

The cytokine interleukin-33 mediates anaphylactic shock

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Anaphylactic shock is characterized by elevated immunoglobulin-E (IgE) antibodies that signal via the high affinity Fcε receptor (FcεRI) to release inflammatory mediators. Here we report that the novel cytokine interleukin-33 (IL-33) potently induces anaphylactic shock in mice and is associated with the symptom in humans. IL-33 is a new member of the IL-1 family and the ligand for the orphan receptor ST2. In humans, the levels of IL-33 are substantially elevated in the blood of atopic patients during anaphylactic shock, and in inflamed skin tissue of atopic dermatitis patients. In murine experimental atopic models, IL-33 induced antigen-independent passive cutaneous and systemic anaphylaxis, in a T cell-independent, mast cell-dependent manner. *In vitro*, IL-33 directly induced degranulation, strong eicosanoid and cytokine production in IgE-sensitized mast cells. The molecular mechanisms triggering these responses include the activation of phospholipase D1 and sphingosine kinase1 to mediate calcium mobilization, Nuclear factor-κB activation, cytokine and eicosanoid secretion, and degranulation. This report therefore reveals a hitherto unrecognized pathophysiological role of IL-33 and suggests that IL-33 may be a potential therapeutic target for anaphylaxis, a disease of considerable unmet medical need.

anaphylaxis | cytokine biology | IL-33

Interleukin (IL)-33 is a new member of the IL-1 family, which includes IL-18 and IL-1β. Like IL-18 and IL-1β, IL-33 was found to have strong immunomodulatory functions (1). However, whereas IL-1β and IL-18 promote proinflammatory and TH1-associated responses, IL-33 induces the production of TH2-associated cytokines and increased levels of serum Ig (1). Moreover, treatment of mice with IL-33 led to high levels of serum IgE and expression of TH2-associated cytokines (1). IL-33 signals via its unique receptor, ST2, and IL-1RAcP (1, 2). ST2, also called T1, DER4 and Fit-2, is highly expressed on mast cells and on TH2 cells (1–4).

The ST2 gene encodes two isoforms of ST2 proteins: ST2L, a transmembrane form, and the soluble ST2 (sST2), a secreted form that can serve as a decoy receptor of IL-33. We have reported that ST2 is associated with important TH2 effector functions (4–6). Treatment of mice with either an antagonistic antibody against ST2 or with an ST2-Fc fusion protein led to enhancement of T helper type 1 (TH1) responses and an inhibitory effect on TH2-associated allergic airway inflammation (6). Interestingly, high levels of sST2 has been found in the sera of patients with acute Asthma (7), and serum elevations of sST2 predict mortality and heart failure in patients with acute myocardial infarction (8, 9). Moreover, a role for IL-33/ST2 has also been suggested for arthritis and atherosclerosis (10, 11). However, the role of IL-33/ST2 in systemic anaphylactic shock (SAS or type I hypersensitivity), is unknown.

The symptoms of type I hypersensitivity are largely attributable to the pharmacologically active materials released by activated mast cells (12, 13). The clinical consequences of type I hypersensitivity triggered by mast cells can range from localized reactions including allergic rhinitis, asthma, atopic dermatitis

and food allergies, to severe life-threatening systemic reactions such as anaphylaxis. Because mast cells are pivotal to allergic reactions and express a high density of ST2, and because recent reports showed that IL-33 can induce mast cells to produce proinflammatory cytokines *in vitro* (14–17), we investigated the potential role of IL-33 in allergic SAS in humans and in murine models of IgE-mediated passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA), the most commonly used models to study specific mast cell responses *in vivo* (18–20).

We show here that IL-33 is markedly elevated in the serum of patients during an anaphylactic shock and in atopic human tissue, suggesting a potential role for IL-33 in human pathology. Moreover, we report here that IL-33 plays a pivotal role in experimental anaphylaxis. In the presence of IgE, IL-33 activates mast cell degranulation through molecular mechanisms that trigger key signal transduction events, including phospholipase D1 and sphingosine kinase-1, to mediate calcium mobilization. Thus, our finding suggests a potential therapeutic target against allergy, an important disease with considerable unmet medical need.

Results

IL-33 Expression Is Increased in Patients with Anaphylaxis and Dermatitis. To investigate a potential role for IL-33 in human atopic allergy, we examined the levels of IL-33 in the serum and tissue from patients during anaphylactic and allergic responses. In atopic patients undergoing surgery, those who developed anaphylactic shock in the operating theater had high levels of IgE and the levels of IL-33 were markedly elevated compared with levels in healthy and atopic control subjects (Figs. 1A, 1B). Immune-staining and real-time-PCR analysis of samples from the skin lesions show that the level of IL-33 mRNA was substantially higher in the skin lesions of patients with atopic dermatitis compared with noninflamed skin samples (Fig. 1C). The presence of IL-33 was also clearly evident in the skin biopsies of atopic dermatitis but absent in healthy skin (Fig. 1C). These data demonstrate that IL-33 is closely associated with clinical allergic and anaphylactic responses and may play an important role in mediating these diseases. To investigate this hypothesis in greater detail we proceeded with the murine models.

IL-33 Triggers Passive Cutaneous Anaphylaxis. We first investigated whether IL-33 could contribute to the anaphylactic response *in vivo* using a murine model of IgE-mediated PCA. One of the key

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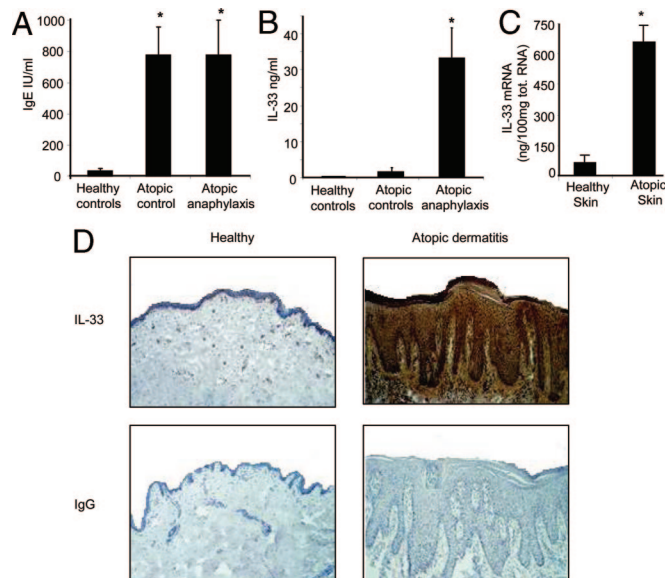


Fig. 1. IL-33 in atopic patients during anaphylaxis and inflammation. (A) Total IgE levels in healthy donors, atopic controls, and atopic patients with anaphylactic shock. (B) IL-33 levels in healthy donors, atopic controls, and atopic patients with anaphylactic shock. Data are mean $\pm$ SD, $n = 5$ for each group. $*P < 0.01$ compared to healthy. (C) Total RNA was extracted from skin biopsy samples of healthy donors and skin lesions of atopic dermatitis patients. IL-33 mRNA was determined by quantitative polymerase chain reaction (Q-PCR). Data are mean $\pm$ SD, $n = 7$, $*P < 0.01$ compared to healthy skin. (D) Immune staining with anti-IL-33 antibody or control IgG. Pictures are representative of skin biopsy samples of seven healthy and seven dermatitis donors.

inflammatory events during an allergic reaction is an increase in vascular permeability (18, 19). Mice were sensitized s.c. with anti-DNP IgE antibody and then challenged intravenously 24 hours later with DNP-HSA, IL-33, or IL-33+DNP-HSA, together with Evans blue. Vascular permeability was visualized by the extent of blue staining of the injection sites at the reverse side of skin sections 60 minutes after challenge. Nonsensitized mice showed no vascular permeability (negative control) and mice sensitized with IgE and challenged with DNP-HSA showed the expected level of vascular permeability (positive control). IL-33 was not able to trigger PCA in nonsensitized mice. In contrast, mice sensitized with the IgE and challenged with IL-33 alone showed a comparable level of vascular permeability as mice challenged with DNP-HSA (Fig. 24). Moreover, IL-33 had an additive effect on the PCA response when injected in conjunction with DNP-HSA (Fig. 24). Thus, in IgE-sensitized mice, IL-33 can trigger an anaphylactic response and, together with the allergen, IL-33 further enhances anaphylaxis, demonstrating a key role for IL-33 in allergy.

To test whether IL-33 could trigger mast cell degranulation *in vivo*, skin samples at the injection sites were removed and examined histologically. The IL-33-challenged, IgE-sensitized mice displayed the number of mast cells and level of degranulation similar to that of the positive control group (Fig. 2B and [supporting information (SI) Fig. S1]). To confirm that the IL-33/ST2 response in the PCA was mediated by mast cells and not by T-lymphocytes, we performed PCA experiments in RAG1^{-/-} mice, which contain no mature T or B cells (21). IL-33 induced a PCA response and mast cell degranulation in RAG1^{-/-} mice indistinguishable from that in the WT mice (Figs. 2C, 2D), demonstrating that the IL-33-induced PCA is independent of T or B cells.

To determine the specificity of IL-33-induced PCA, we tested the effect of IL-33 in ST2^{-/-} mice. DNP-HSA induced PCA and

most cell degranulation in both WT and ST2^{-/-} as expected. In contrast, while IL-33 induced PCA and mast cell degranulation in WT mice, IL-33 did not induce an appreciable level of PCA or mast cell degranulation in ST2^{-/-} mice (Figs. 2E, 2F). Taken together these data demonstrate that IL-33 is capable of triggering mast cell-mediated allergic responses *in vivo* via ST2 signaling.

IL-33 Triggers Systemic Anaphylaxis. We then investigated the role of IL-33 in anaphylactic shock, an acute and systemic allergic condition. Mice injected intravenously with anti-DNP IgE antibody and challenged intravenously 16 hours later with IL-33 developed markedly reduced body temperature, reaching 31 °C 30 minutes after IL-33 challenge (Fig. 3A). The rapid decrease in body temperature was completely prevented by the inclusion of anti-IL-33 antibody or soluble ST2 (sST2, a decoy receptor for IL-33) in the IgE inoculums. Furthermore, anti-IL-33 or sST2 injected 5 minutes after the IL-33 challenge still significantly prevented the drop in body temperature. The effect of IL-33 in the induction of anaphylactic shock and the therapeutic influence of anti-IL-33 and sST2 was also reflected in the plasma histamine levels (Fig. 3B), cytokine and chemokine concentrations (Figs. 3C, 3D), and in immune-cell infiltration into the lungs with concurrent lung inflammation (Fig. 3E, Fig. S2). All these parameters were substantially increased by IL-33 and inhibited by either anti-IL-33 mAb and/or soluble-ST2. Moreover, in IgE sensitized ST2^{-/-} mice, IL-33 failed to induce the anaphylactic response whereas changes in body temperature and increase serum histamine level were observed when the mice were challenged with the DNP-HSA antigen (Fig. 3F). Taken together, these results demonstrate that IL-33 also plays a pivotal role in systemic anaphylaxis.

IL-33 Induces Mast Cell Degranulation *In Vitro*. IL-33 is able to trigger mast cells to release proinflammatory cytokines *in vitro* (14–17). However, attempts to directly induce mast cell degranulation by IL-33 *in vitro* had so far not been met with success. Since individuals with allergy or during certain infections exhibit elevated levels of IgE, we therefore stimulated mast cells with IL-33 in the presence of IgE *in vitro*. Purified mast cells from human and mouse were cultured overnight with IgE and then stimulated with IL-33. Under this culture condition, IL-33 induced the release of β -hexosaminidase (a marker of mast cell degranulation) in a dose-dependent manner (Fig. 4A). In contrast, IL-33 failed to induce mast cell degranulation *in vitro* in the absence of IgE (Fig. 4B), or if the duration of IgE sensitization was shorter than overnight (data not shown). These results are consistent with our findings above that IL-33 could only induce allergy and anaphylactic shock in IgE-sensitized mice. To test the specificity of IL-33, mast cells were purified from the peritoneal cavity of WT or ST2^{-/-} mice sensitized with anti-DNP-HSA IgE. The cells were then cultured with IL-33 *in vitro*. IL-33 triggered degranulation in mast cells from WT mice but not in cells from ST2^{-/-} mice (Fig. 4C). Importantly, IL-33 also triggered mast cells to produce eicosanoids, cytokines and chemokines in an ST2-dependent manner (Figs. 4D–F). Moreover, consistent with our *in vivo* findings above, IL-33 had additive effects with antigen (DNP-HSA) in the induction of mast cell degranulation of WT mice sensitized with IgE (Fig. 4G). Furthermore, mast cells sensitized with IgE express a substantially higher level of ST2 than nonsensitized mast cells (Fig. 4H), perhaps explaining why IL-33 is capable of inducing mast cell degranulation only after IgE sensitization. These results therefore demonstrate that IL-33 is able to directly trigger degranulation and the synthesis of a wide range of mediators (inflammatory cytokines/chemokines and eicosanoids) via ST2.

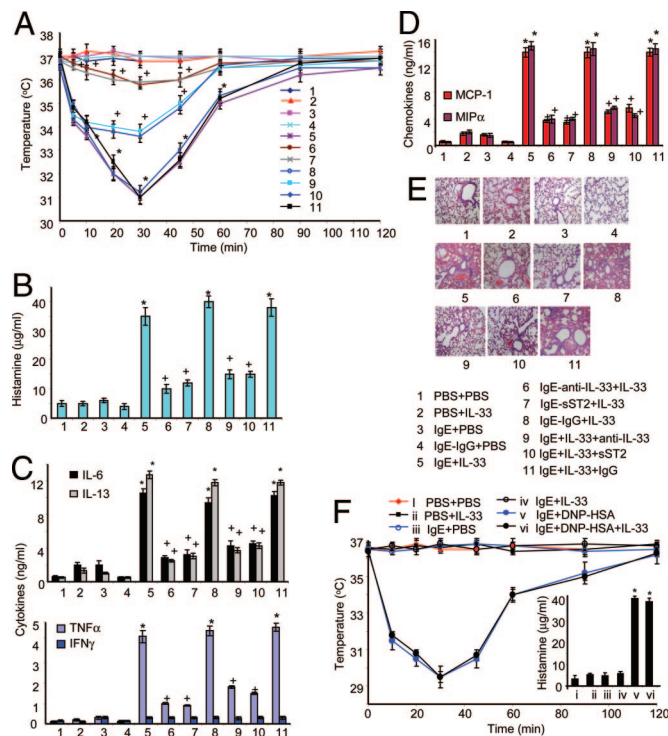
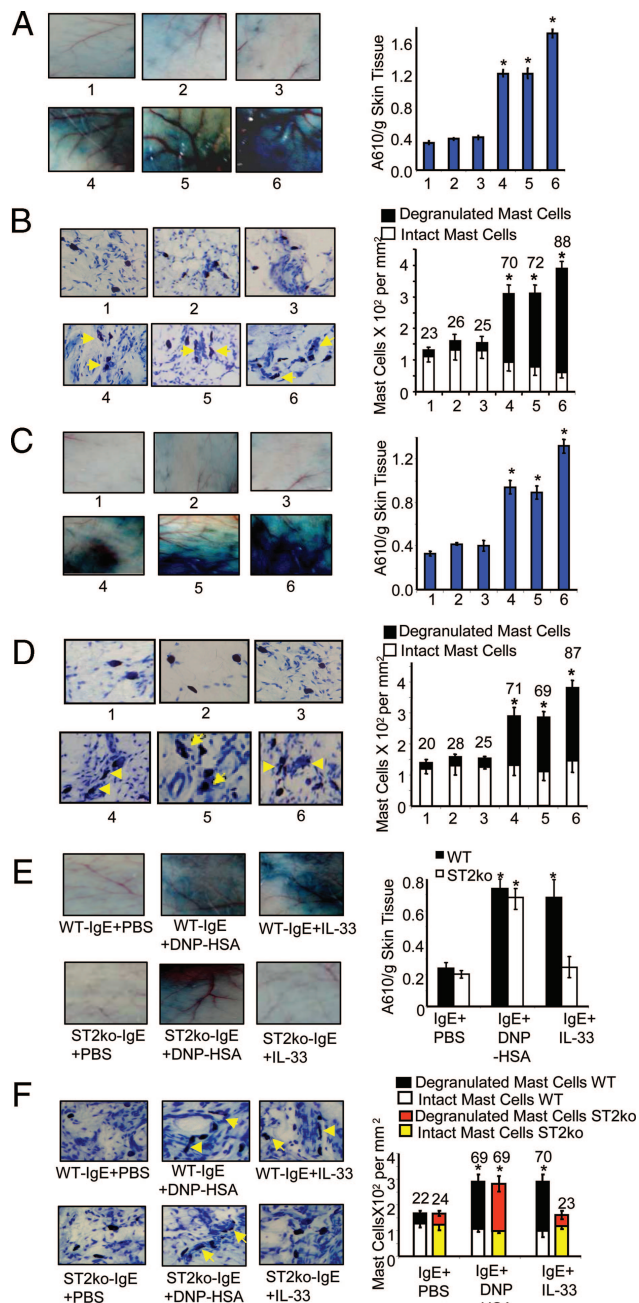


Fig. 3. Systemic anaphylactic shock in mice. For systemic anaphylactic shock in wild-type mice, mice were injected i.v. with the various reagents as indicated in the figure and in Materials and Methods (treatments 1–11). (A) Rectal temperature was measured every 10 minutes after challenge. (B) Serum histamine, (C) plasma cytokines and (D) chemokines were examined at 120 minutes after challenge. Data are mean $\pm$ SEM, $n = 5$, $*P < 0.01$ compared to group 1, $+P < 0.01$ compared with group 5. (E) Lung sections from mice injected i.v. with the various reagents as indicated in the figure and in Materials and Methods (treatments 1–11), were stained with hematoxylin and eosin and examined under light microscopy. The pictures are representative of five mice per group. For full treatment details, please refer to the method, "Induction and Measurement of Anaphylactic Shock." (F) To test the specificity of IL-33-induced systemic anaphylactic shock, ST2^{-/-} mice were injected i.v. with the various reagents as indicated in the figure and in Materials and Methods (treatments 1–6), data are mean $\pm$ SEM, $n = 5$, $*P < 0.01$ compared with group 1.

IgE Sensitization of Mice for IL-33–Mediated Anaphylaxis and Mast Cell Degranulation Is Antigen Independent. To fully characterize the

requirement of IgE sensitization for IL-33-mediated anaphylactic responses, we used three additional murine IgE preparations with different antigen specificity from different sources. Mice sensitized with all of the IgE preparations and challenged with IL-33 showed a comparable level of vascular permeability and *in vivo* mast cell degranulation (Figs. S3A, B). Moreover, mice injected intravenously with all of the IgE antibodies and challenged intravenously 16 hours later with IL-33 developed similar responses, including reduced body temperature, and enhanced levels of serum histamine (Figs. S3C, D). Furthermore, IL-33 also triggered comparable levels of mast cell degranulation in cells sensitized with all of the four IgE preparations *in vitro* (Fig. S3E).

To investigate whether the IgE-sensitized and IL-33-induced anaphylaxis has physiological relevance, we examined the role of IL-33 in a model of ovalbumin (OVA) sensitization, where endogenous IgE are generated after allergen sensitization. After OVA sensitization, the levels of endogenous IgE were increased to similar levels in wild-type, mast cell-deficient (*Kit*^{W-sh/W-sh} mutant) mice (22), and ST2^{-/-} mice (Fig. 5A). However, when challenged with IL-33, only the wild-type mice, but not the mast cell-deficient or the ST2^{-/-} mice, developed anaphylaxis, as

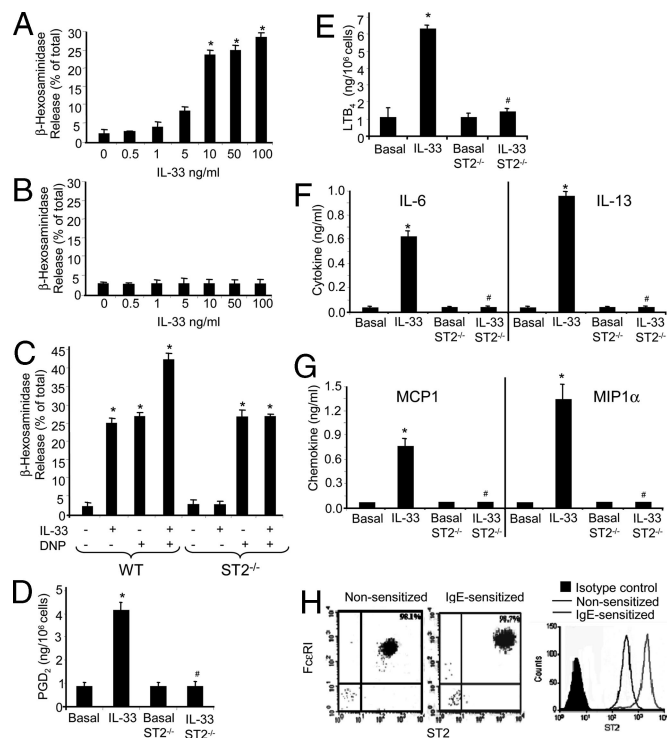


Fig. 4. IL-33 triggers mast cell degranulation and proinflammatory mediators in an ST2-dependent manner. (A) Wild-type mast cells were sensitized with IgE and β -hexosaminidase release was measured after 30 minutes of stimulation with increasing concentration of IL-33. (B) Wild-type mast cells (nonsensitized) were stimulated with increasing concentrations of IL-33, and β -hexosaminidase release was measured after 30 minutes of stimulation. (C) Wild-type and ST2^{-/-} mast cells were sensitized with IgE and β -hexosaminidase release was measured after 30 minutes of stimulation with: 10 ng/ml of IL-33; or 1 μ g/ml of antigen DNP-HSA or a combination of both triggers. PGD₂ (D), LTB₄ (E), cytokines (F), and chemokines (G) production was determined 24 hours after 10 ng/ml of IL-33 stimulation or from wild-type and ST2^{-/-} mast cells. Results are means $\pm$ SD, $n = 3$, and from three independent experiments, $*P < 0.01$ compared to basal level. (H) Cell surface expression levels of Fc ϵ RI and ST2 on nonsensitized and IgE-sensitized murine mast cells. Results are representative from three independent experiments.

shown by a substantial drop in body temperature (Fig. 5B), increase in blood histamine (Fig. 5C), cytokines, and chemokines (Fig. 5D), and lung inflammation (Fig. 5E). Taken together these data demonstrate that in mice with endogenously generated high levels of IgE, IL-33 induces systemic anaphylaxis in a mast cell and ST2-dependent, but antigen-independent, manner.

Molecular Mechanisms for IL-33 Triggered Mast Cell Degranulation.

We then investigated the molecular mechanism by which IL-33 induces mast cell degranulation, focusing on calcium mobilization and phospholipase activation, the hallmarks of mast cell degranulation. IL-33 triggered a rapid cytosolic calcium response in human mast cells in the absence of IgE (Fig. S4A, nonsensitized). However, in mast cells presensitized with IgE, the calcium response was markedly elevated further (Fig. S4A, IgE-sensitized). Similar results were observed for murine mast cells (data not shown). We have previously shown that at least two different pathways are used by antigen and cytokine receptors to trigger cytosolic calcium signals: the classical pathway that activates phospholipase C to generate IP₃ (22, 23), and a novel pathway dependent on the activation of phospholipase D (PLD) and sphingosine kinase (SphK) (24–26). IL-33 triggered the activation and translocation of PLD1 (Fig. S4B) and SphK1 in mast cells (Fig. S4C). In contrast, IL-33 did not induce the

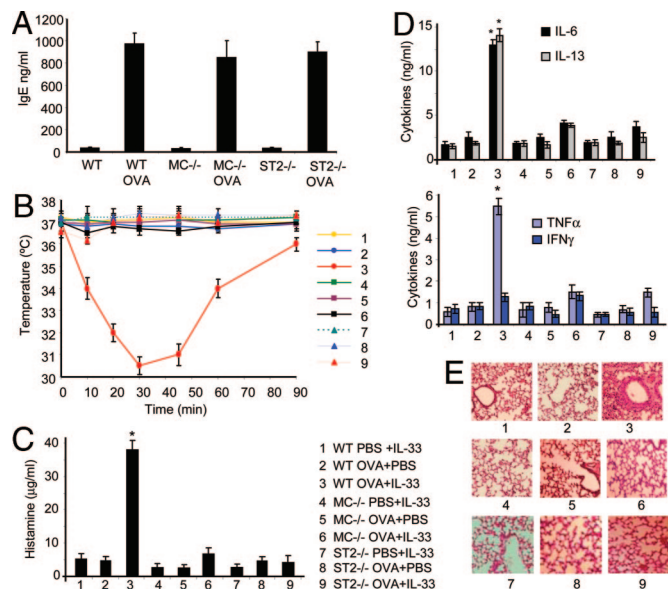


Fig. 5. Effect of IL-33 on ovalbumin (OVA) sensitized mice. (A) OVA induces similar levels of IgE in wild-type (WT), mast cell-deficient (MC^{-/-}) and ST2-null (ST2^{-/-}) mice. (B–E) Mice were treated as follows: treatment 1, WT mice sensitized with PBS and challenged with IL-33; 2, WT mice sensitized with OVA and challenged with PBS; 3, WT mice sensitized with OVA and challenged with IL-33; 4, MC^{-/-} mice sensitized with PBS and challenged with IL-33; 5, MC^{-/-} mice sensitized with OVA and challenged with PBS; 6, MC^{-/-} mice sensitized with OVA and challenged with IL-33; 7, ST2^{-/-} mice sensitized with PBS and challenged with IL-33; 8, ST2^{-/-} mice sensitized with OVA and challenged with PBS; 9, ST2^{-/-} mice sensitized with OVA and challenged with IL-33. (B) Rectal temperature was measured every 10 minutes after IL-33 challenge. (C) Serum histamine, (D) plasma cytokines were examined at 120 minutes after challenge. Data are mean $\pm$ SEM, $n = 5$, $*P < 0.01$ compared to group 1, $+P < 0.01$ compared with group 5. (E) Lung sections from mice euthanized at 120 minutes after challenge were stained with hematoxylin and eosin and examined under light microscopy. Pictures are representative of five mice per group.

production of IP₃ (Fig. S4D). Using anti-sense knockdown for PLD1 and SphK1 (PLD1 was reduced by 90% and SphK1 by 85%, Fig. S4E), we confirmed that PLD1 and SphK1 were required for IL-33-triggered cytosolic calcium signals (Fig. S4F) and mast cell degranulation (Fig. S4G). It should be noted that for mast cell degranulation, the cells were preincubated overnight with IgE. Using siRNA-knockdown of ST2 in human mast cells (Fig. S4H), we showed that, similar to murine mast cells, the IL-33-mediated human mast cell degranulation is also ST2 dependent. Although ST2 knockdown-cells sensitized to IgE degranulated in response to DNP-HSA, they failed to degranulate in response to IL-33 (Fig. S4I). Therefore, IL-33 induces an ST2-mediated mast cell degranulation by the activation of the novel pathway PLD1 and SphK1, but not the classical phospholipase C/IP₃ pathway, leading to calcium mobilization. Similar results were obtained using murine mast cells (data not shown).

IL-33 Triggered Calcium-Dependent Activation of NF κ B, Cytokine, Chemokine, and Eicosanoid Synthesis.

It has been suggested that whilst the production of pro-inflammatory cytokines in mast cells is NF κ B (p50 and p65) dependent (27–29), the induction of Th2 cytokines is NF κ B independent (30). We therefore investigated the effect of IL-33 on the activation of NF κ B and MAPKs in human mast cells. IL-33 triggered the activation of NF κ B (Fig. S5A) and the phosphorylation of ERK1/2 (Fig. S5B) and p38 MAPKs (Fig. S5C). IL-33-stimulated NF κ B activity was abrogated in cells pretreated with the intracellular calcium chelator BAPTA-AM (Fig. S5A). This is in agreement with an earlier observation that Ca²⁺ signal amplitude and frequency play a role

in NF κ B activation (31). In contrast, the IL-33-induced phosphorylation of MAPKs was not affected by BAPTA (Fig. S4B, C). Consistent with this, BAPTA prevented the generation of IL-1 β , IL-3, IL-6, TNF- α , MIP-2, MCP-1, and MIP-1 α synthesis, which are dependent on NF- κ B activity (27), whereas BAPTA had no effect on IL-33-triggered IL-5, IL-13, Eotaxin-2, RANTES or TARC syntheses, which are independent of the NF- κ B pathway (28) (Figs. S5D, E).

Pharmacologically active leukotrienes and prostaglandins are synthesized from arachidonic acid by cytosolic phospholipase A₂ (PLA₂) and released by the breakdown of membrane phospholipids during mast cell degranulation (32, 33). As IL-33 is able to trigger degranulation and elevate the cytoplasmic Ca²⁺ necessary for cPLA₂ activation, we investigated the role of calcium mobilization in IL-33-induced production of leukotrienes and prostaglandins. IL-33 stimulated human mast cells to produce substantial amounts of PGD₂ and LTB₄, the production of which were completely abrogated by the presence of BAPTA (Figs. S5F, S5G). Similar results were obtained using murine mast cells (data not shown).

Together, these results demonstrate that IL-33 induces calcium-dependent NF κ B activation, cytokine/chemokine, and eicosanoid synthesis during mast cell activation.

Discussion

Data reported here suggest that IL-33 is elevated in atopic patients only during an allergic-inflammatory response, and that under the experimental conditions, IL-33 induces acute anaphylactic shock (AS), a disorder of considerable importance and unmet medical need. Furthermore, we show that in the presence of IgE, IL-33 induces AS by rapidly activating mast cell degranulation. Our findings therefore have the following important implications: (i) they reveal a hitherto unrecognized pathophysiological role of IL-33; (ii) they provide evidence that IL-33 induce mast cell degranulation; and (iii) IL-33 may be a potential target for treating allergic shock.

Although mast cell activation and degranulation by IL-33 is independent of the presence of T or B cells, the presence of preformed IgE is critical. *In vitro* the IgE sensitization need to be for at least 16 hours, and shorter sensitization durations failed to prime the mast cells for IL-33-mediated degranulation. This may explain why other studies have failed to show IL-33-triggered degranulation. IL-33 alone is not capable of triggering AS. This requirement of IgE may represent a failsafe control mechanism such that the expression of IL-33 alone, induced by unrelated infection or immunological activation, would not automatically lead to anaphylactic shock or acute allergic response. Conversely, IL-33 can act in an additive fashion with antigen-mediated reaction, to further enhance AS. Thus IL-33 appears to play a critical but controlled role in allergic shock response.

IgE sensitization substantially increased mast cells ST2 expression (Fig. 4H). This was also recently reported by Ho et al. (15). Moreover, the amplitude and duration of the calcium response triggered by IL-33 in IgE-sensitized mast cells was also markedly increased compared to the non-sensitized cells. It is thus likely that IgE receptor occupancy increases ST2 density on mast cells such that IL-33 would then achieve sufficient signaling strength (as shown for the calcium response, Fig. S1A) to induce mast cell degranulation.

Of particular interest is the finding that in human atopic patients IL-33 is substantially elevated during systemic shock and localized allergic reaction, but not in patients without inflam-

mation. Importantly, the systemic anaphylactic shock in mice could be prophylactically and therapeutically attenuated by sST2 (a decoy receptor of IL-33), or by anti-IL-33 antibody. Therefore, our data suggest that endogenous IL-33 may contribute to the pathology of IgE-associated diseases, the importance of which warrants further investigation.

It is well established that mast cells are key effector cells in triggering the immediate Type-I hypersensitivity in allergic patients. Mast cells are predominantly present in the skin, airways, and gastrointestinal tract. Allergen-bound IgE triggers an array of signaling pathways, which lead to degranulation and the release of histamine, proteases, cytokines, as well as *de novo* synthesis of the arachidonic acid metabolites, such as leukotrienes and prostaglandins. These mast cell-generated proinflammatory mediators then cause the immediate symptoms of allergic diseases, such as rhinitis, conjunctivitis and asthma. Despite extensive efforts, the etiology and pathogenesis of most allergic diseases remain poorly understood, and effective therapies with limited side effects are lacking. The key factor that may tip the balance between the physiological and the pathophysiological manifestation of mast cells has long been elusive. Data presented here may go some way to resolve this conundrum. We demonstrate here that, in IgE-sensitized mast cells, IL-33 may be that key factor tipping mast cell activation and degranulation and thus may represent a potential target for treatment of AS.

At this stage it is not known whether the clinical systemic shock and localized allergic reaction stimulated IL-33 production or that IL-33 triggered these clinical symptoms. What is clear, however, is that atopy alone is not sufficient to trigger anaphylaxis. Based on our animal studies, it may be that under conditions where an atopic patient secretes high levels of IL-33 because of a strong inflammatory condition, this IL-33 might then trigger the mast cell responses even in the absence of an IgE-specific allergen. We also showed that IL-33 levels are elevated in atopic dermatitis only in inflamed tissue. This would suggest that during allergic inflammation IL-33 is up-regulated. We screened for patients who were atopic and developed anaphylactic shock in the operating theatre. IL-33 may indeed be elevated in other systemic inflammatory conditions, such as peanut or drug-mediated anaphylactic shock. This is currently being addressed.

Materials and Methods

Clinical Serum Samples and Skin Biopsy Samples. Male and female patients aged >18 years with atopic dermatitis (AD) were enrolled in the study. For full description, see *SI Materials and Methods*.

Induction and Measurement of PCA and AS. Experiments were performed on 8–10-week-old mice. For full details of the procedures, see *SI Materials and Methods*.

In Vitro Assays. Human mast cells were derived from CD34⁺ hematopoietic progenitor cells isolated from umbilical cord blood and cultured as previously described (26, 34). Murine mast cells were isolated from the peritoneal cavity and cultured as previously described (35). For full description on *in vitro* assays, see *SI Materials and Methods*.

Statistical Analysis. Statistical differences between control and treatment groups were calculated using unpaired Student's *t* test. *P* < 0.05 was considered statistically significant.

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Supporting Information

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SI Materials and Methods

Clinical Serum Samples and Clinical Skin Biopsy Samples. Male and female patients aged >18 years with atopic dermatitis (AD) were enrolled in the study. The study was approved by the Ethics Committee, National University Hospital, National University of Singapore. Blood samples were obtained from five atopic patients who developed anaphylactic shock in the operating theater, five atopic control patients, and five healthy volunteers. Levels of IgE and IL-33 in the plasma were measured by enzyme-linked immunosorbent assay (ELISA; IgE ELISA, Bethyl catalog no. E80-108; IL-33 ELISA, Centaur catalog no. 40-288-23146), following the manufacturer's instructions. Skin biopsy samples were obtained from seven patients with AD and seven healthy volunteers with informed consent. For immunohistochemical analysis, 10- μ m cryostat sections were fixed, permeabilized, and immunostained with primary rat-anti-human-IL-33 mAb (R&D catalog no. MAB3625), or an irrelevant rat IgG (control), and the secondary biotinylated anti-rat IgG (Vector Laboratories, catalog no. BA-4000), and R.T.U. Horseradish Peroxidase Avidin (Vector Laboratories, catalog no. A-2704). Sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich) before mounting.

Mice. BALB/c and C57BL/6J wild-type mice were obtained from the Laboratory Animal Holding Unit, National University of Singapore, and the University of Glasgow. ST2^{-/-} mice were generated as previously described (6), and backcrossed to the BALB/c background for 10 generations (5, 11). RAG1^{-/-} mice (B6.129S7-Rag1^{tm1Mom}/J) and mast cell-deficient mice (C57BL/6J-c-kit^{W-sh/W-sh}) (22) were purchased from Jackson Laboratories.

Induction and Measurement of IgE-Mediated PCA. The study was carried out in accordance with the University of Singapore, and the U.K. Home Office Guidelines for animal experimentation. Mice were anesthetized and injected s.c. in the dorsal skin with mouse anti-DNP IgE (20 ng in 20 μ l saline). Saline alone was used as negative control. In additional controls, mice were injected s.c. with three other IgE preparations as stated in the reagent section above. Mice were injected i.v. 24 hours later with 50 μ g of DNP-HSA with 1% Evans blue dye. Some mice were injected with IL-33 (2 μ g/mouse) alone, another group received IL-33 together with DNP-HSA. Vascular permeability was visualized 60 minutes later by the blue staining of the injection sites on the reverse side of the skin (2–4). Skin samples were also harvested and Evans blue extracted from the tissue, using 0.5 ml of formamide at 55 °C for 48 hours and quantified by OD at 610 nm.

Induction and Measurement of Anaphylactic Shock. Wild-type BALB/c mice were treated as follows (all injections i.v.; IgE, 200 μ g; IL-33, 2 μ g; IgG, 100 μ g; monoclonal anti-IL-33, 100 μ g; sST2-Fc, 100 μ g): 1, injected with PBS, challenged 16 hours later with PBS (PBS+PBS); 2, injected with PBS, challenged with IL-33 (PBS+IL-33); 3, injected with IgE and challenged with PBS (IgE+PBS); 4, injected with IgE and IgG, challenged with PBS (IgE-IgG+PBS); 5, injected with IgE, challenged with IL-33 (IgE+IL-33); 6, injected with IgE and anti-IL-33 antibody, challenged with IL-33 (IgE-anti-IL-33+IL-33); 7, injected with IgE and sST2, challenged with IL-33 (IgE-sST2+IL-33); 8, injected with IgE and IgG, challenged with IL-33 (IgE-IgG+IL-33); 9, injected with IgE, challenged with IL-33 and injected 5 minutes later with anti-IL-33 (IgE+IL-33+anti-IL-33); 10, injected with IgE, challenged with IL-33 and injected 5 minutes later with sST2

(IgE+IL-33+sST2); 11, injected with IgE, challenged with IL-33 and injected 5 minutes later with IgG (IgE+IL-33+IgG). Changes in body temperature associated with systemic anaphylaxis were monitored every 10 minutes, by measuring changes in rectal temperature, using a rectal probe coupled to a digital thermometer (Natsume Seisakusyo Co., Tokyo, Japan). At the end of the experiment (120 minutes after challenge), mice were killed and plasma collected and stored at -20 °C until used. IL-6, IL-13, TNF α , IFN γ , MCP-1, and MIP1 α protein levels in the plasma were determined by ELISA (R&D Systems), following the manufacturer's instructions. Histamine levels in the plasma were measured by ELISA (IBL, Hamburg, Germany) following the manufacturer's instructions. Portions of lungs were excised and fixed with 10% buffered formamide (Sigma), dehydrated and embedded in paraffin for histological examination. Sections (5 μ m) were stained with hematoxylin and eosin and evaluated by light microscopy and documented by photographs. In other experiments, ST2^{-/-} mice were injected i.v. with mouse IgE (200 μ g) and challenged i.v. 16 hours later with 50 μ g of DNP-HSA or 2 μ g IL-33 or a combination of both: 1, injected with PBS, challenged with PBS (PBS+PBS); 2, injected with PBS, challenged with IL-33 (PBS+IL-33); 3, injected with IgE, challenged with PBS (IgE+PBS); 4, injected with IgE, challenged with IL-33 (IgE+IL-33); 5, injected with IgE, challenged with DNP-HSA (IgE+DNP-HSA); 6, injected with IgE, challenged with DNP-HSA and IL-33 (IgE+DNP-HSA+IL-33). Changes in body temperature and serum histamine levels were analyzed as above.

Allergen-Induced IgE and Challenge with IL-33. Eight-week-old wild-type, mast cell-deficient, and ST2-null mice were i.p. injected, on days 0 and 14, with 20 μ g of OVA and 2 mg of Alum at a total volume of 200 μ l. Serum was collected from the mice 1 week later, on Day 21, and the concentration of total IgE and OVA-specific IgE was determined by ELISA. OVA-sensitized mice by the above-mentioned method were challenged i.v. with IL-33 (2 μ g) dissolved in 20 μ l PBS, 1 week after the second immunization (day 21). Changes in body temperature associated with systemic anaphylaxis were monitored every 10 minutes. At the end of the experiment (120 minutes after challenge), mice were killed and plasma collected and stored at -20 °C until used. IL-6, IL-13, TNF α , IFN γ , MCP-1, and MIP1 α protein levels in the plasma were determined by ELISA (R&D Systems), following the manufacturer's instructions. Histamine levels in the plasma were measured by ELISA (IBL, Hamburg, Germany) following the manufacturer's instructions. Portions of lungs were excised and fixed with 10% buffered formamide (Sigma), dehydrated and embedded in paraffin for histological examination. Sections (5 μ m) were stained with hematoxylin and eosin and evaluated by light microscopy and documented by photographs.

Histological Analysis of Mast Cells. Skin samples were fixed in 10% formalin and 4- μ m paraffin sections were stained with toluidine blue and examined with an Olympus microscope (model BX 51) using 10x, 40x, and 100x uplan Apo lenses. Mast cell numbers in the skin were calculated by counting the cells under light microscopy in six different sections derived from six mice. The percentage of degranulated mast cells in the skin was calculated by counting the number of cells with >10% of extrusion of granules as previously described (18–20).

In Vitro Assays. Human mast cells were derived from CD34⁺, hematopoietic progenitor, cells isolated from umbilical cord

blood and cultured as previously described (26, 27). Murine mast cells were isolated from the peritoneal cavity and cultured as previously described (38). After culturing for 3 weeks, the purities were $98\% \pm 5\%$ for both human and for murine mast cells, as determined by being double-positive for Fc ϵ RI and ST2. Antisense knockdown of PLD1 and SphK1 was performed as previously reported (25). Briefly, antisense oligonucleotides were purchased from 1st Base (Singapore) 20-mers were synthesized, capped at either end by the phosphorothiorate linkages (the first two and last two linkages), and corresponded to the reverse complement of the first 20 coding nucleotides for PLD1, SphK1, and a scrambled antisense oligonucleotides for controls. The sequences of the antisense oligonucleotides were as follows: PLD1, 5'-CCGTGGCTCGTTTTTCAGTG-3'; PLD1-scrambled control, 5'-TTCCTTGGGTTCGCTTGGA-3'; SphK1, 5'-CCCGCAGGATCCATAACCTC-3'; SphK1-scrambled control, 5'-CTGCTCGCACAGACCACACT-3'.

siRNA gene silencing of ST2 was achieved using the ST2-specific, synthetic, double-stranded siRNA (CCUAGUUA-CACCGUGGAUUDTT and AAUCCAC GGUGUAACUAG-GdT) purchased from Invitrogen. Control scrambled siRNA (ACUCAGUCGAUCUUACGUGdT and CACGUAA-GAUCGA CUGAGUdT) was used. Cells were incubated/transfected with oligonucleotides (1 μ M) mixed with transfection reagent (Oligofectamine; Invitrogen) for a total of 48 hours (for 36 hours before sensitization and for the duration of sensitization).

Mast Cell Degranulation Assay. β -Hexosaminidase released by IL-33-triggered mast cell degranulation was measured by a colorimetric assay as previously described (26, 27).

Cytokine, Chemokine, and Eicosanoid Detection. Supernatants from human and murine mast cells (2×10^6 cells/sample) were collected 24 hours after IL-33 stimulation and stored at -20°C until use. IL-1 β , IL-3, IL-5, IL-6, IL-13, TNF α , MIP-2, Eotaxin 2, MCP1, MIP1 α , RANTES, and TARC were assayed by ELISA (R&D Systems), or using a multiplex immunoassay (Cytokine 20-Plex, Biosource) with the Bio-Plex System (Bio-Rad), following the manufacturer's instructions. PGE2 production was measured by the Biotrak PGE2 system (Amersham, GE Healthcare). LTB4 production was measured by the Biotrak leukotriene B4 enzyme immunoassay system (Amersham, GE Healthcare).

Gel Electrophoresis and Western Blots. Cell lysate, 40 μ g per well, was resolved on 10% polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) under denaturing

conditions and then transferred to 0.45 μ m nitrocellulose membranes as previously stated (26, 27). The blots were probed using the polyclonal anti-SphK1 (Exalpa Biologicals), anti-PLD1 (Santa Cruz Biotechnology), anti-phosphor-ERK1/2, phosphor-p38, anti-ERK1, or p38 antibodies (all BD Biosciences) and anti-GAPDH (Santa Cruz Biotechnologies) for loading control. Bands were visualized using anti-IgG HRP-conjugated secondary antibodies, and the ECL Western Blotting Detection System (Amersham).

Cytosolic Ca²⁺ Measurement. Cytosolic calcium was measured as described previously (26, 27).

Phospholipase C Activity Sphingosine Kinase Assay. Inositol 1,4,5-trisphosphate (IP₃) was measured as described previously (25, 26), using the Biotrak TRK 1000 kit (Amersham, GE Healthcare). SphK activity was measured by a radiometric assay as described previously (26, 27).

NF κ B Activity. NF κ B was analyzed using the Mercury TransFactor-“Profiling Kit-Inflammation” (BD Biosciences), following the manufacturer's instructions. The enzymatic product was analyzed in a standard plate reader as previously described (39).

Flow Cytometry. Cell surface markers (Fc ϵ RI and ST2) were analyzed by FACS Calibur flow cytometry using Cell Quest software (Becton Dickinson). To prevent non-specific binding, all samples were preincubated for 30 minutes with human IgG (Sigma) or CD16/32 (Becton Dickinson) to block the Fc receptors. Aliquots of fresh cells or cultured cells (2×10^5 cells/tube) were suspended in buffer containing 2% fetal calf serum and 0.02% sodium azide in phosphate-buffered saline (staining buffer), human cells were incubated for 20–30 minutes with specific mouse anti-human ST2 antibody (R&D Systems), and anti-mouse PE-conjugated (secondary), as well as with IgE-FITC (Serotec). Mouse cells were stained with FITC-anti-mouse ST2 (MD Bioscience), and mouse IgE (Serotec) followed by anti-IgE-PE conjugated. Cells labeled with FITC- or PE-conjugated mouse isotype-matched antibodies were used as controls.

Microscopy. Translocation of SphK1 and PLD1 was analyzed by fluorescent microscopy. Stimulated cells were fixed at the indicated times and incubated with rabbit polyclonal anti-SphK1 (Exalpa Biologicals), or anti-PLD1 (Santa Cruz Biotechnology), and stained with anti-rabbit FITC conjugated secondary antibody. Another set of cells were incubated with an irrelevant rabbit polyclonal (Santa Cruz Biotechnology), and with the same anti-rabbit FITC conjugated secondary antibody for staining controls.

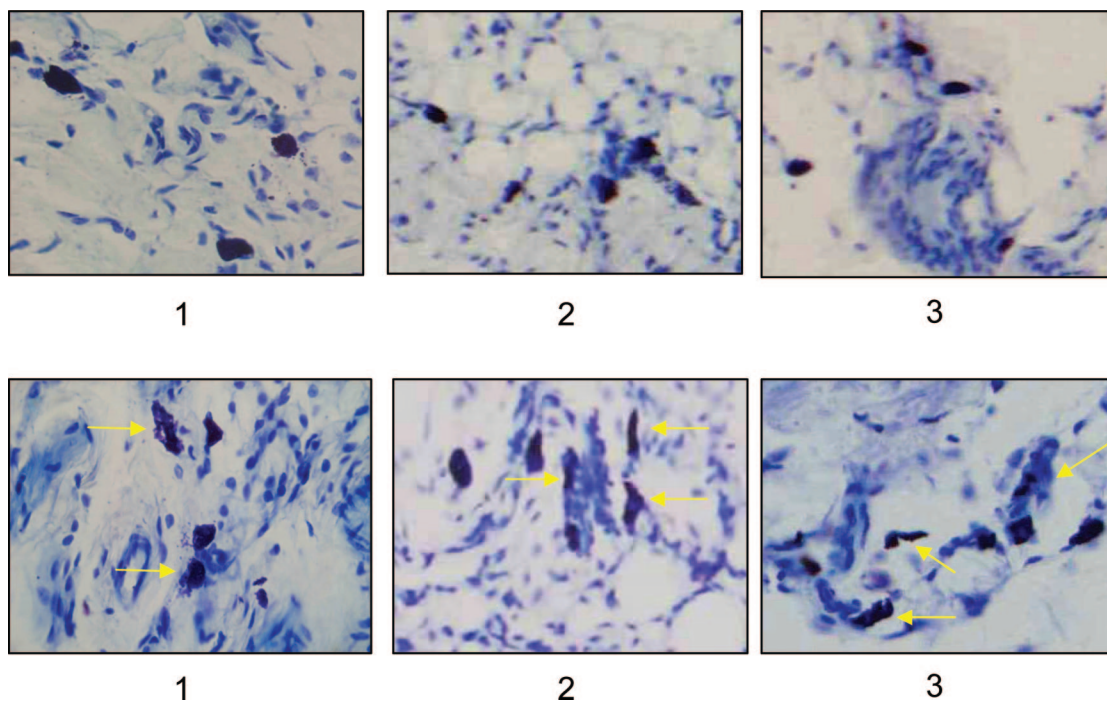
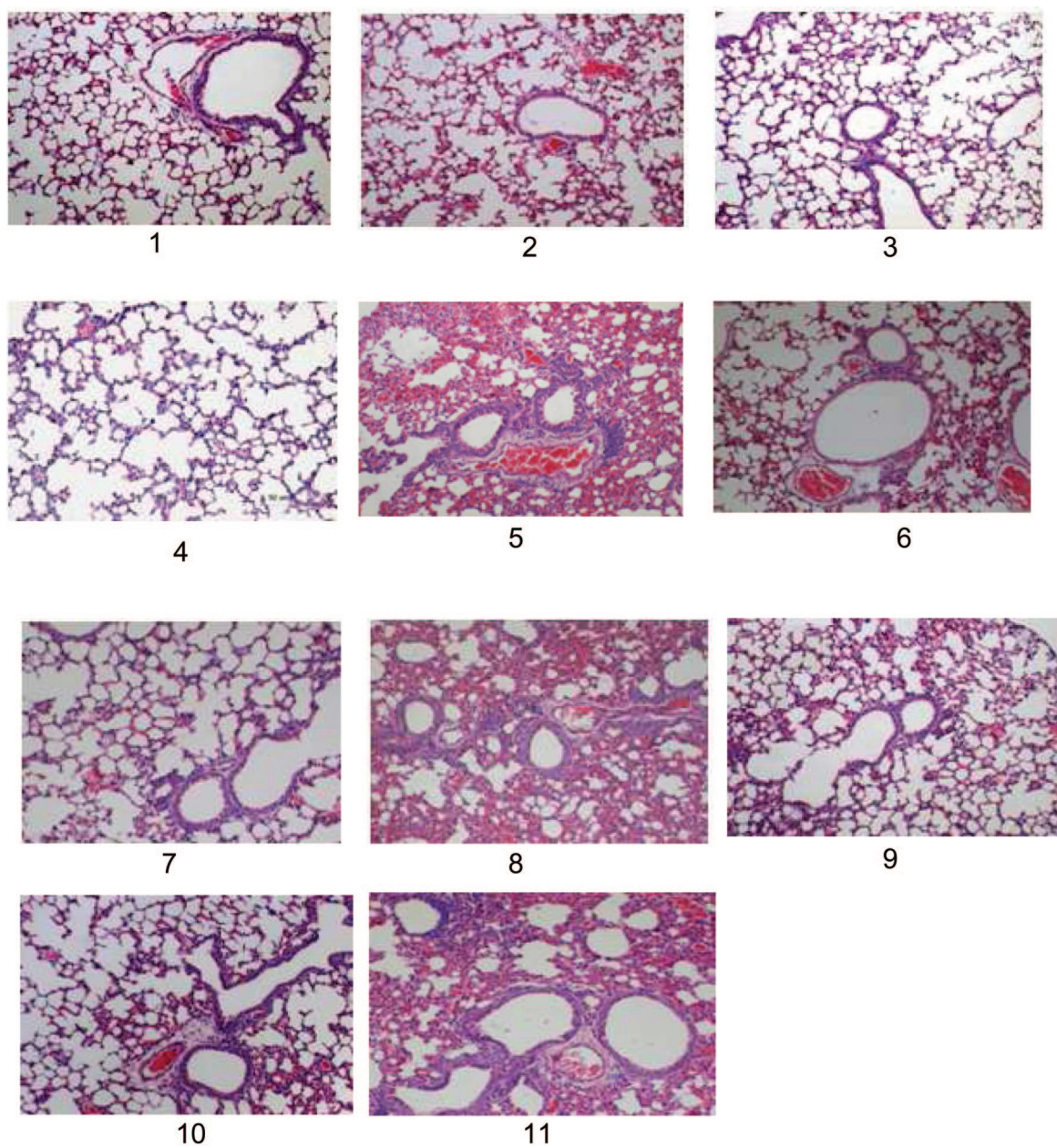


Fig. S1. Enlarged images from Fig. 2B.



- | | |
|---------------|------------------------|
| 1 PBS+PBS | 6 IgE-anti-IL-33+IL-33 |
| 2 PBS+IL-33 | 7 IgE-sST2+IL-33 |
| 3 IgE+PBS | 8 IgE-IgG+IL-33 |
| 4 IgE-IgG+PBS | 9 IgE+IL-33+anti-IL-33 |
| 5 IgE+IL-33 | 10 IgE+IL-33+sST2 |
| | 11 IgE+IL-33+IgG |

Fig. S2. Enlarged images from Fig. 3E.

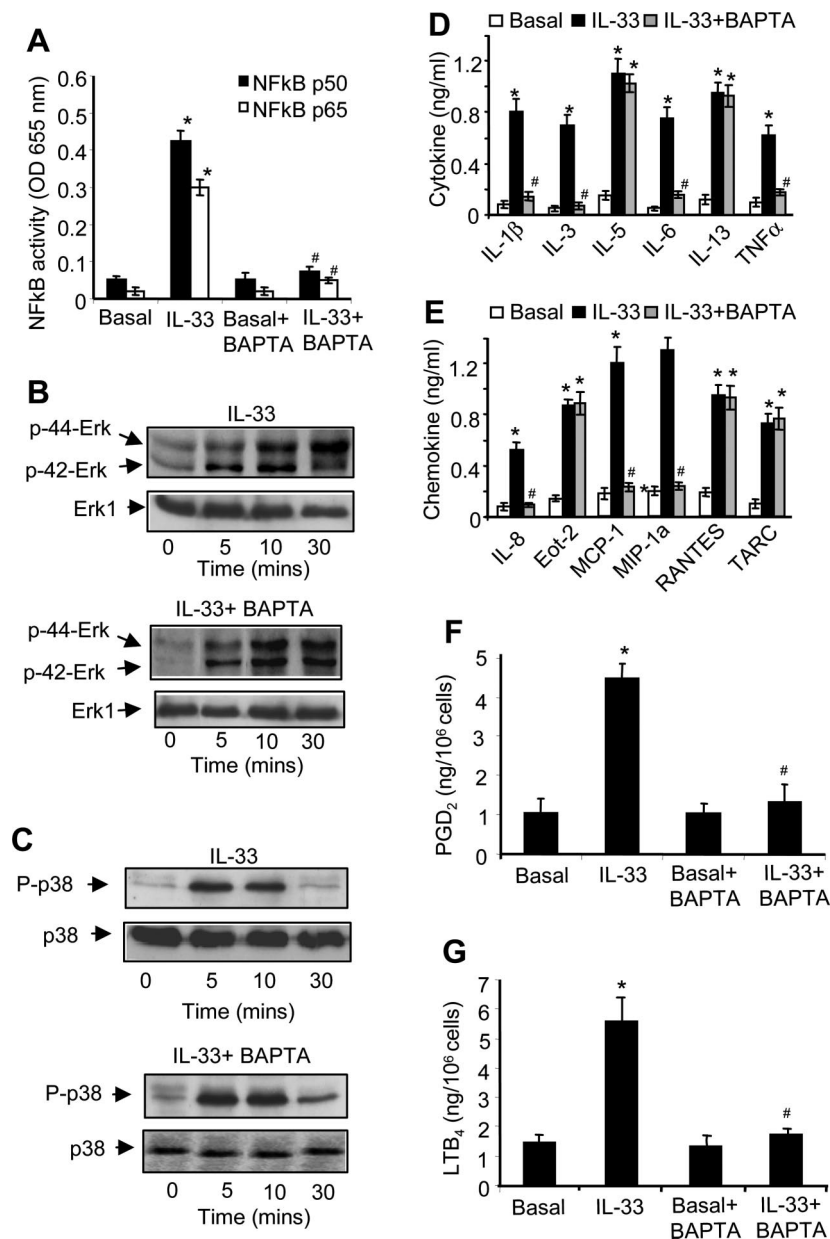


Fig. S5. IL-33-triggered NFκB activation and the production of cytokines/chemokines and eicosanoids are calcium dependent. Nuclear extract from nonstimulated cells (PBS, basal) or IL-33-stimulated (30 minutes) cells were analyzed for NFκB activity (A). Western blots of proteins from IL-33-stimulated human mast cells were carried out for Erk1/2 and p38 MAPKs phosphorylation (B, C). The cytosolic calcium chelator BAPTA inhibited NFκB activity (A) but not MAPKs phosphorylation (B, C). Cytokines (D), chemokines (E), PGD₂ (F), and LTB₄ (G) release was determined from mast cells not stimulated (PBS, Basal) or stimulated with IL-33 (IL-33), or from cells pretreated with BAPTA, not stimulated (Basal+BAPTA) or stimulated with IL-33 (IL-33+BAPTA). Data are means ± SD from three independent experiments. **P* < 0.01 compared with basal level, #*P* < 0.01 compared with cells stimulated with IL-33 alone.

Retraction

IMMUNOLOGY

Retraction for “The cytokine interleukin-33 mediates anaphylactic shock,” by Peter N. Pushparaj, Hwee Kee Tay, Shiau Chen H’ng, Nick Pitman, Damo Xu, Andrew McKenzie, Foo Y. Liew, and Alirio J. Melendez, which appeared in issue 24, June 16, 2009, of *Proc Natl Acad Sci USA* (106:9773–9778; first published June 8, 2009; 10.1073/pnas.0901206106).

The undersigned authors wish to note the following: “The panels in Fig. 2*B1* and 2*D3* are identical and used in a later paper (1), now retracted. The panels in Fig. 3*E4* and 3*E9* are used in two later publications, one that has been retracted (2) and one that has an expression of concern (3). Although we believe the overall message of the paper is correct, we can no longer consider the findings to be reliable. Accordingly, we wish to retract the paper. We sincerely apologize for the inconvenience caused to other investigators.”

**Peter N. Pushparaj
Hwee Kee Tay
Shiau Chen H’ng
Nick Pitman
Damo Xu
Andrew McKenzie
Foo Y. Liew**

1. Pushparaj PN, et al. (2009) Sphingosine kinase 1 is pivotal for Fc ϵ RI-mediated mast cell signaling and functional responses in vitro and in vivo. *J Immunol* 183:221–227.
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