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Natural killer cells in the lung: potential role in asthma and virus-induced exacerbation?

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Shareable abstract (@ERSpublications)

Natural killer cells are essential in lung homeostasis and defence against respiratory viruses; however, their role in asthma and virus-induced exacerbation remains poorly understood and the literature is conflicting <https://bit.ly/3WFtQF>

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Abstract

Asthma is a chronic inflammatory airway disorder whose pathophysiological and immunological mechanisms are not completely understood. Asthma exacerbations are mostly driven by respiratory viral infections and characterised by worsening of symptoms. Despite current therapies, asthma exacerbations can still be life-threatening. Natural killer (NK) cells are innate lymphoid cells well known for their antiviral activity and are present in the lung as circulating and resident cells. However, their functions in asthma and its exacerbations are still unclear. In this review, we will address NK cell activation and functions, which are particularly relevant for asthma and virus-induced asthma exacerbations. Then, the role of NK cells in the lungs at homeostasis in healthy individuals will be described, as well as their functions during pulmonary viral infections, with an emphasis on those associated with asthma exacerbations. Finally, we will discuss the involvement of NK cells in asthma and virus-induced exacerbations and examine the effect of asthma treatments on NK cells.

Introduction

Asthma is a complex chronic airway disease characterised by inflammation and structural changes of the bronchi. Worldwide, an estimated 262 million people suffer from asthma, resulting in 461 000 deaths [1]. However, these figures are most likely underestimated. Asthma is a heterogeneous disease, encompassing different phenotypes defined by clinical characteristics and induced by distinct pathophysiological mechanisms called endotypes. The analysis of the inflammatory processes in the bronchial mucosa of asthma patients allowed the description of two distinct profiles. The T2-high endotype is associated with the production of type 2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, and eosinophilic inflammation, whereas the T2-low endotype is associated with the recruitment of T-helper (Th) 17 cells and neutrophilic inflammation [2]. Despite the improvement of severe asthma management by novel therapies, uncontrolled asthma and asthma exacerbations are still life-threatening. Asthma exacerbations are defined as episodes of deterioration in symptoms requiring an unscheduled visit to healthcare providers and a modification in treatments [3]. They occur despite a good control and/or a satisfactory compliance to anti-inflammatory treatments such as corticosteroids or biologics. Respiratory viral infections are one of the most frequent causes of asthma exacerbations in adults and children [4]. Approximately 50–60% of the viruses associated with exacerbations are rhinoviruses (RVs), especially from the A and C species [5]. Less frequently, other respiratory viruses also contribute to exacerbations, including respiratory syncytial virus (RSV), influenza virus and coronaviruses [6]. Natural killer (NK) cells are crucial actors of the antiviral immune response [7]. The lung is one of the major reservoirs of NK cells in the body, where they represent about 10–20% of lymphocytes [8–10]. However, the functions of NK cells in asthma and its virus-induced exacerbations are still to be deciphered. This review will examine NK cell biology, their functions in lung homeostasis and their role in the antiviral immunity of



the airways. Finally, the current knowledge on the participation of NK cells in the pathogenesis of asthma and its exacerbations will be reported.

Search strategy

Data for this narrative review were identified by searches of PubMed (<https://pubmed.ncbi.nlm.nih.gov>), as well as references from relevant articles and reviews, using the following search terms: natural killer cells AND review/function/activation/migration/subset/dendritic cell/eosinophil/neutrophil/T-cell; natural killer cells AND asthma/lung/airway/lung disease; natural killer cells AND respiratory virus/influenza/rhinovirus/respiratory syncytial virus/severe acute respiratory syndrome coronavirus 2 OR SARS-CoV-2; natural killer cells AND corticosteroids/asthma treatment. Only articles published in English from inception to January 2023 were included.

NK cells: identification, activation and functions

Identification of NK cells

In humans, NK cells are defined as cells lacking the T-cell receptor cluster of differentiation (CD) 3 but expressing CD56 [11]. They can also be characterised as CD3⁻NKp46⁺ [12]. Human NK cells have long been subdivided into two main subsets based on their relative expression of CD56 and CD16, namely CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺. CD56^{bright}CD16⁻ NK cells are predominantly located in human secondary lymphoid organs, lack killer cell immunoglobulin-like receptor (KIR) expression but can produce a large panel of cytokines such as interferon- γ (IFN- γ), IL-10, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor (TNF)- α when stimulated with soluble factors, such as IL-12 and IL-18 [13]. The CD56^{dim}CD16⁺ NK cells are thought to be mainly derived from CD56^{bright}CD16⁻ NK cells through progressive differentiation [14]. The CD56^{dim}CD16⁺ subset comprises 90% of the peripheral blood NK cells. In contrast to the regulatory CD56^{bright}CD16⁻ NK subset, the CD56^{dim}CD16⁺ cells are considered highly cytotoxic as they contain high levels of perforin, granzymes and cytolytic granules, express KIRs and have the capacity to induce antibody-dependent cellular cytotoxicity (ADCC) [15]. They are mostly found at acute inflammatory peripheral sites, while CD56^{bright}CD16⁻ NK cells migrate to chronic inflammatory sites [16]. For a long time, distinction between subsets was made based on their cytotoxic *versus* cytokine production functions. However, the frontier between NK cell subsets is more ambiguous. For example, the kinetics of cytokine production are different, with CD56^{dim}CD16⁺ being an earlier producer [17]. Moreover, the cytokine secretion induced by contact with target cells is greater with CD56^{dim}CD16⁺ than with CD56^{bright}CD16⁻ cells [18]. In mice, NK cells can be detected as CD3⁻NK1.1⁺ (C57BL/6 mice), CD3⁻DX5 (CD49d)⁺ (BALB/c mice) or CD3⁻NKp46⁺ cells (all strains of mice) [19]. NKp46 is currently the most specific marker for mouse NK cells, but it is also expressed by innate lymphoid cell (ILC) type 1, a small population of T-lymphocytes and a subset of ILC3 [20]. Ligands for this activating receptor include viral, bacterial and fungal molecules (ex: hemagglutinin of influenza virus, hemagglutinin neuramidase from human parainfluenza virus type 3), surface molecules induced by intracellular pathogens, a surface protein on healthy pancreatic β -cells awaiting identification, and complement factor P [21]. Mouse NK cells can be divided into four subsets ranging from the most immature to the most mature based on the expression of CD27 and CD11b [22]. Localisation of the distinct NK cell subsets is dependent upon the expression of a specific repertoire of integrins and chemokine receptors, both in humans and mice (nicely reviewed in [23] and [24], respectively). The main characteristics of human and mouse NK cells are summarised in table 1.

NK cell activation

NK cell functions are regulated by surface receptors that integrate inhibitory and activating signals and allow early detection and response to changes in the environment [25]. The negative signals transmitted by the inhibitory receptors tend to be dominant, tipping the balance in favour of an inhibition of NK cells and maintaining tissue homeostasis in physiological conditions [26]. The vast majority of inhibitory receptors recognise major histocompatibility complex (MHC) class I molecules and include the CD94-NKG2A heterodimer and the inhibitory members of KIR in humans or Ly49 in mice [27]. Additional inhibitory receptors, also called checkpoint molecules, include programmed cell death protein 1 (PD-1), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), CD96, T-cell immunoglobulin and mucin domain-containing protein 3 and lymphocyte activation gene-3 (LAG-3) [28]. NK cells express a long list of receptors with activation potential. Apart from the Fc receptor Fc γ R3a (CD16), which triggers ADCC, other receptors, including NKG2D and NKp46, do not activate on their own but need to synergise in order to elicit a cytotoxic response of NK cells [26]. The secretory profile of NK cells increases in magnitude and complexity when different coactivation receptors are co-engaged [18]. Ligands for NK cell-activating receptors include both host and pathogen glycoproteins [29]. Other powerful activators of NK cell functions are cytokines such as IL-12, IL-15, IL-18 and type I IFN [30], but many others including type 2

TABLE 1 Identification of classical natural killer (NK) cells in humans and mice, main functions and localisation, and specific expression of chemokine receptors by subsets

	Humans		Mice	
Identification	CD3 ⁻ CD56 ⁺ [11] CD3 ⁻ NKp46 ⁺ [12]		CD3 ⁻ NK1.1 ⁺ (C57BL/6) CD3 ⁻ DX5 ⁺ (BALB/c) CD3 ⁻ NKp46 ⁺ (all strains) [19]	
Phenotype	CD56 ^{bright} CD16 ⁻	CD56 ^{dim} CD16 ⁺	CD27 ^{high} CD11b ⁺	CD27 ^{low} CD11b ⁺
Main functions	Production of immunomodulatory cytokines in response to IL-1 β , IL-2, IL-12, IL-15, and/or IL-18 [13]	Cytotoxicity [11] Production of IFN- γ upon engagement with target cells [17]	Cytotoxicity +++ [22] Cytokine production +++ [22]	Cytotoxicity + [22] Cytokine production + [22]
Main localisation	Secondary lymphoid organs [13]	Blood, spleen, lung [11]	Secondary lymphoid organs [22]	Blood, spleen, lung [22]
Associated chemokine receptors	CCR7 [23]	CXCR1, CX3CR1, ChemR23, S1P5 [23]	CXCR6 [23]	CX3CR1, S1P5 [23]

CCR7: C-C chemokine receptor type 7; ChemR23: chemerin receptor 23; CX3CR1: CX3C motif chemokine receptor 1; CXCR: C-X-C motif chemokine receptor; IFN: interferon; IL: interleukin; S1P5: sphingosine-1-phosphate receptor 5.

and regulatory cytokines, as well as cytokines belonging to the IL-12 and IL-1 families, all contribute to shape NK cell responses [31].

NK cell functions

NK cells kill stressed, transformed or infected cells through the release of cytolytic granules, which contain perforin and granzymes, and/or by inducing apoptosis, pyroptosis or necroptosis through the activation of cell death receptors (Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4/DR5) on target cells. Whereas apoptosis is defined as nonimmunogenic cell death due to the formation of apoptotic bodies that enclose intracellular content, pyroptosis and necroptosis have been described as immunogenic programmed cell deaths, resulting in the spillage of cellular content. These last two processes may modulate secondary adaptive immune responses [32].

NK cells also shape adaptive immune responses through the production of a large variety of cytokines such as IFN- γ , TNF- α , GM-CSF, IL-10, IL-5 and IL-13, and chemokines such as chemokine (C-C motif) ligand (CCL) 3/macrophage inflammatory protein (MIP)-1 α , CCL4/MIP-1 β , CCL5/regulated on activation, normal T-cell expressed and secreted (RANTES) and chemokine (C-X-C motif) ligand (CXCL) 8/IL-8 [33, 34]. IFN- γ is the signature cytokine of NK cells and is produced in response to target cell recognition and upon stimulation with combination of cytokines, such as IL-18 and IL-12 [35]. In addition, NK cells may regulate immune responses by interacting with other cells of the immune system. Reciprocal interaction has been described with innate immune cells, *i.e.* dendritic cells (DCs) [36–42], monocytes/macrophages [43–47], granulocytes [48–53] and cells from adaptive immunity, *i.e.* T-cells [54–56] and B-cells [57]. The bi-directional interaction between NK cells and DCs is the most studied and was shown to impact activation, maturation and function of both cell types [36–39]. The crosstalk between NK cells and immature DCs may result in either DC maturation or cell death depending on the relative DC/NK cell proportion and DC maturation stage, allowing NK cells to edit DC functions and limit immunopathology [39, 40]. Recently, NK cells were shown to kill immature DCs and T-helper cell type 2 (Th2)-polarising but not Th1-polarising DCs, through NKp30 and DNAX accessory molecule 1 (DNAM-1), thus restricting the pool of DCs involved in Th2 immune responses [41]. Conversely, DC-derived signals *via* cytokines (notably cytokines from the IL-12 family, type I IFN and IL-15) and cell contact (NKp30, NKp46 and NKG2D) are important for activation of NK cells [37, 42]. Regarding granulocytes, NK cell coculture with eosinophils revealed that NK cells are potent inducers of eosinophil activation and apoptosis *in vitro*, suggesting a role for NK cells in the regulation of eosinophilic inflammation [48–50]. Specifically, NK cells induce eosinophil apoptosis [48, 49] *via* direct contact between NK cells and eosinophils, which leads to the apoptosis of eosinophils independently of caspases [49] and NK cell degranulation [48]. The combined action of CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ NK cell subsets is required to induce eosinophil apoptosis [50]. Moreover, NK cells increase the production of mediators such as eosinophil cationic protein and eosinophil-derived neurotoxin [49], but decrease the production of superoxide anions in the presence of lipoxin A4, a mediator of inflammation resolution [50]. In mouse models, eosinophils were shown to inhibit NK cells through metabolic checkpoints [51]. Human

NK cells can also trigger caspase-dependent neutrophil apoptosis *in vitro* through the NKp46 and Fas pathway, which may be involved in the resolution of acute inflammation [52]. Conversely, neutrophils were found to be required for proper NK cell maturation [53].

NK cells in the lung during homeostasis and respiratory viral infections

Lung NK cells at homeostasis

Peripheral NK cells originate from the bone marrow, where they differentiate and mature, although NK cell maturation was also described in secondary lymphoid tissues [58, 59]. Mature NK cells then migrate to the lung [60], possibly in a sphingosine-1-phosphate 5-dependent export through blood flow [9]. The percentage of NK cells among lymphocytes is the highest in the lung compared to other organs [9], supporting a prominent role for these cells in the immunology of the airways. In human lungs, NK cells are located in the parenchyma, are mainly CD56^{dim}CD16⁺ and express KIRs and CD57, but not NKG2A, indicating a well-differentiated phenotype. Highly differentiated NK cells are found at higher frequencies in the lungs compared to matched peripheral blood in adulthood [10] and compared to other tissues in the fetal stage [61]. Only few lung NK cells, mainly among the CD56^{bright}CD16⁻ subset, express CD69, a tissue residency and activation marker [10]. This suggests that the human lung is populated mainly by NK cells circulating between the lung and blood rather than by a stable pool of CD69-expressing tissue-resident cells, which also co-express CD49a and CD103 [62]. As in humans, lung NK cells in mice account for approximately 10% of the lymphocytes, a higher frequency than in other tissues [63], and mainly exhibit a mature phenotype CD27⁻CD11b⁺ [22]. Similar to humans, the majority of lung NK cells in mice are not tissue-resident, as shown by the low percentage of NK cells expressing CD49a and CD69 [64]. Despite a well-differentiated phenotype, both human and mouse lung NK cells are hypofunctional at steady state, in comparison to peripheral blood NK cells (figure 1a). As lungs are continuously exposed to environmental and autologous antigens, this hypofunctional phenotype may contribute to avoid permanent pulmonary inflammation [10]. Lung NK cells obtained from tissues or bronchoalveolar lavage (BAL) can form conjugates with target cells but have an impaired cytotoxic ability [65]. The suppressive effect on NK cells might involve soluble factors produced by alveolar macrophages, such as transforming growth factor (TGF)- β 1 [66] and prostaglandin E2 [67], as well as lipids found in BAL fluid [68], and can be reversed with type I IFN stimulation [69]. Similarly, mouse lung NK cells show lower cytotoxicity compared to spleen NK cells when stimulated by IL-2 or a combination of IL-2, IL-12 and IL-18 [70]. Additionally, lower expression of molecules associated with activation, such as NKp46, NKG2D and CD69, and higher expression of the CD94-NKG2A inhibitory receptor have been observed on lung NK cells compared to spleen and bone marrow NK cells [63].

NK cell involvement in respiratory viral infections

Genetic deficiencies that impact NK cell numbers, phenotype or functions are rare in humans and associated with susceptibility to recurrent viral and bacterial infections of the upper and lower respiratory tract [71], suggesting a role of NK cells in pulmonary host defence. The function of NK cells has been reported in pulmonary infections particularly caused by influenza A virus (IAV), RSV, RVs and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Although IAV, RSV and RV are associated with asthma development and exacerbations [72], it is still unclear if and how SARS-CoV-2 may exacerbate asthma, as there is an overlap between the symptoms of coronavirus disease 2019 (COVID-19) and asthma exacerbation [73–75]. Here, we will briefly examine the activation of NK cells during pulmonary infection induced by these viruses (summarised in figure 1b).

Infants who died of IAV infection have almost no detectable NK cells in their lungs but exhibit large quantities of viral antigen, suggesting that NK cells are necessary to clear up the infection [76]. During mouse IAV infection, NK cells are recruited in the lung draining lymph nodes and to the lungs [77, 78]. The recruitment of NK cells in the lungs is dependent on C-X-C motif chemokine receptor (CXCR) 3 as levels of CXCR3 ligands (CXCL9, CXCL10 and CXCL11) are increased during the infection and CXCR3^{-/-} NK cells fail to migrate in lung tissues. Similarly, the recruitment of CCR5^{-/-} NK cells in the airways and BAL of infected mice is impaired, suggesting that migration to the airway and localisation to the infected epithelium may depend on CCR5 [77]. *Ncr1*-deficient mice exhibit normal NK cell development but are deficient for the NKp46 activating receptor. IAV infection was shown to be lethal in these mice, suggesting that NKp46 plays a critical role in NK cell activation and the control of infection [78]. The beneficial role of NK cells may also involve tissue repair. Indeed, during the recovery phase of IAV infection, a subset of lung NK cells (NCR1⁺NK1.1⁺ROR γ t⁻) was shown to produce IL-22, a cytokine involved in the regeneration of epithelial cells [79]. In contrast, depletion of NK cells, either with antibodies against NK1.1 or asialoganglioside gangliotetraosylceramide (asialo-GM1, ASGM1) or in IL-15^{-/-} transgenic mice, was shown to decrease inflammation and protect against lethal IAV infection, suggesting that NK cells may significantly augment pulmonary inflammation, contributing to the

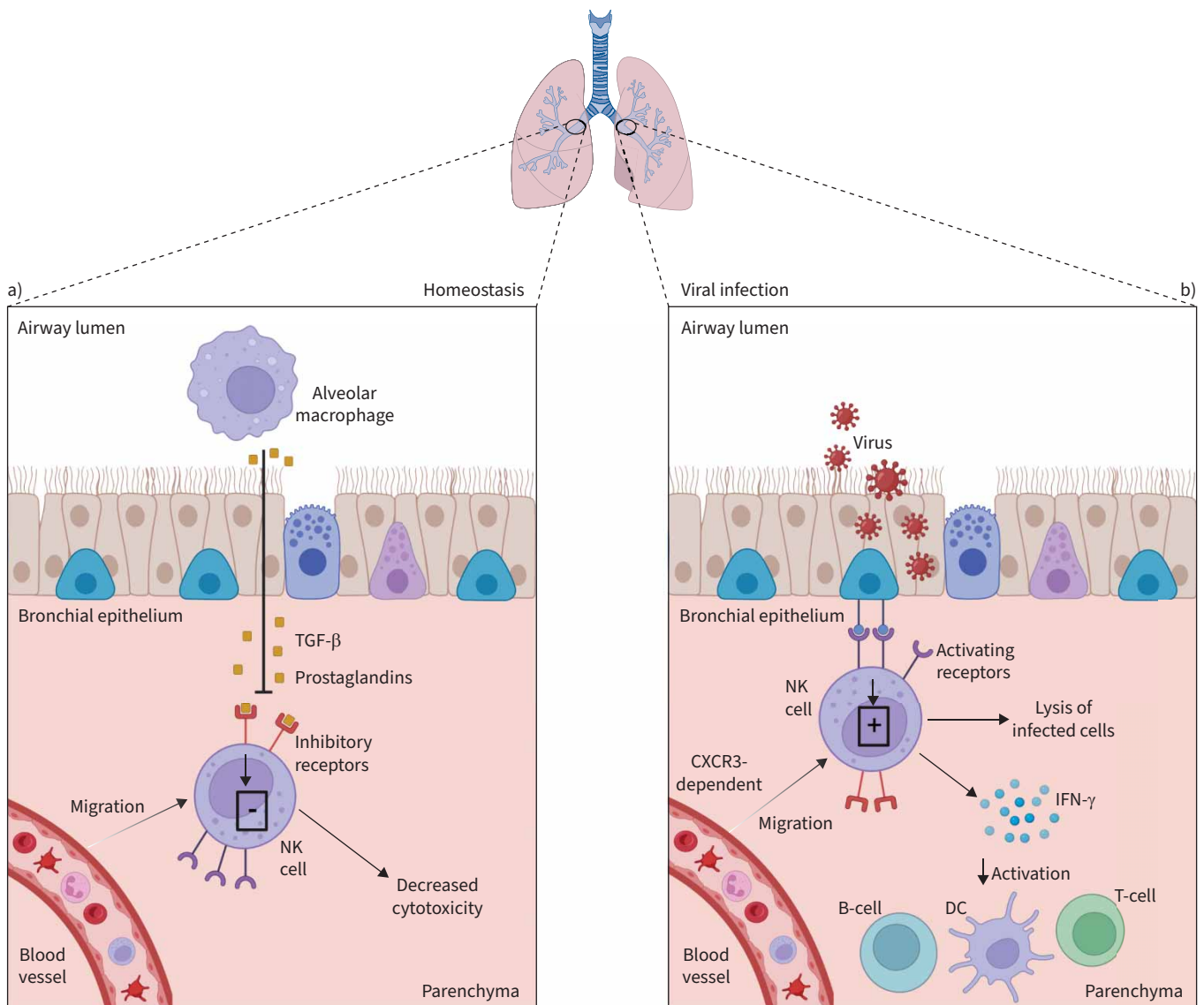


FIGURE 1 Natural killer (NK) cells in the lung at homeostasis and after viral infection. **a)** At homeostasis, alveolar macrophages produce metabolites such as transforming growth factor (TGF)- β 1 and prostaglandin E2 leading to a suppressive effect (-) on NK cells. Indeed, lung NK cells have decreased cytotoxic activity while they can still form conjugates with target cells. This hypofunctional phenotype may allow NK cells to remain tolerant to healthy tissue and avoid permanent pulmonary inflammation. **b)** During viral infection, NK cells migrate from the blood to the lungs in a C-X-C motif chemokine receptor 3 (CXCR3)-dependent manner. Recognition of infected cells, through activating receptors such as Nkp46, results in NK cell activation (+). Activation leads to the lysis of infected cells through apoptosis and to interferon (IFN)- γ production. IFN- γ is involved in the activation of other immune cells (dendritic (DC), T- and B-cells), which participate in viral clearance. Created with BioRender.com.

pathogenesis of IAV infection [80]. Depending on the virus dose, it has been hypothesised that NK cells may play a dual role: they may protect mice against low influenza challenge doses but contribute to pathology and lung tissue injury at higher challenge doses.

Similar involvement of NK cells during the course of RSV infection was reviewed by BHAT *et al.* [81]. As observed in IAV infections, NK cells play a dual role in RSV infection: the initial protective role through natural cytotoxicity is followed by a detrimental one that induces lung injury due to the inhibition of antibody responses and the secretion of pro-inflammatory factors. NK cells may also initially protect against the Th2 response induced by RSV infection [82] that is associated with severe disease [83]. Interestingly, NK cells are susceptible to IAV and RSV infections, which modify NK cell phenotype and functions. For example, RSV was shown to infect human neonate and adult NK cells *in vitro*, although infected NK cells did not release infectious viral particles. RSV-infected NK cells were more prone to

produce IFN- γ than uninfected cells, while the percentage of perforin-secreting cells was not increased, suggesting a shift toward a pro-inflammatory rather than a cytotoxic NK cell state, thus contributing to immunopathology [84].

The precise functions of NK cells during RV infections are less described, as RVs poorly infect mice, limiting the use of mouse models. In humans, experimental RV infection of healthy volunteers increased NK cytolytic activity of peripheral blood mononuclear cells (PBMCs) [85], while RV stimulation of PBMCs *in vitro* induced NK cell activation and degranulation [86, 87]. NK cell activation by RV requires other cells (most likely monocytes and DC) as RV is not able to stimulate purified NK cells [86] and partly depends on type I IFN signalling [87]. In mice, NK cells expressing CD69, IFN- γ and granzyme B accumulate in BAL and lungs after RV infection [88]. The accumulation and cytotoxic activity of NK cells depends upon IL-15, TRAIL and type I IFN signalling [88, 89].

Currently available data demonstrate that SARS-CoV-2 affects NK cell compartments and activation in humans. In acute SARS-CoV-2 infection, the NK cell number decreases in blood while increasing in BAL [90], suggesting homing to the lung, which involves CXCR3, CXCR6 and CCR5 chemokine receptors [91, 92]. The impact of SARS-CoV-2 infection on NK cell phenotype has been recently reviewed [93] and demonstrated that in peripheral blood and BAL from patients with moderate to severe COVID-19, NK cells display signature of highly activated cells [90]. In addition, NK cells in severe COVID-19 exhibit an exhausted phenotype, with increased expression of PD-1, LAG-3 and TIGIT, while the frequency of DNAM-1- and NKG2D-expressing NK cells are decreased. Lymphocyte exhaustion can be defined as an impaired state resulting from antigenic overstimulation. Although T-cell exhaustion has been extensively investigated and discussed, the concept of NK cell exhaustion remains unclear and the exact mechanisms leading to NK cell exhaustion in oncology and chronic infections are poorly defined [94]. Local and systemic inflammation observed in SARS-CoV-2 infection was suggested to impair NK cell effector functions, as shown with the inhibition of TGF- β , which restored NK cell effector functions *in vitro* [95]. In mice, infection with several SARS-CoV-2 variants induced a reduction in lung NK cell numbers [96]. The exact functions of NK cells in SARS-CoV-2 infection have yet to be uncovered, although their dysfunction appears to be linked with disease severity.

In conclusion, NK cells seem to display multiple roles in respiratory viral infections, as they may control viral infection and promote tissue repair, but their proinflammatory activity may be deleterious if uncontrolled.

NK cells: involvement in asthma development and exacerbations, effects of treatments

NK cells in patients with asthma

In the blood and lung compartments, NK cells from asthma patients exhibit phenotypic and functional changes compared to healthy donors [48, 86, 97–101]. The frequency of blood NK cells producing IL-4 is increased in allergic asthma subjects, while peripheral blood IFN- γ ⁺ NK cells are decreased [98]. In allergic asthma patients, the proportion of CD56^{bright}CD16⁺ NK cells is decreased in peripheral blood and exhibits less interaction with DCs *in vitro*, which may participate in the dysregulation of adaptive immunity [99]. The CCL18 chemokine, involved in allergic diseases [102], induces migration and *in vitro* cytotoxicity of NK cells from healthy donors; however, this function is defective in allergic patients [101]. In noneosinophilic neutrophilic asthma, the intracellular expression of granzyme B and IFN- γ is increased in blood NK cells compared to eosinophilic asthma patients and healthy control subjects [103]. In severe asthma, the numbers of peripheral blood NK cells and the ratio of BAL NK cells/CD4⁺ T-lymphocytes are significantly decreased compared to healthy donors. Moreover, NKG2D and CD69 expression by NK cells is increased in severe asthma and positively correlated with the number of blood eosinophils [48, 100]. Blood CD69⁺ NK cells were also found to be negatively correlated with the ratio of forced expiratory volume in 1 s to forced vital capacity [104]. Functionally, NK cells from severe asthma patients induce less eosinophil apoptosis [48] and are less cytotoxic toward K562 cells despite increased production of cytotoxic mediators, in comparison to NK cells from healthy subjects [48, 100]. Therefore, NK cells may interact with several cells involved in asthma. These crosstalks may be dysregulated in asthma patients, resulting in an imbalance between pro- and anti-inflammatory responses (table 2).

Contribution of animal models to the understanding of NK cell functions in asthma

In mouse models of allergic asthma, conflicting results on the role of NK cells have been obtained depending on the allergen sensitisation strategies and the tools used to deplete or inhibit NK cells. Successful specific depletion of NK cells is an important step towards understanding their function in mouse models of asthma. To date, no antibodies are specific for depletion for NK cells. Anti-NK1.1 antibodies also target NK T-lymphocytes [106] and can be used only in some mouse strains such as

TABLE 2 The pro- and anti-inflammatory functions of natural killer (NK) cells and their hypothesised effects in asthma deduced from observations in patients

	Functions of NK cells	Observations in asthma patients	Hypothesised effect in asthma
Pro-inflammatory	IL-4 production [98]	Decreased frequency in atopic asthma [98]	Increased Th2 responses
	Target cell lysis	Decreased in severe asthma [48, 100]	Dysregulated NK cell functions
	RV-induced cytokine production and degranulation [86, 87]	Decreased in severe asthma [86]	
Anti-inflammatory	Maturation of DC producing IL-12 [99]	Decreased in allergic asthma [99]	Increased Th2 responses
	Neutrophil apoptosis [52]	Similar to healthy individuals [48]	No effect on the resolution of neutrophilic inflammation
	Eosinophil apoptosis [48, 49]	Decreased in severe asthma [48]	Delayed resolution of eosinophilic inflammation
	Lipoxin-activated NK cells decrease superoxide anion production by eosinophil [50]	Decreased lipoxin production in severe asthma [105]	Increased damage caused by eosinophils
	Lysis of immature DCs [99]	Decreased in allergic asthma [99]	Dysregulated NK cell functions

DC: dendritic cell; IL: interleukin; RV: rhinovirus; Th: T-helper.

C57BL/6. Anti-ASGM1 antibodies have long been used in BALB/c mice [107]. However, the expression of ASGM1 is not strictly confined to NK cells and is detected on other haematopoietic cells, such as basophils and subsets of NK, CD8⁺ and $\gamma\delta$ T-cells [108]. Moreover, ASGM1 antibodies do not deplete all NK cells [109, 110]. More recently, mice that express Cre recombinase driven by the NKp46 promoter ($Ncr1^{iCre/iCre}$) were generated [111]. Crossing these mice to ROSA-flox-stop-flox-diphtheria toxin A ($ROSA^{DTA/DTA}$) mice results in offspring lacking NKp46⁺NK1.1⁺CD3⁻ cells, which include *bona fide* NK cells, but also subsets of ILC1 and ILC3 [111]. All these tools were used to show that NK cells either have pro-inflammatory properties [110, 112–116], have no effect [117] or promote the resolution or suppression of allergic inflammation [118–121] in mouse models.

In BALB/cByJ mice, ovalbumin (OVA)-induced asthma increased CD3⁻DX5⁺ NK cell numbers in lung draining lymph nodes but not in the lungs. NK cell depletion with an anti-ASGM1 antibody decreased eosinophilia in BAL with no effect on airway hyperresponsiveness [110]. C57BL/6 mice with NK cell deficiency, such as NK cell-deficient mice [122] or mice depleted in Ly49A/D/G cells, exhibit decreased cellular lung infiltrate and airway hyperresponsiveness after OVA sensitisation [114]. In a model of asthma induced by house dust mites (HDMs), NKG2D-deficient mice showed a decreased number of eosinophils in BAL and Th2 cells in the lungs, and decreased serum IgE levels, in comparison to HDM-sensitised wild-type mice. The pro-inflammatory role of NKG2D in experimental allergic asthma depended on granzyme B produced by NK cells, but not on perforin [114, 115]. The role of NK cells has also been highlighted in a model of predisposition to experimental asthma. The offspring of mice exposed before mating to diesel exhaust particles (DEPs) showed increased airway hyperresponsiveness, peribronchial inflammation, mucin hyperproduction, BAL eosinophilia and recruitment of Th2 lymphocytes and ILC2 in the lungs after sensitisation to OVA or HDM. Sensitised offspring of DEP-exposed mothers showed an increased number of lung NK cells, which were more mature and primed for degranulation and production of IL-5 and IL-13. The involvement of NK cells in this model has been shown using NK cell-deficient mice ($Ncr1^{iCre/iCre}$ [111] crossed to $ROSA^{DTA/DTA}$), NK cell transfers or the injection of anti-ASGM1 antibodies. The mechanisms involved include the production of IL-13 and granzyme B by NK cells. The role of granzyme B involved, independently of NK cell cytotoxicity, the production of IL-25 by epithelial cells that induced the activation and recruitment of Th2 and ILC2 cells [116]. Thus, this work describes an endotype of asthma dependent on NK cells, which occurs at the beginning of life following maternal exposure to DEP. The maternal exposure to DEP was a key factor for involvement of NK cells, as the same NK cell-deficient mice still developed all key features of acute or chronic HDM-driven asthma, suggesting that NK cells play no role in the development of experimental allergic asthma [117].

In contrast, OVA sensitisation of *Ncr1*-deficient mice, whose NK cells do not express NKp46, was shown to increase the production of IgE and the number of eosinophils in the airways compared to OVA-sensitised wild-type mice, suggesting that *Ncr1* may reduce allergic pulmonary responses [118]. NK cells are regulated by a wide variety of endogenous eicosanoids such as prostaglandins, leukotrienes, cannabinoid type 2 (CB2), lipoxin-A4 and resolvin-E1. The resolution phase of allergic pulmonary

inflammation was studied in a mouse model of OVA sensitisation after the cessation of OVA aerosols. NK cells accumulated in mediastinal lymph nodes during resolution and their depletion (anti-ASGM1) at the peak of inflammation led to persistent pulmonary allergic inflammation, suggesting a key role for NK cells in the resolution of pulmonary allergic inflammation. Furthermore, the resolution of inflammation by administration of resolvin-E1 was mediated by NK cells [121]. A suppressor role was also shown with other eicosanoid mediators. HDM-sensitised CB2-deficient mice exhibited attenuated allergic inflammation, with high numbers of $\text{IFN-}\gamma^+$ NK cells, but a defect in pulmonary ILC2. NK cell depletion (anti-NK1.1) restores pulmonary allergic inflammation in CB2-deficient mice, whereas the transfer of CB2-deficient NK cells into HDM-sensitised wild-type mice suppressed it. Depletion of NK cells was accompanied by an increase in ILC2, suggesting that NK cells could play a role in limiting ILC2 responses and associated allergic inflammation [119]. The same types of results were obtained with mice deficient for the prostacyclin (PGI_2)/prostacyclin receptor (IP) [120]. The relationship between NK cells and ILC2 is perplexing. Indeed, in a mouse model of pulmonary metastases in mice previously sensitised with *Aspergillus* protease, the anti-tumour function of NK cells was inhibited by ILC2 via eosinophils [51]. The apparent conflicting data obtained in mouse models are actually related to the experimental settings and lead to a better understanding of the global picture. Indeed, it seems that not only the sequence of events, but also the environment leading to the activation of NK cells in the lungs, may play a crucial role in determining their function in allergic pulmonary inflammation. Knowledge on the functions of NK cells in experimental asthma are summarised in figure 2.

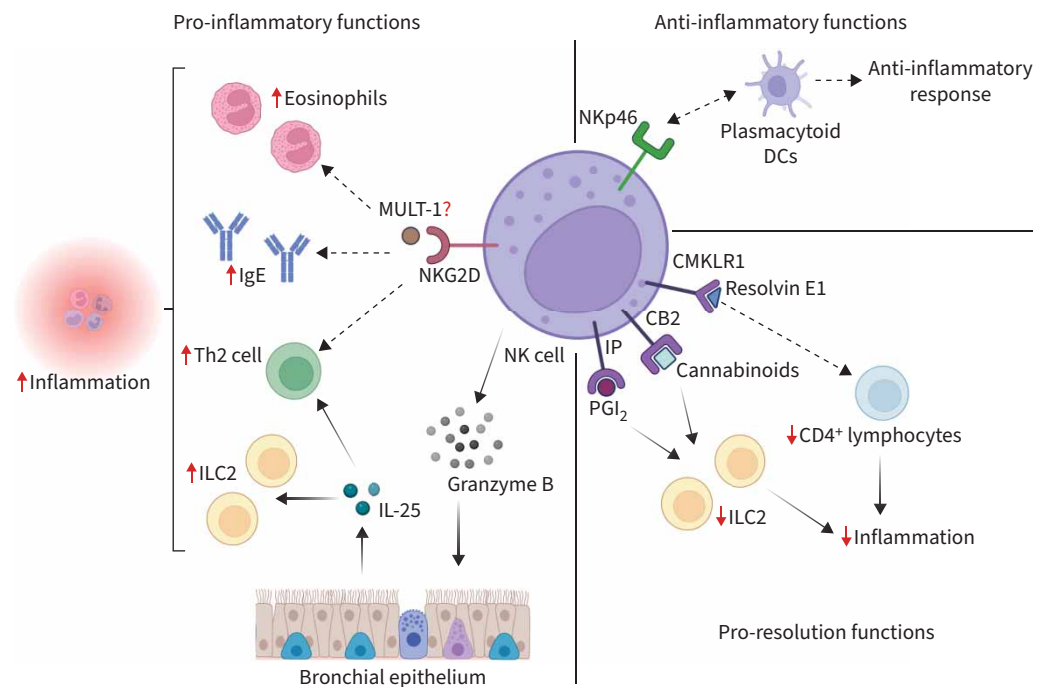


FIGURE 2 Natural killer (NK) cell functions in asthma pathogenesis highlighted by animal models. In many allergic asthma models, NK cells were suggested to play a proinflammatory role. Several studies showed that NKG2D is involved in pathways leading to increased eosinophil numbers in bronchoalveolar lavage, serum IgE production and the recruitment of T-helper 2 (Th2) cells in the lungs. NK cells were also shown to activate airway epithelium through granzyme B, resulting in interleukin (IL)-25 production, subsequently involved in type 1 innate lymphoid cell (ILC1), type 2 innate lymphoid cell (ILC2) and Th2 recruitment and activation. Conversely, NK cells may exert anti-inflammatory functions through the activation of plasmacytoid dendritic cells (DCs) by NKp46. In mice, lung plasmacytoid DCs were shown to suppress T-cell division and effector T-cell generation induced by myeloid DCs, thereby regulating lung inflammation. Finally, NK cells can have pro-resolutive functions through stimulation with endogenous eicosanoids such as prostaglandins, cannabinoids and resolvin-E1. These interactions result in a decrease of ILC2 and Th2 cell responses, leading to the resolution of inflammation. Dotted arrows: hypothetical role/interaction. Created with BioRender.com. CB2: cannabinoid type 2; CMKLR1: chemerin chemokine-like receptor 1; IP: prostacyclin receptor; MULT-1: murine UL16-binding protein-like transcript 1; PGI_2 : prostacyclin.

NK cells during asthma exacerbations

The implication of NK cells in asthma exacerbations is unknown but some observations suggest that their dysregulation may be associated with virus-induced exacerbations. NK cells from severe asthma patients were shown to exhibit abnormal responses to RV *in vitro*. Indeed, stimulation of PBMCs by RV-A9 led to defective activation, cytotoxic capacities and production of the anti-viral cytokine IFN- γ by NK cells from severe asthma patients compared to healthy donors. As all severe asthma patients are treated with inhaled or oral corticosteroids, the confounding effects of this treatment may participate to this defect. However, there was no difference between patients with and without oral corticosteroids on NK phenotype, activation marker expression or degranulation and IFN- γ production, suggesting that the NK cell defect was related to the patient rather than the specific treatment [86]. Surface MHC class I-related chain (MIC) A and B molecules are ligands for the NKG2D, a major activating receptor expressed by all NK cells. MICA and MICB are upregulated on stressed and virus-infected cells and exist in soluble forms [123]. *In vitro* infection of respiratory epithelial cell line (BEAS-2B) by RV-16 was shown to increase the expression of membrane MICA/B and subsequently the release of soluble forms sMICA/B in supernatants. Experimental RV infection of healthy subjects increased sMIC molecule levels in serum, sputum and BAL [124]. MICA and MICB shedding was shown to be a regulatory mechanism that suppresses the function of NKG2D-bearing cells over time [125] and most likely contributes to resolution of the immune response consecutive to RV infection. This mechanism seems to be deficient in asthma patients as no significant changes in sMICA/B levels in sputum or BAL were detected in asthma subjects after RV-16 infection. Asthma patients included in this study were atopic and were not treated with inhaled or oral steroids, suggesting they suffered from mild asthma. Neither NKG2D expression nor the function of NK cells were measured [124]. Nevertheless, NKG2D expression on NK cells was found to be significantly increased in severe but not mild asthma outside of virus-induced exacerbation [48]. Together, these data suggest that NK cells are dysregulated in asthma, conferring abnormal responses to RV infection that might lead to virus-induced exacerbation.

In a murine model of asthma, intranasal administration of the Toll-like receptor 3 agonist poly(I:C) was used to mimic respiratory virus infection and to induce experimental exacerbation. Administered after OVA sensitisation and challenges, poly(I:C) further increased eosinophil and neutrophil numbers in BAL and mucus production in the airways. This exacerbated response was prevented by NK cell depletion with anti-ASGM1 antibody, suggesting a role of NK cells in virus-induced exacerbation of experimental asthma [126].

NK cells and asthma treatments

The most common medications to treat asthma include inhaled or oral corticosteroid, short- or long-acting β_2 agonists and long-acting muscarinic antagonists [127]. Systemic prednisolone treatment was found to decrease the cytotoxicity of peripheral blood NK cells [128]. Intramuscular administration of triamcinolone increased BAL NK cell percentage in asthma patients 3–6 weeks after the injection. Relative numbers of BAL CD56^{bright} NK cells significantly increased, while BAL granzyme A decreased, suggesting that systemic steroid treatment altered the NK cell phenotype [100]. *In vitro*, PBMC treatment with fluticasone propionate or dexamethasone decreases NK cell cytotoxicity [100, 129]. Methylprednisolone on purified NK cells significantly inhibited cytotoxicity, proliferation, IFN- γ production and the expression of NK cell activation markers, showing a direct effect of corticosteroids [130]. Finally, methylprednisolone administration *in vivo* decreases the expression of natural cytotoxicity receptors (NKp30, NKp44 and NKp46) on peripheral blood NK cells [131].

β_2 agonists also affect NK cell functions. Indeed, in an experimental *in vitro* setting with drug incubation followed by cell stimulation with anti-CD16 antibody to mimic ADCC, β_2 -adrenergic agonists (formoterol, levalbuterol, salmeterol and fenoterol) were shown to inhibit NK cell degranulation and the expression of TNF and IFN- γ [132]. Salmeterol also decreased NK cell degranulation in a cytotoxicity assay using the K562 target cell line [133]. Finally, formoterol decreased NKG2D expression and IFN- γ secretion by NK cells *in vitro* [134].

The effect of the biologics, used to treat severe asthma, on NK cells is poorly known. The effect of dupilumab, a humanised anti-IL-4R α , has been evaluated on NK cells from atopic dermatitis patients after 3-month treatment and no modification of the NK cell transcriptomic signature was observed [135]. Benralizumab is a humanised monoclonal antibody, which inhibits the hetero-dimerisation of the IL-5 receptors on eosinophils and binds to the Fc γ IIIa receptor of NK cells through its constant Fc region. Binding was shown to result in the activation of ADCC, leading to the release of perforin and granzymes by NK cells, which induced eosinophil and basophil apoptosis [136]. *In vitro* experiments recently showed that eosinophil apoptosis induced by benralizumab depends on the formation of an eosinophil/NK cell

synapse resulting in ADCC and enhancement of macrophage cytotoxicity by NK cell-derived IFN- γ [137]. Therefore, NK cells are potential key contributors to the efficacy of benralizumab treatment leading to the absence of circulating blood eosinophils.

Conclusions and future perspectives

In conclusion, NK cells are innate immune cells ubiquitous in humans, including in the respiratory tract, where they participate to the homeostasis and the defence against viral infections. Based on their multiple functions, they may affect lung responses during asthma or its exacerbations. Indeed, NK cells interact with several cell types involved in asthma development, inflammation or exacerbations to regulate their functions or induce cell death. In asthma, particularly in severe asthma, NK cell functions are dysregulated. The implication of asthma treatment or physiopathology, including inflammation, in the NK cell defect remains to be fully elucidated, but both are probably involved.

Future studies will need to consider confounding factors such as obesity or cigarette smoke exposure. Obese asthmatic individuals represent a distinct phenotype of asthma that is associated with more severe exacerbations, decreased response to inhaled corticosteroids and poor quality of life. Accumulating evidence has revealed that obesity is associated with alterations in the functionality of several immune cells including NK cells [138]. For instance, diet-induced obesity increases mortality rate in mice after influenza infection, associated with decreased NK cell cytotoxicity [139]. Therefore, obesity associated to asthma may further accentuate NK cell dysfunction and may participate to increased virus-induced exacerbation. Cigarette smoke exposure also has detrimental effects on immune response in general and on NK cell functions in particular. Indeed, smoking exerts dual effects on the frequency and function of NK cells in both mice and humans (see reviews [140, 141]). The influences of cigarette smoking on NK cells may vary, depending on the differential pathological conditions or disease settings and subsets of NK cells with different surface markers. Peripheral blood NK cell activity is reduced in current smokers compared to former or never-smokers in a dose–response manner [142]. In patients with COPD, purified lung NK cells induced apoptosis in autologous epithelial cells, but the level of cytotoxicity is higher in COPD patients than in non-COPD smokers [143]. This suggests that the disease pathogenesis *per se* affects NK cell functions. NK cells are thought to play important roles in the pathological processes of COPD and its exacerbations, although the specific mechanisms are largely unknown. As some asthma patients may exhibit features of COPD, this phenotype must be considered in the study of NK cell functions in asthma.

Future research will also need to define the phenotypes/subsets and functions of NK cells in the various endotypes of asthma. Greater attention should be given to the non-T2 endotype, which is still poorly understood. The relationship between NK cells and the IL-17 axis is not well understood at present. NK cells are capable of IL-17 production [144], although the main IL-17-producing cells in peripheral blood of asthma patients are CD28⁺CD4⁺ T-cells [103]. Conversely, the IL-17 receptor is expressed by NK cells and mice lacking a functional IL-17 pathway exhibit impairment of NK cell-mediated responses [145]. IL-17A was shown to suppress IL-15 signalling during NK cell terminal maturation, thereby constraining NK cell maturation and effector function [146]. It is essential to use several mouse models to mimic different aspects of asthma endotypes to evaluate the involvement of NK cells in this heterogeneous disease. One might consider mouse pre-natal exposure as previously done with pollutants [116] and virus infection either in early life or as an exacerbation inducer.

An understanding of the precise implications of NK cells is needed as it may lead to successful interventions at various stages of the disease. For example, as anti-viral functions of NK cells are defective in severe asthma [86], their restoration may be a way to limit or avoid exacerbations during the viral season.

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