nature immunology

ARTICLES

https://doi.org/10.1038/s41590-018-0067-5

# Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33

Corinne Cayrol[1,2*], Anais Duval[1,2], Pauline Schmitt[1], Stephane Roga[1], Mylène Camus[1], Alexandre Stella[1], Odile Burlet-Schiltz[1], Anne Gonzalez-de-Peredo[1] and Jean-Philippe Girard[1*]

**Allergic inflammation has crucial roles in allergic diseases such as asthma. It is therefore important to understand why and how the immune system responds to allergens. Here we found that full-length interleukin 33 (IL-33$_{FL}$), an alarmin cytokine with critical roles in type 2 immunity and asthma, functioned as a protease sensor that detected proteolytic activities associated with various environmental allergens across four kingdoms, including fungi, house dust mites, bacteria and pollens. When exposed to allergen proteases, IL-33$_{FL}$ was rapidly cleaved in its central 'sensor' domain, which led to activation of the production of type 2 cytokines in group 2 innate lymphoid cells. Preventing cleavage of IL-33$_{FL}$ reduced allergic airway inflammation. Our findings reveal a molecular mechanism for the rapid induction of allergic type 2 inflammation following allergen exposure, with important implications for allergic diseases.**

Allergic diseases such as asthma, atopic dermatitis (eczema), allergic rhinitis (hay fever) and allergic conjunctivitis are common diseases that affect patients from childhood to old age[1,2]. Environmental exposure to allergens has an important role in the exacerbation of these diseases[1,3,4]. The role of pattern-recognition receptors in the detection of conserved molecular structures of pathogens is well established, but comparatively very little is known about the molecular mechanisms involved in allergen detection[1,3]. The recognition of allergen-associated molecular patterns or structures by the pattern-recognition receptor TLR4 (Toll-like receptor 4) has been shown to serve a role[4–6], but other mechanisms are probably involved. Natural allergens are complex mixtures of various constituents and enzymes[1,3,4,7], and the sensing of allergen-associated biochemical and enzymatic activities is believed to have a critical role in the initiation of allergic type 2 inflammation[1,3]. Chitin, a polysaccharide constituent of many allergens and parasites[8], and phospholipase $A_2$ (PLA2), a cytolytic enzyme found in bee venom[9], fungal allergens and house dust mites (HDM)[10], are potent inducers of type 2 immune responses in vivo[8,9].

Many airborne allergens have intrinsic proteolytic activities[7] that can cause the breakdown of epithelial barriers in vivo[1,4]. The sensing of allergen-associated protease activity has thus been proposed to represent an important mechanism of allergen detection for the initiation of allergic inflammation[3,11–13]. Sensor systems for exogenous proteases have been described in *Drosophila* and plant immune systems[14,15], but despite progress[11–13], the mechanisms involved in the detection of allergen proteases in mammals remain incompletely defined.

Interleukin 33 (IL-33) is a tissue-derived nuclear cytokine of the IL-1 family with critical roles in tissue homeostasis and repair, type 2 immunity, viral infection, inflammation and allergy[16–19]. IL-33 activates signaling pathways dependent on the adaptor Myd88 in immune cells expressing the cytokine receptor IL-1RL1 (ST2), including many cell types involved in type 2 immunity, such as mast cells, basophils, eosinophils, the $T_H2$ subset of helper T cells and group 2 innate lymphoid cells (ILC2s)[16–19]. ILC2s, which produce large amounts of the $T_H2$ cytokines IL-5 and IL-13 in response to IL-33 and have critical roles in type-2 immunity, allergic inflammation and eosinophil homeostasis, are major targets of IL-33 in vivo[16–18,20]. Analysis of samples from patients and studies of mouse models support the proposal of an important role for IL-33–ST2 signaling in allergic inflammation in various tissues (lung, nasopharynx and skin) and diseases (asthma, atopic dermatitis, allergic rhinitis and chronic rhinosinusitis)[16–18,20]. *IL33* and *IL1RL1* are major susceptibility loci for asthma, suggestive of a crucial role for this axis in human asthma[16,21,22].

Cells that can produce IL-33, such as endothelial cells in blood vessels and epithelial cells at various barrier tissues, express it constitutively in the nucleus[19,23]. Endogenous IL-33 is released from the nucleus in its full-length form (amino acids 1–270) (IL-33$_{FL}$) after cellular damage or necrotic cell death[24] and is biologically active[24,25]. IL-33 might therefore function as an alarm signal (alarmin) to alert the immune system of damage[16,19,23,24]. The cytokine activity of IL-33 is regulated at multiple levels[16–19]. The oxidation of critical cysteine residues results in the inactivation of IL-33 a few hours after its extracellular release[26]. Apoptotic caspases inactivate IL-33$_{FL}$ during apoptosis[24,25], whereas inflammatory proteases from neutrophils and mast cells process IL-33$_{FL}$ into shorter mature forms containing the C-terminal IL-1-like cytokine domain; the activity of these forms is ~30-fold higher than that of IL-33$_{FL}$[27,28].

Protease-containing allergens induce the rapid release of IL-33 from airway epithelium[29–35], activate lung ILC2s and increase the number of airway eosinophils in an IL-33-dependent manner[28–34,36–39]. However, a role for allergen proteases in the cleavage of IL-33$_{FL}$ and regulation of its biological activity has not been reported. Here we found that IL-33$_{FL}$ functioned as a biochemical sensor of proteolytic activities associated with a large variety of environmental aeroallergens. We propose that the cleavage and

[1]Institut de Pharmacologie et de Biologie Structurale (IPBS), Université de Toulouse, CNRS, UPS, Toulouse, France. [2]These authors contributed equally: Corinne Cayrol and Anais Duval. *e-mail: corinne.cayrol@ipbs.fr; jean-philippe.girard@ipbs.fr

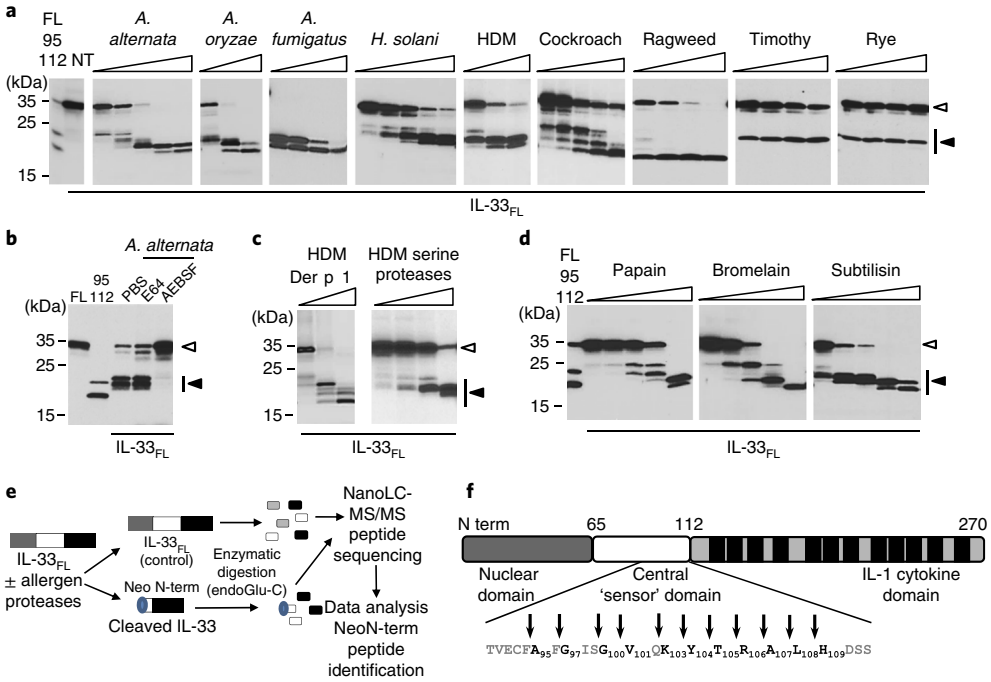


**Fig. 1 | IL-33$_{FL}$ is cleaved by proteases from environmental allergens. a**, Immunoblot analysis of recombinant human IL-33$_{FL}$ after incubation for 1 h at 37 °C with increasing amounts (wedges above blots: up to 1 μg for all except pollens, which were up to 10 μg) of allergen extracts (above blots): left margin (throughout), molecular size in kilodaltons (kDa); far left lane, co-migration of recombinant IL-33$_{FL}$ (FL), IL-33$_{95–270}$ (95) and IL-33$_{112–270}$ (112); NT, not treated (IL-33 left untreated); arrowheads (right margin), IL-33$_{FL}$ (open) or cleaved forms of IL-33 (filled); cropped images. **b**, Immunoblot analysis of the cleavage of recombinant human IL-33$_{FL}$ by *A alternaria* extracts (250 ng) in the presence of PBS, the cysteine protease inhibitor E64 or serine protease inhibitor AEBSF (above lanes): left lanes, migration of IL-33$_{FL}$ (far left) or of IL-33$_{95–270}$ and IL-33$_{112–270}$ (adjacent); cropped images. **c**,**d**, Immunoblot analysis of recombinant human IL-33$_{FL}$ after incubation for 1 h at 37 °C with increasing amounts (wedges) of the allergen proteases (above blots) Der p 1 or HDM serine proteases (125 ng to 1 μg) (**c**) or papain or bromelain (8–125 ng) or subtilisin (1.5–25 ng) (**d**); cropped images. **e**, Protocol for mass spectrometry: ±, with or without; Neo N-term, new amino terminus; endoGlu-C, endoproteinase GluC; nanoLC-MS/MS, nano liquid chromatography and tandem mass spectrometry. **f**, Location of various domains (below diagram) of IL-33 and of cleavage sites (downward arrows) in the central 'sensor' domain of IL-33; above, amino-acid positions. Data are representative of at least two independent experiments.

activation of IL-33$_{FL}$ by allergen proteases has a crucial role in the rapid induction of allergic airway inflammation following exposure to allergens.

## Results

**IL-33$_{FL}$ protein is cleaved by proteases from environmental allergens.** To study the potential regulation of IL-33 by allergen proteases, we incubated recombinant human IL-33$_{FL}$ with extracts of major classes of environmental allergens[4,7], including fungi (*Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus oryzae* and *Helminthosporium solani*), HDM (*Dermatophagoides pteronyssinus*), cockroaches (oriental) and pollens (from ragweed, timothy grass and rye grass). We found that these clinically relevant aeroallergens exhibited IL-33-processing activity and generated shorter forms of the protein (18- to 21-kilodaltons) in a dose-dependent manner (Fig. 1a). Experiments with protease inhibitors (Fig. 1b and Supplementary Fig. 1) and purified proteases (Fig. 1c,d) indicated that IL-33$_{FL}$ was cleaved by both serine proteases and cysteine proteases, including HDM serine proteases and the cysteine protease Der p 1, a major HDM allergen[7] (Fig. 1c). Proteolytic maturation of IL-33$_{FL}$ was also observed with the plant cysteine proteases papain and bromelain and with the bacterial serine protease subtilisin (Fig. 1d), three protease allergens linked to occupational asthma, an allergic lung disease caused by exposure to allergens in the workplace[36–38]. Together these results demonstrated that IL-33$_{FL}$ was processed into shorter mature forms by a wide variety of clinically relevant aeroallergens.

**Mature forms of IL-33 generated by allergen proteases are potent inducers of allergic airway inflammation.** Mass spectrometry (Fig. 1e and Supplementary Fig. 2) identified 11 distinct sites of cleavage by allergen proteases (Supplementary Table 1) and revealed that all these cleavage sites were located in the central 'sensor' domain of IL-33$_{FL}$ (Fig. 1f). Some of the forms generated by allergen proteases (IL-33 containing amino acids 95–270 (IL-33$_{95–270}$), IL-33$_{107–270}$ and IL-33$_{109–270}$) can also be produced by inflammatory proteases from neutrophils[27] and/or mast cells[28]. Other forms are uniquely generated by allergen proteases: IL-33$_{103–270}$ (generated by *A. alternata* and *A. fumigatus*), IL-33$_{106–270}$ (generated by ragweed) and IL-33$_{108–270}$ (generated by fungal allergens, subtilisin and Der p 1). All these processed forms of IL-33 were highly active. For example, IL-33$_{103–270}$ (generated by fungal allergens) and IL-33$_{106–270}$ (generated by ragweed pollen), when produced as recombinant proteins in *Escherichia coli* and injected intraperitoneally into wild type mice, induced airway inflammation and mucus production (Fig. 2a), the proliferation of eosinophils in lungs (Fig. 2b,c) and bronchoalveolar lavage (BAL) fluid (Fig. 2d), and increased IL-5 and IL-13 in BAL fluid (Fig. 2e).

The effects of IL-33$_{103–270}$ and IL-33$_{106–270}$ were also analyzed ex vivo with cultured CD45$^+$Lin$^-$ST2$^+$ ILC2s isolated from lungs (Fig. 2f). To compare the biological activity of the cleaved forms of IL-33 with that of IL-33$_{FL}$, we produced the various proteins in rabbit reticulocyte lysates and precisely quantified them. IL-33$_{103–270}$ and IL-33$_{106–270}$ induced substantial secretion of IL-5 and IL-13 by ILC2s ex vivo when used at low doses ($<0.1$ nM), whereas IL-33$_{FL}$

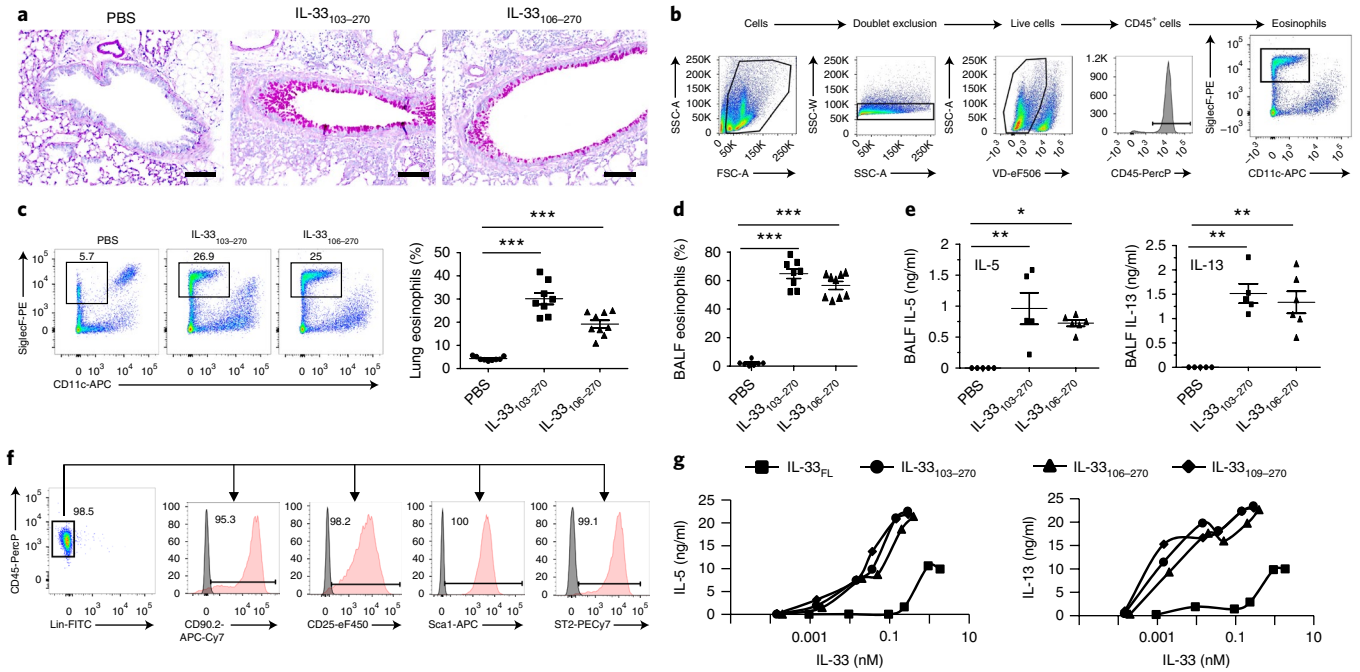


**Fig. 2 | Mature forms of IL-33 generated by allergen proteases are potent inducers of allergic airway inflammation. a**, Microscopy of lung sections obtained from C57BL/6 J wild type mice given intraperitoneal injection of PBS or recombinant human IL-$33_{103-270}$ or IL-$33_{106-270}$ produced in *E. coli* (above images); sections were stained with periodic acid–Schiff and hematoxylin. Scale bars, 100 µm. **b**, Gating strategy for the analysis of cells from mice treated as in **a**. **c**,**d**, Flow cytometry of cells from the lungs of mice treated as in **a** (**c**, left), and frequency of SiglecF$^+$CD11c$^-$ eosinophils (among CD45$^+$ cells) in the lungs (**c**) and BAL fluid (BALF) (**d**) of mice treated as in **a** (above or below plots). Numbers above outlined areas (**c**, left) indicate percent SiglecF$^+$CD11c$^-$ cells. **e**, Concentration of IL-5 (left) and IL-13 (right) in the BAL fluid of mice treated as in **a** (below plots). **f**, Flow cytometry of ILC2s, gating CD45$^+$Lin$^-$ cells (far left), and expression of the alloantigen CD90.2, cytokine receptor CD25, lineage marker Sca-1 and ST2 at the surface of cultured lung ILC2s (middle and right). Number above outlined area (far left) indicates percent CD45$^+$Lin$^-$ cells; numbers above bracketed lines (middle and right) indicate percent cells positive for the marker or receptor. **g**, Concentration of IL-5 and IL-13 (assessing bioactivity) in supernatants of viable CD45$^+$Lin$^-$ ILC2s after treatment with various doses (horizontal axis) of recombinant IL-$33_{FL}$ or mature forms produced in rabbit reticulocyte extracts (key). Each symbol (**c**–**e**) represents an individual mouse; small horizontal lines indicate the mean (±s.e.m.). $^*P = 0.0051$ (**e**, IL-5 and IL-$33_{106-270}$); $^{**}P = 0.0002$ (**e**, IL-13 and IL-$33_{103-270}$), $^{**}P = 0.0004$ (**e**, IL-13 and IL-$33_{106-270}$) or $^{**}P = 0.0008$ (**e**, IL-5, IL-$33_{103-270}$); and $^{***}P < 0.0001$ (one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparisons test). Data are representative of one experiment with $n = 3$ mice (PBS), $n = 2$ mice (IL-$33_{103-270}$) or $n = 2$ mice (IL-$33_{106-270}$) (**a**), three experiments (**b**), five experiments (**f**) or three independent experiments (**g**) or are from three experiments with $n = 9$ mice (PBS), $n = 8$ mice (IL-$33_{103-270}$) or $n = 9$ mice (IL-$33_{106-270}$) (**c**), three experiments with $n = 7$ mice (PBS), $n = 8$ mice (IL-$33_{103-270}$) or $n = 9$ mice (IL-$33_{106-270}$) (**d**) or two experiments with $n = 5$ mice (PBS), $n = 5$ mice (IL-$33_{103-270}$) or $n = 6$ mice (IL-$33_{106-270}$) (**e**).

was active only at higher doses (>1 nM) (Fig. 2g). IL-$33_{109-270}$, a mature form that can be produced both by allergen proteases (fungal allergens, Der p 1 and subtilisin) and inflammatory proteases (cathepsin G and chymase)[27,28], exhibited a potent activity similar to that of IL-$33_{103-270}$ and IL-$33_{106-270}$. We concluded that whatever their N-terminal sequence, the cleavage products had much greater biological activity than that of IL-$33_{FL}$.

**IL-$33_{FL}$ protein functions as a biochemical sensor activated by allergen proteases.** Since cleaved IL-33, but not IL-$33_{FL}$, was active at low doses, we reasoned that IL-$33_{FL}$ might function as a sensor of allergen proteases; i.e., a biochemical sensor activated after proteolytic maturation by allergen proteases. The existence of such host-derived sensors of proteolytic activity that are cleaved by allergen proteases and, once cleaved, activate cells of the innate immune system to induce a $T_H2$ response, has been proposed previously[11]. To test our model (Fig. 3a), we analyzed the effect of allergen proteases on the biological activity of recombinant IL-$33_{FL}$ in an IL-33-dependent cellular bioassay[27,28]. When used at a low dose (<0.1 nM), human IL-$33_{FL}$ exhibited very little biological activity in the absence of allergen proteases (Fig. 3b,c). However, incubation of the same low dose of IL-$33_{FL}$ with the different allergen extracts or allergen proteases resulted in potent activation of the production of IL-5 and IL-13 by ILC2s (Fig. 3b) and secretion of IL-6 by MC/9 mouse mast cells (Fig. 3c). The induced biological activity was associated with proteolytic maturation of IL-$33_{FL}$ by the various allergen proteases, as revealed by immunoblot analysis performed in parallel with the bioassays (Fig. 3b). Together these experiments indicated that IL-$33_{FL}$ functioned as a protease sensor that detected the proteolytic activity of environmental allergens.

**Allergen proteases activate IL-$33_{FL}$ released from primary cells.** We next investigated the regulation of endogenous IL-33 by allergen extracts and allergen proteases. Endothelial cells that constitute important sources of IL-33 both in humans and mice[23,40,41] release IL-33 after mechanical stress[19,24,41]. Indeed, endogenous IL-33 was released extracellularly (Fig. 4a) as an uncleaved IL-$33_{FL}$ preform (Fig. 4b) when confluent monolayers of primary human endothelial cells were collected by scraping, a process known to induce mechanical stress by mimicking the transient sub-lethal membrane disruptions observed in endothelial cells exposed to mechanical forces in vivo[24,42]. When allergen extracts (*A. alternata* or *A. fumigatus*) or allergen proteases (papain or subtilisin) were added to cells collected by scraping, endogenous IL-$33_{FL}$ was processed into shorter mature forms in a dose-dependent manner (Fig. 4b). Functional assays revealed that the proteolytic maturation of endogenous IL-$33_{FL}$ by

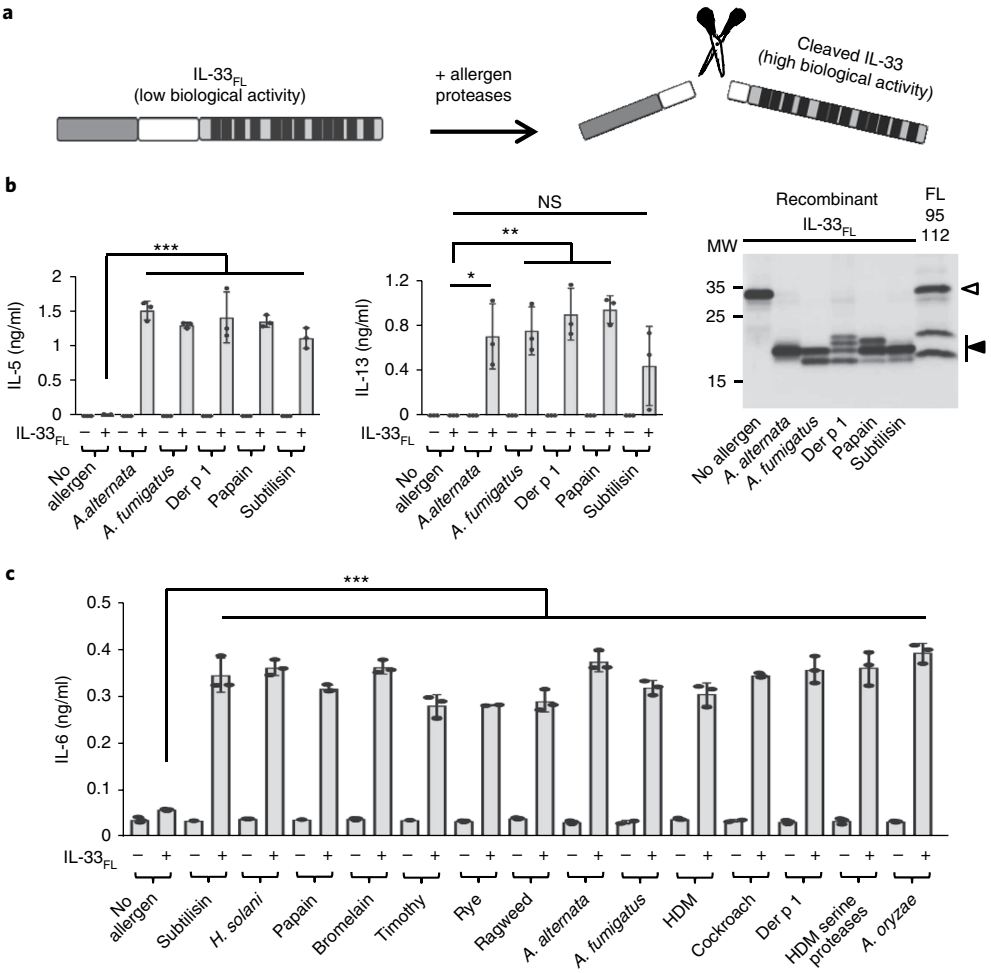


**Fig. 3 | IL-33$_{FL}$ protein functions as a biochemical sensor for allergen proteases. a**, Proposed mode of action for IL-33$_{FL}$ as a sensor of allergen proteases activated through proteolytic maturation. **b**,**c**, Concentration of IL-5 and IL-13 secreted by lung ILC2s (**b**, left and middle) and of IL-6 secreted by MC/9 mast cells (**c**) after treatment with recombinant human IL-33$_{FL}$ that had been incubated for 1h at 37 °C with no allergen or with a fixed amount of allergen extracts of (horizontal axes) *A. alternata* (500 ng (**b**) or 125 ng (**c**)), *A. fumigatus* (250 ng (**b**) or 125 ng (**c**)), *H. solari* (500 ng (**c**)), timothy (1.25 μg (**c**)), rye (2.5 μg (**c**)), ragweed (1.25 μg (**c**)), HDM (250 ng (**c**)) or cockroach (500 ng ng (**c**)) or the allergen proteases Der p 1 (1 μg (**b**) or 250 ng (**c**)), papain (62 ng (**b**) or 31 ng (**c**)), subtilisin (10 ng (**b**) or 6 ng (**c**)), bromelain (31 ng (**c**)), HDM serine proteases (500 ng (**c**)) or *A. oryzae* protease (~0.2 U (**c**)), assessing the bioactivity of recombinant IL-33$_{FL}$, and immunoblot analysis of the cleavage of IL-33$_{FL}$ in the ILC2 bioassay at left (**b**, right; far right lane and arrowheads, as in Fig. 1a; cropped image). Each symbol (**b**,**c**) represents an individual biological replicate. NS, not significant ($P = 0.1311$) (**b**, IL-13 and subtilisin); $^{*}P = 0.0117$ (**b**, IL-13 and *A. alternata*); $^{**}P = 0.0073$ (**b**, IL-13 and *A. fumigatus*), $^{**}P = 0.0019$ (**b**, IL-13 and Der P 1), $^{**}P = 0.0013$ (**b**, IL-13 and papain); and $^{***}P < 0.0001$ (**b**, IL-5; **c**) (one-way ANOVA followed by Dunnett's multiple-comparisons test). Data are representative of two independent experiments with $n = 3$ cultures (**b**, left and middle, **c**; mean ± s.d.) or two independent experiments with similar results (**b**, right).

allergen proteases resulted in induction of the production of IL-6 and IL-13 by MC/9 mast cells (Fig. 4c). We concluded that, similar to recombinant IL-33$_{FL}$, endogenous IL-33$_{FL}$ released from IL-33-producing cells after mechanical stress was cleaved and activated by allergen proteases and allergen-associated proteolytic activity.

We then assessed the ability of allergens to induce the release of endogenous IL-33 from endothelial monolayers in the absence of scraping. We found that *A. alternata* was a potent inducer of IL-33 release (Fig. 5a). Alterations to the endothelial cell monolayers and the presence of trypan blue–positive cells suggested that the release of IL-33 was associated with membrane damage (data not shown). *A. alternata*–induced release of IL-33 was prevented when the allergen extracts were pre-incubated with AEBSF (Fig. 5b), an inhibitor of such release in vivo[33]. Precipitation assays of cell supernatants with soluble IL-33 receptor (ST2-Fc) revealed that both IL-33$_{FL}$ and cleaved IL-33 were detected extracellularly after exposure of intact endothelial monolayers to extracts of *A. alternata* (Fig. 5c). In contrast, uncleaved IL-33$_{FL}$ was the only form of endogenous IL-33 detected extracellularly after cell scraping (Fig. 5c) or after exposure of intact endothelial monolayers to allergen-derived PLA2 (Fig. 5d) or chitin (Fig. 5e), two allergen compounds with no proteolytic activity[8,9]. Experiments with antioxidant glutathione revealed an important role for oxidative stress in the release of IL-33 from primary human cells induced by either *A. alternata* or allergen-derived PLA2 (Supplementary Fig. 3a,b). Together these results indicated that endogenous IL-33 was released from IL-33-producing cells as an uncleaved IL-33$_{FL}$ preform that was not processed extracellularly when the inducer of its release did not contain protease activity (i.e., mechanical stress or non-protease allergens). Furthermore, cleaved IL-33 (in addition to IL-33$_{FL}$) was detected extracellularly only when the allergen exhibited protease activity (i.e., *A. alternata*).

We next studied the kinetics of the *A. alternata*–induced release and cleavage of IL-33 over a 1-hour period. Precipitation assays of cell supernatants with ST2-Fc revealed a two-step mechanism after

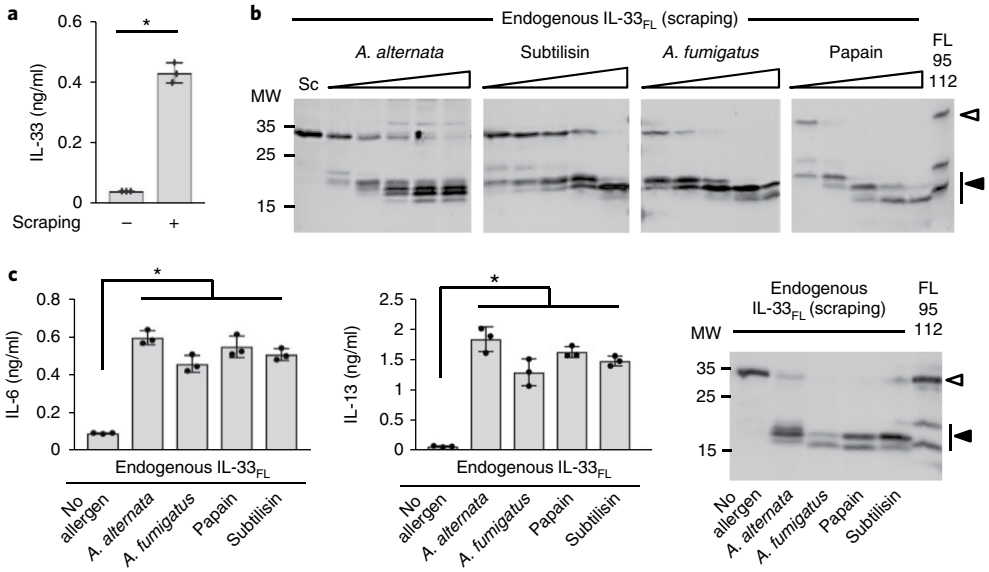


**Fig. 4 | Allergen proteases cleave and activate extracellular IL-33$_{FL}$ released from IL-33-producing primary cells. a**, ELISA of IL-33 in supernatants of primary human endothelial cells collected by scraping (+) or not (−). **b**, Immunoblot analysis of the cleavage of endogenous IL-33$_{FL}$ in cells collected by scraping and incubated for 1 h at 37 °C without allergens (Sc) or with increasing doses (wedges) of *A. alternata* (10–160 μg/ml), subtilisin (66 ng/ml to 3.3 μg/ml), *A. fumigatus* (5–120 μg/ml) or papain (2.5–30 μg/ml) (above blots) (far right lane and arrowheads, as in Fig. 1a; cropped images). **c**, Concentration of IL-6 and IL-13 secreted by MC/9 mast cells after treatment with endogenous IL-33$_{FL}$ that had been incubated with no allergen or with the allergen proteases (horizontal axis) *A. alternata* (120 μg/ml), *A. fumigatus* (120 μg/ml), papain (15 μg/ml) or subtilisin (3.3 μg/ml) (below plots), assessing the bioactivity of endogenous IL-33$_{FL}$, and immunoblot analysis of the cleavage of endogenous IL-33$_{FL}$ during the bioassay (far right; presented as in **b**; cropped image). Each symbol (**a**,**c**) represents an individual technical replicate (**a**) or biological replicate (**c**). *$P < 0.0001$ (unpaired two-tailed Student's *t*-test (**a**) or one-way ANOVA followed by a Dunnett's multiple-comparisons test (**c**)). Data are representative of three experiments (**a**; mean ± s.d.), two independent experiments with similar results (**b**,**c**, far right) or at least two independent experiments with $n = 3$ cultures (**c**, left and middle; mean ± s.d.).

exposure to *A. alternata* (Fig. 5f): extracellular release of an endogenous uncleaved IL-33$_{FL}$ preform (step 1), followed by extracellular processing of IL-33$_{FL}$ into shorter cleavage products (step 2). The release and cleavage steps were tightly coupled, since the uncleaved IL-33$_{FL}$ preform was observed in cell supernatants at 5 min but it was already cleaved by 10 min after its release. Together with our observations obtained with non-protease allergens (Fig. 5d,e), these experiments ruled out the possibility of a role for endogenous intracellular proteases in the cleavage of IL-33$_{FL}$ following exposure to allergens. They also demonstrated that *A. alternata*–induced cleavage of endogenous IL-33$_{FL}$ occurred extracellularly, not intracellularly.

**Endogenous IL-33 is released and cleaved shortly after exposure to allergen proteases in vivo.** We then analyzed the forms of endogenous mouse IL-33 in BAL fluid after intranasal exposure to *A. alternata* allergen proteases. We found that *A. alternata* was a potent inducer of the release of IL-33 and of IL-33-dependent lung eosinophilia (Fig. 6a–c), in agreement with published observations[26,28–30,33,34]. Indeed, intranasal exposure of mice to a single high dose or three low doses of *A. alternata* resulted in the rapid release (15 min to 1 h) of IL-33 into BAL fluid (Fig. 6c and Supplementary Fig. 3c,d). The main form of IL-33 in BAL fluid following exposure to *A alternata* was reported to be uncleaved IL-33$_{FL}$ in one study[33] and cleaved IL-33 in other studies[26,34]. However, the specificity of the immunoblot signals was not confirmed through the use of IL-33-deficient (*Il33*$^{-/-}$) mice in those studies. This is an important issue because it has been shown, in a model of lung inflammation, that the main protein detected in BAL fluid by the most widely used goat antibodies to mouse IL-33 is a non-specific cross-reactive protein with a size similar to that of cleaved IL-33[27]. We thus performed precipitation assays with soluble ST2-Fc receptor, followed by immunoblot analysis with antibody to IL-33 and confirmed the data through the use of *Il33*$^{-/-}$ mice. These assays revealed that the main species of IL-33 in BAL fluid at 1 h after exposure to *A. alternata* was a cleaved form with an apparent molecular weight similar to that of the recombinant mouse protein mIL-33$_{103–266}$ (Fig. 6d). Cleaved IL-33 was not observed in wild type mice treated with PBS or *Il33*$^{-/-}$ mice treated with *A. alternata*, as expected. A smaller cleavage product and IL-33$_{FL}$ were also detected after longer exposure of the immunoblots (Fig. 6e). Cleaved IL-33 was detected in BAL fluid shortly (15 min) after exposure to allergens, but it was not observed when the *A. alternata*–induced release of IL-33 was inhibited by AEBSF[33] (Supplementary Fig. 3e,f). Exposure of mouse alveolar epithelial cells (CMT-64 cells)[34] to *A. alternata* ex vivo resulted in the generation of cleavage products of endogenous IL-33 (Fig. 6f) similar to those observed in vivo.

We then investigated the forms of endogenous mouse IL-33 found in BAL fluid after exposure to a non-protease allergen (allergen-derived PLA2). Precipitation assays with ST2-Fc showed that IL-33$_{FL}$ was the only form detected in BAL fluid 30 min after exposure of wild-type naive mice to PLA2 (Fig. 6g). As expected, no signal was detected in PBS-treated mice or *Il33*$^{-/-}$ mice exposed to PLA2. Thus, these results indicated that endogenous mouse IL-33 was released into BAL fluid as an uncleaved IL-33$_{FL}$ preform when the inducer of its release did not exhibit protease activity. These observations also revealed that IL-33$_{FL}$ released extracellularly following a single intranasal exposure to allergen-derived PLA2 was still in its full-length form in vivo after 30 min, which indicated that it was not cleaved by endogenous proteases from inflammatory cells or lung tissue cells.

Finally, we performed experiments with *A. alternata* extracts treated with alpha-1 anti-trypsin (A1AT), a major serine protease inhibitor found in serum that inhibited the cleavage of IL-33 by *A. alternata* proteases without preventing the *A. alternata*–induced

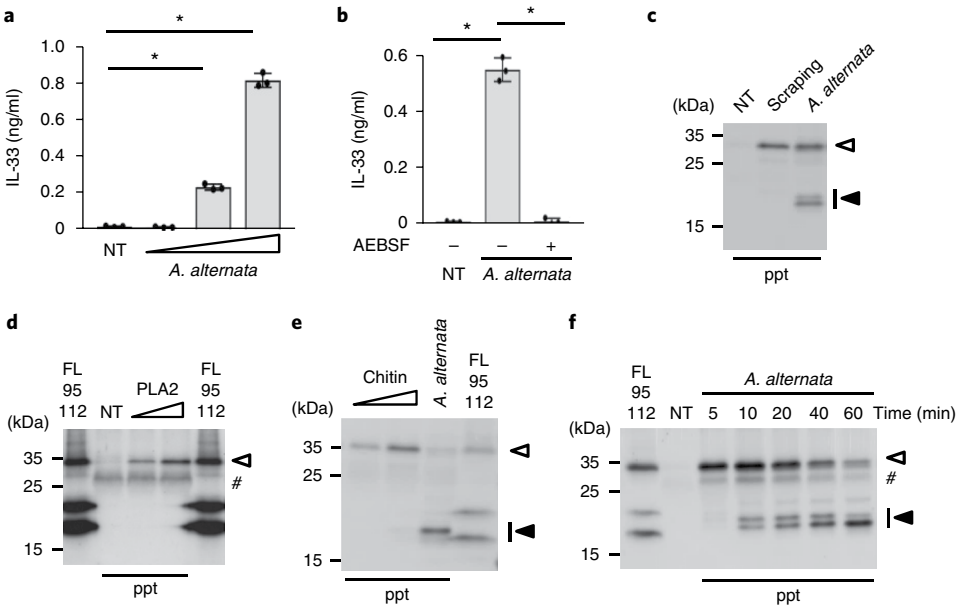


**Fig. 5 | *A. alternata* induces the release of uncleaved IL-33$_{FL}$, which is followed by rapid extracellular cleavage of IL-33. a**,**b**, Concentration of endogenous IL-33 in supernatants of intact monolayers of IL-33-producing primary human endothelial cells without treatment (NT) or at 20 min after exposure to increasing concentrations (wedge) of *A. alternata* (2–20 μg/ml) (**a**) or at 20 min after exposure to *A. alternata* extract treated with AEBSF (2 mM) (+) or not (–) (**b**). **c–f**, Precipitation (ppt) assay (with soluble ST2-Fc) followed by immunoblot analysis of various forms of IL-33 in supernatants of cells obtained 20 min after no treatment (NT), or scraping or treatment with protease-containing allergen *A. alternata* (20 μg/ml) (**c**), of intact monolayers exposed for 60 min to allergen-derived PLA2 (125 or 250 μg/ml (wedge)) (**d**) or chitin beads (5,000 or 8,500 beads per dish (wedge)) (**e**) or of cells treated for 60 min (**e**) or 5–60 min (above lanes) (**f**) with *A. alternata* or not (NT) (far left or right lane (FL, 95, 122) and arrowheads, as in Fig. 1a; #, nonspecific bands; cropped images). Each symbol (**a**,**b**) represents an individual technical replicate. *$P < 0.0001$ (one-way ANOVA followed by Dunnett's (**a**) or Tukey's (**b**) multiple-comparisons test). Data are representative of two independent experiments with $n = 3$ cultures (**a**,**b**; mean ± s.d.) or two independent experiments with similar results (**c–f**).

release of IL-33 (Supplementary Fig. 4a) and thus resulted in the extracellular accumulation of uncleaved IL-33$_{FL}$ (Supplementary Fig. 4b). *A. alternata*–induced allergic inflammation in wild type mice was significantly reduced after treatment of the allergen extracts with A1AT (Supplementary Fig. 4c). Together these results suggested that the cleavage of IL-33 by *A. alternata* proteases was important for induction of allergic airway inflammation in vivo.

**IL-33$_{FL}$ detects proteolytic activity of allergens in vivo.** Mouse IL-33$_{FL}$ (mIL-33$_{1–266}$) can be processed into shorter mature forms by allergen proteases, similar to such processing in humans. However, we noticed an important difference between the two species in the cleavage of IL-33. Incubation with increasing amounts of *A. alternata* (Supplementary Fig. 5a) or for increased periods of time (Supplementary Fig. 5b) resulted in the rapid degradation of recombinant mouse IL-33$_{FL}$ (and its cleaved forms) after cleavage, whereas recombinant human IL-33$_{FL}$ was converted into more-stable mature forms (Fig. 1a and Supplementary Fig. 5b). The human and mouse IL-33$_{FL}$ proteins were produced in exactly the same conditions, incubated with identical amounts of allergen proteases and analyzed in parallel, which indicated that the difference in the sensitivity of the two proteins to degradation was due to differences in their sequence or primary structure, rather than to other factors. We made similar observations with *A. fumigatus*, papain and subtilisin (Supplementary Fig. 5c–e), three other allergens that induced IL-33-dependent eosinophilic airway inflammation[32,36–38] (Supplementary Fig. 6). Together these data indicated that the human and mouse IL-33 proteins were regulated differently by allergen proteases.

Our results suggested that the rapid degradation of mouse IL-33 following cleavage after exposure to an allergen might not mimic perfectly the human situation. We therefore developed a humanized system to further characterize the regulation of IL-33 activity by allergen proteases in vivo. In this system, *Il33*$^{-/-}$ mice were reconstituted with recombinant human IL-33$_{FL}$ administered intranasally in the presence or absence of allergen proteases and the consequences on airway inflammation were determined. IL-33$_{FL}$ was used at a dose similar to that of native IL-33 released in BAL fluid following exposure to allergen (~1 ng; Fig. 6c and Supplementary Fig. 3d). As expected, allergen proteases administered alone did not induce eosinophilia in the lungs or BAL fluid of *Il33*$^{-/-}$ mice (Fig. 7a–c, and Supplementary Fig. 7). However, the addition of human IL-33$_{FL}$ resulted in a substantial increase in eosinophils in the lungs and BAL fluid in response to *A. alternata*, *A. fumigatus* or papain (Fig. 7a–c and Supplementary Fig. 7). At the dose used, IL-33$_{FL}$ did not induce eosinophilic airway inflammation when administered alone, which indicated that in the absence of allergen proteases, it was not activated by endogenous proteases from inflammatory cells or lung-tissue cells. Together with our ex vivo results (Fig. 3), these in vivo observations suggested that IL-33$_{FL}$ acted as an allergen protease sensor that induced airway inflammation following cleavage and activation by protease-containing allergen extracts (*A. alternata* or *A. fumigatus*) or allergen protease (papain).

**Antibody to the IL-33$_{FL}$ sensor domain inhibits allergen protease–induced airway inflammation.** To demonstrate that the increased biological activity of IL-33$_{FL}$ in the presence of allergen proteases was due to its direct cleavage rather than to other mechanisms, we generated a polyclonal antibody to the central 'sensor' domain of human IL-33. This antibody (6.68) efficiently reduced the cleavage (Fig. 8a) and activation (Fig. 8b,c) of IL-33$_{FL}$ by allergen proteases ex vivo. As expected, antibody 6.68, raised against IL-33$_{90–102}$, did not affect bioactivity of the IL-1 cytokine domain (IL-33$_{112–270}$) (Fig. 8d). We next assessed the capacity of this antibody to modulate IL-33-dependent airway inflammation in vivo in the humanized system.

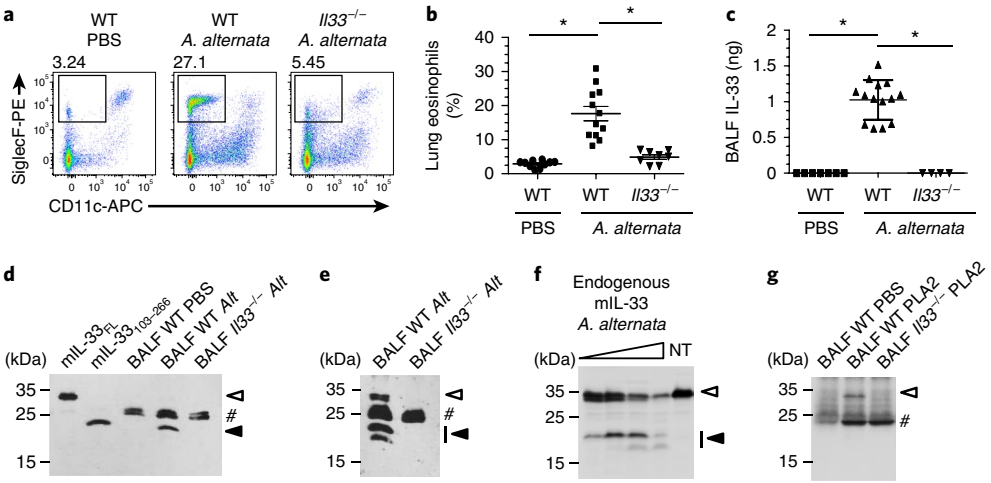


**Fig. 6 | Endogenous IL-33 is released and cleaved shortly after exposure to allergen proteases in vivo. a**, Flow cytometry of cells from the lungs of wild type (WT) or *Il33*$^{-/-}$ C57BL/6 J mice (above plots) 1 h after three intranasal injections of PBS or the protease-containing allergen *A. alternata* (12.5 μg) (above plots). Numbers above outlined areas indicate percent SiglecF$^{+}$CD11c$^{-}$ eosinophils. **b**, Frequency of SiglecF$^{+}$CD11c$^{-}$ eosinophils (among CD45$^{+}$ cells) in the lungs of mice treated as in **a** (below plots). **c**, Quantification of IL-33 in the BAL fluid of wild type and *Il33*$^{-/-}$ C57BL/6 J mice 1 h after one intranasal injection of PBS or *A. alternata* (100 μg) (below plots). **d**,**e**, Precipitation assay (with soluble ST2-Fc) followed by immunoblot analysis (with antibody to mouse IL-33) of recombinant mouse IL-33$_{FL}$ (rmIL-33$_{FL}$) or IL-33$_{103-266}$ (rmIL-33$_{103-266}$) (controls; **d**) or of BAL fluid from mice treated as in **c** (above lanes), with a short exposure (**d**) or long exposure (**e**) of the film (arrowheads, as in Fig. 1a; #, nonspecific bands; cropped images). *Alt*, *A. alternata*. **f**, Immunoblot analysis of endogenous mouse IL-33 (mIL-33) in supernatants of alveolar epithelial (CMT 64) cells collected by scraping and then not treated (NT) or incubated with increasing amounts (wedge) of *A. alternata* extract (10–160 μg/ml) (above lanes) (right margin, as in **e**; cropped image). **g**, Precipitation assay followed by immunoblot analysis of various forms of endogenous mouse IL-33 in BAL fluid of wild type and *Il33*$^{-/-}$mice 30 min after a single intranasal exposure to allergen-derived PLA2 (12.5 μg), a non-protease allergen (above lanes) (right margin, as in **e**; cropped image). Each symbol (**b**,**c**) represents an individual mouse; small horizontal lines indicate the mean (±s.e.m.). *$P < 0.0001$ (one-way ANOVA analysis followed by Tukey's multiple-comparisons test). Data are representative of three experiments (**a**), are pooled from three experiments (**b**; mice per group: $n = 10$ (WT PBS), $n = 12$ (WT *A. alternata*) or $n = 8$ (*Il33*$^{-/-}$ *A. alternata*)), are pooled from six experiments (**c**; mice per group: $n = 7$ (WT PBS,), $n = 14$ (WT *A. alternata*) or $n = 4$ (*Il33*$^{-/-}$ *A. alternata*)) or are representative of at least two independent experiments with similar results (**d**–**g**).

Treatment with antibody 6.68 reduced the accumulation of eosinophils in the lungs (Fig. 8e,f) and BAL fluid (Fig. 8g) in response to co-administered IL-33$_{FL}$ and papain. IL-5 levels in BAL fluid (Fig. 8h), airway inflammation and mucus production (Fig. 8i) were also reduced in the presence of antibody 6.68. Together these results indicated that processing of IL-33$_{FL}$ by allergen proteases was important for IL-33-dependent allergic lung inflammation.

## Discussion

Allergic inflammation is central to the cause of allergic diseases[1,2], and better understanding of how type 2 inflammation starts and is sustained could help guide new treatments for patients. Our findings have identified an important molecular mechanism for the induction of allergic inflammation based on the detection of allergen-associated proteolytic activity by the protease sensor IL-33$_{FL}$. We demonstrated that recombinant and endogenous IL-33$_{FL}$ was cleaved and activated by proteases from environmental aeroallergens central to the development of allergic asthma and by protease allergens linked to occupational asthma. We propose that this IL-33-based protease-sensing mechanism might have an important role in the rapid and efficient activation of ILC2s and the induction of allergic type 2 responses following exposure to allergens.

At low doses, uncleaved IL-33$_{FL}$ had no detectable activity but it became a very potent stimulator of ST2$^{+}$ target cells, such as ILC2s, after cleavage and activation by allergen proteases. IL-33$_{FL}$ thus has all the characteristics of a sensor of allergen proteases[11]. In certain situations, IL-33-producing cells (cellular sensors) that release nuclear IL-33$_{FL}$ (a biochemical sensor) extracellularly might be the first to sense the allergen proteases. In other situations, IL-33$_{FL}$ might sense the proteolytic activity of allergens directly, particularly when it is released independently of allergen proteases (i.e., PLA2- or chitin-induced release) or when it is present extracellularly because of a damaged epithelial barrier (i.e., epithelial injury due to air pollutants and/or oxidative stress). Our data indicated that IL-33$_{FL}$ is probably a critical sensor of proteolytic activities associated with various types of environmental allergens. It might work alone or together with other sensing mechanisms, such as those involved in the direct activation of innate immune cells by allergen proteases[11,13]. Mature IL-33 is a potent activator of basophils[43], and the protease sensor IL-33$_{FL}$ might be one of the 'missing links' in the allergen protease activity–basophil activation axis[11].

Kinetic analyses indicated that the *A. alternata*–induced release of IL-33 and cleavage of IL-33 occurred via a two-step mechanism: release of the uncleaved IL-33$_{FL}$ form shortly after allergen exposure (step 1), rapidly followed by extracellular cleavage into shorter mature forms (step 2). When the inducers of release did not exhibit protease activity (mechanical stress, chitin or PLA2), or when the protease activity of *A. alternata* was blocked with the serine protease inhibitor A1AT, IL-33 was released in the extracellular space as the uncleaved IL-33$_{FL}$ form and cleaved IL-33 was not detected. We made similar observations in vivo. Cleaved forms of endogenous mouse IL-33 were the main forms detected in BAL fluid after a single intranasal exposure to *A. alternata*, whereas uncleaved IL-33$_{FL}$ was the main form detected after exposure to PLA2. Notably, the latter observation indicated that IL-33$_{FL}$ released extracellularly after exposure to a non-protease allergen was not cleaved by endogenous proteases from the lung microenvironment.

Cleavage of IL-33$_{FL}$ by proteolytic activities associated with environmental allergens probably serves a critical role in the initiation of allergic inflammation by lowering the threshold for the activation of IL-33-mediated responses. During more-chronic phases of allergic inflammation and allergic disease, endogenous proteases

secreted by inflammatory cells might also contribute to this. Indeed, serine proteases from mast cells (chymase and tryptase)[28] and neutrophils (cathepsin G and elastase)[27] can cleave IL-33$_{FL}$ and generate hyperactive forms of the cytokine. However, allergen proteases might still have a dominant role in the activation of IL-33 in chronic settings. Indeed, when an allergen has intrinsic proteolytic activity, endogenous host proteases would be less relevant because direct cleavage and activation of IL-33 by allergen proteases would take place rapidly (in < 15 min). In contrast, when an allergen does not have associated proteolytic activity or during viral or bacterial infection, endogenous proteases from mast cells and neutrophils, respectively, might have important roles in the cleavage and activation of IL-33 in the airways.

A loss-of-function mutation in *IL33* that results in ~40% less *IL33* mRNA in heterozygous subjects and lower blood eosinophil counts has been reported to protect such subjects from asthma[22]. Such genetic data suggest that small changes in IL-33 expression have a critical role in susceptibility to allergic diseases. We believe that regulation of the bioactive forms of IL-33 by proteolytic activities associated with environmental aeroallergens is probably equally important, because it could substantially increase the bioactivity of IL-33. Together with genetic factors[16,21,22] and other factors, such as alterations of the airway epithelial barrier[1,2,4], cleavage of IL-33$_{FL}$ by allergen proteases could thus contribute to the initiation and severity of asthma and allergic airway diseases. Indeed, *A. alternata*, a potent inducer of the release[26,29,30,33] and cleavage (our study here) of IL-33, has been linked to the severity of asthma and fatal exacerbation of asthma[44], as well as epidemic asthma associated with thunderstorms[45]. When used at physiological doses similar to those of endogenous IL-33 released into BAL fluid (~1 ng), IL-33$_{FL}$ was a potent sensor of *A. alternata* and other allergen proteases. Relatively high doses of allergen extracts or allergen proteases were needed to induce the release of IL-33 in vitro and IL-33-dependent responses in vivo. Such high concentrations of fungal allergen proteases are believed to be present after inhalation of fungal spores and in situ production in the airways during fungal colonization[46]. Indeed, the levels of *A. alternata* spores in the air can be very high for several days after thunderstorms in the late summer or early autumn, and dispersion of these spores has been shown to coincide with severe asthma episodes in patients that lead to hospital admissions and asthma-related deaths[44,45]. Therefore, although the amount of *A. alternata* allergens that reach the human lungs after inhalation is currently unknown and is difficult to estimate precisely, we believe that the concentrations we used in our experiments appropriately mimic the high levels of *A. alternata* allergens to which asthmatic patients are exposed during epidemic asthma[44,45]. Pollen grains and pollen particles are also released after thunderstorms or heavy rainfalls[45] and could deliver large amounts of allergen proteases locally to the lungs after inhalation. Airway epithelial cells are known to secrete endogenous protease inhibitors, such as the cysteine protease inhibitor cystatin A and the serine protease inhibitor SPINK5[47]. Large local amounts of environmental proteases might thus be needed to create the airway protease–anti-protease imbalance necessary for the cleavage and activation of IL-33 in the lungs. Other mechanisms are probably important for the regulation of IL-33's bioactivity in the lungs[26]. Our results indicated that mouse IL-33 was cleaved by allergen proteases and rapidly degraded following cleavage, whereas cleaved forms of human IL-33 accumulated. This difference might be important for the extrapolation of results from mouse models to humans. IL-33 has important roles in allergic lung inflammation in mice[16–18], but it might be even more crucial for humans. In patients suffering from asthma, IL-33 has been shown to be released into the BAL fluid 10 min after lung challenge with protease-containing allergens (HDM or pollens)[48]. Future studies should analyze the cleavage of IL-33 and IL-33 forms at such early time points after exposure.

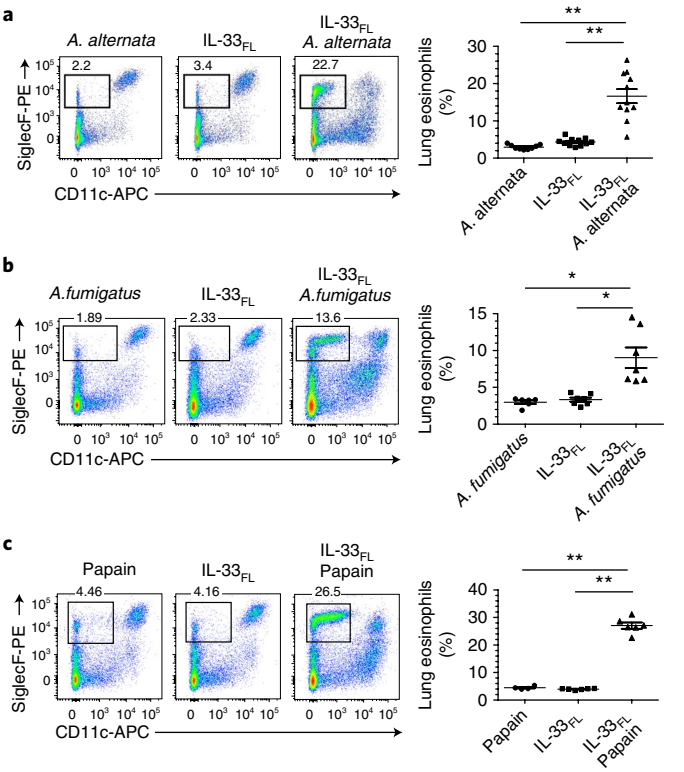


**Fig. 7 | IL-33$_{FL}$ detects proteolytic activity of allergens in vivo.** Flow cytometry of lung cells from *Il33*$^{-/-}$ mice treated intranasally with three injections of *A. alternaria* extract (12.5 μg) (**a**), *A. fumigatus* extract (50 μg) (**b**) or papain (50 μg) (**c**) or IL-33$_{FL}$ alone or together (above plots), without co-incubation of allergens and IL-33$_{FL}$ before administration (left), and frequency of SiglecF$^{+}$ CD11c$^{-}$ eosinophils (among CD45$^{+}$ cells) in the lungs of mice treated as at left (below plots) (right). Numbers above outlined areas (left) indicate percent SiglecF$^{+}$CD11c$^{-}$ eosinophils. Each symbol (right) represents an individual mouse; small horizontal lines indicate the mean (± s.e.m.). *$P$ = 0.0005 and **$P$ < 0.0001 (one-way ANOVA followed by Tukey's multiple-comparisons test). Data are pooled from three experiments (**a**; mice per group: $n$ = 9 (*A. alternata*), $n$ = 11 (IL-33$_{FL}$) or $n$ = 11 (*A. alternata* + IL-33$_{FL}$)) or two experiments (**b**,**c**; mice per group: $n$ = 6 (*A. fumigatus*), $n$ = 7 (IL-33$_{FL}$,) or $n$ = 7 (*A. fumigatus* + IL-33$_{FL}$) (**b**); or $n$ = 4 (papain), $n$ = 6 (IL-33$_{FL}$) or $n$ = 6 (papain + IL-33$_{FL}$) (**c**)).

Our data have demonstrated that a wide variety of environmental allergens and exogenous proteases from fungi, insects, plants and bacteria were able to cleave and activate IL-33$_{FL}$, suggestive of a general mechanism for the regulation of IL-33. This IL-33- and protease-based mechanism of allergen detection might act in concert with other innate sensing mechanisms for the induction of allergic responses, including pathways activated by allergen-derived PLA2[9,10] or chitin[8], and mechanisms based on the pattern-recognition receptor TLR4[5,6,12]. Indeed, whereas some allergens such as *A. alternata* and purified allergen proteases (bacterial subtilisin and plant cysteine proteases) induce IL-33-dependent, TLR4-independent allergic responses[35,37,39], other protease-containing allergens (such as HDM) induce IL-33-dependent[36] and TLR4-dependent[5,6] responses. Interestingly, (indirect) activation of TLR4 by *A. oryzae* protease has been found to initiate both allergic airway disease and antifungal immunity[12]. Protease production was shown to be essential for *Aspergillus*-dependent allergic lung disease and clearance of fungi, and it was thus proposed that allergic inflammation evolved, at least in part, for protection of the organism against fungal airway infection[46]. Fungal allergens are potent inducers of the release and cleavage of IL-33$_{FL}$, and IL-33 is thus probably an important alarm

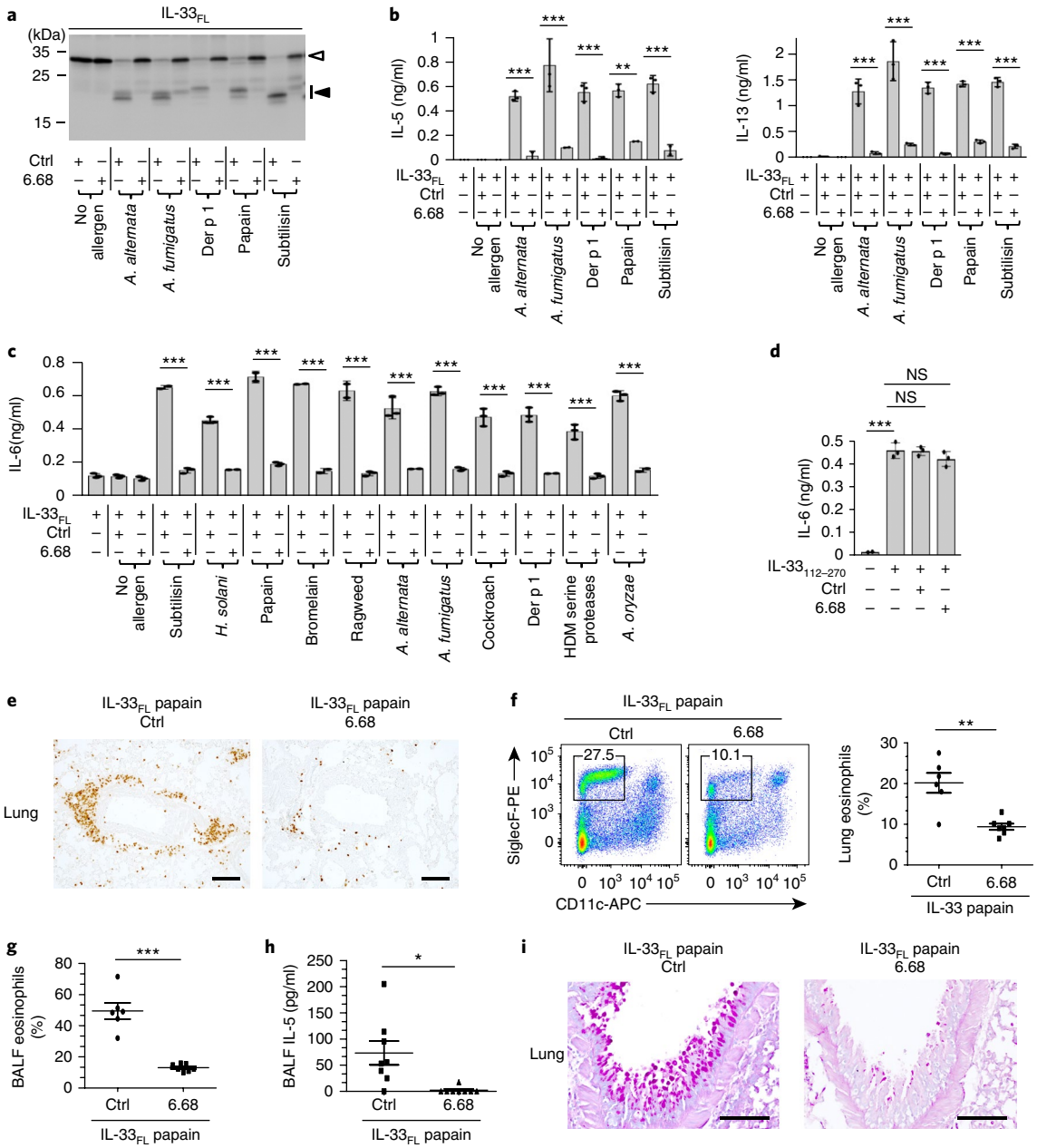


**Fig. 8 | Preventing the cleavage of IL-33$_{FL}$ reduces the activation of ILC2s and allergic airway inflammation. a**, Immunoblot analysis of the cleavage of recombinant IL-33$_{FL}$ pre-incubated with a control antibody (Ctrl) or antibody 6.68 (below lanes) and treated with no allergen or various allergens (below blot) (arrowheads, as in Fig. 1a; cropped image). **b**,**c**, Concentration of IL-5 (left) and IL-13 (right) secreted by ILC2s (**b**) and of IL-6 secreted by MC/9 mast cells (**c**), assessing the bioactivity of IL-33$_{FL}$ pre-incubated with a control antibody or antibody 6.68 (below bars) and treated with no allergen or various allergens (below plots). **d**, Concentration of IL-6 secreted by MC/9 mast cells left untreated (–) or treated with recombinant IL-33$_{112–270}$ pre-incubated with antibody as in **b**,**c** (below bars). **e**, Microscopy of the immunohistochemical staining of eosinophils (marker MBP) in the lungs of *Il33*$^{-/-}$ mice ($n=3$ per group) treated intranasally with three injections of papain (50 μg), plus IL-33$_{FL}$ pre-incubated with a control antibody or antibody 6.68 (above images). **f**,**g**, Flow cytometry of lung cells (**f**, left) and frequency of eosinophils (among CD45$^+$ cells) in the lungs (**f**, right) and BAL fluid (**g**) of mice treated as in **e** (below plots). Numbers adjacent to outlined areas (**f**, left) indicate percent SiglecF$^+$CD11c$^-$ eosinophils. **h**, Concentration of IL-5 in BAL fluid of mice treated as in **e** (below plot). **i**, Microscopy of lung sections from mice ($n=3$ per group) treated as in **e** (above images), stained with periodic acid–Schiff and hematoxylin. Scale bars (**e**,**i**), 100 μm. Each symbol (**b**–**d**,**f**–**h**) represents an individual biological replicate (**b**–**d**) or mouse (**f**–**h**); small horizontal lines (**f**–**h**) indicate the mean (±s.e.m.). NS, not significant ($P=0.999$ (**d**, IL-33$_{112–270}$ no antibody versus IL-33$_{112–270}$ Ctrl Ab) or $P=0.3911$ (**d**, IL-33$_{112–270}$ no antibody versus IL-33$_{112–270}$ 6.68); $^*P=0077$ (**h**); $^{**}P=0.0007$ (**b**) or $^{**}P=0.0009$ (**f**); and $^{***}P<0.0001$ (one-way ANOVA followed by Tukey's multiple-comparisons test (**b**–**d**) or unpaired two-tailed Student's *t*-test (**f**–**h**)). Data are representative of two independent experiments with similar results (**a**) or two independent experiments (**b**–**d**; mean ± s.d. of the following cultures: $n=3$ (Ctrl) or $n=2$ (6.68) (**b**, IL-5); $n=3$ (**b**, IL-13); $n=3$ (**c**, bars 1,2,3,5,6,9,13,14, 16,17,18,19,20,22,23,24) or $n=2$ (**c**, bars 4,7,8,10,11,12,15,21,25); or $n=3$ (**d**)) or are pooled from two experiments (**f**–**i**; mice per group: **f**, $n=6$ (Ctrl) or $n=7$ (6.68); **g**, $n=6$ (Ctrl) or $n=7$ (6.68); **h**, $n=8$).

signal that detects foreign proteolytic activity associated with fungi or other pathogens. Similar to IL-33, IL-1β has been shown to be proteolytically activated by fungal proteases[49] and bacterial proteases[50]. The detection of exogenous proteases by cytokines of the IL-1 family is reminiscent of the innate immune mechanisms described in *Drosophila* that culminate in the activation of Toll following the detection of fungal proteases[14].

In conclusion, we have reported an important mechanism for the regulation of IL-33 by proteases from environmental allergens. The recurrent identification of *IL33* and *IL1RL1* as major susceptibility loci for different forms of asthma[16,21,22], as well our observation that a wide variety of environmental aeroallergens were able to cleave and activate the sensor IL-$33_{FL}$, support the proposal of a crucial role for this mechanism in asthma. Our results could thus have important therapeutic implications. Targeting the 'sensor' domain to prevent the cleavage and activation of IL-$33_{FL}$ might represent a new approach for reducing allergic responses in asthma and other allergic diseases.

## Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41590-018-0067-5.

Received: 5 December 2017; Accepted: 12 February 2018;
Published online: 19 March 2018

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## Acknowledgements

We thank members of the Girard and Burlet-Schiltz laboratories for discussions; the IPBS Anexplo, TRI Imaging and Proteomics core facilities; J. Lee (Mayo Clinic) for antibodies; E. Lefrançais for preliminary observations; J. van Meerwijk and E. Espinosa for comments on the manuscript; F. Viala for iconography; F. Amalric for advice on initial proteomic analyses; and N. Ortega for help with immunohistochemistry and animal ethics. Supported by Agence Nationale de la Recherche (A.D. and M.C.; and ANR-12-BSV3-0005-01 and ANR-16-CE15-0009-01 to J.-P.G.); the French Ministry of Research (P.S.); and Région Midi-Pyrénées, European funds (Fonds Européens de Développement Régional), Toulouse Métropole and the French Ministry of Research with the 'Investissement d'Avenir Infrastructures Nationales en Biologie et Santé program' (Proteomics French Infrastructure project ANR-10-INBS-08) (all for the IBiSA Toulouse Proteomics facility).

## Author contributions

C.C and J.-P.G. conceived of the study, supervised the work and planned experiments; C.C performed the majority of biochemical analyses and cellular assays; A.D. isolated ILC2s and performed in vivo experiments with the help of P.S.; S.R. prepared recombinant proteins and samples for mass spectrometry; M.C. and A.S. performed mass spectrometry under the supervision of O.B.-S. and A.G.d.P.; J.-P.G. wrote the manuscript with input from C.C and A.G.d. P.; and all authors discussed the results and commented on the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41590-018-0067-5.

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**Correspondence and requests for materials** should be addressed to C.C. or J.-P.G.

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## Methods

**Mice.** C57BL/6 J (wild type) mice were purchased from Charles River Laboratories. *Rag2*$^{-/-}$ mice on a C57BL/6 J background (*B6.129-Rag2*$^{tm1Fwa}$) were obtained from EMMA (European Mouse Mutant Archive). *Il33*$^{-/-}$ mice on a C57BL/6 J background (*B6-Il-33Gt*$^{IST10946B6–Tigm–Girard}$) have been previously described[51]. Mice 6–12 weeks of age were used for all experiments; all animals were age- and sex-matched, and then randomized into the different groups. The exact number of animals used in individual experiments is indicated in figure legends. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments or outcome assessment. All mice were maintained in specific-pathogen-free animal facility at IPBS and were handled according to institutional guidelines under protocols approved by the French Ministry of Research and the FRBT (C2EA-01) animal care committee (projects 00663.02 and APAFIS#3873-2016020116301837v3).

**Allergen extracts and protease allergens.** House dust mite extracts (*Dermatophagoides pteronyssinus* # B82), oriental cockroach extracts (*Blatta orientalis* # B60)and pollen extracts (Ragweed, *Ambrosia artemisiifolia* # 56; Timothy, *Phleum pratense* # 28; Rye, *Secale cereale* # 23), and culture filtrate extracts of fungi (*Alternaria alternata*, # M1; *Aspergillus fumigatus*, # M3; *Helminthosporium solani*, # M40) were obtained from Greer Laboratories. HDM serine proteases purified from *D. pteronyssinus* culture medium by benzamidine affinity chromatography, and protease from *Aspergillus oryzae* (EC 232-752-2), were obtained from Indoor Biotechnologies (# NA-DPSP-2, Natural Der p Serine Proteases) and Sigma-Aldrich (# P6110; ≥500 U/g), respectively. Purified Natural Der p 1 (EC 3.4.22.65; Indoor Biotechnologies; # NA-DP1-2), papain from papaya latex (EC 3.4.22.2; Sigma-Aldrich # P4762, ≥10 units/mg protein), bromelain from pineapple stem (EC 3.4.22.32; Sigma-Aldrich # B5144; 5–15 units/mg protein) and subtilisin A protease from *Bacillus licheniformis* (EC 3.4.21.62; Sigma-Aldrich # P5380; 7–15 units/mg solid) were used as sources of purified protease allergens. All allergen extracts and protease allergens were reconstituted in PBS (1 mg/ml) and stored at −80 °C, excepted ragweed, timothy and rye (10 mg/ml; −80 °C), and *A. oryzae* protease (500 U/g; 4 °C).

Allergen-derived phospholipase $A_2$ (PLA2)[9] isolated from bee venom (Sigma) was reconstituted in PBS at 2 mg/ml. Pure chitin beads (New England Biolabs), ranging in size from 50 µm to 70 µm in diameter were prepared by size filtration, washed and resuspended at a concentration of $1 \times 10^5$ chitin beads per ml, as described previously[8].

**Plasmid construction and production of recombinant proteins.** cDNAs encoding human IL-$33_{FL}$ and deletion mutants IL-$33_{95–270}$, IL-$33_{103–270}$, IL-$33_{106–270}$, IL-$33_{109–270}$ and IL-$33_{112–270}$, or mouse IL-$33_{FL}$ and deletion mutants IL-$33_{102–266}$ and IL-$33_{109–266}$, were amplified by PCR with human *IL33* cDNA (NM_033439.3) or mouse *Il33* cDNA (NM_133775.2), respectively, as the template. The PCR fragments thus obtained were cloned into plasmid pcDNA3.1 (Invitrogen). All primer sequences are available upon request. Recombinant IL-$33_{FL}$ protein and IL-33 deletion mutants were produced in vitro in rabbit reticulocyte lysate (RRL) using the TNT T7-coupled Transcription/Translation system (Promega # L4610) according to the manufacturer's instructions. The different IL-33 recombinant forms produced in RRL were quantified by immunoblot analysis and the ChemiDoc MP Imaging system (Biorad). Recombinant human IL-$33_{103–270}$, IL-$33_{106–270}$ and IL-$33_{109–270}$ and mouse IL-$33_{109–266}$ were also produced in *Escherichia coli*. cDNA was subcloned into expression vector pET-15b (Novagen) and proteins were produced in *E. coli* BL21pLysS (Novagen) and were purified using Ni-NTA agarose column (Qiagen) as previously described[27,28]. Recombinant human IL-$33_{103–270}$ and IL-$33_{106–270}$ proteins produced in *E. coli* were used for in vivo analyses after intraperitoneal injection (in microgram amounts). Recombinant human IL-$33_{FL}$ protein could not be produced in *E. coli* because the protein is insoluble and lacks bioactivity when produced *in E. coli*. IL-$33_{FL}$ was thus instead produced in the RRL system (in nanogram amounts) and was compared with mature forms produced in the same RRL system.

**Mammalian cell culture.** Primary human umbilical vein endothelial cells (PraXcell; Promocell) were grown on gelatin-coated Petri dishes (0.2% gelatin, Sigma) in endothelial cell growth medium (ECGM2, Promocell) supplemented with 20% FCS. Mouse CMT 64 lung epithelial cells (isolated from a primary alveogenic lung carcinoma; European Collection of Authenticated Cell Cultures, ECACC 10032301; Sigma-Aldrich) and MC/9 mast cells (ATCC CRL-8306) were grown in Dulbecco's Modified Eagle's Medium (ATCC # 302002) with 10% FBS, supplemented with 10% Rat T-STIM with con A (Corning) for MC/9 cells.

**Flow cytometry.** Lung cell suspension was obtained by lung digestion with 2 mg/ml collagenase D and 0.1 mg/ml DNAse I for 60 min at 37 °C and mashed through a 70-µm cell strainer. Red blood cells were lysed after digestion by applying ACK (ammonium-chloride-potassium) lysing buffer for 2 min. Total cells in the lungs and BAL fluid were counted with a hemocytometer. Single-cell suspensions were stained with combinations of antibodies to the following: CD45 (clone 30F11, BD Biosciences), SiglecF (clone E50-2440, BD Biosciences), CD4 (clone GK1.5, eBioscience), CD19 (clone eBio1D3, BD Biosciences), CD45R (clone RA3-6B2, BD biosciences), NK1.1 (clone PK136, eBioscience), CD3 (clone 17A2, eBioscience), CD11b (clone M1/70, eBioscience), Ter119 (clone Ter119, eBioscience), Ly-6G (clone RB6-8C5, eBioscience), FceRIa (clone MAR-1, eBioscience), CD11c (clone N418, eBioscience), CD90.2 (clone 53–2.1, BD Biosciences), Sca-1 (clone D7, eBioscience), CD25 (clone PC61.5, eBioscience) and CD127 (clone A7R34, eBioscience) and IL-33R (T1/ST2, clone DJ8, MD Biosciences). Eosinophils were characterized with the markers CD45, SiglecF and CD11c using antibodies identified above. ILC2s were identified as $CD45^+$ lineage marker–negative ($Lin^-$) cells ($CD45^+CD4^-CD19^-CD45R^-NK1.1^-CD3^-CD11b^-CD11c^-Ter119^-Ly6G^-FceRI^-$). Antibodies were diluted in FcR block (CD16/CD32 clone 2.4G2; BD biosciences). Isotype-matched control antibodies (rat IgG2a k, clone eBR2a; rat IgG1 k, clone eBRG1) were obtained from eBioscience. Single-stain control antibodies (identified above) were included. Dead cells were excluded by using the fixable viability dye eFluor506 (eBioscience, 1/1000). Samples were acquired on a LSRII flow cytometer using DiVa software (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Isolation and culture of lung ILC2s.** ILC2s were isolated from pooled lungs of IL-33-treated *Rag2*$^{-/-}$ mice as previously described[28]. In brief, $Lin^-$ $CD45^+$ cells were magnetically selected by two successive rounds of depletion of $Lin^+$ 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, StemCell Technologies), followed by positive selection with anti-mouse CD45 microbeads (Miltenyi Biotech). ILC2 phenotype was analyzed by assessing expression of CD90.2, Sca1, CD25, CD127 and IL-33R (ST2) using antibodies identified above. ILC2s were cultured in RPMI medium supplemented with 10% (vol/vol) FCS, 1% penicillin-streptomycin, 50 µM β-mercaptoethanol and 20 ng/ml recombinant mouse IL-2 (R&D Systems). Flow cytometry analysis was performed at day 4 to assess the frequency of $IL\text{-}33R^+$ ILC2s (typically > 99%). Cultured ILC2s were used at day 6 in cellular bioassays.

**Measurements of cytokines.** Cytokine levels in culture supernatants were determined using human Duoset IL-33 and mouse DuoSet IL-5, IL-6 and IL-13 ELISA, according to the manufacturer's instructions (R&D Systems), except that Nessy-1 biotin conjugate (Enzo Life Sciences) was used for detection of human IL-33. The concentrations of IL-5, IL-13 and IL-33 in BAL fluid were analyzed by mouse IL-5 ELISA MAX Deluxe (Biolegend) and mouse IL-13 and IL-33 Quantikine ELISA (R&D Systems).

**Generation of antibody to the IL-33 central domain.** The affinity-purified rabbit polyclonal antibody 6.68 was raised against peptide TVECFAFGISGVQ in the central domain of IL-33, corresponding to amino acids 90–102 of the human sequence (Eurogentec, Seraing, Belgium). Endotoxin levels were < 0.04 EU/µg of protein, as determined by the *Limulus* amebocyte lysate QCL-1000 method (Lonza). Low-endotoxin rabbit polyclonal antibodies (Abcam # ab176094) were used as a control.

**Cleavage of recombinant IL-$33_{FL}$ with allergen extracts and allergen proteases.** Recombinant human IL-$33_{FL}$ protein (2.5 µl RRL lysate) was incubated with increasing amounts of allergen extracts (up to 1 µg for all allergens, excepted pollens, up to 10 µg) or allergen proteases (125 ng to 1 µg for Der p 1 and HDM serine proteases, 8–125 ng for papain and bromelain, and 1.5–25 ng for subtilisin) in 15 µl $PBS^{2+}$ (PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) for 1 h at 37 °C. In some experiments, allergen extracts or protease allergens were pre-incubated (15 min, 37 °C) with cysteine protease inhibitor E64 (50 µM; Sigma), serine protease inhibitor AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; 5 mM; Sigma) or EDTA (Sigma) before incubation with human IL-$33_{FL}$ protein for 1 h at 37 °C. In other experiments, human IL-$33_{FL}$ protein was pre-incubated with 2 µg 6.68 or control antibody (20 min, 37 °C) before incubation with allergen extracts or protease allergens for 1 h at 37 °C. For kinetic studies, recombinant human or mouse IL-$33_{FL}$ proteins (~2.5 ng in 2.5 µl RRL lysate) were incubated with a fixed amount of allergen extracts (500 ng for *A. alternata*, 250 ng for *A. fumigatus*) or protease allergens (125 ng for papain; 8 ng for subtilisin) in 15 µl assay buffer for up to 1 h at 37 °C. Cleavage products were analyzed by SDS-PAGE and immunoblot.

**Cleavage of endogenous IL-33 with allergen extracts and protease allergens.** Primary human endothelial cells (HUVECs) and mouse lung epithelial cells (CMT 64) were selected for these studies because blood vessel endothelium and lung epithelium constitute major sites of IL-33 expression in vivo[23,51]. Cells from confluent monolayers grown in 15-cm Petri dishes were collected by scraping into 2 ml $PBS^{2+}$, were centrifuged 5 min at 260 *g* and were exposed to increasing amounts of allergen extracts (up to 16 µg for *A. alternata* and 12 µg for *A. fumigatus*; ~$3 \times 10^6$ cells) or protease allergens (up to 3 µg for papain and 330 ng for subtilisin; ~$3 \times 10^6$ cells) in 100 µl $PBS^{2+}$ for 1 h at 37 °C. Cells were centrifuged at 5,000 r.p.m., and supernatants were collected for analysis by SDS-PAGE and immunoblot.

**Immunoblot analysis.** Proteins were fractionated by SDS-PAGE, electroblotted and detected with mAb to human IL-33-Cter (305B, 0.5 µg/ml, Cayman Chemical)

or goat antiserum to mouse IL-33-Cter (AF3626, 0.5 µg/ml, R&D), followed by HRP-conjugated goat anti-mouse (V4021, 1/10000; Promega) or donkey anti-goat polyclonal antibodies (V8051, 1/10000; Promega), and finally an enhanced chemiluminescence kit (Amersham ECL Prime; GE Healthcare). ChemiDoc MP Imaging system and Image Lab software (Biorad) were used for imaging immunoblots and quantifying the different IL-33 recombinant forms used in cellular bioassays and in vivo experiments. A standard curve was performed using purified recombinant human IL-$33_{109–270}$ or recombinant mouse IL-$33_{109–266}$. Full scans of immunoblot experiments are provided in Supplementary Fig. 8.

**Sample preparation for identification of cleavage sites by mass spectrometry.** Recombinant human IL-$33_{FL}$ protein was either left untreated (as a control) or incubated with allergen extracts or allergen proteases for 1 h at 37 °C. Cleavage products were analyzed by one-dimensional SDS-PAGE. Processed fragments were excised from the gels and further digested in-gel using a specific proteolytic enzyme to map the central domain of the protein and identify neo N-terminal peptides resulting from allergen cleavage in this region. To confidently identify the neo N-terminal peptide for each allergen-processed IL-33 fragment, a comparative mapping was performed with IL-$33_{FL}$ isolated from the control gel lane. Proteins were reduced in-gel with 25 mM of DTT and were alkylated with 100 mM of chloroacetamide, then were digested by the addition of either 0.6 µg trypsin (Promega) or 1.5 µg V8 endoproteinase Glu-C (endoGlu-C, Promega) in 50 mM ammonium bicarbonate, followed by incubation at 37 °C overnight. Peptides were extracted from the gel by incubation in 50 mM ammonium bicarbonate for 15 min at 37 °C, followed by two incubations in 10% formic acid:acetonitrile (1:1) for 15 min at 37 °C. The resulting peptides were dried in a SpeedVac, and resuspended in 5% acetonitrile and 0.05% TFA for nanoLC-MS/MS analysis.

**NanoLC-MS/MS analysis.** Peptides were analyzed by nanoLC-MS/MS using an UltiMate 3000 RSLCnano system (Dionex) coupled to an Orbitrap Fusion mass spectrometer (ThermoScientific). Peptides were separated on a C-18 column (75 µm inner diameter × 50 cm; packed in-house with Reprosil C18) equilibrated in 95% solvent A (5% acetonitrile and 0.2% formic acid) and 5% solvent B (80% acetonitrile and 0.2% formic acid), using a 5–50% gradient of solvent B in 105 min at 300 nl/min flow rate. The mass spectrometer was operated in data-dependent acquisition mode with the XCalibur software. MS survey scans were acquired in the Orbitrap with a resolution of 120,000. Peptides ions were automatically selected and sequenced either by CID (collision-induced dissociation) in the linear ion trap or by HCD (higher-energy collisional dissociation) in the c-trap with analysis of the fragments in the Orbitrap with a resolution of 30,000.

**Protein identification and quantification.** Raw MS files converted to the mzdb format and were processed with the mzdb-access library (https://github.com/mzdb) to generate peaklists. Data was searched with Mascot (version 2.5.2) against a custom-made database containing all rabbit entries from the UniProtKB database (Swiss-Prot/TrEmbl release 2014-11-06, Oryctolagus cuniculus taxonomy, 22920 entries), plus human $IL33_{FL}$ sequence, as well as C-terminal truncated forms of human IL33 resulting from allergen cleavage in the central domain of the protein at all possible amino acids between Thr64 and Thr114. The search included methionine oxidation as a variable modification, and carbamidomethylation of cysteine as a fixed modification. Specificity of digestion was set for cleavage after lysine or arginine for trypsin-digested samples, and after glutamic acid and aspartic acid (except when followed by proline) for endoGlu-C-digested samples, and two missed cleavages were allowed. The mass tolerance was set to 10 ppm for the precursor ion. It was set to 0.8 Da for fragment ions in CID mode (detection in the ion trap) and 20 mmu in HCD mode (detection in the orbitrap). Validation of identifications was performed through a false-discovery rate set to 1% at protein and peptide-sequence match level, determined by target-decoy search using the in-house-developed software Proline (http://proline.profiproteomics.fr/). Raw MS signal extraction of identified neo N-terminal peptides was performed with Proline across samples treated with different allergens. The mass spectrometry proteomics data have been deposited to the ProteomeXchange[52] Consortium via the PRIDE partner repository[53] with the dataset identifier PXD006081.

**Cellular bioassays for measurement of IL-33 activity.** Mouse ILC2s and MC/9 mast cells were used for cellular bioassays[27,28], since human IL-33 activates mouse target cells as effectively as mouse IL-33 does[18]. For dose–response studies, increasing doses ($5 \times 10^{-4}$ nM to 2 nM) of recombinant human IL-$33_{FL}$ or mature forms IL-$33_{103–270}$, IL-$33_{106–270}$ and IL-$33_{109–270}$ were used to stimulate cultured ILC2s ($5 \times 10^4$ cells per well in 96-well plates; 14 h treatment). For activation assays, recombinant human IL-$33_{FL}$ protein (2.5 µl RRL lysate) or control (2.5 µl 'unprogrammed' RRL lysate; '–' in Fig. 3b,c) was incubated with a fixed amount of allergen extracts (*A. alternata*, 500 ng or 125 ng; *A. fumigatus*, 250 ng or 125 ng; *H. solani*, 500 ng; timothy, 1.25 µg; rye, 2.5 µg; ragweed,.25 mg; HDM, 250 ng; cockroach, 500 ng) or allergen proteases (Der p 1, 1 µg, or 250 ng; papain, 62 ng or 31 ng; subtilisin, 10 ng or 6 ng; bromelain, 31 ng; HDM serine proteases, 500 ng; *A. oryzae* protease, ~0.2 U) in 15 µl $PBS^{2+}$ for up to 1 h at 37 °C. In some experiments, human IL-$33_{FL}$ protein was pre-incubated with the antibody 6.68 or control antibody (2 µg, 30 min, 37 °C) before incubation with allergen extracts or protease allergens. For activation assays with endogenous native IL-33, confluent primary human endothelial cells were collected by scrapping into $PBS^{2+}$, were centrifuged 5 min at 260 *g* and were exposed to allergen extracts (12 µg for *A. alternata* or 12 µg for *A. fumigatus*; ~$3 \times 10^6$ cells) or protease allergens (1.5 µg for papain or 330 ng for subtilisin; ~$3 \times 10^6$ cells) in 100 µl $PBS^{2+}$ for 40 min at 37 °C. The cleavage mixture was diluted in PBS (1/25 for recombinant IL-$33_{FL}$; 1/32 for endogenous IL-33) and 6–24 µl per well was used to stimulate ILC2s ($5 \times 10^4$ cells per well in 96-well plates) or IL-33-responsive MC/9 mast cells ($1 \times 10^5$ to $4 \times 10^5$ cells per well in 96-well plates) for 1 h at 37 °C. Cells were washed and further incubated for 5 h at 37 °C, and supernatants were collected for ELISA (IL-5 and IL-13 for ILC2s; IL-6 and IL-13 for MC/9 cells). The cleavage mixture was analyzed in parallel by immunoblot to verify cleavage of IL-33 by allergen extracts or protease allergens. The specificity of the bioassay for endogenous native IL-33 was validated using a blocking antibody to IL-33R (ST2) (DJ8, MD Biosciences).

**Analysis of allergen-induced IL-33 release in primary cells.** Allergen-induced release of endogenous IL-$33_{FL}$ was analyzed using IL-33-producing primary human endothelial cells grown in complete endothelial cell growth medium 2 (ECGM2, Promocell). Confluent monolayers in 10 cm Petri dishes (~$3 \times 10^6$ cells per dish) were exposed to *A. alternata* extracts (20 µg/ml), allergen-derived PLA2 (125 µg/ml or 250 µg/ml) or chitin beads (5,000 or 8,500 beads per dish) in serum-free ECGM2 on a rocking platform for 1 h at 37 °C. In some experiments, cells were pre-incubated with antioxidant glutathione (GSH, 10 or 20 mM, Sigma) for 40 min at 37 °C before exposure to *A. alternata* extracts (20 µg/ml) or were exposed to *A. alternata* extracts (20 µg/ml) pre-treated with the serine protease inhibitor alpha-1 anti-trypsin (A1AT, 10 or 20 µg/ml, Sigma) for 30 min at 37 °C. For kinetic studies, IL-33-producing cells were exposed to *A. alternata* extracts (20 µg/ml) for 5, 10, 20, 40 or 60 min at 37 °C. Release of endogenous IL-33 into cell supernatants after exposure to the different stimuli was analyzed by ELISA and precipitation assays with human ST2-Fc (R&D). Protease activity in cell supernatants was blocked with 10% FCS. Protein G sepharose beads (GE healthcare, 60 µl) were coated with 1 µg of ST2-Fc overnight at 4 °C in 1 ml PBS containing protease inhibitor cocktail (Roche), 2 mM AEBSF and 20 µM E64, were washed with PBS and then were blocked with 1% BSA for 1 h. Cell supernatants were incubated with ST2-Fc–protein G sepharose beads for 2 h at room temperature in the presence of protease inhibitor cocktail (Roche), 2 mM AEBSF, 20 µM E64 and 10% FCS. After washing, the precipitates were eluted in 30 µl Laemmli buffer and separated by 12% SDS-PAGE to identify endogenous IL-33 forms by immunoblot analysis with monoclonal antibodies B-L33 and B-S33 to human IL-33 (2 µg/ml, Diaclone) and Nessy-1 (1 µg/ml, Enzo Life Sciences). HRP-conjugated goat polyclonal antibodies to mouse (W4021, 1/10000; Promega) were used for detection. Recombinant human IL-$33_{FL}$, IL-$33_{95–270}$ and IL-$33_{112–270}$ were used as controls in the precipitation–immunoblot analyses. Precipitation assays with ST2-Fc were used to analyze the different IL-33 forms, but not for quantitative comparisons, because the amount of IL-$33_{FL}$ could be underestimated due to presumably lower affinity for ST2.

**Induction of allergic airway inflammation.** C57BL/6 J wild type and *Il33*$^{-/-}$ mice were anesthetized by isoflurane inhalation and were treated intranasally with *A. alternata* (12.5 µg), *A. fumigatus* (50 µg), papain (50 µg) or subtilisin (5 µg), in 50 µl PBS, every day for 3 d. In the humanized system, *Il33*$^{-/-}$ mice were treated intranasally with allergen extracts or protease allergens, with recombinant human IL-$33_{FL}$ (0.83 ng, produced in RRL) or with recombinant human IL-$33_{FL}$ alone (0.83 ng), once per day for 3 d. Allergens and IL-$33_{FL}$ were not co-incubated before administration. As a control, 'un-programmed' RRL, instead of recombinant IL-$33_{FL}$, was added to allergen extracts or protease allergens. For blocking experiments, *IL-33*$^{-/-}$ mice were treated intranasally with papain (50 µg), co-administrated with recombinant human IL-$33_{FL}$ (0.83 ng) pre-incubated with 1 µg of 6.68 or control antibody, in 50 µl PBS, every day for 3 d. The effects of human IL-$33_{103–270}$ and IL-$33_{106–270}$ on airway inflammation were analyzed after repeated intraperitoneal administrations over a period of 7 days, of recombinant proteins produced in *E. coli*, as previously described[28]. Lungs and BAL fluid were collected 1 d after the final intranasal or intraperitoneal treatment for flow cytometry, ELISA and histological analyses.

**Analysis of IL-33 forms in BAL fluid.** C57BL/6 J wild type and *Il33*$^{-/-}$ mice were anesthetized by isoflurane inhalation and were treated intranasally with PBS, *A. alternata* (100 µg) or allergen-derived PLA2 (12.5 µg) in 50 µl PBS. In some experiments, the *A. alternata* extract (100 µg) was pre-incubated with AEBSF, an inhibitor of IL-33 release[33], for 30 min before intranasal administration. BAL fluid was collected at selected time points after challenge (15 min, 30 min or 1 h) by lavage with PBS (0.5 ml plus 1 ml) containing protease inhibitor cocktail (Roche), 2 mM AEBSF and 20 µM E64. IL-33 concentration was determined by ELISA using 50 µl of BAL fluid. For precipitation assays, protein G sepharose beads (GE healthcare, 60 µl) were coated with 1 µg of soluble IL-33 receptor (mouse ST2-Fc chimera protein; produced in-house) in 1 ml PBS containing protease inhibitor cocktail (Roche), 2 mM AEBSF and 20 µM E64, overnight at 4 °C, were washed with PBS, and then were blocked with 1% BSA for 1 h. BAL fluid was incubated with ST2-Fc-protein G Sepharose beads for 3 h at 4 °C or 2 h at room temperature

in the presence of 10% FCS. After washing, the precipitates were eluted in 30 µl Laemmli buffer and were separated by 12% SDS-PAGE to identify endogenous IL-33 forms by immunoblot analysis with goat antiserum to mouse IL-33-Cter (AF3626, R&D). Goat TrueBlot: antibody to goat IgG HRP (18-8814-33, 1/10000; Rockland Immunochemicals) or HRP-conjugated donkey polyclonal antibody to goat (V8051, 1/10000; Promega) was used for detection. Recombinant mouse IL-$33_{FL}$ and IL-$33_{102-266}$ were used as controls in the precipitation–immunoblot analyses.

**Histological analysis.** Lungs were fixed in paraformaldehyde 4% overnight at 4 °C. 5-µm paraffin-embedded sections were prepared and stained with periodic acid–Schiff and hematoxylin for analysis of mucus production and cellular inflammation. Immunohistochemical detection of eosinophils was performed with a rat monoclonal antibody to MBP (a gift from J Lee, Mayo Clinic) diluted 1/100 in MAXblock blocking medium (Active Motif). After overnight incubation at 4 °C, sections were washed in PBS for 30 min before incubation with a biotinylated donkey secondary antibody to rat (712-065-150, Jackson Immunoresearch; 1/200) for 1 h at room temperature. Sections were washed with PBS for 30 min and incubated with horseradish peroxidase streptavidin (Zymed; 1/100) for 1 h at room temperature before revelation with DAB substrate (Sigma Aldrich). Images were acquired using a Zeiss AxioImagerM2 microscope coupled to a color Axiocam 503 camera and were processed through Zen software (Zeiss).

**Statistical analysis.** An unpaired two-tailed Student's *t*-test or a two-tailed Mann-Whitney test was used for comparison of independent experimental groups. For experiments containing more than two groups, one-way ANOVA followed by Tukey's test or by Dunnett's test was performed. Statistics were computed with PRISM7 software (GraphPad Software). A $P$ value of less than 0.05 was considered significant.

**Life Sciences Reporting Summary.** Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

**Code availability.** The software Proline (open-source code) developed in-house is freely available at http://www.profiproteomics.fr/proline.

**Data availability.** Mass spectrometry data have been deposited in the ProteomeXchange Consortium repository via PRIDE (https://www.ebi.ac.uk/pride/archive/) with accession code PXD006081. Full scans of immunoblot experiments are provided in Supplementary Fig. 8. Other data that support the findings of this study are available from the corresponding author upon request.

## References

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Corresponding author(s): Jean-Philippe GIRARD

# Life Sciences Reporting Summary

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## Experimental design

1. Sample size

   Describe how sample size was determined.

   For mouse experiments, sample sizes were chosen based on the basis of our previous publications without prior power analysis. Exact numbers of animals used in individual experiments are indicated in figure legends.

2. Data exclusions

   Describe any data exclusions.

   No data were excluded.

3. Replication

   Describe the measures taken to verify the reproducibility of the experimental findings.

   All experiments were reliably reproduced and results are presented as mean +/- SEM or +/- SD as indicated in figure legends. The results presented have been successfully replicated in at least two independent experiments. We have carefully reported the experimental conditions in the Online Methods and indicated precisely the nature of replicates in Figure legends.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   All mice were age and sex-matched and then randomized into the different groups.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   The investigators were not blinded to group allocation during experiments. The experimental observations would be consistent irrespective of blinding. Conclusions were made based on quantitative parameters and statistical significance of the data.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|---|---|
| ☐ | ☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☐ | ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ A statement indicating how many times each experiment was replicated |
| ☐ | ☒ The statistical test(s) used and whether they are one- or two-sided<br>*Only common tests should be described solely by name; describe more complex techniques in the Methods section.* |
| ☐ | ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☐ | ☒ Test values indicating whether an effect is present<br>*Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.* |
| ☐ | ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☐ | ☒ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation) |

*See the web collection on statistics for biologists for further resources and guidance.*

## Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Prism version 7 software (GraphPad) was used to generate graphs and perform statistical analyses
FlowJo software (Tree Star) version10.2 for FACS analyses
ImageLab 5.1 software (Biorad) for western blot analyses
XCalibur (Thermo Scientific)/ Mascot (Matrix Science)/ Proline (in house developed software - see Mat&Med) softwares for proteomic analyses
Zen software (Zeiss) for histological analyses

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

## Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All unique materials are readily available from the authors

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

CD45 (Clone 30F11, 1/1000), SiglecF (Clone E50-2440, 1/200), CD4 (clone GK1.5, 1/2000), CD19 (clone eBio1D3, 1/2000), CD45R (clone RA3-6B2, 1/1000), NK1.1 (clone PK136, 1/300), CD3 (clone 17A2, 1/600), CD11b (clone M1/70, 1/100), Ter119 (clone Ter119, 1/100), Ly-6G (clone RB6-8C5, 1/100), FceRIa (clone MAR-1, 1/100), CD11c (clone N418, 1/300), CD90.2 (clone 53 2.1, 1/600), Sca-1 (clone D7, 1/300), CD25 (clone PC61.5, 1/300), CD127 (clone A7R34, 1/100) were provided by eBioscience, BD biosciences and Biolegend. IL-33R (T1/ST2, clone DJ8, 1/100) was provided by MD Biosciences. Rat IgG2a κ (clone eBR2a, 1/300) and Rat IgG1 κ (clone eBRG1, 1/300) isotype controls were obtained from eBioscience. All these antibodies were validated by manufacturers and largely described in the litterature.
Fixable Viability Dye (eFluor506, 1/1000) was obtained from eBioscience.
IL-33 Abs (mouse 305B, 0.5 microgram/ml, Cayman Chemicals; mouse Nessy 1, 1 microgram/ml, Enzo Life Sciences) against human IL-33 were validated in Western Blot assays in our previous studies using siRNAs directed against human IL-33 (Cayrol and Girard, 2009, 106:9021-9026; Gautier et al., Sci Rep 2016, 6:34255).
IL-33 Abs (B-L33 and B-S33, 2 micrograms/ml, Diaclone) against human IL-33 were validated in the present study using pull down assays and western blot analyses.
IL-33 Ab (6.68; 1 or 2 micrograms in blocking experiments, Eurogentec) against the central domain of human IL-33 was validated in ELISA assays by the manufacturer.
IL-33 Ab (Goat AF3626; 0.5 microgram/ml, R&D System) against mouse IL-33 was validated in Western Blot assays in our previous studies using IL-33 KO mice (Lefrançais et al., PNAS, 2012, 109:1673).
Low endotoxin rabbit polyclonal antibodies were used as isotype controls for 6.68 Ab (1 or 2 micrograms in blocking experiments, Abcam # ab176094).
HRP-conjugated goat anti-mouse or donkey anti-goat polyclonal antibodies were used as secondary antibodies in the immunoblot analyses (1/10000; Promega).
Rat anti-MBP mAb (1/100) was obtained from J Lee (Mayo Clinic). Biotinylated donkey anti-rat antibody (Jackson Immunoresearch; 1/200) was used as a secondary antibody for immunohistochemistry.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Authentified cell lines were obtained from European Collection of Authenticated Cell cultures (ECACC 10032301 for CMT 64) and ATCC (ATCC CRL68306 for MC/9)

b. Describe the method of cell line authentication used.

Authentification was performed by ECACC for CMT 64 cell line (Method: DNA fingerprinting) and ATCC for MC/9 cell line (Method: STR profiling).

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were free of mycoplasma contamination. Testing for mycoplasma contamination was performed by ECACC for CMT 64 cell line and ATCC for MC/9 cell line.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

## Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

C57BL/6J (wild type) mice were purchased from Charles River Laboratories. Rag2-/- mice on a C57BL/6J background (B6.129-Rag2tm1Fwa) were obtained from EMMA (European mouse mutant archive). IL-33-/- mice on a C57BL/6J background have been previously generated in the team (B6-Il-33GtIST10946B6-Tigm-Girard) . Mice aged 6-12 weeks were used for all experiments; all animals were age- and sex-matched, and then randomized into the different groups. Exact numbers of animals used in individual experiments are indicated in figure legends. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment. All mice were maintained in specific pathogen free animal facility at IPBS and handled according to institutional guidelines under protocols approved by the French Ministry of Research and the FRBT (C2EA-01) animal care committee (Projects 00663.02 and APAFIS#3873-2016020116301837v3).

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.

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Corresponding author(s): Jean-Philippe GIRARD

# Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

## Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

## Methodological details

| | |
|---|---|
| 5. Describe the sample preparation. | described in Material and Methods in detail |
| 6. Identify the instrument used for data collection. | described in Material and Methods in detail |
| 7. Describe the software used to collect and analyze the flow cytometry data. | described in Material and Methods in detail |
| 8. Describe the abundance of the relevant cell populations within post-sort fractions. | NA |
| 9. Describe the gating strategy used. | described in Material and Methods and Figure 2 (main figure, not in supplementary information) |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒