

Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells

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Interleukin-33 (IL-33) is an alarmin cytokine from the IL-1 family. IL-33 activates many immune cell types expressing the interleukin 1 receptor-like 1 (IL1RL1) receptor ST2, including group-2 innate lymphoid cells (ILC2s, natural helper cells, nuocytes), the major producers of IL-5 and IL-13 during type-2 innate immune responses and allergic airway inflammation. IL-33 is likely to play a critical role in asthma because the *IL33* and *ST2/IL1RL1* genes have been reproducibly identified as major susceptibility loci in large-scale genome-wide association studies. A better understanding of the mechanisms regulating IL-33 activity is thus urgently needed. Here, we investigated the role of mast cells, critical effector cells in allergic disorders, known to interact with ILC2s in vivo. We found that serine proteases secreted by activated mast cells (chymase and tryptase) generate mature forms of IL-33 with potent activity on ILC2s. The major forms produced by mast cell proteases, IL-33_{95–270}, IL-33_{107–270}, and IL-33_{109–270}, were 30-fold more potent than full-length human IL-33_{1–270} for activation of ILC2s ex vivo. They induced a strong expansion of ILC2s and eosinophils in vivo, associated with elevated concentrations of IL-5 and IL-13. Murine IL-33 is also cleaved by mast cell tryptase, and a tryptase inhibitor reduced IL-33-dependent allergic airway inflammation in vivo. Our study identifies the central cleavage/activation domain of IL-33 (amino acids 66–111) as an important functional domain of the protein and suggests that interference with IL-33 cleavage and activation by mast cell and other inflammatory proteases could be useful to reduce IL-33-mediated responses in allergic asthma and other inflammatory diseases.

cytokine | IL-33 | allergic inflammation | mast cell protease | innate lymphoid cells

Interleukin-33 (IL-33), previously known as nuclear factor from high endothelial venules or NF-HEV (1, 2), is an IL-1 family cytokine (3) that signals through the interleukin 1 receptor-like 1 (IL1RL1) receptor ST2 (4, 5) and induces expression of cytokines and chemokines in various immune cell types, including mast cells, basophils, eosinophils, Th2 lymphocytes, invariant natural killer T, and natural killer cells (3, 4, 6–8). Studies in IL-33-deficient mice indicate that IL-33 plays important roles in type-2 innate immunity and innate-type allergic airway inflammation (9–13). Indeed, IL-33 is a key activator of the recently described group-2 innate lymphoid cells (ILC2s, natural helper cells, nuocytes) (14–17). These cells control eosinophil homeostasis in blood and adipose tissue (18, 19) and produce extremely high amounts of the type-2 cytokines IL-5 and IL-13 in response to IL-33 (14–16). ILC2s also play important roles in allergic airway inflammation (20–24), atopic skin disease (25–28), helminth infection in the intestine (11, 12, 14–16), and influenza virus infection in the lungs (29, 30).

Based on animal model studies and analyses of diseased tissues from patients, IL-33 has been proposed as a candidate therapeutic target for several important diseases, including asthma and other allergic diseases, rheumatoid arthritis, inflammatory bowel

diseases, and cardiovascular diseases (4, 6). IL-33 is likely to play a critical role in asthma because the *IL33* and *IL1RL1/ST2* genes have been reproducibly identified as major susceptibility loci in several independent large-scale genome-wide association studies of human asthma (31, 32).

Despite these important advances into the roles of IL-33, very little is known yet about the mechanisms regulating its activity. Full-length human IL-33 is a 270 amino acid protein localized in the nucleus of endothelial and epithelial cells in blood vessels and epithelial barrier tissues (1, 2, 33, 34), which associates with chromatin (2) and histones H2A–H2B, through a short chromatin-binding motif located in its N-terminal part (amino acids 40–58) (35). IL-33 can be released in the extracellular space upon cellular damage or necrotic cell death (36, 37), and it was thus proposed to function as an alarmin (alarm signal or endogenous danger signal), which alerts the immune system to tissue injury following trauma or infection (33, 36, 37).

Proteases have been shown to regulate IL-33 activity. Full-length IL-33_{1–270} is biologically active but processing by caspases after residue Asp₁₇₈ in the IL-1-like cytokine domain results in its inactivation (36, 37). In contrast, inflammatory proteases from neutrophils, cathepsin G, and elastase, process full-length IL-33 into mature forms that contain an intact IL-1-like cytokine domain and that have an increased biological activity compared

Significance

Interleukin-33 (IL-33) is an IL-1 family cytokine with important roles in type-2 immunity and human asthma. IL-33 is a key activator of the recently described group-2 innate lymphoid cells (ILC2s), which are involved in the initiation of allergic inflammation. Here, we investigated the mechanisms regulating IL-33 activity. We discovered that mast cells, critical effector cells in allergic disorders, secrete proteases, which cleave IL-33 and generate mature forms with increased biological activity. We demonstrate that these mature forms of IL-33 are 30-fold more potent than full-length IL-33 for activation of ILC2s. Our study suggests that inhibition of IL-33 cleavage by mast cell and other inflammatory proteases could be useful to limit IL-33-mediated responses in allergic asthma and other inflammatory diseases.

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with full-length IL-33₁₋₂₇₀ (38). Although neutrophils have been implicated in virus-induced exacerbations of asthma, they are unlikely to be involved in the processing of IL-33 during allergic inflammation. We therefore investigated the possibility that other cell types may be involved in this process. Mast cells, which are widely recognized for their roles as effector cells in allergic disorders, were good candidates because they interact directly with ILC2s *in vivo* (26) and they are strategically positioned close to vessel walls and epithelial surfaces exposed to the environment (39), the major sites of IL-33 expression (33, 34). We now demonstrate that activated human mast cells and purified mast cell proteases, trypsinase and chymase, generate mature forms of IL-33 (IL-33₉₅₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₁₀₉₋₂₇₀), which are ~30-fold more potent than full-length IL-33₁₋₂₇₀ for activation of ILC2s *ex vivo*. These IL-33 mature forms are also potent inducers of ILC2s, eosinophils, and type-2 cytokines *in vivo*. Our study suggests that release of the C-terminal IL-1-like cytokine domain, through proteolytic maturation within the central cleavage/activation domain (amino acids 66–111), is important for full biological activity of IL-33.

Results

Activated Mast Cells Process Full-Length IL-33 into 18- to 21-kDa Products. Because mast cells are located close to IL-33-producing cells in human tissues, we tested the possibility that IL-33 may be a substrate for mast cell proteases. Human mast cells (hMCs) were generated from CD133⁺ peripheral blood precursors cultured in the presence of stem cell factor and IL-6 during 2 mo (40). Mast cells prepared under these conditions have previously been shown to exhibit phenotypic and functional characteristics of connective tissue mast cells, including the abundant expression of mast cell trypsinase and chymase (41). We found that supernatants from activated mast cells can process *in vitro* translated full-length human IL-33₁₋₂₇₀ protein and generate two major cleavage products of ~18–21 kDa and a minor product of ~25/26 kDa (Fig. 1A). Similar cleavage products of full-length IL-33 were observed after mast cell activation with anti-IgE or substance P, two different stimulation pathways resulting in mast cell degranulation. Cleavage of IL-33₁₋₂₇₀ was abrogated in both cases by serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), but not by cysteine protease inhibitor E-64, indicating that mast cell serine proteases are involved in the processing of full-length IL-33 (Fig. 1B and C). Addition of mast cell chymase inhibitors [chymostatin, CS and cathepsin G inhibitor (CGI)] resulted in the disappearance of the 21-kDa product, whereas the 25/26-kDa cleavage product was eliminated in the presence of a trypsinase inhibitor (nafamostat mesylate, NM) (Fig. 1D). Combination of mast cell chymase and trypsinase inhibitors resulted in the disappearance of the 18-kDa cleavage product and a complete inhibition of IL-33 processing by activated mast cells (Fig. 1D). Together, these experiments indicated that full-length human IL-33₁₋₂₇₀ protein is a substrate for endogenous human mast cell serine proteases, trypsinase, and chymase.

To further characterize IL-33 processing by mast cell proteases, we then used purified human mast cell chymase and trypsinase. Purified human mast cell chymase generated 18- and 21-kDa cleavage products, which were identical to those generated by endogenous chymase from activated mast cells treated with trypsinase inhibitor (Fig. 1E). Purified human mast cell trypsinase processed full-length IL-33 into 18- and 25/26-kDa cleavage products, similar to those generated by endogenous trypsinase from activated mast cells treated with chymase inhibitor (Fig. 1F).

We next determined whether cleavage of full-length IL-33 by mast cell proteases also occurs in mice. We found that murine full-length IL-33₁₋₂₆₆ protein, like its human counterpart, is a substrate for activated bone-marrow-derived murine mast cells (Fig. S1A) and endogenous mast cell trypsinase from murine MC/9 mast cells (Fig. S1B).

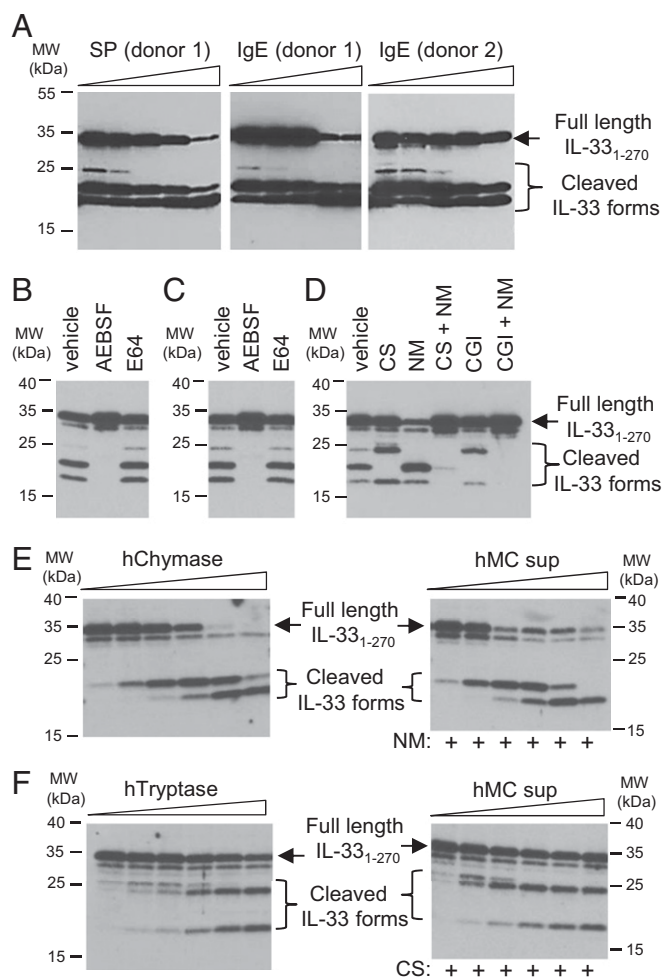


Fig. 1. Serine proteases from activated mast cells process full-length IL-33 into shorter mature forms. (A) *In vitro* translated full-length human IL-33₁₋₂₇₀ was incubated with increasing amounts of supernatants from human mast cells (2.10^2 – 7.10^3 cells, 2 h at 37 °C) activated with substance P (SP) or anti-IgE (IgE). Proteins were migrated on SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. (B–D) Cleavage of IL-33 by mast cells (4.10^2 cells) activated with substance P (B) or anti-IgE (C and D) was performed in the presence of serine protease inhibitor AEBSF, cysteine protease inhibitor E-64, chymase inhibitors CS and CGI, and/or trypsinase inhibitor NM. (E and F) *In vitro* translated IL-33₁₋₂₇₀ was incubated with increasing amounts of purified mast cell chymase (E, Left) or trypsinase (F, Left) or activated mast cell (from 2.10^2 to 2.10^4 cells) supernatants treated with trypsinase inhibitor (E, Right) or chymase inhibitor (F, Right). Proteins were separated on SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. Blots are representative of two independent experiments.

Identification of IL-33 Mature Forms Generated by Mast Cell Proteases. Based on the size of the cleavage products and preferential cleavage sites for mast cell chymase (S01.140; cleavage after F, Y, or L) and trypsinase (S01.143; cleavage after R or K) in the MEROPS peptidase database (merops.sanger.ac.uk/), we then performed mutagenesis studies to precisely map the cleavage sites in full-length human IL-33. We found that human mast cell chymase generates mature forms IL-33₁₀₉₋₂₇₀ and IL-33₉₅₋₂₇₀. Indeed, mutagenesis of leucine 108 and phenylalanine 94 abrogated the formation of the 18- and 21-kDa cleavage products, respectively (Fig. 2A, Left). Similar results were observed for endogenous chymase from activated human mast cells treated with trypsinase inhibitor (Fig. 2A, Right). Mutagenesis studies indicated that the 25/26-kDa cleavage product generated by trypsinase corresponds to two distinct mature forms of IL-33, IL-33₇₉₋₂₇₀, and IL-33₇₂₋₂₇₀.

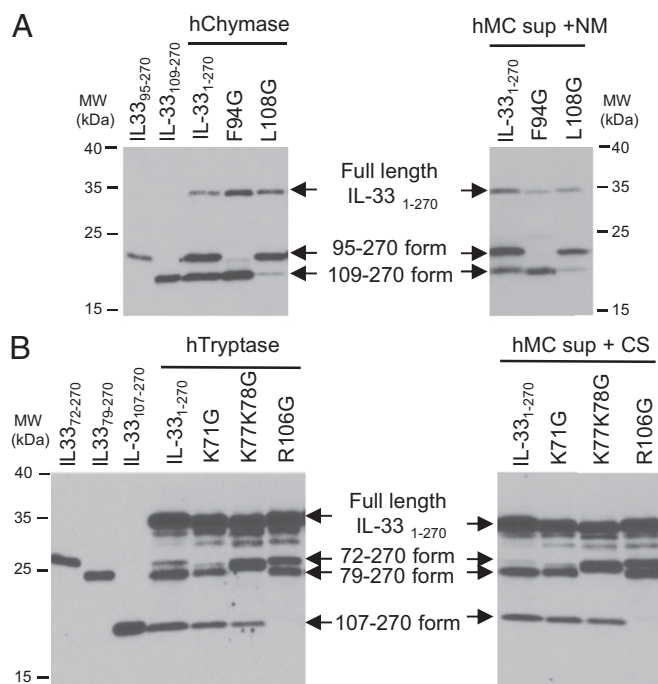


Fig. 2. Identification of IL-33 mature forms generated by mast cell proteases using IL-33 single-point and deletion mutants. (A) Mapping of the chymase cleavage sites. Mutation of L₁₀₈ and F₉₄ to glycine in full-length human IL-33₁₋₂₇₀ inhibits formation of the 18- and 21-kDa chymase cleavage products, respectively. These cleavage products comigrate on SDS/PAGE with in vitro translated proteins IL-33₁₀₉₋₂₇₀ and IL-33₉₅₋₂₇₀. Similar results were observed using purified human chymase (0.9 mU, 30 min at 37 °C) or activated human mast cell (2.10³ cells) supernatant treated with trypsin inhibitor (hMC + NM). (B) Mapping of the trypsin cleavage sites. Mutation of R₁₀₆, K₇₇-K₇₈, and K₇₁ to glycine inhibits formation of the 18-, 25-, and 26-kDa trypsin cleavage products, respectively. These cleavage products comigrate on SDS/PAGE with in vitro translated proteins IL-33₁₀₇₋₂₇₀, IL-33₇₉₋₂₇₀, and IL-33₇₂₋₂₇₀. Similar results were observed using purified human trypsin (1.2 mU, 30 min at 37 °C) or activated human mast cell (7.10² cells) supernatant treated with chymase inhibitor (hMC + CS). Proteins were separated by SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. Blots are representative of at least three independent experiments.

(Fig. 2B). Mutagenesis of arginine 106 revealed that the 18-kDa product generated by mast cell trypsin corresponds to mature form IL-33₁₀₇₋₂₇₀ (Fig. 2B, Left). Similar results were obtained with endogenous trypsin from activated human mast cells treated with chymase inhibitor (Fig. 2B, Right). Together, these experiments indicated that human mast cell proteases process full-length IL-33 into five distinct mature forms, IL-33₁₀₉₋₂₇₀ and IL-33₉₅₋₂₇₀, which are generated by mast cell chymase, and IL-33₁₀₇₋₂₇₀, IL-33₇₉₋₂₇₀, and IL-33₇₂₋₂₇₀, which are generated by mast cell trypsin.

IL-33 Mature Forms Generated by Mast Cell Proteases Are More Active than Full-Length IL-33. We next compared the biological activity of full-length IL-33 and mature forms generated by mast cell proteases. The different proteins were prepared using rabbit reticulocyte lysates and their precise concentrations were determined by quantitative Western blotting. All IL-33 forms were found to induce IL-6 secretion in MC/9 mast cells (Fig. 3A and B). However, dose-responses studies revealed that IL-33 mature forms IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀ have significantly increased biological activity compared with the full-length protein (Fig. 3A). IL-33 mature forms generated by mast cell trypsin, IL-33₇₉₋₂₇₀ and IL-33₇₂₋₂₇₀, also had increased biological activity but it was only twofold higher than that of full-length IL-33 (Fig. 3B). We confirmed these results using an

independent bioassay, IL-5 secretion by KU812 basophil-like cells. Essentially identical results were obtained (Fig. 3C and D). Together, these data indicated that IL-33 mature forms generated by mast cell proteases are more active than the full-length protein, and that removal of the first 94 amino acid residues of IL-33 leads to maximum biological activity of the cytokine.

Mature Forms IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀ Are Potent Activators of ILC2s. We then asked whether IL-33 mature forms IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀, which are the major forms generated by activated human mast cells (Fig. 1A), have direct effects on ILC2s. For that purpose, CD45⁺Lin⁻ ILC2s were isolated from lungs and cultured in media containing IL-2 and IL-7 during 3 d. Phenotypic analysis revealed that cultured CD45⁺Lin⁻ ILC2s express high levels of ST2, CD25, Sca-1, ICOS, and KLRG1 markers (Fig. 4A). These characteristics mirrored those of lung ILC2s in vivo (21, 22). Cultured ILC2s were then stimulated with the different IL-33 forms during 18 h. Analysis of type-2 cytokine secretion revealed that IL-33 mature forms, IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀ induce high levels of IL-5 and IL-13 secretion by ILC2s ex vivo (Fig. 4B). Comparison with full-length IL-33 revealed that IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀ are ~30-fold more potent than full-length IL-33 for induction of type-2 cytokine secretion by ILC2s (Fig. 4C). Together, these results indicated that IL-33 mature forms generated by activated mast cells, IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀, are direct activators of ILC2s, which exhibit a greatly increased potency compared with full-length IL-33.

IL-33 Mature Forms Are Potent Inducers of Type-2 Innate Immune Responses in Vivo. We then analyzed the capacity of IL-33 mature forms generated by mast cell proteases to induce type-2 innate immune responses in vivo. Wild-type mice were injected i.p. with recombinant proteins IL-33₁₀₉₋₂₇₀ and IL-33₉₅₋₂₇₀ or PBS and immune responses in lungs and bronchoalveolar lavage (BAL) fluids were analyzed after 1 wk. We found that IL-33₁₀₉₋₂₇₀ strongly increased the numbers of innate lymphoid cells (CD45⁺Lin⁻ cells; Fig. 5A) and ILC2s (ST2⁺CD90.2⁺CD25⁺CD127⁺Sca1⁺CD45⁺Lin⁻ cells; Fig. 5B and Fig. S24) in lungs, including CD117⁺

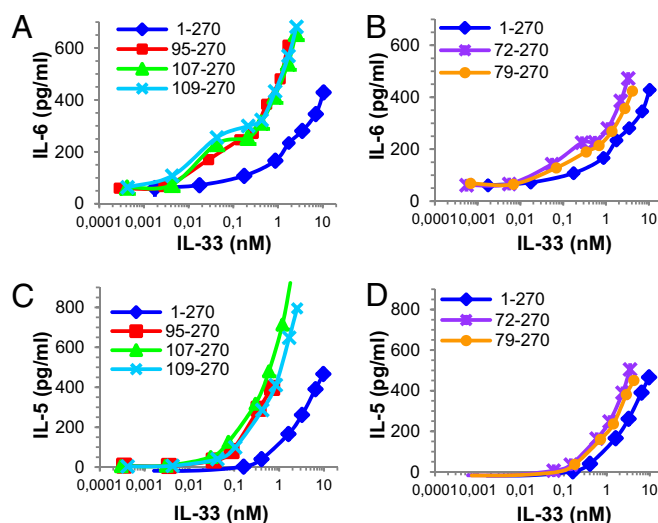


Fig. 3. IL-33 mature forms generated by mast cell proteases have increased biological activity compared with the full-length protein. The capacity of IL-33 mature forms produced in RRL to activate the IL-33-responsive MC/9 mast cells (A and B) and KU812 basophil-like chronic myelogenous leukemia cells (C and D) were analyzed by determining IL-6 (A and B) and IL-5 (C and D) levels in supernatants using ELISA. Results are representative of at least two independent experiments.

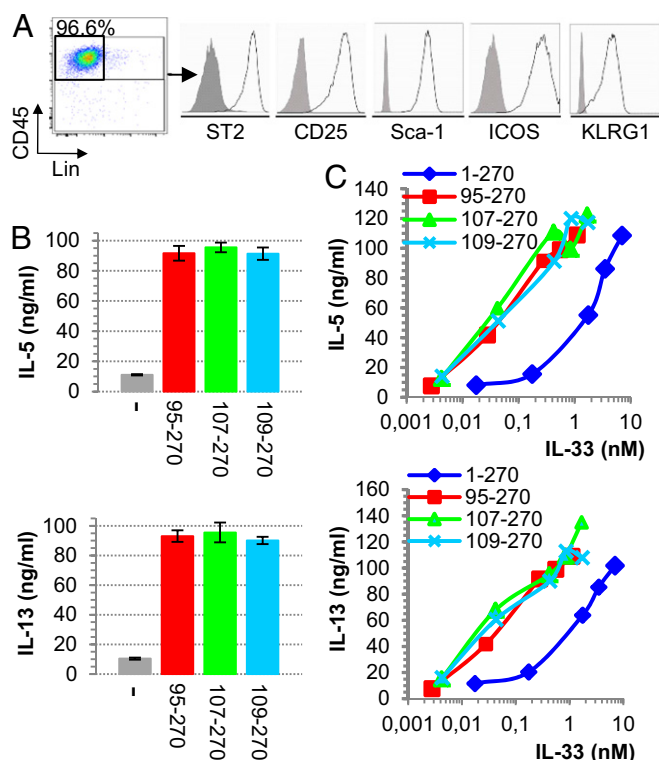


Fig. 4. IL-33 mature forms IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀ are potent activators of ILC2s ex vivo. (A) Representative histograms of ST2, CD25, Sca-1, ICOS, and KLRG1 expression at the surface of ILC2s (black lines) isolated from lungs and cultured for 3 d ex vivo in the presence of IL-2 and IL-7. Isotype controls are shown (shaded histograms). (B) The capacity of IL-33 mature forms produced in RRL (1 μ L lysate) to activate ILC2s was analyzed by determining IL-5 and IL-13 levels in supernatants using ELISA. Results are shown as means and SD of three separate data points. (C) Comparison of the biological activity of IL-33 full-length and mature forms. Various concentrations of the different proteins were used to stimulate ILC2s. IL-5 and IL-13 levels in supernatants were detected by ELISA. Data are representative of three independent experiments.

ILC2s, ICOS⁺ ILC2s, and KLRG1⁺ ILC2s (Fig. S2B). IL-33₁₀₉₋₂₇₀ also increased the numbers of ILC2s in BAL fluids (Fig. S2C). The in vivo expansion of ILC2s induced by IL-33₁₀₉₋₂₇₀ was associated with a strong increase in lung eosinophils (SiglecF⁺CD11c⁻CD45⁺ cells) (Fig. 5C and Fig. S2D), elevated levels of IL-5 and IL-13 in BAL fluids (Fig. 5D) and lungs (Fig. S2E), airway inflammation, and increased mucus production (Fig. 5E). Similarly to IL-33₁₀₉₋₂₇₀, IL-33₉₅₋₂₇₀ was also very efficient for in vivo expansion of ILC2s in lungs and BAL fluids (Fig. 5F). It induced strong expansion of lung eosinophils (Fig. 5G) and greatly increased levels of IL-5 and IL-13 in BAL fluids (Fig. S2F). We concluded that IL-33 mature forms generated by activated mast cells are potent inducers of ILC2s and associated type-2 innate immune responses in vivo.

Finally, we determined the impact of mast cell protease inhibition on the activity of endogenous IL-33 in vivo, using a model of allergic inflammation induced by the clinically relevant fungal allergen *Alternaria alternata* (21). In agreement with previous studies (21), allergic inflammation induced by *Alternaria*, as revealed by airway eosinophilia, was no longer observed in IL-33-deficient mice (Fig. 5H) and ILC2-deficient *Rag2 γ c^{-/-}* mice (Fig. 5I). Interestingly, mast cell tryptase inhibitor NM, which blocks the processing of murine full-length IL-33 by endogenous mast cell tryptase in vitro (Fig. S1), reduced the increase in BAL eosinophils in response to *Alternaria* (Fig. 5J).

Discussion

In this paper, we demonstrate that serine proteases secreted by activated human mast cells generate mature forms of IL-33, which are potent activators of ILC2s. Indeed, we discovered that IL-33 mature forms generated by mast cell proteases, IL-33₁₀₉₋₂₇₀, IL-33₁₀₇₋₂₇₀, and IL-33₉₅₋₂₇₀, are at least 30-fold more potent than full-length human IL-33 for induction of IL-5 and IL-13 secretion by ILC2s. These IL-33 mature forms were also very potent for induction of type-2 innate immune responses in vivo. They induced massive expansion of ILC2s and eosinophils in lungs and high levels of IL-5 and IL-13 secretion in lungs and

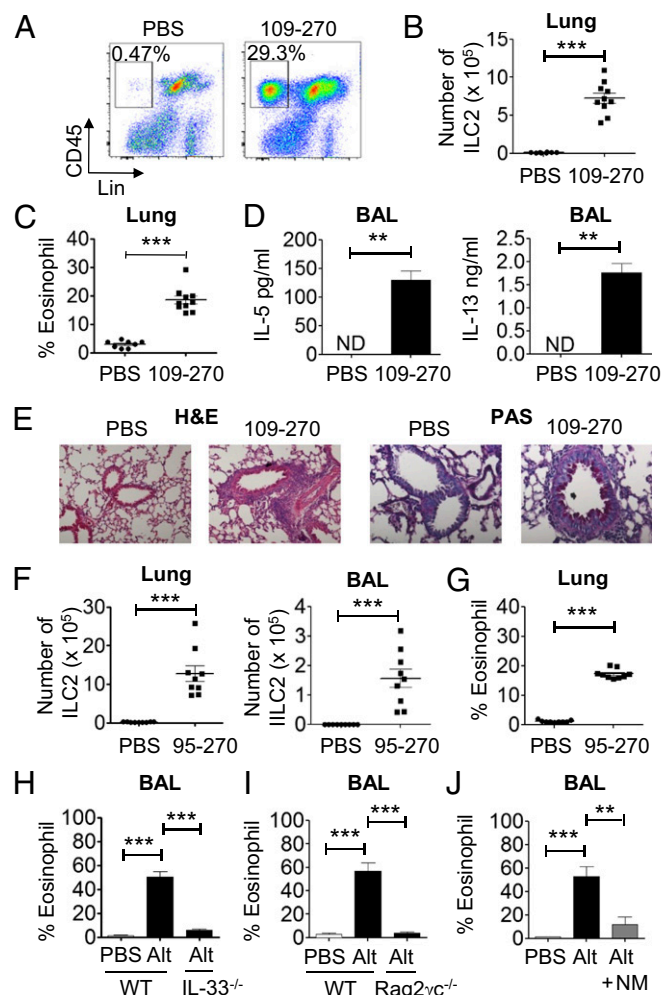


Fig. 5. IL-33-induced airway inflammation in vivo. (A–G) IL-33 mature forms IL-33₁₀₉₋₂₇₀ and IL-33₉₅₋₂₇₀ are potent inducers of type-2 innate immune responses in vivo. (A–E) C57BL/6 mice were injected i.p. with IL-33₁₀₉₋₂₇₀ or PBS. (A) Representative FACS plots of Lin⁻CD45⁺ ILC population from lungs. (B) Number of ST2⁺CD90.2⁺CD25⁺Sca-1⁺CD127⁺ ILC2s in lungs. (C) Frequency of eosinophils (CD45⁺CD11c⁻SiglecF⁺ cells) in lungs. (D) IL-5 and IL-13 levels in BAL fluids. ND, not detected. (E) Staining of lung tissue sections with hematoxylin/eosin (H&E) and periodic acid-Schiff (PAS). (F and G) C57BL/6 mice were injected i.p. with IL-33₉₅₋₂₇₀ or PBS. (F) Number of ILC2s in lungs and BAL fluids. (G) Frequency of lung eosinophils. $n = 8$ –10 mice, two experiments. (H–J) Tryptase inhibitor NM reduces IL-33-dependent airway eosinophilia in a model of allergic inflammation. The fungal allergen *Alternaria alternata* (Alt) or PBS was administered i.n. to wild-type (WT) mice (H–J), IL-33^{-/-} (H), or ILC2-deficient *Rag2 γ c^{-/-}* (I) mice or wild-type mice treated with tryptase inhibitor NM (J). Frequency of eosinophils in BAL fluids is shown. $n = 5$ –8 mice, two experiments. ** $P < 0.005$, *** $P < 0.001$ compared with PBS (B–D and F–J) or Alt (H–J) control, unpaired Student t test.

BAL fluids. Murine IL-33 was also cleaved by mast cell tryptase, and a tryptase inhibitor reduced airway eosinophilia in a model of allergic inflammation dependent on endogenous IL-33 and ILC2s. Together, these results indicate that cleavage of full-length IL-33 by mast cell proteases may represent an important step in the activation of type-2 innate immune responses in vivo.

We found that the two major serine proteases produced by activated mast cells, chymase and tryptase, are involved in the processing of IL-33. Both proteases generate IL-33 mature forms with potent activity on ILC2s. Therefore, although mast cell proteases are differentially expressed in different mast cell subsets (42), all types of mast cells, including mucosal and connective tissue mast cells, may be able to generate highly active mature forms of IL-33. Mast cells are located close to IL-33-producing cells in tissues (33, 34, 39), and they interact very closely with ILC2s in vivo (26). Mast cells and the proteases they secrete are thus likely to play an important role in the activation of the IL-33 alarm signal during inflammation.

We show that mast cell chymase and tryptase can cleave IL-33 in its central domain and generate five distinct mature forms of the cytokine (Fig. 6). During the course of this study, we observed that granzyme B, another protease secreted by human mast cells upon activation (43), can process full-length IL-33 into shorter mature forms, including a 18-kDa form corresponding to IL-33_{111–270} (Fig. S3). In addition, we reported previously that neutrophil proteases, cathepsin G and elastase, can also process full-length IL-33 within the central domain and generate mature bioactive forms of the cytokine (38). Therefore, multiple proteases are able to activate IL-33 by cleavage within its central domain. This apparent redundancy suggests that cleavage/activation is important for regulation of IL-33 biological activity. For instance, mast cell proteases may be critical for activation of IL-33 in allergic asthma and allergic inflammation, whereas neutrophil proteases may play a role in virus-induced exacerbations of asthma and other inflammatory or infectious conditions. Interestingly, processing of IL-33 by mast cell (Fig. S1) and neutrophil (38) proteases was also observed in mouse, despite a lack of primary sequence conservation of the central domain between IL-33 orthologs (2).

Our study identifies the central cleavage/activation domain of IL-33 (amino acids 66–111) as a functional domain of the protein, located between the N-terminal chromatin-binding nuclear domain (amino acids 1–65) (2, 35) and the C-terminal IL-1-like cytokine domain (amino acids 112–270) (4). The nuclear domain plays a critical role in the regulation of IL-33 cytokine activity, and its deletion has recently been shown to result in constitutive extracellular release of the protein, multiorgan inflammation, and death of the organism (44). The cleavage/activation domain is likely to represent another important domain for regulation of IL-33 biological activity in vivo. For instance, although full-length IL-33 may initiate the immune response after cellular damage (36, 37), cleavage of IL-33 by inflammatory proteases

within the activation domain will potentiate the response through the generation of the highly active mature forms containing the IL-1-like cytokine domain.

The increased biological activity of IL-33 mature forms compared with the full-length protein indicates that the nuclear domain inhibits IL-33 cytokine activity. An important role of IL-33 cleavage within its central domain thus appears to be to release the C-terminal IL-1-like cytokine domain from the inhibitory effect of the N-terminal nuclear domain. Although the molecular mechanism of this inhibitory effect is currently unknown, charge is likely to be important. The IL-1-like cytokine domain of IL-33 is highly acidic (isoelectric point < 5), whereas the nuclear domain of IL-33 is highly basic (isoelectric point > 10). Acidic residues are critical for binding to ST2 (5), and the basic nuclear domain may thus interfere some way with the binding of the acidic cytokine domain to the ST2 receptor.

We found many sites of cleavage for inflammatory proteases within the central activation domain of IL-33 (Fig. 6). In contrast, we did not observe cleavage of the IL-1-like cytokine domain by human mast cell chymase and tryptase (Fig. S4), suggesting that the IL-1 cytokine fold protects from cleavage. Therefore, although mouse mast cell chymase MCP4/MCPT4 has been reported to cleave and degrade the IL-1-like cytokine domain of IL-33 (45, 46), this probably only occurs in the presence of very high concentrations of the proteases.

In conclusion, we have identified an important functional domain of IL-33, the cleavage/activation domain within its central part. Cleavage of IL-33 by mast cell proteases chymase and tryptase, within this activation domain generates mature forms of the cytokine with highly increased biological activity toward ILC2s. These IL-33 mature forms induce strong expansion of ILC2s and eosinophils, and very high levels of type-2 cytokine secretion by ILC2s. A tryptase inhibitor reduced IL-33-dependent allergic airway inflammation, indicating that mast cell proteases may play an important role in the regulation of IL-33 biological activity in vivo. Together, these results suggest that interference with IL-33 cleavage/activation by mast cell proteases could reduce IL-33-mediated type-2 responses in allergic asthma and other types of allergic inflammation (allergic rhinitis, atopic dermatitis). Inhibitors targeting the central activation domain of IL-33 may turn out to also be useful for other inflammatory diseases, because activation by inflammatory proteases, through cleavage within the central domain, appears to be a general mechanism for regulation of IL-33 activity during inflammation.

Materials and Methods

SI Materials and Methods provides details regarding culture of mast cells, plasmid constructions, protein production, Western blot analysis, flow cytometry, and histology.

Protein Cleavage Assays with Mast Cell Proteases. In vitro translated proteins produced in rabbit reticulocyte lysate (RRL) were incubated with mast cell chymase [0.14–4.5 milliunits (mU); Sigma] or tryptase (0.24–12 mU; Calbiochem) in 15 μ L assay buffer (2 μ L RRL lysate + 5 μ L PBS) for 30 min at 37 °C. Cleavage assays with activated mast cells (6.10^3 – 3.10^4 mast cells) were performed for 1–2 h at 37 °C using hMC supernatants. In some experiments, mast cell protease supernatants were preincubated (20 min, 37 °C) with cysteine protease inhibitor E64 (50 μ M; Sigma), serine protease inhibitor AEBSF (8 mM; Calbiochem), tryptase inhibitor NM (1 μ M; Enzo Life Sciences), or chymase inhibitors CS (100 μ M; Sigma) and CGI (50 μ M; Calbiochem), before adding in vitro translated proteins. Cleavage products were analyzed by SDS/PAGE and Western blot.

IL-33 Activity Assays and ELISA. In vitro translated full-length IL-33 or mature forms (up to 6 μ L lysate per well, 18-h treatment) were used to stimulate IL-33-responsive MC9 mast cells (10^5 cells per well in 96-well plates), KU812 basophil-like chronic myelogenous leukemia cells (ATCC; 5×10^5 cells per well in 96-well plates), and cultured ILC2s (5×10^4 cells per well in 96-well plates). Cytokine levels in culture supernatants were determined using DuoSet IL-6, IL-5, and IL-13 ELISA (R&D Systems). The concentration of IL-5

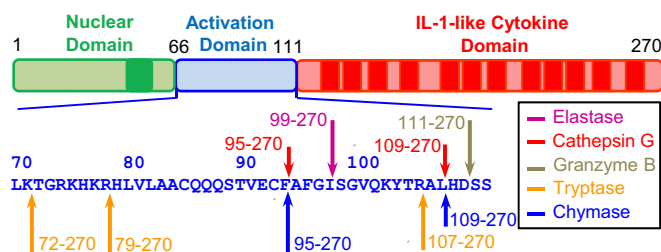


Fig. 6. Primary structure of human IL-33. The nuclear (amino acids 1–65), cleavage/activation (amino acids 66–111), and IL-1-like cytokine (amino acids 112–270) domains are indicated. The different mature forms of IL-33 and the sequences surrounding the cleavage sites for inflammatory proteases are shown.

and IL-13 in BAL fluids and lung homogenate supernatants were analyzed by ELISA (ELISA MAX Deluxe Sets, Biolegend for IL-5 and Quantikine, R&D Systems for IL-13) according to the manufacturer's instructions.

In Vivo Treatment of Mice. C57BL/6 mice (Charles River) were treated daily i.p. with 4 μ g recombinant human IL-33_{95–270}, IL-33_{109–270}, or PBS for 7 d. Twenty-four hours after the last injection, BAL fluids and lungs were collected for ELISA, flow cytometry, and histological analyses. For induction of allergic airway inflammation, C57BL/6 wild-type (Charles River), IL-33^{−/−} (34), or Rag2^{γc} (47) mice were anesthetized by isoflurane inhalation and 12.5 μ g *A. alternata* extract (Greer Laboratories) was administered intranasally (i.n.) in 50 μ L PBS on days 0, 1, and 2. Tryptase inhibitor NM was used in some experiments (2 mg/kg, administered i.n. on days 0, 1, and 2). BAL fluids were collected 24 h after the final *Alternaria* challenge. All mice were handled according to institutional guidelines under protocols approved by the Institut de Pharmacologie et de Biologie Structurale (IPBS) and Fédération de Recherche en Biologie de Toulouse (FRBT) (C2EA-01) animal care committees (project no. 00663.01).

Isolation and Culture of ILC2 Cells. Lineage-negative cells from lungs were enriched by magnetically depleting lineage-positive cells using biotin-conjugated

antibodies to CD5, CD11b, CD19, CD45R, Ly-6G/C, Ter119, and 7–4 and Easysep D magnetic particles (Mouse Hematopoietic Progenitor Cell Enrichment kit; Stem Cell Technologies). Subsequently, Lin[−]CD45⁺ cells were selected by using magnetic beads conjugated to anti-mouse CD45 monoclonal antibody (Miltenyi Biotec). Lung CD45⁺Lin[−] ILC2s were cultured at a density of 3.10⁵ cells/mL in RPMI medium complemented with 10% (vol/vol) FCS, 1% penicillin/streptomycin, 50 μ M β -mercaptoethanol, 20 ng/mL recombinant mouse IL-2 (rmIL-2), and 10 ng/mL rmIL-7 (R&D Systems). After 72 h, surface markers of lung ILC2s were analyzed by flow cytometry.

Statistical Analyses. The Student *t* test was used for comparison between two groups. All data are represented as mean $\pm$ SEM.

ACKNOWLEDGMENTS. We thank members of the J.-P.G. laboratory for help with animal experiments and the Infrastructures en Biologie, Santé et Agronomie (IBISA) Toulouse Réseau Imagerie (TRI)-IPBS, and Anexplo-IPBS facilities. This work was supported by grants from Fondation ARC [fellowships to E.L. and E.M. and Fondation ARC Programme SL20110603471 (to J.-P.G.)], Agence Nationale pour la Recherche (ANR-12-BSV3-0005-01), and Fondation pour la Recherche Médicale (fellowship to E.L.).

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Supporting Information

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SI Materials and Methods

Animals. C57BL/6 wild-type and Rag2^{-/-} mice were purchased from Charles River Laboratories. IL-33^{-/-} mice have been previously described (1). Rag2^{γc}^{-/-} mice from the National Institute of Allergy and Infectious Diseases (2) were obtained via Taconic Biosciences. All mice were handled according to institutional guidelines under protocols approved by the IPBS and FRBT (C2EA-01) animal care committees (project no. 00663.01).

Plasmid Construction and Protein Production. IL-33 deletion mutants were amplified by PCR using the human (NM_033439) IL-33/NF-HEV cDNA as template. The PCR fragments, thus obtained, were cloned into plasmid pcDNA3.1 (Invitrogen). The human IL-33_{K71G}, IL-33_{K77K78G}, IL-33_{F94G}, IL-33_{R106G}, and IL-33_{L108G} mutants were generated by PCR and cloned into the same expression vector. All primer sequences are available upon request. Wild-type and mutant IL-33 proteins were synthesized in vitro in rabbit reticulocyte lysate (RRL) using the TNT-Coupled Transcription/Translation system (Promega). For expression in *Escherichia coli*, cDNAs encoding mature forms of IL-33₉₅₋₂₇₀ and IL-33₁₀₉₋₂₇₀ were subcloned into expression vector pET-15b (Novagen). Recombinant proteins were produced in *E. coli* BL21pLysS (Novagen) and purified using Ni-NTA agarose column (Qiagen). His-Tag was removed by cleavage with thrombin and proteins were further purified by gel filtration (FPLC; GE Healthcare). Endotoxin levels were <0.01 endotoxin units/μg of protein as determined by the *Limulus* amoebocyte lysate QCL-1000 method (Lonza).

Culture and Activation of Mast Cells. Human mast cells (hMCs) were obtained from CD133⁺ peripheral blood precursors cultured in the presence of stem cell factor (SCF) and IL-6 as previously described (3). After 2 mo of culture, cells were tested phenotypically (tryptase⁺, CD117⁺, FcεRI⁺) and functionally (β-hexosaminidase release in response to FcεRI cross-linking) before use for experiments. Supernatants from activated mast cells, treated with either anti-IgE (2.5 μg/mL) or substance P (10 μmol/L) for 30 min, were collected and used for IL-33 cleavage assays. Mouse bone marrow mast cells (BMMCs) (4) were obtained from femur and tibia bone marrow cell suspensions cultured for 10 wk in DMEM (ATCC) in the presence of IL-3 (10 ng/mL) and SCF (50 ng/mL). BMMCs or MC/9 mast cells (ATCC) were activated by treatment with PMA (20 nM to 10 μM) and ionomycin (2 μM) for 3 h, and supernatants were collected.

Protein Cleavage Assays with Mast Cell Proteases. In vitro translated proteins produced in rabbit reticulocyte lysate (RRL) were incubated with mast cell chymase (0.14 mU to 4.5 mU, Sigma) or tryptase (0.24 mU to 12 mU, Calbiochem) in 15 μL assay buffer (2 μL RRL lysate + 5 μL PBS) for 30' at 37 °C. Cleavage assays with activated mast cells (6.10³–3.10⁴ mast cells) were performed for 1h-2h at 37 °C using hMCs, BMMCs, or MC/9 mast cells supernatants. In some experiments, mast cell proteases supernatants were preincubated (20 min, 37 °C) with cysteine protease inhibitor E64 (50 μM; Sigma), serine protease inhibitor AEBSP (8 mM; Calbiochem), tryptase inhibitor Nafamostat mesylate (NM, 1 μM; Enzo Life Sciences) or chymase inhibitors chymostatin (CS, 100 μM; Sigma) and CGI (50 μM; Calbiochem), before adding in vitro translated proteins. Cleavage products were analyzed by SDS/PAGE and Western blot.

Western Blot Analysis. Proteins were fractionated by SDS/PAGE, electroblotted and detected with mAb to human IL-33-Cter (305B, 1/1,000, Alexis Biochemicals) or goat antiserum to mouse IL-33-Cter (AF326, 1/500; R&D), followed by HRP-conjugated goat anti-mouse or donkey anti-goat polyclonal antibodies (1/10000; Promega), and finally an enhanced chemiluminescence kit (Amersham ECL Prime; GE Healthcare). Quantitative Western blots were performed to quantify the six IL-33 forms used in cellular bioassays, using a Typhoon TRIO instrument (Amersham, GE Healthcare). A standard curve was performed using purified recombinant IL-33₉₅₋₂₇₀ protein.

IL-33 Activity Assays and ELISA. In vitro translated full-length IL-33 or mature forms (up to 6 μL lysate per well, 18-h treatment) were used to stimulate IL-33-responsive MC/9 mast cells (10⁵ cells per well in 96-well plates), KU812 basophil-like chronic myelogenous leukemia cells (ATCC; 5 × 10⁵ cells per well in 96-well plates) and cultured ILC2s (5 × 10⁴ cells per well in 96-well plates). Cytokine levels in culture supernatants were determined using DuoSet IL-6, IL-5, and IL-13 ELISA (R&D Systems). The concentration of IL-5 and IL-13 in BAL fluids and lung homogenate supernatants were analyzed by ELISA (ELISA MAX Deluxe Sets, Biolegend for IL-5 and Quantikine, R&D Systems for IL-13) according to the manufacturer's instructions.

In Vivo Treatment of Mice. C57BL/6 mice were treated daily i.p. with 4 μg recombinant human IL-33₉₅₋₂₇₀, IL-33₁₀₉₋₂₇₀, or PBS for 7 d. 24 h after the last injection, BAL fluids (three lavages of lungs with three aliquots of 0.6 mL of PBS) were collected and stored at -80 °C for cytokine assays. Upper right lungs were collected and homogenized in 1 mL of PBS with Ultra-Turrax (IKA Laboratory). Lung homogenates were centrifuged at 16000 rpm for 15 min at 4 °C and the supernatants were stored at -80 °C for cytokine analysis. Protein concentrations in lung supernatants were quantified using NanoDrop (Nanodrop 1000 Spectrophotometer; Thermo Fischer). To obtain lung single-cell suspensions, lower right lungs were digested with 2 mg/mL collagenase D and 0.1 mg/mL DNase I for 60 min at 37 °C and meshed through a 70-μm cell strainer. Leukocyte population was enriched by using a Lympholyte M gradient (Cedarlane Laboratories) at 1,300 rpm for 20 min at room temperature. The total number of cells in lungs and BAL fluids was counted with a hemocytometer. Left lung was fixed in PFA 4% overnight at 4 °C for histological analysis. For induction of allergic airway inflammation, mice were anesthetized by isoflurane inhalation and 12.5 μg *Alternaria alternata* extract (Greer Laboratories) was administered intranasally (i.n.) in 50 μL PBS on days 0, 1, and 2. Tryptase inhibitor NM was used in some experiments (2 mg/kg, administered i.n. on days 0, 1, and 2). BAL fluids were collected 24 h after the final *Alternaria* challenge and analyzed by flow cytometry.

Analysis of Cell Surface Markers by Flow Cytometry. ILC2s were identified in lung single-cell suspensions and BAL fluids using a combination of FITC-conjugated antibodies allowing the exclusion of T, B, natural killer (NK), NKT, mast cells, basophils, dendritic cells and macrophages. Lineage antibodies included CD4 (clone GK1.5), CD19 (clone 1D3), CD45R (clone RA3-6B2), NK1.1 (clone PK136), CD3 (clone 17A2), CD11b (clone M1/70) (BD Bioscience), Ter119 (clone Ter119), Ly-6G (clone RB6-8C5), FcεRIα (clone MAR-1) and CD11c (clone N418) (eBioscience). Antibodies CD45-PerCp (clone 30F11), CD90.2-APCCy7 (clone 53-2.1) (BD Bioscience), Sca-1-APC (clone D7),

CD25-eFluor450 (clone PC61.5), CD127-Pe (clone A7R34), KLRG1-APC (clone 2F1), ICOS-PE (clone 7E-17G9) (eBioscience) and T1/ST2 biotinylated (clone DJ8, MD Biosciences) followed by streptavidin-PeCy7 staining (BD Bioscience) were also included for further characterization of ILC2s. Eosinophils were identified by using CD45-PerCp (clone 30F11), SiglecF-Pe (clone E20-2440) (BD Bioscience) and CD11c-APC (clone N418) (eBioscience). Antibodies were diluted in a FcR blocker (CD16/CD32 clone 2.4G2; BD bioscience) for 30 min in the dark at 4 °C. Isotype and single-stain controls were included. Dead cells were excluded by using viability dye eFluor506. Samples were acquired on a LSRII flow cytometer using DiVa software (BD Bioscience) and analyzed using FlowJo software (Tree Star).

Isolation and Culture of ILC2 Cells. ILC2s were isolated from pooled lungs of Rag2^{-/-} mice treated with daily i.p. injections of 4 µg of rhIL-33_{95–270} for 7 d. Lineage-negative cells were enriched by magnetically depleting lineage-positive cells using biotin-conjugated antibodies to CD5, CD11b, CD19, CD45R, Ly-6G/C, Ter119, and 7–4 and Easysep D magnetic particles (Mouse

Hematopoietic progenitor cell enrichment kit; Stem Cell Technologies). Subsequently, Lin⁻ CD45⁺ cells were selected by using magnetic beads conjugated to anti-mouse CD45 monoclonal antibody (Miltenyi Biotech). Purity of Lin⁻ CD45⁺ cells was analyzed by staining isolated cells with lineage antibodies and antibodies against ST2, CD25, CD127, Sca-1 and CD90.2. Lung CD45⁺ Lin⁻ ILC2s were cultured at a density of 3.10⁵ cells/mL in RPMI medium complemented with 10% FCS, 1% Penicillin/Streptomycin, 50 µM β-mercaptoethanol, 20 ng/mL recombinant mouse IL-2 (rmIL-2) and 10 ng/mL rmIL-7 (R&D Systems). After 72 h, surface markers of lung ILC2s were analyzed by flow cytometry.

Histology. Histological evaluation was performed on formalin-fixed mouse tissues. Five µm paraffin-embedded sections of the lung were prepared and stained with hematoxylin and eosin for morphological evaluation. Periodic-acid-Schiff (PAS) staining was used to detect the presence of mucus in lung tissue sections.

Statistical Analyses. The Student *t* test was used for comparison between two groups. All data are represented as mean ± SEM.

1. Pichery M, et al. (2012) Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: In situ analysis using a novel IL-33-LacZ gene trap reporter strain. *J Immunol* 188(7):3488–3495.
2. Cao X, et al. (1995) Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity* 2(3):223–238.

3. Gaudenzio N, Laurent C, Valitutti S, Espinosa E (2013) Human mast cells drive memory CD4⁺ T cells toward an inflammatory IL-22⁺ phenotype. *J Allergy Clin Immunol* 131(5):1400–1407.
4. Gurish MF, et al. (1992) Differential expression of secretory granule proteases in mouse mast cells exposed to interleukin 3 and c-kit ligand. *J Exp Med* 175(4):1003–1012.

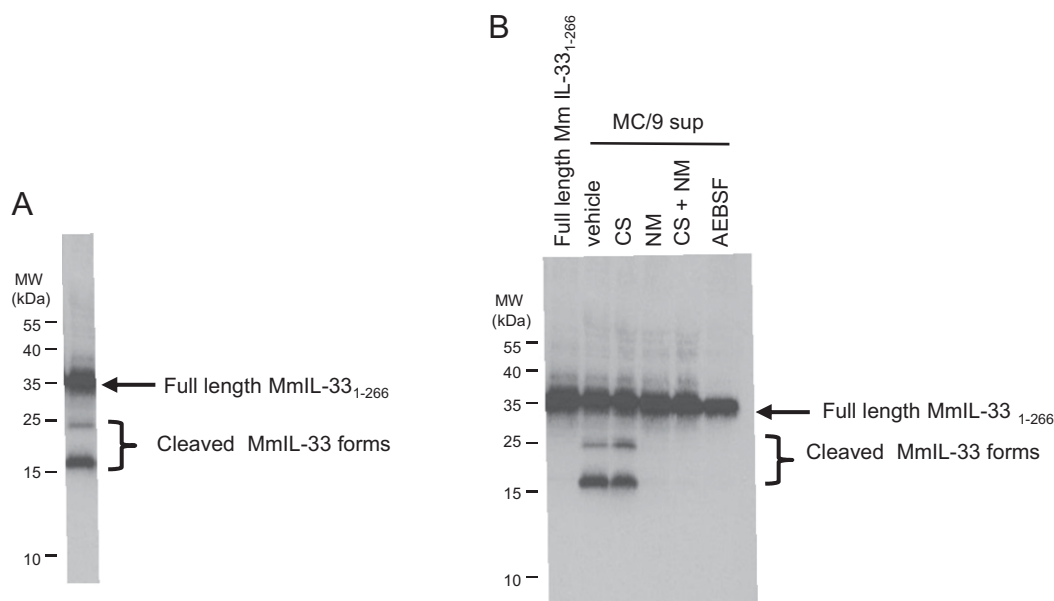


Fig. S1. Murine full-length IL-33 protein is a substrate for mast cell proteases. In vitro translated full-length murine IL-33_{1–266} (2.5 µL lysate) was incubated with supernatants of activated mouse bone-marrow-derived mast cells (BMMCs) or MC/9 mast cells for 1 h at 37 °C. (A) Cleavage of full-length IL-33 by supernatants from activated bone-marrow-derived murine mast cells. Murine mast cells were differentiated from bone marrow multipotential stem cells by culture in the presence of SCF and IL-3, and release of serine proteases from secretory granules was induced by stimulation with ionomycin and PMA. (B) Murine full-length IL-33 protein is processed by mast cell tryptase. Activated MC/9 mast cells were pretreated with vehicle, chymase inhibitor chymostatin (CS), tryptase inhibitor nafamosat mesylate (NM), or serine protease inhibitor AEBSF for 1 h at 37 °C. Proteins were migrated on SDS/PAGE and revealed by Western blot with goat anti-mouse IL-33-CTer antibodies.

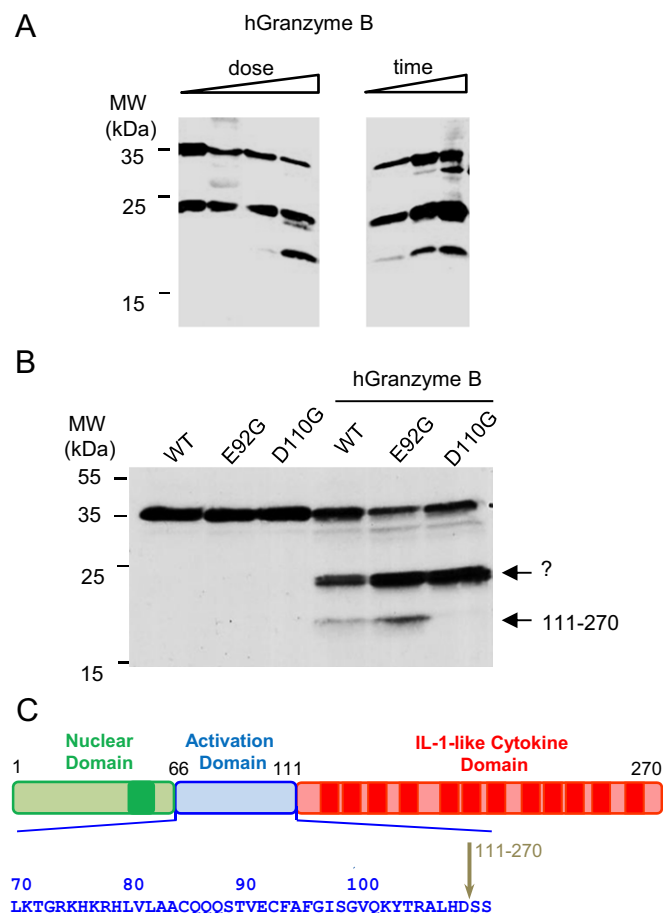


Fig. S3. Human full-length IL-33 protein is a substrate for granzyme B. (A) In vitro translated full-length human IL-33₁₋₂₇₀ was incubated with purified human granzyme B (*Left*) increasing amounts up to 0.3 units; (*Right*) increased time from 5 min to 1 h. Proteins were separated by SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. Blots are representative of two independent experiments. (B) Mutation of D₁₁₀ to glycine in full-length IL-33 inhibits formation of the 18-kDa granzyme B cleavage product. In vitro translated full-length human IL-33₁₋₂₇₀ or single point mutants IL-33_{E92G} (E92G) and IL-33_{D110G} (D110G) were incubated with purified human granzyme B (0.3 units, 1 h at 37 °C). (C) Mapping of the granzyme B cleavage site in the central domain of IL-33 (amino acids 66–111). The sequence surrounding the granzyme B cleavage site is shown.

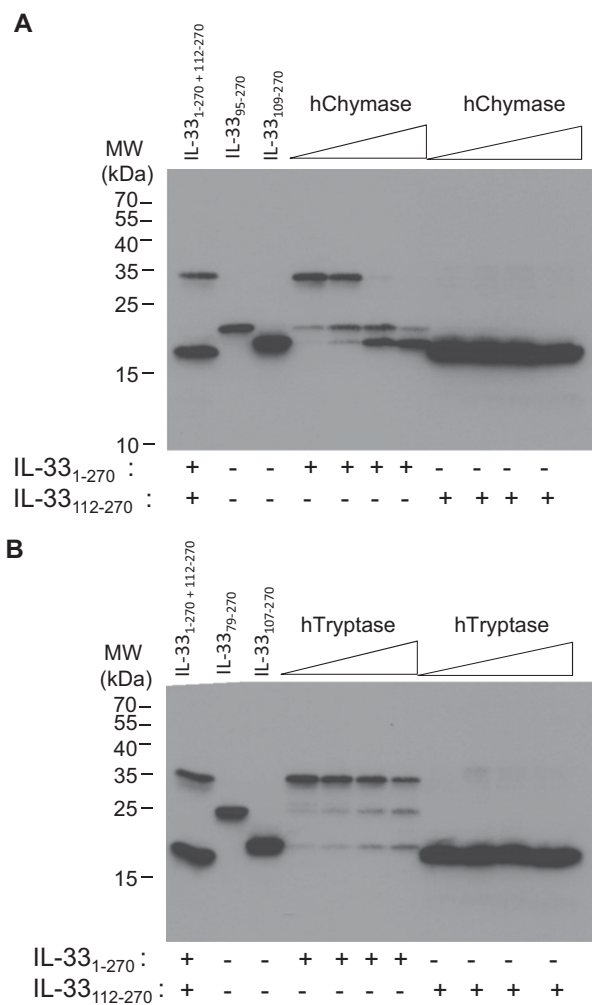


Fig. S4. The IL-1-like cytokine domain of IL-33 is not cleaved by mast cell proteases. (A and B) In vitro translated full-length human IL-33₁₋₂₇₀ and IL-1-like cytokine domain IL-33₁₁₂₋₂₇₀ were incubated with increasing amounts of purified human mast cell chymase (A) or tryptase (B). Proteins were separated on SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. The IL-33 mature forms generated by mast cell chymase (A, IL-33₉₅₋₂₇₀ and IL-33₁₀₉₋₂₇₀) and tryptase (B, IL-33₇₉₋₂₇₀ and IL-33₁₀₇₋₂₇₀) were comigrated on the gels. Blots are representative of two independent experiments.