**A**

FIG 3. Ca^{2+} mobilization through PAF-R in PB-derived MCs in response to PAF. **A**, Ca^{2+} mobilization in MCs in response to PAF. **B**, Effect of the PAF-R antagonist CV-6209 on PAF- or calcium ionophore A23187–induced Ca^{2+} mobilization. The *y-axis* and *x-axis* show intracellular free calcium levels and the incubation time after stimulation, respectively. *Arrows* indicate the time point of addition of PAF or calcium ionophore A23187. The data are representative of similar results obtained from 3 independent experiments. The vehicle used was 0.1% chloroform.
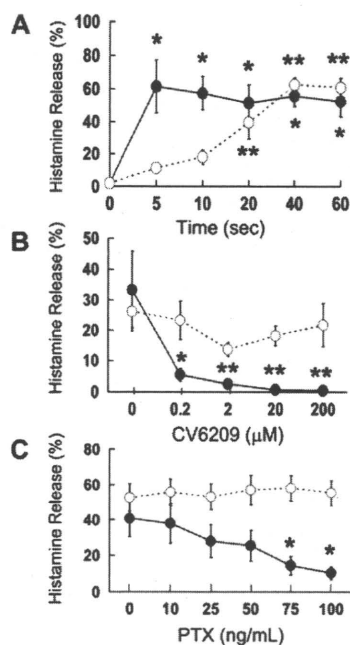
FIG 4. PAF-induced histamine release from PB-derived MCs is rapid and inhibited by pretreatments with CV-6209 and PTX. **A,** Kinetics of histamine release induced by PAF (10^{-7} mol/L, *solid circles*) or IgE/anti-IgE (30 μg/mL, *open circles*; n = 3). **B** and **C,** Effects of CV6209 (n = 6) and PTX (n = 7) on PAF- or IgE/anti-IgE–induced histamine release. Data shown are means ± SEMs. *P < .05 and **P < 0.01 compared with 0 seconds (Fig 4, *A*). *P < .05 and **P < .01 compared with nontreatment (Fig 4, *B* and *C*).

Effect of lentiviral short hairpin RNA silencing of PLCγ1 and PLCβ2 on FcεRI- and PAF-R–mediated degranulation

mRNA for PLCγ1 and PLCβ2 were knocked down by using the lentiviral short hairpin RNA (shRNA) silencing technique to clarify the roles of PLCγ1 and PLCβ2 on PAF- and IgE/anti-IgE–induced histamine release. These mRNA were selectively knocked down (Fig 6, *A*). Reduction of PLCγ1 and PLCβ2 levels in MCs significantly inhibited both IgE/anti-IgE– and PAF-induced histamine release (Fig 6, *B*). In particular, reduction of PLCβ2 completely inhibited it.

Effects of BAPTA/AM and Go6976 on histamine release from MCs in response to PAF or IgE/anti-IgE

Because PLC activation leads to intracellular Ca^{2+} level increase and PKC activation,[23] we investigated whether these intracellular events are necessary for degranulation. Exposure of cells to BAPTA/AM almost completely inhibited PAF- or IgE/anti-IgE–induced degranulation (Fig 7, *A*). Removal of extracellular Ca^{2+} from the solution of PB-derived MCs completely inhibited IgE/anti-IgE–induced degranulation, although only partially, but significantly inhibited PAF-mediated degranulation. The augmented activation of PLC and increase in intracellular Ca^{2+} levels would likely extend to enhanced activation of conventional PKCs (α, βI, βII, and γ). Both IgE/anti-IgE– and PAF-induced degranulation were effectively blocked by preincubation with Go6976, an inhibitor of Ca^{2+}-dependent isozymes of PKC (Fig 7, *B*).

DISCUSSION

In this study we have demonstrated for the first time that human lung MCs and PB-derived MCs release histamine in response to PAF through the PAF-R. Activation of the PAF-R–coupled PTX-sensitive G protein $G_{\alpha i}$ in PB-derived MCs leads to degranulation through activation of both PLCγ1 and PLCβ2, which are common features of both FcεRI- and PAF-R–mediated MC activation. This has important implications for our understanding of the mechanisms of acute allergic responses, in particular anaphylaxis.

PAF is a phospholipid (1-o-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) that is released by a variety of human cells, including platelets and MCs.[26] It has been reported that mean serum PAF levels were 1.5×10^{-9} mol/L (805 pg/mL) in patients with anaphylaxis and were correlated with the severity of anaphylaxis.[8] Mean serum PAF levels in patients with grade 3 anaphylaxis were more than 10 times greater than the levels in control patients (approximately 2.5×10^{-9} mol/L). However, these concentrations are likely to be an underestimate because of rapid degradation[27] and lower than those achieved locally within tissue. Nevertheless, 10^{-9} mol/L PAF induced 20% to 30% of total histamine release from some lung MC preparations (see Fig E1). Therefore the PAF concentrations required for human lung MC activation correspond to physiological concentrations measured during an anaphylactic response.

MCs are known to play a central role in human anaphylaxis, as supported by the observation that there are increased concentrations of MC-specific β-tryptase in the sera of anaphylactic patients.[3,4] It is often assumed that the systemic MC response after local exposure occurs as a result of the dissemination of allergen through the bloodstream. Although this is possible, the rapid nature of events after oral exposure suggests that other factors might be important. Increased concentrations of PAF appear to be important for human anaphylaxis, although its biological role is not known. Our finding that PAF can directly induce human MC degranulation and arachidonic acid metabolism and can potentiate IgE-dependent mediator release provides a mechanism whereby PAF directly amplifies the effects of allergen exposure. The source of PAF in human anaphylaxis is uncertain but in part might come from MCs.[28] Thus we suggest that PAF generated locally by MCs and perhaps other cells in response to MC activation at the point of allergen contact leads to an exaggerated MC response at more distal sites, such as the airway. We therefore propose a PAF amplification loop as a key factor leading to airway MC degranulation on exposure to oral allergens.

We did not find any effect of PAF on human skin MCs, which is in keeping with a lack of PAF-R expression in these cells. However, PAF can induce MC degranulation through the stimulation of neural reflexes,[9] and because skin MCs can be activated by neuropeptides,[29] PAF might still be relevant to skin MCs. Whether cardiac and gut MCs respond to PAF requires further investigation.

Because every GPCR downstream signaling pathway can have its own unique pharmacology in a particular cell depending on the pathway stimulated by the unique ligand receptor conformation or complex involved,[30] we investigated the signaling pathway of PAF-R on human MCs. Previous reports have demonstrated that the PAF-R interacts with multiple G proteins, including $G_{\alpha i}$, $G_{\alpha o}$, and $G_{\alpha q/11}$, leading to simultaneous stimulation of distinct signaling pathways.[24] Histamine release was blocked by approximately 80% by using PTX at 100 ng/mL in PB-derived MCs, suggesting a dominant role for $G_{\alpha i}$. Further studies will be required to
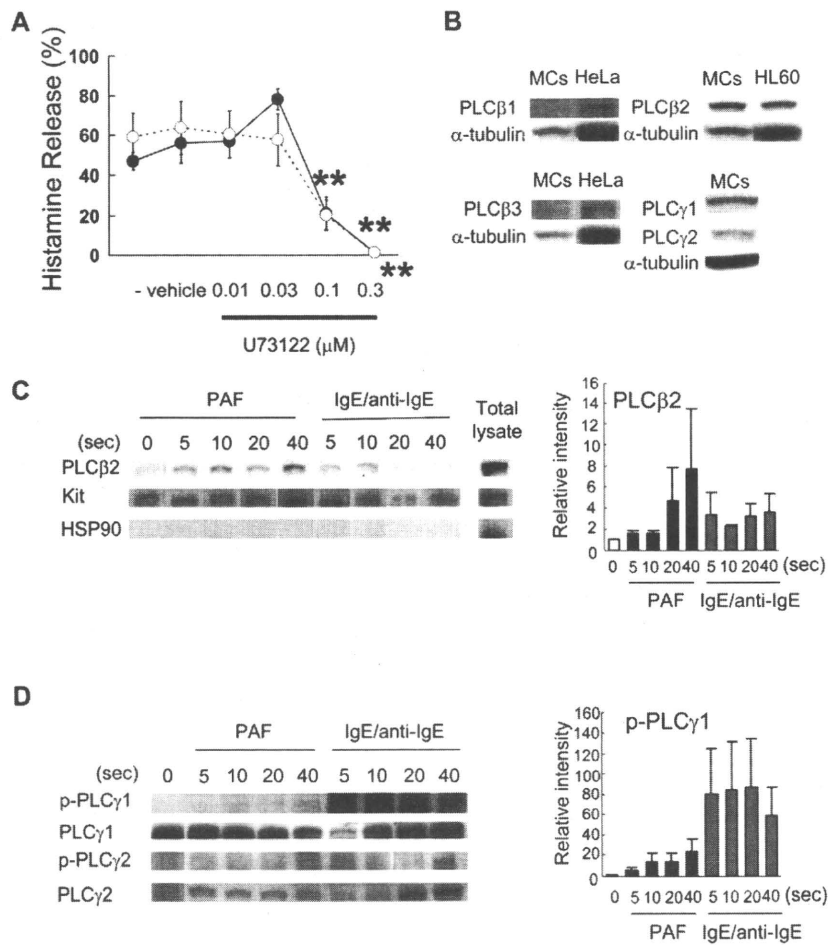
FIG 5. Activation of PLCγ1 and PLCβ2 in PB-derived MCs by PAF. **A,** Effect of U73122 on PAF-induced *(solid circles)* or IgE/anti-IgE–induced *(open circles)* histamine release (n = 4). **B,** Expression of PLCβ and PLCγ in MCs. HeLa cells and HL-60 cells were used as positive controls. (PLCβ1, PLCβ2, and PLCβ3, n = 3; PLCγ1 and PLCγ2, n = 6). **C,** Membrane translocation of PLCβ2 in response to PAF or IgE/anti-IgE. Anti-Kit was used to verify the membrane protein loading, and anti–heat shock protein *(HSP)* 90 was used to verify the absence of cytosolic proteins. **D,** Phosphorylation of PLCγ1 (n = 5) and PLCγ2 (n = 4) in response to PAF or IgE/anti-IgE. Data shown are means ± SEMs. *$P < .05$ and **$P < .01$ compared with vehicle (0.1% dimethyl sulfoxide). The data (Fig 5, *C* and *D*) were generated by scanning the blots and then normalizing to the response at 0 seconds (n = 3). –, Buffer alone; *p-PLCγ,* anti–phospho-PLCγ.

investigate interactions of the $G_{\alpha q}$ protein with PAF-R and involvement of $G_{\alpha q}$ for PAF-R–mediated degranulation in human MCs. Nilsson et al[31] demonstrated that PAF induced a transient increase in intracellular Ca^{2+} concentrations and induced significant migratory responses in the human leukemia MC line HMC-1. Both PAF-induced calcium mobilization and migration of HMC-1 cells were shown to require activation of PTX-sensitive G protein. Although we have not investigated the migratory response of primary MCs to PAF, there is a possibility that PAF induces migration and degranulation of MCs simultaneously, which might result in augmented inflammation.

Several reports have characterized the ability of PAF to activate tyrosine kinases with subsequent activation of PLCγ1, an isozyme of phospholipase that is activated by tyrosine phosphorylation in B cells, platelets, and PAF-R–transfected cell lines.[24] In PAF-R–transfected RBL-2H3 cells, PAF did not activate PLCγ,[32] whereas in human umbilical vein endothelial cells PAF activated PLCβ3 through its $G_{\alpha q}$-coupled receptor.[33] In human MCs IgE-dependent activation induces activation of both PLCγ1[34] and

PLCβ2, although not PLCβ3.[15] We found that PAF-R activation also leads to degranulation through activation of both of PLCγ1 and PLCβ2. Therefore the isozymes of phospholipase that are activated in response to PAF are dependent on the cell type, and PAF-R– and FcεRI-mediated histamine release in human MCs share a common pathway using activation of both PLCγ1 and PLCβ2. The main difference in the cell-signaling pathway between PAF-R– and FcεRI-mediated histamine release is therefore upstream of PLC. This is supported by the observation that PAF-mediated degranulation was rapid (maximal at 5 seconds), and it did not completely require extracellular Ca^{2+}, which is required for sustained activation of the tyrosine kinase syk by IgE-dependent MC activation.[35]
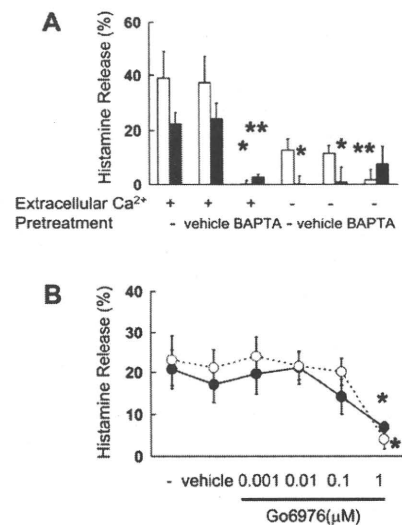
We found that the cytokines induced by PAF-activated MCs were also induced by IgE/anti-IgE–activated MCs (see Fig E2), but PAF-induced cytokine mRNA upregulation was only transient. We measured IL-8 and PAI-1 levels in the supernatant of activated MCs after stimulation with PAF or IgE/anti-IgE. The amounts of IL-8 and PAI-1 produced by PAF-activated MCs
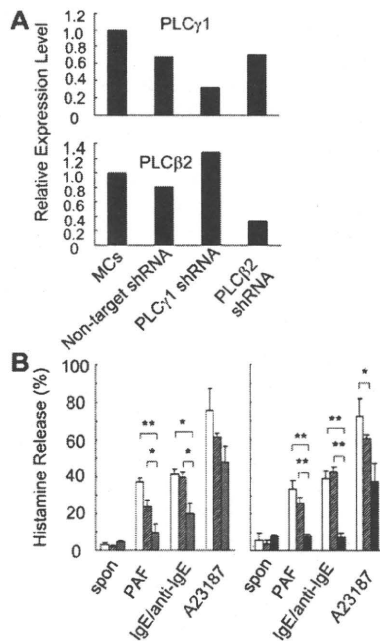
FIG 6. Lentiviral shRNA silencing of PLCγ1 and PLCβ2 reduces FcεRI- and PAF-R-mediated degranulation. **A**, Assessment for loss of PLCγ1 and PLCβ2 mRNA expression in nontransduced PB-derived MCs, cells transduced with nontargeted shRNA, or lentiviral shRNA constructs for PLCγ1 and PLCβ2. **B**, Degranulation from nontransduced MCs *(open bars)*, MCs transduced with nontargeted shRNA *(hatched bars)*, or lentiviral shRNA constructs for PLCγ1 *(gray bars)* and PLCβ2 *(solid bars)*, which were stimulated with PAF (10^{-7} mol/L), IgE/anti-IgE (30 μg/mL), or calcium ionophore A23187. Data shown are means ± SEMs of 4 independent experiments. *$P < .05$ and **$P < .01$.

FIG 7. Effects of BAPTA/AM and Go6976 on PAF- or IgE/anti-IgE–induced histamine release. **A**, Effect of BAPTA/AM on PAF-induced *(open bars)* or IgE/anti-IgE–induced *(solid bars)* histamine release in the presence or absence of extracellular Ca^{2+} (n = 6). **B**, Effect of Go6976 on PAF-induced *(solid circles)* or IgE/anti-IgE–induced *(open circles)* histamine release (n = 10). Data shown are means ± SEMs. *$P < .05$ and **$P < .01$ compared with vehicle (0.03% dimethyl sulfoxide) in the presence of extracellular Ca^{2+} (Fig 7, *A*). *$P < .05$ compared with vehicle (0.1% dimethyl sulfoxide; Fig 7, *B*).

were much smaller than those produced by IgE/anti-IgE–activated MCs (Fig 1, *E* and *F*). The IL-8 production by PAF-activated MCs was significantly inhibited by CV-6209, but IL-8 production by IgE/anti-IgE–activated MCs was not, indicating the IL-8 production by PAF-activated MCs was mediated through PAF-R (data not shown). These data indicate that PAF-induced activation of MCs predominantly affects the early phase of allergic responses, which are the key feature of anaphylaxis.

Two common MC phenotypes recognized in human subjects are identified by their protease content. One population contains only tryptase (MC$_T$), whereas a second contains tryptase, chymase, carboxypeptidase A3, and cathepsin G (MC$_{TC}$).[36] The MC$_T$ phenotype is typically found at mucosal surfaces, such as the nasal and bronchial epithelium in patients with rhinitis and asthma, respectively, and the bronchial lamina propria in states of both health and disease.[36,37] In contrast, the MC$_{TC}$ phenotype favors connective tissues, such as normal skin,[36] the airway smooth muscle bundles in patients with asthma,[38] and atherosclerotic lesions.[39] Our histochemical analyses of human lung specimens showed that PAF-R was preferentially expressed in the MC$_T$ phenotype (Fig 2, *C* and *D*). This study also shows the marked heterogeneity of human MCs, as demonstrated by the difference in GPCR expression in the lung and skin. Our microarray analysis of comparison of GPCR expression between human lung and skin MCs revealed specific expression of *PAFR* and *MRGX2* transcripts in lung and skin MCs, respectively. The expression of *MRGX2* in the cord blood–derived cultured MCs has been reported.[40] The ligands for *MRGX2* are basic neuropeptides, such as substance P, vasoactive intestinal peptide, and cortistatin. Taken together with previous evidence,[8-10,31] our findings support the presence of 2 pathways for a PAF amplification loop in the generation of anaphylaxis: one provided by the direct activation of airway MCs contributing to bronchospasm and laryngeal edema and perhaps MCs in other organs, such as the heart, and the second mediated through neural reflexes in organs such as the skin.

In summary, PAF activates human lung and PB-derived MCs directly and potentiates IgE-dependent human MC activation. The effects are predominantly evident with respect to degranulation and arachidonic acid metabolism rather than cytokine mRNA expression or IL-8 release. This study therefore provides a mechanism that links the increased PAF levels found in patients with anaphylaxis to the pathophysiology of the disease. This strengthens the case for targeting PAF in the treatment of anaphylaxis.

Clinical implications: Blocking of PAF-R on human lung MCs might offer a novel approach to the prevention and treatment of anaphylaxis.

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METHODS

Generation of adult PB progenitor–derived MCs

Lineage-negative (CD4⁻, CD8⁻, CD11b⁻, CD14⁻, CD16⁻, and CD19⁻) mononuclear cells were selected from the PB mononuclear cells and cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and IMDM containing recombinant human stem cell factor (PeproTech EC Ltd) at 200 ng/mL, recombinant human IL-6 (PeproTech EC) at 50 ng/mL, and recombinant human IL-3 (Intergen, Inc, Purchase, NY) at 1 ng/mL, as previously described.[E1] On day 42 of culture, methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in IMDM containing stem cell factor at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS. The MCs used in the experiments were 12 to 16 weeks old. High MC purity (>99%) was obtained by using Kit-positive magnetic selection with anti-CD117 mAb (clone AC126, Miltenyi Biotec GmbH). MC numbers and purity were determined by counting after metachromatic staining with toluidine blue or by means of tryptase staining (clone AA1, Dako).

Isolation of human PB mononuclear cells

Human PB mononuclear cells were isolated by means of centrifugation of whole blood on a Ficoll-Isopaque density gradient (Nycomed, Oslo, Norway).

Cell lines

Jurkat, HeLa, and HL60 cells were purchased from American Type Culture Collection (Manassas, Va). Jurkat and HeLa cells were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS and 1% penicillin-streptomycin, and HL60 cells were cultured in IMDM supplemented with 10% FCS and 1% penicillin-streptomycin.

Isolation of RNA and RT-PCR

Isolation of total RNA and RT-PCR were performed as previously described.[E2] Amplification was performed for 30 cycles. Each cycle included denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C for *PAFR* and at 64°C for *GAPDH*, and extension for 1 minute at 72°C. After the final cycle, the samples were incubated for 10 minutes at 72°C. The sequences of the primers for *PAFR* were 5'-CGGACATGCTCTTCTTGAT CA-3' for the 5' primer and 5'-GTCTAAGACACAGTTGGTGCTA-3' for the 3' primer. The sequences of the primers for *GAPDH* were 5'-TGATGACA TCAAGAAGGTGGTGAAG-3' for the 5' primer and 5'-TCCTTGGAGGCC ATGTGGGCCAT-3' for the 3' primer.

GeneChip expression analysis

Human genome-wide gene expression was assessed by using the Human Genome U133 Plus 2 probe array (GeneChip; Affymetrix, Santa Clara, Calif), which contains an oligonucleotide probe set for approximately 66,996 full-length genes and expressed sequence tags (ESTs), according to the manufacturer's protocol (Expression Analysis Technical Manual and as in previous reports).[E2] The data obtained were analyzed with Genespring software version GX 7.3 (Silicon Genetics, San Carlos, Calif). The average difference values for all genes on a given chip were divided by the median of all measurements on that chip to normalize the staining intensity variations among chips. Data were considered significant when (1) expression changed by at least 4-fold (activation program) and (2) increased gene expression included at least 1 "present absolute call" (Affymetrix algorithm). Based on those normalized values, the genes were classified as upregulated or downregulated.

Histamine release assays

The degree of MC degranulation was monitored by measuring the histamine released into the extracellular fluid after activation with either an enzyme immunoassay kit (Immunotech A; Beckman Coulter Co, Marseille, France) or a radioenzymatic assay, as described previously.[E3] The net percentage of histamine release was calculated from the ratio of each sample, with spontaneous release subtracted to total histamine.

PGD₂ assay

The PGD₂ MOX EIA kit (Cayman Chemical, Ann Arbor, Mich) was used to measure the amounts of PGD₂ in the supernatants.

ELISA for IL-8 and PAI-1

Human IL-8 and PAI-1 were measured with ELISA kits. The ELISA kits for IL-8 and PAI-1 were purchased from R&D Systems, Inc (Minneapolis, Minn), and from AssayPro (Brooklyn, NY). The assay sensitivities of human IL-8 and PAI-1 were 31.2 and 78 pg/mL, respectively.

Measurement of intracellular calcium

Cells were suspended in HBSS supplemented with 1% FCS and 1 mmol/L $CaCl_2$. Indo-1AM (3.6 μg/mL, Invitrogen) containing F127 (Sigma-Aldrich) and FCS was added, and the cells were incubated at 37°C for 30 minutes in the dark. After washing, the cells were resuspended in HBSS supplemented with 1% FCS and 1 mmol/L $CaCl_2$ and stimulated with PAF or calcium ionophore A23187. In some experiments MCs were preincubated with CV-6209 or BN52021 at 37°C for 15 minutes before stimulation. The fluorescence intensity of intracellular Indo-1AM was monitored and analyzed with an LSR system (BD, Franklin, NJ).

Immunoblotting

Cells were lysed in SDS sample buffer containing protease inhibitor cocktail (Roche, Indianapolis, Ind) and phosphatase inhibitor cocktail set I (Calbiochem). The whole-cell lysates were boiled for 5 minutes. In some experiments membrane fractions were prepared as previously described.[E4] Briefly, the lysates were centrifuged to remove intact cells and nuclei. The recovered supernatants were centrifuged at 20,000g for 30 minutes at 4°C. Proteins in the pellet fractions were resuspended in SDS sample buffer containing protease inhibitor cocktail and phospatase inhibitor cocktail set I and put on ice for 30 minutes. The samples were then boiled for 5 minutes and centrifuged at 15,000g for 15 minutes at 4°C. The recovered supernatants were saved as membrane fractions. Proteins were separated by means of electrophoresis on 4-20% or 8% Tris-Glycine Gel (Invitrogen). The separated proteins were then transferred onto polyvinylidene difluoride membranes (GE Healthcare, Uppsala, Sweden), blocked with TBS containing 5% nonfat dry milk and 0.05% Tween-20 for 1 hour at room temperature, and then probed with antibodies against PAF-R (Alexis Biochemicals, San Diego, Calif); PLCβ1 and PLCβ2 (Santa Cruz Biotechnology, Santa Cruz, Calif); PLCβ3, PLCγ1, PLCγ2, and phospho-PLCγ2 (Cell Signaling Technology, Danvers, Mass); and phospho-PLCγ1 (Invitrogen) overnight at 4°C. After washing and incubation with the horseradish peroxidase–linked secondary antibody (GE Healthcare), the blotted membranes were visualized with ECL plus Western Blotting Detection Reagents (GE Healthcare). Membranes were stripped by means of incubation in Re-Blot plus Strong Antibody Stripping Solution (Chemicon, Temecula, Calif) for 15 minutes at room temperature. After washing, membranes were blocked with Block Ace (Dainippon, Inc, Osaka, Japan) and reprobed with antibodies against α-tubulin (Sigma-Aldrich), Kit (R&D systems, Inc), heat shock protein 90 (BD Transduction Laboratories, San Diego, Calif), PLCγ1, and PLCγ2. The heat shock protein 90 and α-tubulin were used as positive controls of intracellular and protein expression, respectively. Bound proteins were quantified with ImageJ 1.39u software.

Immunohistochemical analysis

The study was conducted with the approval of the Ethics Committee of Dokkyo Medical University School of Medicine, and written informed consent for participation was obtained from each of the subjects in accordance with the Helsinki Declaration of the World Medical Association. Bronchial tissue samples were collected from the subcarinal region between the right lower lobe and middle lobe bronchi (origin of the right B6 bronchus) in the asthmatic subjects by using a pair of standard forceps during fiberoptic bronchoscopy, as previously described.[E5] Each biopsy specimen was immediately placed in OCT medium, snap-frozen in liquid nitrogen, and stored at −80°C until cryostat sectioning. The cells were dual stained with fluorescein

isothiocyanate–conjugated anti-tryptase mAb (clone AA1, Dako) or fluorescein isothiocyanate–conjugated anti-chymase mAb (clone CC1; MBL, Tokyo, Japan) and rabbit anti-human PAF-R antibody (Cayman Chemical).[E6] PAF-R$^+$ cells were visualized by means of incubation with Alexa Flour 647–conjugated anti-rabbit IgG (Invitrogen, Tokyo, Japan).

Lentivirus shRNA vector construction and gene transduction

The lentiviral expression plasmid with the sense and antisense oligonucleotide sequence for construction of 2 PLCγ1 (GenBank accession no. NM_002660) and 2 PLCβ2 (GenBank accession no. NM_004573) shRNAs were purchased from Sigma-Aldrich. Packaging vector (MISSION lentiviral packaging mix, Sigma-Aldrich), the lentiviral expression vector (pLKO<-puro) with PLC shRNA (MISSION shRNA plasmid DNA) or MISSION nontarget shRNA control vector (Sigma-Aldrich), or lentiviral expression vector encoding enhanced green fluorescent protein (EGFP) were cotransfected into 293FT packaging cells with TransIT (Takara Bio, Inc, Shiga, Japan). After 6 hours, the culture supernatants were centrifuged at 3,000 rpm for 10 minutes and filtered with a 0.45-μm filter. The filtered supernatants were centrifuged at 9,000 rpm overnight at 4°C, and the released virus was kept on ice for 1 hour. This step concentrated the supernatants from 220 mL to 1 mL. Transduction of human MCs (1 × 10^4) was conducted by resuspending the cells in 100 μL of virus containing IMDM. Two days after infection, the medium was changed to virus-free IMDM containing stem cell factor at 100 ng/mL and IL-6 at 50 ng/mL with 2% FCS, and antibiotic selection (1 mg/mL puromycin) was performed for 2 days.
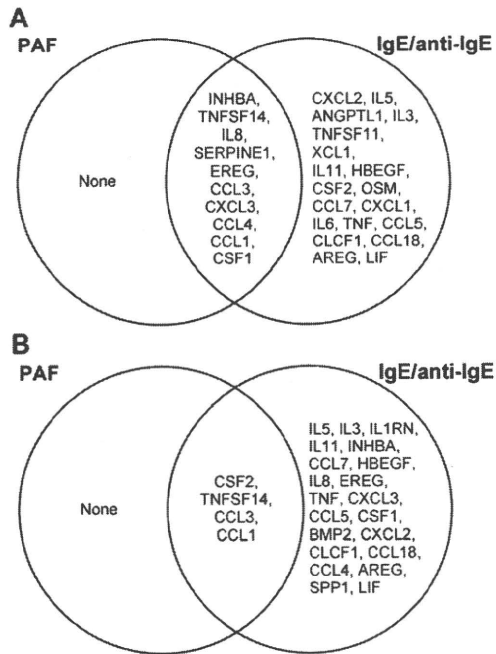
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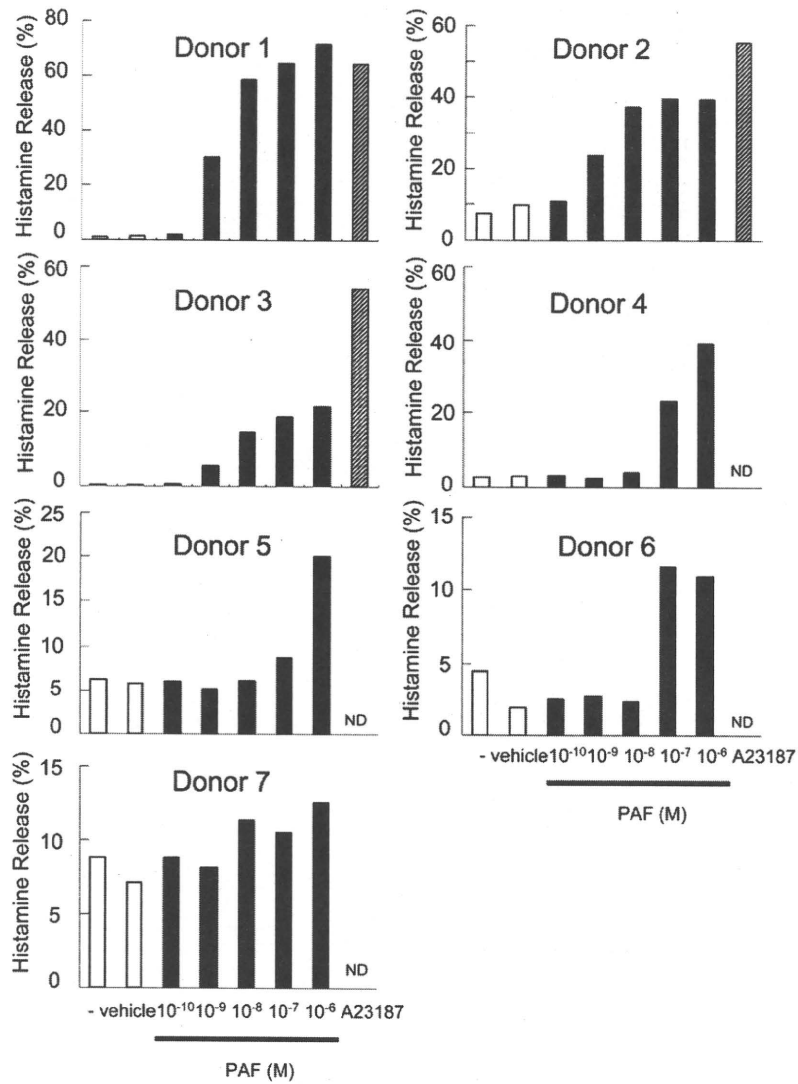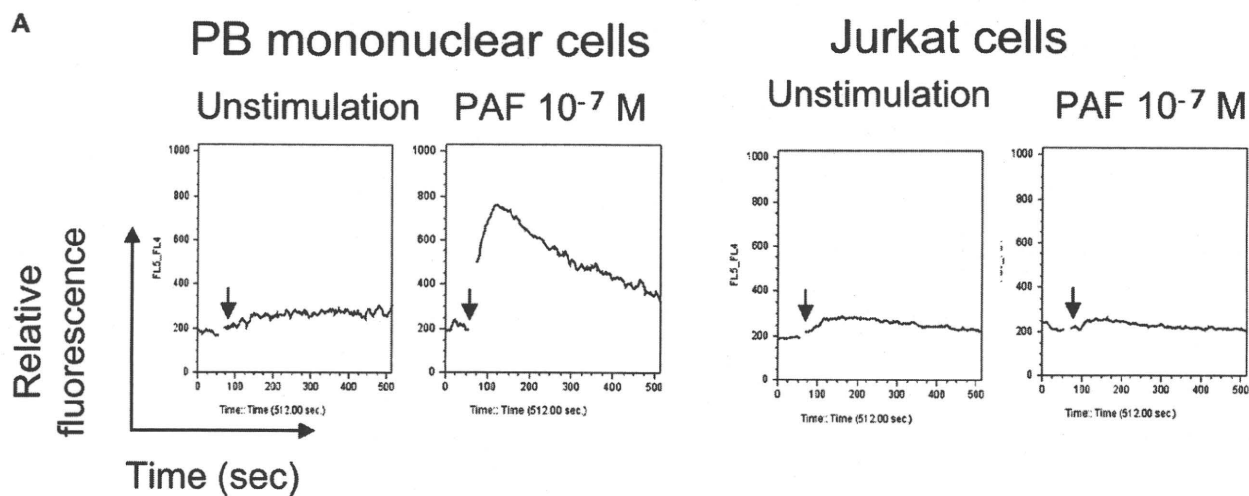
FIG E1. Variable response of histamine release from human lung MCs in response to PAF (n = 7). *ND*, Not determined.
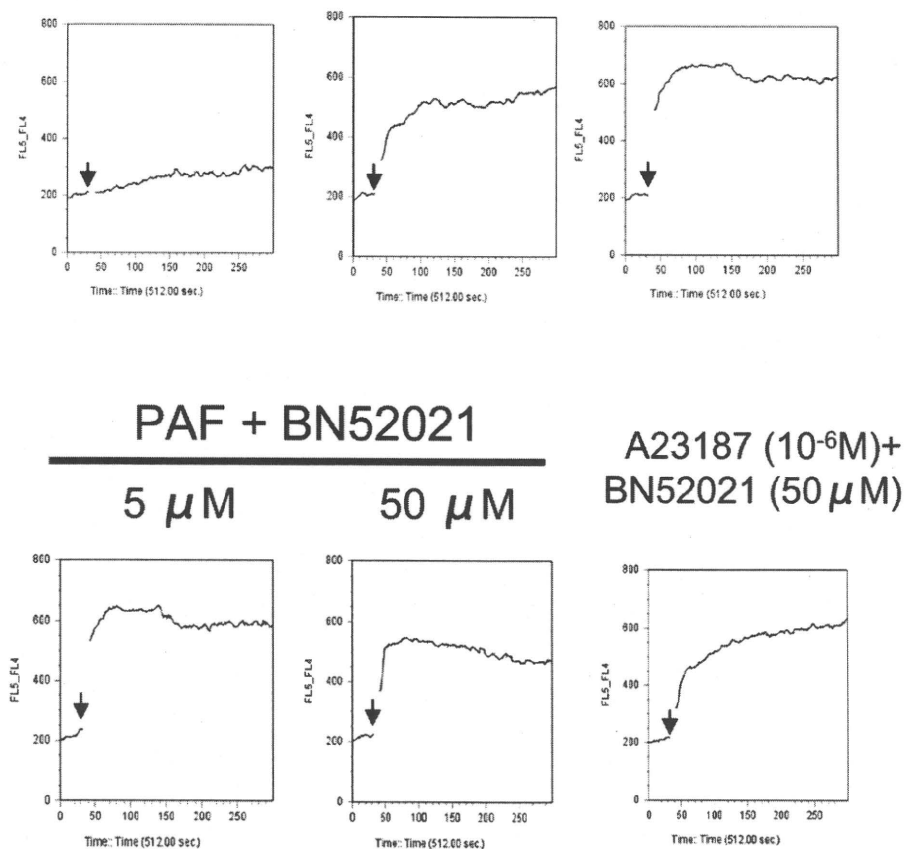
A

PAF **IgE/anti-IgE**

None

INHBA,
TNFSF14,
IL8,
SERPINE1,
EREG,
CCL3,
CXCL3,
CCL4,
CCL1,
CSF1

CXCL2, IL5,
ANGPTL1, IL3,
TNFSF11,
XCL1,
IL11, HBEGF,
CSF2, OSM,
CCL7, CXCL1,
IL6, TNF, CCL5,
CLCF1, CCL18,
AREG, LIF

B

PAF **IgE/anti-IgE**

None

CSF2,
TNFSF14,
CCL3,
CCL1

IL5, IL3, IL1RN,
IL11, INHBA,
CCL7, HBEGF,
IL8, EREG,
TNF, CXCL3,
CCL5, CSF1,
BMP2, CXCL2,
CLCF1, CCL18,
CCL4, AREG,
SPP1, LIF

FIG E2. PAF-R– or FcɛRI-mediated cytokine gene expression profiles in human PB-derived MCs. Human MCs were activated with PAF (10^{-7} mol/L) or IgE/anti-IgE (3 μg/mL) for 1 hour **(A)** and 4 hours **(B)**. Each experiment was performed with 3 independent donors, and the data are the average of 3 separate analyses. Overlapping sets of PAF- and IgE/anti-IgE–induced cytokine genes are shown. Stimulus-induced cytokine genes were selected if the ratio of the relative expression level between those with and without the stimulus was greater than 4.0.

A

PB mononuclear cells

Unstimulation   PAF 10⁻⁷ M

Jurkat cells

Unstimulation   PAF 10⁻⁷ M

Relative fluorescence

Time (sec)

B

Chloroform A23187 (10^{-6}M) PAF (10^{-8} M)

PAF + BN52021

5 μM   50 μM

A23187 (10^{-6}M)+ BN52021 (50 μM)

FIG E3. Ca^{2+} mobilization through PAF-R in PB-derived MCs in response to PAF. **A,** Ca^{2+} mobilization in Jurkat cells (n = 3) and PB mononuclear cells (n = 3) in response to PAF. **B,** Effect of the PAF-R antagonist BN52021 on PAF- or calcium ionophore A23187–induced Ca^{2+} mobilization (n = 3). Intracellular free calcium levels *(y-axis)* were monitored with the LSR system. The *x-axis* shows the incubation time after stimulation. *Arrows* indicate the time point of addition of PAF or calcium ionophore A23187. The data are representative of similar results obtained from 3 independent experiments with MCs from different donors.
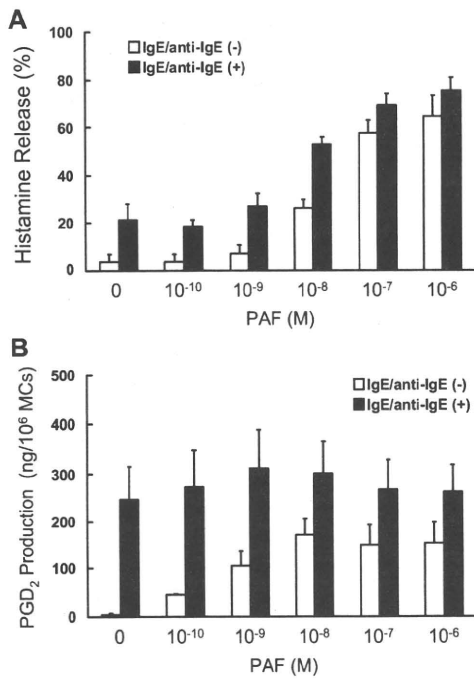
FIG E4. The additive effect of PAF on IgE-dependent histamine release and PGD$_2$ production by MCs. MCs were sensitized with IgE for 16 hours and then washed and challenged with PAF (10^{-10} to 10^{-6} mol/L) with (*IgE/anti-IgE [+]*, *solid bars*) or without (*IgE/anti-IgE [−]*, *open bars*; 0.3 μg/mL) anti-IgE for 30 minutes for histamine release (**A**, n = 3) and PGD$_2$ production (**B**, n = 4). Data shown are means ± SEMs.

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.

nterleukin-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

*Department of Allergy and Immunology, National Research Institute for Child Health and Development; ‡Department of Pediatrics, Keio University, School of Medicine, Tokyo; †Department of Dermatology, Fujita Health University, School of Medicine, Toyoake; and §Department of Dermatology, Nagoya University, School of Medicine, Nagoya, Japan

1A.Y. and K.O. contributed equally to this work.

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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'-CTGTC-TGGCCCTGAATTTGC-3'; antisense, 5'-AGCAGAGTGGCCTCAATC-CA-3'), sST2 (sense, 5'- CTGTCTGGCCCTGAATTTGC-3'; antisense, 5'-TGGAACCACACTCCATTCTGC-3'), IL-8 (sense, 5'-GTCTGCTAG-CCAGGATCCACAA-3'; antisense, 5'-GAGAAACCAAGGCACAGTGG-AA-3'), IL-6 (sense, 5'- CAATAACCACCCCTgACCCA-3'; antisense, 5'-GCGCAGAATGAGATGAGTTGTC-3'), STAT6 (sense, 5'-TCTGACCG-GCTGATCATTGG-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. 3D) 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. 1A, 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. 1A, 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. 1B, 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. 1B, 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. 1B, 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
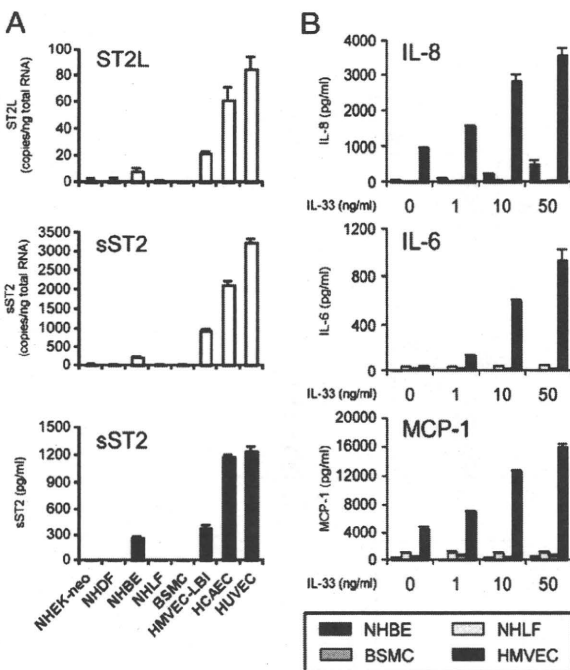
FIGURE 1. *A*, Expression of mRNA for ST2L and sST2 in cultured human primary cells. Total RNA was isolated from various human primary cells in growth phase, and the levels of mRNA for ST2L and sST2 were measured by quantitative real-time PCR (open bars). Concentrations of secreted sST2 protein in the 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
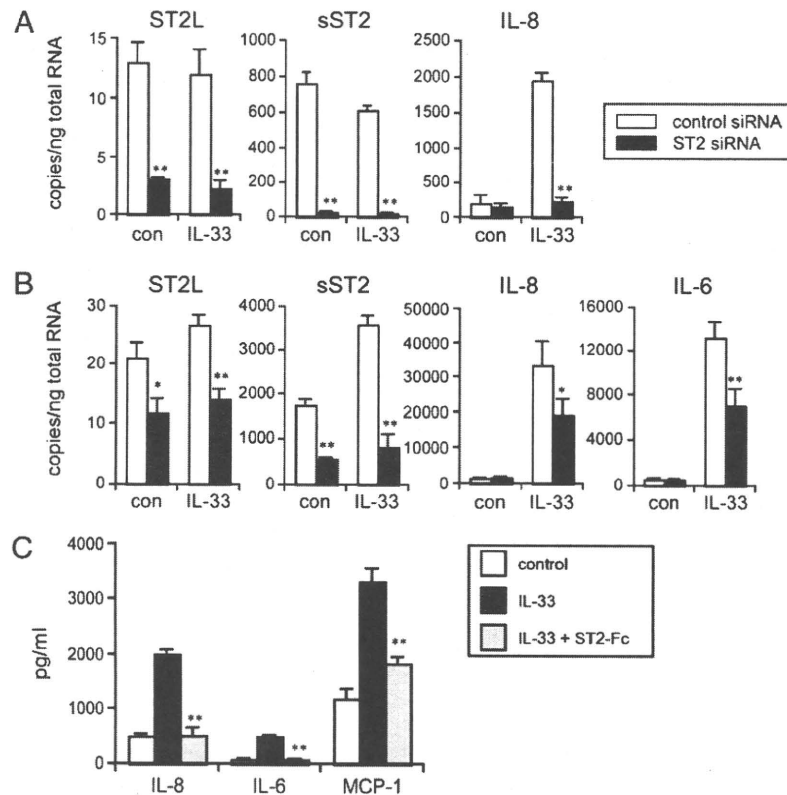
FIGURE 2. IL-33 mediates inflammatory responses via the ST2 receptor on HMVEC-LBl and NHBEs. *A* and *B,* Cultured NHBEs (*A*) and HMVEC-LBl (*B*) were transfected with siRNA against ST2 (solid bars) or nontargeting control siRNA (open bars) at 5 nM (NHBEs) or 10 nM (HMVEC-LBl). The transfected cells 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. 7A). 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. 7B). 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. 1A). Furthermore, there was good correspondence between the ST2 distribution (Fig. 1A) and IL-33 responsiveness (Fig. 1B) 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. 1A) or responsiveness to IL-33 (Fig. 1B), suggesting that
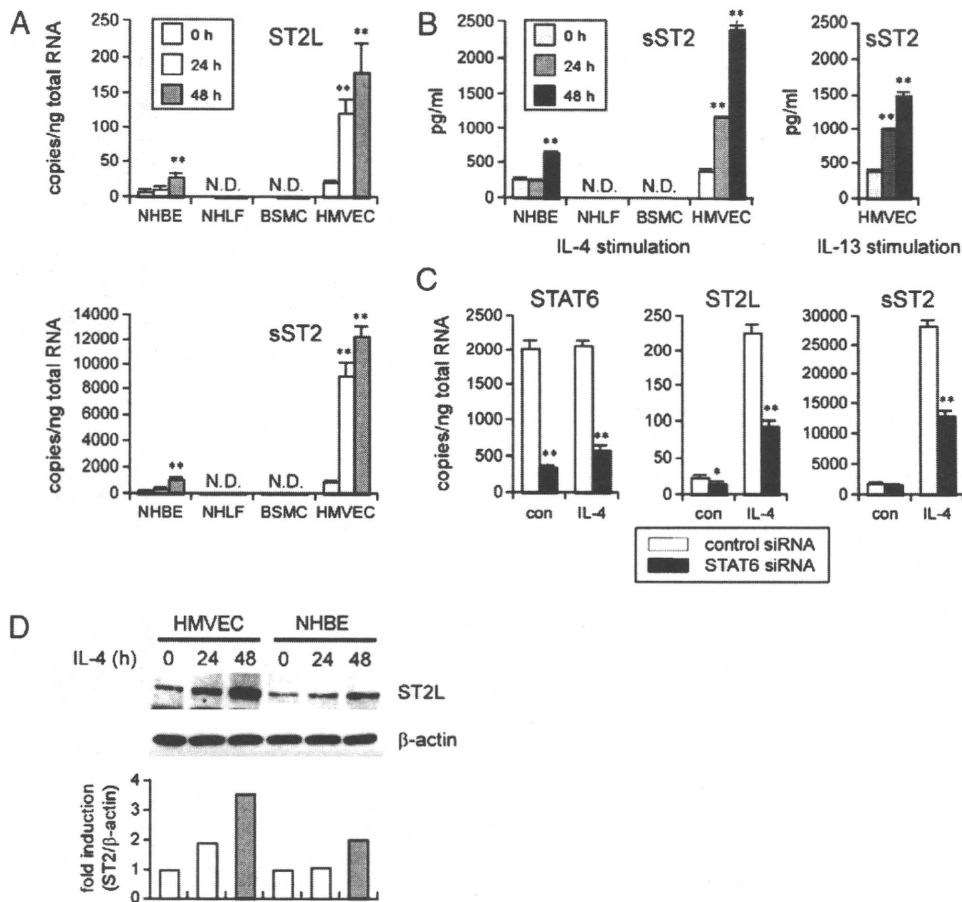
FIGURE 3. Effects of Th2 cytokines on the expression of ST2 in cultured lung tissue cells. Cells were treated with 10 ng/ml IL-4 for the indicated periods. *A*, The levels of mRNA for ST2L and sST2 are shown. *B*, The accumulated sST2 protein levels after 10 ng/ml IL-4 treatment (*left graph*) or 10 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
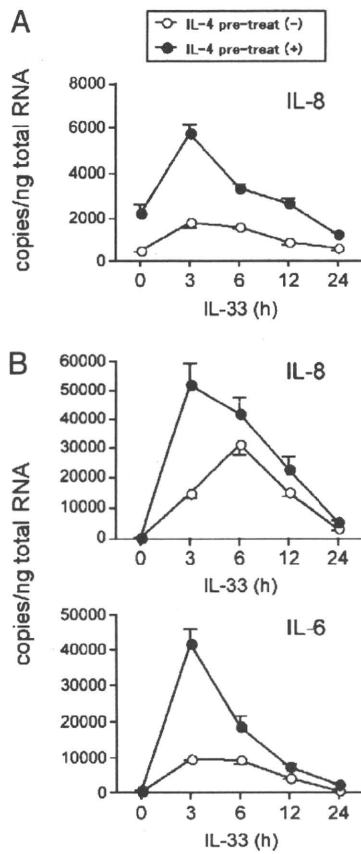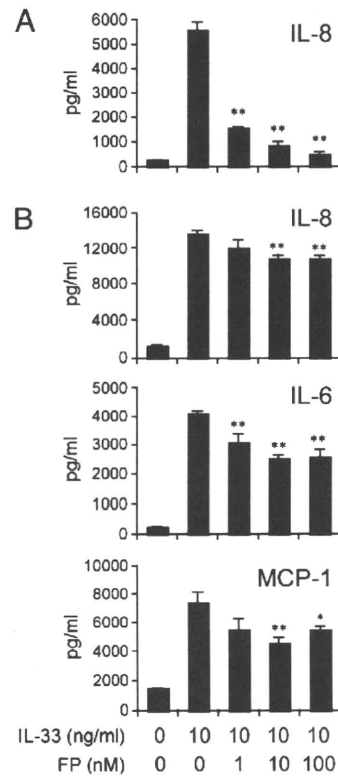
FIGURE 4. IL-33–mediated responses were further enhanced by IL-4 pretreatment of NHBEs and HMVEC-LBl. Cultured NHBEs (A) or HMVEC-LBl (B) were treated with 10 ng/ml IL-4 for 48 h, and then the cells were washed twice with HBSS and replaced with fresh medium containing 10 ng/ml IL-33 for the indicated 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. 5A), 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. 5B), which were found to be the main IL-33–targeted cells among the lung tissue cells (Fig. 1B). 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
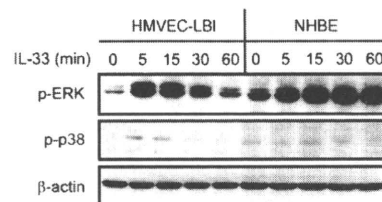
FIGURE 6. IL-33–induced phosphorylation of MAPK in cultured HMVEC-LBl and NHBEs. Whole-cell lysates were examined at the indicated time points after stimulation with 10 ng/ml IL-33 for the expression of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and β-actin (as a loading control).

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.
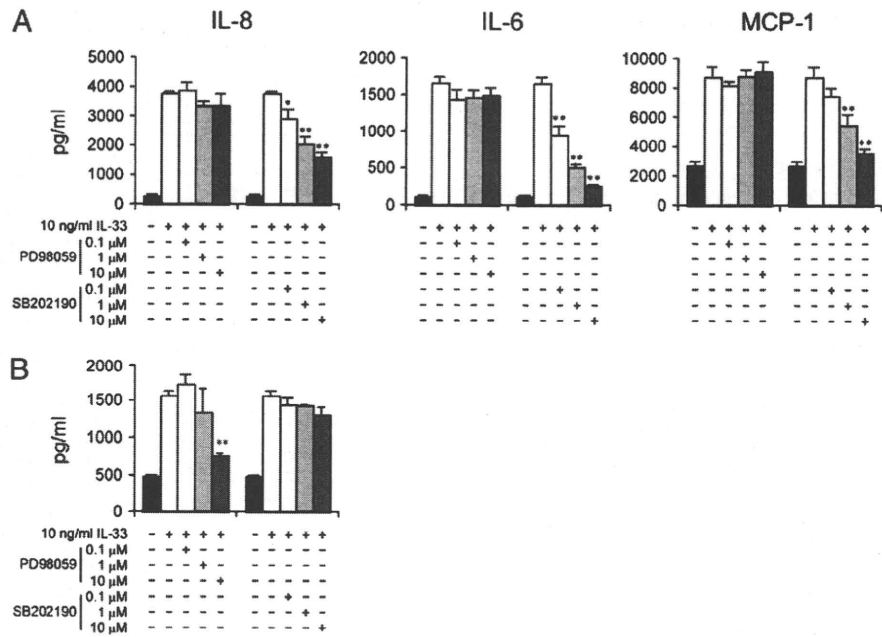
airway hypervascularity in severe asthma, an element of airway remodeling resulting from accelerated angiogenesis, responds poorly to corticosteroid treatment and is clinically involved in reduced lung function (27–29). We previously showed that autocrine CXCR2 chemokines, such as IL-8, are indispensable 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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Review

AAIR
Allergy, Asthma & Immunology Research

IL-33 and Airway Inflammation

Keisuke Oboki,[1] Susumu Nakae,[2] Kenji Matsumoto,[1] Hirohisa Saito[1]*

[1]Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan
[2]Frontier Research Initiative, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Interleukin-33 (IL-33) is the 11th member of IL-1 cytokine family which includes IL-1 and IL-18. Unlike IL-1β and IL-18, IL-33 is suggested to function as an alarmin that is released upon endothelial or epithelial cell damage and may not enhance acquired immune responses through activation of inflammasome. ST2, a IL-33 receptor component, is preferentially expressed by T-helper type (Th) 2 cells, mast cells, eosinophils and basophils, compared to Th1 cells, Th17 cells and neutrophils. Thus, IL-33 profoundly enhances allergic inflammation through increased expression of proallergic cytokines and chemokines. Indeed, IL-33 and its receptor genes are recognized as the most susceptible genes for asthma by several recent genome-wide association studies. It has also recently been shown that IL-33 plays a crucial role in innate eosinophilic airway inflammation rather than acquired immune responses such as IgE production. As such, IL-33 provides a unique therapeutic way for asthma, i.e., ameliorating innate airway inflammation.

Key Words: IL-33; ST2; host defense; allergy; autoimmunity; chronic disease; mast cell; basophil; eosinophil

INTRODUCTION

Identification of a nuclear protein and an orphan receptor as IL-33 and IL-33 receptor

Interleukin-33 (IL-33), a member of the IL-1 cytokine family, is considered to be crucial for the induction of T-helper type (Th) 2 cell-dominant immune responses such as host defense against nematodes and allergic diseases.[1] IL-33 was originally identified as "DVS27", a gene upregulated in vasospastic cerebral arteries after subarachnoid hemorrhage[2] and as a nuclear factor, "nuclear factor from high endothelial venules (NF-HEV)", which is expressed in endothelial cell nuclei.[3]

IL-33 receptor was first identified as an IL-1 receptor-like molecule and termed as ST2 (the gene symbol was termed as *IL-1RL1*) by Tominaga in 1989.[4] ST2 was subsequently found to be preferentially expressed in Th2 cells and started to attract many researchers involved in allergy.[5] In 2005, DVS27 was rediscovered as the 11th member in the IL-1 family of cytokines, which includes IL-1α, IL-1β, and IL-18, by computationally searching for the sequences containing β-trefoil structure seen in IL-1- and FGF-like proteins, and termed as IL1F11 or IL-33.[6]

IL-33 receptor and signal transduction

As the receptors for the other IL-1-related cytokines, the IL-33 receptor is formed heterodimeric molecules consisting of ST2 and IL-1 receptor accessory protein (IL-1RAcP; Fig. 1). IL-1RAcP is also known as a common component of receptors for IL-1α, IL-1β, IL-1F6, IL-1F8, and IL-1F9.[1]

The two major products of ST2 genes (*IL1RL1*), i.e., transmembrane form ST2 (ST2 or ST2L) and soluble form ST2 (sST2) are produced by alternative splicing under the control of two distinct promoters. ST2 is considered to be the functional component for induction of IL-33 bioactivities, while sST2 act as a decoy receptor for IL-33 like soluble IL-1Rs for IL-1.[1]

The signal transduction downstream of IL-33 receptor is mediated by common adapter molecules to that of the other IL-1 receptor family such as IL-1R and IL-18R. The binding of IL-33 to IL-33 receptor results in the recruitment of MyD88 to the Toll-interleukin-1 receptor domain in cytoplasmic region of ST2, leading to the induction of inflammatory mediators by activating transcription factors such as NF-κB and AP-1 through IRAK, TRAF6 and/or MAP kinases, like other IL-1 family receptor or Toll-like receptor (TLR) activation.[6]

Correspondence to: Hirohisa Saito, MD, PhD, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan.
Tel: +81-3-5494-7027; Fax: +81-3-5494-7028; E-mail: hsaito@nch.go.jp
Received: December 31, 2010; Accepted: January 4, 2011
• There are no financial or other issues that might lead to conflict of interest.