Alarmins and innate lymphoid cells 2 activation: a common pathogenetic link connecting RSV bronchiolitis and later wheezing/asthma?

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Abstract

Severe 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 anti-viral immune response. The airway epithelium, the first target of respiratory syncytial virus (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. ILC2, reflect the innate counterparts of Th2 cells and, when activate, 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.

1. INTRODUCTION

Respiratory syncytial virus (RSV) is a common and highly contagious pathogen inducing acute respiratory tract infections in infants [1]. Although in many cases symptoms remain mild, a subset of individuals develops severe RSV-associated bronchiolitis [1,2]. 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 [3,4]. This phenomenon is not always associated to atopic sensitization but is thought to be related to long-term immune response alterations and neuromuscular tone instability in the airways [3,4]. 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 [5]. 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 [3,5]. However, BECs may also be programmed to release mediators that activate a Th2 immune response, aggravating the infection, and contributing to airway damage [6]. These mediators include thymic stromal lymphopoietin (TSLP), IL-33, the high mobility group box 1 (HMGB1) and IL-25

[6,7]. 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 [7-12]. 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 [13]. Through DNA methylation and histone ubiquitination, RSV can induce epigenetic modifications in BECs that favor Th2 responses [14], promoting post bronchiolitis wheezing [15]. 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 pro-inflammatory cytokine, and oxidative stress-related molecules [16]. 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 [17]. 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, as shown in infants with bronchiolitis [18].

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 [10,11]. 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 [9-11,19]. Being tissue-resident elements, ILC tend to proliferate locally following environmental challenges [9-11,18]. 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 [19-21]. 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 $CD4^+$ Th1, Th2 and Th17 cells [9,10,18-21]. 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 [22-25]. ILC1 express the transcription factor Tbx21 and, in response to IL-12, IL-15, and IL-18, produce IFN-γ and TNF-α (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 [26]. ILC3 express the transcription factor RORγt and, in response to IL-23 and IL-1β, release IL-17 and IL-22 [20,27]. ILC subsets are not 'fixed' and exhibit considerable functional plasticity depending on the inflammatory milieu [28]. For example, ILC2 can transdifferentiate into ILC1 or ILC3 in response to IL-1β, IL-12 and TGF-β but this switch is bi-directional, as these transdifferentiations can be reversed by IL-4 (figure 1B) [28]. Additionally, TLR-2 activation can promote the production of the Th2 cytokines IL-5 and IL-13 by ILC3, which suggests that these cells might be able to differentiate into ILC2 (figure 1C) [29]. Finally 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 [30], 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 [8]. 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 [8]

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 [31-33]. IL-5 recruits and enhances survival of eosinophils, major component of the Th2 inflammatory infiltrates [34,35]. IL-13 increases goblet cell mucin expression and development of airway hyperresponsiveness [36]. 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 [37].

3.1 Thymic Stromal Lymphopoietin (TSLP)

Clinical and experimental studies showed that TSLP, a cytokine primarily expressed by epithelial cells, is induced by RSV infections [39,40]. 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 [41]. 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 to healthy control [42]. In wild-type mice, TSLP levels were increased 12 hours after infection and significant increase in IL-13–producing ILC2 and in lung IL-13 levels was observed on day 4 after infection [43]. 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 [43]. Gender differences may also impact on TSLP-induced immune alterations following early-life RSV infection [44]. As compared to neonatal female mice, neonatal male mice infected with RSV exhibited higher viral loads and lower IFNβ production and delayed infection resolution, on day 4 post-infection. At 4 weeks post-infection, neonatal male 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 [44]. These changes in male mice were associated with an increased susceptibility to allergic exacerbation upon allergen challenge at 4 weeks of age [44].

3.2 Interleukin-33 (IL-33)

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 [45-47]. 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 [41]. In BALB/c mice intranasal inoculation with RSV induced IL-33 and IL-13 production, and increased eosinophil recruitment in the lung [48]. In these animals, production of IL-13 occurred involving the IL-33/ST2 pathway, since incubation of lung cells with anti-ST2 antibody diminished IL-13-producing cell frequency [48]. 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 [43]. 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$^+$ ILC2. However, IL-13 concentrations were significantly lower in RSV-infected IL-33 KO mice than in RSV-infected wild-type mice [43], highlighting the role for IL-33 in ILC2 activation. 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 [1]. 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 [49]. 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 [49]. 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 significantly reductions of Th2 inflammation and airway hyperresponsiveness following RSV reinfection [49]. 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 [50].

3.3 High Mobility Group Box 1 (HMGB1)

HMGB1 is one of the most important damage-associated molecular patters acting as a proinflammatory cytokine [51]. It promotes and perpetuate the immune responses in infectious and non-infectious inflammatory diseases [51,52]. HMGB1 levels were found to be elevated in nasopharyngeal aspirates of hospitalized infants with RSV bronchiolitis, and significantly higher in the moderate-severe group [53]. In immortalized and primary human BECs, the RSV-induced HMGB1 expression was decreased when the cultures were exposed to glycyrrhizin, a specific HMGB1 inhibitor [54]. A dose dependent decreased of the HMGB1 positive cell numbers was also detected, associated with significant reduction in viral replication [54]. 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 [53]. 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 [53]. In IRF7-deficient ($^{-/-}$) 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 [55]. Increase of both the levels of the potent airway smooth muscle (ASM) mitogen TGF-β and ASM cell proliferation was also detected, suggesting that ASM alterations could initiate the response to a severe RSV infection in early-life [55]. Anti-HMGB1 antibodies ablated lung ILC2 numbers and ASM growth *in vivo* , and inhibited ILC2-mediated ASM cell proliferation in a co-culture model [55]. 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 to control antibody or mice not given antibody [56,57].

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 [58]. IL-17RB receptor is mainly expressed on ILC2, and its activation is involved in type 2 effector response [58-61]. 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 [61]. Treatment with an anti-IL-25 antibody significantly reduced Th2 cytokine production, mucus-associated *gob5* gene expression and airway hyperresponsiveness. Moreover, IL-17RB$^{-/-}$ mice showed increased clearance of the virus and diminished pathology [61]. In a different set of experiments, IL-17RB$^{-/-}$ mice were sensitized to allergen, infected with RSV during the active allergic responses, and then challenged with allergen [62]. As compared to wild-type mice, decreased inflammatory response, cytokine production and IL-13 and *gob5* gene expression was detected in IL-17RB$^{-/-}$ mice, possibly reflecting enhanced clearance of RSV, leading to decreased immune activation [61]. Immune and morphological responses to RSV infection were also evaluated in wild-type and NK cell-depleted BALB/c mice [62]. NK cells play a critical role in the development of an effector immune response in RSV infection [63]. 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 [61]. 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 [62].

4. GUT MICROBIOTA, RSV INFECTION, ALARMINS AND ILC2

Gut microbiota composition might affect the severity of respiratory virus infections, but the interaction can be bidirectional, since infections can induce gut dysbiosis [64,65]. The high concentration of microorganism- and pathogen-associated molecular patterns physiologically present in the gut and the huge amounts of cytokines and chemokines produced during dysbiosis can activate local ILC that can then migrate to other sites of the body [19-21]. In an integrated microbiota dysbiosis mice model, it was demonstrated that gut

microbiota can modulate ILC2 directional migration to the lung via regulation of select cytokines [66]. In these mice, *Proteobacteria*abundance was associated with increased IL-33 production which promoted ILC2 migration and accumulation in the lung. Blocking the IL-33 receptor with anti-ST2 antibodies, abolished the observed increased percentages of lung ILC2 in this animal model [65]. Moreover, tissue and circulating ILC2 can recognize microbial ligands through their TLR, and directly produce a variety of cytokines, chemokines which can fight or promote infections [19-21, 67-70]. 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 [71]. 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 [71]. 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 [18]. Out 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 [18]. 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 3A) [72]. Interestingly, many members of the *Bacteroidetes* phylum use mucus as an energy source [73] and Muc5ac mucin levels were significantly increased in the airways and colon of RSV-infected mice, but not in control mice. Changes in gut microbiota composition following RSV infection may also indirectly regulate ILC2 function 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 [73]. Compared with the CON group, the OVA group had lower abundance of both*Bacteroidetes* and *Firmicutes* (figure 3B), whereas higher abundance of these phyla was detected in the OVA + RSV group, compared with the OVA group (figure 3C). 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 [74]. *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 [74]. Aggravation of bronchial hyperresponsiveness to methacholine and airway inflammation was observed in asthmatic mice following RSV infection-induced alteration of gut microbiota. 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 [75]. Whether probiotics and/or bacterial derived products, potentially involved in immune training, could affect gut dysbiosis, prevent GIT epithelial dysfunction and the related negative influence on the immune system is an interesting hypothesis that needs to be adequately addressed and demonstrated [76,77].

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. 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.

CONFLICT OF INTEREST. The author declares no conflicts of interest.

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

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Figure legends.

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-γ and TNF-α. 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 RORγt and in response to IL-23 and IL-1β, release IL-17 and IL-22. B. ILC2 can transdifferentiate into ILC1 or ILC3 in response to IL-1β IL-12 and TGF-β and these trans differentiations can be reversed by Th2 cytokines, as IL-4. C.

Toll-like-receptor-2 ligands can promote the production of the Th2 cytokines IL-5 and IL-13 by ILC3 cells, which suggests that these cells might be able to differentiate into ILC2 cells.

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. IL-5 is involved in eosinophils recruitment and in their survival enhancement. IL-13 increases goblet cell mucin and airway responsiveness.

Figure 3. Changes in gut microbiota community composition following RSV infection and allergen sensitization in mice. A. RSV infection was associated with significant increase in the relative abundance of *Bacteroidetes* and a corresponding decrease in *Firmicutes* . B. In ovalbumin (OVA) sensitized mice, lower abundance of both *Bacteroidetes* and *Firmicutes* infection was detected. C. 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 IL-33.

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