

Platelets constitutively express IL-33 protein and modulate eosinophilic airway inflammation

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Background: Although platelets play a key role in allergic inflammation in addition to their well-established role in hemostasis, the precise mechanisms of how platelets modulate allergic inflammation are not fully understood. IL-33 is an essential regulator of innate immune responses and allergic inflammation.

Objective: We sought to determine the expression of IL-33 protein by platelets and its functional significance in airway inflammation.

Methods: IL-33 protein in human platelets, the human megakaryocyte cell line MEG-01, and bone marrow-derived mouse megakaryocytes was detected by using Western blot analysis and fluorescent immunostaining. We examined the functional relevance of IL-33 protein in platelets by comparing platelet-intact and platelet-depleted groups in a murine model of IL-33-dependent airway eosinophilia elicited by intranasal administration of papain. We further compared the additive effect of administration of platelets derived from wild-type versus IL-33-deficient mice on the papain-induced eosinophilia. **Results:** Platelets and their progenitor cells, megakaryocytes, constitutively expressed IL-33 protein (31 kDa). Papain-induced IL-33-dependent airway eosinophilia in mice was significantly attenuated by platelet depletion. Conversely, concomitant administration of platelets derived from wild-type mice but not IL-33-deficient mice enhanced the papain-induced airway eosinophilia.

Conclusions: Our novel findings suggest that platelets might be important cellular sources of IL-33 protein *in vivo* and that platelet-derived IL-33 might play a role in airway inflammation. Therefore platelets might become an attractive novel therapeutic target for asthma and probably allergic inflammation. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Airway inflammation, asthma, eosinophil, IL-33, papain, platelet

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Blood platelets, which are produced by cytoplasmic fragmentation of bone marrow megakaryocytes, are essential for primary hemostasis and to repair damaged blood vessels. Experimental and clinical studies have revealed that in addition to their well-established role in hemostasis, platelets actively participate in immune-inflammatory processes, including the pathogenesis of bronchial asthma.¹ Asthma is a disease of chronic airway inflammation. Histopathologically, asthma is characterized by infiltration of the airway walls by various inflammatory cells, including eosinophils, and by airway remodeling with increased angiogenesis, vascular permeability, and plasma exudation. Platelets have been found to be crucially involved in all these processes. For instance, in a murine model of ovalbumin (OVA)-induced allergic lung inflammation, infiltration of eosinophils into the airways after OVA challenge was significantly reduced in platelet-depleted mice.² Conversely, transfusion of platelets to thrombocytopenic mice completely restored OVA-induced pulmonary eosinophil trafficking, indicating an essential role for platelets in leukocyte recruitment in patients with allergic

Abbreviations used

AM:	Alveolar macrophage
BAL:	Bronchoalveolar lavage
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HBSS:	Hank balanced salt solution
HMVEC-L:	Human microvascular endothelial cells from the lung
ILC2:	Group 2 innate lymphoid cell
IL1RL1:	IL-1 receptor-like 1
IM:	Intestinal macrophage
MDC:	Macrophage-derived chemokine
MIP:	Macrophage inflammatory protein
OVA:	Ovalbumin
qPCR:	Quantitative PCR
TARC:	Thymus and activation-regulated chemokine

inflammation. The same group also demonstrated that depletion of platelets markedly reduced airway remodeling elicited by chronic exposure of mice to OVA.³

Also, in human subjects platelet activation in patients with allergic asthma has been documented,⁴⁻⁷ and Kowal et al⁸ showed that bronchial allergen challenge in asthmatic patients resulted in activation of peripheral blood platelets. Duarte et al⁹ recently demonstrated that circulating levels of platelet-derived microparticles were significantly increased in asthmatic patients compared with control subjects.⁹

Hence platelets are evidently essential for the pathogenesis of asthma, and therefore inhibition of platelet activation might represent a novel therapeutic approach for asthmatic patients. In fact, Barr et al¹⁰ previously demonstrated that long-term treatment with low-dose aspirin, which preferentially inhibits platelet aggregation, can reduce the risk of newly diagnosed adult-onset asthma, although asthmatic patients have traditionally been advised to avoid using aspirin. More recently, prasugrel, an antiplatelet drug that targets the platelet P2Y₁₂ receptor, was shown to be useful in treating asthmatic patients in a randomized, double-blind, placebo-controlled crossover study.¹¹ However, the precise mechanisms of how platelets regulate allergic inflammation are not fully understood.

Several recent large-scale genome-wide association studies showed that both IL-33, a member of the IL-1 family of cytokines, and its receptor, IL-1 receptor-like 1 (IL1RL1)/ST2, which are located on different chromosomes, are closely associated with asthma onset without regard to race.¹²⁻¹⁵ Indeed, IL-33 was shown to be functionally involved in the pathogenesis of asthma by provoking strong type 2 immune responses in various inflammatory cells, including group 2 innate lymphoid cells (ILC2),^{16,17} T_H2 cells,¹⁸ eosinophils,¹⁹ basophils,^{20,21} dendritic cells,²² and mast cells.^{23,24} Thus IL-33 is a fundamental regulator of allergic inflammation. Although IL-33 was initially described as a nuclear factor expressed in high endothelial venules,²⁵ subsequent studies showed that it is preferentially and constitutively expressed in the nuclei of such tissue structural cells as epithelial and endothelial cells and that it is released by necrotic cells after tissue injury, trauma, or both (see Nakae et al²⁶), such as high mobility group box-1. Therefore IL-33 is thought to exert its biological functions as a damage-associated molecular pattern molecule to provoke local inflammation. These findings suggest that the degree of tissue damage might define the level of IL-33 activity in local tissues. However, there might be some other IL-33 activation mechanism *in vivo* in addition to its release from necrotic cells because genome-wide association studies¹²⁻¹⁴ suggest that the

degree of IL-33/IL1RL1 signaling and/or their expression levels in local tissues play key roles in the onset of asthma. Therefore we hypothesized that there might be an essential cellular source of IL-33 protein besides its release from damaged tissues.

Importantly, we previously reported that IL-33 can also effectively promote inflammatory responses in human vascular endothelial cells in the airway through the IL1RL1/ST2 receptor.²⁷ Because platelets play key roles in endothelial function, we hypothesized that platelets are one of the important cellular sources of IL-33 protein and that platelet activation might play a pivotal role in IL-33-dependent airway inflammation. In the present study we show that platelets constitutively express IL-33 protein, and we also confirm the functional relevance of platelet-derived IL-33 in a murine IL-33-dependent airway eosinophilia model elicited by intranasal administration of protease allergen.²⁸ To the best of our knowledge, this is the first study to demonstrate the expression of IL-33 by platelets and its functional significance in airway inflammation.

METHODS

Isolation of human platelets

Human blood from healthy volunteers was mixed with a 1:9 volume of 3.8% sodium citrate solution (Sysmex, Hyogo, Japan). Platelet-rich plasma was obtained by means of centrifugation at 300g for 10 minutes at room temperature. Platelets were purified from platelet-rich plasma with a Sepharose 2B column (Sigma-Aldrich, St Louis, Mo) in PIPES buffer (5 mmol/L PIPES, 1.37 mmol/L NaCl, 4 mmol/L KCl, and 0.1% glucose, pH 7.0).²⁹

Sample collection and all procedures were approved by the Ethics Committee of the National Research Institute for Child Health and Development in accordance with the ethical standards of the Helsinki Declaration of 1975. Informed consent was obtained from all volunteers.

Cell culture and preparation of nuclear and cytosol extracts

Human megakaryoblast cell line MEG-01 and COS-7 cells, an African green monkey kidney fibroblast-like cell line, were obtained from the American Type Culture Collection (Manassas, Va) and cultured in RPMI 1640 and Dulbecco modified Eagle medium supplemented with 10% FBS, respectively. The nuclear and cytosol fractions of MEG-01 were extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, Ill), according to the manufacturer's instructions.

Generation of full-length IL-33 expression vector and transient transfection into COS-7 cells

Based on the human IL-33 sequence (NM_033439), full-length IL-33 was amplified from human universal reference cDNA (Agilent Technologies, Palo Alto, Calif) by using reverse transcription PCR with the following primers containing restriction sites. The forward primer contained a *Bam*HI site and the ATG codon 5'-CGGATCCACCATGAAGCCTAAATGAAGTATTCAACCAAC-3', and the reverse primer contained a stop codon and the *Eco*RI site 5'-ACCGAATTCCTAAGTTTCAGAGAGCTTAAACAAGATATTTC-3'. After purification, the amplified PCR product was subcloned into the pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, Calif) and verified by means of sequencing. The IL-33/pcDNA expression plasmid or the empty pcDNA plasmid was transfected into COS-7 cells by using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. After 72 hours of transfection, the cells were harvested and counted.

Western blotting

For Fig 1, A, transfected COS-7 cells (1×10^5 cells/lane), human platelet extracts (1×10^6 cells/lane), and cytosol (1×10^5 cells/lane) and nuclear

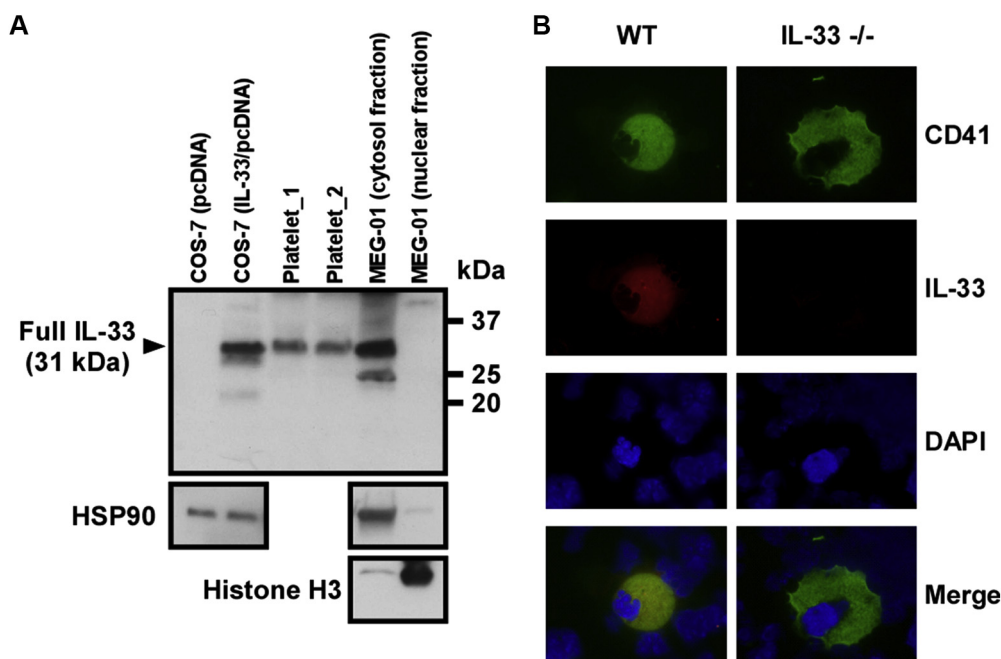


FIG 1. IL-33 protein expression in platelets and megakaryocytes. **A**, Western blotting analysis for IL-33. COS-7 cell lysates transiently transfected with full-length IL-33-expressing vector or empty vector (1×10^5 cells/each lane), human platelet extracts from 2 distinct donors (1×10^6 cells/each lane), and cytosol (1×10^5 cells/lane) and nuclear (4×10^5 cells/lane) fractions of MEG-01 cells were loaded, and Western blotting was performed with rabbit polyclonal antibodies for human IL-33, heat shock protein 90 (as a loading control for COS-7 cells and a cytosol fraction control for MEG-01 cells), and histone H3 (as a nuclear fraction control for MEG-01 cells). **B**, Expression of IL-33 protein in murine megakaryocytes. Murine megakaryocytes were isolated from bone marrow cells of C57BL/6N (wild-type [WT]) and IL-33-deficient (IL-33^{-/-}) mice. After fixation with cold methanol/acetone (1:1), cells were stained for platelet integrin CD41 (green) and IL-33 (red), and nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue).

(4×10^5 cells/lane) fractions of MEG-01 cells were lysed in NuPAGE LDS sample loading buffer (Life Technologies, Carlsbad, Calif) containing 5% (vol/vol) β -mercaptoethanol and heated at 95°C for 3 minutes. Samples were separated by using SDS-PAGE (5-15% Ready Gels J; Bio-Rad, Hercules, Calif) and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Western blotting was performed with rabbit polyclonal antibodies for human IL-33 (MBL, Nagoya, Japan), heat shock protein 90 (E289; Cell Signaling Technology, Danvers, Mass), and histone H3 (Cell Signaling Technology), according to the manufacturers' instructions.

Mice

C57BL/6N wild-type mice were obtained from Sankyo Lab (Tsukuba, Japan). IL-33-deficient mice (accession no. CDB0631K, <http://www.clst.riken.jp/arg/mutant%20mice%20list.html>) on the C57BL/6N background were generated, as described previously.²⁸ C57BL/6N wild-type mice and IL-33-deficient female mice (approximately 6-7 weeks of age) were used in all experiments. Mice were housed under specific pathogen-free conditions at the National Research Institute for Child Health and Development. The animal protocols used in this study were approved by the Institutional Review Board of the National Research Institute for Child Health and Development.

Preparation and immunostaining of murine megakaryocytes

Whole bone marrow cells were isolated from the leg bones of C57BL/6N wild-type and IL-33-deficient mice. Megakaryocytes were enriched from whole bone marrow cells by means of negative selection with biotin-labeled anti-CD4, CD8, CD19, Gr-1, F4/40, and TER-119 mAbs (Becton, Dickinson and Company, Franklin Lakes, NJ) and BD IMag Streptavidin Particles Plus-DM (Becton, Dickinson and Company). The enriched megakaryocytes were

fixed in methanol/acetone (1:1) at -20°C, blocked with 10% normal rat serum, and stained with anti-mouse IL-33 polyclonal goat IgG antibody (R&D Systems, Minneapolis, Minn) and Alexa Fluor 594-labeled donkey anti-goat IgG (red; Thermo Fisher Scientific, Waltham, Mass) or fluorescein isothiocyanate-labeled anti-mouse CD41 (green; Becton, Dickinson and Company). The stained cells were mounted with Vectashield mounting medium containing 4'-6-diamidino-2-phenylindole dihydrochloride (blue; Vector Laboratories, Burlingame, Calif), and images were acquired with a fluorescence microscope BZ-9000 (Keyence, Tokyo, Japan) equipped with an objective lens ($\times 100$ magnification).

Platelet depletion, intranasal platelet administration, and airway inflammation in mice

Platelet depletion was accomplished by intraperitoneal injection of anti-mouse CD41 rat mAb (MWR30; Becton, Dickinson and Company; 5 μ g/200 μ L of sterile PBS) or isotype control rat IgG1 κ (Becton, Dickinson and Company) twice on days 0 and 2 (Fig 2, A). For papain-induced airway inflammation, mice were subjected to anesthesia with 2.5% isoflurane (Wako, Osaka, Japan) for 5 minutes and treated intranasally with papain (Wako; 25 μ g/20 μ L of sterile PBS) or PBS alone once per day for 3 days (Fig 2, A).²⁸ Twenty-four hours after the last administration of papain, bronchoalveolar lavage (BAL) fluid was collected from each mouse by using the following procedure. The mouse was killed with an overdose of isoflurane, and then the trachea was cannulated with a 21-gauge needle and lavaged 3 times with 1 mL of Hank balanced salt solution (HBSS; Thermo Scientific) containing 2% FCS. The collected BAL fluid (total volume, 0.8 mL) was centrifuged at 1000g for 5 minutes, and the BAL cells were resuspended in 200 μ L of HBSS. The numbers of total WBCs, eosinophils, macrophages, neutrophils, and lymphocytes in the BAL fluid were counted with a hemocytometer (Sysmex XT-1800i).

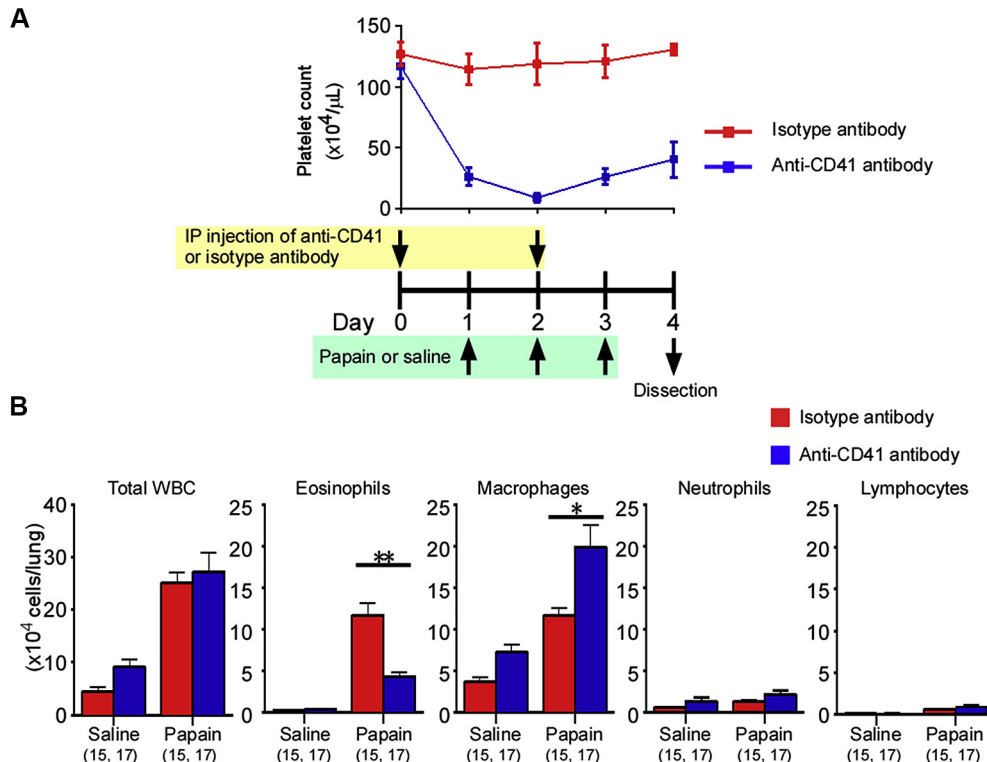


FIG 2. Platelet depletion resulted in a significant decrease in papain-induced eosinophilic inflammation in the murine airway. **A**, Study design and platelet numbers in blood during the experiment. *IP*, Intraperitoneal. **B**, Twenty-four hours after the last administration, BAL fluid was collected from each mouse, and total WBCs, eosinophils, macrophages, neutrophils, and lymphocytes in each fluid were counted. Data show means $\pm$ SDs (isotype antibody + saline, $n = 10$; isotype antibody + papain, $n = 15$; anti-CD41 antibody + saline, $n = 11$; and anti-CD41 antibody + papain, $n = 17$). * $P < .05$ and ** $P < .01$.

For concomitant intranasal administration of platelets and papain, blood from wild-type or IL-33-deficient mice was mixed with a 1/9 volume of 3.8% sodium citrate solution (Sysmex). Platelet-rich plasma was obtained by means of centrifugation at 100g for 10 minutes at room temperature, washed with sterile PBS (without Ca^{2+} and Mg^{2+}), and then suspended in the same PBS (1×10^4 platelets/ μL). Purity of the platelets from each mouse type was greater than 95%, as assessed based on forward-scatter and side-scatter characteristics and expression of CD41 by using flow cytometry, and the contamination rate by CD45 $^{+}$ leukocytes was less than 0.6%. Then 2×10^5 platelets/20 μL (in sterile PBS) from wild-type or IL-33-deficient mice were intranasally administered immediately after each intranasal papain treatment once per day for 3 days.

Quantitative PCR

Twenty-four hours after the last administration of papain, lungs were removed from each mouse after PBS perfusion. Total RNA extraction, cDNA synthesis, and quantitative PCR (qPCR) were performed, as previously described.³⁰ Primer sets for 5 murine genes were synthesized at Fasmac (Kanagawa, Japan): *IL4* (sense, 5'-TCC AAG GTG CTT CGC ATA TTT T-3'; antisense, 5'-CAG CTT ATC GAT GAA TCC AGG C-3'), *IL5* (sense, 5'-CCC TCA TCC TCT TCG TTG CAT-3'; antisense, 5'-ATG TGA TCC TCC TGC GTC CAT-3'), *IL13* (sense, 5'-GGC AGC AGC TTG AGC ACA TT-3'; antisense, 5'-GGC ATA GGC AGC AAA CCA TG-3'), *IFN γ* (sense, 5'-GAA CTG GCA AAA GGA TGG TGA-3'; antisense, 5'-TGT GGG TTG TTG ACC TCA AAC-3') and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; sense, 5'-CCC ACT CTT CCA CCT TCG ATG-3'; antisense, 5'-AGG TCC ACC ACC CTG TTG CT-3'). Quantified concentrations of the purified PCR products of IL-4, IL-5, IL-13, IFN- γ , and GAPDH were serially diluted and used as standards to determine the exact copy numbers of the target genes. Aliquots of cDNA equivalent to 2 ng of the total RNA samples were

used for each qPCR. mRNA expression levels were normalized to GAPDH levels in each sample.

Statistical analysis

The primary end point in this study was the decrease in papain-induced eosinophil counts in the lung by platelet depletion, whereas other end points, including WBCs, macrophages, neutrophils, and lymphocytes, were exploratory. Therefore the sample size was determined in accordance with our earlier study for eosinophil analysis.²⁸ Because eosinophil numbers in the lung were expected to be lower in the platelet-depleted group compared with those in the control group, we hypothesized that the SD of eosinophil counts was 8 and 2 for the isotype and anti-CD41 antibody (platelet depletion) groups, respectively, and the difference in eosinophil counts between 2 groups would be 8. Fifteen samples were required per arm to achieve a power of 0.9 with a significance level of .05 in the primary analysis. However, we found that papain-treated mice were prone to bleeding and occasionally died. For that reason, we used more than 15 mice in the anti-CD41 plus papain group. All data are presented as means $\pm$ SDs. Differences between groups were analyzed by using a 2-sided Welch *t* test and considered significant at a *P* value of less than .05. No adjustments for multiple comparisons were made for analyses of exploratory end points.

RESULTS

Platelets express full-length IL-33 protein

First, we examined the expression of IL-33 protein by using Western blot analysis in human platelets, the purity of which was 98.9%, as assessed by the expression of CD41 and size estimation (side scatter) by using flow cytometry (data not shown). Fig 1, A,

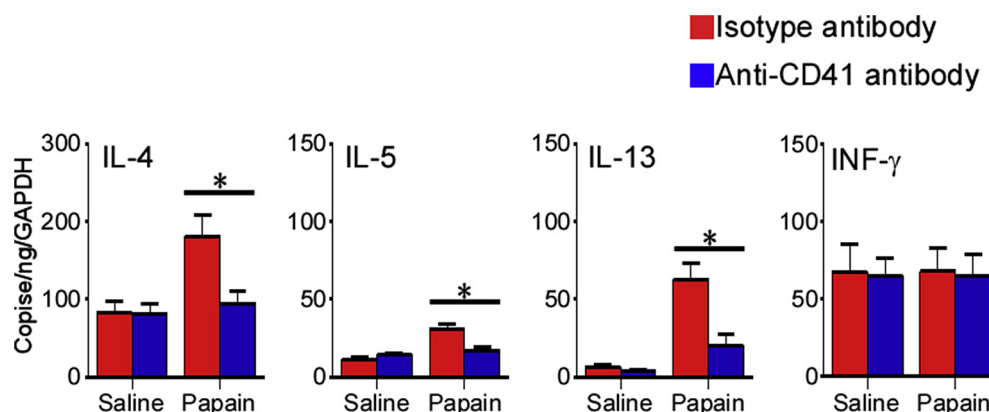


FIG 3. Effects of platelet depletion on the expression of cytokines/chemokines in the lung. mRNA was isolated from the lung at 24 hours after the last administration. Expression of IL-4, IL-5, IL-13, and IFN- γ was determined by using qPCR. Data show means $\pm$ SDs (isotype antibody + saline, $n = 8$; isotype antibody + papain, $n = 6$; anti-CD41 antibody + saline, $n = 8$; and anti-CD41 antibody + papain, $n = 6$). * $P < .05$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

shows that IL-33 protein was predominantly expressed in its full-length form (31 kDa) in accordance with the band detected in the COS-7 cells transiently transfected with full-length IL-33-expressing vector that served as the source of full-length IL-33 protein. Platelets are anuclear membrane-bound cellular fragments derived from megakaryocytes, and we demonstrated expression of full-length IL-33 protein and possibly its degradation product in the cytosol, but not nuclear, fraction of MEG-01 cells, a human megakaryoblast cell line. We further confirmed expression of IL-33 protein in the cytoplasm of bone marrow-derived megakaryocytes from wild-type but not IL-33-deficient mice²⁸ by means of fluorescent immunostaining (Fig 1, B).

Effect of platelet depletion on IL-33-dependent airway eosinophilic inflammation in mice

We previously generated and studied IL-33-deficient mice and found that IL-33 is essential for papain-induced eosinophilic inflammation in the airway.²⁸ Importantly, we also showed that heat-inactivated papain (at 100°C for 15 minutes) cannot induce airway eosinophilia at all, indicating that the protease activity of papain is necessary for induction of airway inflammation in this model. Therefore we assume that the protease activity of papain can cause barrier disruption in the epithelium, leading to release of IL-33 protein and activation of its target cells, thereby promoting airway eosinophilia. To further elucidate the functional relevance of IL-33 in platelets in addition to its being recognized in the epithelium, we examined the effects of platelet depletion on the papain-induced airway eosinophilic inflammation model, which is fully dependent on IL-33. Platelet depletion was accomplished by using intraperitoneal injection of anti-CD41 antibody twice, on days 0 and 2, and we found that circulating platelet numbers were significantly reduced for up to 96 hours compared with mice treated with isotype control antibody (Fig 2, A). Histopathologic analysis revealed that papain-induced airway inflammation was significantly attenuated by anti-CD41 treatment but not by isotype control IgG (see Fig E1 in this article's Online Repository at www.jacionline.org). Of note, platelet depletion resulted in a significant decrease in eosinophil, but not neutrophil, counts in papain-treated BAL fluid (Fig

2, B). In contrast, mice treated with anti-CD41 antibody showed increased numbers of macrophages in BAL fluid.

Effects of platelet depletion on papain-induced cytokine expression in the lungs and blood of mice

To evaluate the immunologic effects of platelet depletion on the lung, we investigated the mRNA expression levels of cytokines, such as IL-4, IL-5, IL-13, and IFN- γ . We found significantly reduced mRNA expression for IL-4, IL-5, and IL-13, but not IFN- γ , in the lung (Fig 3). Because cytokine proteins were never detected in BAL fluid treated with papain, probably because of its potent proteolytic activity, we used a multiplex assay to examine the effects of platelet depletion on serum levels of various cytokines and chemokines. Among 43 kinds of cytokines/chemokines tested, levels of only 4 molecules (ie, IL-5, thymus and activation-regulated chemokine [TARC], macrophage-derived chemokine [MDC], and IL-27) were increased by papain treatment, and these levels were significantly lower in the platelet-depleted group (see Fig E2 in this article's Online Repository at www.jacionline.org).

Effect of concomitant administration of platelets with papain on airway inflammation in mice

Platelets have been reported to contain a number of proteins, including growth factors, adhesion molecules, inflammatory cytokines/chemokines, and their receptors.³¹ Therefore some factors other than IL-33 in platelets can also participate in the papain-mediated eosinophil inflammation in the airway (Fig 2, B), even though the model is fully IL-33 dependent.²⁸ To further confirm the functional involvement of IL-33 in platelets, we examined whether concomitant administration of platelets with papain can enhance papain-induced airway inflammation. Of note, combined treatment with wild-type platelets, but not IL-33-deficient platelets, significantly enhanced papain-induced eosinophil numbers, as well as total WBC numbers, in the BAL fluid (Fig 4). In contrast, combined treatment with platelets derived from either wild-type or IL-33-deficient mice showed no statistically significant effects on the numbers of macrophages, neutrophils, or lymphocytes in the airway.

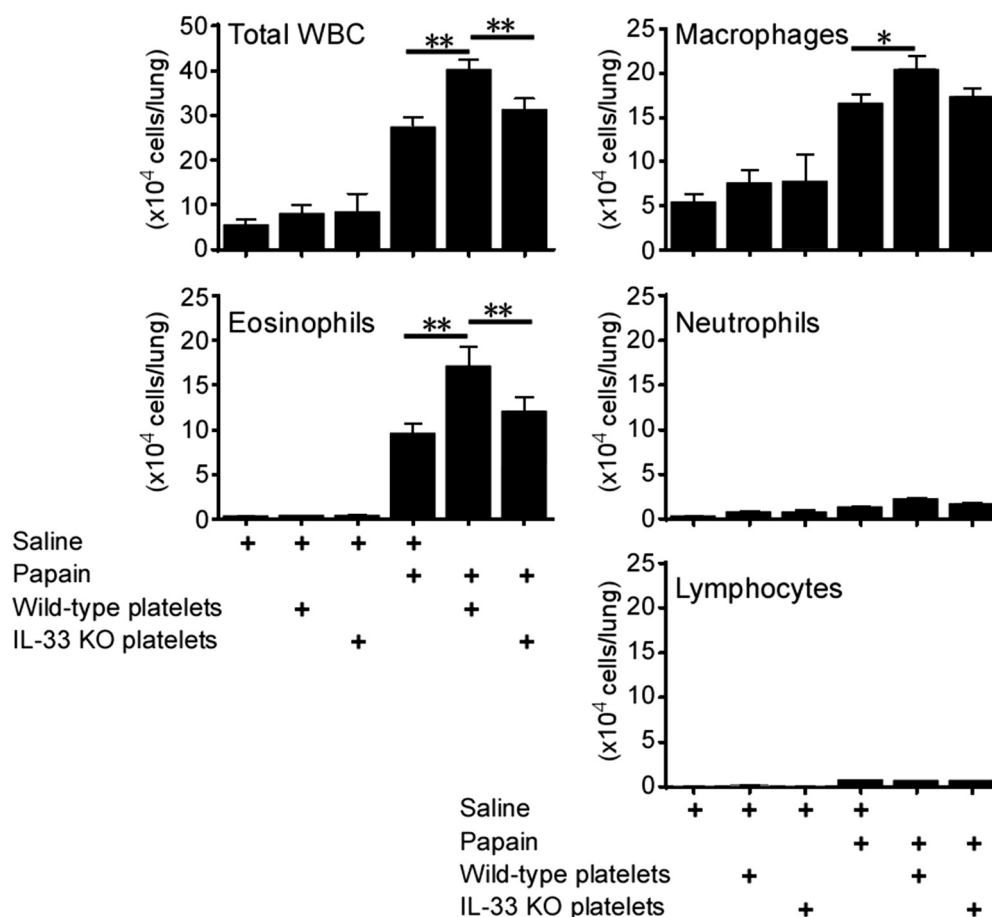


FIG 4. Effects of concomitant administration of platelets with papain on the airway inflammation. Platelets from wild-type or IL-33-deficient mice were intranasally administered immediately after each papain treatment once per day for 3 days. Twenty-four hours after the last administration, BAL fluid was collected from each mouse, and total WBCs, eosinophils, macrophages, neutrophils, and lymphocytes in each fluid were counted. Data show means $\pm$ SDs (saline, $n = 10$; saline + wild-type platelets, $n = 5$; saline + IL-33 $[-/-]$ platelets, $n = 5$; saline + papain, $n = 18$; papain + wild-type platelets, $n = 20$; and papain + IL-33 $[-/-]$ platelets, $n = 15$). * $P < .05$ and ** $P < .01$. KO, Knockout.

DISCUSSION

Platelet abnormalities in patients with allergy and asthma were initially described nearly half a century ago,³² and subsequent clinical and experimental studies have confirmed platelets as important players in the development of allergic inflammation.^{1-8,33-35} First, we demonstrated that platelets and their progenitor cells, megakaryocytes, constitutively express IL-33 protein (Fig 1), an IL-1 family cytokine. IL-33 has attracted attention as a critical cytokine in the development of allergic diseases because of its potent ability to induce type 2 immunity and its strong genetic association with asthma.^{12-15,26} IL-33 was originally identified as a nuclear protein, called NF-HEV.²⁵ The amino terminus of full-length IL-33 protein contains a predicted nuclear localization sequence and a homeodomain-like helix-turn-helix DNA binding motif.³⁶ Indeed, subsequent studies revealed that IL-33 is preferentially and constitutively expressed in the nuclei of such tissue structural cells as epithelial and endothelial cells.^{37,38} Importantly, we found that IL-33 protein was predominantly expressed in human platelets as their full-length form (31 kDa), matching the band detected in the COS-7 cells transiently transfected with full-length IL-33-expressing vector,

which served as the source of full-length IL-33 protein (Fig 1, A). IL-33 was initially thought to be biologically activated by caspase-1 cleavage, such as other IL-1 family cytokines (eg, IL-1 β and IL-18).¹⁸ However, several subsequent studies demonstrated that IL-33 is not a direct substrate for caspase-1 *in vivo* and that the full-length IL-33 protein (31 kDa) can bind to IL-33 receptor and is fully active under physiologic conditions.³⁹⁻⁴¹ Therefore we speculated that IL-33 protein detected in platelets (31-kDa form) might be biologically active and that platelets might be one of the important cellular sources of IL-33 protein, contributing to the pathogenesis of allergies and asthma.

To date, we have repeatedly tried to detect IL-33 protein in the supernatants of activated platelets under various conditions. However, we have not been successful, despite using several commercially available IL-33 ELISA kits. Several reasons can be suggested for this failure. Most importantly, the sensitivity of the anti-IL-33 antibody for intact IL-33 protein might be too low. We have also never detected IL-33 protein in the cell supernatants of bronchial epithelial cells or MEG-01 cells using those ELISA kits, even when the cells were activated by a wide variety of stimulants, such as pathogen-associated molecular patterns and

proinflammatory cytokines. This might be the weakest point and greatest limitation of this study. Therefore we attempted to determine whether activated platelets can actually release bioactive IL-33 using an alternative method.

We demonstrated previously that human microvascular endothelial cells from the lung (HMVEC-Ls) are the primary targets of IL-33, resulting in selective induction of expression of high levels of various neutrophil-attracting CXCR2 chemokines, including IL-8.²⁷ Then we measured the level of IL-8 mRNA expression in HMVEC-Ls using an *in vitro* “endothelium-platelet” coculture system to investigate whether thrombin-activated human platelets can release bioactive IL-33. Consequently, we found that thrombin-activated platelets, but not thrombin or platelets alone, significantly enhanced IL-8 expression in this coculture system, which was almost completely inhibited by concomitant neutralization using a soluble ST2-Fc chimera (see Fig E3 in this article’s Online Repository at www.jacionline.org). These results suggest that thrombin-activated platelets can release active IL-33 protein.

We also demonstrated expression of IL-33 protein in the cytosol, but not nuclear, fraction of MEG-01 cells, a human megakaryoblast cell line (Fig 1, A), as well as in the cytoplasm of bone marrow–derived megakaryocytes from wild-type but not IL-33–deficient mice (Fig 1, B). These observations make sense because platelets are cytoplasmic fragments of megakaryocytes, but further studies need to clarify the biological mechanisms of cytoplasmic localization of IL-33 protein in megakaryocytes. Nevertheless, Nile et al⁴² reported that the IL-33 protein was found mainly in the cytoplasm of human monocytes, and thus cytoplasmic localization of IL-33 protein might be a unique characteristic of myeloid cells but not tissue structural cells.

We next used a murine model of IL-33–dependent airway eosinophilia to elucidate whether platelets play a role in airway inflammation. We previously generated IL-33–deficient mice and showed that IL-33 is essential for papain-induced airway eosinophilia in mice.²⁸ Papain, which is a cysteine protease with homology with the house dust mite allergen Der p 1,⁴³ is known to be a cause of occupational asthma in human subjects.⁴⁴ In mice inhalation of papain can induce strong airway eosinophilia, even in the absence of acquired immune cells, such as T cells, B cells, and natural killer T cells. Most importantly, papain-induced airway eosinophilia is almost completely abolished in IL-33–deficient mice,²⁸ indicating that the papain-induced innate airway inflammation is fully IL-33 dependent. Therefore we examined how platelet depletion by anti-CD41 treatment affected the IL-33–dependent airway inflammation model in mice and found that it resulted in a significant decrease in eosinophilic inflammation in the papain-treated murine airway (Fig 2, B). These results suggest that the IL-33–dependent airway eosinophilia might be at least partly mediated by platelet-derived IL-33 protein. On the other hand, we reproducibly demonstrated a significant increase in the number of macrophages among BAL cells after anti-CD41 treatment. To further identify the phenotype of macrophages increased in the platelet-depleted group (Fig 2, B), CD45⁺ alveolar macrophages (AMs; F4/80⁺CD11c⁺) and intestinal macrophages (IMs; F4/80⁺CD11c[−])⁴⁵ in BAL fluid were determined by using flow cytometry (see Fig E4, A, in this article’s Online Repository at www.jacionline.org), and the total number of AMs and IMs per lung was presented (see Fig E4, B). We found that AMs were the main macrophages in BAL fluid of saline-treated control animals, regardless of anti-CD41 treatment. Papain treatment further increased the number of AMs,

especially in platelet-intact mice. Importantly, papain treatment resulted in an increased number of IMs in both the platelet-intact and platelet-depleted groups, whereas the increasing rate in the platelet-depleted group was more significant. These results suggest that increased numbers of macrophages in the BAL fluid of platelet-depleted mice (Fig 2, B) might be due to IMs recruitment into the lung. Furthermore, percentages of the CD206⁺ M2 macrophage subset⁴⁶ in the IMs of BAL fluid were determined by using anti-CD206 (Fig E4, B). As a consequence, we found that IMs increased in the platelet-intact group were partly M2 macrophages, whereas IMs increased in the platelet-depleted group were not M2 macrophages at all, although the underlying mechanism has not been clarified.

In agreement with the attenuation of eosinophilic inflammation in the lung by anti-CD41 treatment, platelet depletion significantly reduced mRNA expression for IL-4, IL-5, and IL-13, but not IFN- γ , in the lung (Fig 3). IL-5, but not IL-4 or IL-13, was also detected in the sera of the papain-treated mice and was reduced by anti-CD41 treatment (see Fig E2). We should have determined cytokine/chemokine levels in BAL fluid of mice. However, we were unable to detect any cytokine/chemokine proteins in BAL fluid treated with papain, probably because of its potent proteolytic activity. We then measured serum levels of 43 cytokines/chemokines using a multiplex assay system. However, only 4 molecules (IL-5, TARC, MDC, and IL-27) of 43 analytes were detected with statistical significance, whereas the other 39 molecules could not be detected at all (Fig E2). Both TARC and MDC can induce platelet activation through their receptor, CCR4, which has been identified in platelets.⁴⁷ Fujisawa et al⁴⁸ reported that platelets contained TARC protein and released it on stimulation with thrombin. Thus significant attenuation of blood levels of TARC and MDC by platelet depletion (see Fig E2) might reflect the levels, numbers, or both of activated platelets. Activated platelets are also known to release abundant functional IL-27.⁴⁹ According to a platelet mRNA expression profile found in Gene Expression Omnibus (GSE11524),⁵⁰ platelets express IL-5 mRNA. However, we do not know how much IL-5 protein is contained in platelets.

We previously demonstrated that papain-induced airway eosinophilia was profoundly impaired in IL-4/IL-13 double-deficient mice, as well as in IL-33–deficient mice, even though increased papain-induced airway inflammation was observed in Rag-2–deficient mice, which lack T cells, B cells, and natural killer T cells.²⁸ These results strongly suggest that IL-4, IL-13, or both derived from innate inflammatory cells, but not T cell–derived IL-4, IL-13, or both, are important for the event. Recently, a number of IL-5– and IL-13–producing innate lymphoid cells, now termed ILC2s,⁵¹ which include natural helper cells, nuocytes, and innate type 2 helper cells, were identified.^{16,52,53} These cells constitutively express IL-33 receptor, IL1RL1/ST2, and can produce larger amounts of IL-5 and IL-13 than basophils and mast cells in response to IL-33. Indeed, Halim et al⁵⁴ demonstrated that lung natural helper cells were required for papain-induced eosinophil infiltration in the murine airway. More recently, the same group reported that ILC2-derived IL-13 was critical for papain-induced T_H2 cell differentiation, as well as eosinophilia in the lung.⁵⁵ They also showed that papain-induced ILC2 activation and T_H2 cell differentiation were IL-33 dependent.⁵⁵ Motomura et al⁵⁶ demonstrated that basophil-derived IL-4 is essential for ILC2-produced IL-5, IL-13, and eotaxin, resulting in airway eosinophilia in this model. Together with our data showing that

platelet depletion significantly reduced mRNA expression of IL-4, IL-5, and IL-13 (Fig 3), we speculate that platelet-derived IL-33 can play pivotal roles in papain-induced airway eosinophilia at least in part through activation of ILC2s, basophils, or both, resulting in platelet-dependent upregulation of IL-4, IL-5, and IL-13. However, a functional association between platelet-derived IL-33 and ILC2s or basophils still needs to be demonstrated.

Platelets have been reported to contain a number of proteins, including growth factors, adhesion molecules, inflammatory cytokines/chemokines, and their receptors.³¹ Therefore we cannot conclude that the effects of platelet depletion on reduced airway eosinophilia were directly and functionally caused by IL-33 depletion, despite use of an IL-33-dependent model. To further confirm the functional relevance of IL-33 in platelets, we investigated whether concomitant administration of platelets would have an additive effect on papain-induced airway inflammation. In contrast to platelet depletion (Fig 2, B), combined treatment with wild-type platelets, but not IL-33-deficient platelets, significantly enhanced papain-induced eosinophil counts, as well as total WBC counts, in the BAL fluid (Fig 4). On the other hand, combined treatment with platelets derived from wild-type and IL-33-deficient mice showed no statistically significant effects on the numbers of macrophages, neutrophils, or lymphocytes in the airways. These results suggest that platelets might be significant cellular sources of IL-33 protein in papain-induced and IL-33-dependent airway eosinophilia in addition to their well-established roles in blood coagulation and hemostasis. We also examined the effects of adoptive transfer of human platelets as sources of IL-33 protein in the platelet-depleted, papain-induced airway inflammation model on an IL-33-deficient background. Consequently, we found that human platelets restored the papain-induced airway eosinophilia on an IL-33-deficient background (see Fig E5 in this article's Online Repository at www.jacionline.org), further supporting the hypothesis that platelet-derived IL-33 might be an important contributor to papain-induced airway eosinophilia *in vivo*.

In summary, a number of previous reports revealed that platelets actively participate in the pathogenesis of bronchial asthma. In the present study we show that platelets and their progenitor cells, megakaryocytes, constitutively express IL-33 protein (31 kDa). Papain-induced IL-33-dependent airway eosinophilia in mice was significantly decreased by platelet depletion. Conversely, concomitant administration of papain with platelets derived from wild-type mice, but not from IL-33-deficient mice, enhanced the papain-induced eosinophilia in the airway. Although IL-33 has been shown to be a fundamental regulator of allergic inflammation, the exact cellular sources of functional IL-33 protein, especially *in vivo*, remain poorly understood. Our novel findings suggest that platelets might be an important cellular source of IL-33 protein and that platelet-derived IL-33 could play a role in type 2 inflammation in the airway. Greater understanding of the precise molecular mechanisms of platelet-derived IL-33 in the pathophysiology of asthma can lead to platelets becoming an attractive novel therapeutic target for asthma.

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Key messages

- Platelets and their progenitor cells, megakaryocytes, constitutively express IL-33 protein, which is an essential regulatory molecule of allergic inflammation.
- Platelet-derived IL-33 protein is functionally involved in protease-induced airway eosinophilic inflammation in mice, suggesting that it might be a useful therapeutic target for asthma.

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METHODS

Histology

Lungs were harvested 24 hours after the last administration of papain or saline (Fig 2, B) and fixed in Carnoy solution. The fixed tissue was embedded in paraffin, sliced into 5-mm sections, and stained with hematoxylin and eosin.

Luminex multiplex assay of cytokine/chemokine concentrations in murine serum

Twenty-four hours after the last administration of papain or saline, blood serum was collected from each mouse and later assayed for the following 43 cytokines and chemokines: IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-20, IL-21, IL-22, IL-23, IL-25, IL-27, IL-28 β , IL-33, IFN- γ , TNF- α , IFN-inducible protein 10, keratinocyte-derived cytokine, monocyte chemoattractant protein 1, monocyte chemoattractant protein 5, macrophage inflammatory protein (MIP) 1 α , MIP-1 β , MIP-2, MIP-3 α , MIP-3 β , RANTES, erythropoietin, Exodus-2, TARC, Fractalkine, MDC, tissue inhibitor of metalloproteinase 1, granulocyte colony-stimulating factor, and GM-CSF by using customized Milliplex kits (Millipore, St Charles, Mo) in accordance with the manufacturer's instructions.

In vitro "endothelial-platelet" coculture assay to evaluate IL-33 bioactivity

Primary HMVEC-Ls were purchased from Lonza (Walkersville, Md) and cultured in EGM-2MV BulletKit culture medium (Lonza). HMVEC-Ls were washed twice with sterile HBSS (Thermo Scientific) and replaced with fresh EGM-2MV medium alone (control), EGM-2MV medium containing 10 IU/mL thrombin (Sigma-Aldrich), 4×10^4 purified human platelets, or 5 μ g/mL recombinant human ST2-Fc chimera protein (R&D Systems) for IL-33 neutralization, each alone and in combination, as indicated in Fig E3. Six hours after treatment, the level of mRNA for IL-8 was determined by means of qPCR, according to our previous report.^{E1}

Flow cytometry

Macrophages in BAL fluid were identified by means of flow cytometry with the following antibodies: CD45–allophycocyanin-Cy7 (clone30-F11),

CD11c–phycoerythrin-Cy7 (clone N418), F4/80–peridinin-chlorophyll-protein complex Cy5.5 (clone BM8) and CD206–phycoerythrin (clone C068C2; BioLegend, San Diego, Calif). Dead cells were excluded by means of propidium iodide staining, and live cells were analyzed on a Gallios Flow Cytometer (Beckman Coulter, Fullerton, Calif) with Kaluza v1.2 software (Beckman Coulter).

Adoptive transfer of human platelets in the platelet-depleted, papain-induced airway inflammation model in IL-33-deficient mice

The study design of platelet depletion and papain-induced airway inflammation in IL-33-deficient mice is shown in Fig 2, A. On both days 1 and 3, purified human platelets (purity > 98%), as assessed based on expression of CD41 and size estimation by using flow cytometry, were intravenously administered (2.5×10^7 platelets/500 μ L of sterile PBS) to the mice. Twenty-four hours after the last administration of papain and human platelets, BAL fluid was collected from each mouse, and the numbers of WBCs, eosinophils, macrophages, neutrophils, and lymphocytes in the BAL fluid were counted with a hemocytometer (Sysmex XT-1800i). This experimental protocol is feasible because of the following 2 points: (1) the anti-mouse CD41 rat mAb used for platelet depletion in mice (MWReg30; Becton, Dickinson and Company) does not cross-react with human CD41 and (2) human IL-33 can react with mouse ST2 receptor.^{E2}

Statistical analysis

All data are presented as means $\pm$ SDs. Differences between groups were analyzed by using a 2-sided Welch *t* test and considered significant at a *P* value of less than .05.

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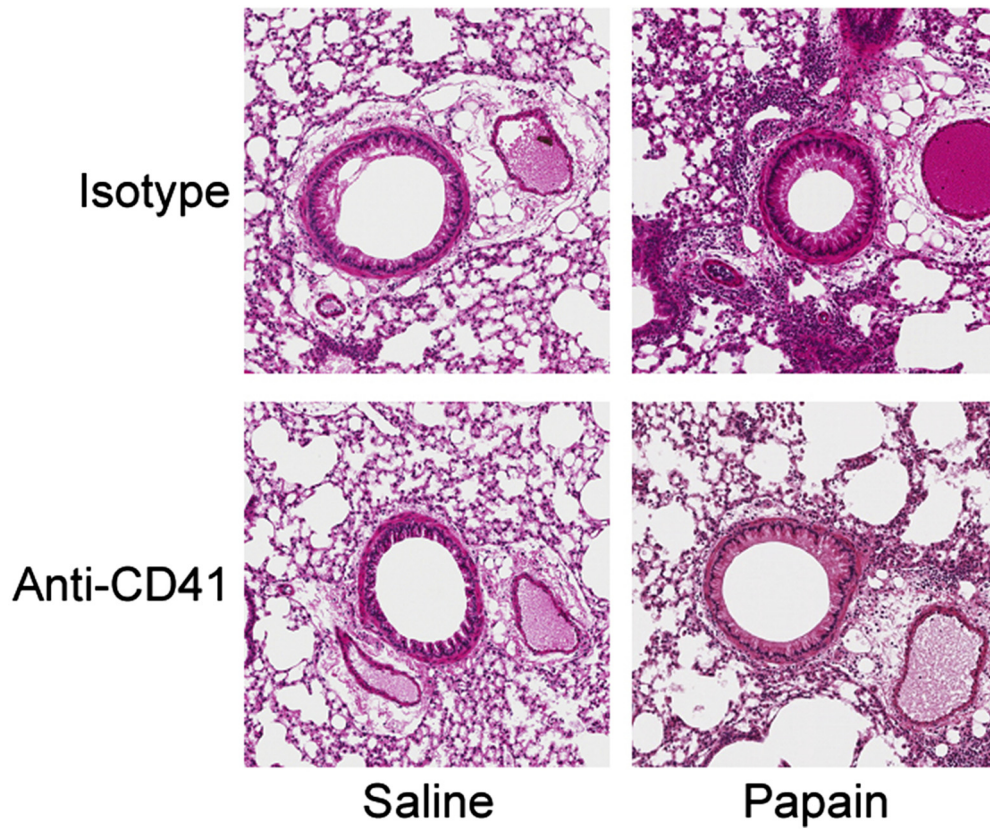


FIG E1. Papain-induced airway inflammation was attenuated by anti-CD41 treatment. Lungs were harvested 24 hours after the last administration of papain or saline and stained with hematoxylin and eosin.

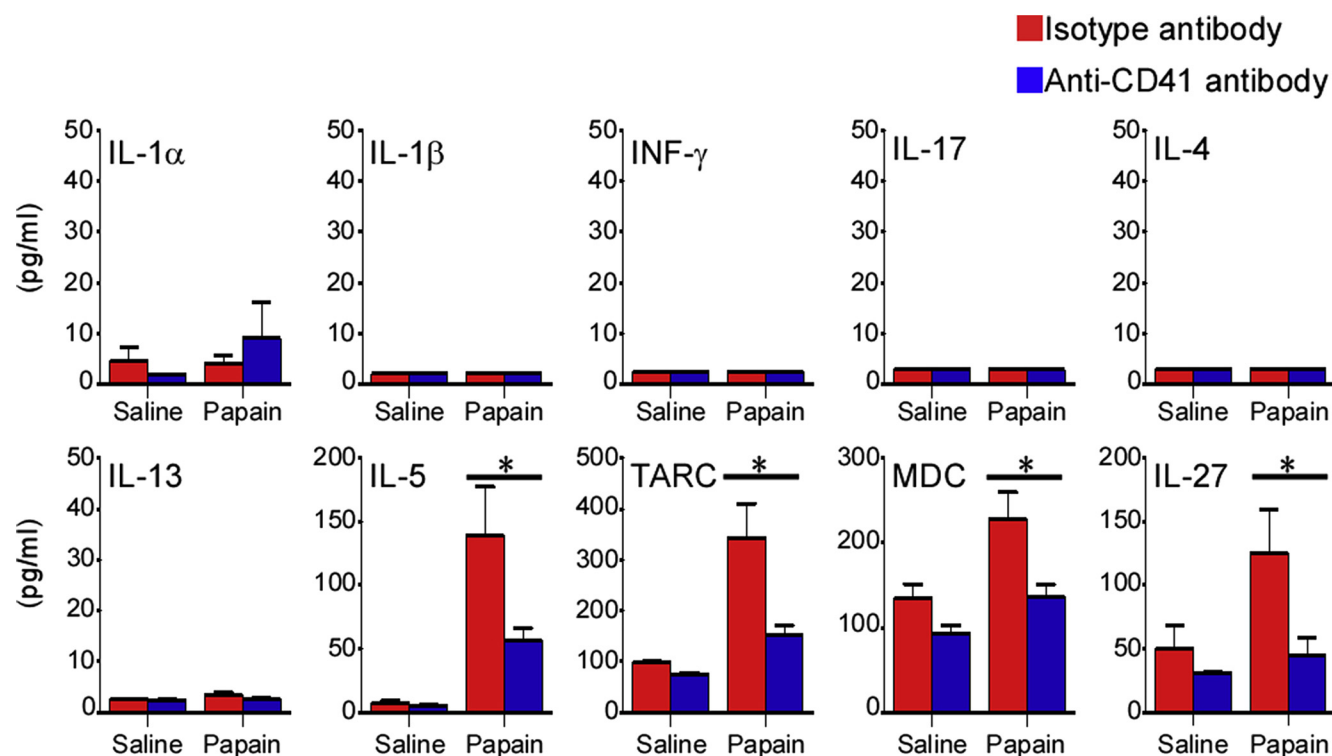


FIG E2. Effects of platelet depletion on expression of cytokines/chemokines in blood serum. Blood serum was collected from each mouse at 24 hours after the last administration of papain or saline. Concentrations of 43 kinds of cytokines/chemokines (listed in the Methods section in this article's Online Repository) in sera were measured by using the multiplex assay. Only 4 molecules (IL-5, TARC, MDC, and IL-27) of the 43 analytes were detected with statistical significance, whereas the other 39 molecules could not be detected at all. Despite being negative, the data for IL-1 α /IL-1 β , INF- γ , IL-17, and IL-4/IL-13 are shown as representatives of proinflammatory, T_H1, T_H17, and T_H2 cytokines, respectively, in addition to those for IL-5, TARC, MDC, and IL-27. Data show means $\pm$ SDs (isotype antibody + saline, n = 3; isotype antibody + papain, n = 5; anti-CD41 antibody + saline, n = 5; and anti-CD41 antibody + papain, n = 5). Differences between groups were analyzed by means of ANOVA with the Bonferroni *post hoc* test and considered significant at a *P* value of less than .05. **P* < .05.

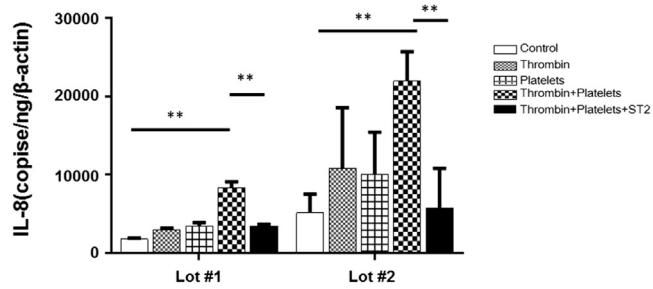


FIG E3. *In vitro* “endothelium-platelet” coculture assay to evaluate IL-33 bioactivity. HMVEC-Ls were treated with thrombin, purified human platelets, and recombinant human ST2-Fc chimera protein, either alone or in combination for 6 hours, and mRNA levels for IL-8 were measured by using qPCR. Data from 2 different lots of HMVEC-Ls (Lots #1 and #2) from 2 individual donors were shown as means $\pm$ SDs of triplicate or quadruple samples. ** $P < .01$.

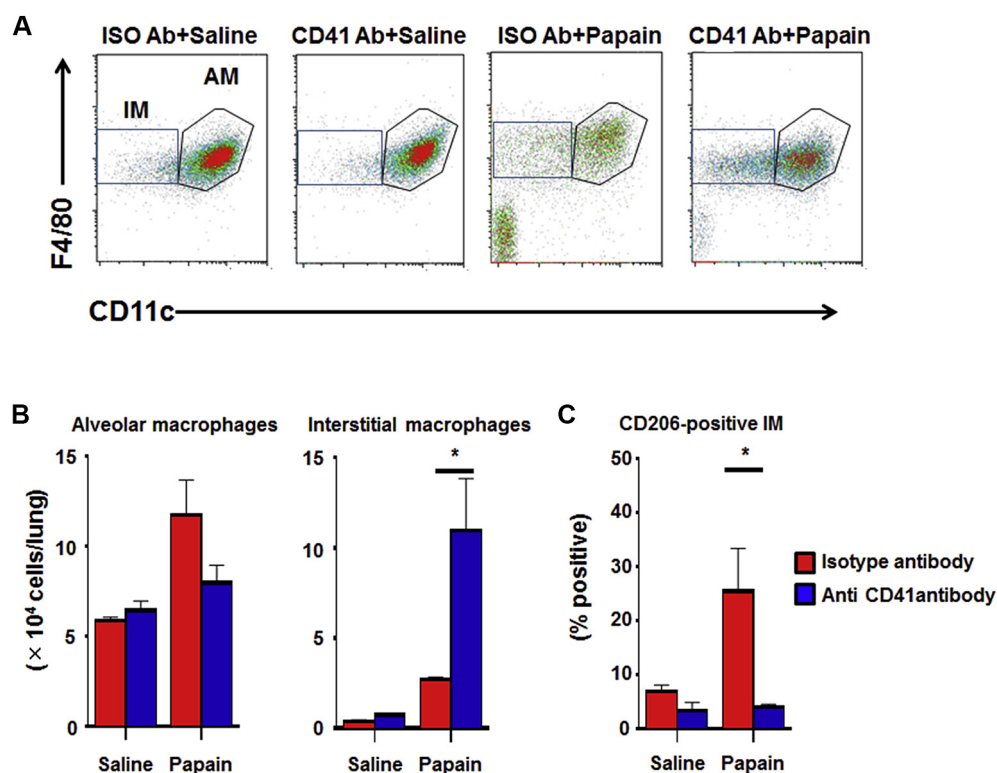


FIG E4. Flow cytometric analysis of macrophages. Twenty-four hours after the last administration of papain or saline, BAL fluid was collected from each mouse. **A**, CD45⁺ AMs (F4/80⁺CD11c⁺) and IMs (F4/80⁺CD11c⁻) in BAL fluid were determined by means of flow cytometry. *Ab*, Antibody. **B**, Total numbers of AMs and IMs per lung are shown. **C**, Percentages of the CD206⁺ M2 macrophage subset in IMs from each BAL fluid sample were determined by using anti-CD206. Data show means $\pm$ SDs (isotype antibody + saline, $n = 5$; isotype antibody + papain, $n = 5$; anti-CD41 antibody + saline, $n = 5$; and anti-CD41 antibody + papain, $n = 5$). * $P < .05$.

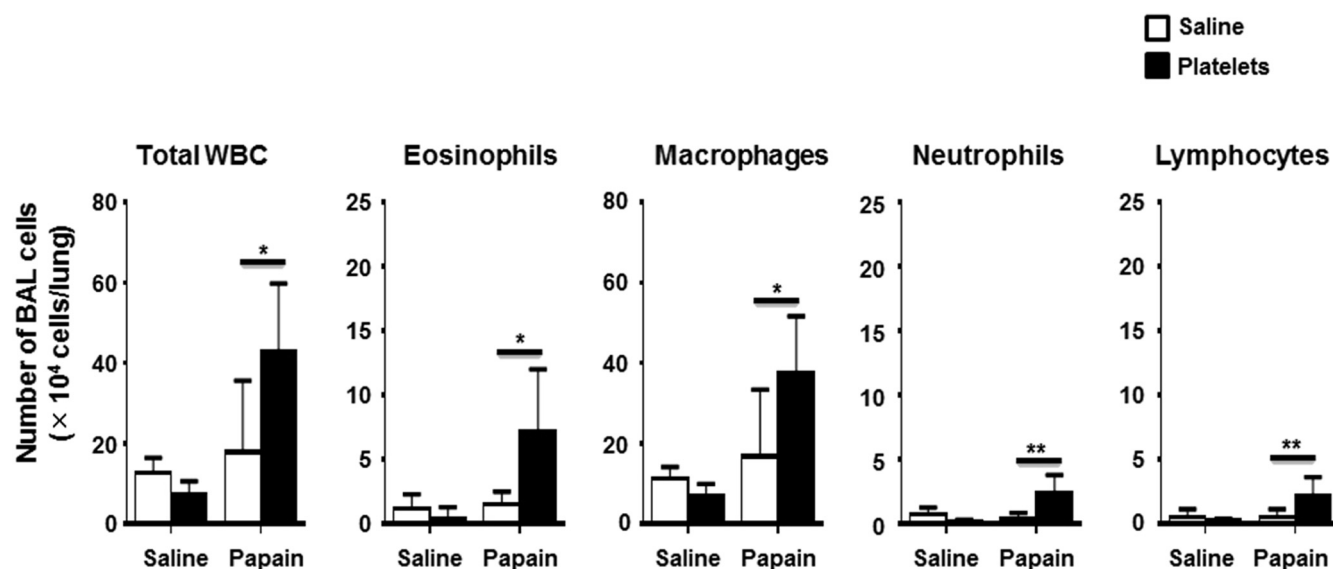


FIG E5. Effects of adoptive transfer of human platelets in IL-33-deficient mice. The study design of platelet depletion and papain-induced airway inflammation in IL-33-deficient mice was the same as described in Fig 2, A. On both days 1 and 3, purified human platelets were intravenously administered (2.5×10^7 platelets/500 μ L of sterile PBS). Twenty-four hours after the last administration of papain and human platelets, BAL fluid was collected from each mouse, and the numbers of total WBCs, eosinophils, macrophages, neutrophils, and lymphocytes in BAL fluid were counted. Data show means $\pm$ SDs (saline, $n = 5$; saline + human platelets, $n = 4$; papain, $n = 5$; and papain + human platelets, $n = 7$). * $P < .05$ and ** $P < .01$.