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Title: IL-33 in the developing lung – roles in asthma and infection

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Abbreviations: AHR, Airway hyperresponsiveness; **IgE, Immunoglobulin E**; IL, Interleukin; IFN, Interferon; RV, Rhinovirus, RSV, respiratory syncytial virus; ILC2, type 2 innate lymphoid cell; MAPK, Mitogen-activated protein kinase; MyD88, Myeloid differentiation primary response 88; NFκB, Nuclear factor kappa-light-chain enhancer of activated B cells; PVM, Pneumonia Virus of Mice; Th2, T helper type 2; Treg, T regulatory; TSLP, thymic stromal lymphopoietin

Abstract

It has become increasingly clear that interleukin-33 (IL-33) plays a crucial role in initiation of type 2 immunity. The last decade of intense research has uncovered multiple mechanisms through which IL-33 targets key effector cells of the allergic immune response. Recently, IL-33 has been implicated in shaping the immune system of the lungs early in life, at a time which is crucial in the subsequent development of allergic asthma. In this review, we will address the current literature describing the

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role of IL-33 in the healthy and diseased lung. In particular, we will focus on the evidence for IL-33 in the development of immune responses in the lung, including the role of IL-33-responsive immune cells that may explain susceptibility to allergic sensitisation at a young age and the association between genetic variants of IL-33 and asthma in humans. Finally, we will indicate areas for potential therapeutic modulation of the IL-33 pathway.

Interleukin-33 (IL-33) is a critical mediator of allergic immune responses, and has been implicated in multiple allergic and inflammatory diseases. In this article, we will first review the current knowledge of the basic biology of expression, release and control of IL-33, the dynamic control of IL-33 responses during lung development in early life, its cellular targets in the lung, and its roles in allergic asthma and respiratory viral infection. Finally, we will discuss the implications that these findings have for clinical intervention.

IL-33 Biology

IL-33 is an “alarmin” cytokine – i.e. it is constitutively expressed by endothelial cells, and in epithelial cells of barrier sites such as the intestine, skin and lung ¹. It is released upon necrotic cell death, and due to its expression pattern it is thus well-placed at barrier sites to detect toxic stimuli, whether they be intrinsic (e.g. during ischemia), or extrinsic, as occurs on parasitic or viral infection, or inhalation of toxic stimuli such as cigarette smoke, diesel particulates or protease-containing allergens ². IL-33 is a potent inflammatory mediator, and as such it is kept under tight regulation. In the following sections we will focus first on the mechanisms by which IL-33 is induced and activated, then the mechanisms by which its activity is inhibited and controlled.

Activation and release of IL-33:

The full-length IL-33 protein consists of a N-terminus chromatin-binding domain, a central region containing multiple protease cleavage sites, and a C-terminus receptor-binding domain (**Figure 1**). IL-33 lacks the signal peptide required for conventional secretion, and is instead transported to the nucleus of the expressing cell, where it is anchored to chromatin through its N-terminus chromatin-binding domain. Thus IL-33 is only released during necrosis, cytolysis and release of the nuclear contents ³. The strong binding of IL-33 to chromatin appears to be a mechanism to slow its release from necrotic cells, thus extending its effective half-life of release ⁴. It was initially hypothesized that

nuclear IL-33 could be acting as a transcription factor, however recent studies indicate that nuclear IL-33 does not affect gene expression within the IL-33-expressing cell ⁴. Conversely, deletion of the IL-33 chromatin-binding domain leads to uncontrolled IL-33 release, and lethal multi-organ inflammation ⁵. Recently, it was shown that full-length IL-33 can be released from necrotic cells in complex with histones, and that IL-33-histone complexes have increased activity compared to full-length IL-33 alone ⁴. The activity of IL-33 can be further increased by protease cleavage between the chromatin-binding and receptor-binding domains ⁶. This cleavage occurs via a range of cysteine- and serine-proteases, which can be intrinsic to the IL-33-expressing cell, released by recruited mast cells or neutrophils, or present in allergens ^{6,7}. An interesting context for IL-33 study is NETosis (i.e. formation of Neutrophil Extracellular Traps), where recruited neutrophils release genomic DNA and histones, as well as neutrophil proteases such as elastase and cathepsin G – IL-33 release in the presence of these products would be highly active due to synergistic signaling with neutrophil histones, and/or processing by neutrophil proteases. Of note, NETosis is associated with asthma exacerbations ⁸.

IL-33 expression is also upregulated during allergic responses. This may occur at the level of cell-intrinsic protein expression, with factors (including IL-33 receptor signaling itself) upregulating IL-33 ⁹, or at the cellular population level, with epithelial cell proliferation resulting in a larger pool of IL-33-producing cells ¹⁰. Thus, epithelial cells may “remember” previous allergic stimuli, resulting in exacerbated IL-33 responses on subsequent stimuli, and a vicious cycle of allergic responsiveness.

Inhibition or downregulation of IL-33 activity:

The activity of IL-33 is controlled at multiple levels. IL-33 is a member of the IL-1 family of cytokines: its C-terminal receptor-binding domain shows significant structural homology to other IL-1-family members, such as IL-1 β and IL-18 ². IL-1 β and IL-18 are produced as inactive pro-forms, which are processed into an active mature form by caspases – proteases with critical roles in apoptosis (programmed cell death) or in inflammation (in the “inflammasome” an intracellular complex formed during inflammatory responses). Although caspases also cleave IL-33, in contrast to IL-1 β and IL-18, this cleavage occurs within the receptor binding domain, effectively inactivating IL-33 in the context of apoptosis and inflammasome activation ¹¹.

A further critical control mechanism of IL-33 is its oxidative state. IL-33 contains 4 cysteines in its receptor-binding domain. In the reducing environment of the nucleus, these cysteines are kept in a reduced, unbound state, with no disulphide bond formation. However, as the cytokine is released into the oxidative extracellular environment (in response to allergen stimulation or spontaneous necrosis in culture of mouse or human lung tissue), the reduced cysteines in IL-33 bind each other in a process of oxidation and disulphide bond formation. This results in a conformational change of the cytokine, rendering it incapable of binding to its receptor. This process occurs rapidly in vitro and in vivo – within 1 h of IL-33 release, the cytokine is effectively inactivated ¹².

IL-33 is also regulated at the level of receptor signaling. The IL-33 receptor consists of the “suppression of tumorigenicity 2” (ST2) protein and the IL-1 receptor accessory protein (IL-1RAcP) ¹³. IL-33 binds with high affinity to ST2 and then recruits IL-1RAcP, mediating signaling through the signaling proteins MyD88, MAPK, and NF- κ B ². In order to signal to T cells to induce antigen-independent IL-13 production (see “IL-33 responsive cells in the lung”), the IL-33 receptor must also interact with the epidermal growth factor receptor (EGFR) ¹⁴, while in mast cells, the IL-33 receptor must interact with c-kit ¹⁵, adding to specificity of responses dependent on the responding cell. As with all IL-1 family cytokines, IL-33 signalling is negatively regulated by the single Ig IL-1-related receptor (SIGIRR), an IL-1-family receptor with no ligand, and constitutive negative regulatory signalling ¹⁶. ST2 and IL-1RAcP can be produced as membrane-bound receptors, or as soluble sST2 and soluble IL1RAcP forms which bind active IL-33, inhibiting its activity in solution, and further adding to the tight spatiotemporal regulation of this cytokine ¹⁷. These multiple regulatory pathways imply that the cytokine has potentially dangerous effects which must be controlled at multiple checkpoints: at the levels of transcription, after translation and in responder cells (**Figure 2**).

IL-33-responsive cells in the lung

ST2 is expressed on a number of innate and adaptive immune cells in the lung, including mast cells, eosinophils, basophils, macrophages, type 2 innate lymphoid cell (ILC2s), CD4⁺ T helper type 2 (Th2) cells and T regulatory (Treg) cells ¹⁸. Activation of these cells by IL-33 is strongly associated with type 2 (pro-allergic) immune responses ².

In basophils and mast cells, IL-33 stimulation induces IL-4 production which is important for immunoglobulin E (IgE) production by B cells in the context of allergic diseases^{19,20}. Furthermore, IL-33 enhances mast cell degranulation and is capable of inducing cytokine production independently of IgE-FcεRI signaling²¹. M2 polarized alveolar macrophages develop under the influence of IL-4, IL-13 or IL-33 and contribute to tissue remodeling in asthma, producing CCL24 (Eotaxin-2) and CCL17 (TARC) to recruit eosinophils and T cells, respectively²². Eosinophils themselves are also directly stimulated by IL-33, causing degranulation, increased cell survival and eosinophil lineage commitment by precursor cells^{23,24}.

Naïve CD4⁺ T cells are not IL-33-responsive, but on differentiation to either a Treg or Th2 phenotype, they upregulate ST2. Antigen-specific Th2 cells produce IL-13 in an IL-33-dependent manner and IL-5-producing memory Th2 cells are critical targets of IL-33 in airway inflammation^{25,26}. Responses of Treg to IL-33 are context-dependent – in colitis responses dominated by a Th1/Th17 phenotype, IL-33 signalling increases Treg suppressive capacity²⁷, while in a Th2 milieu, ST2⁺ Treg cells lose their suppressive capacity in response to IL-33 stimulation *in vivo* and increase their expression of ST2, type 2 cytokines and the canonical Th2 transcription factor GATA-3²⁸.

Type 2 innate lymphoid cells (ILC2)

The response to IL-33 is often dominated by ILC2s, and these cells have critical roles in responses in the lung. ILC2s rapidly produce large amounts of IL-5 and IL-13 upon IL-33 activation. Located near epithelial barriers, ILC2s are in a prime position to respond to epithelial-derived signals such as IL-33. In the tissue, ILC2s are long-lived and can undergo local expansion upon infection and IL-33 stimulation^{29,30}. Furthermore, airway allergen exposure in adult mice was recently found to induce IL-33-dependent migration of ILC2 progenitors to the lung from the bone marrow³¹, further increasing the pool of lung ILC2s.

An emerging field with important implications in ILC2 biology is innate immune memory³². ILC2s in the lungs of adult mice that were previously exposed to allergens acquired a gene signature similar to memory T cells, and produced more type 2 cytokines upon IL-33 stimulation compared to ILC2s from naïve lungs³³. Likewise, after allergic stimulus, IL-33-producing epithelial cells proliferate and/or upregulate IL-33 expression^{9,10} increasing the pool of stored IL-33 and increasing IL-33

release upon subsequent stimulus. The fact that the epithelium-ILC2 axis possesses memory features likely play a role in long-lasting type 2 immunity and highlights the importance of understanding immune mechanisms early in life.

In addition to the immune system, IL-33 receptor signalling has been implicated in interactions with the nervous system. ST2-expressing sensory neurons of the skin respond to IL-33 from epidermal keratinocytes, mediating itch in contact allergy³⁴. Neuronal networks have a protective role in the airways where chemical and physical stimuli initiate responses such as coughing and potentiate activation of both immune cells and neurons. Intriguingly, crosstalk between neuronal cells and ILC2s have been reported to occur at mucosal barriers³⁵, however the understanding of interactions between neuronal cells and ILC2s (or other IL-33-responsive cells) in the lung is still very limited.

Therefore, the release of IL-33 is kept under tight control, and in the lung IL-33 activates a coordinated response through a range of immune and non-immune cells, which can lead to type 2 inflammation in allergic disease. The role of IL-33 in early life has received a great deal of interest in recent years, as IL-33 appears to play a critical role in the development of lung immune responses.

IL-33 in lung development

IL-33 expression changes dynamically during lung development. In mice, IL-33 levels and IL-33-producing epithelial cell numbers in the developing lung increase rapidly at birth, reducing to baseline levels from the third week of life to adulthood^{36,37}. This rapid increase in IL-33 levels at birth appears to be due to the mechanical damage caused by replacement of liquid in the developing lung with gas inflation at the first breath, and could be replicated by exposure of late-stage murine embryos to a vacuum³⁷. This early-life IL-33 release leads to the development of a type 2 environment in the developing lung, which reaches a peak during the alveolarization phase of lung development at days 7-14 of life in mice, thought to be equivalent to 2 to 3 years of age in humans³⁶. During this alveolarization phase of lung development the lung epithelium undergoes final differentiation and remodeling, and ILC2s, mast cells, eosinophils and basophils accumulate in the lung in an IL-33-dependent manner^{36,37}. The pro-allergic environment of the lung at this critical time point has functional consequences as mice sensitized at day 14 of life have a much-increased response to allergen challenge in later life than those sensitized as adults³⁶. Furthermore, IL-33-

dependent IL-13 production by ILC2s in early life results in a lasting M2 phenotype of alveolar macrophages, in turn leading to reduced immunity to bacterial lung infections³⁷. Thus early-life IL-33 release may be designed to prevent excessive inflammation in response to exposure to inhaled bacteria.

Even earlier in lung development, at day 3 of life, mice are also predisposed to allergic responsiveness, however at this timepoint IL-33 and ILC2 IL-13 responses are dispensable for airway hyperresponsiveness (AHR), while AHR is absolutely dependent on IL-13 production by lung CD4+ T cells³⁸. Interestingly, in mice lacking IL-13 expression specifically in CD4+ T cells, only AHR was ablated while IL-33 levels, ILC2 cytokine production, and eosinophilia remained identical to wild-type mice³⁸. Thus other cytokines may be involved in the initiation of allergy in very early life while IL-33 is required later, and into adulthood. These studies highlight the importance of precise definitions regarding “neonatal” mouse studies (which can range from day 3 to 14 of life) and the dynamic nature of IL-33 responses during lung development.

The importance of IL-33 in early life is therefore age- and context-dependent, and can lead to the development of airway allergy. In the following sections we will discuss how lung IL-33 can affect allergic sensitization, and the development of asthma, with a focus on early-life stimuli.

IL-33 in asthma

The inflammatory response in asthma involves many different specialized immune cells and structural cells of the airways which contribute to considerable disease heterogeneity. Nevertheless, most asthmatic children and over 50% of adults with asthma display evidence of ongoing type 2 airway inflammation with atopy, increased number of airway and blood eosinophils, mucus overproduction and high IgE levels³⁹. Type 2 immunity is stimulated by epithelial barrier damage to activate wound healing and tissue remodeling. Regulation of these reparative responses restores tissue homeostasis, however, in asthma, epithelial dysfunction and uncontrolled type 2 inflammation result in chronic disease with airway remodeling and bronchial hyperresponsiveness.

As a potent inducer of type 2 immunity, there is a strong positive relationship between IL-33 and asthma. Gene polymorphisms in *IL33* and *IL13/1* (encoding ST2) are associated with an increased risk of developing asthma ⁴⁰⁻⁴², whereas a loss-of-function mutation in the *IL33* gene is associated with reduced levels of blood eosinophils and lower asthma prevalence ⁴³. Similarly, interruption of IL-33 signaling in adult mice results in decreased basal levels of eosinophils ²⁴ and allergen-induced eosinophil infiltration and airway hyperresponsiveness are attenuated in neonatal or adult mice that lack IL-33 or ST2 ^{44,45}. Conversely, administration of IL-33 in mouse models exacerbates allergen-induced airway responses ⁴⁶.

Studies of human asthma support the importance of IL-33: elevated IL-33 levels correlate with increased asthma severity in both children and adults ^{45,47-49}, while expression of IL-33 in bronchial epithelium and airway smooth muscle cells correlates with AHR in asthma ⁵⁰, and an increased frequency of IL-33-responsive ILC2s are observed in patients with severe disease ⁴⁷. Furthermore, alternative splicing of the IL-33 transcript generates an isoform that retains signaling capacity and can (unlike the conventional isoform of IL-33) be secreted. Detection of this alternatively-spliced isoform is strongly associated with type 2 inflammation in stable asthma ⁵¹.

A study that compared the relative contribution of type 2 cytokines by ILC2s and CD4+ T cells in the airways found that even though CD4+ T cells were more abundant than ILC2s in sputum from adult asthmatic subjects, the ILC2s produced proportionally more cytokines and were the predominant source of IL-5 and IL-13 ⁵². This balance may be altered in infants, as ILC2 prevalence is increased in neonatal mice ³⁶ (see “IL-33 in lung development”), although this has not yet been directly measured in humans.

In addition to type 2 cytokines, IL-33-mediated activation of ILC2s induces production of amphiregulin, a growth factor that promotes collagen production in fibroblasts ⁵³, and induces proliferation and differentiation in epithelial cells ⁵⁴. Therefore amphiregulin can both enhance tissue repair following infection or injury, and increase tissue remodeling and fibrosis. Amphiregulin plays an important role in airway tissue remodeling and it is found at higher levels in induced sputum from children with asthma ⁵⁵.

The initial stimuli for IL-33 production, leading to the development of asthma, can be inhaled allergens, or infectious stimuli. Severe respiratory viral infections in early life are particularly strongly associated with the subsequent development of asthma, an interaction in which IL-33 may also play a critical role.

IL-33 in respiratory viral infection

As a sensitive indicator of epithelial damage, IL-33 is released during viral infection. The first study showing IL-33's role in a respiratory viral infection was in influenza of adult mice, in which airway hyperresponsiveness was found to be T cell independent, but IL-33, ILC2 and IL-13-dependent⁵⁶. Inhaled smoke can increase preformed IL-33 levels in an adult mouse model, resulting in a further exacerbated IL-33-mediated inflammatory response to influenza⁵⁷. Conversely, this influenza IL-33-ILC2 axis is also critical for correct healing and remodeling of the lung after infection, especially in neonatal mice and humans, through induction of ILC2 amphiregulin production^{53,58}. Thus it appears that in influenza infection, IL-33 has a pathological role early in infection, where it exacerbates pathogenic inflammation, and a protective role later as it aids in healing of the lung.

Respiratory Syncytial Virus (RSV) is a human respiratory virus which infects almost all children by 2 years of age. In a proportion of infants infected, RSV can cause severe bronchiolitis, and this severe disease has been linked to an increased risk of asthma^{59,60}. Evidence for a causative role of RSV bronchiolitis in the development of asthma comes from studies in which timing of birth correlated with development of asthma, with a birth date 4 months prior to peak winter virus season conferring the highest risk of asthma⁶¹. Furthermore, prophylactic treatment with an anti-RSV monoclonal antibody reduces incidence of reported wheeze to age 6, although this difference did not extend to physician-diagnosed asthma or lung function^{62,63}. There remains significant controversy in this field as to whether the association between RSV and asthma is causative or correlative, however these results suggest that RSV bronchiolitis, although not altering baseline lung function, could predispose the lung to increased incidence or severity of wheeze.

Attention has therefore focused on the role of RSV in early life, and its potential to lead to asthma through induction of type 2 immune responses. Increased IL-33 is seen in nasopharyngeal aspirates of infants with RSV bronchiolitis^{64,65}, while Single Nucleotide Polymorphisms (SNPs) in *IL13* are

implicated in susceptibility to both RSV bronchiolitis⁶⁶ and asthma⁴⁰. In mouse models of infection, RSV causes release of IL-33⁶⁷ and expansion of ILC2s⁶⁸. Interestingly, this response is much increased in RSV-infected 5-day-old neonatal mice (compared to adult mice), leading to allergic responses in later life which could be blocked with anti-IL-33 administration to neonates, or replicated with recombinant IL-33 administration to adults⁶⁵. Pneumonia Virus of Mice (PVM) is a murine virus which is related to RSV, and in neonatal mouse models coadministration of PVM and low dose allergen can also cause IL-33 release, allergic sensitization and asthma-like pathology in later life⁶⁹.

IL-33 release induced by viral infection also affects anti-viral Interferon (IFN) responses, and blockade of IL-33 during PVM infection of neonatal mice increases lung type I, II and III IFN responses^{69,70}, consequently reducing viral titre. Conversely, studies in mice have found that virus-induced IFN- γ dampens ILC2 activation and expression of the type 1 interferon (IFN) receptor on human and mouse ILC2s mediates suppression of type 2 cytokine production by the ILC2s⁷¹. Therefore, anti-viral and allergic responses are counter-regulatory, adding complexity to the association between viral infections and asthma.

Viral stimuli and damage are therefore capable of inducing IL-33 release, and this can have lasting effects on the development and exacerbation of asthma. These effects seem particularly potent in infants, due to increased IL-33 responses during development of the lung.

Clinical implications of IL-33 blockade

Clinical and genetic studies of allergy in children and adults, and animal model experiments using neonatal and adult mice have demonstrated an important role of IL-33 in the development of allergic immune responses at an early age. This has led to the development of a series of clinical trials aimed at blocking the IL-33 pathway in asthma (NCT03207243) and chronic obstructive pulmonary disease (NCT03546907; NCT03615040) in adults. Although these trials are aimed at treatment of established disease, mouse models appear to indicate that the role of IL-33 may be more critical in the initiation phase.

In humans, premature infants often receive supplemental oxygen, and can also suffer from lung developmental defects, as well as a higher risk of developing asthma⁷². A recent study showed that hyperoxia (i.e. supplemental 80% oxygen) resulted in increased inflammation when mice were subsequently exposed to allergens. Supplemental oxygen increased expression of IL-33, and the pro-allergic effect was dependent on IL-33 and ILC2s⁷³. Oxidative stress has also been shown to induce IL-33 release from human bronchial epithelial cells in vitro, with inhibitors of reactive oxygen species, or inhibitors of the oxidative stress pathways, reducing IL-33 release⁷⁴. Finally, in a safety study focusing on reducing oxygen saturation targets during bronchiolitis, reducing the oxygen saturation target from 94% to 90% was not only safe, but also proved to reduce the time taken for recovery, discharge, and return to normal feeding⁷⁵. These studies have implications for supportive supplemental oxygen treatment of infants, with high oxygen saturation potentially increasing subsequent allergic sensitization, and slowing recovery.

Clinical and experimental evidence supports the role of IL-33 in the initiation of allergic responses after neonatal viral infection, therefore an effective prophylactic against the development of allergic asthma may be to block IL-33 in infants at risk of developing asthma, especially during respiratory viral infection and supplemental oxygen administration.

IL-33 therefore provides a critical link between allergic sensitization, viral infection, and the development of asthma during early life. IL-33 may also represent a target for intervention to prevent or treat allergic asthma. However, IL-33 also has protective roles in many disease states (for instance in some infections and in resolution of damage), therefore treatments designed to block its effects may need to be approached with caution, to ensure that context-dependent deleterious side-effects are avoided.

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Figure 1: IL-33 processing and release.

The full-length (FL) IL-33 protein is stored in the nuclear compartment of the cell and consists of a N-terminal chromatin-binding domain (CBD), a protease-sensitive domain (PSD) and a receptor binding domain (RBD). During programmed cell death (apoptosis), intracellular caspases cleave IL-33 in the RBD, inactivating the protein and avoiding immune activation. IL-33 can also be inactivated after release due to formation of disulphide bonds in the oxidative environment outside of the nucleus, resulting in altered protein conformation. FL-IL-33 released by necrotic cells acts as an alarmin with high biologic activity. FL-IL-33 in complex with histones has higher activity compared to FL-IL-33 alone, and extracellular proteases from mast cells, neutrophils and allergens cleave IL-33 in the PSD, releasing a cleaved version of IL-33 with even more potent activity.

Figure 2: IL-33 signalling and downstream immune responses

IL-33 signals through a receptor complex consisting of transmembrane ST2 and IL-1R accessory protein (IL-1RAcP). Through alternative splicing, the ST2 gene product can also be expressed as a soluble form (sST2) which acts as a decoy receptor for IL-33. Upon binding to the receptor complex, IL-33 activates MyD88-dependent pathways leading to induction of type 2 cytokines and other inflammatory mediators in target cells. SIGIRR is a negative regulator which disrupts MyD88 signalling. Secretion of the type 2 cytokines IL-4, IL-5 and IL-13, and amphiregulin (Areg) through IL-33-mediated activation promotes production of allergen-specific immunoglobulin E (IgE), eosinophil expansion and tissue infiltration, goblet cell hyperplasia and mucus overproduction, and tissue remodelling by increased deposition of extracellular collagen.

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