

COX-1 mediates IL-33-induced extracellular signal-regulated kinase activation in mast cells: Implications for aspirin sensitivity

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Background: Classical FcεRI-induced mast cell (MC) activation causes synthesis of arachidonic acid (AA)-derived eicosanoids (leukotriene [LT] C₄, prostaglandin [PG] D₂, and thromboxane A₂), which mediate vascular leak, bronchoconstriction, and effector cell chemotaxis. Little is known about the significance and regulation of eicosanoid generation in response to nonclassical MC activation mechanisms.

Objectives: We sought to determine the regulation and significance of MC-derived eicosanoids synthesized in response to IL-33, a cytokine critical to innate type 2 immunity.

Methods: We used an *ex vivo* model of mouse bone marrow-derived mast cells and an IL-33-dependent *in vivo* model of aspirin-exacerbated respiratory disease (AERD). **Results:** IL-33 potently liberates AA and elicits LTC₄, PGD₂, and thromboxane A₂ production by bone marrow-derived mast cells. Unexpectedly, the constitutive function of COX-1 is required for IL-33 to activate group IVa cytosolic phospholipase A₂ with consequent AA release for synthesis of all eicosanoids, including CysLTs. In contrast, COX-1 was dispensable for FcεRI-driven CysLT production. Inhibition of COX-1 prevented IL-33-induced phosphorylation of extracellular signal-related kinase, an upstream effector of cytosolic phospholipase A₂, which was restored by exogenous PGH₂, implying that the effects of COX-1 required its catalytic function. Administration of a COX-1-selective antagonist to mice completely prevented the generation of both PGD₂ and LTC₄ in a model of AERD in which MC activation is IL-33 driven.

Conclusions: MC-intrinsic COX-1 amplifies IL-33-induced activation in the setting of innate type 2 immunity and might help explain the phenomenon of therapeutic desensitization to aspirin by nonselective COX inhibitors in patients with AERD. (*J Allergy Clin Immunol* 2018;■■■■:■■■■-■■■■.)

Key words: Eicosanoids, mast cells, IL-33, aspirin-exacerbated respiratory disease, cyclooxygenase asthma

Abbreviations used

AA:	Arachidonic acid
AERD:	Aspirin-exacerbated respiratory disease
BMMC:	Bone marrow-derived mast cell
cPLA ₂ :	Cytosolic phospholipase A ₂
ERK:	Extracellular signal-regulated kinase
5-LO:	5-Lipoxygenase
LT:	Leukotriene
Lys-ASA:	Lysine-aspirin
MAPK:	Mitogen-activated protein kinase
MC:	Mast cell
MEK:	Mitogen-activated kinase kinase
PG:	Prostaglandin
R _L :	Lung resistance
SCF:	Stem cell factor
TX:	Thromboxane

Mast cells (MCs) are tissue-resident effector cells that initiate and propagate inflammation in both innate and adaptive immunity. Classical allergen-induced MC activation results from cross-linkage of the tetrameric high-affinity Fc receptor for IgE (FcεRI) occupied by antigen-specific IgE. MCs activated through this mechanism degranulate and rapidly release preformed mediators, such as histamine and proteases, in patients with anaphylaxis, rhinitis, and allergic asthma.¹ Simultaneously, MCs release arachidonic acid (AA) from cell membrane phospholipids through a calcium-dependent group IVa phospholipase A₂ (also known as cytosolic phospholipase A₂ [cPLA₂]),² which requires serine phosphorylation by extracellular signal-related kinase (ERK), p38 mitogen-activated protein kinase (MAPK), or both.³ Both calcium flux and MAPK-dependent phosphorylation are necessary for cPLA₂ to translocate from the cytosol to the intracellular membranes.

Once liberated by cPLA₂, AA is converted through COX-1 and/or COX-2 to prostaglandin (PG) H₂, which in turn is converted by hematopoietic PGD₂ synthase to PGD₂ and by thromboxane (TX) synthase to TXA₂.^{4,5} MCs also metabolize AA to leukotriene (LT) A₄ by means of 5-lipoxygenase (5-LO)-dependent oxidation.⁶ LTA₄ is converted by LTA₄ hydrolase to LTB₄⁷ and by LTC₄ synthase to LTC₄,⁸ the parent of the cysteinyl leukotrienes (CysLTs) LTD₄ and LTE₄. PGD₂, TXA₂, CysLTs, and LTB₄ cooperate to elicit bronchoconstriction,⁹ changes in vascular tone and permeability,¹⁰ priming of endothelial adhesion pathways,¹¹ and recruitment and activation of bloodborne hematopoietic cells to the tissues.¹² Consequently, AA-derived eicosanoids are strongly implicated in the clinical and physiologic manifestations of type 1 hypersensitivity and likely also contribute to IgE-dependent adaptive immunity to helminths.¹³ The mechanisms regulating their production are of substantial importance.

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Although the biochemistry of FcεRI-dependent eicosanoid generation by MCs is well studied, comparatively little is known about how this process is regulated during nonclassical, antigen-independent MC activation. MCs can be activated by proteases,¹⁴ complement fragments,¹⁵ lipids,¹⁶ LPS,¹⁷ and endogenous cytokines.¹⁸ MCs express the IL-1 receptor family member ST2, which binds the potent type 2 cytokine IL-33.¹⁹ Like other IL-1 receptor family members, ST2 activates ERK, p38, and c-Jun terminal kinase and induces nuclear factor κB-dependent transcriptional activity through the canonical myeloid differentiation primary response gene-88 pathway.²⁰ Release of IL-33 from injured or activated epithelial and endothelial cells initiates type 2 immune responses to allergens,^{21,22} helminths,^{23,24} and viruses²⁵ by activating cells of the innate immune system (including MCs).²⁶ Tissue levels of IL-33 (and expression of ST2) are strongly upregulated in patients with diseases characterized by robust type 2 immunopathology and increased numbers of activated tissue MCs,^{27,28} such as severe asthma^{29,30} and chronic rhinosinusitis.³¹ Although MCs generate abundant quantities of IL-5 and IL-13 in response to IL-33 *ex vivo*,¹⁹ little is known about whether IL-33-driven, ST2-dependent MC activation elicits eicosanoid generation; how this process is regulated; and the nature of the physiologic consequences.

Aspirin-exacerbated respiratory disease (AERD) is a distinctive clinical syndrome characterized by severe type 2 respiratory tract immunopathology, asthma, chronic rhinosinusitis, and idiosyncratic reactions to COX-1-active drugs (but generally not COX-2-selective drugs)³² characterized by MC activation and high-level synthesis of both COX (PGD₂) and 5-LO (LTC₄) products.³³ Paradoxically, COX-1 inhibitors also elicit “desensitization,” permitting subsequent administration of COX-1 inhibitors without eliciting symptoms or MC activation and daily administration of aspirin is therapeutic.³⁴ IL-33 is strongly expressed in the sinonasal tissues of patients with AERD.³⁵

Recently, we demonstrated that MC activation (with attendant release of LTs and PGD₂) in a mouse model of AERD depends on IL-33 and ST2.³⁵ Therefore we undertook this study to understand the mechanisms by which IL-33 induces lipid mediator generation by MCs. We found that IL-33 induces AA release from mouse bone marrow-derived mast cells (BMMCs), resulting in concomitant generations of PGD₂, TXA₂, and LTC₄. Unexpectedly and in contrast to classical MC activation, IL-33-induced AA release and synthesis of all eicosanoids hinges critically on the constitutive activity of COX-1. COX-1 activity is necessary for IL-33-dependent phosphorylation of ERK, but not p38, and is essential for downstream ERK-mediated phosphorylation of cPLA₂. Treatment of AERD-like mice with SC560, a selective COX-1 inhibitor, elicits desensitization to aspirin and prevents IL-33-driven MC activation and production of both PGD₂ and LTC₄ *in vivo*.

Our findings reveal a previously unrecognized role for COX-1 in regulating signaling events in MCs downstream of IL-33 and ST2. This mechanism might affect both the therapeutic benefits of COX inhibitors, including desensitization to COX-1 inhibitors, and treatment with aspirin in patients with AERD, which prevents increases in MC-dependent eicosanoids with aspirin challenge and improve outcomes.^{34,36}

METHODS

Reagents

Chemicals were purchased from Sigma-Aldrich (St Louis, Mo), unless otherwise stated. Murine IL-33, IL-3, and stem cell factor (SCF) were from PeproTech (Rocky Hill, NJ). Selective COX-1 inhibitors (SC560 and FR122047), PGH₂, PGD₂-MOX, TXB₂, and CysLT EIA kits were purchased from Cayman Chemical (Ann Arbor, Mich). The MAPK kinase inhibitor U0126 and the p38 inhibitor SKF-86002 were obtained from Cell Signaling Technologies (Danvers, Mass). *Dermatophagoides farinae* was obtained from Greer Laboratories (Lenoir, NC; XPB81D3A25).

Cell culture and transfection

BMMCs were derived from wild-type mouse bone marrow cells cultured at 37°C in a humidified incubator with 5% CO₂ in RPMI 1640 supplemented with 10% FBS, glutamine (4 mmol/L), sodium pyruvate (1 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), nonessential amino acids (10 mL/L), and β-mercaptoethanol (50 μmol/L) in the presence of mouse recombinant IL-3 (10 ng/mL) and SCF (10 ng/mL).³⁷ BMMCs were studied at 4 to 6 weeks when virtually all cells were stained with toluidine blue and used for experiments within 6 weeks after achieving purity. For downregulation of endogenous COX-1, BMMCs were transfected with SMART pool ON-TARGETplus Ptgsl or a pool of 4 nontargeting small interfering RNAs as a control (300 nmol/L; L-040883-01-0005, D-001810-10-05; Dharmacon, Lafayette, Colo). Electroporation was performed by using an MAXA Nucleofector II (Lonza, Basel, Switzerland) with mouse macrophage cell buffer (VPA1009; Lonza) and program T001. Cells were suspended in MC culture medium immediately after electroporation for 24 hours before further experiments. Downregulation of endogenous COX-1 was confirmed by quantitative PCR (qPCR) of COX-1 transcripts. Human MCs were derived from umbilical cord blood progenitor cells by using a combination of recombinant SCF (100 ng/mL), IL-6 (50 ng/mL), and IL-10 (10 ng/mL; R&D Systems, Minneapolis, Minn), as described previously,³⁸ and primed for 3 days with IL-4 (10 ng/mL) before activation.³⁹

Cell stimulation

BMMCs (1.5×10^6 cells/mL) were stimulated with IL-33 (10 ng/mL) or sensitized overnight with mouse IgE SPE-7 (0.2 μg/mL). Excess IgE was washed off the next day before dinitrophenyl-human serum albumin was used (1 μg/mL). In some conditions COX-1-specific antagonists (10 nmol/L SC560 or 300 nmol/L FR122047) were added. Both supernatants and cell pellets were collected for measurement of eicosanoid production and COX-1 and COX-2 transcripts over time. In some experiments supernatants were analyzed for eicosanoids by using mass spectrometry at the University of California at San Diego Lipid Maps Core Facility (www.ucsd-lipidmaps.org).

qPCR

RNA was extracted from BMMC pellets by using the RNeasy Plus Mini Kit (Qiagen, Germantown, Md). Levels of COX-1 and COX-2 transcription over time were measured by using RT2 SYBR Green qPCR Master Mix with primers designed specifically to COX-1 or COX-2 mRNA. Relative quantities of mRNA were determined by comparison with glyceraldehyde-3-phosphate dehydrogenase.

Western blotting

Total BMMC lysates were prepared by centrifuging cells into ice-cold cell lysis buffer (RIPA, 5 mmol/L EDTA, and 1× complete protease inhibitor cocktail; Roche, Mannheim, Germany) and incubation on ice for 10 minutes with repeated vortexing, followed by denaturing in boiling Laemmli sample buffer. Proteins were resolved by using SDS-PAGE and transferred onto polyvinylidene difluoride by means of wet transfer. Membranes were blocked

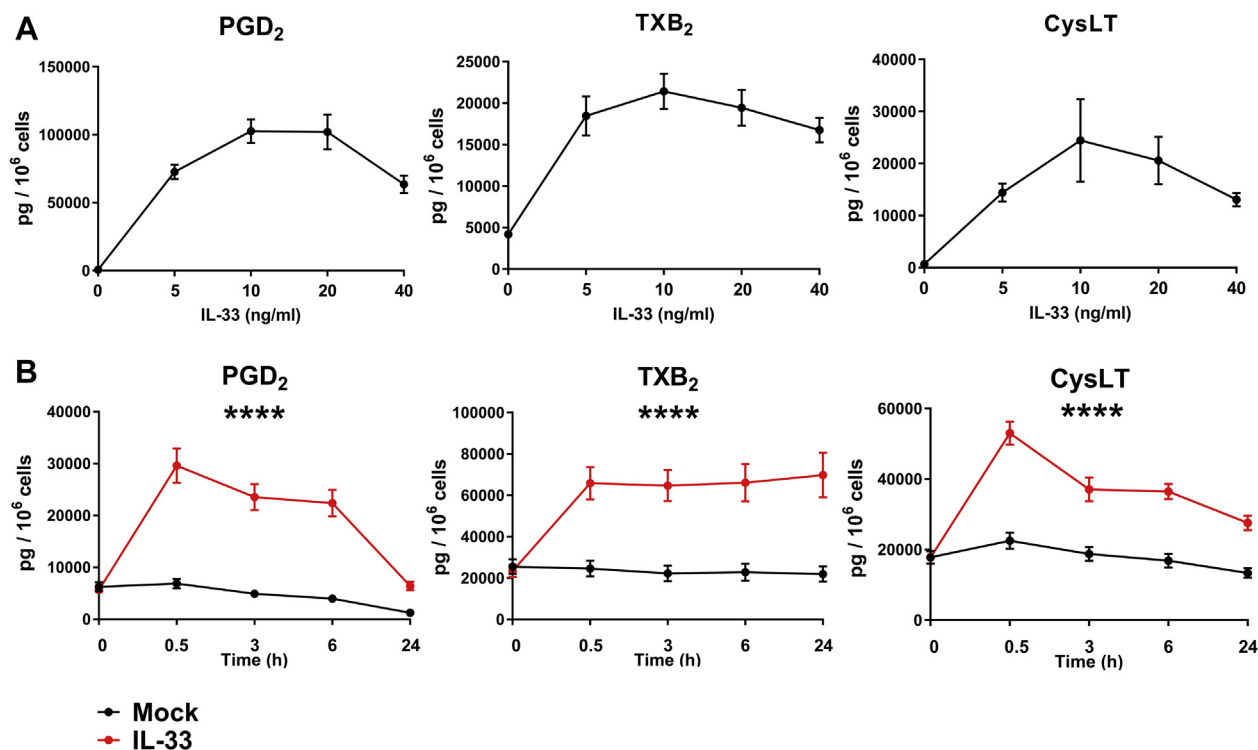


FIG 1. IL-33 induced eicosanoid generation by BMMCs. **A**, Cells were stimulated with IL-33 at various concentrations. Media were collected at 3 hours, and the indicated lipid mediators were analyzed by ELISA. **B**, Time course of IL-33-induced eicosanoid production at 10 ng/mL. Results are means $\pm$ ranges pooled from 2 independent experiments for Fig 1, A, and means $\pm$ SEMs from 10 independent experiments for Fig 1, B. **** $P < .0001$ across all time points by using 2-way ANOVA.

in TBS and 0.05% Tween-20 containing 5% blotting-grade blocker. The following primary antibodies were used (Cell Signaling Technology, Danvers, Mass) at the manufacturer's recommended concentrations: phospho-p44/42 MAPK (Erk1/2) 197G2, p44/42 MAPK (Erk1/2), phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK antibody, phospho-cPLA₂ and cPLA₂. Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, Calif). Detection was done with Amersham ECL (Amersham, Piscataway, NJ) and Kodak X-ray film (Kodak, Rochester, NY). Where required, membranes were stripped in Restore Western Blot Stripping Buffer (Thermo Scientific, Waltham, Mass) and reprobed. For densitometric analysis, films were scanned, and band intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, Md).

ELISA analysis of lipids

Cultured BMMC supernatants stimulated with IL-33 or IgE were analyzed for PGD₂, TXB₂, and CysLTs with Cayman ELISA assays, according to the manufacturer's protocol.

Mice

All animal protocols were approved by the Animal Care and Use Committee of Brigham and Women's Hospital. Wild-type C57BL/6 mice were purchased from Charles River (Wilmington, Mass). C57BL/6 mice lacking microsomal prostaglandin E synthase-1 (*Ptges*^{-/-} mice) were from Dr Shizuo Akira (Osaka University, Japan).⁴⁰ Animals were housed for at least 1 week at the Building for Translational Medicine before experiments were done. *Ptges*^{-/-} mice were primed with 6 intranasal doses of *D. farinae* (3 μ g) before lysine-aspirin (Lys-ASA) challenge.⁴¹ Animals receive 2 doses of SC560 (or vehicle control) intraperitoneally (10 mg/kg) at 48 and 24 hours before challenge with aerosolized Lys-ASA. Measurement of lung resistance

(R_L), collection of bronchoalveolar lavage fluid, and measurements of PGD₂ and CysLT levels were carried out as described previously.⁴¹

Statistics

Statistical analysis was done with the unpaired Student *t* test for 2 samples or 1-way ANOVA for 3 or more samples, followed by appropriate multiple comparison tests, as appropriate. Data with irregular distribution or unequal variance were assessed by using the unpaired Student *t* test with the Welch correction or by using nonparametric tests, either the Mann-Whitney test for 2-sample analysis or the Kruskal-Wallis test for 3 or more samples, as appropriate. *P* values of less than .05 were considered statistically significant.

RESULTS

IL-33 induces generation of both COX and 5-LO products by BMMCs

To determine whether MCs generated lipid mediators in response to stimulation with IL-33, we activated BMMCs with IL-33 over a range of concentrations (5–40 ng/mL) for 30 minutes. Supernatants were analyzed by using respective commercial ELISAs specific for detection of PGD₂, TXB₂ (a stable metabolite of TXA₂), and CysLTs. IL-33-stimulated BMMCs generated all 3 mediators at 30 minutes, with the optimal effect at 10 ng/mL (Fig 1, A). IL-33-induced PGD₂, TXA₂, and CysLT generation peaked at approximately 0.5 to 3 hours (Fig 1, B). Concentrations of PGD₂ in media decreased after 3 hours, likely reflecting conversion to stable metabolites. TXB₂ and CysLT levels remained stable after 3 hours.

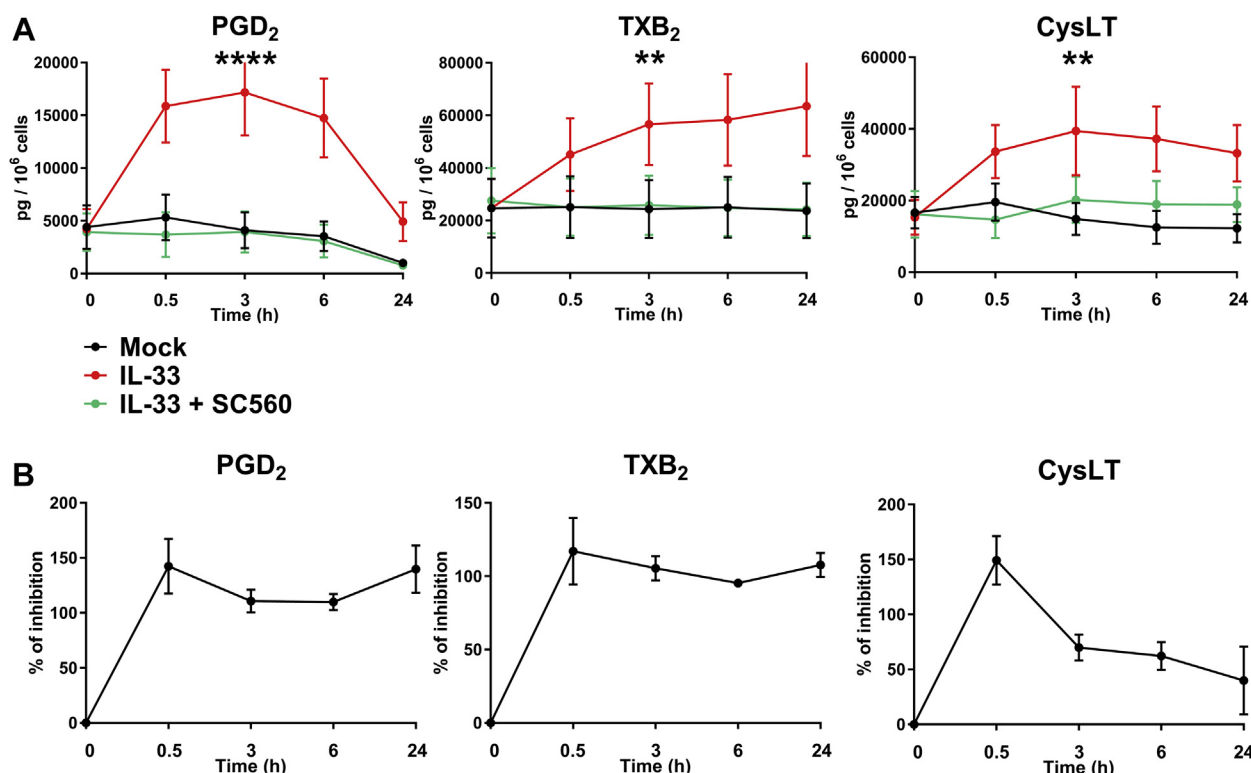


FIG 2. Effect of COX-1 blockade on IL-33-induced eicosanoid production. BMMCs were stimulated with IL-33 (10 ng/mL) in the presence of the COX-1 antagonist SC560 (10 nmol/L) or vehicle control. **A**, PGD₂, TXB₂, and CysLTs levels were measured in culture supernatants harvested at the indicated time points. **B**, Net percentage inhibition (subtracting quantities present in the mock control) for each experiment was calculated at each time point. Data are means $\pm$ SEMs from 5 independent experiments. ** $P < .01$ and **** $P < .0001$ comparing SC560-treated samples with control samples across all time points by using 2-way ANOVA.

COX-1 function is necessary for IL-33-induced expression of COX-2 by BMMCs

We next sought to determine which COX isoforms were necessary for IL-33 to induce PGD₂ and TXA₂ production by BMMCs. BMMCs were stimulated with IL-33 in the absence or presence of SC560, a selective inhibitor of COX-1. SC560 markedly suppressed the IL-33-induced production of PGD₂ and TXA₂ (Fig 2, A, left and center panels), approaching complete inhibition throughout the time course (Fig 2, B). Unexpectedly, SC560 also prevented CysLT generation in response to IL-33 (Fig 2, A, right panel), with complete inhibition at the 30-minutes time point and 50% to 75% thereafter (Fig 2, B). In contrast, treatment of the cells with the selective COX-2 inhibitors valdecoxib and SC236 attenuated generation of PGD₂ and TXA₂ but not of CysLTs (see Fig E1 in this article's Online Repository at www.jacionline.org). Stimulation of BMMCs with IL-33 did not change COX-1 expression levels but induced a sharp and transient increase in expression of COX-2 mRNA at 0.5 hours, decreasing to baseline levels by 6 hours (see Fig E2, A, in this article's Online Repository at www.jacionline.org). IL-33-induced upregulation of COX-2 mRNA was completely blocked by SC560 (see Fig E2, A) but not by SC236 (see Fig E2, D).

To verify that the effects of SC560 were not off target, we performed similar studies with a second COX-1 selective inhibitor, FR122047, and studied the effects of small interfering

RNA knockdown of COX-1 on both LTC₄ production and inducible COX-2 expression in response to IL-33. Both FR122047 and COX-1 knockdown (approximately 70% efficiency; see Fig E3, B, in this article's Online Repository at www.jacionline.org) resulted in diminished IL-33-mediated production of PGD₂, TXA₂, and LTC₄ (see Fig E3, A and C) and expression of COX-2 mRNA (see Fig E2, B and C).

To determine whether the effects of COX-1 blockade were stimulus specific, we treated passively sensitized BMMCs with SC560 before challenge with specific allergen. SC560 suppressed FcεRI-mediated production of both PGD₂ and TXA₂ by BMMCs (Fig 3, left and middle panels). However, in sharp contrast to its effects on IL-33-induced activation, it did not alter the FcεRI-mediated production of LTC₄ (Fig 3, right panel) and did not alter the induced expression of COX-2 in response to this stimulus (see Fig E4 in this article's Online Repository at www.jacionline.org).

Exogenous PGH₂ reverses COX-1-mediated inhibition of IL-33-induced MC activation responses and induces eicosanoid formation

COX-1 and COX-2 catalyze conversion of AA to PGH₂, the immediate precursor of all bioactive prostaglandins. To determine whether inhibition of IL-33-driven MC activation by SC560 was due to a loss of COX-1 catalytic activity and not to an off-target

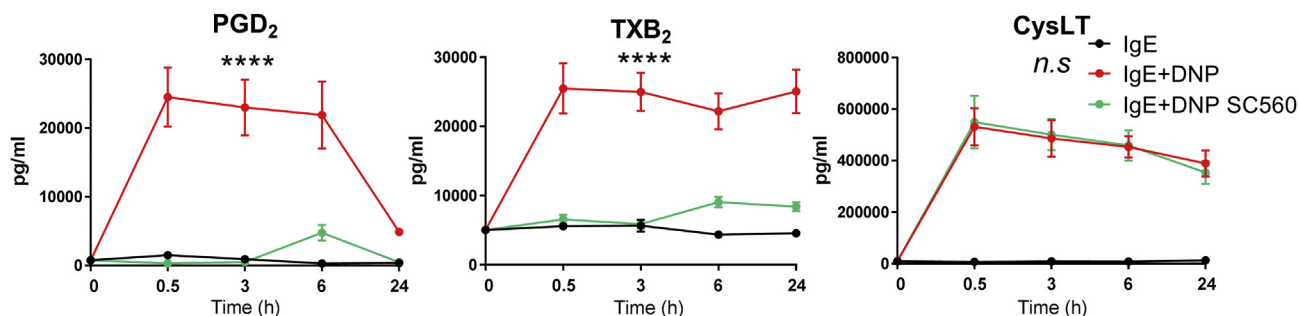


FIG 3. Effect of COX-1 inhibition on IgE-induced eicosanoid generation. BMMCs were sensitized with IgE (SPE-7, 0.2 μ g/mL) overnight before antigen stimulation (1 μ g/mL) in the presence or absence of SC560. Eicosanoid levels were measured in culture supernatants harvested at the indicated time points. Results are means $\pm$ SEMs from 3 independent experiments. **** P < .0001 comparing SC560-treated samples with control samples across all time points by using 2-way ANOVA. DNP-HSA, (2,4)-Dinitrophenyl-human serum albumin; n.s., not significant.

effect of the drug, we added PGH₂ (the product of COX-mediated AA metabolism) to some samples of BMMCs stimulated with IL-33 in the absence or presence of SC560. We also treated some samples of cells with PGH₂ in the absence of other stimuli or inhibitors. Exogenous PGH₂ restored production of both PGD₂ (Fig 4, A) and TXA₂ (Fig 4, B) by SC560-treated BMMCs stimulated with IL-33. PGH₂ also restored the IL-33-mediated generation of CysLTs in a dose-dependent manner (Fig 4, C) and also elicited production of all 3 lipids in the absence of IL-33 (Fig 4). Exogenous PGH₂ restored the IL-33-induced upregulation of COX-2 mRNA expression that was inhibited by SC560 and induced some COX-2 expression in the absence of IL-33 (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast to PGH₂, neither PGD₂ nor the TP receptor selective agonist U46619 restored IL-33-induced COX-2 expression or production of CysLTs in cells treated with SC560 (not shown).

COX-1 inhibition selectively blocks IL-33-mediated AA release and formation of all AA-derived eicosanoids but not release of ω -3 fatty acids

To identify the control points at which COX-1-derived PGH₂ controls IL-33-induced eicosanoid formation by MCs and to verify the surprising ELISA results, we analyzed a broad spectrum of lipid mediators generated by BMMCs stimulated with IL-33 in the absence or presence of SC560 by using a mass spectrometry-driven lipidomic approach. Consistent with the ELISA data, supernatants from the IL-33-activated BMMCs contained substantial quantities of PGD₂ and TXB₂ (Fig 5, A), total 5-LO products, LTB₄, and LTC₄ (reflected by the stable conversion product LTE₄; Fig 5, B) at 30 minutes. At 24 hours, PGD₂ levels had decreased, whereas corresponding levels of the PGD₂ metabolite PGJ₂ had increased (data not shown) and levels of TXB₂ and LTE₄ remained constant. Total quantities of both COX and 5-LO pathway products were reduced markedly in samples from BMMCs that were stimulated with IL-33 in the presence of SC560 compared with controls (Fig 5, A and B). Stimulation with IL-33 induced rapid (30 minutes) release of AA (Fig 5, C, left panel), as well as the ω -3 fatty acids eicosapentaenoic acid (Fig 5, C, center) and (to a lesser extent) docosahexaenoic acid (Fig 5, C, right). Treatment of the cells

with SC560 markedly suppressed release of AA but not eicosapentaenoic acid or docosahexaenoic acid release (Fig 5, C).

IL-33-induced activation of ERK, but not p38, depends on COX-1-derived PGH₂

cPLA₂, the major enzyme responsible for AA release in MCs, requires phosphorylation of p38 and/or ERK MAPKs for its translocation and activation.^{3,42} p38 and ERK can also facilitate transcriptional induction of COX-2.^{43,44} Because COX-1 blockade altered both the release of AA and the induction of COX-2 mRNA expression by IL-33-stimulated BMMCs, we speculated that COX-1-derived PGH₂, conversion products, or both can regulate MAPK activation (and consequently cPLA₂ activation) downstream of IL-33 stimulation. Stimulation of BMMCs with IL-33 induced modest phosphorylation of cPLA₂ at 30 minutes (52% $\pm$ 15% over baseline, n = 5; Fig 6, A), corresponding to the peak of mediator generation. IL-33 also induced strong phosphorylation of ERK at 30 minutes that decrease at 3 hours and returned to baseline at 6 hours (Fig 6, B). Similar kinetics were observed for phosphorylation of mitogen-activated kinase kinase (MEK; not shown), as well as p38 (Fig 6, C). Treatment with SC560 eliminated the transient IL-33-induced phosphorylation of cPLA₂ (Fig 6, A), MEK (data not shown), and ERK (Fig 6, B) in all 5 experiments performed but not p38 (Fig 6, C). Addition of PGH₂ to the IL-33-stimulated cells treated with SC560 restored ERK phosphorylation and induced phosphorylation of ERK and cPLA₂, even in otherwise unstimulated cells (Fig 6, D, and see Fig E6 in this article's Online Repository at www.jacionline.org). In contrast, PGH₂ tended to decrease the phosphorylation of p38 (see Fig E6). Treatment of the cells with the MEK inhibitor U0126 prevented production of CysLTs, PGD₂, and TXA₂ stimulated with IL-33 (Fig 6, E). Similar results were obtained with the ERK inhibitor FR 180204 (10 μ mol/L; see Fig E7, A, in this article's Online Repository at www.jacionline.org). In contrast, the p38 inhibitor SKF-86002 blocked IL-33-induced production of PGD₂ and TXA₂ but had no effect on production of LTC₄ (see Fig E7, B). Both U0126 and SKF-86002 blocked the IL-33-induced expression of COX-2 nearly completely (data not shown). IL-33-stimulated human cord blood-derived MCs also exhibited strong ERK phosphorylation that was blocked by SC560 but did not show CysLT generation (data not shown).

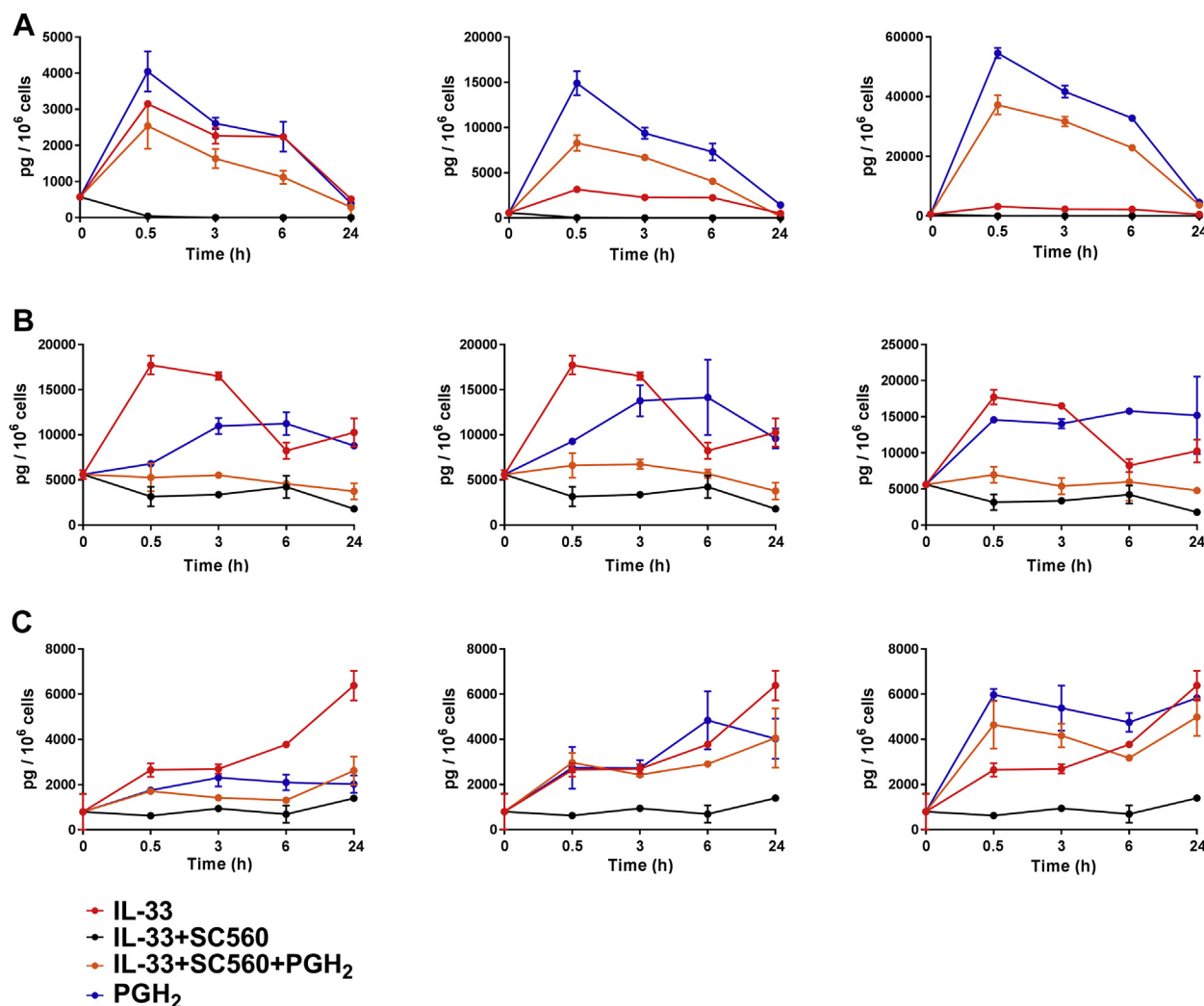


FIG 4. Effect of exogenous PGH₂ on eicosanoid production alone or in the presence of IL-33 with COX-1 blockade. BMMCs were stimulated with IL-33 (10 ng/mL) with or without SC560 (10 nmol/L). PGH₂ was added to some samples at the indicated concentrations. PGD₂ (A), TXA₂ (B), and CysLT (C) levels were measured in culture supernatants harvested at the indicated time points. Results are means of duplicates from a single representative experiment repeated twice with the same trends.

COX-1 inhibition blocks eicosanoid production in response to Lys-ASA challenge in a mouse model of AERD

To determine whether IL-33-driven MC activation and eicosanoid formation *in vivo* depend on COX-1, we studied the effect of pharmacologic inhibition of COX-1 in AERD-like *Ptges*^{-/-} mice after establishment of allergic airway inflammation by means of repetitive intranasal priming by *D. farinae*.⁴¹ We administered SC560 or vehicle control intraperitoneally daily for the 48 hours before challenge and then challenged the mice with inhaled Lys-ASA. As expected, vehicle control-treated mice showed sharp increases in airway resistance (R_L ; Fig 7, A) when challenged with Lys-ASA accompanied by sharply increased bronchoalveolar lavage fluid levels of both PGD₂ and CysLTs (Fig 7, B). In contrast, SC560-treated mice showed dramatically reduced changes in R_L and CysLT and PGD₂ levels when challenged with Lys-ASA.

To determine whether the absence of cell-intrinsic microsomal prostaglandin synthase-1 altered the response of BMMCs to IL-33, we activated wild-type and *Ptges*^{-/-} BMMCs with IL-33. *Ptges*^{-/-} BMMCs generated similar quantities of LTC₄, PGD₂, and TXA₂ in response to IL-33, as did wild-type controls (data not shown). SC560 blocked production of all 3 mediators by the *Ptges*^{-/-} BMMCs (see Fig E8 in this article's Online Repository at www.jacionline.org).

DISCUSSION

MCs are strategically located at tissue interfaces with the external environment and the microvasculature to serve sentinel functions in both adaptive and innate immunity. Their role in IgE-dependent type 1 hypersensitivity, a process involved in all allergic diseases, is undisputed. This role includes mobilization of AA for conversion to potent eicosanoids in response to allergen exposure. LTC₄ and its metabolites, LTD₄ and LTE₄, are powerful

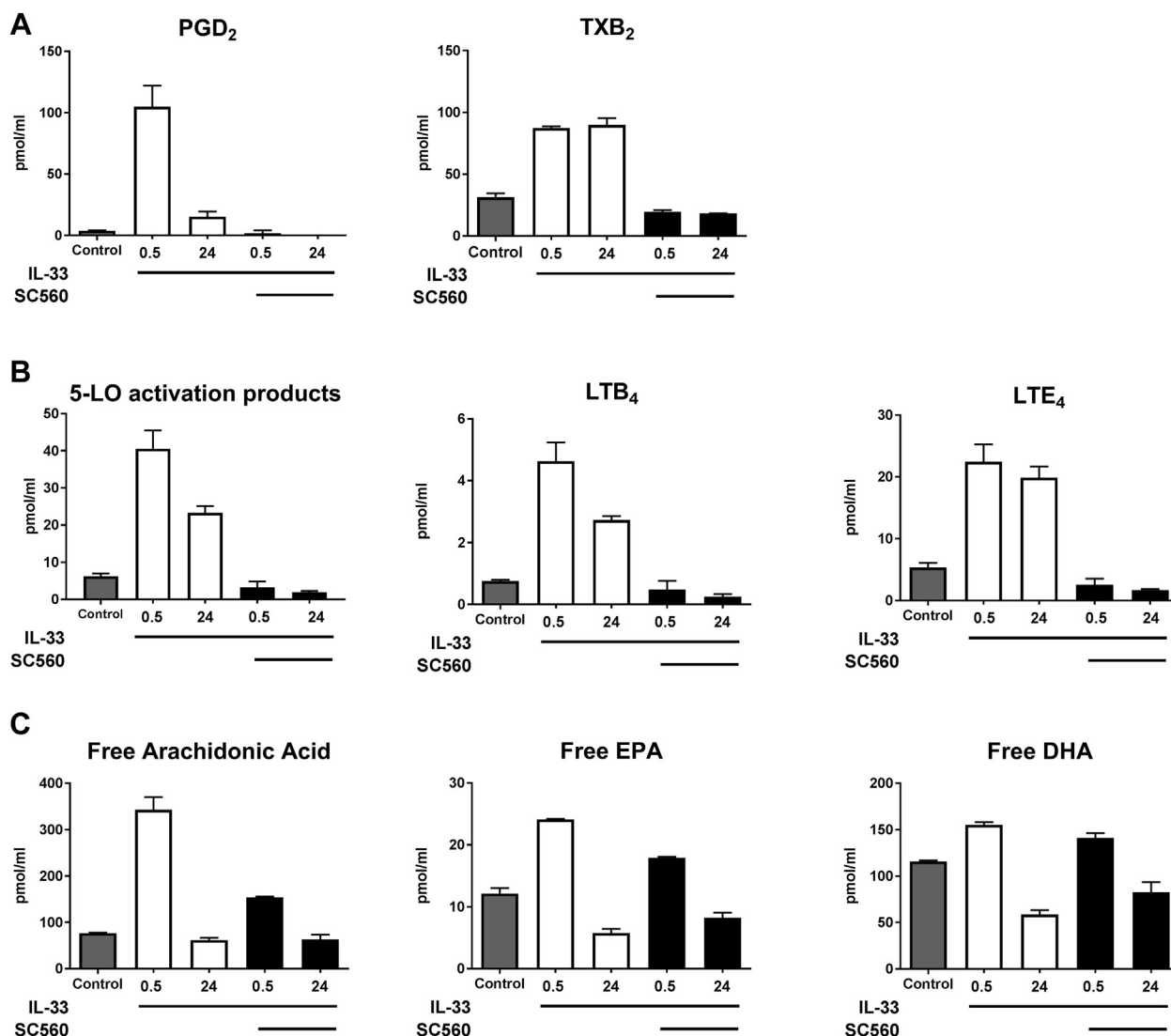


FIG 5. Lipidomic profiling of IL-33-stimulated BMMCs. BMMCs (1.5×10^6 cells/mL) were stimulated with IL-33 (10 ng/mL) or medium control in the absence or presence of SC560 (10 nmol/L). Culture supernatants obtained at 0.5 and 24 hours were analyzed by using mass spectrometry. **A**, Effects of COX-1 blockade with SC560 on IL-33-induced PGD₂ and TXA₂ (reflected by the stable metabolite TXB₂). **B**, Effects of COX-1 blockade on total 5-LO activation-derived products (consisting of LTB₄; LTE₄; 14,15-LTD₄; 5-HETE; 5-HEPE; and 5-HETrE), LTB₄, and CysLTs (reflected by the stable metabolite LTE₄). **C**, Effect of COX-1 blockade on IL-33-driven AA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) release by BMMCs. Results are means $\pm$ ranges from biological replicate samples.

smooth muscle constrictors that also elicit rapid increases in vascular permeability and mucous secretion through 3 different receptors.⁴⁵⁻⁴⁸ LTB₄ is a potent chemoattractant for neutrophils and effector memory T cells.^{7,12} PGD₂ can drive chemotaxis of eosinophils, basophils, and T_H2 cells⁴⁹ and can also directly induce T_H2 cytokine production from T_H2 cells⁵⁰ and innate group 2 lymphocytes.⁵¹ TXA₂ activates platelets and endothelial cells, facilitating both thrombosis and leukocyte recruitment.^{11,52} The clinical efficacy of 5-LO inhibitors,⁵³ CysLT receptor antagonists,⁵⁴ and COX inhibitors⁵⁵ validates the importance of AA metabolites in patients with allergic disease and inflammation in general.

Recently, MCs have been implicated strongly in various models of innate type 2 immunity initiated by endogenous

cytokines (IL-33, thymic stromal lymphopoietin, and IL-25),²⁴ and IL-33 potently induces cytokine generation by MCs (IL-5 and IL-13) without eliciting exocytosis of histamine.¹⁹ We undertook this study to determine whether IL-33-dependent MC activation elicits eicosanoid synthesis and, if so, whether this process is regulated differently from the calcium- and MAPK-dependent cPLA₂ activation reported for MC activation induced by the classical mechanism.

IL-33 signals through ST2, an IL-1 receptor family member. Although IgE-dependent PGD₂ and TXA₂ production by MCs occurs dominantly through COX-1,^{56,57} signaling through IL-1 receptor family members can induce COX-2 expression in MCs and other cell types, resulting in sustained PG production.⁴⁴ As expected, IL-33 induced production of both PGD₂ and TXA₂ by

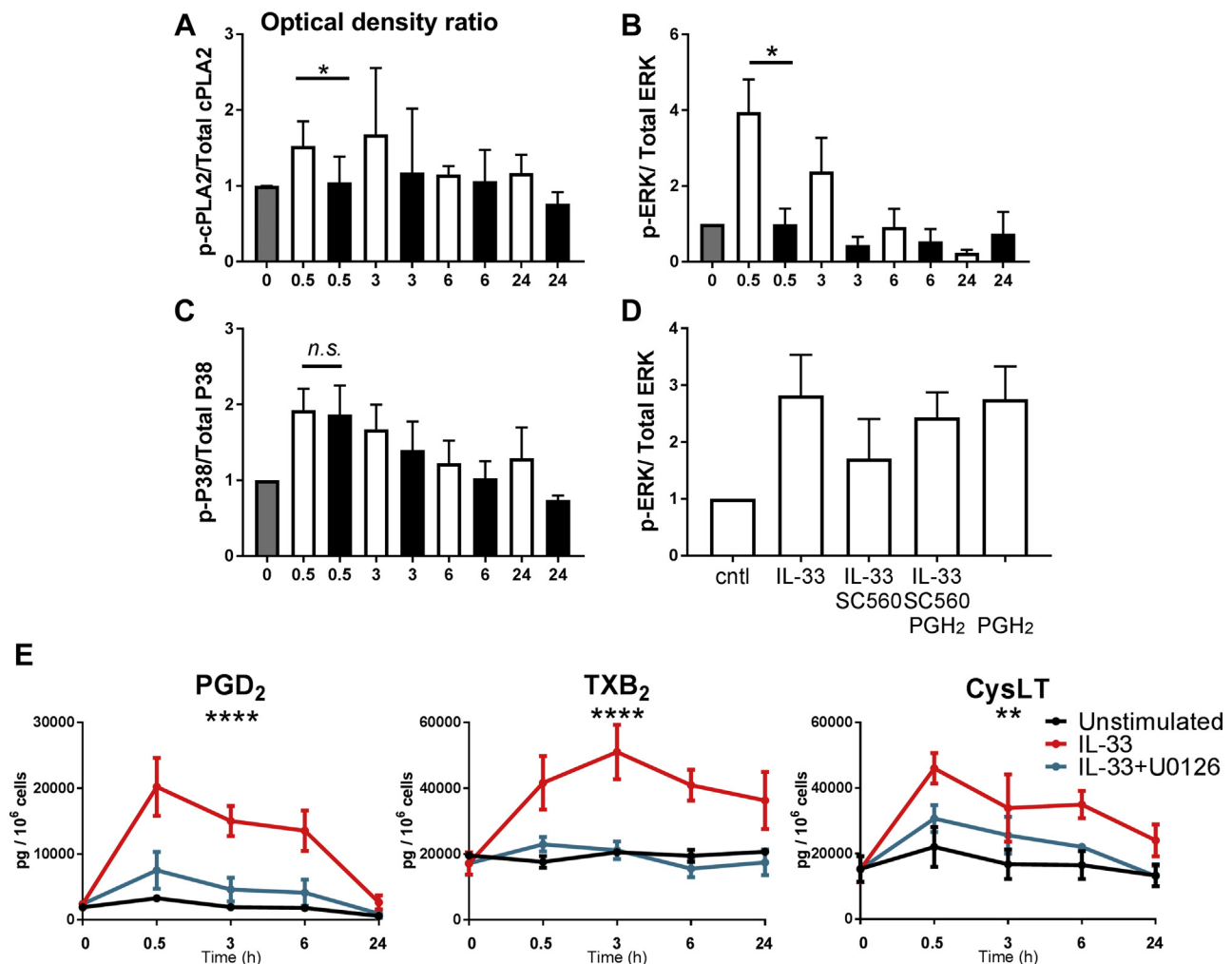


FIG 6. Effect of COX-1 activity on IL-33-induced MAPK-dependent activation of cPLA₂. **A-C**, Western blots generated from BMMCs stimulated with IL-33 for the indicated time points in the absence or presence of SC560 were probed for phosphorylated and total cPLA₂ (Fig 6, A), ERK (Fig 6, B), and p38 (Fig 6, C). Blots from representative experiments are displayed (left), and quantitative densitometric results (corrected for total proteins, mean $\pm$ SEM) are shown (right) from 3 to 5 experiments. * $P < .05$. n.s., Not significant. **D**, Effect of exogenous PGH₂ on ERK activation in unstimulated or IL-33-stimulated BMMCs in the absence or presence of COX-1 blockade. Results are representative of 2 experiments with similar results. **E**, Effect of ERK inhibition with U0126 (1 μ mol/L) on IL-33-driven eicosanoid production by BMMCs. Results are means $\pm$ SEMs from 3 independent experiments. ** $P < .01$ and **** $P < .0001$ across all time points by using 2-way ANOVA.

BMMCs, although with rapid kinetics that were consistent with the actions of constitutive COX-1 (Fig 1).

Two unexpected observations formed the basis of our subsequent inquiries. First, both pharmacologic blockade (using 2 different inhibitors) and targeted knockdown of COX-1 not only decreased generation of PGD₂ and TXA₂ (Fig 2) but also blocked induced expression of COX-2 (see Figs E2 and E3). In contrast, selective inhibition of COX-2 decreased PGD₂ and TXA₂ production but did not affect COX-2 expression (see Fig E2).

Second, and surprisingly, COX-1 activity (Fig 2 [but not COX-2 activity, see Fig E1]) was necessary to produce LTC₄ (as reflected by the effects of both COX-1 inhibitors tested on levels of CysLTs) in response to IL-33, differing sharply from Fc ϵ RI-induced activation (Fig 3). The fact that these functional responses were restored by

exogenous administration of PGH₂ (Fig 4) confirmed that the effects did not reflect off-target actions of SC560 and that PGH₂ derived from COX-1, but not that derived from COX-2, permitted at least 1 step critical to elicit both CysLT and COX-2-driven PGD₂ generation by MCs activated by IL-33 but not Fc ϵ RI cross-linkage. Moreover, the fact that neither PGD₂ itself nor the TP receptor agonist AU46619 mimicked the effect of PGH₂ implies the involvement of a minor PG or a distinct role for PGH₂ itself in regulating intracellular responses to an extracellular signal.

Of the multiple PLA₂ enzymes capable of releasing AA from the sn-2 position of membrane phospholipids, cPLA₂ is indispensable for the productions of both LTC₄ and PGD₂ with classical MC activation.⁴² cPLA₂ translocates from the cytosol of resting cells to intracellular membranes with activation and carries out its enzymatic function after it is phosphorylated by

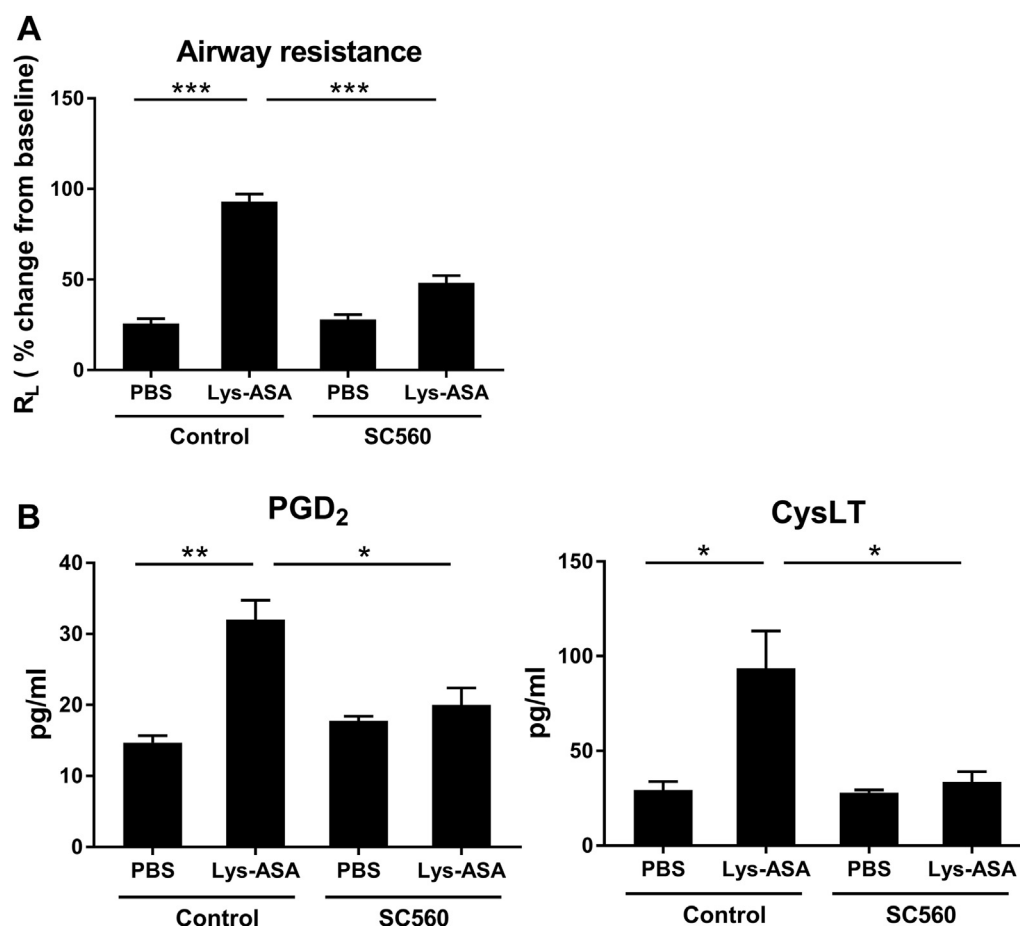


FIG 7. Effect of COX-1 inhibition *in vivo* on Lys-ASA-induced changes in airway physiology and MC-dependent eicosanoid generation in AERD-like mice. **A**, *D. farinae*-primed *Ptges*^{-/-} mice were challenged with Lys-ASA through inhalation. R_L was monitored continuously for 45 minutes, and peak change from baseline was recorded for each animal. The indicated groups received intraperitoneal doses of SC560 or vehicle control 48 and 24 hours before challenge. **B**, PGD₂ and CysLTs levels detected in bronchoalveolar lavage fluid collected from the same mice 45 minutes after challenge. Results are means $\pm$ SEMs from 3 to 5 mice per group in one of 2 experiments performed with similar results. * $P < .05$, ** $P < .01$, and *** $P < .001$.

ERK and/or p38 MAPKs,³ particularly in response to stimuli that also elicit calcium flux.²

Several lines of evidence in our study suggest that COX-1 activity is necessary to elicit ERK-dependent cPLA₂ activity in response to IL-33. First, IL-33-induced AA release itself (and release all downstream AA-derived products, including LTE₄, LTB₄, and combined 5-LO pathway products) was sharply reduced by treatment of BMMCs with SC560 (Fig 5). In contrast, release of ω -3 fatty acids (which are not the preferred substrates for cPLA₂) were minimally to negligibly affected by COX-1 inhibition.

Second, the modest cPLA₂ phosphorylation and strong ERK phosphorylation induced by IL-33 were eliminated by treatment of the cells with SC560, whereas p38 phosphorylation was unaffected (Fig 6). SC560 also suppressed IL-33-induced ERK activation by human cord blood MCs.

Third, both cPLA₂ and ERK activation were restored by the administration of exogenous PGH₂ (see Fig E6), which was also able to induce ERK activation (Fig 6, D) and cPLA₂ phosphorylation (see Fig E6, A) on its own, which is consistent with its ability to induce CysLT production even without IL-33

(Fig 4). It is likely that COX-1-derived PGH₂ provides a requisite signal that is essential for IL-33 to initiate AA release and the surge in both COX and 5-LO products characteristic of MC activation. Because most previous studies of eicosanoid production focused on Fc ϵ RI-dependent activation mechanisms, in which COX-1 is required for PGD₂ production but not needed to activate cPLA₂, this requirement for COX-1 in regulating ERK and cPLA₂ activity and LTC₄ production had not been recognized previously. Previous studies demonstrating IL-33-induced PGD₂ generation by MCs^{58,59} had neither examined CysLT production nor recognized the proximal role of COX-1-derived PGH₂ in initiating eicosanoid synthesis detailed in our study.

Airway MC activation produces airflow obstruction that is largely attributable to the contractile effects of the CysLTs.⁶⁰ Although allergen inhalation in sensitized hosts elicits Fc ϵ RI-dependent MC activation and the accompanying release of both CysLTs and PGD₂,^{61,62} challenge of patients with AERD with COX-1 inhibitors (but not COX-2 inhibitors) results in MC activation through an idiosyncratic mechanism that requires depletion of COX-1-derived PGE₂.⁶³ Notably, COX-1 inhibitors also elicit a subsequent state of “desensitization”

in which subsequent challenge with aspirin (or any other COX-1–active drug) does not elicit characteristic MC activation and CysLT release.³⁶ In *Ptges*^{−/−} mice both aspirin and ketorolac (but not the COX-2–selective inhibitor celecoxib) provoke AERD-like reactions⁴¹ that depend on IL-33 release, which is absolutely required for MC activation in this model.¹ Although the time frame of the aspirin challenge protocol used in our study does not permit serial invasive assessment of lung function in response to sequentially administered agents, it seems likely that SC560 also elicits a reaction (caused by PGE₂ depletion), followed by desensitization. This desensitization might be due, at least in part, to “stabilization” of MCs through inhibition of cell-intrinsic COX-1 and impairment of their responses to endogenous IL-33 (Fig 7). Because IL-33–driven MC activation might be central not only to AERD but also to severe asthma, chronic sinusitis, and other diseases, our findings have potential implications for the targeted use of COX inhibitors in patients with other pathologic conditions. Our findings might also help explain the therapeutic effects of desensitization to aspirin in patients with AERD, which prevents incremental mediator release in response to rechallenge.³⁶

Key messages

- IL-33 activates MCs to release AA and generate eicosanoids involved in allergic disease.
- In contrast to classical IgE-dependent MC activation, IL-33 requires COX-1 activity to activate cPLA₂ and induce AA release.
- COX-1 inhibition can limit IL-33–driven MC activation in patients with AERD and could explain part of the mechanism of aspirin desensitization.

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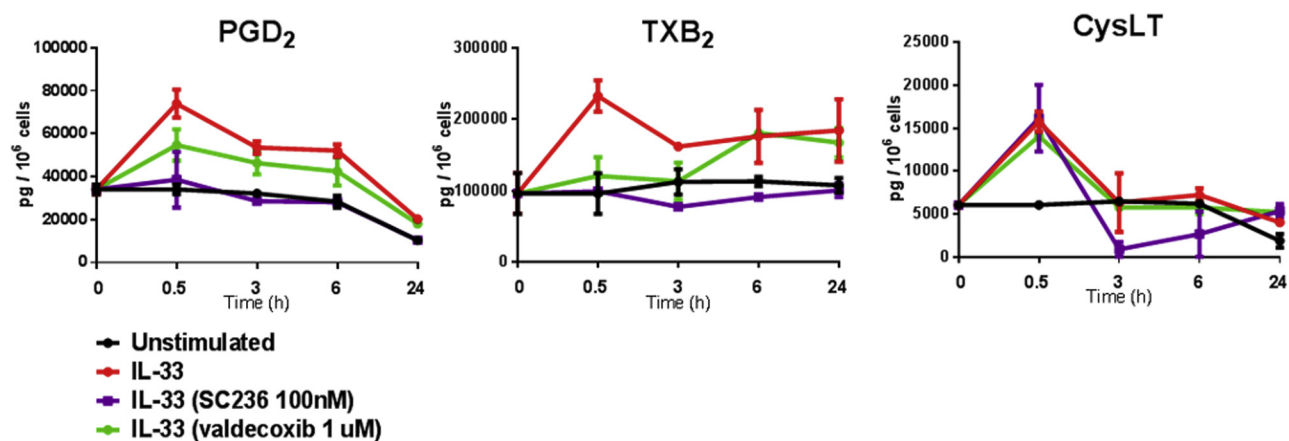


FIG E1. Effects of COX-2 inhibition on IL-33-induced PGD₂, TXB₂, and CysLT production. SC236 (100 nmol/L) or valdecoxib (1 μmol/L) was added to the indicated samples of IL-33-treated BMMCs. Lipid mediators were measured by means of ELISA. Results are means ± ranges of duplicates from a single experiment representative of 2 performed.

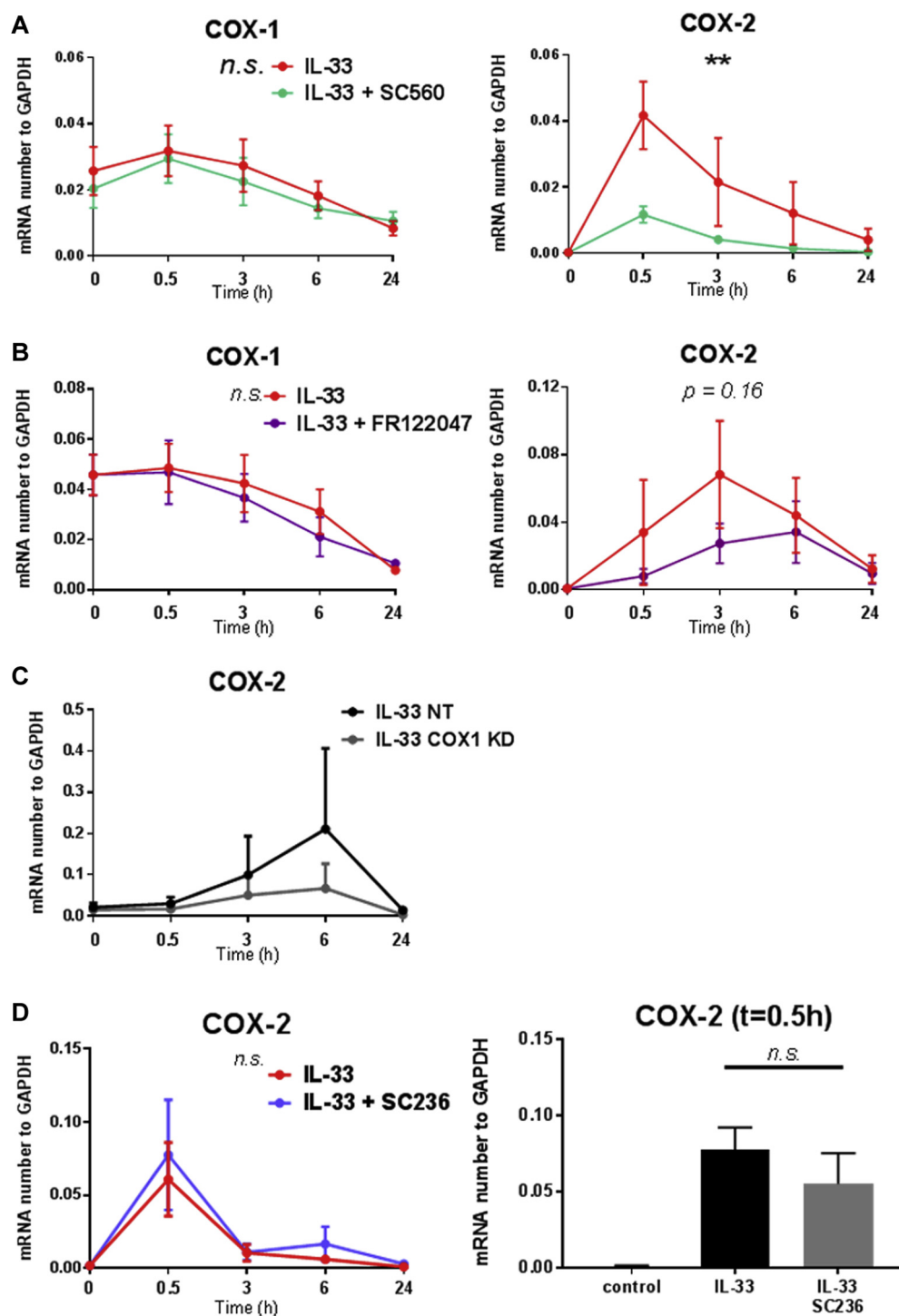


FIG E2. qPCR analysis of COX mRNA corrected for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in IL-33-stimulated BMMCs with COX-1 blockade, endogenous COX-1 small interfering RNA depletion, and COX-2 blockade. BMMCs were stimulated with IL-33, and samples were harvested for RNA at the indicated time points. **A**, Effect of the COX-1 inhibitor SC560 (10 nmol/L). **B**, Effect of the COX-1 inhibitor FR122047 (300 nmol/L). **C**, Effect of knockdown of COX-1 with small interfering RNA. **D**, Effect of the COX-2 inhibitor SC236 (100 nmol/L). Results in Fig E2, **A** and **B**, are means $\pm$ SEMs from 5 and 3 independent experiments, respectively. Results in Fig E2, **C**, indicate means $\pm$ ranges from 2 experiments, and results in Fig E2, **D**, are from 2 independent experiments for the time course and 4 for the 0.5-hour time point. ** $P < .001$ for the full time course by using 2-way ANOVA in Fig E2, **A**, and the Student *t* test when comparing IL-33-treated BMMCs with or without SC236 at 0.5 hours in Fig E2, **D**. n.s., Not significant.

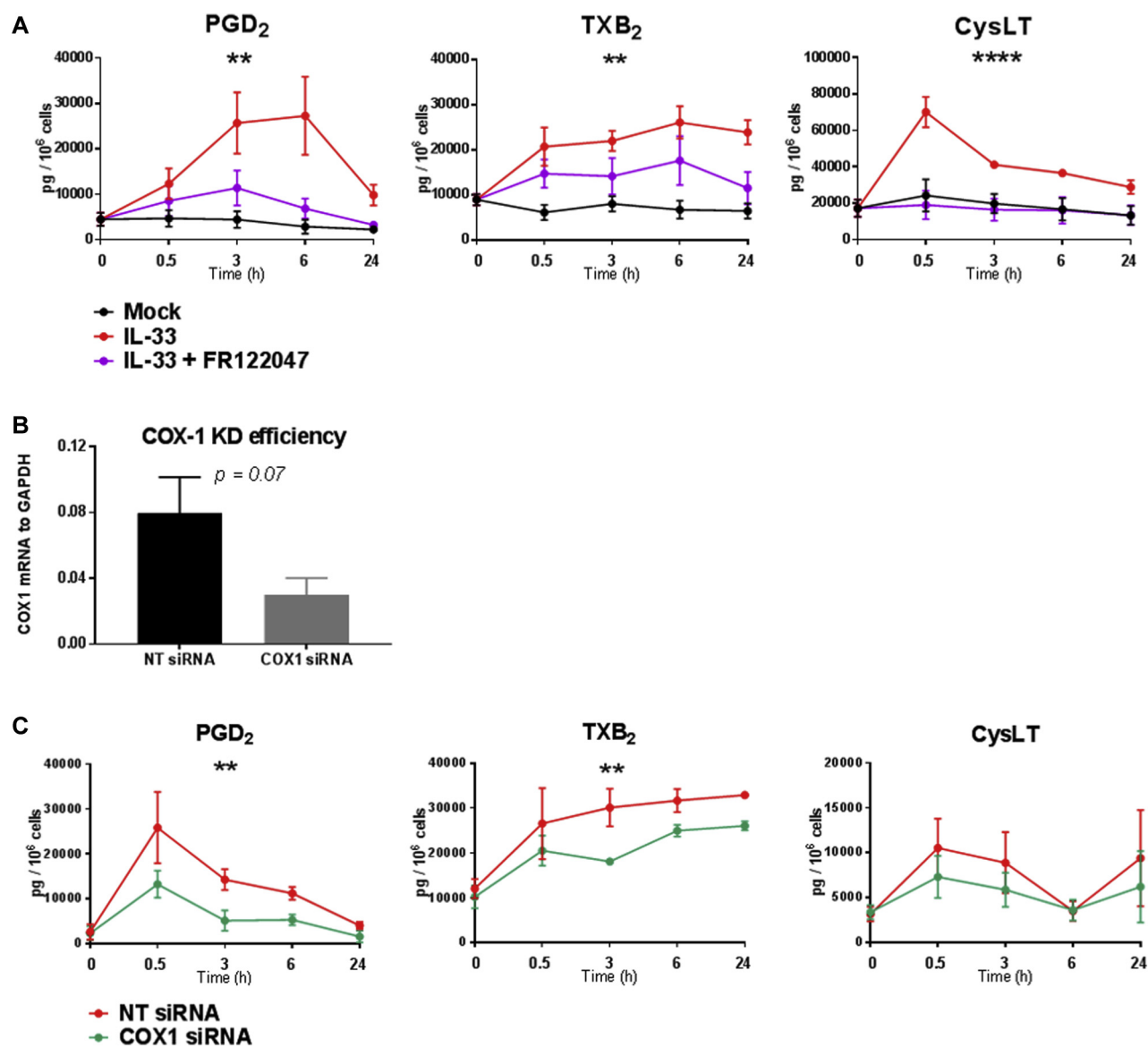


FIG E3. Effects of the COX-1 antagonist FR122047 and small interfering RNA-mediated knockdown of COX-1 on IL-33-induced PGD₂, TXA₂, and CysLT production. **A**, Quantities of eicosanoids generated by using mock- and IL-33-stimulated BMDCs treated with FR122047 (300 nmol/L) or vehicle for the indicated times. **B**, Efficacy of COX-1 knockdown. **C**, Effects of COX-1 knockdown on eicosanoid production by IL-33-stimulated BMDCs. Results are means $\pm$ SEMs from 3 experiments each. ** $P < .01$ and **** $P < .0001$ across time points by using 2-way ANOVA.

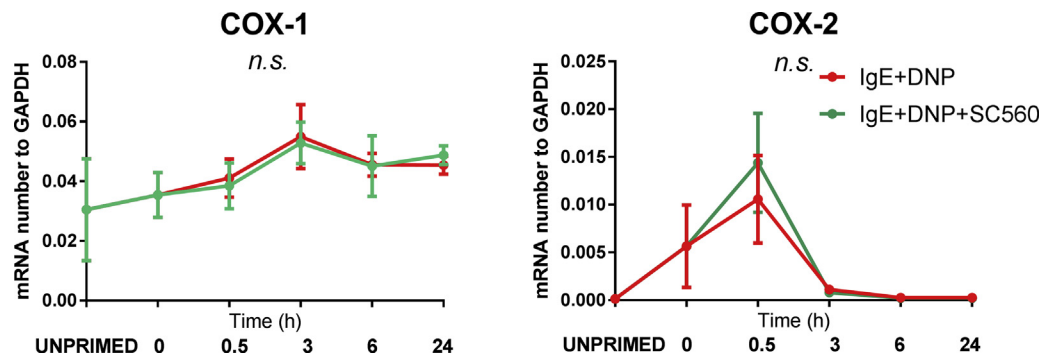


FIG E4. Lack of requirement for COX-1 function in IgE-induced expression of COX-2 mRNA expression by BMMCs. After overnight sensitization with IgE (SPE-7; 0.2 μ g/mL), BMMCs were activated with dinitrophenyl-human serum albumin (1 μ g/mL) in the presence or absence of SC560 (10 nmol/L). Expression of COX-1 and COX-2 mRNA was measured by using qPCR. Results are means $\pm$ SEMs from 3 independent experiments. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *n.s.*, not significant.

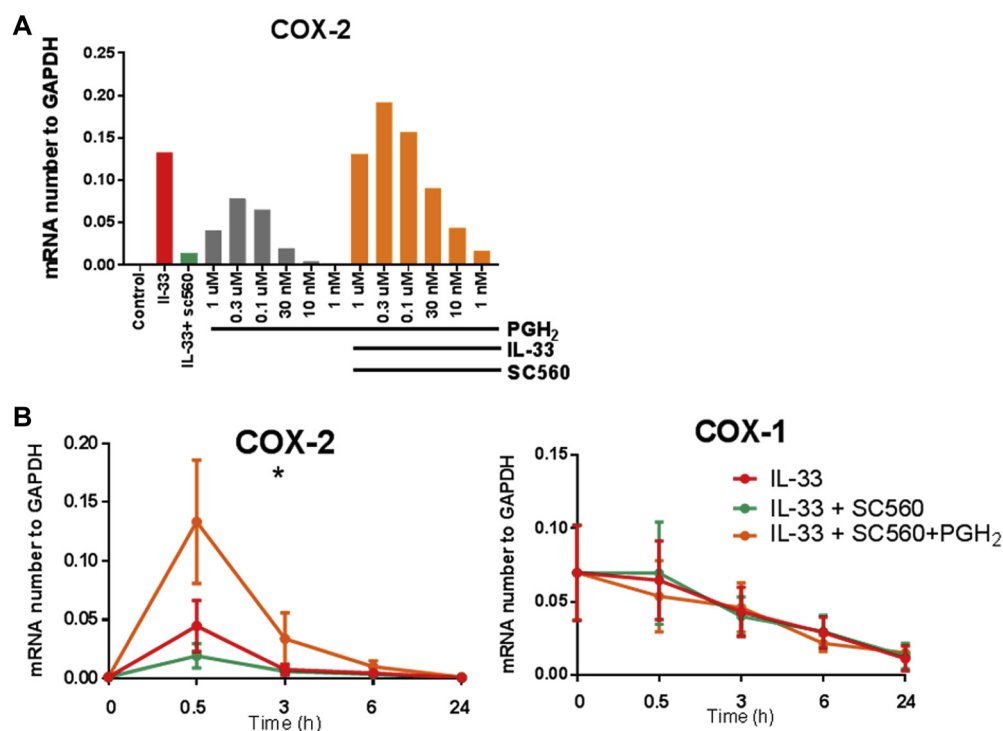


FIG E5. Exogenous PGH₂ restores COX-1-dependent COX-2 induction. **A**, Dose-dependent effect of PGH₂ on basal and IL-33-induced COX-2 expression. BMMCs were stimulated for 0.5 hours with various concentrations of PGH₂ alone or with IL-33 (10 ng/mL) and SC560 (10 nmol/L). **B**, Time-dependent effects of PGH₂ (300 nmol/L) on COX-2 and COX-1 mRNA expression by BMMCs stimulated with IL-33 in the presence of the COX-1-specific inhibitor SC560. Results in Fig E5, **B**, are means $\pm$ SEMs from 3 independent experiments. $P = .02$ between IL-33 plus SC560 and IL-33 plus SC560 plus PGH₂ for COX-2 induction at 0.5 hours. $*P < .05$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

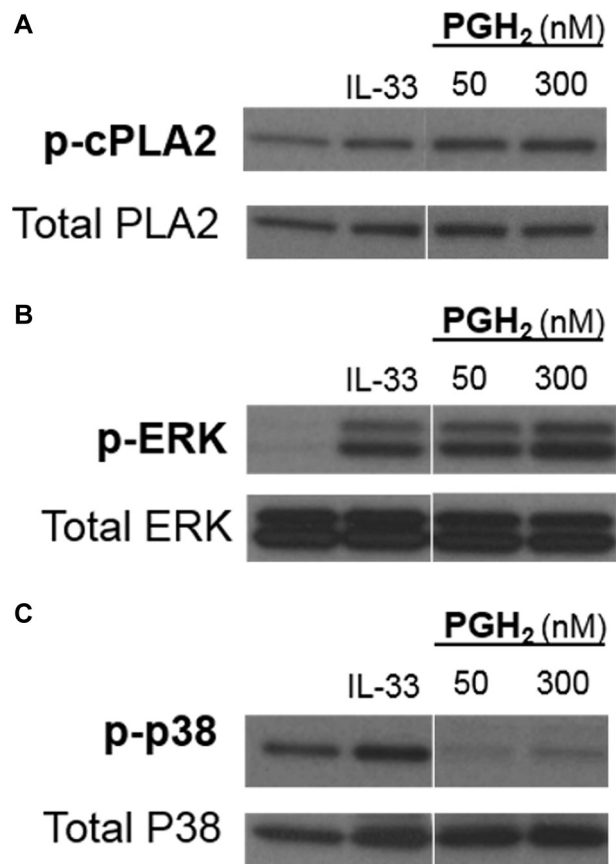


FIG E6. Effect of exogenous PGH₂ on cPLA₂ (A), ERK (B), and p38 (C) phosphorylation at 0.5 hours. Results are representative blots from one of 2 individual experiments with similar results.

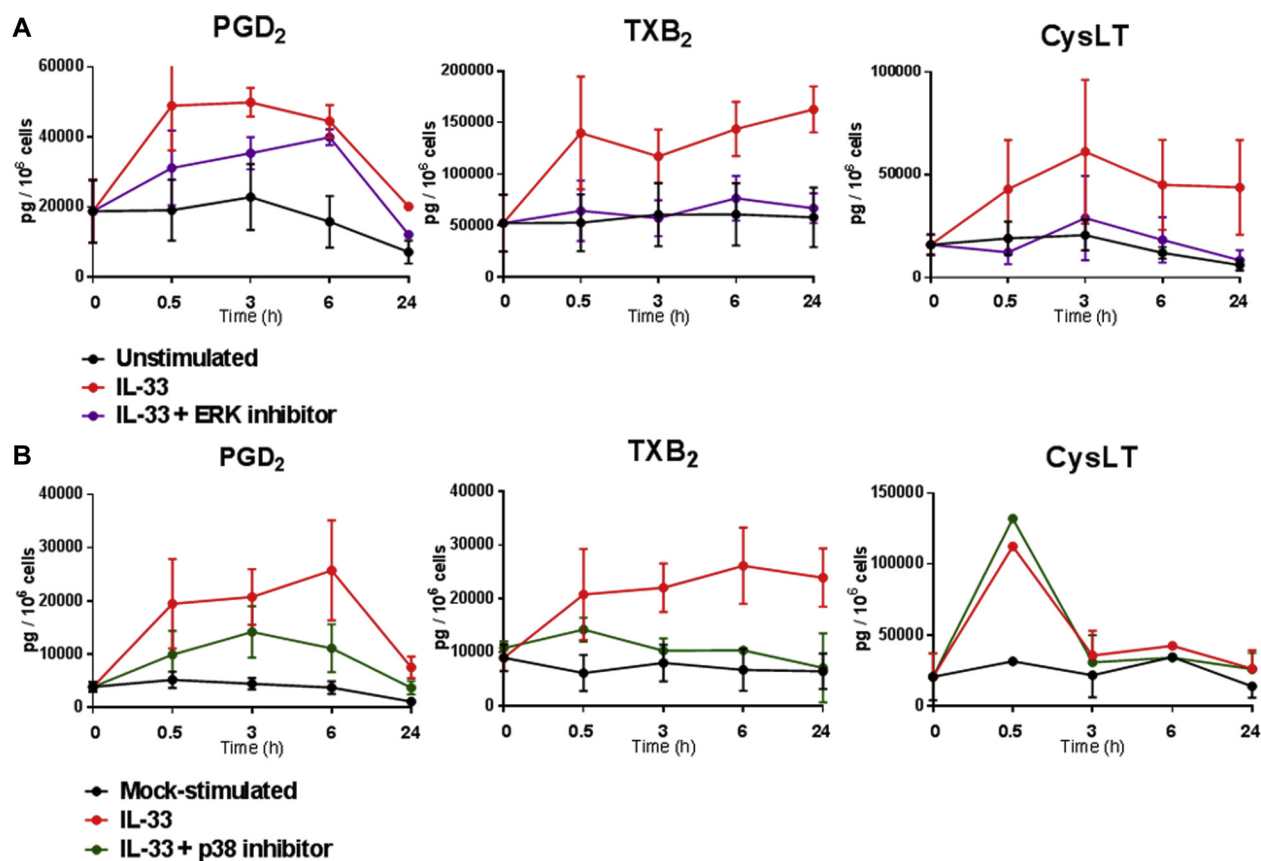


FIG E7. Effect of ERK or p38 inhibition on IL-33–induced PGD₂, TXA₂, and CysLT production. BMMCs were stimulated with IL-33 (10 ng/mL) in the presence or absence of the selective ERK inhibitor FR 180204 (10 μ mol/L; **A**) or in the presence or absence of the p38 inhibitor SKF-86002 (1 μ mol/L; **B**). Eicosanoid levels at the indicated time points were measured by means of ELISA of culture supernatants. Data are means $\pm$ ranges from 2 experiments.

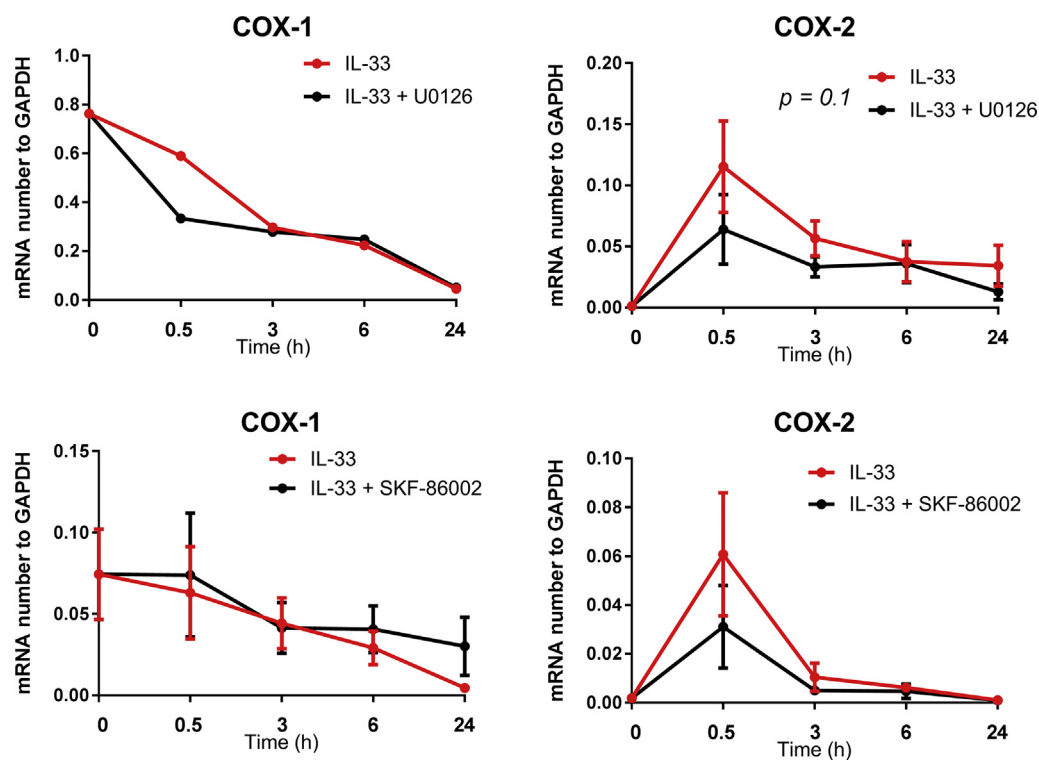


FIG E8. Effects of COX-1 blockade on IL-33–induced PGD₂, TXA₂, and CysLT production by BMMCs from *Ptges*^{−/−} mice. *Ptges*^{−/−} BMMCs were stimulated with IL-33 (10 ng/mL) in the presence or absence of the COX-1 antagonist SC560 (10 nmol/L). PGD₂, TXB₂, and CysLTs levels were measured at the indicated time points. Results are means ± ranges from duplicates of a single experiment representative of 2.