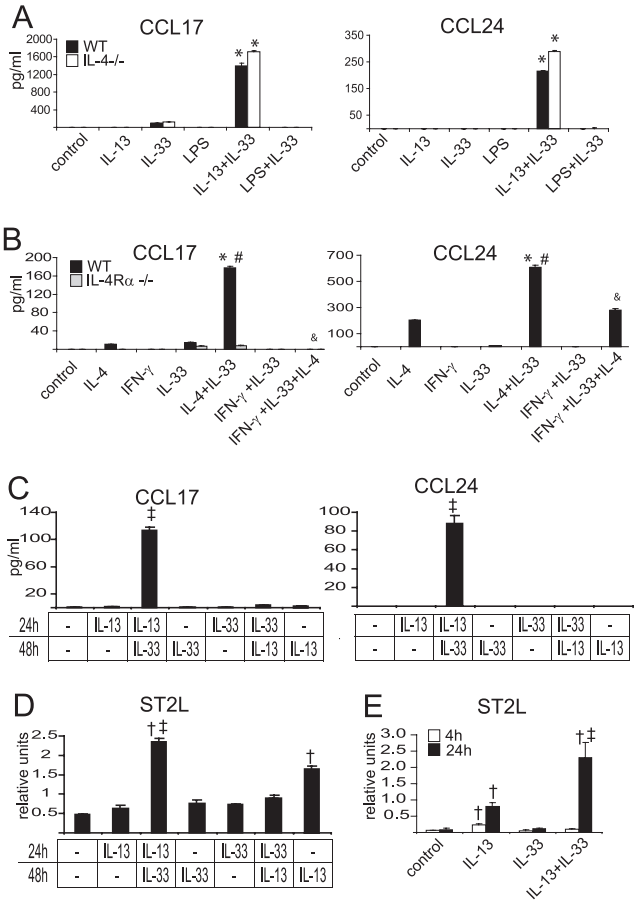

FIGURE 2. IL-13 increases macrophage responsiveness to IL-33 by increasing ST2L expression. *A* and *B*, BMM from BALB/c (*A*), IL-4$^{-/-}$ (*A*), or IL-4R$\alpha^{-/-}$ (*B*) mice were harvested and stimulated as described in Fig. 1. *C* and *D*, BMM from BALB/c mice were preincubated with IL-13 or IL-33 or medium alone. After 24 h cells were washed and stimulated with IL-33 or IL-13. After 48 h CCL24 and CCL17 production (*C*) and ST2L (*D*) expression were analyzed by ELISA and qPCR, respectively. *E*, BMM were stimulated with IL-13 or IL-33 or combination of these cytokines. After 4 or 24 h, total RNA was isolated and expression of ST2L was evaluated by qPCR. Data are means ± SEM of three wells and are representative of at least two independent experiments. *, $p < 0.05$, IL-33-treated vs other samples; #, $p < 0.05$, WT vs relevant gene-deficient mice; &, $p < 0.05$, IFN-γ- vs non IFN-γ-treated samples; †, $p < 0.05$ cytokine treated vs control; ‡, $p < 0.05$ IL-33-treated vs other samples.

macrophages to IL-33 and not vice versa. We then investigated the mechanism by which IL-13 may potentiate macrophage responsiveness to IL-33. qPCR analysis revealed that IL-13, but not IL-33 alone, triggered ST2L expression in macrophages (Fig. 2, *D* and *E*). This was observed as early as at 4 h of incubation with IL-13. The peak of ST2L expression was detected at 24 h of incubation (Fig. 2*E*). Interestingly, IL-33, together with IL-13 (Fig. 2*E*) or with IL-13-pretreated macrophages (Fig. 2*D*), further increased ST2L expression. Collectively, these data demonstrate that IL-13/IL-4Rα signaling is crucial for IL-33-driven AAM amplification by inducing the expression of ST2L, which could be further increased by the presence of IL-33.

IL-33 changes the quiescent phenotype of alveolar macrophages toward AAM in vivo

Alveolar macrophages are the predominant immune effector cells resident in the alveolar spaces and airways and are responsible for activating inflammatory responses. Given the prominent effect of

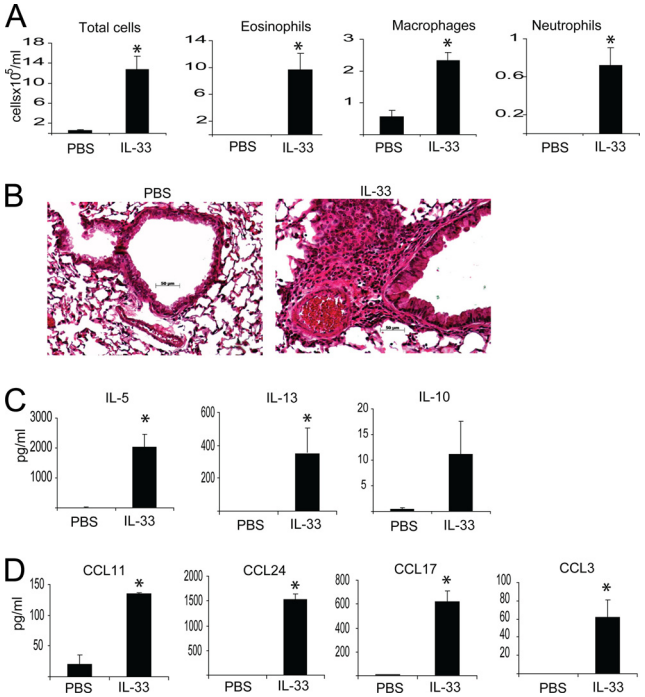

FIGURE 3. Type 2 chemokines are induced in IL-33-triggered airway inflammation. BALB/c mice were inoculated i.n. with IL-33 (4 μg/mouse) or PBS for 6 consecutive days ($n = 8$). Differential cell count in the BAL fluid (*A*), lung histology (*B*), BAL cytokines (*C*), and BAL chemokines (*D*) were assayed 24 h later. Data are means ± SEM and are representative of at least three independent experiments. *, $p < 0.05$, IL-33-treated vs control mice.

IL-33 on IL-13/IL-4 induced AAM differentiation in vitro, we investigated if IL-33 changes the quiescent phenotype of alveolar macrophages toward AAM during airway inflammation. Naive mice were administered with IL-33 (4 μg i.n.) or PBS for 6 consecutive days. Mice receiving IL-33 showed increased BAL eosinophil, macrophage, and neutrophil cell counts (Fig. 3*A*) as well as marked cellular infiltration in the tissue compared with control PBS-treated mice (Fig. 3*B*). IL-33 also triggered high levels of IL-5 and IL-13 expression in the BAL cells (Fig. 3*C*). Other cytokines were undetectable (IL-4, GM-CSF, IFN-γ, IL-1α/β, IL-2, IL-6, IL-12, and IL-17) or low and unchanged (TNF-α) (data not shown). Reflecting the cellular infiltrates, high levels of type 2 chemokines were detected. CCL11, CCL24 (eosinophil, basophil, and mast cell chemoattractants), CCL17 (memory Th2 and NKT cell chemoattractants), and CCL3 (monocyte chemoattractant) were significantly increased in mice treated with IL-33, compared with the control PBS-treated group (Fig. 3*D*). Type 1 chemokines CXCL10 (IP-10), CXCL2 (KC), CCL2 (MCP-1) and CXCL9 (MIG) were not detectable in any of the mice (data not shown). Similar cellular, cytokine, and chemokine profiles were obtained when IL-33 was administered over a shorter time course (3 consecutive days), only of lower magnitudes. IL-33 elicited no airway inflammation in ST2$^{-/-}$ mice (data not shown). In subsequent experiments, we administered IL-33 for 3 days and the mice were culled on day 6. BAL cells from IL-33-treated or PBS-treated control WT mice were harvested and the expression of M1 and AAM activation markers were evaluated. As previously reported (28, 32, 33), alveolar macrophages from untreated mice had a quiescent phenotype characterized by low to moderate levels of TLR2, TLR4, MR, ST2L, and IL-4Rα (Fig. 4, *A* and *B*). Following IL-33 administration, alveolar macrophages polarized to an AAM phenotype with marked increase in the expression of MR and IL-4Rα.

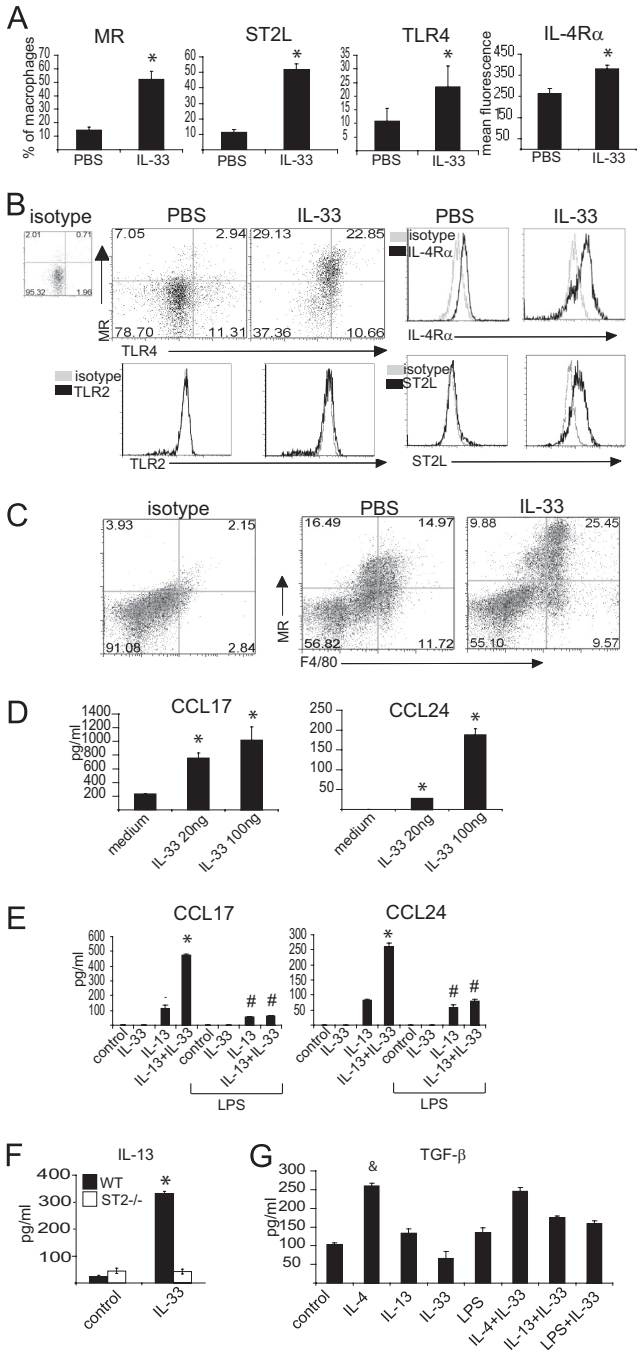

FIGURE 4. IL-33 polarizes alveolar macrophages toward AAM in vivo. BALB/c mice ($n = 5$) were inoculated i.n. with IL-33 (4 μg/mouse) or PBS for 3 consecutive days and mice were culled on day 6. *A* and *B*, BAL cells were gated for F4/80$^+$CCR3$^-$ macrophages and the expression of MR, ST2L, TLR4, IL-4Rα, and TLR2 were analyzed by FACS. Quantitative evaluation (*A*) and representative staining (*B*) are shown. Lungs of PBS- or IL-33-treated mice were digested and the cell surface markers of lung macrophages were analyzed by FACS (*C*). *D*, *E*, and *G*, BAL F4/80$^+$CCR3$^-$ macrophages from IL-33- (*D*) and PBS- (*E* and *G*) treated mice were sorted by FACSAria and cultured in the presence of IL-13 (10 ng/ml), IL-4 (10 ng/ml), IL-33 (20 or 100 ng/ml), LPS (10 ng/ml), or a combination of these reagents for 48 h. *F*, Whole BALB/c lungs were cut into pieces and cultured with or without IL-33 (20 ng/ml) for 48 h. Culture supernatants were analyzed for chemokines or cytokines by ELISA. Data are means ± SEM and are representative of at least three independent experiments. *, $p < 0.05$, IL-33-treated vs control mice; #, $p < 0.05$, LPS vs non-LPS samples; &, $p < 0.05$, IL-4-treated vs other samples.

TLR4 and ST2L were also increased. In contrast, IL-33 did not affect the expression of M1 associated receptor, TLR2 (Fig. 4, *A* and *B*). Similarly, IL-33 markedly induced MR expression by lung parenchyma macrophages (Fig. 4*C*). Moreover, FACS-sorted alveolar macrophages from IL-33-primed mice produced CCL17 spontaneously, which was further increased by IL-33 stimulation in vitro. IL-33 also induced CCL24 production in these cells (Fig. 4*D*). In contrast, consistent with their quiescent phenotype, alveolar macrophages isolated from untreated mice did not produce chemokines spontaneously nor did these cells produce significant amounts of CCL17 or CCL24 in response to IL-33 in vitro (Fig. 4*E*).

To investigate the conditions that render alveolar macrophages responsive to IL-33 activation in IL-33-primed mice, alveolar macrophages isolated from naive mice were stimulated with IL-33 in the presence of either IL-13 or IL-4 (AAM stimuli) or IFN-γ (a M1 stimulus) or LPS (a known stimulator of ST2 expression) (19). IL-13 triggered modest production of CCL17 and CCL24 by these cells, whereas IL-33 together with IL-13 (to a lesser extent IL-4) strongly amplified CCL17 and CCL24 production (Fig. 4*E* and data not shown). In contrast, IFN-γ and LPS not only were unable to induce CCL17 and CCL24 production but also inhibited IL-33 plus IL-13-induced chemokine productions (data not shown and Fig. 4*E*). Because, in contrast to IL-13, IL-4 was not detected in BAL cells of mice with IL-33-induced airway inflammation (Fig. 3), we focused on IL-13 in our further studies. Since IL-13/IL-4Rα signaling was indispensable for IL-33 amplification of AAM polarization (Fig. 4*E* and Fig. 2), we investigated the source of IL-13 in the lungs after IL-33 stimulation. Macrophages, fibroblasts, or the whole lung explants were cultured with IL-33 for 2 days and the concentrations of IL-13 in the culture supernatants were assayed by ELISA. Macrophages and fibroblasts did not produce significant amounts of IL-13 when cultured with IL-33 (data not shown). However, whole lung explants from naive WT but not ST2$^{-/-}$ mice released a substantial amount of IL-13 upon IL-33 stimulation (Fig. 4*F*), suggesting that IL-13 production can be stimulated by IL-33 locally in the lung cells (other than macrophages) and that the IL-13 then synergizes with IL-33 in AAM polarization.

It is thought that alveolar macrophages are critically involved in the maintenance of immune tolerance to harmless environmental Ags (28, 32, 34), likely by constitutive expression of TGF-β (32). To test whether IL-33 affects the production of TGF-β and IL-10, alveolar macrophages were stimulated as before. In contrast to IL-4, IL-33 and IL-13 alone did not affect TGF-β production. In apparent contrast to AAM-associated chemokine production, IL-33 did not have a synergistic effect with IL-4 or IL-13 on TGF-β production (Fig. 4*G*). IL-10 was not detectable in these cultures (data not shown).

Other chemokines found in the BAL cells during IL-33-induced airway inflammation (CCL11 and CCL3) were not released by AAM upon IL-33 stimulation (data not shown). In contrast, fibroblasts and mast cells released substantial amounts of CCL11 and CCL3, respectively, when cultured with IL-33 (Ref. 35 and data not shown).

Taken together, these data suggest that IL-33 is involved in the polarization of the quiescent form of alveolar macrophages to the alternatively activated macrophages with the production of AAM-associated chemokines in vivo.

IL-33 induced airway inflammation and alveolar AAM polarization is IL-13-dependent

To confirm the contribution of IL-13 to IL-33-induced airway inflammation, IL-13-neutralizing Abs or control IgG were coadministered with IL-33. Neutralization of IL-13 significantly decreased the total cell and eosinophil counts in the BAL fluid by 45 and

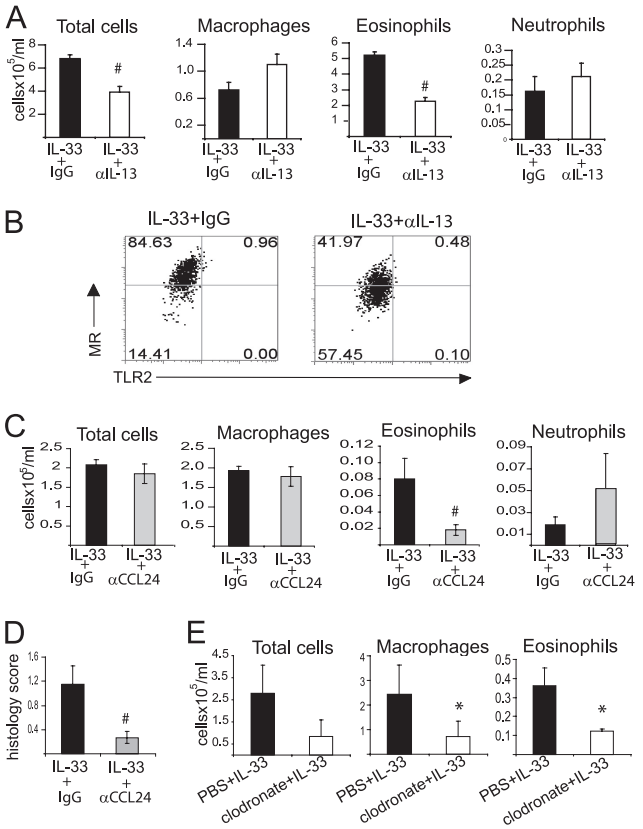

FIGURE 5. Macrophages, IL-13, and AAM-derived CCL24 mediate IL-33-induced airway inflammation. *A–D*, BALB/c mice were treated i.n. with IL-33 (4 μg/mouse) or PBS for 3 consecutive days. Some mice also received anti-IL-13 (*A* and *B*) or anti-CCL24-neutralizing Abs (*C* and *D*) (20 μg/mouse) for 5 consecutive days. The control group received isotype-matched rat IgG (all groups $n = 5$). Mice were sacrificed on day 6. *A* and *C*, BAL cell counts are shown. *B*, Representative staining for MR and TLR2 of alveolar macrophages from anti-IL-13 Abs and IgG-treated mice is shown. *D*, Tissue inflammation of anti-CCL24 Abs and IgG treated mice is shown. *E*, Clodronate ($n = 5$) or control ($n = 5$) liposomes (both 40 μl/mouse) were administered i.n. 72 and 24 h before i.n. IL-33 inoculation (3 consecutive days, 4 μg/mouse/day). All mice were sacrificed on day 6 and BAL cells were analyzed. Data are means ± SEM. *, $p < 0.05$, clodronate vs control liposomes; #, $p < 0.05$ neutralizing Abs vs IgG-treated mice.

55%, respectively (Fig. 5*A*). Importantly, anti-IL-13-neutralizing Abs markedly decreased the percentage of MR^+ alveolar macrophages compared with the control group (54 ± 8% and 82 ± 2%, respectively, $p < 0.05$). The percentage of $TLR2^+$ macrophages were not changed (Fig. 5*B*). These data demonstrate that IL-13 is required, at least partially, for IL-33-induced airway inflammation and differentiation of alveolar macrophages toward AAM.

Alveolar macrophages and AAM-derived CCL24 mediate IL-33-induced airway inflammation

To determine the contribution of AAM-derived CCL24 (eotaxin-2) to IL-33-induced eosinophilia, CCL24-neutralizing Abs or control IgG were coadministered with IL-33. Neutralization of CCL24 reduced BAL eosinophila by 80% (Fig. 5*C*) and also strongly reduced tissue eosinophilia (Fig. 5*D*), indicating that macrophage-derived CCL24 is, at least in part, responsible for this process. To further confirm that alveolar macrophages are important in the initiation of IL-33-induced airway inflammation, mice were treated with cytotoxic clodronate or control liposomes followed by IL-33 administration. This route of liposome administration affects alveolar but not lung parenchyma macrophages (28). Intranasal administration of clodronate liposomes depleted ~80% of alveolar macrophages compared with the control group. IL-33 induced significantly less severe eosinophilia in alveolar macrophage-depleted mice compared with control mice (Fig. 5*E*), indicating that IL-33-driven AAM activation of alveolar macrophages is likely responsible for IL-33-induced airway eosinophilia.

$ST2^{-/-}$ mice show an impaired polarization of AAM during allergic airway inflammation

Next, we investigated the role of endogenous IL-33/ST2 in AAM polarization during OVA-induced airway inflammation. We have shown recently that $ST2^{-/-}$ mice developed reduced allergic airway inflammation compared with WT mice. This was associated with reduced differentiation of IL-5^+ T cells. However, the levels of IL-4 and IL-13, the classical inducers of AAM and chemokines, were similar in WT and $ST2^{-/-}$ mice (18). Therefore, we investigated the possibility that IL-33/ST2L-regulated local differentiation of AAM and production of type 2 chemokine (CCL17, CCL11, and CCL24) may significantly contribute to the allergic airway inflammation in WT mice but not $ST2^{-/-}$ mice. To test this possibility, WT and $ST2^{-/-}$ mice were sensitized with OVA and challenged 9 days later for 3 consecutive days with either intranasal OVA or PBS. BAL cells were analyzed after first, second, and third Ag challenge and chemokines were examined after the last challenge. As we reported previously (18), there was a less pronounced increase in total cell, macrophage, and eosinophil accumulation in the BAL cells of $ST2^{-/-}$ mice compared with WT mice (total cells in Fig. 6*A* and in Ref. 18). No chemokine was detected in PBS-challenged WT or $ST2^{-/-}$ mice. In contrast, all mice sensitized and challenged with OVA had an increased level of CCL11, CCL24, and CCL17 in the BAL cells. Consistent with the lower number of cell infiltration in the BAL cells, the levels of CCL11, CCL24, and CCL17 were significantly lower in $ST2^{-/-}$ mice compared with WT mice (Fig. 6*B*). Other chemokines were unchanged (CCL3, CXCL3) or undetectable (CXCL10, CCL2, CXCL9, and CCL1) in WT or $ST2^{-/-}$ mice.

Next, we isolated RNA from lungs of WT and $ST2^{-/-}$ mice 3 h after the first and second challenge and assayed for M1 and AA macrophage-associated genes by qPCR. There was no difference in the expression of iNOS and CCL5 (M1 macrophage-associated genes) between WT and $ST2^{-/-}$ mice at any time point. In contrast, the expression of arginase I and Ym1 (specific AAM markers) was markedly reduced at both time points in $ST2^{-/-}$ mice compared with that of the WT mice (Fig. 6*C*).

Taken together, these data indicate that IL-33/ST2 signaling is an important pathway in the amplification of AAM polarization and chemokine production in the allergic airway inflammation.

Asthma patients express IL-33 in the lung epithelial cells

To investigate the relevance of our findings to clinical disease, we evaluated the expression of IL-33 protein in the bronchial biopsies by immunohistochemistry. IL-33 protein was present in the lung biopsies of both asthma patients and healthy controls. However, the number of IL-33^+ cells was significantly more abundant in asthma patients compared with healthy individuals (Fig. 7). Staining appeared confined to bronchial epithelial cells. These results suggest that local IL-33 might be available for the activation of alveolar macrophages in asthma patients, thereby contributing to the clinical outcome.

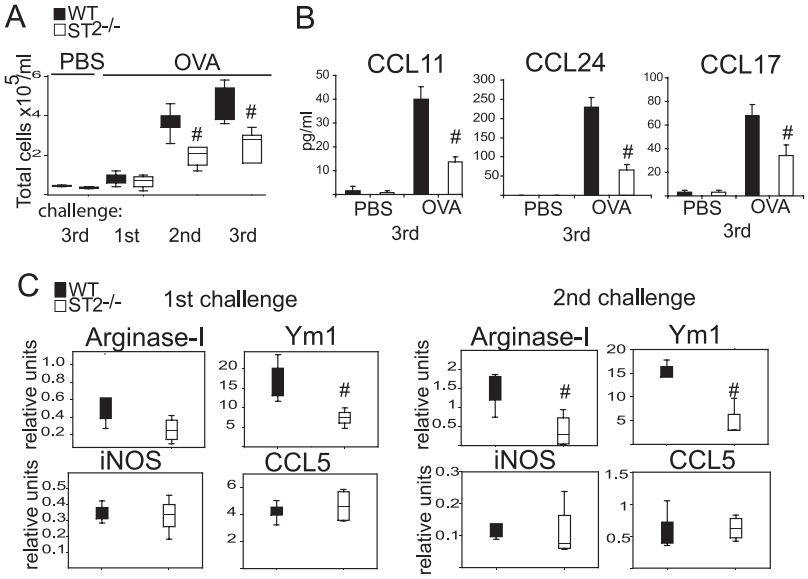

FIGURE 6. ST2$^{-/-}$ mice show an impaired development of AAM during allergic airway inflammation. WT and ST2$^{-/-}$ mice were sensitized (i.p.) with 100 μg of OVA in 2% alum on day 1 and then challenged (i.n.) on days 9, 10m and 11 with 10 μg of OVA or PBS. *A*, Three hours after the first and second OVA challenge and 24 h after the third OVA challenge, differential cell counts in the BAL fluid were performed. *B*, Chemokine concentrations in BAL cells were measured 24 h after the last Ag challenge. *C*, Three hours after the first and second OVA challenge, lungs of BALB/c and ST2$^{-/-}$ mice were collected, followed by RNA isolation and qPCR for arginase I, Ym1, iNOS, and CCL5. Data are presented as percentage of 18S expression (relative units). Data are means ± SEM (*A* and *B*, $n = 8$; *C*, $n = 5$) and are representative of at least three independent experiments. #, $p < 0.05$, WT vs ST2$^{-/-}$ mice.

Discussion

Data presented in this report demonstrate that IL-33 is a powerful enhancer of alternatively activated macrophage development during innate and adaptive immune responses. Alveolar macrophages, resident cells of the lungs, have a distinct phenotype compared with other resident macrophages (33). In the lungs, edema and inflammation can lead to the thickening of alveolar walls and compromise gas exchange, and therefore the immune defense of this barrier has to be tightly regulated (33). To avoid damage to alveoli, alveolar macrophages are generally maintained in a quiescent state that resembles the M2c phenotype (28, 34). It is thought that these cells produce suppressive cytokine such as TGF-β and inhibit the initiation of adaptive immunity to harmless Ags (32). However, in the presence of pathogens or damaged tissues, alveolar macrophages are likely responsible for the initiation of the inflammatory response (36–39). We showed here that IL-33 synergizes with endogenous IL-13/IL-4Rα signal to induce the polarization of quiescent TGF-β-producing macrophages toward AAM-producing CCL24 and CCL17, leading to inflammation in the lung. It is also likely that parenchyma macrophages are differentiated into AAM phenotype by IL-33, as they showed an enhanced expression of MR in IL-33-treated mice. The proposed mechanism of action of IL-33 in enhancing AAM polarization is summarized in Fig. 8. Importantly, IL-33 enhances IL-13-induced CCL24 and CCL17 production by human macrophages. Given the presence of IL-33 in serum of some asthma patients (40) and in theirs lungs (Fig. 7), it is likely that IL-33-driven macrophage activation may contribute to clinical disease.

Although IL-33 can also synergize with IL-4 in AAM differentiation in vitro, IL-4-deficient mice administered with IL-33 could mount airway inflammation indistinguishable from that of the WT mice. This indicates that IL-4 is marginally involved in IL-33-induced airway inflammation (G. Murphy et al., manuscript in

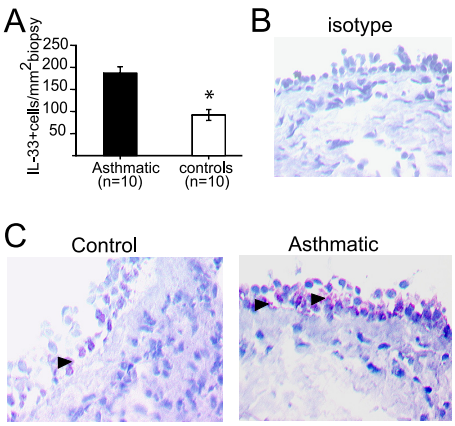

FIGURE 7. Asthma patients express more IL-33 in the lung epithelial cells compared with healthy controls. *A–C*, Frozen sections of lung biopsies of asthmatic patients and healthy donors were stained with anti-IL-33 Ab. Quantitative evaluation (*A*) and representative staining (*B* and *C*) are shown. Positive signal is violet. Data are means ± SEM ($n = 10$); *, $p < 0.05$, asthmatic vs controls.

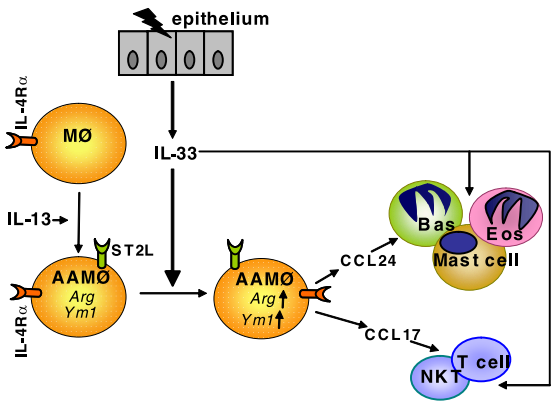

FIGURE 8. Schematic representation of the contribution of IL-33 to AAM polarization and airway inflammation. IL-33 is released by endothelial or epithelial cell damage during trauma or infection (9, 43). By acting on alveolar macrophages, which are located proximal to epithelial surfaces, IL-33 synergizes with IL-13/IL-4Rα signal to induce polarization of quiescent TGF-β-producing macrophages toward AAM. IL-33 strongly amplifies the expression of arginase I and Ym1 as well as the production of CCL24 and CCL17, which may further recruit inflammatory cells. This process, together with the contribution of IL-33 to polarization of IL-5^{+} T cells (18), stimulation of memory Th2 cells (8, 46), and direct activation of basophils (14, 15), eosinophils (15, 47), and mast cells (48) may lead to a pathogenic outcome such as excessive type 2 inflammation. Eos indicates eosinophil; Bas, basophil; AAMϕ, alternatively activated macrophages; ArgI, arginase I; Ym1, chitinase-like lectin.

preparation). Moreover, in contrast to IL-13 (Fig. 5), the lack of endogenous IL-4 did not affect alternative activation of alveolar macrophages in IL-33-treated mice (WT mice, 43 ± 15% MR^+ macrophages; IL-$4^{-/-}$ mice, 51 ± 17% MR^+ macrophages; $p <$ 0,05). Thus, although both IL-4 and IL-13 shared IL-4Rα, IL-13 is the predominant cytokine for the synergism with IL-33 in the polarization of AAM in vivo.

IL-33 also enhances TLR4 expression on macrophages, rendering them more responsive to LPS stimulation, which was recently confirmed by Espinassous et al. (41). LPS and IFN-γ strongly inhibited the IL-33-induced AAM activation and type 2 chemokine production. This finding provides an additional explanation for an early report showing a beneficial role of LPS in treatment of allergic airways inflammation (42). It is also likely that an increased endotoxin sensitivity in $ST2^{-/-}$ mice (19) could partially result from a shift in the macrophage activation from an alternatively activated to classically activated program in these mice. On the other hand, it also has been shown that short but not prolonged exposure of macrophages to IL-33 can enhance LPS-induced cytokine production (41), indicating that IL-33 is able to modulate an innate activation of macrophages. Taken together, IL-33 may have a very complex role in macrophage biology where both the time of exposure and local environment (LPS, IL-13) are likely to determine the activation outcome.

In vivo, IL-33 is likely induced by a variety of antigenic and environmental stimuli. IL-33 is expressed in substantial levels in the lungs of mice primed with OVA plus alum in epithelial cells and macrophages (18) and in patients with asthma (40), principally by epithelial cells (Fig. 7). IL-33 is also constitutively expressed in various normal human tissues, particularly in endothelial cells and epithelial cells exposed to the environment, such as skin, gastrointestinal tract, and lungs (9). It has been suggested that IL-33 may be released by cells undergoing necrosis or by inflammasome activation (9, 41, 43–45). Thus, IL-33 might function, similar to the prototypic "alarmin" HMGB1, to alert the immune system after endothelial or epithelial cell damage during trauma or infection (9, 43). Therefore, alveolar macrophages may play an important role in response to IL-33 released during lung infection or trauma, as they are located proximal to epithelial surfaces. Consistent with this notion, recent studies indicate that, in contrast to macrophages, other cells expressing ST2, including mast cells, NK cells, and basophils, are not indispensable for IL-33-induced airway inflammation (17).

The ability of IL-33 to enhance the polarization of macrophages toward the AAM lineage and the subsequent production of type 2 chemokines further empowers AAM to recruit inflammatory cells to enhance the immune response. This process, together with the contribution of IL-33 to the polarization of IL-5^+ T cells (18), activation of Th2 cells (8, 46), basophils (14, 15), eosinophils (15, 47), and mast cells (35, 48) may lead to a pathogenic outcome such as excessive type 2 inflammation and airway hyperreactivity (8, 17, 18, 46) (Fig. 8). A recent study showed that resolution of allergic inflammation and airway hyperreactivity is indeed dependent on disruption of the ST2/IL-33 pathway (46).

In summary, this report defines a previously unrecognized role for IL-33/ST2 in type 2 immunity, acting as an important enhancer for the development of alternatively activated macrophages as well as an amplifier of chemokine production. IL-33 is widely expressed in endothelial cells and in the epithelial barrier. Our findings may therefore have implications for a wide range of immune responses where persistent AAM activation was found to play a crucial role (6, 49–52).

Disclosures

The authors have no financial conflicts of interest.

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