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*

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

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Supported in part by National Institute of Biomedical Innovation grant ID10-43 (to K.M.), grants from the National Center for Child Health and Development #26-9 (to K.M.) and #26-46 (to M.E.-S.), and JSPS KAKENHI grant no. 23591666 (to A.M.).

Disclosure of potential conflict of interest: E. Inoue has consultant arrangements with Tokyo Women's Medical University, is employed by Kitasato University, and has received payment for lectures from Merck Serono. H. Saito is employed by the National Center for Child Health & Development, Japan; has received grants from the Ministry of Health, Labour & Welfare, Japan, and the Japan Society for Promotion of Science; has received payment for lectures from Teijin Pharma, Merck Sharp and Dohme KK, Nippon Boehringer Ingelheim, Shiseido, Ono Pharmaceutical, GlaxoSmithKline KK, Pfizer Japan, Novartis Pharma KK, Kyowa Hakko Kirin, Kyorin Pharmaceutical, Daiichi Sankyo, and AstraZeneca KK; has received payment for development of educational presentations from Toho University School of Medicine; and has received travel support from the Japanese Society of Pediatric Allergy & Clinical Immunology, the Japanese Society of Allergology, and the World Allergy Organization. K. Matsumoto has received grants from the National Institute of Biomedical Innovation (Grant ID10-43) and a grant from the National Center for Child Health and Development (#26-9); is employed by the National Research Institute for Child Health and Development; has received payment for lectures from Merck Sharp and Dohme KK, Ono Pharmaceutical, GlaxoSmithKline, Kyorin Pharmaceutical, Ohtsuka Pharmaceutical, Mitsubishi Tanabe Pharma, AstraZeneca KK, Siemens Healthcare, Abbott Japan, Sumitomo Dainippon Pharma, Maruho, and Teijin Pharma; and has received payment for development of educational presentations from Gifu Pharmaceutical University. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 4, 2014; revised November 29, 2015; accepted for publication January 7, 2016.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2016.01.032

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 al[8] showed that bronchial allergen challenge in asthmatic patients resulted in activation of peripheral blood platelets. Duarte et al[9] 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 al[10] 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 $P2Y_{12}$ 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 al[26]), 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 studies[12-14] 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 300*g* 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*H1 site and the ATG codon 5′-CGGATCCACCATGAAGCCTAAAATGAAGTATTCAAC CAAC-3′, and the reverse primer contained a stop codon and the *Eco*RI site 5′-ACCGAATTCCTAAGTTTCAGAGAGCTTAAACAAGATATTTTC-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