Natural Killer Cells and Host Defense Against Human Rhinoviruses Is Partially Dependent on Type I IFN Signaling

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Rhinovirus (RV), the causative agent of the common cold, causes only mild upper respiratory tract infections in healthy individuals, but can cause longer lasting and more severe pulmonary infections in people with chronic lung diseases and in the setting of immune suppression or immune deficiency. RV-infected lung structural cells release type I interferon (IFN-I), initiating the immune response, leading to protection against viruses in conjunction with migratory immune cells. However, IFN-I release is deficient in some people with asthma. Innate immune cells, such as natural killer (NK) cells, are proposed to play major roles in the control of viral infections, and may contribute to exacerbations of chronic lung diseases, such as asthma. In this study, we characterized the NK cell response to RV infection using an in vitro model of infection in healthy individuals, and determined the extent to which IFN-I signaling mediates this response. The results indicate that RV stimulation in vitro induces NK cell activation in healthy donors, leading to degranulation and the release of cytotoxic mediators and cytokines. IFN-I signaling was partly responsible for NK cell activation and functional responses to RV. Overall, our findings suggest the involvement of NK cells in the control of RV infection in healthy individuals. Further understanding of NK cell regulation may deepen our understanding of the mechanisms that contribute to susceptibility to RV infections in asthma and other chronic lung diseases.

Keywords: natural killer cell, rhinovirus, type I interferon, antiviral immunity, cytokine production, degranulation

INTRODUCTION

Respiratory viruses, particularly rhinoviruses (RV), typically cause only mild, self-limited infections in healthy individuals, but the consequences of infection can be much more serious in people with chronic lung diseases and in the setting of immune suppression or immune deficiency (Glezen et al., 2000; Versluys et al., 2010; Costa et al., 2011; Jacobs et al., 2013). For example, RV are implicated in both the induction of asthma and exacerbations of established asthma, representing the most common cause of asthma-related death (Longini et al., 1984; Beasley et al., 1988; Gern et al., 1996; Papadopoulos et al., 2002a; Papadopoulos et al., 2002b; Hansbro et al., 2008). Asthmatics are no
more likely than healthy individuals to develop a cold, but are substantially more likely for the infection to spread to the lower respiratory tract, causing more severe symptoms (Corne et al., 2002). This raises important questions regarding the mechanisms by which healthy people are able to mount an effective host response to RV. Understanding these processes that occur in healthy individuals might help to explain why RV infections can have such severe outcomes in people with asthma.

Bronchial epithelial cells (BECs) are the primary point of contact between infecting respiratory viruses and the host (Bals and Hiemstra, 2004). During infections, BECs release type I (IFN-I) and III IFNs, which are integral for the induction of innate immune response, conferring protection against viruses in conjunction with migratory immune cells (Hansbro et al., 2008; Message et al., 2008). Some investigators report that RV-induced IFN-I release is deficient in asthma (Gern et al., 2000; Corne et al., 2002; Wark et al., 2005; Hansbro et al., 2008), though other reports have been unable to confirm these findings (Sykes et al., 2014; Xi et al., 2015). Cell-mediated immunity to RV is also important in the control and clearance of infection and may be abnormal in asthmatics (Message and Johnston, 2001). Innate immune cells, such as natural killer (NK) cells have been proposed to play a major role in both asthma (Message et al., 2008; Culley, 2009), and the control of viral infections in general (Biron et al., 1999; Biron and Brossay, 2001; Moretta, 2002; Vivier et al., 2008). Increased cell-surface CD69 expression is widely used as a marker of NK cell activation (Gern et al., 1996; Vering et al., 2000; Draghi et al., 2007; Barnig et al., 2013).

NK cells can lyse target cells through the directed exocytosis of cytolytic granules containing perforin and granzyme at the immunological synapse between the NK cell and target cell (Robertson and Ritz, 1990; Cooper et al., 2001a; Biron et al., 1999; Biron and Brossay, 2001; Moretta, 2002; Vivier et al., 2008). The release of these cytolytic granules can be indicated through the presence of cell surface CD107a (Alter et al., 2004; Bryceson et al., 2010).

In addition to cytolytic function, NK cells produce chemokines and cytokines that can recruit other immune cells, promote cellular resistance to infection, and influence the formation of adaptive immunity (Robertson and Ritz, 1990; Biron et al., 1999; Biron and Brossay, 2001; Moretta, 2002; Vivier et al., 2008). An important cytokine produced by NK cells is IFN-γ, with known immunoregulatory effects (Gern et al., 1996; Cooper et al., 2001a; Cooper et al., 2001b; Colucci et al., 2003; Maroof et al., 2008; Strowig et al., 2008; Bi et al., 2017).

With the strong association between respiratory viral infections and asthma exacerbations, it has been speculated that inappropriate immune activation, possibly due to defective IFN-I response, may be involved in the susceptibility of asthmatics to more persistent and severe infections (Wark et al., 2005; Message et al., 2008; Culley, 2009).

Therefore, this study aimed to determine whether RV can activate NK cells, and the extent to which NK cell activation and function in vitro are IFN-I dependent.

**MATERIALS AND METHODS**

**Participants**

All volunteers completed a detailed questionnaire regarding respiratory symptoms, prior medical diagnoses, and medication use. Healthy participants had no symptoms or prior self-reported physician diagnoses of respiratory disease (including asthma) and had not experienced respiratory infection symptoms within the preceding month. All participants underwent skin prick testing (SPT) against a panel
of common allergens (Aspergillus fumigatus, Alternaria, grass pollen, house dust mite, and dog and cat dander) to determine allergic status. Prior to sample collection, volunteers were asked to abstain from antihistamine use for 72 h. The study was approved by the Human Ethics Committees at The University of Queensland and the Princess Alexandra Hospital, and all participants provided written consent.

**Rhinovirus Generation and Titration**

RV16 stocks were generated by passage in Ohio HeLa cells, as previously described (Sanders et al., 1998). RV16 was purified over Optiprep gradient (Sigma-Aldrich) as described by Xi et al. (2015). Viral titer was determined using TCID50, and RV16 at an MOI of 1 was used in culture (Pritchard et al., 2012).

**PBMC Isolation and Culture**

Human peripheral blood was collected into heparinized tubes (BD Vacutainer) from 12 healthy volunteers (mean age 21.6 ± 2.8 years, 67% female, 25% atopic). Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density gradient centrifugation with Lymphoprep (Stemcell Technologies). PBMC suspensions (4×10⁶ cells/ml) were prepared in media (RPMI 1640 (Gibco) supplemented with 5% autologous plasma, 2-ME, penicillin, streptomycin, and glutamine). 500 µL of the PBMC suspensions (2×10⁶ cells) were seeded on a 24-well flat bottom plate. IFN-I signaling was blocked in PBMC cultures using recombinant B18R protein (100 ng/ml, eBioscience), alongside a media-only control, for 1 h at 37°C with 5% CO₂. B18R acts as a decoy receptor for IFN-I with high specificity and affinity for all known subtypes of the IFN-I family, blocking IFN-I in vivo.

**Flow Cytometry**

Flow cytometry was used to identify immune cell populations and their expression of activation and function markers. Cell cultures were centrifuged, cell pellets were washed with PBS and labeled with live/dead (L/D) aqua viability dye (1:500 in PBS, Thermo Fisher Scientific) for 30 min on ice, protected from light, to allow for the exclusion of dead cells. Cells were surface stained with the following Abs: CD3-FITC, CD14-PerCP-Cy5.5, CD19-APC, CD56-PE-Cy7, and CD69-APC-Cy7 (Table S1); for 30 min on ice, protected from light. Cell were then fixed and permeabilised using the Cytofix/ Cytoperm™Fixation/Permeabilisation kit (BD Biosciences) and stained intracellularly with IFNγ-PE and GzymB-BV421 Abs (Table S1) for 30 min at room temperature, protected from light. Stained cells were then fixed in 2% paraformaldehyde. A total of 200,000 events per sample were collected using LSRFortessa X-20 (BD Biosciences) and the results were analyzed using FlowJo software (v10.7, Tree Star, Inc.). NK cells were identified as CD3− CD14− CD19− CD56− lymphocytes, activated NK cells as CD3− CD14− CD19− CD56− CD69+, and degranulating NK cells as CD3− CD14− CD19− CD56− CD107a+ (Herberman and Ortaldo, 1981; Robertson and Ritz, 1990; Bancroft, 1993; D’Arena et al., 1998; Vering et al., 2000; Alter et al., 2004; Papamichail et al., 2004; Draghi et al., 2007; Bryceson et al., 2010; Wang et al., 2012; Barnig et al., 2013).

**RESULTS**

**RV16, But Not IFN-I Signaling, Causes Minor Changes in NK Cell Populations**

To determine whether RV could activate NK cells, and the extent to which NK cell activation and functional changes are IFN-I dependent, PBMCs from healthy volunteers were cultured for 1 h in the presence or absence of B18R to block IFN-I signaling, prior to culture for 24 h in the presence or absence of RV16 stimulation. Following this, flow cytometry was used to identify and analyze NK cell subpopulations (CD56dim and CD56bright) following RV16 stimulation (Figure 1A). These changes in the distribution of the CD56− NK cell frequency that was less apparent when IFN-I signaling was blocked (Figure 1B).

**RV16 Induces Intense NK Cell Activation, Which Is Partly Dependent on IFN-I Signaling**

NK cell activation was assessed based on cell surface CD69 expression. Both an increase in the frequency of CD69+ cells and the expression intensity of CD69 can be used to assess NK cell activation. RV16 induced a significant increase in the frequency of CD69+ NK cells, as well as an increase in the expression intensity of CD69, when compared to media-only controls (Figure 1C). However, IFN-I signaling can also contribute to NK cell activation, as evident by the increase in CD56+ CD107a+ NK cells observed in the presence of B18R (Figure 1D). These changes in the distribution of CD56dim and CD56bright NK cells were not substantially altered when IFN-I signaling was blocked.
cell activation (Draghi et al., 2007; Du et al., 2010; Souza-Fonseca-Guimaraes et al., 2012; Barnig et al., 2013). RV16 stimulation of PBMC for 24 h led to substantial and significant increases in the proportion of NK cells expressing CD69, though this occurred to a lesser extent in the absence of IFN-I signaling (Figure 2A, left). Blocking IFN-I signaling had a larger impact on the percentage of CD69+ cells in the CD56bright subset (Figure 2A, right) than in the CD56dim subset (Figure 2A, middle). RV16 also increased the median fluorescent intensity (MFI) of CD69 surface expression on NK cells (Figure 2B), especially the CD56dim subset (Figure 2B, middle).

RV16 Induces NK Cell Cytolytic Granule Release Which Is Partly Dependent on IFN-I Signaling

NK cell degranulation was assessed based on CD107a surface expression. CD107a lines the cytolytic granules that are secreted during cytolysis, and appearance at the cell surface is upregulated following stimulation, correlating with target cell lysis (Alter et al., 2004; Bryceson et al., 2005; Bryceson et al., 2010). The presence of CD107a at the cell surface is an indicator of NK cell release of cytotoxic granules (Haworth et al., 2011; Barnig et al., 2013). RV16 stimulation of PBMC cultures resulted in significant increases in the proportion of CD56+ NK cells expressing cell surface CD107a (Figure 3A, left). Blocking of IFN-I signaling in vitro led to a lower frequency of degranulating CD56+ NK cells, both in the presence and absence of RV16 stimulation. These trends were also observed in both the CD56dim (Figure 3A, middle) and CD56bright NK cell subsets (Figure 3A, right). Stimulation with RV16 had no significant effect on the MFI of CD107a surface expression on CD56+ NK cells (Figure 3B, left). There were no significant changes in the level of CD107a surface expression in the CD56dim NK cell population (Figure 3B, middle). However, in the CD56bright NK cell population (Figure 3B, right), blocking of IFN-I signaling resulted in a small increase in the MFI of CD107a surface expression, in both the presence and absence of RV16 stimulation (Figure 3C).

Figure 1: RV16 altered NK cell populations, in an IFN-I independent manner. PBMCs from healthy people (n=12) were cultured in vitro with B18R (100 ng/ml) for 1 h to block IFN-I signaling, alongside a media-only control (UT), prior to stimulation with RV16 (MOI = 1), alongside an unstimulated control (US) for 24 h. (A) Percentage of lymphocytes, (B) total CD56+ NK cells, (C) and NK cell subsets (CD56dim and CD56bright) were evaluated using flow cytometry. Raw dot plots are representative of all 12 healthy donors. Each colored symbol represents data from one donor, lines represent medians. Data are representative of three experiments. *p<0.05, ***p<0.001 by Wilcoxon matched-pairs signed rank tests. RV16, rhinovirus 16; IFN-I, type I interferon; NK, natural killer; PBMC, peripheral blood mononuclear cell; UT, untreated; MOI, multiplicity of infection; US, unstimulated; SSC-A, side scatter-area.
RV16 Induces Small Changes in Both the Percentage of GzymB-Producing NK Cells and Their Intracellular GzymB

The cytolytic granules released through directed exocytosis contain proteins, such as GzymB, which is important in NK cell-mediated apoptosis of target cells (Shresta et al., 1995). RV16 stimulation of PBMC cultures resulted in small, but statistically significant, increases in the proportion of CD56+ NK cells producing GzymB (Figure 4A, left). This was most apparent in the CD56bright NK cell population (Figure 4A, right). Blocking of IFN-I signaling in vitro with B18R did not significantly alter the frequency of GzymB-producing NK cells (Figure 4A). RV16 stimulation also increased the intracellular GzymB MFI of CD56+ NK cells (Figure 4B, left), with changes observed in both the CD56dim (Figure 4B, middle) and CD56bright NK cell subsets (Figure 4B, right). Blocking of IFN-I signaling caused only minor changes in these responses.

RV16 Induces IFNγ-Producing NK Cells in an IFN-I Dependent Manner

NK cells are known to produce IFNγ in response to other viruses, including influenza viruses (Du et al., 2010). IFNγ activates multiple pathways associated with direct antiviral functions and immunoregulation, and promotes downstream protective immune responses (Biron and Brossay, 2001). Herein, we have found that RV16 stimulation resulted in a significant increase in the frequency of IFNγ-producing CD56+ NK cells (Figure 5A, left), and in the intracellular IFNγ MFI (Figure 5B, left). These trends were observed in both CD56dim (Figure 5A, middle; 5B, middle) and CD56bright NK cell subsets (Figure 5A, right; 5B, right). The increase in frequency of IFNγ-producing cells due to RV16 stimulation was most prominent in the CD56bright NK cell subset (Figure 5A, right). When IFN-I was blocked, there was a significant decrease in the frequency of RV16-stimulated IFNγ-producing CD56+ NK cells, which was reflected in both the CD56dim and CD56bright NK cell subsets (Figure 5A). This indicated that the RV16-stimulated increase in the frequency of IFNγ-producing NK cells was only partially dependent on IFN-I signaling.

IFN-I Signaling Is Involved in the RV16-Stimulated Release of GzymB and IFNγ

ELISA techniques were used to quantify RV16-stimulated GzymB and IFNγ release. Herein, we found that RV16 stimulation of PBMCs resulted in significant GzymB and IFNγ release into the culture supernatants (Figure 6). When IFN-I signaling was blocked, there was a decrease in the concentration of released GzymB, in both the presence and absence of RV16 stimulation (Figure 6A). In the presence of RV16 stimulation, blocking of IFN-I signaling also decreased IFNγ concentrations in culture supernatant (Figure 6B).

NK Cells Make a Large Contribution to the Production of GzymB and IFNγ

As there are multiple cell types in PBMC (other than NK cells) that can respond to viral stimuli, the amount of GzymB and IFNγ in culture supernatant cannot be wholly attributed to NK cells (Hornung et al., 2002). Thus, we next determined the relative levels of GzymB and IFNγ produced by other cells types (T and NKT cells) versus NK cells. In order to do this, surface staining was used to identify T cells and NKT cells (Figure S1), and the iMFI was calculated for GzymB and IFNγ production for each of
these cell types, as described by Darrah et al. (Darrah et al., 2007). We then scaled this relative to the size of each population by multiplying the iMFI by the frequency of each cell type of the total lymphocyte population.

We found that RV16 stimulation significantly upregulated the relative iMFI of GzymB+ cells in NK cells, T cells, and NKT cells, both in the presence and absence of IFN-I signaling (Figure 7A). In the NK cell and NKT cell populations, the relative iMFI of GzymB+ cells increased when IFN-I signaling was blocked in the absence of RV16 stimulation (Figure 7A, left and right). In all the conditions tested, the relative iMFI of GzymB+ cells was highest in NK cells (Figure 7A, left). It is worth noting that, unlike in the ELISA results, there was no significant decrease in RV16-stimulated GzymB when IFN-I signaling was blocked.

RV16 stimulation significantly upregulated the relative iMFI of IFNγ+ cells in all three of the populations tested, in both the presence and absence of IFN-I signaling (Figure 7B). In RV16-stimulated cultures, blocking of IFN-I signaling resulted in a significant decrease in the relative iMFI of IFNγ+ NK cells and T cells, but not NKT cells (Figure 7B). The trends observed in NK cells and T cells reflect the levels of IFNγ detected in the culture supernatant (Figure 7B, left and middle). Stimulation with RV16 caused the largest change in the relative iMFI of IFNγ+ cells in the NK cell population (Figure 7B, left).

**DISCUSSION**

This study aimed to investigate the possible role for NK cells in the immune response to RV infection and determine the extent to which RV16-induced activation and function of NK cells in vitro is dependent on IFN-I signaling. The key findings to emerge were that in cultured PBMCs from healthy people, RV16 stimulation affected NK cell activation and function, in a manner that was partially regulated by IFN-I signaling. IFN-I signaling partly contributed to RV-stimulated NK cell activation. Blocking of IFN-I signaling in PBMC cultures prior to RV16 stimulation reduced, but did not eliminate, NK cell activation.
The magnitude of the effect varied between donors, but was generally modest.

IFN-I has previously been shown to play both a direct and indirect role in the activation of NK cells (Biron et al., 1999; Cooper et al., 2001b; Hansbro et al., 2008). RV16 induces PBMCs to release IFN-I into the supernatant within 24 h (Khaitov et al., 2009). DC-mediated activation of NK cells involves both IFN-I dependent and independent mechanisms (Benlahrech et al., 2009). This is consistent with the NK cell activation observed in this study.

NK cells can be activated by several stimuli, including interactions with APCs, and cytokines, including IL-2, IL-12,
IL-15, and IL-18 (Orange and Biron, 1996a; Biron et al., 1999; Biron and Brossay, 2001; Cooper et al., 2001b; Moretta, 2002; He et al., 2004; Schoenborn and Wilson, 2007; Vivier et al., 2008). RV infection induces IL-15 expression from DCs and BECs, and can activate NK cells, inducing IFNγ production, independent of IFN-I signaling (Jayaraman et al., 2014; Xi et al., 2017; Kronstad et al., 2018). RV16-stimulated activation of NK cells in PBMC cultures where IFN-I signaling is blocked, suggests that RV16 can activate NK cells via IFN-I-independent mechanisms, similar to what has been reported for other viruses (Gary-Gouy et al., 2002; Hornung et al., 2002). Further experiments are required to analyze the exact nature of these IFN-I-independent mechanisms involved in RV16-stimulated NK cell activation.

Some respiratory viruses, such as influenza, can directly interact with NK cells to elicit immune responses (Ennis et al., 1981; Sirens et al., 2004; Hwang et al., 2013). The use of PBMC cultures in this study did not allow us to determine the contribution of direct interactions between RV16 and NK cells, nor whether RV16 activates NK cells indirectly via other cells, such as APCs. Future experiments could determine this by studying purified NK cells.

RV16-stimulated NK cell degranulation was reduced when IFN-I signaling was blocked in vitro, as shown in Figure 3. This correlates with previous research into murine models of viral infection, with IFN-I signaling shown to contribute to the degranulation of NK cells during MCMV infection (Orange

![FIGURE 6](image1) RV16 stimulated PBMCs release GzymB and IFNγ; this occurs to a lesser extent when IFN-I signaling is blocked. PBMCs from healthy people (n=12) were cultured in vitro with B18R (100 ng/ml) for 1 h, prior to stimulation with RV16 (MOI = 1), alongside an unstimulated control (US) for 24 h. ELISAs were performed on cell-free supernatants to determine GzymB and IFNγ concentrations. (A) Concentration of GzymB released into cell culture media by PBMCs. (B) Concentration of for IFNγ released into cell culture media by PBMCs. Each colored symbol represents data from one donor, lines represent medians. Data are representative of three experiments. *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon matched-pairs signed rank tests. IFN-I, type I interferon; GzymB, granzyme B; IFNγ, interferon gamma; PBMC, peripheral blood mononuclear cell; RV16, rhinovirus 16; UT, untreated; MOI, multiplicity of infection; US, unstimulated; ELISA, enzyme-linked immunosorbent assay.

![FIGURE 7](image2) NK cells are responsible for producing a large amount of the GzymB and IFNγ seen in RV16-stimulated PBMCs. PBMCs from healthy people (n=12) were cultured in vitro with B18R (100 ng/ml) for 1 h, prior to stimulation with RV16 (MOI = 1), alongside an unstimulated control (US) for 24 h. Expression of cell surface markers and intracellular cytokine production was determined by flow cytometry. (A) iMFI GzymB-producing NK cells, T cells, and NKT cells, scaled to account for their population size. (B) iMFI IFNγ-producing NK cells, T cells, and NKT cells, scaled to account for their population size. Each colored symbol represents data from one donor, lines represent medians. Data are representative of three experiments. *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon matched-pairs signed rank tests. NK, natural killer; GzymB, granzyme B; IFNγ, interferon gamma; RV16, rhinovirus 16; PBMC, peripheral blood mononuclear cell; IFN-I, type I interferon; UT, untreated; MOI, multiplicity of infection; US, unstimulated; iMFI, integrated median fluorescence intensity; NKT, natural killer T.
and Biron, 1996b; Nguyen et al., 2002). Interestingly, blocking IFN-I signaling reduced the proportion of CD107a+ NK cells both in virus-stimulated and unstimulated cultures. The latter observation may be attributed to autologous DCs inducing NK cell degranulation, as described by others (Hornung et al., 2002; Walwyn-Brown et al., 2018). Alternatively, it is possible there is a certain amount of constitutive IFN-I signaling that produces low-level NK cell degranulation. It has been suggested that CD107a protects degranulating cells from their own cytolytic granules, providing a basis for constitutive CD107a expression (Cohnen et al., 2013). There were no notable differences between the degranulation of CD56dim and CD56bright NK cell subsets.

GzymB is a cytotoxic mediator released from NK cells to cause lysis of target cells (Fehniger et al., 2007). While RV16 stimulation increased the frequency of GzymB-producing NK cells, blocking of IFN-I signaling did not have a significant impact. Despite the frequency of GzymB-producing NK cells not changing significantly, the amount of intracellular GzymB (which was significantly increased in response to viral stimulation) was lower in the absence of IFN-I signaling. Previous studies have observed similar trends in murine vaccinia virus infection, where the addition of IFN-I directly blocks IFN-I signaling. Importantly, NK cells were observed to produce more GzymB than T cells or NKT cells.

Stimulation by viruses and cytokines induces NK cell production of IFNγ (Cooper et al., 2001b; Papadopoulos et al., 2002b; Maroo et al., 2008). RV16 stimulation significantly increased both the frequency of IFNγ-producing NK cells and their level of intracellular IFNγ. Blocking IFN-I signaling resulted in a reduction in the frequency of IFNγ-producing NK cells in RV16-stimulated cultures. This indicates that IFN-I signaling plays a role in RV16-stimulated IFNγ production by NK cells, and is consistent with previous studies on the role of IFN-I on NK cell IFNγ in other viral infections (Biron et al., 1999; Gary-Gouy et al., 2002; Martinez et al., 2008; Kronstad et al., 2018). Notably, almost three times as many CD56bright NK cells were producing IFNγ in response to RV16 stimulation, than CD56dim NK cells. RV16 stimulation also significantly increased the amount of released IFNγ, and this was also partly dependent on IFN-I signaling. Importantly, NK cells were found to be more responsive to both RV16 stimulation and IFN-signaling, than T cells or NKT cells. Subsequent studies could elucidate the variations in NK cell surface receptor repertoire, specifically the density of IFNAR, that may contribute to the differential effects of RV on the CD56dim and CD56bright NK cell subsets (Cooper et al., 2001a; Sedlmayr et al., 2004).

The limitations of this study must be acknowledged. Firstly, the use of PBMCs as opposed to lung immune cells for in vitro experiments. However, NK cells in the lung seem to primarily consist of circulating rather than tissue resident cells (Marquardt et al., 2017). In addition to this, RV stimulation of PBMCs has been shown to be a suitable in vitro model in which to observe immune responses (Message and Johnston, 2001; Papadopoulos et al., 2002b; Hornung et al., 2002; Xi et al., 2015; Xi et al., 2017). It is also worth noting that the sample size of this study was small (n=12); despite this, there was enough statistical power identify significant differences between groups. The age of participants in this study (21.6 ± 2.8 years) was also restricted. Future studies should be conducted in larger cohorts, with a broader age range, to confirm these findings and account for interindividual variation in response to viral stimulation. This study only assessed NK cell response at a single time point with a single MOI of one serotype of human RV. There are over 150 serotypes of human RV (Glanville and Johnston, 2015). These serotypes are categorized into major and minor subtypes, based on their method of cell entry; however, even serotypes that share a common method of cell entry can follow different endocytic pathways and release of viral genome at different locations within the infected cells (Blaas and Fuchs, 2016), leading to diversity in the elicited immune response (Wark et al., 2009). Despite this, recognition of pathogen associated molecular patterns that are highly conserved across RV serotypes, such as ssRNA, by pattern recognition receptors triggers the activation of the innate immune response (Triantafillou et al., 2011). Activation of PBMCs in response to RV stimulation has also been shown to be dose-dependent (Gern et al., 1996). Future studies using multiple MOIs and different serotypes of RV could address the question of dose-dependent and serotype-dependent differences in NK cell responses. It is also important to acknowledge that immune cells, including NK cells, do not interact with RV in circulation, but at the airway epithelium. Thus, further studies should be conducted in co-cultures of respiratory epithelial cells and NK cells, in order to observe the NK cell response to virally infected respiratory epithelial cells. Future studies should also assess the levels of RV-specific neutralizing antibodies in the serum of each participant. A deficiency in RV-specific neutralizing IgG antibodies, specifically those targeting the VP1 viral capsid protein, has previously been associated with increased risk of exacerbations in patients with COPD (Yerkovich et al., 2012). Future studies should examine whether variations in RV-induced NK cell activation are associated with susceptibility to colds. A study of this nature would require a large cohort of participants to have sufficient study power.

In conclusion, we demonstrated that RV16 stimulates NK cells in vitro, and that this response is partially, but not completely, regulated by IFN-I signaling. These results established that RV16 stimulation of PBMCs leads to NK cell activation, degranulation, cytotoxic mediator production, cytokine production, and the release of cytotoxic mediators and cytokines into the culture supernatant. These aspects of NK cell activation and function were all partially dependent on IFN-I signaling. While deficient IFN-I signaling may play some role in the susceptibility of asthmatics to more persistent and severe infections, our findings also indicate that further studies need to examine other cytokines and APC function, and how this impacts on NK cell function. This study provides an important
foundation for future studies into NK cell activation and function in asthma.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENTS

The studies involving human participants were reviewed and approved by The University of Queensland Human Research Ethics Committee and Metro South Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and JU contributed to the conception and design of the study. SH and YX conducted the experiments and statistical analysis. SH wrote the manuscript. YX and JU contributed to the revision of the manuscript and approved the submitted version. All authors contributed to the article and approved the submitted version.

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