

IL-33 synergizes with IgE-dependent and IgE-independent agents to promote mast cell and basophil activation

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Abstract

Objective Mast cell and basophil activation contributes to inflammation, bronchoconstriction, and airway hyperresponsiveness in asthma. Because IL-33 expression is inflammation inducible, we investigated IL-33-mediated effects in concert with both IgE-mediated and IgE-independent stimulation.

Methods Because the HMC-1 mast cell line can be activated by GPCR and RTK signaling, we studied the effects of IL-33 on these pathways. The IL-33- and SCF-stimulated HMC-1 cells were co-cultured with human lung fibroblasts and airway smooth muscle cells in a collagen gel contraction assay. IL-33 effects on IgE-mediated activation were studied in primary mast cells and basophils.

Result IL-33 synergized with adenosine, C5a, SCF, and NGF receptor activation. IL-33-stimulated and SCF-stimulated HMC-1 cells demonstrated enhanced collagen gel contraction when cultured with fibroblasts or smooth muscle cells. IL-33 also synergized with IgE receptor activation of primary human mast cells and basophils.

Conclusion IL-33 amplifies inflammation in both IgE-independent and IgE-dependent responses.

Keywords ST2 signaling · HMC-1 · IgE receptor · Adenosine receptors · RTK signaling

Introduction

Interleukin (IL)-33 is a potent activator of immune cell types, inducing cytokine and chemokine production by mast cells, basophils, NK, iNKT, and T cells [1–4]; cell adhesion and survival of eosinophils and mast cells [5, 6]; migration of Th2 cells [7]; and the in vitro maturation of human mast cell progenitors [8]. IL-33 is primarily expressed by epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells [1], and expression is induced under inflammatory conditions [1, 9, 10]. Acting through the cell surface ST2 receptor in association with IL-RACp, IL-33 triggers activation of TRAF6, Myd88, and MAPK signaling pathways in responding cells [1, 11–13]. Cell surface ST2 promotes Th2 cytokine production, which is impaired following exposure to soluble ST2 protein, neutralizing ST2 antibodies, and in ST2-deficient mice [14–17]. This suggests a role for IL-33 in amplifying Th2 responses, which is supported by evidence of IL-33-driven Th2 activation in mouse models of allergic inflammation [18–20], immunity to nematodes [21], and hepatic fibrosis [22]. Importantly, IL-33 can induce lung inflammation and airway hyperresponsiveness in RAG2-deficient mice [23], suggesting that IL-33 may be a potent activator of antigen-independent pathways in disease. Activation of mast cells by IL-33 may contribute to its role in disease states, as has been shown in a collagen-induced arthritis model [24]. IL-33 enhanced cytokine production by primary human

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mast cells in response to IgE receptor cross-linking [6], but mast cells may also respond to IL-33 in an antigen- and IgE-independent manner [25]. For example, IL-33 was found to stimulate primary human mast cell cytokine production responses to PMA and TSLP [26].

To further explore effects of IL-33 on IgE-dependent and IgE-independent mast cell activation, we have examined responses to a range of stimuli in HMC-1 cells, primary human mast cells, and human peripheral blood basophils. Responses to SCF, adenosine analogs, and C5a were studied in the HMC-1 cell line. Although they lack the high affinity IgE receptor, HMC-1 cells serve well as a model for IgE-independent mast cell activation pathways [27–30]. They respond to the mast cell maturation factor, SCF, which induces receptor tyrosine kinase (RTK) c-kit signaling to mediate a range of mast cell responses, including cytokine and chemokine production [31]. HMC-1 cells also respond to adenosine, a potent bronchoconstrictive agent in asthmatics [32], through G protein-coupled adenosine receptors [33–37]. We also examined responses to the anaphylatoxin C5a, which potentiates cysLT production in human lung tissues [38], contributes to allergic inflammation [39], and triggers HMC-1 activation through the G-protein-coupled C5aR [40].

The effects of IL-33 on these IgE-independent responses were compared to effects on IgE-dependent activation of primary human mast cells and basophils. These studies allowed us to investigate the synergistic interactions of IL-33 with other mast cell and basophil activators. We confirmed the functional consequences of these interactions by using a model of collagen gel contraction in which fibroblast and smooth muscle responses are driven by mast cell activation.

Materials and methods

Cells and reagents

The human mast cell line, HMC-1, was obtained from J.H. Butterfield, M.D. (Mayo Clinic, Rochester, MN, USA) and cultured in Iscove's medium with 10% defined, iron-supplemented calf serum and 1.2 mM α -thioglycerol. IL-33 was purchased from Axxora (San Diego, CA), stem cell factor and NECA (5'-N-ethylcarboxamidoadenosine) from Sigma-Aldrich (St. Louis, MO, USA), human ST2-Fc from R&D Systems (Minneapolis, MN, USA) and Axxora, and IL-1 α from Chemicon (Temecula, CA, USA). Western blot supplies were from Bio-Rad (Hercules, CA, USA) and GE Healthcare (Piscataway, NJ, USA). All antibodies were from Cell Signaling Technology (Danvers, MA, USA), except for actin antibody from Santa Cruz Biotechnology

(Santa Cruz, CA, USA), and phospho-p38 antibody and U0126 from Calbiochem (La Jolla, CA, USA). Human IgE was obtained from Millipore (Billerica, MA, USA), anti-human IgE antibody from KPL (Gaithersburg, MD, USA), and anti-CD14 and anti-CD117 from BD (Franklin Lakes, NJ, USA). IL-6, CXCL8, CCL4, and CCL2 were quantitated using Cytosets (R&D Systems), IL-4 using ELISA kit (R&D Systems), and IL-13 using the ultra-sensitive ELISA kit (BioSource, Camarillo, CA, USA). Limits of detection were 5 pg/ml for IL-4, IL-6, and CXCL8; 25 pg/ml for CCL2; 8 pg/ml for CCL4; and 1.5 pg/ml for IL-13.

RT-PCR

RNA was treated with the RQ1 DNase system (Promega) and used to amplify total ST2 (ST2 common) with a generic primer set, or ST2L and sST2 with isoform transcript-specific primers, using AccessQuick RT-PCR kit from Promega. Primers were as follows: ST2 common (forward: 5'-CCA GCT GAA GTT GCT GAT TCT GGT A-3'/reverse: 5'-CCT TTT CCA AAA CAA GCA GAG CAA G-3', product size 500 bp), ST2L (forward: 5'-AGG CTT TTC TCT GTT TCC AGT AAT CGG-3'/reverse: 5'-GGC CTC AAT CCA GAA CAT TTT TAG GAT GAT AAC-3', product size 454 bp), and sST2 (forward: 5'-AGG CTT TTC TCT GTT TCC AGT AAT CGG-3'/reverse: 5'-CAG TGA CAC AGA GGG AGT TCA TAA AGT TAG A-3', product size 659 bp).

Quantitative RT-PCR

HMC-1 cells were stimulated as indicated for 4 h, followed by RNA isolation. IL-13 and CXCL8 mRNA were measured by TaqMan analysis using standard cycling conditions with 10 ng RNA/reaction, 600 nM forward and reverse primers, and 300 nM probe. B2M mRNA was measured for normalization. IL-13 forward: 5'-AAG GTC TCA GCT GGG CAG TTT-3'; IL-13 reverse: 5'-AAA CTGGGC CAC CTC GAT T-3'; IL-13 probe: 5'-CCA GCT TGC ATG TCC GAG ACA CCA-3'. CXCL8 forward: 5'-GGA AGA AAC CAC CGG AAG GA-3'; CXCL8 reverse: 5'-AGA GCC ACG GCC AGC TT-3'; CXCL8 probe: 5'-CCA TCT CAC TGT GTG TAA ACA TGA CTT-3'.

HMC-1 cell activation for cytokine and chemokine production and signaling studies

HMC-1 cells were pretreated for 15 min with ST2-Fc or control IgG-Fc, followed by 18 h stimulations as indicated, in 96-well plates containing 2×10^5 cells/well in serum-free medium. Culture supernatants were assayed for cytokines and chemokines.

For analysis of signaling pathways, HMC-1 cells were stimulated for 15 min as indicated and quenched by the addition of three volumes of ice-cold 1 mM sodium orthovanadate in PBS. Cells were washed in ice-cold PBS and lysed using modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM NaF) containing protease inhibitors (Roche) and Phosphatase Inhibitor Sets I and II (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined by Bradford assay (BioRad, Hercules, CA, USA) and equivalent protein lysates were separated by 10% Tris-Glycine SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA), transferred to nitrocellulose membranes, and analyzed by Western blot. Densitometry for quantifying phospho-protein signals was normalized to β -actin or total protein signals.

For NFAT-luciferase assays, HMC-1 cells were transiently transfected with pNFAT-Luc reporter construct (Stratagene, La Jolla, CA, USA) using Lipofectamine 2000 (Invitrogen) for 16 h (overnight) in T25 flasks containing 5×10^6 cells in 5 ml of antibiotic-free Iscove's medium. All transfections were set up in batch format in one flask, and 96-well plates were seeded with 2×10^5 cells/well of transfected cells prior to stimulation. For MEK1/2 inhibitor studies, 1 μM U0126 (0.02% DMSO in-assay concentration) was added to cells 30 min prior to stimulation. Cells were then stimulated overnight, lysed in Promega cell lysis buffer, and analyzed using the Luciferase Assay system from Promega.

Type I collagen gel contraction co-culture model

Human airway smooth muscle cells (HASM) were purchased from ScienCell Research Laboratories (San Diego, CA, USA) and maintained in a defined Smooth Muscle Growth Medium (SmGM) (Lonza). Confluent smooth muscle cells were serum-starved for 24 h in basal smooth muscle medium and treated with 5 ng/ml TGF- β for 2 days. Human fetal lung fibroblasts (HFL-1) were purchased from ATCC (Manassas, VA, USA) and were grown in DMEM containing 10% fetal bovine serum (Hyclone, Logan UT). Collagen lattices were prepared in 24-well plates by mixing neutralized bovine type I collagen (Organogenesis, Canton, MA, USA) with HASM or HFL-1 (2.5×10^5 cells/ml). HMC-1 (2.5×10^5 cells/ml) were added as indicated, and gels were solidified overnight at 37°C 10% CO_2 . Conditioned medium from HMC-1 cells stimulated for 24 h was added to the incubation medium as indicated. Polymerized gels were released into a 6-well plate with 3 ml of basal SmBm (HASM) or serum-free DMEM (HFL-1) and treated with 10 ng/ml IL-33 and 50 ng/ml SCF. Gels were photographed post-release, and the degree of collagen gel contraction was quantified by

measuring the area using LaserPix software (BioRad, Hercules, CA, USA). Data are shown on a scale of 40–100% of initial gel area.

Primary mast cell activation

To generate human primary mast cells, CD34-positive progenitors were purified from PBMC, as previously described [41]. Cells were cultured in Iscove's medium containing glutamine (Lonza, Walkersville, MD, USA), 100 ng/ml SCF (R&D Systems, Minneapolis, MN, USA), 3% 20 $\times$ concentrated conditioned medium generated from HCC2157 BL lymphoblastoid cell line (ATCC), and 10 pg/ml GM-CSF (R&D Systems) at 10^4 cells/ml. Culture media also contained 30% charcoal-treated fetal bovine serum, 50 $\mu\text{g/ml}$ iron-saturated holo-transferrin, 1 $\times$ penicillin-streptomycin, and 10^{-5} M β -mercaptoethanol from Sigma (St. Louis, MO, USA). At 6 weeks, the culture was depleted of macrophages by magnetic bead separation using CD14 antibody (Invitrogen, Carlsbad, CA, USA), and cell phenotype and purity were evaluated by Wright stain and flow cytometry for CD117 $^+$ /CD14 $^-$ cells. The purity of the primary mast cells was >95% Fc ϵ RI $^+$ /CD11b $^-$. Cells were sensitized overnight with 0.2 $\mu\text{g/ml}$ human IgE, then treated with 10 $\mu\text{g/ml}$ anti-human IgE (KPL, Gaithersburg, MD, USA) for 30 min at 37°C in the presence or absence of 10 ng/ml IL-33 for degranulation studies, in a 96-well plate at 10^4 cells/well in 200 μl serum-free Iscove's medium containing 0.005% human serum albumin (Sigma). Beta-hexosaminidase released into the supernatants was quantitated by incubation for 2 h at 37°C with an equal volume of 1.3 mg/ml nitrophenyl *N*-acetyl- β -D-glucosaminide (Sigma) in 0.08 M sodium citrate pH 4.5. The reaction was stopped with 1 M NaOH, and the absorbance was read at 405 nm. To determine the total cellular β -hexosaminidase (maximum release), cells were lysed in 0.5% Triton X-100. Leukotrienes C4/D4/E4 production was quantitated from same cell supernatants using CAST ELISA (Buhlmann) from ALPCO Diagnostics (Windham, NH, USA). For cytokine production, cells were sensitized overnight with 0.1 $\mu\text{g/ml}$ human IgE and then stimulated for 24 h with or without 10 $\mu\text{g/ml}$ anti-human IgE antibody and 10 ng/ml IL-33, alone or in combination, in the presence of 20 ng/ml SCF, at 5×10^4 cells/well in a 96-well plate.

Basophil histamine release and cytokine production assays

Blood from healthy subjects was drawn into sodium heparin tubes. Collection and usage of blood from human donors were in accordance with the regulations of Wyeth Research. Basophils were enriched in the granulocyte fraction following sedimentation of red blood cells and

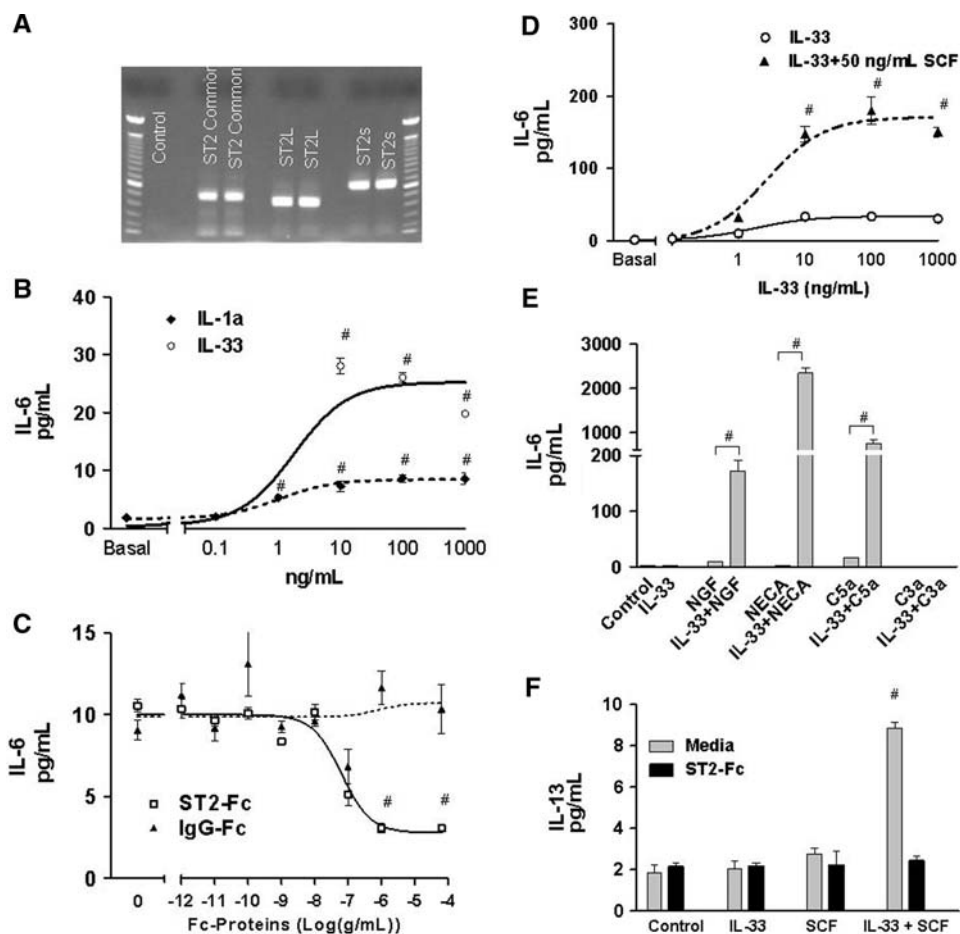
platelets with 4.5% dextran (ICN, Irvine, CA, USA) in 0.9% saline in 10 mM HEPES, pH 7.4. Purity averaged around 80% by IgE Receptor+/CD203c+/IL-3R+ staining by FACS. Cells were washed into PACM buffer (25 mM PIPES, pH 7.2, 110 mM NaCl, 5 mM CaCl₂, 2.5 mM MgCl₂) with 0.005% human serum albumin (Sigma), and treated with 10 ng/ml IL-3 for 10 min at 37°C. Cells were challenged with 1 µg/ml anti-human IgE, 10 ng/ml IL-33, or both for 30 min in 96-well plates at 37°C. Cell culture supernatants were analyzed by histamine ELISA (Beckman Coulter, Marseille, France). Background histamine release was determined by challenging cells with PACM. Total histamine release was determined by lysis of cells with 0.1% Triton X-100. Histamine concentration was converted to % maximum release using the equation: (sample – background)/(total – background) × 100. For cytokine production, basophils were purified from buffy coats obtained from normal healthy donors (Massachusetts General Hospital, Boston, MA, USA) using the Basophil Isolation Kit (Miltenyi). Cells were stimulated in RPMI with 10% serum for 24 h with 1 µg/ml anti-human IgE antibody or 10 ng/ml IL-33, or in combination, at

5 × 10⁴ cells/well in a 96-well plate, and cytokine production in culture supernatant was measured.

Experimental replicates and statistical analysis

All cytokine production experiments were set up to include three to eight wells per condition, and each experiment was independently repeated a minimum of three times with exceptions stated below; representative experiments are shown. Western blot studies were done three times for each, except for pJNK, which was done two times, and representative data are shown. U0126 inhibition of CXCL8 production and phosphorylation of c-jun were done two times each. For primary mast cell studies, two donors were used to generate mast cells from PBMCs. For basophil histamine release experiments, four independent experiments with four donors were done. For basophil cytokine production, two independent experiments from two donors were done. Data are presented as mean ± SEM for Figs. 1, 2, and 4. In Figs. 5 and 6 data are presented as mean ± SD. The *t*-test results below 0.05 are considered significant (**p* < 0.05; ***p* < 0.01; #*p* < 0.005).

Fig. 1 HMC-1 cells express ST2, and ST2 signaling synergizes with SCF and pro-inflammatory mediators to induce IL-6 and IL-13 production. **a** ST2L and sST2 mRNA expression analysis in HMC-1 cells using RT-PCR with either common ST2 primers or isoform-specific primers. IL-6 concentrations were measured as described in the “Materials and methods” section after HMC-1 cell stimulations as follows: **b** increasing concentrations of IL-33 or IL-1α (from 0.1 to 1,000 ng/ml), **c** 10 ng/ml IL-33 and ST2-Fc or human IgG control, **d** IL-33 in the presence or absence of 50 ng/ml SCF, **e** 10 ng/ml IL-33 with 50 ng/ml NGF, 1 µM NECA, 10 nM C5a, or 10 nM C3a. IL-13 was measured after stimulations as follows: **f** 10 ng/ml IL-33 and/or 50 ng/ml SCF in the presence or absence of 1 µg/ml ST2-Fc. Statistical significance (#*p* < 0.005) was calculated by *t* tests comparing induced to uninduced (**b**), ST2-Fc to IgG-Fc (**c**), IL-33 to IL-33 with SCF (**d**), and either mediator alone to two mediators (**e**, **f**)



Results

IL-33 amplifies mast cell cytokine and chemokine production

We examined the IL-33 receptor ST2 expression in HMC-1 cells by RT-PCR and found that the alternatively spliced membrane-associated long-form ST2L and soluble-form sST2 [42] are both expressed (Fig. 1a). We next investigated IL-6 and IL-13 production in response to IL-33 stimulation. We found that IL-33 induced low amounts of IL-6 production in a dose-dependent manner and was somewhat more potent than IL-1 α for IL-6 production (Fig. 1b). IL-33-induced IL-6 production was ST2-dependent, as it was blocked in the presence of ST2-Fc (Fig. 1c). SCF alone did not elicit IL-6 production, but IL-33 synergized with SCF to generate eightfold to tenfold higher IL-6 concentration over that produced in response to IL-33 alone (Fig. 1d). In addition to SCF, IL-33 amplified IL-6 production together with mast cell-activating agents, NECA (5'-N-ethylcarboxamidoadenosine), a pan-selective adenosine receptor agonist [33–37], C5a [43], and NGF [27], but not with C3a (Fig. 1e). C3a has been shown to activate HMC-1 cells [44] but did not lead to IL-6 production, despite inducing calcium signaling (data not shown). These results suggest that distinct GPCR-activating pathways may play unique inflammatory roles in mast cells.

As was seen for IL-6 production, IL-33 synergized effectively with the RTK activator SCF to induce IL-13 production (Fig. 1f) and IL-13 mRNA expression (data not shown). IL-33 also synergized with adenosine receptor

activators C5a and NECA to induce IL-13 (data not shown). For both IL-6 and IL-13 production, the combination of IL-33 with C5a or NECA induced relatively more cytokine than the combination of IL-33 and SCF or NGF, suggesting a stronger amplification of cytokine production in the presence of GPCR activators as compared to RTK activators.

We next examined IL-33 effects on chemokine production in response to either RTK activation with SCF or GPCR activation with NECA. Both SCF (Fig. 2a) and NECA (Fig. 2b) synergized with IL-33 to induce CXCL8 (IL-8) and CCL4 (MIP-1 β). The IL-33 synergistic responses were dependent on ST2, as shown by ST2-Fc-specific blockade of the amplified response (Fig. 2a, b). A concomitant enhancement of CXCL8 mRNA expression was confirmed (data not shown). Again the chemokine production response to the combination of IL-33 and NECA was of higher magnitude than that induced by the combination of IL-33 and SCF (note scale of Fig. 2a versus b). This observation also held true for IL-33 synergy with C5a, in comparison to IL-33 with NGF (Fig. 2c). Collectively, these results suggest a stronger amplification of signaling from ST2-GPCR co-activation than ST2-RTK co-activation.

IL-33 synergies enhance JNK and ERK activation

We further explored the signaling basis underlying the IL-33-induced synergies in HMC-1 cells by investigating MAPK activation. A time course study was performed to identify the optimal time point for MAPK activation downstream of IL-33 stimulation, which was found to peak

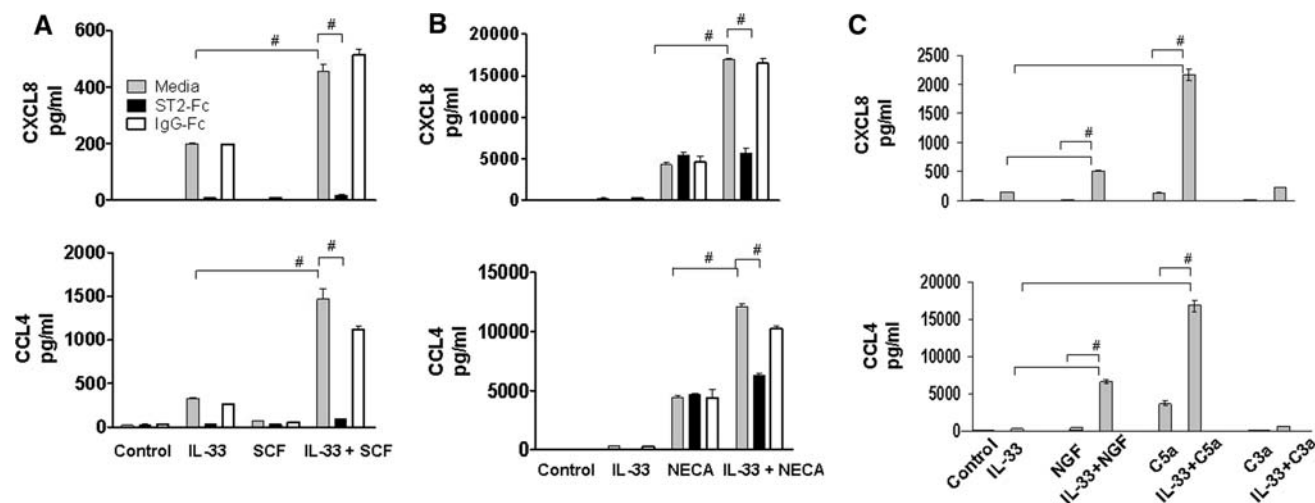


Fig. 2 IL-33 synergizes with RTK and GPCR signaling to induce chemokine production. HMC-1 cells were stimulated using the mediator concentrations described in Fig. 1 unless otherwise stated: **a** IL-33 and SCF alone or in combination. **b** IL-33 and NECA alone or in combination. **c** IL-33 in combination with either 50 ng/ml NGF,

10 nM C5a, or 10 nM C3a. Cell supernatants were collected and analyzed for CXCL-8 and CCL4 content by ELISA. Statistical significance ($\#p < 0.005$) was determined by *t*-tests comparing ST2-Fc to IgG control (**a**, **b**), IL-33 alone to two mediators (**a**, **c**), and NECA alone to IL-33 with NECA (**b**)

at 15 min (data not shown), as demonstrated previously [1]. The IL-33-induced p38 phosphorylation was not modulated by either NECA or SCF co-stimulation (Fig. 3a), whereas both SCF and NECA enhanced IL-33-mediated JNK phosphorylation (Fig. 3b). Neither SCF nor NECA alone significantly activated either p38 or JNK (Fig. 3a, b). In contrast, IL-33, SCF, and NECA each induced ERK phosphorylation, with NECA producing the strongest response. The combination of IL-33 with either SCF or NECA resulted in an enhancement of ERK phosphorylation relative to either mediator alone (Fig. 3c). Similar results for ERK activation were observed with IL-33 and C5a co-activation (data not shown).

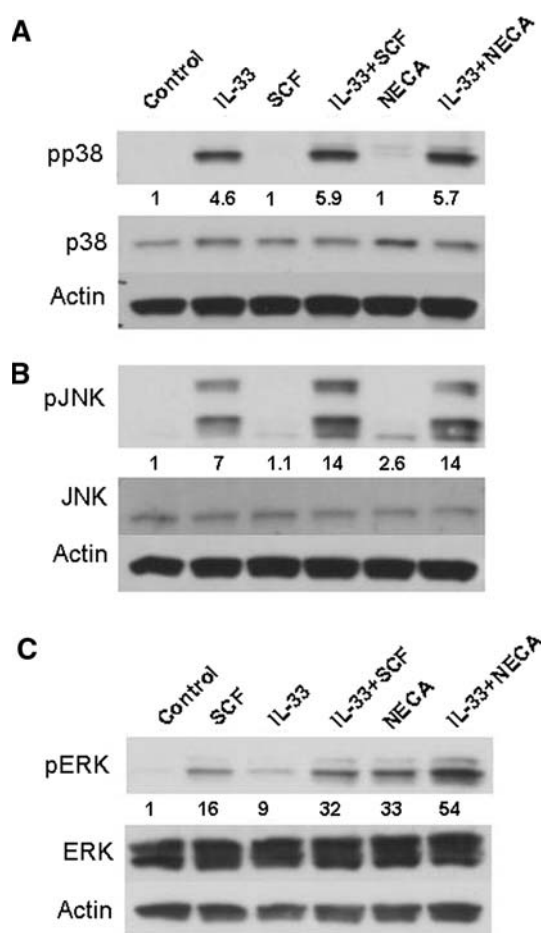


Fig. 3 Adenosine receptor and c-kit signaling enhances ST2-induced MAPK activation. Lysates from HMC-1 cells, stimulated in the activation conditions as stated in Fig. 1 where indicated, were analyzed using Western blots probed with pp38 (a), pJNK (b), or pERK (c) antibodies. Blots were washed and re-probed for total p38, JNK, and ERK. Actin blots verify equal protein loading. Quantitation from densitometric analysis for phospho-protein signals was normalized by cell numbers seeded. Fold change over control conditions is indicated for representative experiments as stated in the “Materials and methods” section

IL-33 and adenosine receptor co-stimulation induces an ERK-dependent NFAT response

Cytokine production by mast cells in response to NGF [27] and adenosine receptor activation [33] has been shown previously to involve NFAT signaling. Therefore, we asked if IL-33 modulates NFAT activation in synergy with GPCR or RTK activators. IL-33 alone did not induce NFAT activation and did not affect NFAT activation downstream of the RTK activator, SCF (Fig. 4a). However, IL-33-enhanced NECA induced NFAT activation in an ST2-dependent manner (Fig. 4a). We also compared NFAT activation in response to IL-33 together with NGF or C5a or C3a, and found synergistic NFAT activation only for IL-33 with C5a (data not shown).

ERK-mediated effects on the NFAT transcription complex have been described previously [45–47], so we utilized the MEK kinase inhibitor U0126 to assess if the synergistic NFAT activation in response to IL-33 and NECA was sensitive to ERK blockade. While the NECA-induced NFAT activation signal was partially dependent on ERK signaling, the IL-33 synergy was abolished in the presence of U0126 (Fig. 4b). Thus, IL-33 enhanced NECA-induced NFAT activation by an ERK-dependent mechanism. Because IL-33 did not directly induce calcium signaling in these cells (data not shown), its potentiation of NECA-induced NFAT activity most likely occurred by a calcium-independent pathway. Thus, the synergy between IL-33 and GPCR activators leading to enhanced NFAT signaling may involve cross-talk with MAPK.

Because ERK activation was induced by IL-33, NECA, and SCF (Fig. 3c) and IL-33 synergized with either NECA or SCF to induce CXCL8 production (Fig. 2a, b), we asked if U0126 affected CXCL8 production by HMC-1 cells. We found that U0126 significantly reduced CXCL8 production induced by either IL-33 alone, NECA alone, IL-33 and NECA, or IL-33 and SCF (Fig. 4c). Consistent with IL-33 being the least effective ERK activator of the three mediators (Fig. 3c), the inhibition of IL-33-induced CXCL8 was the most modest, at 26% inhibition. IL-33 synergy with SCF was reduced by U0126 inhibition to the amount of signal with IL-33 alone, and NECA synergy with IL-33 was reduced to that of NECA alone. These observations are consistent with functional involvement of ERK activation in the synergistic induction of chemokine production by IL-33 and NECA or IL-33 and SCF.

IL-33 and SCF co-stimulation of HMC-1 mast cells induces contraction of collagen gels embedded with fibroblasts or smooth muscle cells

Activated HMC-1 cells are able to drive contraction of collagen gels co-embedded with human lung fibroblasts

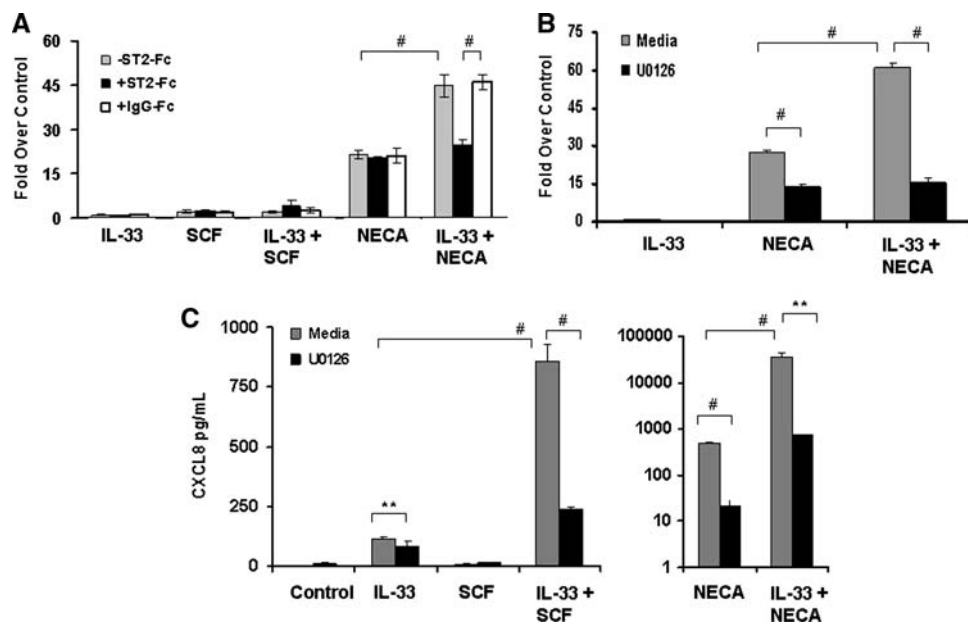


Fig. 4 IL-33 amplifies GPCR-induced NFAT activation. HMC-1 cells transfected with pNFAT-Luc reporter construct were re-seeded as described in the “Materials and methods” section, and stimulated in the activation conditions as stated in Fig. 1, after which cell lysates were assayed for NFAT activation (relative fluorescent units) and reported as fold change over control: **a** IL-33, SCF, and NECA, in the presence of either ST2-Fc or IgG-Fc. **b** IL-33 and NECA in the

presence or absence of 1 μ M U0126. **c** HMC-1 cells were stimulated using the mediator concentrations described in Fig. 1 in the presence or absence of 1 μ M U0126, and chemokine production was measured as described for Fig. 2. Statistical significance (** $p < 0.01$, # $p < 0.005$) was calculated by *t*-tests comparing ST2-Fc to control (**a**), two mediators to one mediator alone (**a–c**), and inhibitor to control (**b, c**)

(HFL-1) [41] or human airway smooth muscle cells (HASM) [48], as a model for remodeling changes associated with asthma. To examine functional consequences of the synergistic activation of HMC-1 by IL-33 and SCF, HMC-1 cells were co-embedded in collagen gels with either HFL-1 or HASM, and contractile responses were measured as a decrease in gel surface area. SCF treatment resulted in gel contraction in this model (Fig. 5a), but NECA did not (data not shown). The combination of IL-33 and SCF produced a greater degree of contraction than either mediator alone, both for gels embedded with HMC-1 and HASM (Fig. 5a), and those embedded with HMC-1 and HFL-1 (Fig. 5b). The gel contraction activity could be transferred in the conditioned medium of HMC-1 cells treated with IL-33 and SCF (Fig. 5a, b), confirming that the response was a consequence of mast cell activation and indicating that soluble agents released by the activated mast cells mediated the contraction in this model.

IL-33 enhances IgE receptor-mediated activation in primary mast cells and basophils

To address effects of IL-33 on IgE-dependent mast cell responses, primary human mast cells were derived from progenitors in peripheral blood, as described [41]. These cells produced low concentrations of CXCL8 in response to either IL-33 or anti-IgE. The combination of IL-33 and IgE

receptor cross-linking synergized to drive increased production of CXCL8 (Fig. 6a). Similarly, IL-33 augmented IgE-mediated degranulation (Fig. 6b) and leukotriene synthesis (Fig. 6c) by primary human mast cells.

In addition to mast cells, human basophils express ST2 and produce cytokines in response to IL-33 stimulation [2, 3, 4]. We found that IL-33 significantly enhanced IgE receptor-mediated degranulation of basophils in peripheral blood (Fig. 6d). For analysis of cytokine production, basophils were enriched to 80% purity from peripheral blood by negative selection, as described in the “Materials and methods” section. Cells were stimulated for 24 h at 37°C with anti-IgE in the presence or absence of IL-33, and supernatants were assayed for IL-4 and IL-13. Previous studies have shown that under conditions of short-term stimulation with anti-IgE, basophils and not T-cells are the major IL-4-producing cell type in PBMC [49]. IL-33 significantly enhanced production of both IL-4 and IL-13 in response to IgE receptor cross-linking (Fig. 6e, f).

Discussion

In asthma and other inflammatory disease states, both infiltrating leukocytes and tissue-resident cells, including fibroblasts, smooth muscle cells, and endothelia, undergo coordinated activation, with release of cytokines and

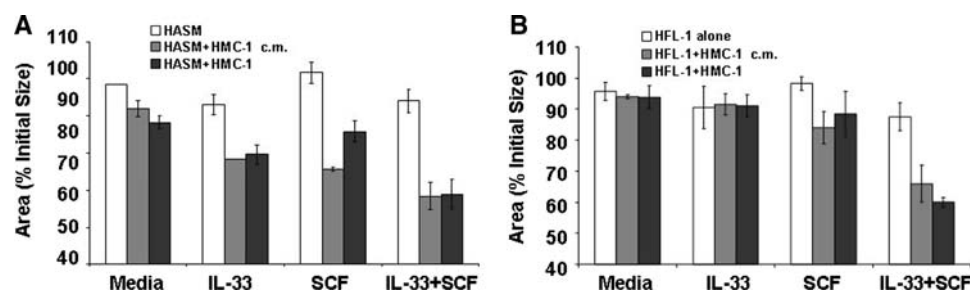


Fig. 5 HMC-1 cells stimulated with IL-33 and SCF induce HASM cell and HFL-1 cell contraction in collagen gels. Three-dimensional collagen gel lattices were generated with **a** HASM or **b** HFL-1 in the presence (black bars) or absence (white bars) of HMC-1 cells stimulated as described in the “Materials and methods” section. Gels containing HASM or HFL-1 cells were also incubated with conditioned medium from resting or 24-h-stimulated HMC-1 cells (grey bars). Gel surface area was measured and expressed as a percentage

of the initial area, pre-incubation. For gels co-embedded with HASM or HFL-1 and HMC-1 cells, contraction induced by the combination of IL-33 and SCF was significantly greater than the contraction induced by either agent alone ($*p < 0.05$). For conditioned media, combination treatment produced significantly greater contraction than either agent alone for HFL-1 cells ($*p < 0.05$) but not for HASM ($p = 0.06$)

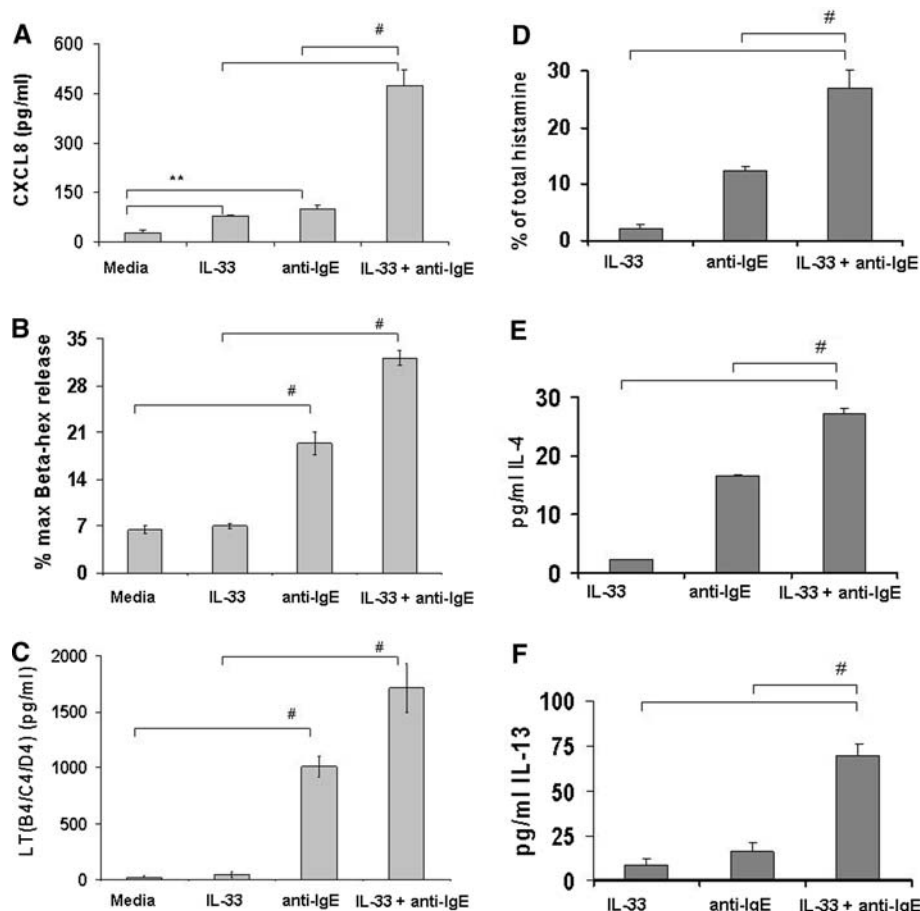


Fig. 6 IL-33 potentiates IgE-mediated activation of primary human mast cells and basophils. **a** CXCL8 production from mast cells was measured after 24 h stimulation with 10 μ g/ml anti-human IgE antibody and 10 ng/ml IL-33. Statistical significance ($**p < 0.01$, $\#p < 0.005$) was calculated by *t*-tests comparing with and without IgE cross-linking in each of the conditions shown, as well as with and without IL-33. **b** β -hexosaminidase release and **c** leukotriene (C4/D4/E4) production were assayed after 30-min incubation with 0.03 μ g/ml anti-human IgE antibody and 10 ng/ml IL-33. Statistical significance

($**p < 0.01$, $\#p < 0.005$) was calculated by *t*-tests comparing media to IgE-mediated, and IL-33 to IL-33 with IgE receptor cross-linking. **d** Histamine release, **e** IL-4 production, and **f** IL-13 production from human peripheral blood basophils were assayed with 1 μ g/ml anti-human IgE antibody and 10 ng/ml IL-33, as described in the “Materials and methods” section. Statistical significance ($\#p < 0.005$) was calculated by *t*-tests comparing both mediators to either mediator alone

chemokines. Under these conditions, cross-talk among secreted mediators likely contributes to escalation of inflammation. IL-33 is produced by fibroblasts and other tissue-resident cell types and interacts with the cell surface receptor complex consisting of ST2 and IL-1RAcP to induce activation of Th2 cells, mast cells, eosinophils, and basophils [4, 50–52]. The alternatively spliced receptor isoform sST2 is found in elevated concentrations in serum from patients with inflammatory and cardiovascular diseases, in particular in acute asthma in children as well as in exacerbations in adults [53, 54]. In a mouse model of allergic inflammation, sST2 blocks the effects of IL-33 administration [55]. Therefore, sST2 may be a decoy receptor for the IL-33 signaling complex. In addition, the single Ig IL-1 receptor-related molecule (SIGIRR/Toll IL-1R8) may also act to modulate the IL-33 response through an interaction with cell surface ST2 [56]. IL-33 has emerged as a key regulatory cytokine in promoting tissue inflammation. Neutralization of IL-33 and ST2 ameliorates inflammation in a mouse model of asthma [18]. Conversely, administration of IL-33 exacerbates disease in a mouse model of arthritis [24] and induces lung hyperplasia and airway hyperresponsiveness in the absence of an adaptive immune system [23].

We propose that a major role of IL-33 in driving tissue inflammation may be to synergize with other activation signals to amplify immune activation in asthma and other disease states. Mast cells are likely targets for IL-33 responses *in vivo*. They express very high levels of ST2 and can be directly activated by IL-33 to produce cytokines, including IL-13 and CXCL8, which are critical mediators of pathology in asthma [57, 58]. Our findings using the HMC-1 cell line, primary human mast cells, and peripheral blood basophils show that in addition to its direct activity, IL-33 synergizes with IgE-dependent and IgE-independent mast cell activation signals.

HMC-1 cells are a well studied model for IgE-independent mast cell activation pathways [27, 30, 33, 34, 36, 37]. We now demonstrate their expression of ST2, including the ST2L membrane form, and sST2. In response to IL-33 alone, HMC-1 cells produce low concentrations of cytokines and chemokines. When combined with GPCR activators or RTK ligands, however, IL-33 induced a striking and potent synergistic response in HMC-1 cells. In general, the synergy with GPCR activators, including NECA and C5a, was more pronounced than that with RTK ligands such as SCF and NGF. Given the amplification of IL-33-induced responses resulting from the above described synergies, sST2 may be a mechanism for mast cells to limit the scope of this response by IL-33 binding, thereby providing a mechanism to prevent excessive immune activation.

To address the mechanism for these synergistic responses, we examined activation of signaling pathways downstream of IL-33, SCF, C5a, and NECA stimulation in HMC-1 cells. IL-33 alone induced activation of p38, JNK, and ERK. The GPCR agonists NECA and C5a also resulted in ERK phosphorylation and effectively induced NFAT activation and CXCL8 production. IL-33 synergistically amplified all of these responses. A role for NFAT activation in mast cell cytokine production in response to GPCR activation has been reported previously [33, 59, 60], and the current findings suggest cross-talk downstream of ST2 and adenosine receptor signaling pathways, resulting in potentiation of the response. Interestingly, the synergistic NFAT activation was sensitive to ERK inhibition, as has been described downstream of TCR activation, and for fibroblasts treated with mitogen [45–47]. The RTK agonist SCF also induced ERK activation in HMC-1 cells and synergized with IL-33 to drive CXCL8 production. Unlike the GPCR agonists, however, neither SCF nor NGF triggered NFAT activation, either alone or in combination with IL-33. While the potent induction of CXCL8 seen with the combination of IL-33 and SCF did not involve NFAT, it was associated with a synergistic induction in phosphorylation of both JNK and ERK and was blocked by the MEK inhibitor U0126. Thus, ERK was a key mediator of IL-33 synergies with either SCF or NECA, leading to enhanced production of CXCL8. Because MEK inhibition with U0126 reduced chemokine levels to those seen with IL-33 or NECA alone, rather than resulting in complete blockade, an ERK-independent pathway may also contribute to the synergy. In addition to signaling cross-talk, mechanisms such as cross-regulation of receptor expression could contribute to synergies between IL-33 and GPCR agonists or RTK ligands.

In these studies, primary mast cells were used to study IgE-dependent responses, while IgE-independent responses, including those to SCF, were examined in HMC-1 cells. Unlike primary mast cells, HMC-1 cells do not require SCF for their survival and are maintained in the absence of this growth factor. Although HMC-1 have a mutation in *c-kit* resulting in ligand-independent activation [61], they nevertheless respond to SCF by production of cytokines and chemokines [62, 63]. IL-33 was found to synergize with SCF and other HMC-1 activators, leading to enhanced cytokine and chemokine production. An additional effector activity could be seen in a model of collagen gel contraction. Gels co-embedded with HMC-1 and human airway smooth muscle cells or fibroblasts underwent contraction in response to SCF, and this response was enhanced by addition of IL-33. Within the asthmatic lung, mast cells infiltrate the smooth muscle layer [64, 65] and contribute to smooth muscle hypertrophy, extracellular matrix deposition, and hyper-contractility, all of which

underlie airway remodeling [66]. Our findings suggest that IL-33, derived from fibroblasts or smooth muscle cells under pro-inflammatory conditions, may synergize with fibroblast-derived SCF to exacerbate changes associated with airway remodeling.

HMC-1 have been very well characterized as a model for human mast cell activation in numerous studies but represent an immature phenotype due to the lack of appreciable IgE receptor expression [67]. In addition to the fact that they are primary cells, we considered one advantage of blood-derived mast cells to be their responsiveness to IgE cross-linking. The demonstration that primary human mast cells, activated through IgE receptor cross-linking, showed enhanced chemokine production, degranulation, and leukotriene release in the presence of IL-33 confirmed the role of IL-33 as a synergistic activator of mast cell effector responses. IL-33 synergy with IgE receptor signaling, resulting in enhanced CXCL8 and IL-13 production, has recently also been reported for cord-blood-derived human mast cells [6]. However, in contrast to our findings, this study found no effect of IL-33 on IgE-mediated degranulation or leukotriene synthesis. These differences may be a consequence of the source of primary mast cells derived from peripheral blood in our study, as opposed to those derived from cord blood. The relevance of these observations to asthma would be strengthened by analysis of IL-33 effects on human lung mast cells, which was beyond the scope of the current study. Compared to human mast cells derived from skin, cord blood, or peripheral blood, lung mast cells express a distinct panel of chemokine receptors [68], which direct their infiltration into the airway smooth muscle layer [69, 70]. Given that IL-33 is highly expressed by bronchial smooth muscle [1], and the current demonstration that IL-33 synergistically drives chemokine production by mast cells, infiltration of airway smooth muscle by even a small number of activated mast cells may result in an amplification cascade of IL-33-driven chemokine production, leading to further infiltration of mast cells.

In addition to mast cells, basophil numbers are increased in asthmatic airways, and there is evidence supporting basophil degranulation in asthma [71]. We found that IL-33 also synergized with IgE receptor cross-linking to drive basophil activation responses, including degranulation and cytokine synthesis, in agreement with recent findings [3, 4].

In conclusion, our findings demonstrate that IL-33 has the capacity to synergistically activate both IgE-dependent and IgE-independent mast cell and basophil responses, leading to changes that are associated with lung remodeling. These include release of histamine and leukotrienes, production of cytokines, and the induction of fibroblast and smooth muscle cell contractile responses. The demonstration that release of mast cell-derived mediators can be

enhanced in the presence of IL-33 suggests that therapeutic modulation of IL-33 may be a promising strategy for treatment of atopic disease.

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