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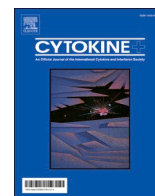
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# Interleukin-33 (IL-33): A critical review of its biology and the mechanisms involved in its release as a potent extracellular cytokine

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## ABSTRACT

Interleukin-33 (IL-33), a member of the IL-1 family, is an alarmin cytokine with crucial roles in tissue homeostasis and repair, type 2 immunity, allergic and non-allergic inflammation, viral infection, and cancer. IL-33 is abundant in the nuclei of tissue-derived cells, including endothelial cells from blood vessels, epithelial cells from barrier tissues, and fibroblastic stromal cells from various tissues. IL-33 is released upon cell damage or tissue injury and activates Myd88-dependent signaling pathways in cells expressing the ST2 (IL-1RL1) receptor. Analysis of patient samples and studies in murine models support an important role of IL-33/ST2 signaling in allergic inflammation in different tissues (lung, nasopharynx, skin) and diseases (asthma, chronic rhinosinusitis, allergic rhinitis, atopic dermatitis). *IL33* and *IL1RL1/ST2* are among the most highly replicated susceptibility loci for asthma. However, the IL-33/ST2 pathway is also important in non-allergic inflammation. Indeed, targets of IL-33 include immune cells involved in both type 2 and type 1 immunity and regulatory responses, such as group 2 innate lymphoid cells (ILC2s), mast cells, regulatory T cells (Tregs), Th2 cells, basophils, eosinophils, macrophages, dendritic cells (DCs), neutrophils, Th1 cells, CD8 T cells, NK and iNKT cells.

In the main part of this review, we discuss the basic biology of the IL-33 protein (molecular characteristics, nuclear localization, cellular sources *in vivo*), and its mechanisms of release, and bioactive forms in various contexts. Importantly, we alert the scientific community to the problems of specificity of IL-33 reagents, we explain why studies without specificity controls with IL-33-deficient cells are misleading to the field and lead to unnecessary controversy, and we make recommendations to generate reliable results. In the final part, we review the genetic and environmental regulation of IL-33 in allergic airway inflammation and asthma, and we highlight recent studies showing clinical efficacy of anti-IL-33 antibodies in asthma and chronic obstructive pulmonary disease (COPD).

## 1. Introduction

Allergic diseases such as asthma, allergic rhinitis and eczema are common diseases that affect many patients from childhood to adulthood. Indeed, according to estimates from the World Health Organization, there are currently 235 million people with asthma worldwide. While several genes have been associated with asthma in different large-scale genetic studies, the genes encoding interleukin-33 (*IL33*) and its receptor ST2 (*IL1RL1*) were among the very few genes (<5 genes) reproducibly identified as major susceptibility loci in all genome-wide association studies of asthma [1,2], supporting a critical role of the IL-33/ST2 axis in human asthma. Numerous studies in animal models and clinical data have conclusively shown the critical role of IL-33 in allergic inflammation [1,3–5]. However, the IL-33/ST2 pathway is also

important in non-allergic inflammation. Indeed, a crucial role of IL-33 in patients suffering from chronic obstructive pulmonary disease (COPD) has recently been uncovered in the clinics.

IL-33 is an alarmin cytokine, released upon tissue injury [5]. Tissue-derived immune cells, such as ILC2s, mast cells and Tregs, express the ST2 receptor constitutively and are major targets of IL-33 *in vivo* [3–5]. Furthermore, IL-33 activates additional subsets important in type 2 immunity and allergic inflammation, such as Th2 cells, basophils, eosinophils, M2 macrophages, and DCs [3–5]. Other immune cells, including NK, iNKT cells and neutrophils, express ST2 constitutively, and its levels further increase upon stimulation [6–8]. In other cell types, ST2 is inducible and dynamically expressed depending on the tissue microenvironment. For instance, IL-12 induces the ST2 receptor on Th1 cells and CD8 T cells, and IL-33 plays important roles in the activation of

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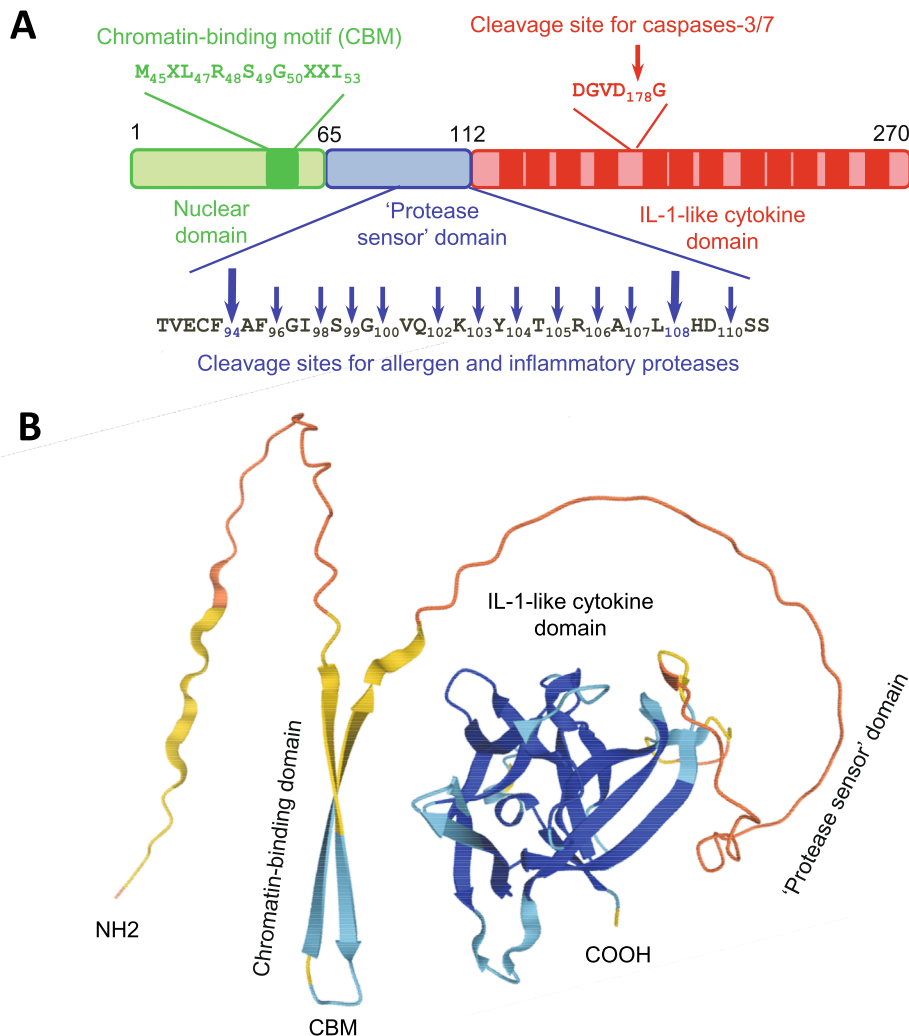
these cells [3,5]. IL-33 is thus a pleiotropic cytokine with critical roles in both type 2 and type 1 immunity, allergic and non-allergic inflammation, and regulatory responses [3,5]. We refer the reader to comprehensive reviews about the roles of IL-33 in the activation of various immune cell subsets and the pathophysiology of many diseases [3,4]. Here, we focus on the fundamental properties of the IL-33 protein (nuclear localization, constitutive and inducible expression in tissue-derived cells, release upon cell damage and tissue injury, bioactive forms) and emphasize its crucial roles in allergic inflammation and asthma, and COPD. We explain that most controversies regarding IL-33 protein expression, localization, release and function, are easily resolved when one considers separately studies that include appropriate controls from studies that lack such controls. Finally, we make recommendations to generate reliable results. We strongly believe that it is important to include specificity controls early in a project to avoid wasting time, effort and money studying a protein that is cross-reactive with anti-IL-33 antibodies but distinct from the true IL-33 protein.

## 2. IL-33, a chromatin-associated cytokine from the IL-1 family

Full-length human IL-33 protein (IL-33<sub>FL</sub>) is a 270 amino-acid protein belonging to the IL-1 cytokine family, discovered by our team in 2003 as a nuclear factor of high endothelial venule (NF-HEV) endothelial cells [9]. IL-33<sub>FL</sub> protein is composed of two evolutionary conserved domains (Fig. 1A), the N-terminal nuclear domain and the C-terminal IL-1-like cytokine domain, separated by a divergent ‘protease

sensor’ domain [5,10]. The C-terminal domain (cytokine domain, aa 112–270) possesses cytokine activity [11] and has a three-dimensional structure similar to interleukin 1 (IL-1), with 12  $\beta$ -strands arranged in a  $\beta$ -trefoil fold [12,13]. It binds to a heterodimer formed by its specific primary receptor ST2 and the co-receptor IL-1 receptor accessory protein (IL-1RAcP). Surface charge complementarity is critical for IL-33-ST2 interaction [13]. Crystal structure of the IL-33 signaling complex revealed that IL-33 stabilizes ST2 in a conformation allowing recruitment of IL-1RAcP to form ternary complexes [14]. Juxtaposition of the ST2 and IL-1RAcP cytoplasmic Toll-IL-1R (TIR) domains in the ternary complexes then results in signal transduction through MyD88-dependent signaling pathways [3]. Thus IL-33 shares many canonical features with other IL-1 cytokines (structure, receptor use, signaling), and is a *bona fide* member of the IL-1 subfamily and the larger IL-1 family [15,16].

Endogenous IL-33 is a chromatin-associated nuclear factor *in vivo* [17]. The N-terminal part of the protein (nuclear domain, aa 1–65) is necessary and sufficient to bring IL-33 into the nucleus [17,18]. It comprises a chromatin-binding motif (CBM) that allows attachment of IL-33 to chromatin through the histones H2A/H2B acidic pocket [18]. The IL-33 chromatin-binding domain containing the CBM adopts a tight hairpin structure (Fig. 1B), stabilized by an array of intramolecular hydrogen bonds, similar to that of Kaposi sarcoma LANA’s CBM [18]. The critical residues for association with chromatin and histones are identical in IL-33 and LANA CBM (MXLRSG motif, Fig. 1A), providing a unique example of molecular mimicry of a chromatin-associated



**Fig. 1.** Primary and three-dimensional structure of IL-33 protein. (A) Primary structure of the human IL-33 protein (aa 1–270) showing its three domains: the N-terminal nuclear domain, the central ‘protease sensor’ domain and the C-terminal IL-1-like cytokine domain. The chromatin binding motif (CBM) and the cleavage sites for proteases involved in IL-33 activation (inflammatory and allergen proteases) or inactivation (caspases 3 and 7) are indicated. IL-33<sub>35-270</sub> and IL-33<sub>109-270</sub> forms that are generated by various allergen proteases, mast cell proteases and neutrophil proteases, are likely to be major mature forms of IL-33 protein *in vivo*. (B) Three-dimensional (3D) structure of the full-length mouse IL-33 protein (aa 1–266) showing the hairpin structure of the N-terminal chromatin-binding domain and CBM and the IL-1-like fold of the C-terminal cytokine domain. The structure of full-length mouse IL-33 protein was predicted using AlphaFold (<https://alphafold.ebi.ac.uk/entry/Q8BVZ5>), an artificial intelligence-based system developed by DeepMind that predicts a protein’s 3D structure from its amino acid sequence. Human and rat IL-33 proteins are predicted to exhibit a similar 3D structure, including the evolutionary conserved N-terminal CBM hairpin structure (human, <https://alphafold.ebi.ac.uk/entry/O95760>; rat, <https://alphafold.ebi.ac.uk/entry/Q66H70>). Experimentally determined NMR and/or X-ray crystallography structures are available for the C-terminal IL-1 like cytokine domains of human and mouse IL-33 proteins (Protein Data Bank ID: 2kl1, 4kc3, 5vi4).

cytokine by a DNA tumor virus [18]. The central ‘protease sensor’ domain of IL-33 (aa 66 to 111 in humans) [10] is a cleavage and activation platform for a large number of proteases, both endogenous inflammatory proteases [19,20] and exogenous allergen proteases [10,21].

### 3. IL-33, a tissue-derived cytokine: Cellular sources of nuclear IL-33 *in vivo*

#### 3.1. Endothelial cells

IL-33 is abundant in the nuclei of endothelial cells from both large and small blood vessels in almost all human organs at steady state [22,23], indicating that the endothelium is a major cellular source of IL-33 in the human body [5]. Such widespread expression of IL-33 in the endothelium along the vascular tree suggests a role for the endothelium as an important sentinel for immune surveillance. It could provide a means of rapidly alerting immune cells to tissue damage in any organ of the body. Constitutive expression of IL-33 in blood vessels does not occur in mice, indicating an important species-specific difference [24]. This difference appears to be due to a 5-kb enhancer that is highly active in endothelial cells and is present in the human *IL33* gene but absent in the mouse *Il33* gene [25]. However, IL-33 protein is constitutively expressed in a few murine microvascular beds (liver, kidney, lung, adipose tissue) [24,26–30] and induced in others during inflammation (heart, colon) [24,31,32]. The first conditional knock out (KO) of the *Il33* gene in mouse, performed in endothelial cells (Table 1), revealed that endothelium-derived IL-33 induces systemic inflammation after myocardial pressure overload [31]. Furthermore, endothelial IL-33 contributes to kidney and liver ischemia–reperfusion injury and acts as an alarmin in these settings [28,30].

#### 3.2. Epithelial cells

In addition to endothelium, we observed strong nuclear expression of IL-33 in epithelial cells of tissues exposed to the environment [22,24]. Indeed, epithelial cells of barrier tissues (bronchus, nasopharynx, skin, stomach, salivary gland, vagina, etc...), constitute major sources of IL-33 protein (Fig. 2) [22,24]. For instance, nasal epithelium in the respiratory tract [33,34] and glandular epithelial cells of the stomach and salivary glands [22,24] express high levels of nuclear IL-33 in both species. However, important species-specific differences exist in other tissues [5]. IL-33 expression is constitutive in mouse keratinocytes but inducible in human keratinocytes [24,35]. Alveolar type II pneumocytes (ATII) are the major sources of IL-33 in murine lung [36,37] whereas airway basal epithelial cells and endothelial cells are the major sources of IL-33 in human lungs [22,38,39]. Thus, although IL-33 is abundant in alveoli in both species, its cellular sources are not the same. IL-33 derives from ATII epithelial cells in mice and alveolar endothelial cells in humans (Fig. 2).

Epithelial cell-derived IL-33 plays critical roles in allergic inflammation and type 2 immunity [3,5]. For instance, IL-33 produced by nasal epithelial cells and alveolar ATII cells mediates experimental allergic rhinitis [33] and type 2 immunity in nematode-infected mice [36], respectively. Recently, the use of conditional KO approaches confirmed the important role of epithelium-derived IL-33 in several tissues and disease processes (Table 1). For instance, such studies demonstrated the crucial role of keratinocyte-derived IL-33 in mouse models of atopic march and gastrointestinal allergy [40].

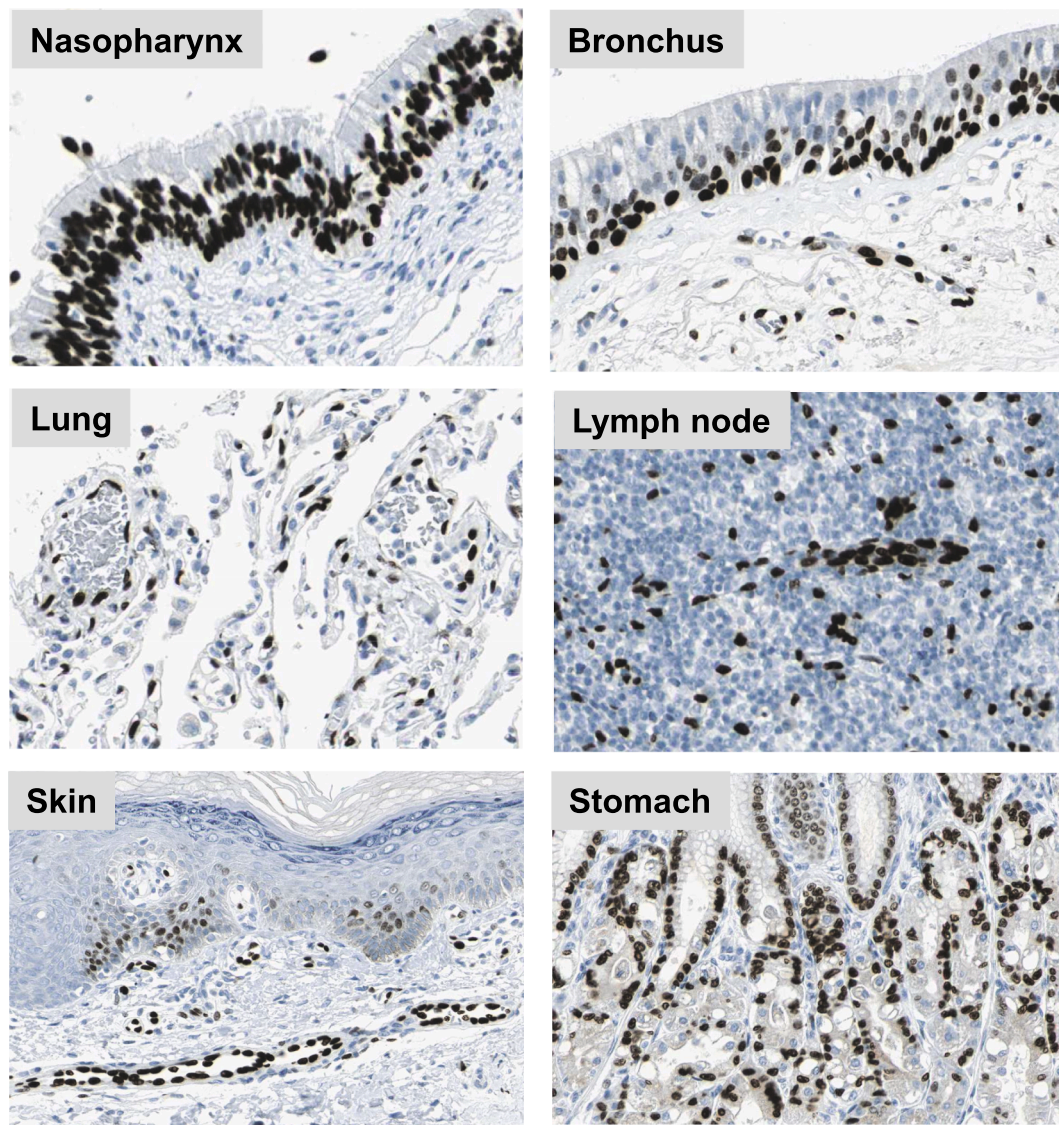
#### 3.3. Stromal cells

Fibroblastic stromal cells, fibroblast-like cells and myofibroblasts represent other important sources of IL-33 protein in both human and mouse tissues at steady state and during inflammation, particularly in diseases associated with tissue fibrosis, mucosal healing and wound

**Table 1**  
Conditional knock out of *Il33* gene in tissue-derived cells.

Cell type - Tissue	Mouse line	Associated features	References
<b>Endothelial cells</b>			
Endothelium	<i>Tek-RFP-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Critical role of endothelial cell-derived IL-33 in systemic inflammation	[31]
Lymphatic endothelium	<i>Prox1-CreERT2. Il33<sup>fllox/fllox</sup></i>	No role of lymphatic endothelium-derived IL-33 in CD8 T cell responses to viral infection in lymph nodes	[42]
<b>Epithelial cells</b>			
Skin (keratinocytes)	<i>K14-Cre. Il33<sup>fllox/fllox</sup></i>	Crucial role of keratinocyte-derived IL-33 in food anaphylaxis after mechanical skin injury (tape stripping)	[79]
Skin (keratinocytes)	<i>K14-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Critical role of keratinocyte-derived IL-33 in atopic dermatitis-like skin disease (TSLP-driven atopic march model)	[40]
Lung and skin (basal epithelial cells)	<i>Krt5-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Crucial role of basal cell-derived IL-33 in skin epithelial barrier and postviral lung disease	[67]
Gut (intestinal epithelial cells)	<i>Villin-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Critical role of epithelial cell-derived IL-33 in anti-helminth immunity	[57]
<b>Fibroblastic stromal cells</b>			
Adipose tissue (mesenchymal stromal cells)	<i>Pdgfra-Cre. Il33<sup>fllox/fllox</sup></i>	Crucial role of PDGFR $\alpha$ + stromal cell-derived IL-33 in maintenance of Tregs in visceral adipose tissue	[43]
Lung (adventitial stromal cells, ASCs)	<i>Gli1-CreER<sup>T2</sup>. Cre. Il33<sup>fllox/fllox</sup></i>	Impaired helminth-driven induction of Th2 cells after ASC-specific deletion of <i>Il33</i>	[29]
Lung (fibroblastic stromal cells, FSCs)	<i>Ccl19-Cre. Il33<sup>fllox/fllox</sup></i>	Crucial role of FSC-derived IL-33 in pulmonary expansion of effector memory CD8 <sup>+</sup> T cells (adenovirus vaccine)	[110]
Subcutaneous tumor (fibroblastic stromal cells, FSCs)	<i>Cxcl13-Cre. Il33<sup>fllox/fllox</sup></i>	Crucial role of FSC-derived IL-33 in sustained anti-tumor CD8 <sup>+</sup> T cell responses	[111]
Synovium (fibroblast-like synoviocytes)	<i>Col1a2-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Decreased synovitis after synovial fibroblast-specific deletion of <i>Il33</i>	[112]
<b>Other cell types</b>			
Brain (astrocytes)	<i>hGFAP-Cre. Il33<sup>fllox/fllox</sup></i>	Critical role of astrocyte-derived IL-33 in microglial synapse engulfment	[49]
Brain (neurons)	<i>Synapsin-Cre. Il33<sup>fllox/fllox</sup></i>	Crucial role of neuronal-derived IL-33 in microglial remodeling of the extracellular matrix	[50]
Cartilage (chondrocytes)	<i>Acan-CreER<sup>T2</sup>. Il33<sup>fllox/fllox</sup></i>	Cartilage specific ablation of <i>Il33</i> decreases synovitis and attenuates osteoarthritis	[112]

repair [3,5]. We showed that fibroblastic reticular cells (FRCs) in the T cell areas of peripheral lymphoid organs (lymph nodes, spleen, tonsils, appendix) express high levels of nuclear IL-33 protein at baseline (Fig. 2) [22,24]. Conventional and conditional KO approaches uncovered critical roles of FRC-derived IL-33 in CD8<sup>+</sup> T cell responses to viral infection in both spleen [41] and lymph nodes [42].



**Fig. 2. Nuclear alarmin IL-33 is abundant in endothelial cells, epithelial cells and fibroblastic stromal cells from human tissues.** Endogenous IL-33 protein is present at high levels in the nuclei of endothelial cells from blood vessels in bronchus, lung alveoli, skin and lymph node (HEV blood vessel). In addition, IL-33 is abundant in the nuclei of respiratory epithelial cells in the nasopharynx and bronchus, glandular epithelial cells in the stomach, and some keratinocytes in skin. Fibroblastic stromal cells such as the fibroblastic reticular cells (FRCs) in lymph nodes, also express high levels of nuclear IL-33. Images were downloaded from the Human Protein Atlas [108,109] and are available at <https://www.proteinatlas.org/ENSG00000137033-IL33/tissue/>

The use of various *Il33* promoter-driven citrine, eGFP or mCherry reporters (Table 2) showed that PDGFR $\alpha$ <sup>+</sup>Sca-1<sup>+</sup> fibroblastic stromal cell populations are important sources of IL-33 in multiple murine tissues including lung, adipose tissue, skeletal muscle, liver and pancreas [29,43–46]. Such cells that express mesenchymal cell markers and are often located within perivascular niches, have variously been termed adventitial stromal cells (ASCs), mesenchymal stromal cells (mSCs), mesenchymal stem cells, adipose stem and progenitor cells (ASPCs), multipotent stromal cells (MSCs) and fibro-adipogenic progenitors (FAPs) [29,43–45,47]. Although PDGFR $\alpha$ <sup>+</sup>Sca-1<sup>+</sup> fibroblast-like stromal cells are heterogeneous both within and between tissues, the IL-33<sup>+</sup> subsets play important roles in the regulation of ILC2s and Tregs, in both lung and adipose tissue [29,43–45].

Interestingly, *Il33* promoter-driven reporter mice revealed expression of IL-33 in mesothelial stromal cells expressing both epithelial and mesenchymal markers [29,44]. Mesothelial cells form a squamous epithelium-like layer or mesothelial membrane protecting visceral organs and body cavities (peritoneal, pleural and pericardial cavities) from

physical damage [43,44]. Mesothelium-derived IL-33 acts as an alarmin and induces rapid ILC2s and eosinophil responses upon the breach of the peritoneal barrier by the intestinal helminth parasite *Nippostrongylus brasiliensis* [44].

### 3.4. Other cell types

Using an *Il33* promoter-driven  $\beta$ -galactosidase reporter, we observed that expression of IL-33 is very high in mouse brain at baseline [24]. Glial cells (astrocytes, oligodendrocytes), but not microglia, are the major sources of the IL-33 protein in the brain [48,49]. Oligodendrocyte-derived IL-33 function as an alarmin rapidly released following brain injury to promote recovery [48]. Astrocyte-derived IL-33 promotes microglial synapse engulfment and neural circuit development [49]. In addition to glial cells, a subset of neurons express IL-33 in the hippocampus, and neuronal IL-33 induces microglial remodeling of the extracellular matrix [50]. Strikingly, up to 30% of isolated murine brain cells are IL-33<sup>+</sup> [48]. Although human astrocytes express IL-33

**Table 2**  
*Il33* promoter reporter transgenic mice lines.\*

Reporter	Genomic construct	References <sup>†</sup>
<i>mIl33-LacZ</i> reporter	βgeo genetrap cassette inserted in intron 1 of the mouse <i>Il33</i> locus	[24,27,38,49]
<i>mIl33-citrine</i> reporter	GFP-derived citrine reporter inserted downstream of the ATG start codon of mouse <i>Il33</i>	[37,45,113]
<i>mIl33-GFP</i> reporter	GFP-neo cassette inserted downstream of the ATG start codon of mouse <i>Il33</i> (deletion of neo cassette required for GFP expression)	[42,114,115]
<i>mIl33-eGFP</i> reporter	EGFP reporter inserted into the 3' UTR of the mouse <i>Il33</i> locus	[43,44,56]
<i>mIl33-mCherry</i> reporter	H2B-mCherry cassette inserted in intron 4 of the mouse <i>Il33</i> locus	[29,49,50,67,116]
<i>hIL33-E2-Crimson</i> reporter	E2-Crimson reporter inserted downstream of the ATG start codon of human <i>IL33</i> in a « humanised » transgenic mouse model (166 kb human BAC, bacterial artificial chromosome)	[25]

\* Activity of *Il33* promoter reporter does not necessarily mean that endogenous IL-33 protein is produced in the cells.

<sup>†</sup> Some representative studies are indicated. The lists are not exhaustive.

protein [51], it is currently unknown whether the human brain contains such high levels of IL-33-expressing cells and whether these cells perform similar functions to those described in mice. Species-specific differences could exist.

Immune cells, such as macrophages, mast cells, DCs, were suggested as important cellular sources of IL-33 protein *in vivo* [52–54]. However, single-cell RNA-seq databases (see for instance the Single Cell Portal at

[https://singlecell.broadinstitute.org/single\\_cell](https://singlecell.broadinstitute.org/single_cell)) clearly show that tissue-derived cells (including endothelial, epithelial and stromal cells), rather than immune cells, are the major cellular sources of IL-33 in human and mouse tissues, both at steady state and during various disease processes (inflammation, infection, cancer). Small numbers of immune cells were weakly positive in studies using *Il33* promoter-driven citrine, GFP or mCherry reporters [29,42,45]. However, *Il33* promoter-driven reporters measure activity of *Il33* promoter, not expression of IL-33 protein, and although *Il33* mRNA is detectable in CD45<sup>+</sup> immune cells, its levels remain considerably lower than those found in structural cells and tissues [38,42,44,55]. Moreover, engulfment of *bona fide* IL-33 producing cells by immune cells could possibly contribute to the low staining of immune cells observed in some studies with *Il33* promoter-driven reporters. Importantly, we believe that the flow cytometry assay (staining with rat anti-mouse IL-33 mAb, # clone 396118) [52,54] that has been widely used to claim IL-33 protein expression in macrophages, mast cells and DCs, is non-specific. We confirmed the staining of CD45<sup>+</sup> immune cells with this assay but we observed the same positive staining with immune cells isolated from *Il33*-deficient mice (E. Lefrançois and JP Girard, unpublished data; see also product review on [https://www.rndsystems.com/products/mouse-il-33-pe-conjugated-antibody-396118\\_ic3626p#product-reviews](https://www.rndsystems.com/products/mouse-il-33-pe-conjugated-antibody-396118_ic3626p#product-reviews): mast cells isolated from *Il33*-deficient mice were positive with rat anti-mouse IL-33 mAb clone 396118). This critical specificity control with cells isolated from *Il33* KO mice was lacking in the previous studies [52,54]. A specific flow cytometry assay has been used in other studies (staining with goat anti-mouse IL-33 antibodies using *Il33*-deficient cells as controls for gating) (Table 3), that confirmed *bona fide* expression of endogenous IL-33 protein in fibroblastic stromal cells, lymphatic endothelial cells and oligodendrocytes [42–44,48,56]. When analyzing IL-33 expression by flow cytometry, it is thus important to use specific assays that include appropriate specificity controls (Table 3). The same remarks apply to other techniques. For example, IL-33 protein expression in mouse monocytes/macrophages has been claimed on the basis of western blot analyses with goat antibodies to mouse IL-33 (R&D # AF3626).

**Table 3**  
Recommendations for validation of endogenous IL-33 protein expression in cellular systems and/or *in vivo* models.\*<sup>†</sup>

Techniques	Expected results	Specificity controls	References <sup>‡</sup>
<b>Immunofluorescence or Immunohistochemistry</b> Primary cells or cell lines Tissue sections <sup>§</sup>	Nuclear staining with specific anti-IL-33 antibodies Loss of nuclear signal in <i>IL33</i> Knock-out (KO) or Knock-down (KD) cells or <i>Il33</i> KO tissues	<i>IL33</i> KO/KD cells (human, mouse), <i>Il33</i> KO tissues (mouse)	[22–24,27–30,33–35,42,48–50,62,117,118]
<b>Western blot</b> Primary cells or cell lines Fluids or tissues <sup>  </sup>	Detection of a 33–35 kDa band (full-length IL-33 protein) <sup>¶</sup> with specific anti-IL-33 antibodies Loss of the 33–35 kDa band <sup>¶</sup> in <i>IL33</i> KO/KD cells (extracts, supernatants) or <i>Il33</i> KO fluids and tissues (extracts)	<i>IL33</i> KO/KD cells (human, mouse), <i>Il33</i> KO fluids or tissues (mouse)	[10,19,23,24,36,62,69,115,119]
<b>Flow cytometry</b> Primary cells or cell lines	Fluorescence signal with specific anti-IL-33 antibodies Loss of fluorescence signal in <i>IL33</i> KO/KD cells (critical controls for gating)	<i>IL33</i> KO/KD cells (human, mouse)	[42–44,48,56]
<b>ELISA</b> Primary cells or cell lines Fluids or tissues	Positive signal with specific IL-33 ELISA assay Loss of the signal in <i>IL33</i> KO/KD cells (extracts, supernatants) or <i>Il33</i> KO fluids and tissues (extracts)	<i>IL33</i> KO/KD cells (human, mouse), <i>Il33</i> KO fluids or tissues (mouse)	[10,28,30,31,37,40,44,45,79,113]

\* Activity of *Il33* promoter (*LacZ*-, citrine-, GFP- or mCherry-reporter mice) or detection of *Il33* mRNA (qPCR) does not necessarily mean that endogenous IL-33 protein is produced in the cells.

<sup>†</sup> Phenotypic (gene expression) or functional (adoptive cell transfer experiments) changes in cells isolated from *Il33*-deficient mice can occur in cells that do not produce the endogenous IL-33 protein, because IL-33 is a pleiotropic cytokine and many cell types (expressing or not ST2) can be affected in *Il33*-deficient tissue microenvironments.

<sup>‡</sup> Some representative studies are indicated. The lists are not exhaustive.

<sup>§</sup> For human tissues, since *IL33* KO tissues are not available, we recommend the use of several independent antibodies, each validated with *IL33* KD primary human cells.

<sup>||</sup> Immunoprecipitation followed by western blot analysis could be necessary for detection of endogenous IL-33 protein in fluids.

<sup>¶</sup> Specific bands of 18–22 kDa corresponding to cleavage products of endogenous IL-33 protein can also be detected in certain conditions (extracellular fluids or tissues).

However, we have previously shown that these antibodies can give both specific and non-specific signals [10,19,24]. They cross-react in western blots with an abundant nonspecific protein that is induced under inflammatory conditions and is similar in size to the bioactive forms of IL-33 [10,19]. None of the previous studies claiming IL-33 expression in monocytes/macrophages included specificity controls (IL-33 KD/KO cells). Most studies have used nonspecific tools. We believe that monocytes/macrophages are not a relevant source of IL-33 protein.

Recently, it has been claimed that CD11c<sup>+</sup> myeloid antigen-presenting cells in lymph nodes and spleen (cDC1s and cDC2s), small intestine and colon, represent an important source of IL-33 protein [57,58]. The authors used various assays, including the non-specific flow cytometry assay with rat anti-mouse IL-33 mAb (clone 396118), and showed strong cytoplasmic staining of CD11c<sup>+</sup> DCs of the lamina propria with goat anti-mouse IL-33 antibodies [57,58]. However, IL-33 protein localizes to the nucleus of *bona fide* IL-33 producing cells [5,17,18]. Moreover, we previously showed that the same goat anti mouse IL-33 antisera (R&D # AF3626) gives specific nuclear staining (staining lost in *Il33* KO mice) of *bona fide* IL-33 producing cells (ATII cells, keratinocytes, FRCs, ...) and non-specific cytoplasmic staining of lamina propria cells (staining still observed in *Il33* KO mice) [24]. Unfortunately, the critical control with *Il33* KO tissue was not included in the former study [57]. Moreover, we and others have previously demonstrated using appropriate specificity controls (tissues from *Il33* KO mice) that non-hematopoietic cells (i.e. FRCs and endothelial cells) are the major sources of IL-33 in lymph nodes and spleen, and that the IL-33 protein is not expressed in DCs in lymphoid tissues [22,24,41,42]. Since the proof of the actual expression of IL-33 protein in DCs was not brought with accurate controls, it remains unclear to us why the authors observed some changes in Tregs in *CD11c-Cre.Il33<sup>fl/fl</sup>* mice [57,58], but these changes may be related to differences between *CD11c-Cre* and *Il33<sup>fl/fl</sup>* transgenic lines (microbiota, genetic polymorphisms, other factors...).

### 3.5. Inflammation

IL-33 is constitutively expressed at high levels in human and mouse tissues in the steady state [5,22,24]. However, it is important to point out that IL-33 expression is upregulated in various human diseases and inflammatory processes (allergic and non-allergic inflammation, infection with parasites or viruses, ...) [1,3,5]. Most of the time, the same tissue-derived cells that expressed nuclear IL-33 at baseline (i.e. endothelial, epithelial and/or fibroblastic stromal cells), are the major producers of the cytokine during inflammation [1,3,5]. The increased expression of IL-33 is due to increased levels of IL-33 in individual cells and/or increased numbers of these tissue-derived IL-33 producing cells, often in an IL-33-dependent amplification loop [37,59]. However, *de novo* induction of IL-33 expression occurs in other cellular subsets, including several subsets of fibroblast-like cells in human diseases associated with tissue fibrosis or mucosal ulceration [3,5].

## 4. Essential role of IL-33 nuclear localization

Nuclear localization (or nuclear sequestration) of IL-33 is a fundamental property of the protein, that allows regulation of its cytokine activity, and is essential for survival of the organism [5]. Indeed, deletion of the N-terminal chromatin-binding nuclear domain results in constitutive release of the cytokine in serum, and leads to tissue eosinophilia, multi-organ inflammation (lymphadenopathy, lung asthma-like pathology, severe enterocolitis, arthritis, cardiac inflammation) and early lethality in a knock-in (KI) mouse model [60]. Importantly, death of the mice was prevented in the absence of ST2, indicating that the lethality was due to the potent extracellular cytokine activity of IL-33, and not due to the loss of a putative nuclear function of the protein [60]. Analysis of the same KI mouse transgenic line in a model of age-related macular degeneration revealed spontaneous release of IL-33 in

the retina and ST2-dependent retinal cell loss [61]. Keeping IL-33 in the nucleus of producing cells is thus essential to maintain tissue homeostasis and protect the body from uncontrolled lethal inflammation. These observations are likely to explain why the N-terminal nuclear domain of IL-33 is evolutionary conserved, similar to the C-terminal IL-1-like cytokine domain [5].

## 5. Lack of a cell-intrinsic nuclear role or nuclear function of IL-33

Although IL-33 is very abundant in the nuclei of producing cells [22,24] and binds to the acidic pocket of histones H2A-H2B dimer on chromatin [17,18], it does not appear to regulate gene or protein expression. We performed high-resolution mass spectrometry analyses [62] to analyze a putative role of nuclear IL-33 in the regulation of protein expression in primary human endothelial cells, a major source of IL-33 protein in human tissues [22,23]. We found that extracellular IL-33 cytokine, and not endogenous nuclear IL-33, regulates protein expression in endothelial cells [62]. Importantly, in order to obtain reliable experimental results, we used two independent RNA silencing strategies to knockdown endogenous IL-33 protein expression. Although these two RNA silencing strategies were state-of-the-art and each combined multiple siRNAs, we observed some off-target effects [62]. We believe that off-target effects and insufficiently controlled experiments, and not a cell intrinsic role of IL-33, explain the differences observed in several reports after knockdown of endogenous IL-33 expression. Indeed, using carefully controlled knockdown experiments, we did not find any evidence for a role of nuclear IL-33 in the regulation of NF-kappaB subunits [62], contrary to previous studies performed using a single siRNA.

Consistent with our observations in endothelial cells, studies using epithelial cells found no evidence for a role of nuclear IL-33 in regulating gene expression. Inducible expression of nuclear IL-33 in oesophageal epithelial cells lacking ST2 had no reproducible impact on gene expression [63]. Epithelial cells isolated from the intestine of transgenic mice expressing nuclear IL-33 in the intestinal epithelium exhibited transcriptional changes [64]. However, ST2 deficiency completely abrogated IL-33-induced gene expression changes in the epithelial cells indicating that the transcriptional effects are due to activation of the epithelial cells by extracellular IL-33, and not by intracellular nuclear IL-33 [64]. In addition, RNA sequencing performed on sorted FRCs from lymph nodes and fibroblastic stromal cells from adipose tissues revealed no significant changes in gene expression in cells isolated from wild type versus *Il33* KO mice [42]. These findings argued against a nuclear and cell-intrinsic role of IL-33 in lymph node FRCs and adipose tissue fibroblastic stromal cells, despite the abundant expression and nuclear localization of the protein in these *bona fide* IL-33-producing cells. Similarly, the transcriptome of astrocytes isolated from the brain of *Il33* KO mice was unchanged, arguing against cell-autonomous roles of IL-33 in astrocytes [49]. Thus the available evidence indicate that nuclear IL-33 does not regulate gene or protein expression and has no cell-intrinsic role in *bona fide* IL-33 producing cells, including endothelial cells, epithelial cells, fibroblastic stromal cells and astrocytes.

A cell-intrinsic role of IL-33 in regulating the transcriptional landscape of Foxp3<sup>+</sup> Tregs in tumors was recently proposed [65]. However, a major issue is that the authors have not demonstrated that Tregs are *bona fide* producers of IL-33 protein. Indeed, they did not show expression of IL-33<sub>FL</sub> protein (~33–35 kDa) by western blot, nor demonstrate nuclear expression of IL-33 in Foxp3<sup>+</sup> Tregs by immunofluorescence or immunohistochemistry. Nuclear expression of endogenous IL-33 in *bona fide* IL-33 producing cells is easy to detect [5,22,24]. Actually, the authors did not perform any of the assays previously used to validate *bona fide* IL-33 producing cell types (Table 3). Many other groups have shown that tissue-resident Foxp3<sup>+</sup> Tregs express high levels of ST2 and are important targets of IL-33, but are not cellular sources of the protein. For instance, immunofluorescence studies showed that Foxp3<sup>+</sup> Tregs are

located in close proximity to IL-33 producing stromal cells in skeletal muscle, liver and pancreas, but do not express endogenous IL-33 protein themselves [29,46]. Hatziioannou et al presented some data with *Foxp3-YFP/Cre.II33<sup>flox/flox</sup>* mice [65], however the *Foxp3-YFP/Cre* line utilized is leaky and can lead to gene deletion in non-Tregs cells, particularly in stromal cell areas of lymph nodes [66], and deletion of *Il33* may have occurred in tumor stromal cells. We thus believe that *Foxp3<sup>+</sup>* Tregs are not a relevant source of IL-33 protein, and that the transcriptional and functional differences between *Foxp3<sup>+</sup>* Tregs isolated from wild type and *Il33* KO mice [65] are due to phenotypic alterations of Tregs in the *Il33*-deficient tumor microenvironment. IL-33 is a pleiotropic cytokine and the IL-33/ST2 pathway regulates many cell types in the tissue microenvironment [3–5]. Phenotypic alterations are likely to occur in many cell types (expressing or not expressing ST2) in an *Il33* KO background. Changes in gene expression or functional differences in adoptive cell transfer experiments are therefore not sufficient to claim IL-33 protein expression and nuclear function in the cells. When convincing evidence that the analyzed cells are *bona fide* producers of endogenous IL-33 protein is lacking, we recommend to use the term ‘cells isolated from *Il33*-deficient mice’, rather than the term ‘IL-33-deficient cells’ that would be misleading for the field.

A nuclear role of IL-33 has been proposed in some studies based on phenotypic differences between *Il33* KO and *St2* KO mice. However, as discussed in one of our previous reviews, some of the discrepancies can probably be attributed to the different background of the strains of mice used [3]. Alternatively, differences in microbiota or other factors may play a role. Another possibility is that the lack of ST2 expression in immune cells from *St2* KO mice may increase signaling through other IL-1R/Toll-like receptor family members that use the same signaling components (receptors for IL-1 family cytokines, TLRs) [3]. This later scenario may explain the phenotypic differences between *Il33* KO and *St2* KO mice recently reported in a mouse model of postviral lung disease [67]. In carefully controlled experiments, the authors showed that IL-33-derived from basal epithelial cells is required for basal cell proliferation, type 2 inflammation, mucus production and accumulation of IL-33 responsive immune cells [67]. The authors provided experimental evidence for activation of the EGFR/Stat3 pathway in the basal epithelial cells, and the lack of ST2 expression in these cells [67]. The results are thus compatible with a bidirectional cross talk between ST2<sup>+</sup> basal cells releasing IL-33 and activated ST2<sup>+</sup> immune cells (lung ILC2s or other immune cells) releasing EGFR ligands, such as amphiregulin, for increased basal cell proliferation. Such a cross talk between IL-33-activated ILC2s producing amphiregulin and EGFR<sup>+</sup> epithelial cells occurs in a model of intestinal damage and inflammation [68]. As discussed above, the lack of phenotype in *St2* KO mice in this postviral lung disease model would then be explained by increased signaling through other IL-1Rs or TLRs in the *St2* KO immune cells.

Based on the evidence discussed above, that nuclear IL-33 does not regulate gene or protein expression and has no cell-intrinsic role in *bona fide* IL-33 producing cells, we believe that there could be other explanations for gene expression changes in *Il33*-deficient cells observed in some studies. For instance, autocrine signaling when IL-33 producing cells express ST2 or paracrine signaling and bidirectional dialogue with neighboring cells when IL-33 producing cells do not express ST2. In any case, phenotypic and transcriptional differences between *Il33* KO and *St2* KO mice and tissues/cells are clearly insufficient to claim a nuclear function or nuclear role of IL-33, because there are several other reasons that may explain the discrepancies.

## 6. IL-33 release upon cell damage and tissue injury

IL-33 functions as a nuclear alarmin, an alarm signal released from the nuclei of producing cells upon cell injury to alert the immune system of the damage [1,5]. We were the first to propose and to demonstrate the mode of action of IL-33 as an alarmin, using primary human endothelial cells, a *bona fide* IL-33 producing cell-type [22,69]. We based our initial

hypothesis on the observation that, unlike inducible cytokines, IL-33 is present at high levels in normal human tissues at steady state [22]. We showed the release of endogenous nuclear IL-33 protein in the extracellular space after endothelial cell damage or mechanical injury [69]. Release of full-length IL-33 protein by primary human endothelial cells was observed after induction of cellular necrosis by several cycles of freezing and thawing, damage to the cell membrane by non-ionic detergents, or mechanical wounding of endothelial cells by cell scraping (transient sublethal membrane disruptions) or cell scratching (*in vitro* model of wound healing) [69]. Release of endogenous IL-33 protein was also observed after treatment of the endothelial cells with *Alternaria alternata*, a potent fungal allergen, chitin, a polysaccharide constituent of many allergens and parasites, or phospholipase A2 (PLA2), a cytolytic enzyme found in bee venom, fungal allergens and house dust mites (HDM) [10]. Extracellular IL-33 protein was detected 5 min after exposure to *Alternaria* and correlated with the presence of trypan blue-positive cells, suggestive of membrane damage [10]. Experiments in mice revealed that endogenous IL-33 protein is rapidly released in bronchoalveolar lavage (BAL) fluids after intranasal exposure to *Alternaria* (15 min) or bee venom PLA2 (30 min) [10] or after intravenous administration of oleic acid (2 h), which results in damage to the lung alveolar epithelium [19]. Rapid and transient release (15 min to a few hours) of endogenous IL-33 protein in BAL fluids after *Alternaria* treatment was observed in other studies [70–73] and found to be inhibited by HpARI, a protein secreted by a helminth parasite [74]. Importantly, in all our experiments, we validated the specificity of IL-33 detection in cell supernatants and BAL fluids using *Il33* KD cells (human primary cells) or *Il33* KO tissues (mouse tissues) [10,19,69]. During our studies, we observed that some widely used mouse IL-33 ELISA assays give positive signals with *Il33* KO fluids. Others have reported specificity issues with human IL-33 ELISA assays [75,76]. Thus, specificity controls are crucial and studies asserting IL-33 protein release in the absence of specificity controls should be viewed with caution, as the possibility that the positive signal with the IL-33 ELISA is due to a cross-reactive protein is not unlikely. Although serum IL-33 levels have been measured in various human inflammatory and non-inflammatory disorders [77], we believe that many studies are not reliable because of specificity issues with ELISA assays. Specificity controls are also crucial when analyzing IL-33 protein release by western blot analysis. For instance, we previously showed in models of allergic and non-allergic lung inflammation that the main protein detected in BAL fluids by the most widely used goat antibodies to mouse IL-33 (R&D # AF3626) is a non-specific cross-reactive protein with a size similar to that of IL-33 bioactive forms [10,19]. Whatever the cellular system or *in vivo* model used, validation of the specificity of IL-33 signals from the beginning of the study is thus critical (Table 3).

Consistent with our observations, many studies have confirmed the rapid release of endogenous IL-33 protein upon cell damage and tissue injury. For instance, mechanical skin injury (tape stripping to mimic injury caused by scratching) induced release of endogenous mouse IL-33 protein in skin explants and serum [78,79]. Similarly, kidney or liver ischemia–reperfusion injury [28,30], spinal cord injury [80], and intranasal administration of ragweed pollen [33], induced release of endogenous IL-33 protein in plasma, cerebrospinal fluids, and nasal lavage fluids, respectively. In all studies, levels of extracellular IL-33 rapidly increased after the injury (1 h) but this increase was transient, and IL-33 levels in the various fluids returned to baseline after a few hours (baseline levels 24 h after the injury in all contexts). Interestingly, such a rapid and transient increase of IL-33 protein levels in serum, plasma, urine and BAL fluids has been observed in human patients after kidney and liver ischemia–reperfusion injury [30,81], severe trauma [82,83] and asthmatic lung exposure to protease-containing allergens (HDM, pollens) [84]. Thus, IL-33 acts as an alarmin released upon tissue injury in various contexts.

IL-33 release also occurs after tissue damage due to parasite or viral infection. For instance, endogenous IL-33 protein is detectable in

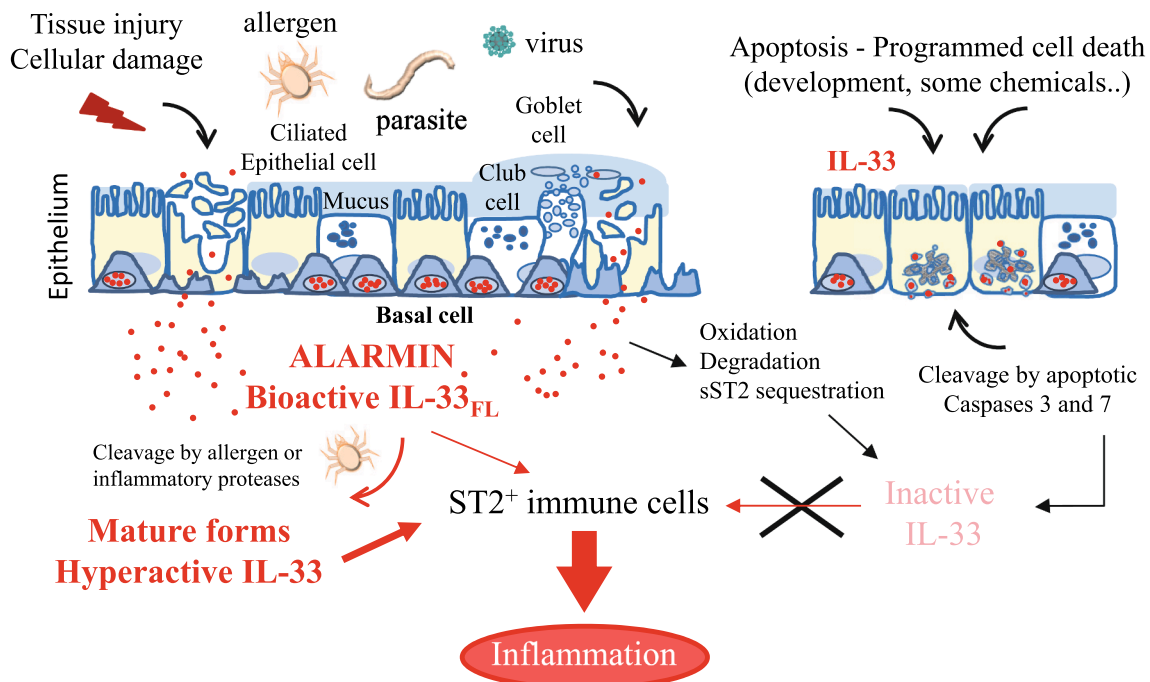


peritoneal fluids and serum 4 h after intraperitoneal infection with *Nippostrongylus brasiliensis* [44]. Similarly, release of IL-33 occurs in the peritoneal cavity after *Toxoplasma gondii* infection and IL-33 levels correlate with levels of parasite replication [56]. Imaging revealed that areas of parasite replication inversely correlated with the presence of IL-33<sup>+</sup> cells [56]. Similarly, after influenza viral infection in the lungs, there is a strong spatial correlation between influenza-infected lung areas and a local reduction of IL-33 staining [39]. The authors proposed that virus-mediated cellular damage induces extracellular release of IL-33 [39].

Thus, the available evidence indicates that cell damage, tissue injury, exposure to allergens, and infection with parasites or viruses are major mechanisms of IL-33 release *in vivo* (Fig. 3). Although high levels of extracellular IL-33 are invariably associated with cell damage and/or tissue injury, it remains unclear whether IL-33 can be released at low levels in the absence of cell death, for instance during tissue homeostasis. We previously proposed that under certain circumstances, IL-33 could be released from cells subjected to mechanical forces *in vivo* after a 'near cell death experience' followed by cell recovery or death at later time points [5]. Several studies claimed the release of endogenous IL-33 from living cells, but to the best of our knowledge, none of these studies included the appropriate controls (specificity of IL-33 detection, proof of the absence of low levels of cell death). For instance, we believe that the mechanism proposed to mediate unconventional IL-33 secretion by DCs [57] is irrelevant because DCs are not *bona fide* producers of the endogenous IL-33 protein (see paragraph 3.4). Other studies analyzed IL-33 release after overexpression of IL-33 protein fused to fluorescent protein or tetracycline tags. However, ectopic expression and epitope-tagging are likely to interfere with the normal behavior of the IL-33 protein. Although these approaches can be useful to investigate some aspects of IL-33 biology (mutagenesis of protein motifs), we believe that they are not appropriate to model release of endogenous IL-33 protein.

Deletion of exons 3 and 4 by alternative splicing of *IL33* mRNA has been proposed to result in the generation of truncated IL-33 proteins that are actively secreted [85,86]. However, this was only shown using overexpression and epitope-tagging approaches [85,86]. Amplification by PCR is required to detect splice variants of *IL33* mRNA, probably because these variants are minor components in primary cells. A western blot analysis suggested higher levels in airways of the truncated IL-33 form produced by alternative splicing, compared to IL-33<sub>FL</sub> [86]. However, the fact that the alternatively spliced *IL33* mRNAs are present at ~500 fold lower levels than the full length *IL33* mRNA in human lung tissues [86], raises doubts about the specificity of the western blot assay used by the authors. Endogenous truncated IL-33 proteins corresponding to alternatively spliced mRNAs have never been detected in primary cells or tissues using specific western blot assays. Furthermore, if truncated cytoplasmic forms of IL-33 corresponding to alternative splicing were produced at significant levels *in vivo*, it is likely that their expression would have been detected in some cells during immunofluorescence/immunohistochemistry studies. However, during the past 20 years, we, and others have never observed specific cytoplasmic staining for IL-33 (staining validated using KD/KO cells or tissues) in cells or tissues of human and mouse origin (Table 3). Alternative splicing leading to synthesis of truncated cytoplasmic IL-33 forms is thus unlikely to be a relevant and general mechanism of IL-33 release.

Adoptive cell transfer experiments revealed functional differences between immune cells isolated from WT and *IL33* KO mice in several studies. As discussed above (paragraph 5), we believe that these differences are due to phenotypic alterations of immune cells isolated from an *IL33* KO tissue microenvironment, and not to IL-33 production and release by immune cells.



**Fig. 3. Mode of action and regulation of IL-33 alarmin in the airways.** After tissue injury or cellular damage, IL-33 is released from the nuclei of producing cells in a full-length form (IL-33<sub>FL</sub>) that can be cleaved extracellularly by proteases from environmental allergens and/or proteases from inflammatory cells (mast cells, neutrophils). IL-33<sub>FL</sub> is biologically active at high doses, but has little activity at low doses. However, its cleavage results in the generation of hyperactive mature forms which exhibit 30-to-60 fold higher activity than IL-33<sub>FL</sub>. IL-33<sub>FL</sub> and the hyperactive mature forms activate various populations of tissue-resident or newly recruited immune cells expressing the ST2 receptor for induction of type 2 and/or type 1 inflammatory responses depending on the context (homeostasis, infectious or inflammatory disease). IL-33 is a very potent cytokine and several mechanisms regulate its activity: sequestration in the nuclei of producing cells; inactivation by apoptotic caspases 3 and 7 during apoptosis; extracellular inactivation by oxidation of cysteine residues critical for correct folding of the cytokine domain; sequestration by soluble sST2 receptor in biological fluids.

## 7. Bioactive forms of IL-33: full-length protein and mature forms

The initial dogma was that IL-33<sub>FL</sub> was an inactive precursor of the cytokine that required cleavage and activation by caspase 1/inflammasomes for biological activity [11]. However, we [10,19,20,69], and others [87–89] demonstrated that IL-33<sub>FL</sub> (IL-33aa1-270 in humans; IL-33aa1-266 in mice) is biologically active and induces ST2-dependent responses in target cells. We also showed that apoptotic caspases cleave IL-33<sub>FL</sub> in the C-terminal IL-1-like cytokine domain [69], in agreement with two other studies [87,89]. Cleavage occurs after residue Asp 178 (Asp 175 in mouse) at a DGVD<sub>178</sub>G consensus site of cleavage for caspase-3 (Fig. 1A), and caspases 3 and 7 mediate the cleavage. We [69], and others [87,89] showed, using *bona fide* ST2-dependent assays with appropriate controls, that the two caspases cleavage products of human IL-33 (IL-33aa1-178 and IL-33aa179-270 in humans) do not induce significant ST2-dependent responses, contrary to IL-33<sub>FL</sub>. Thus, cleavage of IL-33 protein by caspases results in its inactivation during apoptosis (Fig. 3). This evolutionary conserved mechanism of IL-33 regulation is likely to be important to avoid alerting the immune system unnecessarily after physiological programmed cell death, as opposed to pathological un-programmed cell death.

A recent study claimed that the caspase cleavage product of human IL-33 (IL-33aa1-178) lacking the two-thirds of the IL-1-like cytokine domain is a bioactive form of IL-33 that induces ST2-dependent responses [90]. Surprisingly, the three previous studies that consistently and convincingly demonstrated, using appropriate assays for both human and mouse IL-33 proteins, that this caspase-cleavage product does not induce ST2-dependent responses [69,87,89], were not discussed, nor even mentioned in this study. A major issue is that the authors did not include the appropriate controls in their bioassays [90]. Indeed, the authors only used PBS or medium alone as controls and did not show in any of their *in vitro* and *in vivo* experiments the critical controls with necrotic supernatants from un-transfected cells and un-programmed wheat germ extracts. Moreover, the authors did not show bioactivity in a *bona fide* ST2-dependent bioassay and did not validate their assays with St2 KO cells (*in vitro* experiments) or mice (*in vivo* experiments). Furthermore, the authors claimed that the C-terminal part of IL-33 (residues 179–270; deletion of two-thirds of the IL-1-like cytokine domain) is dispensable for ST2-dependent activity [90]. However, a previous carefully controlled study showed that C-terminal residues 205–270, that are deleted in an inactive form of IL-33 found in individuals that have reduced blood eosinophil counts and are protected from asthma and allergic rhinitis [91,92], are essential for binding to ST2 and induction of ST2-dependent responses by IL-33 [91]. In another series of experiments, Brusilovsky et al describe a role of the ripoptosome in IL-33 cleavage, but these findings are questionable because in all experiments the authors coinoculated cells with the cell-permeable proteasome inhibitor MG-132, that can induce spontaneous ripoptosome activation [93]. Finally, the authors performed a conditional KO of caspase 8 in bronchial epithelial cells to support a role of the ripoptosome and caspase 8 in the release of bioactive IL-33. However, AT2 cells in the alveoli, not bronchial epithelial cells, are the cellular sources of IL-33 protein in mouse lungs at baseline and after *Alternaria* exposure (see above, paragraph 3.2). Moreover, mice deficient for caspase-7, that acts downstream of caspase 8 show increased levels of IL-33 and type-2 allergic inflammation following allergen exposure [94]. Therefore, we believe that the findings and claims of Brusilovsky et al [90] are not reliable, and that there is absolutely no evidence left for IL-33 bioactive forms generated by intracellular proteolytic maturation. Furthermore, contrary to the authors claim, ripoptosome activation is not a relevant mechanism of IL-33 release, because the caspase-cleavage products are biologically inactive.

Although cleavage of IL-33<sub>FL</sub> by caspases results in its inactivation [69,87,89], we demonstrated that mechanisms increasing IL-33 activity also exist [10,19,20]. IL-33<sub>FL</sub> is biologically active [69,87–89]. However, its cleavage in the central domain by various proteases from

inflammatory cells, including neutrophil elastase and cathepsin G, and mast cell chymase and trypsin (Fig. 1), results in the generation of cleavage products of 18–21 kDa (mature forms) containing the C-terminal IL-1-like cytokine domain [19,20]. The biological activity of these mature forms is ~30- to 60-fold higher than that of IL-33<sub>FL</sub> [19–21]. An important role of IL-33<sub>FL</sub> cleavage within its central domain could be to release the C-terminal IL-1-like cytokine domain from the inhibitory effect of the N-terminal nuclear domain. Acidic residues within the IL-1-like cytokine domain (isoelectric point < 5) are critical for binding to ST2 [13], and the basic nuclear domain (isoelectric point > 10) may thus interfere some way with the binding of the acidic IL-1-like cytokine domain to the ST2 receptor.

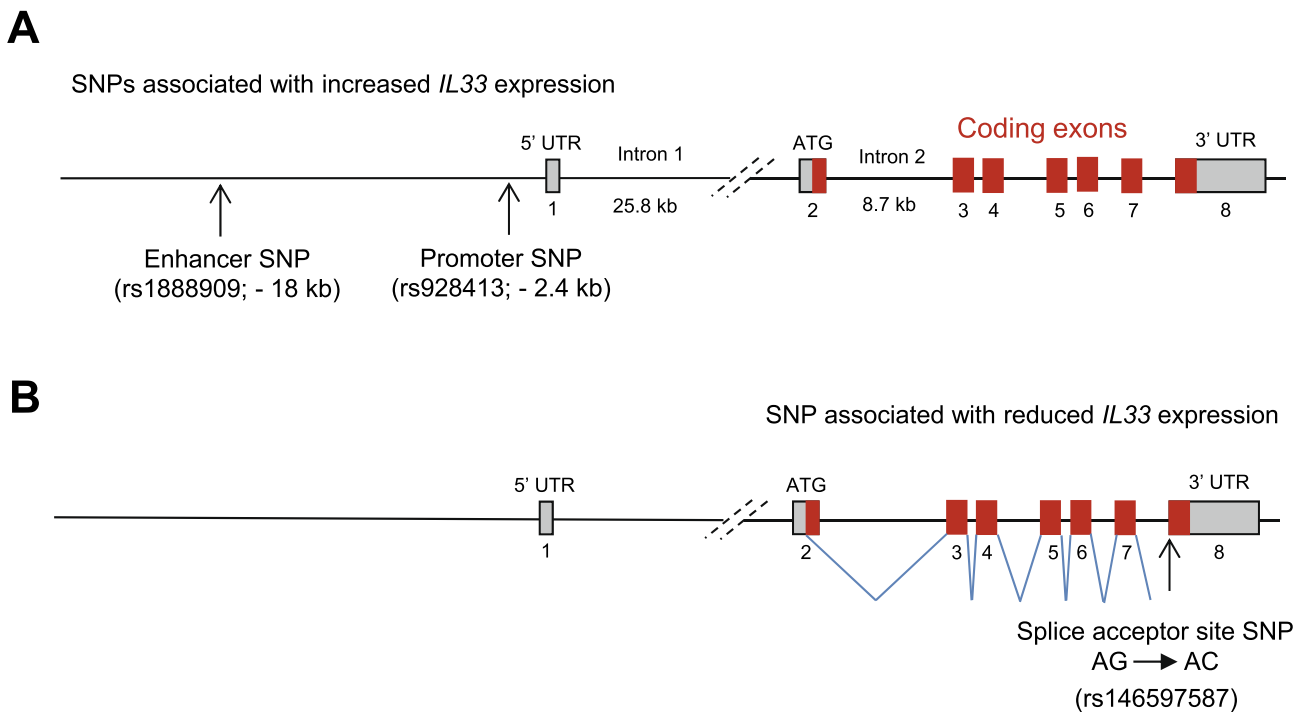
Recently, we discovered that IL-33<sub>FL</sub> functions as a protease sensor cleaved in its central ‘sensor’ or ‘activation’ domain by exogenous proteases from various environmental allergens, across four kingdoms, including fungi, house dust mites, bacteria and pollens [10]. We identified many sites of cleavage by allergen proteases within the IL-33 central sensor domain, including two sites cleaved by pollen and fungal allergen proteases, that are also cleaved by inflammatory proteases from neutrophils (cathepsin G) and mast cells (chymase), corresponding to IL-33 mature forms IL-33<sub>95-270</sub> and IL-33<sub>109-270</sub> (Fig. 1). Whatever their N-terminal sequence, the mature forms of IL-33 generated by allergen proteases had much greater biological activity than that of IL-33<sub>FL</sub> [10]. We detected cleaved forms of endogenous IL-33 proteins in human endothelial cell supernatants, 10 min after exposure to *Alternaria ex vivo*, and in mouse BAL fluids, 15 min after intranasal administration of the allergen extract [10]. Our findings revealed a critical mechanism for the rapid induction of allergic inflammation following allergen exposure [10], with important implications for allergic diseases (see below, paragraph 8.2 and Figs. 4,5). Processing of endogenous IL-33 by inflammatory and allergen proteases has been shown *in vivo* in the lungs (the identity of endogenous IL-33 protein was validated using *IL33*-deficient mice) [10,19]. In contrast, although calpain proteases can cleave IL-33<sub>FL</sub> *in vitro*, calpain activity was not required for cleavage of endogenous IL-33 protein in BAL fluids after exposure to *Alternaria in vivo* [21].

Proteolytic maturation of IL-33<sub>FL</sub> by inflammatory and/or allergen proteases is an important regulatory mechanism that results in enhanced cytokine activity [10,19–21]. However, inflammatory and/or allergen proteases, when present in large amounts, could also be involved in IL-33 protein degradation and termination of its activity [5]. Indeed, we observed that mouse IL-33 protein was particularly sensitive to degradation by allergen proteases [10]. IL-33 is rapidly released upon cell damage and tissue injury (see paragraph 6). However, the protein is no longer detectable in extracellular fluids after a few hours [30,33,70,73,80,83]. Proteolytic degradation is thus likely to be an important mechanism that limits IL-33 bioactivity *in vivo*. Extracellular oxidation of the protein could also be important for termination of IL-33 bioactivity [72].

## 8. IL-33 in disease: Focus on genetic and environmental regulation of IL-33 in allergic airway inflammation and asthma

### 8.1. *IL33* in genetic susceptibility to asthma

Genetic studies indicate that the IL-33/ST2 axis plays a crucial role in susceptibility to asthma [1,95]. Indeed, the genes *IL-33* (9p24) and *IL1RL1* (2q12), encoding ST2, were reproducibly identified as top hits in all major genome-wide association studies of asthma [2,95–99]. *IL33* and *IL1RL1* single nucleotide polymorphisms (SNPs) are particularly associated with blood eosinophils counts in the general population [96,100], eosinophilic asthma [96,100], and frequent virus-induced exacerbations in severe childhood-onset asthma [98]. Most *IL33* SNPs previously associated with asthma are located in close proximity to each other in the main *IL33* promoter/enhancer region [25,100] (Fig. 4A). Such observations suggested that specific genetic variation at the *IL33*



**Fig. 4. Genetics: regulation of *IL33* expression levels in asthma.** (A–B) Structure of the human *IL33* gene. The gene contains eight exons, and is located on chromosome 9 at 9p24.1. Exon 1 (also designated exon 1a), corresponding to the main promoter active in tissue-derived cells, is located 25.8 kb upstream of exon 2 containing the AUG codon. An alternative exon 1b, located 4.6 kb upstream of exon 2 has also been described. Most *IL33* single nucleotide polymorphisms (SNPs) associated with asthma in genome wide association studies (rs1342326, rs2381416, rs1888909, rs992969, rs3939286, rs928413) are located in the main promoter and enhancer regions in a linkage disequilibrium block of 41 kb in European ancestry individuals [25] (A). A rare loss of function mutation in a splice acceptor site SNP before exon 8 results in reduced levels of *IL33* mRNA and IL-33 protein in heterozygotes [91,101] (B).

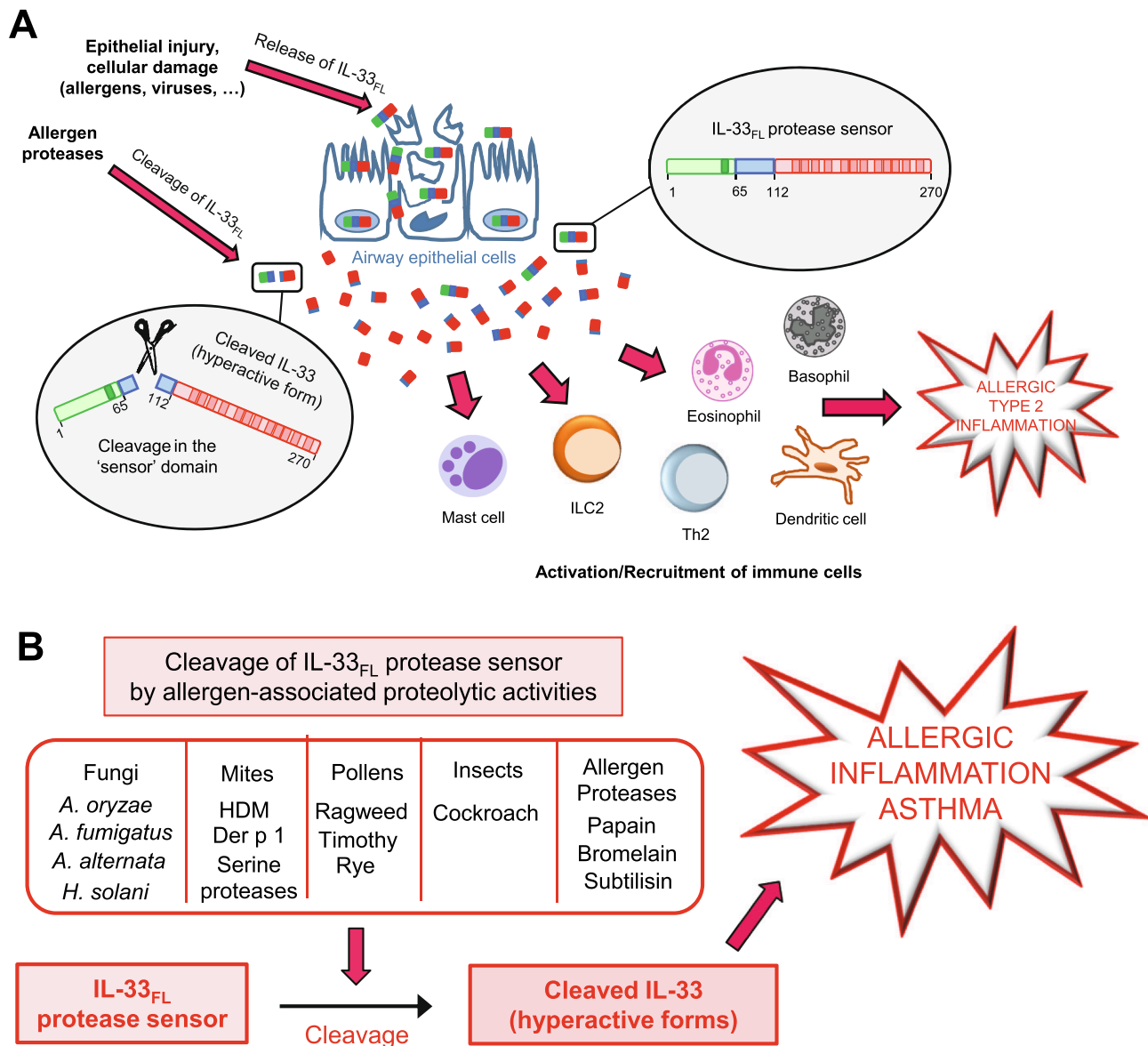
locus regulates eosinophil numbers and asthma through modulation of *IL33* transcription and *IL33* mRNA levels in producing cells. The risk allele of one SNP located in the *IL33* promoter (rs928413), 2.4 kb upstream the main *IL33* transcription start site, associates with higher level of blood eosinophils, higher risk of eosinophilic asthma, and higher *IL33* mRNA levels in bronchial epithelial brushes [100]. Interestingly, the risk allele of another SNP (rs1888909), 18 kb upstream the main *IL33* transcription start site, is located in a 5 kb enhancer essential for *in vivo* activity of the human *IL33* promoter in endothelial cells and basal epithelial cells, the two major cellular sources of IL-33 in human lungs [25]. Individuals who carried the asthma risk allele at rs1888909 had higher *IL33* transcript (bronchial epithelium, nasal epithelium) and IL-33 protein (plasma) levels compared to non-carriers of this allele [25].

A rare loss-of-function mutation in *IL33* that disrupts a canonical splice acceptor site before the last coding exon (Fig. 4B) results in about 40% lower total *IL33* mRNA levels in adipose tissue and a 46% reduction in total IL-33 protein levels in serum in heterozygotes [91,101]. The protein encoded by the mutant allele lacks the last 66 C-terminal residues that are essential for binding to ST2 and activation of IL-33 target cells [91]. Heterozygotes carrying the mutation have reduced blood eosinophils counts and are protected from asthma and allergic rhinitis [91,92]. Human homozygotes for the splice site mutation ( $n = 9$ ) neither showed reduced life expectancy nor increased susceptibility to other diseases [91]. A predicted very low level of IL-33 is thus compatible with long life.

Together, the human genetic studies of asthma-associated SNPs and loss-of-function mutation in *IL33* indicate that changes in *IL33* expression have a critical role in regulating blood eosinophil levels and susceptibility to allergic diseases. Deletion of *IL33* or *St2* in mice leads to significant reduction in blood eosinophils at baseline [91,102], and to reduced type 2 inflammation in different models of allergic diseases [1,3,4], thus mimicking human observations.

## 8.2. Regulation of *IL-33* protein bioactivity by proteases from environmental allergens

Genetic factors, such as the *IL33* and *IL1RL1* asthma-associated SNPs, play important roles in asthma. However, the environment is also critical. Indeed, we demonstrated a direct regulation of human IL-33 protein bioactivity by environmental allergens [10]. We found that human IL-33<sub>FL</sub> is cleaved and activated by proteases from environmental aeroallergens central to the development of allergic asthma, including fungi (*Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Helminthosporium solani*), house dust mites (HDM, *Dermatophagoides pteronyssinus*), cockroaches (oriental) and pollens (ragweed, timothy, rye) (Fig. 5). Using various biochemical assays and protease inhibitors, we showed that IL-33<sub>FL</sub> is cleaved by serine proteases from *Alternaria*, *Aspergillus*, HDM and timothy pollen, HDM cysteine protease Der p1, and serine and cysteine proteases associated with occupational asthma such as papain, bromelain and subtilisin [10]. Another group claimed that *Alternaria*-derived cysteine protease activities rather than serine proteases mediate the cleavage of IL-33<sub>FL</sub> [21], however cysteine protease inhibitors had no effect on IL-33 processing in BAL fluids *in vivo* [21], and *Alternaria* extracts were found to contain serine but not cysteine protease activities [71]. We showed that the hyperactive mature forms of IL-33 generated by allergen proteases are potent inducers of allergic airway inflammation *in vivo* and have an increased capacity to activate production of type 2 cytokines by lung ILC2s compared to IL-33<sub>FL</sub> protein [10]. Since cleaved IL-33, but not IL-33<sub>FL</sub>, was active at low doses, we reasoned that IL-33<sub>FL</sub>, when present at low levels in extracellular fluids *in vivo*, may have little activity on its own, and may rather function as an allergen protease sensor, i.e. a biochemical sensor activated after proteolytic maturation by allergen proteases. We provided experimental evidence (*in vivo*, *ex vivo*) showing that both recombinant and endogenous human IL-33<sub>FL</sub> protein induce potent type 2 responses after cleavage and activation by allergen proteases [10]. Kinetic analyses



**Fig. 5. Environment: regulation of IL-33 bioactivity by environmental allergens.** (A) Role of IL-33<sub>FL</sub> as an allergen protease sensor. IL-33<sub>FL</sub> alarmin is released from airway epithelial cells after tissue injury or cellular damage induced by allergens (proteases, PLA2, chitin,...) or other factors (viruses,...). In the presence of allergen proteases, IL-33<sub>FL</sub> does not accumulate at high levels extracellularly because of its rapid cleavage by allergen proteases. Therefore, when cellular damage is due to an allergen protease or a protease-containing allergen, there is a tight coupling of IL-33<sub>FL</sub> release and cleavage. Cleaved IL-33 forms generated by allergen proteases are hyperactive in various biological assays and can induce potent stimulation of ILC2s and other immune cells involved in allergic type 2 inflammation (Th2 cells, mast cells, eosinophils, basophils, dendritic cells, type 2 macrophages). When allergens do not contain proteolytic activity, IL-33<sub>FL</sub> could still induce type 2 inflammation, but only when present in large amounts because it has little activity at low concentration. Inflammatory proteases released by inflammatory cells (i.e. mast cell chymase or tryptase) could also contribute to cleavage and activation of IL-33<sub>FL</sub>. (B) Detection of allergen-associated biochemical (proteolytic) activities by IL-33<sub>FL</sub> protease sensor. IL-33<sub>FL</sub> is processed into shorter mature forms containing the C-terminal IL-1-like domain, by a wide-variety of environmental allergens and exogenous proteases from fungi, insects, plants and bacteria. IL-33 mature forms generated by allergen proteases are potent inducers of allergic airway inflammation *in vivo* and could play important roles in asthma.

indicated that *A. alternata*-induced IL-33 release and cleavage occur in a two-step mechanism: release of uncleaved IL-33<sub>FL</sub> form shortly after allergen exposure (step 1), rapidly followed by extracellular cleavage into shorter mature forms (step 2). Thus, we believe that IL-33<sub>FL</sub> does not accumulate in the airways following exposure to allergens containing proteolytic activity, due to its rapid cleavage and activation by allergen proteases (Fig. 5). We proposed that this IL-33-based protease sensing mechanism could play an important role in the rapid and efficient activation of ST2<sup>+</sup> immune cells (ILC2s, mast cells, ...) and induction of allergic type 2 responses following allergen exposure.

When the inducers of release did not exhibit protease activity

(mechanical stress, chitin, PLA2), or when *A. alternata* protease activity was blocked with serine protease inhibitor alpha-1 antitrypsin, IL-33 was released in the extracellular space as an uncleaved IL-33<sub>FL</sub> form and cleaved IL-33 was not detected [10]. Uncleaved IL-33<sub>FL</sub> was the major form detected in BAL fluids 30 min after a single intranasal exposure to PLA2 *in vivo*, indicating the absence of cleavage by endogenous proteases at this early time point [10]. IL-33<sub>FL</sub> could be the bioactive form when released at high levels in extracellular fluids. At later time points, and in chronic phases of allergic inflammation and allergic disease, endogenous proteases released from inflammatory cells (mast cells, neutrophils) could contribute to IL-33<sub>FL</sub> cleavage. However,

when allergens themselves contain protease activity, endogenous host proteases will be less relevant because direct cleavage and activation of IL-33 by allergen proteases will occur rapidly (<15 min). Cleavage of IL-33<sub>FL</sub> by proteolytic activities associated with environmental allergens is likely to play a critical role in the induction of allergic inflammation by lowering the threshold for activation of IL-33-mediated responses (Fig. 5).

Thus, both genetic factors (SNPs) and environmental factors (allergen proteases) contribute to the critical role of the IL-33/ST2 pathway in allergic airway inflammation and asthma. Our observations that a wide variety of environmental aeroallergens are able to cleave and activate the IL-33<sub>FL</sub> protease sensor, support a crucial role for this mechanism in asthma.

## 9. IL-33 in the clinics: anti-IL-33 antibodies for therapy of asthma and COPD

### 9.1. Targeting IL-33 for therapy of asthma and other allergic diseases

The results of the first clinical trials of monoclonal antibodies targeting IL-33 (Itepekimab, human IgG4 mAb, Sanofi-Regeneron Pharmaceuticals) and ST2 (Astegolimab, human IgG2 mAb, Genentech) in patients with asthma have recently been reported [103,104]. In a randomized phase 2 clinical trial that included 296 individuals, IL-33 blockade with itepekimab improved asthma control and quality of life, as compared to placebo in patients with moderate-to-severe asthma [103]. Itepekimab treatment led to increased levels of IL-33 in serum, which is reflective of a longer half-life of IL-33 when it is bound to itepekimab [103]. Such increased levels of IL-33 in serum occur in healthy volunteers administered with itepekimab, suggesting continuous release and degradation of IL-33 in serum during homeostasis in healthy individuals [105]. Itepekimab therapy reduced blood eosinophil counts [103]. In a phase 1 study, itepekimab injected subcutaneously (4 doses over a one month period) reduced blood eosinophil counts by 35–40% [105]. A similar reduction of blood eosinophil counts was observed after treatment with the anti-ST2 antibody astegolimab in a double blind, placebo controlled study of patients (n = 502) with inadequately controlled, severe asthma [104]. Interestingly, anti-ST2 mAb reduced asthma exacerbation rates in a broad population of patients with severe asthma, including those with low blood eosinophils who have limited treatment options [104]. Further clinical trials of anti-IL-33 and anti-ST2 mAbs in asthmatic patients with elevated (type 2-high) or low (type 2-low) blood eosinophils are required. Even if the improvement of asthma control with itepekimab was not better than with dupilumab (anti-IL-4R $\alpha$ ) [103], targeting the IL33/ST2 pathway may benefit patients that do not benefit from dupilumab. Although some clinical trials with anti-ST2 antibodies were discontinued, a phase 2 clinical trial in asthma recently started for another anti-IL-33 mAb (Tozorakimab, Astra Zeneca).

Studies in preclinical mouse models show that IL-33 plays critical roles in allergic inflammation in various tissues. Anti-IL-33 mAbs could thus be useful for therapy of allergic diseases affecting different tissues sites. Phase 2a clinical trials with another anti-IL-33 mAb (Etokimab, IgG1; AnaptysBio) suggest potential roles of IL-33 in atopic dermatitis [106] and peanut allergy [107]. However, these studies only included a limited number of patients (n = 12 in atopic dermatitis; n = 20 in peanut allergy). Additional studies are thus required.

### 9.2. Targeting IL-33 for therapy of COPD

An important role of IL-33 in COPD emerged from analyses of mouse pre-clinical models and human clinical samples [38,39]. Lung IL-33 levels increase in animal models of COPD (cigarette-smoke induced COPD, postviral chronic obstructive lung disease) and in patients with severe COPD [38,39]. In humans, IL-33 expression localizes to the lung microvasculature and to a subset of airway basal cells, particularly

prominent in areas of epithelial hyperplasia and mucous remodeling in COPD patients [38]. Cigarette smoke and infection are the major drivers of COPD. Loss of *IL33* protects mice from smoke-induced exaggerated inflammatory responses to influenza virus infection, suggesting that IL-33 plays critical roles in pathogen-induced exacerbations of COPD [39].

Genetic analyses demonstrated association of loss of function in *IL33* (splice site mutation rs146597587-C) with reduced COPD risk, and gain of function in *IL33* and *IL1RL1* with increased risk [101]. Moreover, anti-IL-33 mAb itepekimab was found to reduce chronic airway type 1 (neutrophilic) and type 2 (eosinophilic) inflammation in a humanized mouse model of persistent and exacerbating airway disease characterized by a mixed inflammatory phenotype [59]. IL-33 blockade with itepekimab was thus tested in a randomized phase 2 clinical trial that included 343 COPD patients. Although the study did not meet the primary endpoint in the overall population, subgroup analysis showed that itepekimab reduced exacerbation rate and improved lung function in former smokers with COPD, who are an important clinical subset [101]. Phase 3 clinical trials are awaited to confirm the efficacy of itepekimab in former smokers with COPD.

## 10. Concluding remarks

We discovered IL-33 as an endothelial cell nuclear factor and visualized the expression of the endogenous protein in human tissues almost two decades ago [9]. Over the past 20 years, we have tested many commercially available reagents, including antibodies for immunofluorescence/immunohistochemistry, flow cytometry, and western blot analyses, and ELISA assays. We believe that it is our responsibility to alert researchers working on IL-33 and newcomers in the field of the issues with specificity of IL-33 reagents and the absolute necessity to perform appropriate specificity controls in each experimental system and model analyzed. Fortunately, there are many solid articles on IL-33 based on sound science with appropriate controls. Unfortunately, there are also several studies published recently that made claims that are misleading for the field, because they used inappropriate tools and did not carry out the necessary checks. The use of nonspecific tools in the absence of appropriate controls leads to unnecessary controversy and a significant waste of resources. We thus think it is time to sound the alarm even louder than in our previous reviews. Our final words: ‘Please include specificity controls in your future IL-33 studies. This will have at least two benefits. First, and most importantly, you will get reliable results, and second, you will validate tools or models that may be useful to others working in this field.’

### Author contributions

The two authors wrote and discussed the implications of the manuscript. They prepared Tables and Figures, and approved the submitted version of the manuscript.

### CRediT authorship contribution statement

**Corinne Cayrol:** Conceptualization, Validation, Writing – review & editing, Visualization. **Jean-Philippe Girard:** Conceptualization, Validation, Writing – review & editing, Visualization.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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