

## REVIEW ARTICLE

# Alarmins and innate lymphoid cells 2 activation: A common pathogenetic link connecting respiratory syncytial virus bronchiolitis and later wheezing/asthma?

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**Abstract**

Severe respiratory syncytial virus (RSV) infection in infancy is associated with increased risk of recurrent wheezing in childhood. Both acute and long-term alterations in airway functions are thought to be related to inefficient antiviral immune response. The airway epithelium, the first target of RSV, normally acts as an immunological barrier able to elicit an effective immune reaction but may also be programmed to directly promote a Th2 response, independently from Th2 lymphocyte involvement. Recognition of RSV transcripts and viral replication intermediates by bronchial epithelial cells brings about release of TSLP, IL-33, HMGB1, and IL-25, dubbed “alarmins.” These epithelial cell-derived proteins are particularly effective in stimulating innate lymphoid cells 2 (ILC2) to release IL-4, IL-5, and IL-13. ILC2s, reflect the innate counterparts of Th2 cells and, when activated, are potent promoters of airway inflammation and hyperresponsiveness in RSV bronchiolitis and childhood wheezing/asthma. Long-term epithelial progenitors or persistent epigenetic modifications of the airway epithelium following RSV infection may play a pathogenetic role in the short- and long-term increased susceptibility to obstructive lung diseases in response to RSV in the young. Additionally, ILC2 function may be further regulated by RSV-induced changes in gut microbiota community composition that can be associated with disease severity in infants. A better understanding of the alarmin-ILC interactions in childhood might provide insights into the mechanisms characterizing these immune-mediated diseases and indicate new targets for prevention and therapeutic interventions.

**KEYWORDS**

airway epithelium, epigenetics, gut microbiota, high mobility group box 1, IL-25, IL-25, IL-33, IL-33, innate immunity, respiratory syncytial virus, Th2 lymphocytes, thymic stromal lymphopoietin

## 1 | INTRODUCTION

Respiratory syncytial virus (RSV) is a common and highly contagious pathogen inducing acute respiratory tract infections in infants.<sup>1</sup> Although in many cases, symptoms remain mild, a subset of individuals develops severe RSV-associated bronchiolitis.<sup>1,2</sup> The severity of the disease in infancy, thought to be related to the physiological immaturity of the immune system, is also linked to an increased risk of recurrent wheezing in later life.<sup>3,4</sup> This phenomenon is not always associated with atopic sensitization but is thought to be related to long-term immune response alterations and neuromuscular tone instability in the airways.<sup>3,4</sup> The airway epithelium, the primary target of RSV infection, is an important component of the antiviral response, acting both as a physical fence and as an immunological barrier able to elicit inflammatory and immune mediate responses.<sup>5</sup> Recognition of RSV transcripts and viral replication intermediates by bronchial epithelial cells (BECs) induces the production of type I and type III IFNs and other cytokines, all geared to mount an effective antiviral reaction.<sup>3,5</sup> However, BECs may also be programmed to release mediators that activate a Th2 immune response, aggravating the infection, and contributing to airway damage.<sup>6</sup> These mediators include thymic stromal lymphopoietin (TSLP), IL-33, the high mobility group box 1 (HMGB1) and IL-25.<sup>6,7</sup> Often characterized as “alarmins,” these epithelial cell-derived proteins regulate a broad spectrum of immune cell populations and are particularly potent in eliciting and activating type 2 innate lymphoid cells (ILC2) in a variety of lung disorders, including RSV bronchiolitis and recurrent wheezing/asthma in childhood.<sup>7-12</sup> It is therefore possible that, following a first severe respiratory infection in early life, long-term epithelial cell progenitors or persistent epigenetic modifications leading to alarmin-induced ILC2 activation may be involved in the ongoing increased susceptibility to obstructive lung diseases.<sup>13</sup> Through DNA methylation and histone ubiquitination, RSV can induce epigenetic modifications in BECs that favor Th2 responses,<sup>14</sup> promoting postbronchiolitis wheezing.<sup>15</sup> In RSV-infected BEC cultures, upregulation of the expression of the epigenetic regulator histone deacetylase2 (HDAC2) was associated with increased viral replication and production of proinflammatory cytokine, and oxidative stress-related molecules.<sup>16</sup> Evaluating cells obtained by nasal brushing at age 6 in preterm infants, who received RSV immunoprophylaxis with palivizumab or placebo during their first RSV season, demonstrated that palivizumab had global and persistent effects on nasal DNA methylation patterns, relevant innate immune viral response, and viral budding genes.<sup>17</sup> These data suggest that RSV-induced epigenetic modification in airway cell functions could represent a common pathogenetic imprint in severe RSV bronchiolitis and subsequent recurrent wheezing/asthma in childhood. Finally, ILC2 activation and disease severity can also be regulated through the production of alarmins by RSV-induced gut microbiome dysbiosis. Indeed, specific gut microbial profiles have been related to increased RSV bronchiolitis severity,<sup>18</sup> which is characterized by elevated numbers of ILC2s and higher levels of alarmins in the airways.<sup>19</sup>

### Key Message

In response to a respiratory syncytial virus infection (RSV), the airway epithelium releases TSLP, IL-33, HMGB1, and IL-25. These molecules, dubbed “alarmins,” stimulate ILC2 to release Th2 cytokines, promoters of airway inflammation and hyperresponsiveness in RSV bronchiolitis and childhood wheezing/asthma. Long-term epithelial progenitors or epigenetic modifications of airway epithelium governing the alarmin production may explain the increased susceptibility to obstructive lung diseases following RSV infection. Alarmin-ILC interactions may represent a new target for prevention and therapeutic interventions.

## 2 | THE INNATE LYMPHOID CELLS (ILC)

To maintain tissue homeostasis and integrity, lungs are protected by a complex network of highly specialized immune cells that include ILC.<sup>10,11</sup> Creating a first-line defense system, these cells play diverse roles in inflammation and infection, including promotion of innate immunity, acquisition of adaptive immunity, maintenance of epithelial barrier function and tissue repair.<sup>9-11,20</sup> Being tissue-resident elements, ILC tend to proliferate locally following environmental challenges.<sup>9-11,20</sup> However, there is increasing evidence that, in inflammatory disorders, ILC migrating from other organs could contribute to the expansion and the modulation of the local cell pools.<sup>20-22</sup> Based on distinctive phenotypic markers, transcription factors, and effector function, ILC can be divided into three subsets: ILC1, ILC2, and ILC3. These ILC subsets share many phenotypic, morphological, and functional features with CD4<sup>+</sup> Th1, Th2, and Th17 cells.<sup>9,10,20-22</sup> However, in contrast with T-cells, regulated by antigen presentation via antigen-presenting cells, ILC are mainly regulated by soluble factors such as cytokines, chemokines, neuronal factors, and other inflammatory mediators.<sup>23-26</sup> ILC1 express the transcription factor Tbx21 and, in response to IL-12, IL-15, and IL-18, produce IFN- $\gamma$  and TNF- $\alpha$  (Figure 1A). ILC2 express the transcription factor GATA-3 and, in response to the alarmins TSLP, IL-25, HMGB1 and IL-33, secrete the classical type 2 effector cytokines IL-4, IL-5, and IL-13.<sup>25-27</sup> ILC3 express the transcription factor ROR $\gamma$ t and, in response to IL-23 and IL-1 $\beta$ , release IL-17 and IL-22.<sup>21,25-27</sup> ILC subsets are not “fixed” and exhibit considerable functional plasticity depending on the inflammatory milieu.<sup>28,29</sup> For example, ILC2 can transdifferentiate into ILC1 or ILC3 in response to IL-1 $\beta$ , IL-12, and TGF- $\beta$ , but this switch is bi-directional, as these transdifferentiations can be reversed by IL-4 (Figure 1B).<sup>28,29</sup> Additionally, ILC3 can transdifferentiate into IFN- $\gamma$ -producing ILC1, in response to IL-12 and IL-23 (Figure 1C).<sup>28-30</sup> Former immune challenges may have a role on ILC2s plasticity. In BALB/c mice, influenza A infection downregulated GATA-3 expression in lung resident ILC2s, with a concomitant switch into IFN- $\gamma$  producing ILC1 cells, a conversion induced by IL-12 and IL-18.<sup>31</sup> ILC2 cells clustered within inflamed areas

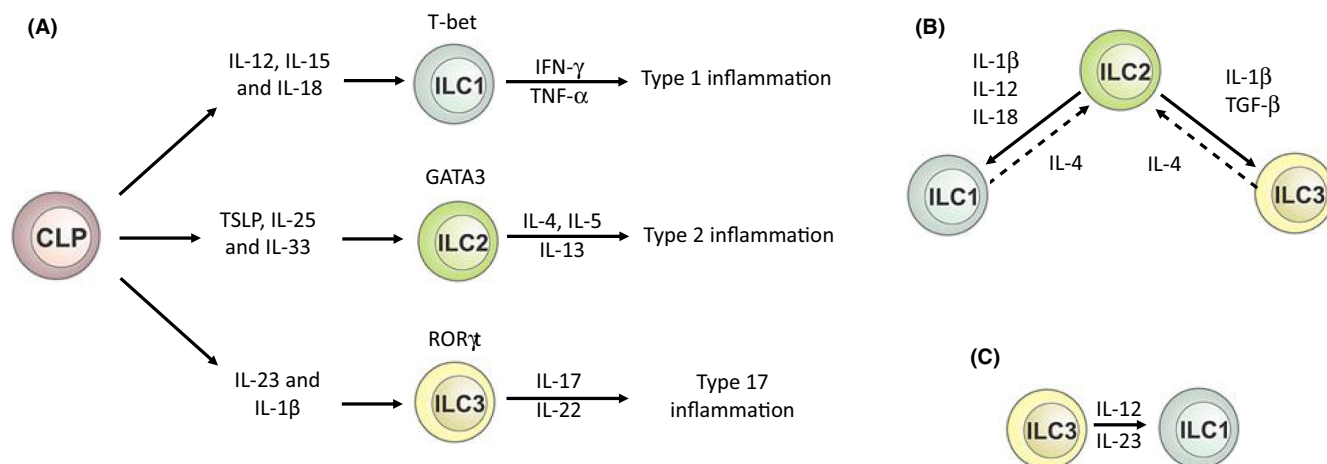


**FIGURE 1** Innate lymphoid cells. (A) ILC can be divided into three subsets ILC1, ILC2, and ILC3. ILC1 express the transcription factor T-bet and, in response to IL-12, IL-15, and IL-18, produce IFN- $\gamma$  and TNF- $\alpha$ . ILC2 express the transcription factor GATA-3 and, in response to the alarmins TSLP, IL-25, HMGB1 and IL-33, secrete IL-4, IL-5 and IL-13. ILC3 express the transcription factor ROR $\gamma$ t and in response to IL-23 and IL-1 $\beta$ , release IL-17 and IL-22. (B) ILC2 can transdifferentiate into ILC1 or ILC3 in response to IL-1 $\beta$ , IL-12, IL-18 and TGF- $\beta$  and these transdifferentiations can be reversed by Th2 cytokines, as IL-4. (C) ILC3 can transdifferentiate into IFN- $\gamma$ -producing ILC1, in response to IL-12 and IL-23

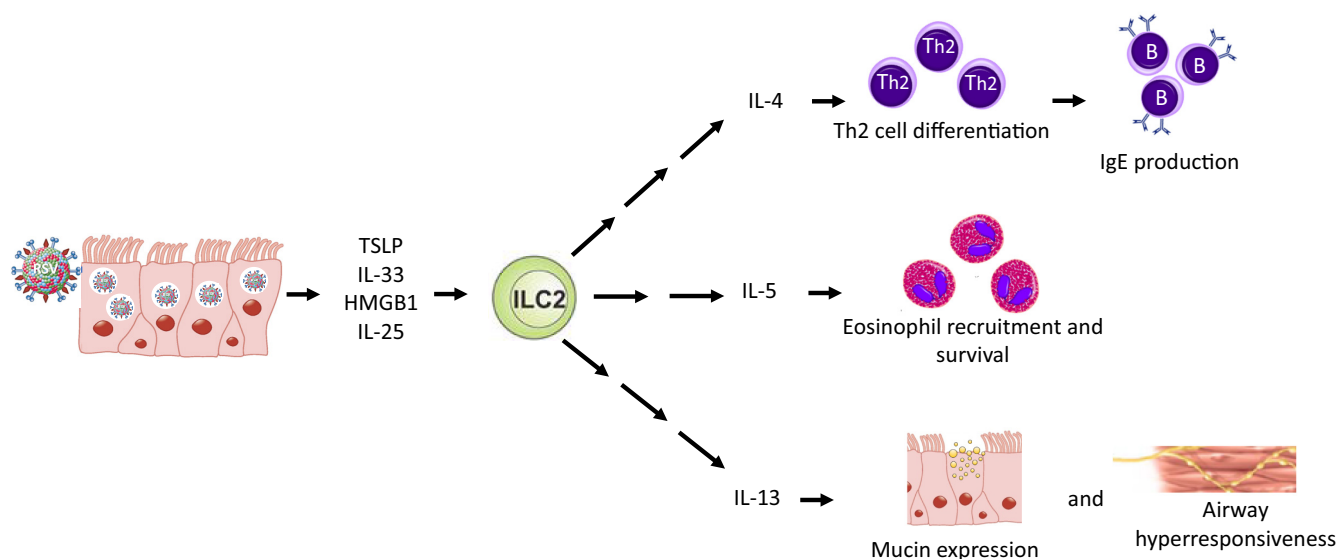


**FIGURE 2** Following RSV infection, through the release alarmins BECs stimulate ILC2 to release IL-4, IL-5, and IL-13. IL-4 drives Th2 cell differentiation and promotes B cell antibody class switch to IgE producing plasma cells. IL-5 is involved in eosinophils recruitment and in their survival enhancement. IL-13 increases goblet cell mucin and airway responsiveness

in mice lung and acquired an ILC1-like phenotype.<sup>31</sup> Interestingly, early reports suggest that ILC2 are significantly more potent than Th2 cells, producing greater than 10-fold the amount of Th2 cytokines on a per-cell basis,<sup>32</sup> explaining why ILC2 may have a significant role in several inflammatory disorders and, possibly, in reduced response to corticosteroids. Increased proportions of sputum and blood ILC2 were detected in children with severe therapy-resistant asthma.<sup>8</sup> In these children, ILC2s were significantly reduced *in vitro* when cultured with steroids and *in vivo* following intramuscular triamcinolone. Systemic corticosteroids, but not maintenance inhaled steroids, resulted in improved symptom control and exacerbations.<sup>8</sup>

Finally, ILC2 could acquire memory-like properties, as shown in a study on allergen-stimulated C57BL/6 mice.<sup>33</sup> After resolution of type 2 inflammation, a population of ILC2s that persisted in the lungs and the draining mediastinal lymph nodes of these animals displayed memory-like characteristics. Indeed, these persistent ILC2s responded more vigorously than naive ILC2s to unrelated allergen challenge and exhibited a gene expression profile similar to that of memory T-cells.<sup>33</sup> The hypothesis that epigenetic mechanisms might be involved in ILC memory is supported by a study on C57BL/6 mice.<sup>34</sup> Repetitive exposure to *Alternaria* allergen extract generated a form of memory that elicited an asthma-like response upon

a subthreshold recall challenge 3–15 weeks later, and this memory was associated with the presence of ILC2 in the lung.<sup>34</sup> It is therefore possible that memory-like properties of ILC2s acquired after RSV infection in infancy could be involved in the pathogenesis of the increased risk of recurrent wheezing in early childhood.

### 3 | ALARMINs, ILC ACTIVATION IN RSV INFECTION AND WHEEZING/ASTHMA

In response to RSV, other viruses as well as allergens and other triggers involved in wheezing/asthma pathogenesis, airway epithelium can directly stimulate ILC2 to release IL-4, IL-5, and IL-13 through the release of alarmins (Figure 2). IL-4 drives Th2 cell differentiation and promotes B cell antibody class switch to IgE-producing plasma cells.<sup>35–37</sup> IL-5 recruits and enhances survival of eosinophils, major component of the Th2 inflammatory infiltrates.<sup>34,35,38,39</sup> IL-13 increases goblet cell mucin expression and development of airway hyperresponsiveness.<sup>40</sup> Thus, the alarmins IL-33, IL-25, TSLP, and HMGB1, through ILC2 activation, can play a significant role in promoting the progression of Th2 type response in infants with RSV bronchiolitis and in increasing the risk of developing recurrent wheezing/asthma and, possibly, susceptibility to allergic exacerbation in later life.<sup>11,41</sup> Several clinical and experimental studies have evaluated the role alarmins in RSV infections. The different experimental details of those performed on mice are summarized in Table 1.

#### 3.1 | Thymic stromal lymphopoietin (TSLP)

Clinical and experimental studies showed that TSLP, a cytokine primarily expressed by epithelial cells, is elevated in the bronchial mucosa of asthmatic patients,<sup>42,43</sup> and its expression is upregulated by RSV infections.<sup>44,45</sup> Evaluating infants hospitalized with bronchiolitis (70% due to RSV), and matched healthy infants at their primary care appointments, García-García et al showed upregulation of TSLP levels in nasopharyngeal aspirates of the bronchiolitis

group.<sup>44</sup> Among RSV-infected infants, those who needed ICU admission presented more frequently detectable and higher TSLP concentrations. Moreover, when primary BEC cultures obtained by cytologic brushings from children were exposed to RSV, a rapid and significantly higher TSLP production was detected in asthmatics, as compared with healthy controls.<sup>45</sup> In wild-type mice, TSLP levels were increased 12 h after infection and significant increase in IL-13-producing ILC2, and in lung, IL-13 levels was observed on day 4 after infection.<sup>46</sup> TSLPR knockout (KO) mice did not mount an IL-13-producing ILC2 response to RSV infection, displayed reduced lung IL-13 protein levels, decreased airway mucus and hyperreactivity, as compare with wild-type mice.<sup>46</sup> Gender differences may also impact on TSLP-induced immune alterations following early-life RSV infection.<sup>47</sup> As compared with neonatal female mice, neonatal male mice infected with RSV exhibited higher viral loads, lower IFN $\beta$  production and delayed infection resolution, on day 4 post-infection. At 4 weeks post-infection, neonatal male mice but not female mice had higher TSLP and IL-33 levels in their lungs, increased IL-13 gene expression in lung ILC2, airway reactivity, and mucus secretion.<sup>47</sup> These changes in male mice were associated with an increased susceptibility to allergic exacerbation upon allergen challenge at 4 weeks of age.<sup>47</sup>

#### 3.2 | Interleukin-33 (IL-33)

IL-33 is a nuclear cytokine that is constitutively expressed in structural cells like type 2 pneumocytes in mice and lung epithelial cells in humans and can be induced in hematopoietic cells.<sup>48,49</sup> Binding to and signaling through its ST2 receptor, IL-33 drives the production of Th2-associated cytokines in asthma and other allergic diseases but also acts as ILC2 activator during viral infections.<sup>49–51</sup> In infants <2 years of age, hospitalized with bronchiolitis during the RSV season, increased amounts of IL-33 were detected in the upper airways, more frequently in infants with RSV and HRV coinfections than in those with either infection alone.<sup>44</sup> In BALB/c mice, intranasal inoculation with RSV induced IL-33 and IL-13 production, and increased eosinophil recruitment in the lung.<sup>52</sup> In these animals, production

TABLE 1 Summary of murine studies addressing alarmin response to RSV infection

| Alarmin        | Mouse strain                                        | Age                  | Virus strain | Sample              | Expression peak post infection | Ref. |
|----------------|-----------------------------------------------------|----------------------|--------------|---------------------|--------------------------------|------|
| TSLP and IL-33 | Female BALB/c                                       | 8–12 weeks           | RSV 01/2-20  | Lung tissue         | 12 h                           | 46   |
| TSLP and IL-33 | Male & female BALB/c                                | 7 days               | RSV A2 (L19) | Lung tissue         | Up to 4 weeks                  | 47   |
| IL-33          | IL33 <sup>f/f</sup> LysM <sup>Cre</sup> BALB/c mice | -----                | RSV A2 L19   | BAL                 | Day 3 and 6                    | 48   |
| IL-33          | Female BALB/c                                       | 6-weeks              | RSV A2       | BAL                 | Day 3                          | 52   |
| IL-33          | BALB/c                                              | 5 days and 6–8-weeks | RSV A2       | Lung tissue         | 24 h                           | 53   |
| HMGB1          | BALB/c                                              | 6–8-weeks            | RSV A2       | BAL and lung tissue | Day 21–30                      | 57   |
| HMGB1          | C57BL/6.Irf7 <sup>-/-</sup>                         | 7–73 days            | PVM          | BAL and lung tissue | Day 3–14                       | 59   |
| IL-25          | C57BL/6 IL-17RB <sup>-/-</sup>                      | -----                | RSV L19      | Lung tissue         | Day 4–8                        | 65   |
| IL-25          | Male BALB/c                                         | 8–10-weeks           | RSV A2       | Lung tissue         | Day 2–4                        | 66   |

of IL-13 occurred involving the IL-33/ST2 pathway, as incubation of lung cells with anti-ST2 antibody diminished IL-13-producing cell frequency.<sup>52</sup> To further determine the role of IL-33 in activating ILC2 during RSV infection, a study on wild-type and IL-33 KO mice was performed.<sup>46</sup> On day 4 after infection, both wild-type and IL-33 KO mice showed a significant inflammatory response to RSV, with a significant and similar increase in the total numbers of lung IL-13<sup>+</sup> ILC2. However, IL-13 concentrations were significantly lower in RSV-infected IL-33 KO mice than in RSV-infected wild-type mice,<sup>46</sup> highlighting the role for IL-33 in ILC2 activation. RSV can trigger IL-33 production from multiple sources, such as lung structural cells but also myeloid cells. To elucidate the roles of ILC2 myeloid cells in RSV infection-triggered pathophysiology, myeloid-specific, IL-33 knockout (IL33<sup>f/f</sup> LysM<sup>Cre</sup>) mice were employed.<sup>48</sup> ILC2s myeloid cell-derived IL-33 was required for airway inflammation and hyperresponsiveness, IL-13 production and local and peripheral eosinophilia.<sup>48</sup> Age-variable effects of alarmins on ILC2 can also explain why chronological age at time of infection is an important risk factor for severe RSV bronchiolitis.<sup>1</sup> In the lungs of neonatal, but not of adult mice, infection with RSV induced a rapid IL-33 expression and an increase in ILC2 numbers.<sup>53</sup> Administration of IL-33 to adult mice during RSV infection induced lung disease, whereas blocking IL-33 with antibodies during infection or using IL-33 receptor KO neonatal mice inhibited Th2 inflammation, airway hyperresponsiveness, and mucus overproduction.<sup>53</sup> In this study, wild-type mice were reinfected with RSV at 4 weeks post-primary infection. In neonatal mice, neutralizing IL-33 with antibodies during primary infection resulted in significant reductions of Th2 inflammation and airway hyperresponsiveness following RSV reinfection.<sup>53</sup> Moreover, adoptive transfer of ILC2 from donor wild-type mice or administration of IL-33 to IL-33-deficient mice was crucial for the development of airway inflammation and hyperresponsiveness following RSV infection.<sup>53</sup> Notably, in newborn mice lung, a number of profound changes related to ILC2 occur with the first breath.<sup>54</sup> The sudden inflation of the previously liquid-filled lungs caused considerable mechanical stress and potential tissue injury, which resulted in IL-33 induction. The immediate IL-33 upregulation was closely followed by a wave of IL-13-producing ILC2, with the coincident appearance of alveolar macrophages (AMs) characterized by the anti-inflammatory M2 phenotype. ILC2s continued to maintain the M2 AM phenotype during adult life at the cost of a delayed response to infections.<sup>54</sup>

### 3.3 | High mobility group box 1 (HMGB1)

High mobility group box 1 is one of the most important damage-associated molecular patterns acting as a proinflammatory cytokine.<sup>55</sup> It promotes and perpetuates the immune responses in infectious and noninfectious inflammatory diseases.<sup>55,56</sup> HMGB1 levels were found to be elevated in nasopharyngeal aspirates of hospitalized infants with RSV bronchiolitis, and were significantly higher in the moderate-severe group.<sup>57</sup> In immortalized and primary human BECs, the RSV-induced HMGB1 expression was decreased when the

cultures were exposed to glycyrrhizin, a specific HMGB1 inhibitor.<sup>58</sup> A dose-dependent decrease of the HMGB1-positive cell numbers was also detected, associated with significant reduction in viral replication.<sup>58</sup> In BALB/c mice infected with RSV, type 2 cytokines IL-4, IL-5, and IL-13 were found to be gradually increased in bronchoalveolar lavage from days 14 to 30 post-infection.<sup>57</sup> HMGB1 expression was localized to bronchiolar low columnar/cuboidal cells found in the small airways. Treatment with anti-HMGB1 antibody significantly reduced HMGB1 levels and IL-4, IL-5, and IL-13 concentrations, suppressed the inflammatory cell infiltration, and decreased the severity scores.<sup>57</sup> In IRF7-deficient (<sup>-/-</sup>) mice, characterized by defective antiviral immunity, infection with PVM (a mouse-specific pathogen belonging to the same genus as RSV) promoted epithelial HMGB1 expression, associated with an increase in the numbers of IL-13-producing ILC2.<sup>59</sup> Increase of both the levels of the potent airway smooth muscle (ASM) mitogen TGF- $\beta$  and ASM cell proliferation was also detected, suggesting that ASM alterations could initiate the response to a severe RSV infection in early life.<sup>59</sup> Anti-HMGB1 antibodies ablated lung ILC2 numbers and ASM growth *in vivo*, and inhibited ILC2-mediated ASM cell proliferation in a co-culture model.<sup>59</sup> In this study, it was demonstrated that the genetic ablation of RAGE (receptor for advanced glycation end products), one of the receptors ligated by HMGB1, was also protective, diminishing ILC2s numbers, type-2 cytokine production and ASM cell proliferation.<sup>59</sup> Finally, several other mouse studies using different inducible models of asthma demonstrated that anti-HMGB1 antibody treatment reduced IL-4, IL-5, and IL-13, as well as airway mucus compared with control antibody or mice not given antibody.<sup>60,61</sup>

### 3.4 | Interleukin-25 (IL-25)

IL-25 is a member of the interleukin-17 cytokine family that recognizes a receptor composed of IL-17RB and IL-17RA subunits.<sup>62</sup> IL-17RB receptor is mainly expressed on ILC2, and its activation is involved in type 2 effector response.<sup>62-64</sup> In C57BL/6J mice, exposure to RSV induced the expression of IL-25 and IL-17RB lung transcripts and of other potentially pathogenic cytokines, including IL-13.<sup>65</sup> Treatment with an anti-IL-25 antibody significantly reduced Th2 cytokine production, mucus-associated *gob5* gene expression and airway hyperresponsiveness. Moreover, IL-17RB<sup>-/-</sup> mice showed increased clearance of the virus and diminished pathology.<sup>65</sup> In a different set of experiments, IL-17RB<sup>-/-</sup> mice were sensitized to allergen, infected with RSV during the active allergic responses, and then challenged with allergen.<sup>65</sup> As compared with wild-type mice, decreased inflammatory response, cytokine production and IL-13 and *gob5* gene expression was detected in IL-17RB<sup>-/-</sup> mice, possibly reflecting enhanced clearance of RSV, leading to decreased immune activation.<sup>65</sup> Immune and morphological responses to RSV infection were also evaluated in wild-type and NK cell-depleted BALB/c mice.<sup>66</sup> NK cells play a critical role in the development of an effector immune response in RSV infection.<sup>67</sup> Depletion of NK cells led to increased IL-25 expression in the respiratory epithelium, higher IL-4,

IL-13 and eotaxin lung mRNA levels and higher serum IgE, tissue eosinophil numbers and mucus-secreting cells. This increased Th2 pathology was reflected in a delayed viral clearance in the later stages of infection.<sup>66</sup> RSV-induced Th2 responses were IL-25 dependent. Treatment of NK-depleted mice with anti-IL-25 antibodies led to attenuation of the Th2 cell responses, suppression of inflammation, and histopathological changes in the lungs.<sup>66</sup>

## 4 | GUT MICROBIOTA, RSV INFECTION, ALARMINs AND ILC2

The gut microbiota, the microorganisms that live in the digestive tracts, provide local and systemic signals, essential for the development and education of the innate and adaptive immune systems, reducing the predisposition and the severity of respiratory infections and promoting tolerance to allergens.<sup>68</sup> This modulation shapes immune responses locally but also at distal organs, including the lung. Changes in the constituents of the gut microbiome can result in alteration of the immune responses and homeostasis in the airways.<sup>68,69</sup>

### 4.1 | Gut microbiota and inhibition of Th2 inflammation

Gut microbe structural components and their metabolic products, generated by the biotransformation of exogenous compounds, can activate local ILC that can then migrate to other sites of the body.<sup>70</sup> Tissue and circulating ILC can recognize gut microbial ligands and metabolic products through their toll-like receptors and produce a variety of cytokines and chemokines which can fight or promote inflammation and infections.<sup>71-73</sup> The main gut microbiota metabolites are short-chain fatty acids (SCFAs), that serve as an energy source for intestinal epithelial cells but also can directly act on leukocytes, promoting their differentiation and activation.<sup>74,75</sup> A mouse model of IL-33-induced Th2 inflammation demonstrated that SCFAs derived from fermentation of dietary fibers by the gut microbiota inhibited pulmonary ILC2 functions and the subsequent development of airway hyperreactivity.<sup>75</sup> SCFAs modulated GATA3 expression, oxidative phosphorylation, and glycolytic metabolic pathways in pulmonary ILC2 and this phenotype modification was associated with increased IL-17a secretion and linked to enhanced neutrophil recruitment to the airways. Therefore, strategies aimed at modulating metabolomics and microbiota in the gut in early life could be useful in preventing Th2 lung inflammation and airway hyperreactivity.<sup>75</sup> Lack of specific taxa in gut microbiota is associated with systemic inflammation and immune dysregulation early in life. Human breastmilk contains abundant human milk oligosaccharides (HMOs), not digestible by humans (which lack the necessary glucosidases) but metabolized by specialized microbes, including *Bifidobacterium* and *Lactobacillus* strains.<sup>76</sup> HMOs, which can bind to cell surface receptors expressed on epithelial and immune effector

cells, are involved in the modulation of neonatal gut immunity. In breastfed infants, supplemented with *Bifidobacterium infantis*, intestinal Th2 cytokines were silenced and IFN- $\beta$  was induced, providing a functional link between beneficial microbes and immunoregulation during the first months of life.<sup>77</sup> Moreover, offspring of BALB/c mice supplemented with *Lactobacillus johnsonii* exhibited reduced airway mucus and Th2 cell-mediated response to RSV infection.<sup>78</sup> Maternal supplementation resulted in a consistent gut microbiome in mothers and their offspring, and exhibited decreased inflammatory metabolite production and reduced Th2 cytokine production and lung inflammation following RSV infection.<sup>78</sup> Finally, a study on C57BL/6 mice showed that the generation of virus-specific CD4 and CD8 T cells and antibody responses, following respiratory influenza virus infection, is critically regulated by a normal commensal microbiota composition dominated by *Lactobacillus spp.*<sup>79</sup> Interestingly, in 2-5-year-old children, consumption of *Lactobacillus paracasei* Lpc-37 or *Bifidobacterium lactis* HN019 was found to be associated with higher levels of selected SCFAs in fecal samples.<sup>80</sup> It is therefore possible that breastfeeding and *Lactobacillus* and/or *Bifidobacterium* supplementation could promote the production of high SCFAs concentrations in the gut, resulting in inhibition of pulmonary ILC2 functions and in reduced Th2 inflammation following RSV infection (Figure 3A).

### 4.2 | Specific gut microbial profile, ILC2 functions and RSV severity

Modifications in microbiota composition can modulate ILC2 functions and induce alteration of the immune responses to viral respiratory infections, leading to different clinical outcome. In stool samples collected from infants hospitalized during a bronchiolitis season, four microbiota profiles were detected: *Escherichia*-dominant, *Bifidobacterium*-dominant, *Enterobacter/Veillonella*-dominant, and *Bacteroides*-dominant.<sup>81</sup> The proportion of infants with bronchiolitis (related to RSV infection in 65% of them) was lowest in the *Enterobacter/Veillonella*-dominant profile and highest in the *Bacteroides*-dominant profile.<sup>81</sup> To determine whether a specific gut microbial profile could be associated with RSV severity, stool samples were collected in 95 infants during an RSV season: 37 were healthy babies and 58 were hospitalized with RSV bronchiolitis.<sup>18</sup> Of the RSV-positive infants, 53 remained in the pediatric ward and 5 later moved to the pediatric intensive care unit. There was a significant enrichment in *Bacteroides*, and a decrease in *Firmicutes* in RSV infants vs. healthy controls. In addition, infants with severe RSV disease had slightly lower alpha diversity (richness and evenness of the bacterial community) of the gut microbiota compared to infants with moderate RSV and controls. Beta diversity (overall microbial composition) was significantly different between all RSV patients compared to controls.<sup>18</sup> These results were confirmed in BALB/mice in which, after RSV infection, showed a significant increase in the relative abundance of *Bacteroidetes* and a corresponding decrease in *Firmicutes* was detected (Figure 3B).<sup>82</sup> Changes in

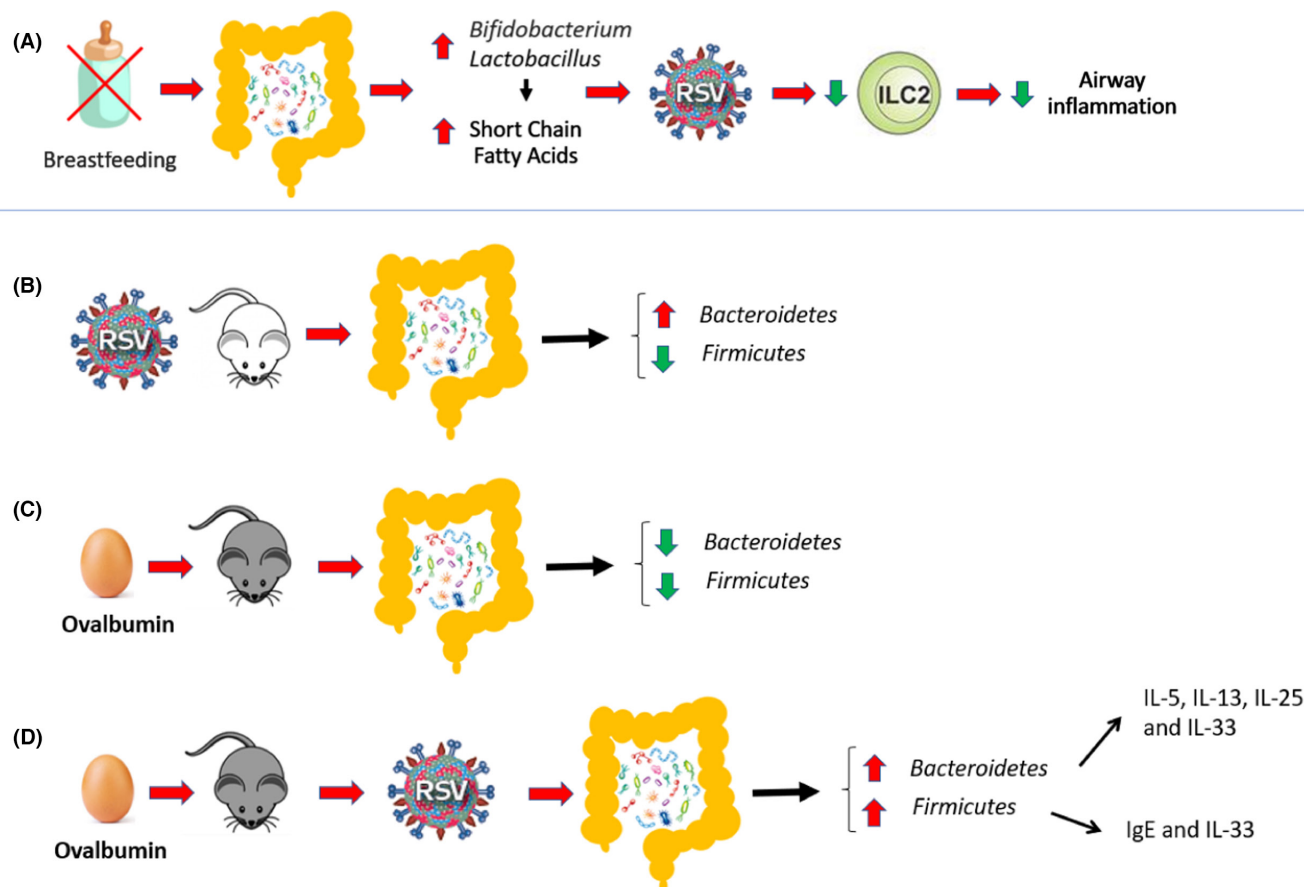


**FIGURE 3** Changes in gut microbiota community composition, RSV infection and allergen sensitization. (A) Breastfeeding and *Lactobacillus* and/or *Bifidobacterium* supplementation can promote the production of high SCFAs concentrations in the gut, resulting in inhibition of pulmonary ILC2 functions and in reduced Th2 inflammation, following RSV infection. (B) In mice, RSV infection was associated with a significant increase in the relative abundance of *Bacteroidetes* and a corresponding decrease in *Firmicutes*. (C) In ovalbumin (OVA) sensitized mice, lower abundance of both *Bacteroidetes* and *Firmicutes* infection was detected. (D) In mice, previously sensitized to OVA, RSV infection was associated with higher abundance of both *Bacteroidetes* and *Firmicutes*. *Bacteroidetes* were most significantly associated with IL-4, IL-5, IL-13, IL-25, and IL-33 production and *Firmicutes* with IgE and IL-33

gut microbiota composition following RSV infection may also indirectly regulate ILC2 cytokine release through the production of alarmins. 6-to-8-week-old BALB/c male mice were randomly divided into a control (CON) group, an ovalbumin (OVA) sensitized group, and an OVA + RSV group.<sup>83</sup> Compared with the CON group, the OVA group had lower abundance of both *Bacteroidetes* and *Firmicutes* (Figure 3C), whereas higher abundance of these phyla was detected in the OVA + RSV group, compared with the OVA group (Figure 3D). RSV-infected asthmatic mice had increased expression of IL-25 and IL-33 and of the Th2 cytokines IL-4, IL-5, and IL-13.<sup>83</sup> *Prevotellaceae\_NK4A136\_group*, which belongs to the *Bacteroides* species, was significantly associated with IgE, IL-33, IL-25, IL-5, and IL-13 levels, whereas *Lachnospiraceae\_NK4A136\_group*, which belongs to the *Firmicutes* species, was significantly associated with IgE and IL-33 levels.<sup>83</sup> Aggravation of bronchial hyperresponsiveness to methacholine and airway inflammation was observed in asthmatic mice following RSV infection-induced alteration of gut microbiota.<sup>83</sup> Interestingly, in a study comparing children with recurrent respiratory tract infections (RRTI) and a healthy control group, distinct gut

bacterial community structures between the groups were observed with an enrichment in *Bacteroidetes* in the RRTI group.<sup>84</sup> Whether probiotics and/or bacterial derived products, potentially involved in immune training, could affect gut dysbiosis, prevent gastrointestinal epithelial dysfunction and the related negative influence on the immune system is an interesting hypothesis that needs to be adequately addressed and demonstrated.<sup>85,86</sup>

## 5 | CONCLUSIONS

In response to RSV infection BECs can release alarmins, mediators effective in stimulating ILC2 to produce Th2 cytokines, promoters of airway inflammation and hyperresponsiveness in RSV infection. Long-term epithelial progenitors or persistent epigenetic modifications of BECs, following RSV bronchiolitis in infants, may play a pathogenetic role on the ongoing increased susceptibility to obstructive lung diseases in childhood. Experimental studies suggest that alarmin-induced ILC2 activation, which can be modulated by

gut dysbiosis, may represent a common pathogenetic imprint in RSV bronchiolitis and later recurrent wheezing. Exhaustive research in human subjects is required to determine how human ILC2s mediate pathology in wheezing and asthma. A better understanding of the pathways involved in alarmin production by airway epithelial cells and on the alarmin-ILC2 interactions might provide insights into the mechanisms characterizing these immune-mediated diseases. The position of alarmins at the top of the inflammatory cascade makes them a promising prevention and therapeutic targets.

## AUTHOR CONTRIBUTIONS

**Giovanni Arturo Rossi:** Conceptualization (lead); Supervision (equal); Writing – review & editing (lead). **Stefania Ballarini:** Conceptualization (supporting); Validation (supporting); Writing – review & editing (supporting). **Pietro Salvati:** Data curation (supporting); Formal analysis (supporting); Validation (supporting). **Oliviero Sacco:** Investigation (equal); Validation (equal); Writing – review & editing (supporting). **Andrew Colin:** Conceptualization (equal); Supervision (equal); Writing – review & editing (equal).

## CONFLICT OF INTEREST

The author declares no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this manuscript.

## ETHICAL APPROVAL

Because of category of the manuscript, a review, approval from the ethical committee was not necessary.

## PEER REVIEW

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