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IL-33 Mediates Inflammatory Responses in Human Lung Tissue Cells

Akiko Yagami,*,†,1 Kanami Orihara,*,1 Hideaki Morita,*,‡ Kyoko Futamura,*,§ Noriko Hashimoto,* Kenji Matsumoto,* Hirohisa Saito,* and Akio Matsuda*

IL-33 is a member of the IL-1 family and mediates its biological effects via the ST2 receptor, which is selectively expressed on Th2 cells and mast cells. Although polymorphic variation in *ST2* is strongly associated with asthma, it is currently unclear whether IL-33 acts directly on lung tissue cells at sites of airway remodeling. Therefore, we aimed to identify the IL-33–responsive cells among primary human lung tissue cells. ST2 mRNA was expressed in both endothelial and epithelial cells but not in fibroblasts or smooth muscle cells. Correspondingly, IL-33 promoted IL-8 production by both endothelial and epithelial cells but not by fibroblasts or smooth muscle cells. Transfection of ST2 small interference RNA into both endothelial and epithelial cells significantly reduced the IL-33–dependent upregulation of IL-8, suggesting that IL-33–mediated responses in these cells occur via the ST2 receptor. Importantly, Th2 cytokines, such as IL-4, further enhanced ST2 expression and function in both endothelial and epithelial cells. The IL-33–mediated production of IL-8 by epithelial cells was almost completely suppressed by corticosteroid treatment. In contrast, the effect of corticosteroid treatment on the IL-33–mediated responses of endothelial cells was only partial. IL-33 induced activation of both ERK and p38 MAPK in endothelial cells but only ERK in epithelial cells. p38 MAPK was required for the IL-33–mediated responses of endothelial cells, whereas ERK was required for IL-33–mediated IL-8 production by epithelial cells. Taken together, these findings suggest that IL-33–mediated inflammatory responses of lung tissue cells may be involved in the chronic allergic inflammation of the asthmatic airway. ***The Journal of Immunology*, 2010, 185: 5743–5750.**

Interleukin-33 is a newly identified member of the IL-1 family that is a ligand for the orphan IL-1 family receptor ST2 (also called IL1RL1, DER4, Fit-1, or T1) (1). Over the past decade, numerous studies established that the ST2 receptor is a selective marker on both murine and human Th2 cells (2). Recent studies have demonstrated that ST2 is also expressed on mast cells (3, 4), eosinophils (5, 6), and basophils (7), but not on Th1 cells or neutrophils. IL-33 potently drives the production of proinflammatory Th2-associated cytokines, including IL-4, IL-5, and IL-13, by in vitro polarized Th2 cells (1), mast cells (3, 4, 8), and basophils (9). These hematopoietic cells also produce other inflammatory cytokines and chemokines, including IL-6 and IL-8, via IL-33 stimulation (3, 4, 6, 8, 9). More recently, Allakhverdi et al. (10) demonstrated that circulating $CD34^+$ hematopoietic progenitor cells expressed ST2 and responded to IL-33 by rapidly releasing high levels of Th2-associated cytokines. Furthermore, IL-33 not only drives the production of cytokines/chemokines by various hematopoietic cells but also directly activates eosinophils (5, 6), basophils (7), and dendritic cells (11). These activities suggest potential roles for IL-33 in Th2-associated immune responses, and thus IL-33 is thought to be closely associated with allergic inflammatory diseases, including asthma.

Indeed, a very recent article reported increased IL-33 levels in the bronchoalveolar lavage fluid from subjects with moderate asthma compared with that in mild asthmatics and controls without asthma (12). The same group also reported that bronchial epithelium (12) and airway smooth muscle cells (13) from asthmatics expressed elevated levels of IL-33 compared with that in healthy controls. Furthermore, a recent genome-wide association study showed that a single-nucleotide polymorphism in *ST2/IL1RL1* was most strongly associated with asthma in a collection of 10 different populations (14). A single-nucleotide polymorphism in *IL-33* that showed a suggestive association with the circulating eosinophil count was also significantly associated with atopic asthma (14). These findings further support the pathophysiological relevance of the IL-33/ST2 pathway to asthma.

Lung tissue cells as well as a number of inflammatory cells are known to participate in airway inflammatory responses and play important roles in the pathogenesis of asthma. Chronic inflammation in the lung leads to persistent structural alterations in the airway wall (i.e., airway remodeling), which is thought to cause irreversible airflow obstruction and exacerbation of asthma (15). Airway remodeling consists of several structural alterations, such as goblet cell hyperplasia, subepithelial fibrosis, smooth muscle cell hypertrophy/hyperplasia, and angiogenesis in the lung (15).

However, it is currently unclear whether IL-33, a pro-Th2 cytokine, acts directly on lung tissue cells at sites where airway remodeling occurs. We therefore designed this study to identify IL-33–responsive cells among human lung tissue cells and found

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Abbreviations used in this paper: BSMC, bronchial smooth muscle cell; FP, fluticasone propionate; HCAEC, human coronary artery endothelial cell; HMVEC-LBl, human microvascular endothelial cells from lung blood vessels; NHBE, normal human bronchial epithelial cell; NHLF, normal human lung fibroblast; siRNA, small interference RNA; sST2, soluble ST2; ST2L, membrane-bound ST2.

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that IL-33 acts directly on pulmonary microvascular endothelial cells and epithelial cells, but not on smooth muscle cells or fibroblasts, via the ST2 receptor. More importantly, we found that Th2 cytokines, such as IL-4 and IL-13, significantly enhanced ST2 expression and function in both endothelial and epithelial cells. These findings suggest that IL-33–mediated inflammatory responses in lung tissue cells may be crucially involved in the chronic allergic inflammation of the asthmatic airway.

Materials and Methods

Reagents

Recombinant human IL-33 was purchased from PeproTech (Rocky Hill, NJ). Recombinant human ST2-Fc chimera was purchased from R&D Systems (Minneapolis, MN). PD98059 and SB202190 were purchased from Calbiochem (La Jolla, CA). Fluticasone propionate was purchased from Sigma (St. Louis, MO).

Primary human cell culture, treatment, and transfection

Normal human bronchial epithelial cells (NHBEs), normal human lung fibroblasts (NHLFs), bronchial smooth muscle cells (BSMCs), human microvascular endothelial cells from lung blood vessels (HMVEC-LBl), neonatal normal human epidermal keratinocytes, normal human dermal fibroblasts, normal HUVECs, and normal human coronary artery endothelial cells (HCAECs) were purchased from Lonza (Walkersville, MD) and maintained exactly as recommended by the manufacturer. NHBEs were cultured in flasks or plates coated with type I collagen (Iwaki, Tokyo, Japan). All the experiments described in this study were performed using second- or third-passage cells in 70–80% confluent monolayers unless otherwise noted.

All the cells were treated with different concentrations of IL-33 for up to 24 h or with 10 ng/ml IL-4 for up to 48 h. In some experiments, NHBEs and HMVEC-LBl were treated with different concentrations of PD98059 or SB202190 for 30 min prior to stimulation with IL-33 (Fig. 7).

Both the SAGM BulletKit and EGM-2MV BulletKit (Lonza), which are optimized for use with NHBEs and HMVEC-LBl, respectively, contain hydrocortisone. Therefore, experiments examining the effects of fluticasone propionate (Fig. 5) were performed after hydrocortisone deprivation for 24 h, as previously described (16). All other experiments described in this study were performed using a complete medium suited for each type of cell (Lonza).

NHBEs and HMVEC-LBl were seeded at 5×10^4 cells/well in 12-well culture plates and cultured until the cells reached 50–60% confluence. Then, the cells were transfected with small interference RNA (siRNA) against ST2 (No. SI00114618; Qiagen, Valencia, CA), STAT6 (No. SI02662905; Qiagen), or nontargeting control siRNA (No. 1027281; Qiagen) at 5 nM (NHBEs) or 10 nM (HMVEC-LBl) using HiPerFect transfection reagent (Qiagen) in accordance with the manufacturer's instructions. The transfected cells were further grown for 48 h and then stimulated with the indicated cytokine(s).

Quantitative real-time PCR

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as previously described (16, 17). Primer sets for six genes were synthesized at Fasmac (Kanagawa, Japan): ST2L (sense, 5′-CTGTCTGGCCCTGAATTTGC-3′; antisense, 5′-AGCAGAGTGGCCTCAATCCA-3′), sST2 (sense, 5′- CTGTCTGGCCCTGAATTTGC-3′; antisense, 5′-TGGAACCACACTCCATTCTGC-3′), IL-8 (sense, 5′-GTCTGCTAGCCAGGATCCACAA-3′; antisense, 5′-GAGAAACCAAGGCACAGTGGAA-3′), IL-6 (sense, 5′- CAATAACCACCCCTgACCCA-3′; antisense, 5′-GCGCAGAATGAGATGAGTTGTC-3′), STAT6 (sense, 5′-TCTGACCGGCTGATCATTGG-3′; antisense, 5′-CCAATCTCTGAGTCGCTGAAGC-3′), and β-actin (sense, CCCAGCCATGTACGTTGCTAT-3′; antisense, 5′-TCACCGGAGTCCATCACGAT-3′). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of ST2L, soluble ST2 (sST2), IL-8, IL-6, STAT6, and β-actin were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each real-time PCR. The mRNA expression levels were normalized to the β-actin level in each sample.

ELISA

The concentrations of the sST2, IL-8, IL-6, and MCP-1 proteins in cell-free supernatants were measured with specific ELISA kits (R&D Systems) in accordance with the manufacturer's instructions.

Western blotting

Cells were seeded into 6-well plates at 1×10^5 cells/well and cultured until subconfluent (2 or 3 d). The cells were then treated for the indicated time periods with 10 ng/ml IL-4 (for ST2 blotting, see Fig. 3*D*) or 10 ng/ml IL-33 (for phospho-MAPK blotting, see Fig. 6). Whole-cell lysates were extracted with 200 μl NuPAGE sample buffer (Invitrogen, Carlsbad, CA) containing 5% 2-ME and lysed by sonication. Equal amounts of whole-cell lysates were separated by SDS-PAGE (5–15% Ready Gels J; Bio-Rad, Hercules, CA) gel electrophoresis and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Immunoblotting was performed using the following Abs: clone 97203, mouse mAb for ST2/IL-1R4 (R&D Systems); clone D13.14.4E, rabbit mAb for phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); rabbit polyclonal Ab for phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Danvers, MA); and clone AC-15, mouse mAb for β-actin (Sigma), in accordance with the manufacturers' instructions.

Statistical analysis

All data are presented as the mean ± SD. Differences between groups were analyzed using ANOVA with Bonferroni's post hoc test and were considered to be significant when $p < 0.05$.

Results

Preferential expression of ST2 among lung tissue cells

The ST2 gene encodes, by alternative splicing, both membrane-bound ST2L, which is a receptor for IL-33, and sST2, which is a decoy receptor for IL-33 (18). We first examined the expression of ST2 mRNA in lung tissue cells and other human primary cells. We found that both ST2L and sST2 were preferentially expressed in microvascular endothelial cells (HMVEC-LBl) and airway epithelial cells (NHBEs), but not in lung fibroblasts (NHLFs), smooth muscle cells (BSMCs), epidermal keratinocytes (neonatal normal human epidermal keratinocytes), or normal human dermal fibroblasts (Fig. 1*A*, open bars). ST2 mRNA expression was also observed in other human endothelial cells, such as umbilical vein endothelial cells (HUVECs) and coronary artery endothelial cells (HCAECs), suggesting that ST2 is characteristically expressed in human vascular endothelial cells. We further confirmed that the secreted sST2 level in the culture supernatant of each type of cell correlated well with its respective mRNA level (Fig. 1*A*, solid bars).

IL-33–mediated inflammatory responses in lung tissue cells

Because we had elucidated the cell type distribution pattern of ST2L expression, we next examined the biological significance of ST2 expression in lung tissue cells. We examined the ability of IL-33 to induce the production of various cytokines/chemokines by those cells. Consequently, we found that there was good correspondence between the ST2L mRNA distribution and IL-33 responsiveness. More specifically, neither NHLFs nor BSMCs, which did not express ST2L mRNA, responded to IL-33 (Fig. 1*B*, yellow and green bars). In NHBEs, IL-33 induced IL-8 production, detected in the supernatants of 24-h cultures in a dose-dependent manner (Fig. 1*B*, *upper graph*, blue bars). However, IL-33 induced neither IL-6 nor MCP-1 production by NHBEs. Of note, HMVEC-LBl showed dose-dependent, enhanced production of IL-6 and MCP-1 in addition to IL-8 in response to treatment with IL-33 for 24 h (Fig. 1*B*, red bars). Thus, IL-33 induced stronger responses in HMVEC-LBl than in NHBEs in accordance with the levels of ST2 expression in each type of cell. Although we looked for production of other cytokines/chemokines, including IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α, IL-1β, and IP-10, none were found in either HMVEC-LBl or NHBEs.

IL-33 mediates inflammatory responses via the ST2 receptor in lung tissue cells

To elucidate the role of ST2 in IL-33–mediated inflammatory responses in lung tissue cells, we depleted ST2 mRNA by using

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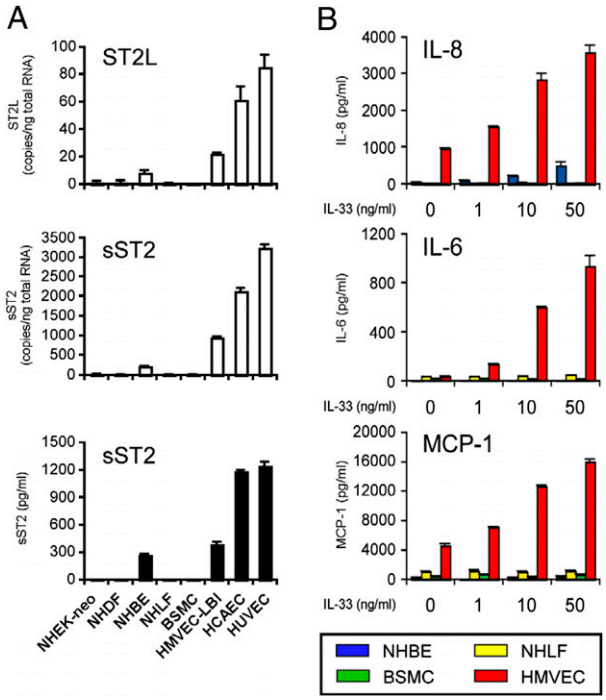

FIGURE 1. *A*, Expression of mRNA for ST2L and sST2 in cultured human primary cells. Total RNA was isolated from various human primary cells in growth phase, and the levels of mRNA for ST2L and sST2 were measured by quantitative real-time PCR (open bars). Concentrations of secreted sST2 protein in the culture supernatants were quantified by ELISA (solid bars). *B*, IL-33–mediated cytokine-chemokine production by lung tissue cells. NHBEs (blue), NHLFs (yellow), BSMCs (green), and HMVEC-LBl (red) were treated with the indicated concentrations of IL-33 for 24 h. Protein concentrations in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of experiments using at least two different lots from individual donors of NHBEs, NHLFs, BSMCs, and HMVEC-LBl.

siRNA specific for ST2 (No. SI00114618; Qiagen), designed to target a site within the sequence shared by ST2L and sST2. NHBEs (Fig. 2*A*) and HMVEC-LBl (Fig. 2*B*) were transfected with siRNA against nontargeting control siRNA or ST2 and then stimulated with IL-33 for 6 h. Control experiments demonstrated that both ST2L and sST2 mRNA were significantly suppressed by the ST2 siRNA compared with the levels of ST2L and sST2 transcripts, respectively, in nontargeting control siRNA-transfected cells. Induction of IL-8 (NHBEs, HMVEC-LBl) and IL-6 (HMVEC-LBl) by IL-33 was significantly inhibited by the transfection of ST2 siRNA, suggesting that IL-33–mediated responses in these cells occur via an ST2-dependent pathway. Furthermore, we found that IL-33–mediated responses in HMVEC-LBl were IL-33 specific because they were almost completely suppressed by simultaneous treatment with IL-33 and recombinant ST2-Fc chimera (Fig. 2*C*).

Th2 cytokines enhance the expression and function of ST2 in lung tissue cells

IL-33 is a potent inducer of Th2 immunity, and we thus examined the effects of Th2 cytokines such as IL-4 on the expression and function of ST2 in lung tissue cells. As shown in Fig. 3*A*, both ST2L mRNA and sST2 mRNA were significantly upregulated by 10 ng/ml IL-4 treatment in a time-dependent manner. Importantly, this IL-4–mediated upregulation of the ST2 genes was observed in IL-33–responsive cells such as NHBEs and HMVEC-LBl but not in the IL-33–unresponsive cells such as NHLFs and BSMCs. We further confirmed that the sST2 protein levels accumulated in the culture supernatants of NHBEs and HMVEC-LBl in response to IL-4 treatment correlated well with their respective sST2 mRNA levels (Fig. 3*B*, *left graph*).

IL-13 is another Th2 cytokine that plays a prominent role in the pathogenesis of allergic inflammation. IL-13 and IL-4 share many functional properties, stemming from the fact that they share the α subunit of the IL-4R. In fact, we found that IL-13 also induced sST2 production by HMVEC-LBl (Fig. 3*B*, *right graph*). IL-4 or IL-13 stimulation of cells leads to activation of multiple signaling pathways via IL-4R α, one of which involves a transcription factor, STAT6. Therefore, to examine the role of STAT6 on IL-4–induced expression of ST2, we depleted STAT6 mRNA by using siRNA for STAT6 (No. SI02662905; Qiagen). The siRNA for STAT6 or nontargeting control siRNA was transfected into HMVEC-LBl. The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-4 for 24 h. The efficiency of STAT6 mRNA depletion was more than 70% compared with the level of STAT6 transcripts in control siRNA-transfected cells, which was confirmed by real-time PCR (Fig. 3*C*, *left graph*). Transfection of STAT6 siRNA significantly reduced the IL-4–dependent upregulation of both ST2L mRNA and sST2 mRNA (Fig. 3*C*, *right two graphs*), suggesting that STAT6 is required for IL-4–enhanced expression of ST2 genes.

To confirm the IL-4–enhanced expression of ST2 at the protein level, whole-cell lysates from IL-4–stimulated HMVEC-LBl and NHBEs were subjected to SDS-PAGE followed by immunoblotting with an anti-ST2 Ab or an anti–β-actin Ab as a loading control. We found that IL-4 significantly enhanced ST2L protein in the whole-cell lysates of both HMVEC-LBl and NHBEs (Fig. 3*D*), in parallel with upregulation of ST2L mRNA in these cells (Fig. 3*A*, *upper graph*).

We next examined whether IL-33–mediated responses of lung tissue cells were further enhanced by IL-4 pretreatment. NHBEs and HMVEC-LBl were pretreated with 10 ng/ml IL-4 for 48 h and then stimulated with 10 ng/ml IL-33 for the indicated periods. IL-4–pretreated cells showed significantly enhanced IL-33–mediated responses, including the induction of IL-8 and IL-6 mRNA (Fig. 4). Thus, Th2 cytokines significantly enhanced ST2 expression and function in both lung endothelial and epithelial cells.

Effects of corticosteroid on IL-33–mediated responses in epithelial and microvascular endothelial cells

Currently, inhaled corticosteroids are a first-line therapy and known to be one of the most effective therapies available for asthma (19). Therefore, we next examined the effect of corticosteroid on the responses of both NHBEs and HMVEC-LBl to IL-33. Fluticasone propionate (FP) treatment showed significant attenuation of IL-33–mediated IL-8 production by NHBEs even at a low FP concentration (1 nM) (reduction to 28% of the production in the absence of corticosteroid), and the production was almost completely suppressed by 100 nM FP treatment (Fig. 5*A*). In contrast, FP treatment showed only partial attenuation of IL-33–mediated IL-6, IL-8, and MCP-1 production by HMVEC-LBl (reduction to 80, 63, and 74% of the respective production in the absence of corticosteroid) even at a high concentration of FP (100 nM) (Fig. 5*B*). The higher levels of IL-8 production by NHBEs compared with the results observed in Fig. 1*B* may be due to the hydrocortisone deprivation before IL-33 stimulation. Because IL-33–mediated IL-8 production by NHBEs was sensitively inhibited by corticosteroid treatment, we presume that

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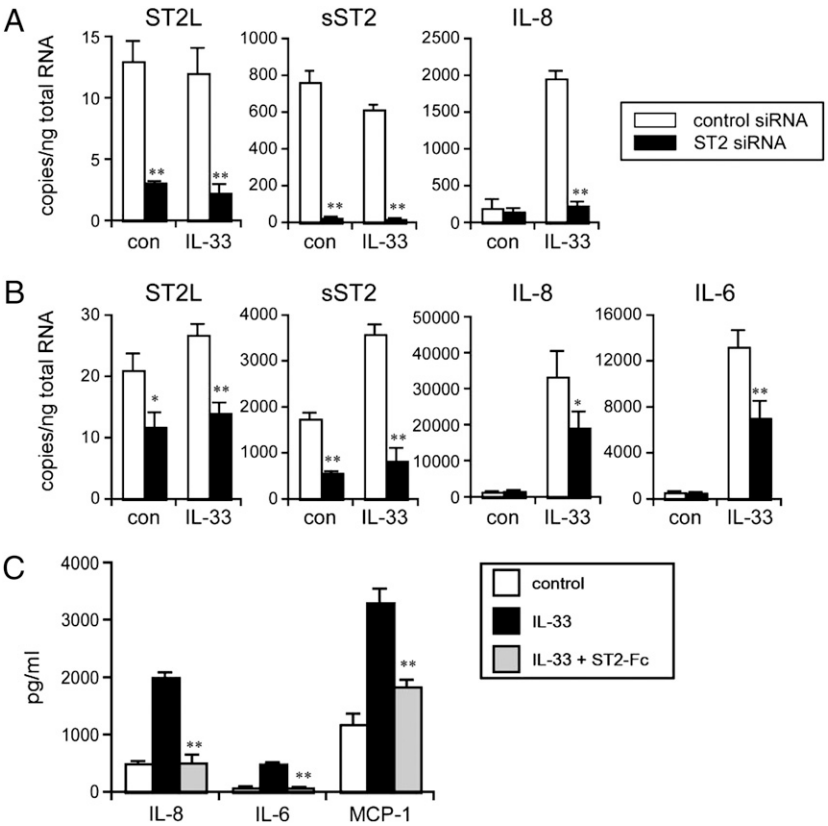

FIGURE 2. IL-33 mediates inflammatory responses via the ST2 receptor on HMVEC-LBl and NHBEs. *A* and *B*, Cultured NHBEs (*A*) and HMVEC-LBl (*B*) were transfected with siRNA against ST2 (solid bars) or nontargeting control siRNA (open bars) at 5 nM (NHBEs) or 10 nM (HMVEC-LBl). The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-33 for 6 h. The levels of mRNA for ST2L, sST2, IL-8, and IL-6 were determined by real-time PCR. Data are shown as the mean ± SD of triplicate samples and are representative of two individual experiments. $*p < 0.05$; $**p < 0.01$ compared with nontargeting control siRNA. *C*, IL-33–mediated responses in cultured HMVEC-LBl were IL-33 specific. HMVEC-LBl were stimulated in the presence of 10 ng/ml IL-33 and 10 μg/ml neutralizing ST2-Fc chimera for 24 h. Protein concentrations in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of two individual experiments. $**p < 0.01$ compared with 10 ng/ml IL-33.

IL-33 robustly enhanced IL-8 production in the absence of corticosteroid.

IL-33–induced phosphorylation of MAPK in epithelial and microvascular endothelial cells

We next sought to evaluate the signaling pathways involved in the IL-33 responses in both NHBEs and HMVEC-LBl. Although the signaling pathways activated by IL-33 remain poorly understood, it was reported that IL-33–mediated IL-8 production by human mast cells is mediated by a signaling pathway involving p38 MAPK (8). Therefore, we investigated whether IL-33 induces phosphorylation of MAPK, including ERK and p38, in NHBEs and HMVEC-LBl. In HMVEC-LBl, transient phosphorylation of both ERK and p38 was observed after 5 to 15 min treatment with IL-33 (Fig. 6). In contrast, in NHBEs, phosphorylation of ERK was observed for up to 60 min of treatment with IL-33, whereas constitutive phosphorylation of p38 was unaffected.

Effects of ERK and p38 MAPK inhibitors on IL-33–mediated responses in microvascular endothelial cells and epithelial cells

To verify which MAPK was involved in the IL-33–mediated responses in HMVEC-LBl, the cells were treated with various concentrations of ERK inhibitor PD98059 or p38 inhibitor SB202190 for 30 min prior to treatment with IL-33. IL-33–mediated productions of IL-8, IL-6, and MCP-1 were dramatically and dose-dependently reduced by the addition of p38 inhibitor SB202190 but not by ERK inhibitor PD98059 (Fig. 7*A*). These results clearly indicate that p38 MAPK is required for IL-33–mediated responses in HMVEC-LBl.

In contrast, IL-33–mediated production of IL-8 by NHBEs was significantly reduced by the addition of ERK inhibitor PD98059, but not by p38 inhibitor SB202190 (Fig. 7*B*). This indicates that, conversely from HMVEC-LBl, ERK is required for IL-33–mediated IL-8 production by NHBEs.

Discussion

In this study, we found that IL-33, a pro-Th2 cytokine, acts directly on pulmonary microvascular endothelial cells and epithelial cells and mediates inflammatory responses.

First, our investigation of lung tissue cells found that both ST2L and sST2 were preferentially expressed in microvascular endothelial cells (HMVEC-LBl) and airway epithelial cells (NHBEs) but not in either lung fibroblasts (NHLFs) or smooth muscle cells (BSMCs) (Fig. 1*A*). Furthermore, there was good correspondence between the ST2 distribution (Fig. 1*A*) and IL-33 responsiveness (Fig. 1*B*) among these lung tissue cells. This suggests that IL-33 mediates its actions via the ST2 receptor on both HMVEC-LBl and NHBEs. Indeed, depletion of ST2 mRNA significantly reduced the IL-33–mediated responses of these cells (Fig. 2).

Neither NHLFs nor BSMCs showed any expression of ST2 (Fig. 1*A*) or responsiveness to IL-33 (Fig. 1*B*), suggesting that

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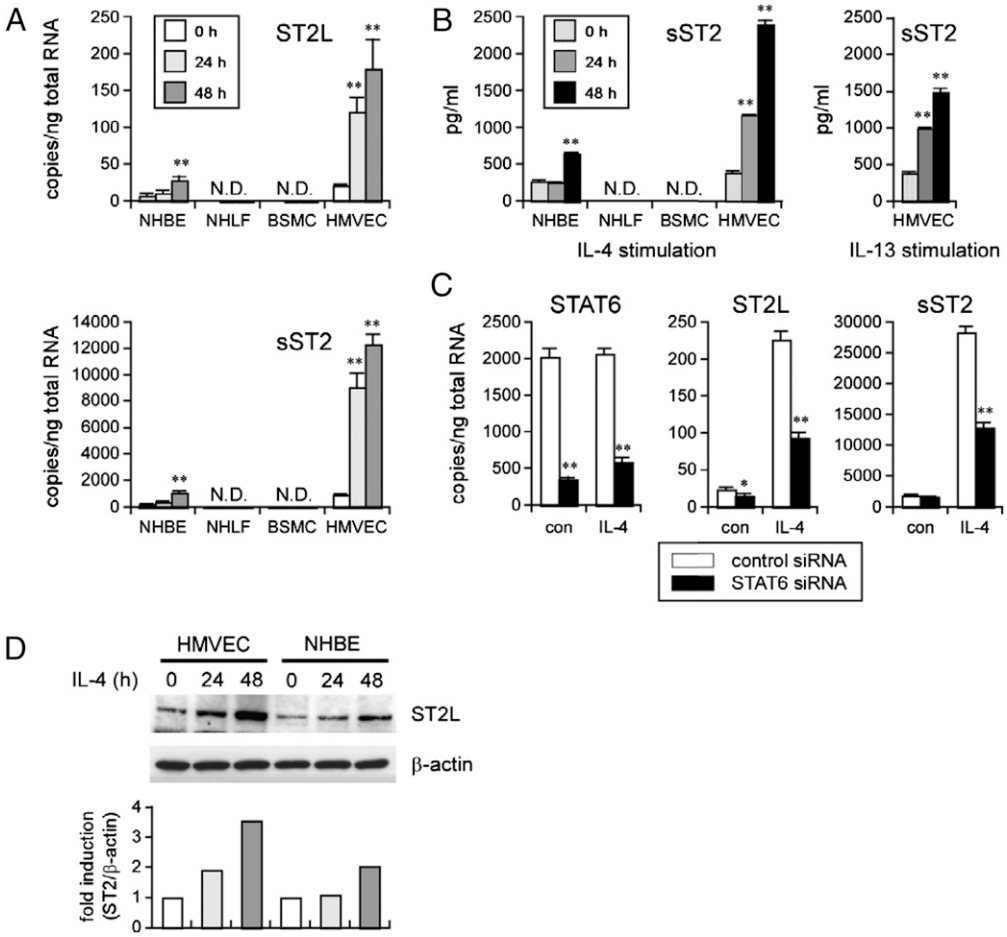

FIGURE 3. Effects of Th2 cytokines on the expression of ST2 in cultured lung tissue cells. Cells were treated with 10 ng/ml IL-4 for the indicated periods. *A*, The levels of mRNA for ST2L and sST2 are shown. *B*, The accumulated sST2 protein levels after 10 ng/ml IL-4 treatment (*left graph*) or 10 ng/ml IL-13 treatment (*right graph*) for the indicated periods are shown. $**p < 0.01$ compared with no cytokine treatment (0 h). *C*, STAT6 is required for IL-4–enhanced expression of ST2 in HMVEC-LBl. Cultured HMVEC-LBl were transfected with siRNA against STAT6 (solid bars) or nontargeting control siRNA (open bars). The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-4 for 24 h. The levels of mRNA for STAT6, ST2L, and sST2 were determined by real-time PCR. $*p < 0.05$; $**p < 0.01$ compared with nontargeting control siRNA. *D*, Whole-cell lysates from IL-4–stimulated HMVEC-LBl and NHBEs were harvested, and ST2L and β-actin were analyzed by Western blotting. The fold induction of ST2L protein was determined by densitometry and normalized to the respective β-actin level (*lower graph*). Data are shown as the mean ± SD of triplicate samples (*A*–*C*) and are representative of three (*A*, *B*) or two (*C*, *D*) individual experiments.

IL-33 does not act directly on these cells in the asthmatic airway. Of note, ST2 was preferentially expressed in vascular endothelial cells, including HUVECs and HCAECs (Fig. 1*A*). These observations are consistent with recent reports of sST2 secretion by human venous and arterial endothelial cells (20, 21).

IL-33 drives production of Th2-associated cytokines, including IL-4, IL-5, and IL-13, by various hematopoietic cells (1, 3, 4, 8–10). Unlike in those hematopoietic cells, IL-33–mediated cytokine-chemokine production by the lung tissue cells was rather limited (Fig. 1*B*), and we found no production of Th2-associated cytokines (data not shown). It was recently reported that the ST2/IL-33 pathway is necessary not only for the development of an allergic inflammatory response but also for its maintenance (22). Thus, the actions of IL-33 on lung tissue cells may not contribute to the development of allergic inflammation but rather to the maintenance of chronic inflammation. It should be noted that Th2 cytokines, such as IL-4, significantly enhanced ST2 expression (Fig. 3) and function (Fig. 4) in both lung endothelial and epithelial cells. These findings are important when considering chronic inflammation in the lung and suggest that allergic individuals may be more susceptible to IL-33–mediated inflammatory responses of lung tissue cells than nonallergic individuals. Aoki et al. (21) recently reported that IL-33 stimulated secretion of IL-6 and IL-8 by HUVECs. Notably, they showed that ST2 gene expression in HUVECs was growth-dependent and was downregulated when the cells were differentiated to form vascular structures on an extracellular membrane matrix in vitro, whereas vascular endothelial growth factor gene expression was not downregulated. These results suggest that blood vessels normally would not respond to IL-33. In contrast, Th2-inflamed lung blood vessels and/or epithelium seem to be potential targets for the actions of IL-33.

Although the results of this study were limited to in vitro experiments, several reports by others have shed light on the in vivo roles of IL-33 by exogenous administration of recombinant IL-33 to mice (1, 23–25) or by transgenic overexpression of IL-33 in mice (26). Those studies have independently provided evidence that excessive expression of IL-33 in vivo might lead to an increase in the number of inflammatory cells in the airway via release of endogenous Th2 cytokines and chemokines. Notably, Zhiguang et al. (26) showed that pulmonary inflammation with infiltration of inflammatory cells was observed around the blood vessels in the airway of IL-33–transgenic mice, supporting our conclusion from this study that pulmonary endothelial cells can be direct targets of IL-33. Furthermore, both administration and

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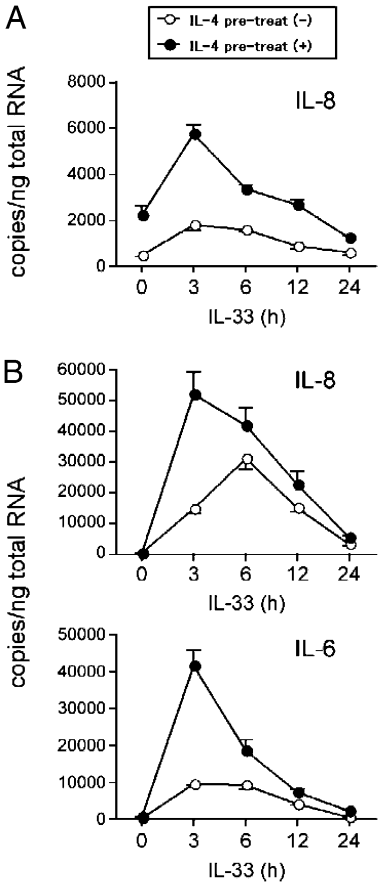

FIGURE 4. IL-33–mediated responses were further enhanced by IL-4 pretreatment of NHBEs and HMVEC-LBl. Cultured NHBEs (*A*) or HMVEC-LBl (*B*) were treated with 10 ng/ml IL-4 for 48 h, and then the cells were washed twice with HBSS and replaced with fresh medium containing 10 ng/ml IL-33 for the indicated periods. The levels of mRNA for IL-8 and IL-6 were determined by real-time PCR. Data are shown as the mean ± SD of triplicate samples and are representative of three individual experiments.

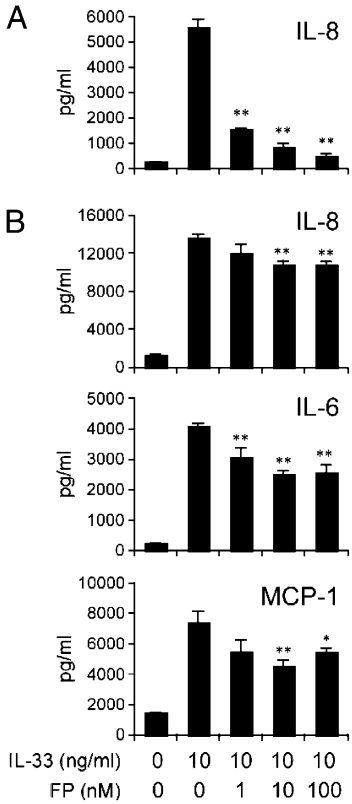

FIGURE 5. Effects of corticosteroid on IL-33–mediated responses in NHBEs and HMVEC-LBl. Cultured NHBEs (*A*) and HMVEC-LBl (*B*) were simultaneously treated with IL-33 and FP for 24 h at the indicated concentrations. Concentrations of IL-8, IL-6, and MCP-1 in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of at least three individual experiments. $*p < 0.05$; $**p < 0.01$ compared with 10 ng/ml IL-33.

transgenic overexpression of IL-33 in mice led to increased numbers of neutrophils as well as eosinophils in the airway (23, 25, 26). Neutrophils are not regarded as direct target cells of IL-33 because they have few ST2 receptors on their surface. Therefore, we surmise that IL-33 can promote neutrophil infiltration in the airway through IL-33–induced release of neutrophil chemoattractants, including IL-8 family members, by lung tissue cells.

Today, inhaled corticosteroids are a first-line anti-inflammatory treatment and known to be one of the most effective therapies available for asthma (19). Indeed, FP treatment showed significant attenuation of IL-33–mediated IL-8 production by NHBEs even at a low FP concentration (1 nM), and that production was almost completely abrogated by 100 nM FP treatment (Fig. 5*A*), suggesting that corticosteroids are capable of effectively reducing IL-33–mediated airway epithelial inflammation. In contrast, FP treatment was only partially effective against IL-33's actions on microvascular endothelial cells (Fig. 5*B*), which were found to be the main IL-33–targeted cells among the lung tissue cells (Fig. 1*B*). We recently showed that corticosteroid treatment was also only weakly effective on TNF-α–mediated microvascular inflammation, including chemokine production (16). In addition, corticosteroid enhanced TNF-α–mediated leukocyte adhesion to pulmonary microvascular endothelial cells via upregulation of cell-surface expression of ICAM-1 and VCAM-1 (16). Taken together, those various findings suggest that the poor effect of corticosteroid on TNF-α/IL-33–mediated inflammatory responses is not only a specific feature of airway microvessels but is also crucially involved in the refractoriness seen in the asthmatics. Notably, Préfontaine et al. (13) recently demonstrated that dexamethasone fails to abolish TNF-α–induced IL-33 upregulation in primary human airway smooth muscle cells, further suggesting a contribution of IL-33 as well as TNF-α to the refractory phenotype of certain asthmatics treated with corticosteroids.

Persistent chronic inflammation in the lung leads to structural alterations in the airway wall (i.e., airway remodeling), which is thought to cause irreversible airflow obstruction and exacerbation of asthma (15). Recent compelling evidence has demonstrated that

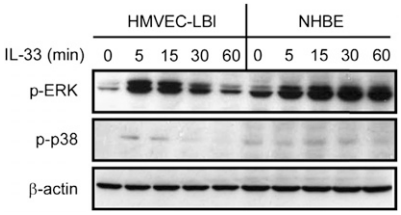

FIGURE 6. IL-33–induced phosphorylation of MAPK in cultured HMVEC-LBl and NHBEs. Whole-cell lysates were examined at the indicated time points after stimulation with 10 ng/ml IL-33 for the expression of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and β-actin (as a loading control).

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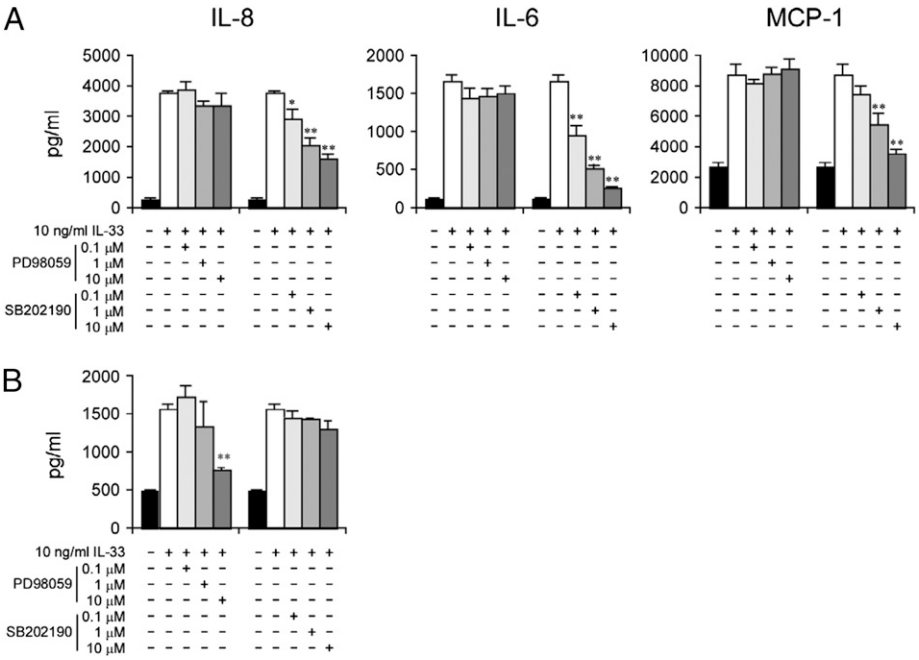

FIGURE 7. Divergent effects of ERK inhibitor (PD98059) and p38 MAPK inhibitor (SB202190) on IL-33–mediated responses in HMVEC-LBl and NHBEs. Cultured HMVEC-LBl (*A*) and NHBEs (*B*) were treated with the indicated concentrations of PD98059 or SB202190 for 30 min prior to stimulation with 10 ng/ml IL-33 for 24 h. Data are shown as the mean ± SD of triplicate samples and are representative of three individual experiments. $*p < 0.05$; $**p < 0.01$ compared with 10 ng/ml IL-33.

airway hypervascularity in severe asthma, an element of airway remodeling resulting from accelerated angiogenesis, responds poorly to corticosteroid treatment and is clinically involved in reduced lung function (27–29). We previously showed that autocrine CXCR2 chemokines, such as IL-8, are indispensable for lung angiogenesis in a corticosteroid-insensitive manner (16, 17, 30). As shown in our current study, IL-33 can induce IL-8 production by pulmonary endothelial and epithelial cells (Fig. 1*B*), suggesting that IL-33 is involved in lung angiogenesis and the resultant airway hypervascularity. As a matter of fact, Choi et al. (31) recently demonstrated that IL-33 promotes angiogenesis and vascular permeability by stimulating endothelial NO production via the ST2 receptor.

As shown in Fig. 1*B*, both IL-6 and MCP-1, which were also secreted by IL-33–stimulated pulmonary microvascular endothelial cells, are known to be critically involved in allergic inflammation (32–34). Therefore, these proinflammatory mediators originating from IL-33–stimulated pulmonary microvessels may also play roles in the maintenance of chronic allergic inflammation of the asthmatic airway.

Although IL-33/ST2 signaling pathways remain poorly understood, it could be expected that the signaling molecules are similar to those for other IL-1 family cytokines. Indeed, it was reported that IL-33, as well as IL-1β, can enhance MAPK (ERK and p38) phosphorylation in both murine (1) and human (8) mast cells. We confirmed that IL-33 can activate MAPK (ERK and p38) phosphorylation in HMVEC-LBl (Fig. 6). Moreover, as was reported for IL-33–induced IL-8 production by human mast cells (8), IL-33–induced production of each of IL-8, IL-6, and MCP-1 by HMVEC-LBl was dramatically and dose-dependently reduced by treatment with a p38 inhibitor, SB202190, but not with an ERK inhibitor, PD98059 (Fig. 7*A*). This suggests that the IL-33–mediated signaling pathway in human microvascular endothelial cells is similar to that in human mast cells. In contrast, ERK, but not p38, is required for IL-33–mediated IL-8 production by NHBEs (Fig. 7*B*). Because ST2L mRNA remained at a lower level in NHBEs than in HMVEC-LBl (Fig. 1*A*), we initially considered that NHBEs respond only partially to IL-33 and produce only IL-8 (not IL-6 or MCP-1) (Fig. 1*B*) simply due to a smaller number of ST2 protein molecules on the surface of NHBEs compared with that of HMVEC-LBl. However, as described above, we found a distinct difference between these cells in their requirements for MAPK in the IL-33–mediated signaling pathway. These mechanistic differences between HMVEC-LBl and NHBEs in their IL-33–mediated signaling pathways should be further elucidated. Nevertheless, these observations suggest that, contrary to our initial expectation, IL-33–mediated responses in NHBEs use signal transduction pathways that are distinct from the pathways in HMVEC-LBl and human mast cells.

The main sources of IL-33 involved in the pathogenesis of asthma remain controversial. IL-33 was originally identified as NF-high endothelial venules, which is an NF preferentially expressed in high endothelial venules (35). The same group also reported that endothelial cells constitute a major source of IL-33 mRNA in chronically inflamed tissues from patients with rheumatoid arthritis and Crohn's disease (36). Furthermore, they showed abundant nuclear expression of IL-33 in endothelial cells from both large and small blood vessels in most normal human tissues (37), suggesting that endothelial cells constitute major sources of IL-33 in vivo. Indeed, we also confirmed expression of IL-33 mRNA and protein in whole-cell lysates, but not culture supernatants, of HMVEC-LBl by real-time PCR and ELISA, respectively (data not shown). Although further studies are clearly needed, we speculate that endogenous IL-33 released from inflamed and/or injured blood vessels acts on neighboring vessels as an endogenous "danger signal" (37), leading to chronic inflammatory responses. Notably, release of the IL-33 "danger signal" by damaged/injured endothelial cells has recently been demonstrated (38), lending further support to the endogenous "danger signal" hypothesis.

In conclusion, IL-33, a pro-Th2 cytokine, acts directly on pulmonary microvascular endothelial cells and epithelial cells (among lung tissue cells), which express its ST2 receptor. Importantly, Th2 cytokines significantly enhanced ST2 expression and function in both endothelial and epithelial cells. Furthermore, the responses of those cells, especially microvascular endothelial cells, to IL-33 are almost refractory to corticosteroid treatment, and we thus anticipate that IL-33 and/or its receptor, ST2, may be able to be exploited as a novel target for development of curative drugs for refractory asthma.

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Disclosures

The authors have no financial conflicts of interest.

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