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Age predicts cytokine kinetics and innate immune cell activation following intranasal delivery of IFN γ and GM-CSF in a mouse model of RSV infection

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in young children and is further associated with increased healthcare utilization and cost of care in the first years of life. Severe RSV disease during infancy has also been linked to the later development of allergic asthma, yet there remains no licensed RSV vaccine or treatment. Pre-clinical and clinical studies have shown that disease severity and development of allergic asthma are associated with differences in cytokine production. As a result, stimulation of the innate host immune response with immune potentiators is gaining attention for their prospective application in populations with limited immune responses to antigenic stimuli or against pathogens for which vaccines do not exist. Specifically, macrophage-activating cytokines such as interferon gamma (IFN γ) and granulocyte colony-stimulating factor (GM-CSF) are commercially available immune potentiators used to prevent infections in patients with chronic granulomatous disease and febrile neutropenia, respectively. Moreover, an increasing number of reports describe the protective function of IFN γ and GM-CSF as vaccine adjuvants. Although a positive correlation between cytokine production and age has previously been reported, little is known about age-dependent cytokine metabolism or immune activating responses in infant compared to adult lungs. Here we use a non-compartmental pharmacokinetic model in naïve and RSV-infected infant and adult BALB/c mice to determine the effect of age on IFN γ and GM-CSF elimination and innate cell activation following intranasal delivery.

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Keywords

Interferon gamma; Granulocyte macrophage-colony stimulating factor; Infant; Cytokine kinetics; Respiratory syncytial virus; Age-dependent

1. Introduction

Few diseases have a greater effect on the health of children during their first years of life than viral lower respiratory tract infections. Respiratory syncytial virus (RSV) alone is responsible for approximately 800,000 childhood outpatient medical visits each year in the United States [1]. The World Health Organization estimates an annual mortality rate of ~160,000 worldwide due to RSV bronchiolitis or pneumonia, with more inclusive all-cause mortality rates approaching 600,000 each year. Moreover, hospital expenditures for bronchiolitis-related care in children under 2 years of age exceeded \$1.7 billion in 2009, emphasizing the significant public health and financial burden of viral bronchiolitis [2]. Conventional immunization strategies that depend on antigen specificity and conditioning of the adaptive immune response continue to be a primary focus of ongoing research, yet there remains no licensed vaccine for RSV or many other viral pathogens associated with childhood bronchiolitis [3]. Infants, unlike older children and adults, have a limited capacity to sustain an efficient memory response to vaccination due to limited prior antigen exposure and a suppressive lung environment that favors production of Th2-polarizing cytokines [4–6]. Therefore, non-clonal stimulation of innate immunity is a viable strategy to prevent or treat viral-mediated infant bronchiolitis.

Stimulation of the innate host immune response with immune potentiators is gaining attention for their prospective application in populations with limited immune responses to antigenic stimuli or against pathogens for which vaccines do not exist. Macrophage-activating cytokines such as interferon gamma (IFN γ) and granulocyte colony-stimulating factor (GM-CSF) are commercially available immune potentiators used to prevent infections in patients with chronic granulomatous disease and febrile neutropenia, respectively [7–9]. Moreover, an increasing number of reports describe the protective function of IFN γ and GM-CSF as vaccine adjuvants [10–13] [14, 15]. Despite the reported anti-viral activity, systemic toxicity with immune potentiating cytokines discourages their use in vulnerable patient populations, including young children. In adults, however, inhaled delivery of IFN γ or GM-CSF largely bypasses systemic exposure and was shown to be well tolerated in clinical studies among healthy adults and adults with lung disease [16–19]. A recent study further showed that local cytokine delivery to the lungs of influenza-infected mice improved disease outcomes to a greater degree when cytokine was retained in the airway using quantum dots [20]. Yet, pulmonary absorption of immune potentiating cytokines from the infant lung remains unknown and limits the utility of this therapeutic strategy in young children.

We have shown that intranasal delivery of IFN γ cuts viral infection time by half, blocks mucus production, and prevents the accumulation of apoptotic cells in a neonatal mouse model of RSV infection [21, 22]. Moreover, depletion of alveolar macrophages with

clodronate liposomes further demonstrated that alveolar macrophages are critical mediators of viral clearance in the infant airway [21]. Harker and colleagues further showed enhanced innate immune cell activation and viral clearance following infection with recombinant RSV expressing IFN γ [23]. Similarly, Burkeyev and colleagues demonstrated that recombinant RSV expressing GM-CSF attenuated RSV replication in adult mice [24]. However, it remains unclear how age affects cytokine-mediated cellular activation or how efficiently the infant compared adult lung environment clears pulmonary cytokines. This report expands on our previous findings and current understanding of how age influences cytokine kinetic parameters and innate immune cell activation in the lung. The goal of this work is to inform immune potentiating cytokine therapy and cytokine-stimulating vaccine strategies that target pulmonary viruses in the infant airway.

Alveolar macrophages constitute 90–95% of the cellular repertoire of the lung at baseline, help to maintain homeostasis, and serve as the first line of defense against inhaled pathogens [20]. They clear invading organisms through phagocytosis and microbicidal enzymes while regulating inflammatory responses through secretion of pro- and anti-inflammatory cytokines. Pulmonary dendritic cells (DCs) further contribute to the anti-viral cytokine milieu in the lung during viral infection and are essential for antigen presentation and initiation of adaptive immunity. However, efficient anti-viral cytokine production and engagement of the adaptive immune response depends largely on AM and DC maturation, suggesting that the Th2-dominant infant lung environment limits maturation and activation of innate immune cells and their anti-viral activity [25–27]. Thus, local exposure to pro-inflammatory cytokines in the lung is critical for proper innate cell maturation and disease resolution. A positive correlation between cytokine production and age has previously been reported [28], but much less is known about age-dependent cytokine metabolism or immune activating responses in infant compared to adult lungs. Using a non-compartmental pharmacokinetic model coupled with flow cytometric analysis, we show here that infant BALB/c mice eliminated IFN γ and GM-CSF at slower rates than their adult counterparts following a single, weight-based intranasal dose. Despite a prolonged residence time in the lung, activation of antigen presenting cells was reduced in infants compared to adults, suggesting that cytokine-mediated cellular activation is concentration- rather than time-dependent. We further show that innate immune cells in infant compared adult lungs are differentially activated by IFN γ and GM-CSF, suggesting that age is an important consideration when choosing an immune potentiating cytokine for therapeutic or vaccine prevention strategies.

2. Materials and Methods

2.1. Mice, virus, IFN γ and GM-CSF dosing

Balb/cJ mice (The Jackson Laboratory, Bar Harbor, ME) (6–8 weeks) were bred in-house, as described previously [22], or used for experimental purposes. Mice younger than 7 days of age are considered infants therefore, infant studies used mice at post-natal day (PND) 4–7. All mice were maintained in pathogen-free facilities in the Division of Laboratory Animal Resources at the University of Pittsburgh (Pittsburgh, PA). Experiments and animal handling were performed according to protocols approved by The University of Pittsburgh

Institutional Animal Care and Use Committee. Where indicated, mice were infected intranasally (i.n.) at PND 4–7 with RSV Line 19 (RSV L19, Martin Moore, Emory University, Atlanta, GA) (5×10^5 pfu/g, $\sim 1.5 \times 10^6$ pfu in 10 μ l) under isoflurane anesthesia. The pharmacokinetic studies administered a single dose of recombinant murine IFN γ (Peptotech, Rocky Hill, NJ) (16ng/g, infants 10 μ l, adults 100 μ l) or GM-CSF (50ng/g, infants 10 μ l, adults 100 μ l) or vehicle (PBS) and doses were administered i.n. to RSV-naïve infant and adult mice. In additional studies, RSV-infected infant mice were administered i.n. IFN γ (16ng/g) or PBS on days 1, 3, and 5 post-infection), i.n. GM-CSF (50ng/g) or PBS daily beginning 1 day post-infection under isoflurane anesthesia. Where indicated, litters were weighed and an average infant weight was calculated and graphed. At the indicated time points, left lungs were harvested and snap frozen for viral titer determination using a standard hematoxylin-eosin (H&E) plaque assay, as previously described [22].

2.2. Pharmacokinetics

Following a single dose of IFN γ or GM-CSF, RSV-naïve infant and adult mice were culled using 100% isoflurane at 0, 0.5, 1, 2, 4, 6, 8, 12 (IFN γ only), 18, 24, and 48 (IFN γ only) hours post-dose. Right lungs were weighed and snap-frozen in liquid nitrogen. Samples were stored at -80° C until homogenization, protein quantification using a BCA protein assay (ThermoFisher, Waltham, MA), and cytokine analysis, as previously detailed [21]. Blood was collected following cardiac puncture (adults) or severing of the abdominal aorta (infants). Following clotting, blood samples were centrifuged ($1,000 \times g$ for 15 minutes at 4° C) and the supernatants were transferred and centrifuged ($10,000 \times g$ for 10 minutes at 4° C). Resulting serum was stored (-80° C) until cytokine analysis. Lung samples were diluted to a protein concentration of 500 μ g/mL and serum was diluted as needed prior to cytokine analysis using a Bio-Plex Pro mouse cytokine assay (Bio Rad, Hercules, CA). Samples were analyzed using a Luminex® 200™ Total System machine (Luminex Corp, Austin, Tx). IFN γ and GM-CSF concentrations vs. time were plotted following a single i.n. dose and a non-compartmental analysis (NCA) was used to determine pharmacokinetic parameters. Using visual assessment, the terminal elimination rate constant, k_{el} , was calculated by log-linear regression of at least three time points in the terminal phase of lung and serum concentration vs. time plots. The elimination half-life, $t_{1/2}$, was calculated as $\ln 2/k_{el}$. The area under concentration (AUC) versus time curves for lung and serum from time zero to infinity, $AUC_{0 \rightarrow \infty}$, were also calculated using the linear trapezoidal rule, $AUC_{last \rightarrow \infty}$ was extrapolated by calculating C_{last}/k_{el} . $AUC_{0 \rightarrow \infty}$ was calculated as the sum of AUC_{last} and C_{last}/k_{el} . Other pharmacokinetic parameters calculated include IFN γ total body clearance, estimated as Dose/AUC.

2.3. Flow cytometry

Following a single dose of IFN γ or GM-CSF, the left lung was collected from infant and adult mice and processed for flow cytometry as described previously [22]. Additionally, right lungs were collected from infant mice and processed similarly for flow cytometry following RSV infection and IFN γ or GM-CSF treatment. Briefly, lungs were minced and enzyme digested (collagenase, 1mg/mL, Sigma Aldrich, St. Louis, MO) for 1 hour at 37° C. A single cell suspension was prepared by pushing lung samples through a 70 micron mesh screen. Cells were enumerated following red blood cells lysis using a hypotonic lysis buffer.

Cells from the lung digest ($0.5\text{--}1 \times 10^6$) were surface stained with murine antibodies against CD16/32 (2.4G2, BD), CD11c (N418, Biolegend), CD11b (M1-70, BD), MHCII (AMS-32.1, BD), and CD86 (GL-1, BD). Cells were fixed in 1% paraformaldehyde prior to analysis using an LSRII or LSR Fortessa (BD Biosciences, San Jose, CA), managed by the University of Pittsburgh United Flow Core (Pittsburgh, PA). Using FSC/SSC, lymphocytes were excluded and large granular cells were isolated and analyzed using the antibodies previously mentioned using FlowJo software (FLOWJO, Ashland, OR).

2.4. Statistical analysis

Results are presented as the mean $\pm$ SEM of at least 3 mice per group. Statistical analysis was performed using GraphPad Prism software (La Jolla, CA) using either a Kruskal-Wallis 1-way ANOVA, a 2-way ANOVA, or an unpaired t-test as indicated in the figure legends.

3. Results

3.1. IFN γ elimination rates are reduced in infants compared to adults following intranasal delivery

Cytokines are broken down by proteolysis into individual amino acids or are otherwise distributed out of the central compartment from which they are produced and secreted. To determine if these elimination processes are age-dependent in the local lung environment, a single, weight-based dose of IFN γ was delivered intranasally to adult and infant BALB/c mice. A total of 16ng/g of body weight was delivered providing 1.52ng and 1.07ng of IFN γ per mg of lung in infants and adults, respectively (Table 1). IFN γ concentrations were then determined at nine separate time points from digested lung tissue and blood ranging from time zero through 48-hours post-dose.

A non-compartmental pharmacokinetic analysis was performed to elucidate age-dependent differences for key pharmacokinetic parameters (Table 2). Adult mice demonstrated a rapid increase in IFN γ concentrations in the lung, peaking one hour after intranasal delivery (Fig. 1A; Table 2). The majority of IFN γ was cleared from the lungs by 12 hrs post-administration, which is further reflected by a mean residence time (MRT) of only 20 hours. Terminal elimination from adult lungs was calculated from 12 to 48 hrs post-dose and was used to generate the elimination half-life ($T_{1/2}$) of 14 hours, suggesting that IFN γ would be cleared from the lungs within 56–70 hours after delivery (4–5 half-lives) (Fig. 1C, solid line). The terminal elimination half-life was 16 hours in infant mice as demonstrated by the reduced slope (Fig. 1C, dashed line). Consistent with lung, terminal elimination of IFN γ from the blood was also more rapid in adult compared to infant mice (Fig. 1D). Moreover, the average IFN γ area under the concentration time curve (AUC) in the blood of adult animals was markedly reduced compared to the lung (Fig. 1A), suggesting that the majority of IFN γ is metabolized directly in the lung. A closer look at the first 4 hours after intranasal delivery revealed a 2-hour delay in peak blood concentrations from that achieved in the lung (Fig. 1A, inset), indicating that pulmonary absorption does not occur immediately.

Many age-dependent differences in IFN γ kinetics were noted between infants and adults. Specifically, AUCs and peak concentrations achieved in infant lungs were dramatically

lower than that achieved in adults (Fig. 1B; Table 2), despite identical weight-based dosing. This discrepancy may reflect reduced efficiency in cytokine delivery to the narrow infant airways or higher lung to body weight ratios in infants compared to adults (Fig. S1). Unlike adults, infants demonstrated bi-modal IFN γ peak concentrations (Cpk) in the lung 1 and 8-hrs after delivery (Fig. 1B), possibly due to stimulation of endogenous IFN γ -secreting immune cells. Twelve and 48 hours were used to calculate the elimination $T_{1/2}$ of 16 hours, indicating ~64–80 hours would be required to clear IFN γ from infant lungs. Consistent with their longer $T_{1/2}$, infants compared to adults had a more gradual terminal elimination slope reflective of a reduced rate of IFN γ clearance from infant compared to adult lungs (Fig. 1C; Table 2). Interestingly, IFN γ Cpk in the blood of infants was delayed by 6 hours compared to the 2-hour delay in adults, indicating that pulmonary absorption occurs more slowly in infants. Unlike cytokine clearance in the lung, only 31% of total IFN γ measured in the blood was cleared by 4 hours in infant mice, which is further reflected in the extended MRT of 102hrs compared to a MRT of 3.73hrs in adults. Together, these data describe important age-dependent differences in IFN γ kinetics following intranasal delivery, including reduced AUC, Cpk, and pulmonary clearance in infants compared to adults.

3.2. GM-CSF elimination rates are lower in infants compared to adults following intranasal delivery

To determine if age-dependent differences in cytokine kinetics were specific to IFN γ or if they may be more broadly applied, GM-CSF was delivered intranasally to adult and infant BALB/c mice. GM-CSF concentrations were determined at seven different time points ranging from 0 to 24 hours. Once again, a non-compartmental pharmacokinetic analysis was used to define age-dependent differences following administration of a single, 50ng/g dose of GM-CSF to each age group (Table 3). Infant and adult mice received comparable doses of GM-CSF with an average of 4.78ng and 3.52ng per mg of lung weight, respectively. Intranasal GM-CSF resulted in a rapid Cpk (0.5hrs) and high overall AUCs in the lung, averaging 1391.5ng*hr/ml. Cytokine removal was also rapid with 71% of GM-CSF cleared within the first hour and nearly all cytokine removed by 4 hours post-delivery with a $T_{1/2}$ of 12h in adult animals (Fig. 2A). As shown by the inset in Figure 2A, the time with which GM-CSF concentrations distribute into blood parallel that of the lung, albeit at lower overall concentrations (Fig. 2A). Terminal elimination curves for adults, showed a steeper slope compared to infants which is further reflected in adult versus infant clearance rates in the lung of 0.72 and 0.33 ml/hr, respectively (Fig. 2C; Table 3).

As with IFN γ , the average GM-CSF AUC and Cpk in infant lungs were lower than that of adult mice (Fig. 2B; Table 3), suggesting reduced cytokine exposure. Moreover, clearance from infant compared to adult lungs was reduced which is further reflected by a longer MRT among infants and a $T_{1/2}$ of 16h in infants. Similar to adult mice, nearly all GM-CSF is cleared from infant lungs by 4 hours post-dose, suggesting extensive and rapid removal. As was also true for IFN γ , infant pulmonary absorption of GM-CSF was delayed showing a Cpk at 4 hours as opposed to 1 hour in adults (Fig. 2A–B; Table 3). Taken together, these data show similar kinetic differences for intranasal GM-CSF as was shown with IFN γ apart from a more rapid removal of GM-CSF from infant murine blood compared to IFN γ and

suggest that age-dependent differences in cytokine kinetic parameters should be considered following cytokine-mediated immune stimulation in the lung.

3.3. Age predicts differential expression of activation markers following intranasal IFN γ and GM-CSF

To determine if age-based differences in cytokine exposure and clearance elicited differential stimulation, the expression of CD11b, MHCII, and CD86 activation markers on CD11c+ innate immune cells were determined following intranasal delivery of IFN γ and GM-CSF. Flow cytometry was used to measure activation marker expression in infant and adult murine lungs following a single weight-based dose of each cytokine (Fig. 3). GM-CSF increased CD11b-expressing cells as early as 4hrs post-dose, reflective of the rapid pulmonary absorption observed in adult compared to infant mice. By 8 hours, adult and infant mice increased CD11b-expressing cells following GM-CSF and IFN γ . Forty-eight hours after cytokine delivery, adults showed a resurgence of CD11b-expressing cells, suggesting that endogenous GM-CSF-producing cells were stimulated more efficiently in adults than in infants. Conversely, IFN γ more than GM-CSF increased MHCII-, and CD86-expressing cells in the infant lung which can be observed almost exclusively 24 hours after delivery. Interestingly, MHCII is largely more responsive to cytokine delivery than CD86 in both infant and adult lungs. Additionally, adult mice show persistent increases in MHCII-expressing cells following both IFN γ and GM-CSF which may reflect, in part, generally higher AUCs observed in the adult animals. Overall, these data demonstrate that age predicts differential responses to immune stimulating cytokines and that concentration rather than time may be a more important predictor of innate immune cell stimulation.

To compare adult and infant cellular immune responses over time following intranasal IFN γ or GM-CSF, the relative frequency of CD11c+ cells expressing CD11b, MHCII, or CD86 were determined (Fig. 4). CD11b-expressing cells were greater in infant compared to adult lungs at baseline (Fig. 4A), yet both infant and adult CD11b-expressing cells increased over time. However, CD11b-expressing cells remained elevated through 48 hours in infant compared to adult lungs. Alternatively, adults increased CD11b-expressing cells more than infants following intranasal delivery of GM-CSF, suggesting that age predicts differential responses to distinct cytokines (Fig. 4D). Overall increases in MHCII- and CD86-expressing cells were greater in adult compared to infant lungs (Fig. 4B–C; E–F). Interestingly, infants increased MHCII- and CD86-expressing cells more in response to IFN γ than to GM-CSF, whereas adults were more responsive to GM-CSF than to IFN γ . These age-based differences likely reflect the extent to which adult peak cytokine levels exceeded that of infants in the lung (differences between adult and infant cytokine levels: IFN γ 18.98ng/ml; GM-CSF 282.35ng/ml), further emphasizing the importance of cytokine concentration or exposure to efficient immune activation.

3.4. Age predicts differential cytokine production following intranasal IFN γ and GM-CSF

To further elucidate the differential age-dependent effects of intranasal IFN γ or GM-CSF on innate immune cell stimulation, an array of chemokines and cytokines were quantified from adult and infant lungs normalized to protein concentration. The chemokines KC and MIP-1 α , were increased compared to baseline (time = "0") levels in both age groups given

IFN γ (Fig. 5A–B). Despite the observed increase in KC and MIP-1 α , the total number of cells in the lung did not increase, which may reflect an insufficient chemokine gradient necessary to recruit inflammatory cells [29] (Fig. S2A). Moreover, no significant differences in KC and MIP-1 α were detected between infant and adult groups. Alternatively, adults but not infants increased IL-5 and RANTES, responsible for the recruitment of eosinophils and neutrophils, respectively, suggesting that IFN γ promotes greater IL-5 and RANTES production in adult compared to infant lungs (Fig. 5C–D). High levels of IFN γ were detected in infant and adult mice following intranasal delivery of the recombinant cytokine (Fig. 5E). Intranasal IFN γ further stimulated production of TNF α in infant mice, which together may account for the dual signaling required for innate immune activation (Fig. 5E–F). Other pro-inflammatory cytokines including IL-1 β and IL-6 were increased in infant and adult mice in response to intranasal IFN γ , suggesting effective innate immune cell stimulation following direct cytokine delivery (Fig. 5G–H). Specifically, IL-1 β , a cytokine recently linked to the regulation of RSV replication [30], was significantly increased in infant lungs with an increasing trend also observed in adult animals (Fig. 5G). Moreover, adult mice responded to intranasal IFN γ with greater production of IL-1 β compared to infants 0.5h and 1h after cytokine delivery. Lastly, the pro-inflammatory cytokine IL-6 was increased over baseline in infant and adult mice, though the extent of production was greater overall in adult mice (Fig. 5H).

Following intranasal administration of GM-CSF, production of KC and MCP-1 was significantly increased in both adult and infant mice, though a marked increase in KC was noted in infant mice (Fig. 6A–B); MCP-1 was further increased in adult lungs compared to infants at each time point evaluated. Adult mice generated significantly more IL-5 and RANTES over baseline levels as well as compared to infant levels at times 0–1h after delivery (Fig. 6C–D). Inflammatory cytokines, IL-1 β , TNF α , and IL-6 each increased over baseline in infant mice treated with intranasal GM-CSF (Fig. 6E–H), however, IFN γ production was more responsive to GM-CSF exposure in adult compared to infant mice (Fig. 6E). These findings are consistent with the enhanced frequency of activation marker expression in adults compared to infants shown in Figure 4. Despite the observed increase in chemokine production, namely KC, MCP-1, IL-5, and RANTES, following IFN γ and GM-CSF delivery, granulocyte recruitment was not increased and weight gain was not delayed (Fig. S2A–B). Additionally, no increases in IL-10, IL-13, or IL-4 were observed in adults or infants after cytokine delivery, indicating that neither IFN γ nor GM-CSF stimulated Th2-type cytokine production early after intranasal delivery (*data not shown*).

3.5. Cytokine-mediated innate immune stimulation reduces viral titers without impairing weight gain in infant mice

Both IFN γ and GM-CSF increased the frequency of activation markers and cytokine production in uninfected animals. To determine the extent to which intranasal cytokine delivery increased cellular activation in infected animals, RSV- or mock-infected infant mice were treated with intranasal IFN γ or PBS on days 1, 3, and 5 post-infection as previously described [22]. Lungs were harvested at 4 and 7 dpi for flow cytometric analysis. The relative frequency of CD11b-expressing CD11c⁺ cells did not change at 4 or 7 dpi (Fig. 7A, E). However, at 4 dpi, IFN γ increased the percent of MHCII-expressing CD11c⁺ cells in

naïve compared to RSV-infected mice (Fig. 7B). Alternatively, the percent of CD86-expressing CD11c⁺ cells was increased in both naïve and RSV-infected mice compared to uninfected controls (Fig. 7B–C). By 7 dpi, IFN γ delivery increased the relative frequency of MHCII- and CD86-expressing cells in RSV-infected mice compared to uninfected controls; this delayed activation suggests that RSV infection reduces the activation efficiency of innate immune cells following local IFN γ exposure (Fig. 7B–C; F–G). RSV-infected infant mice treated with PBS alone failed to increase MHCII or CD86 expression on CD11c⁺ cells in PBS-treated mice that remained uninfected. These data show that intranasal IFN γ increased the activation of CD11c⁺ cells compared to PBS alone during RSV infection. Interestingly, IFN γ -mediated reductions in viral titers did not directly correlate with the increased frequency of activation markers at 4dpi as MHC II and CD86 frequency on CD11c⁺ cells was similar between mice receiving PBS or IFN γ despite reductions in viral titers occurring only in mice that received IFN γ . However, by 7dpi, a trending increase in the frequency of MHC II and CD86 expression on CD11c⁺ cells was evident in mice receiving IFN γ compared to PBS alone (Fig. 7D, H). Intranasal GM-CSF markedly increased CD11c⁺ cells expressing CD11b by 4 and 7 dpi in RSV-infected infant mice compared to naïve mice (Fig. 8A and E). Unlike with IFN γ delivery, expression of CD86 on CD11c⁺ cells was significantly reduced in infant mice treated with intranasal GM-CSF at 4 dpi. Similarly, MHCII expression appeared lower compared to PBS-treated animals, though statistical significance was not achieved (Fig. 8B–C). By 7dpi, CD86 expression remained low in the GM-CSF-treated group, whereas the frequency of MHCII expression increased to similar levels observed in the PBS-treated group in RSV-infected infant mice (Fig. 8F and G), suggesting the observed increase was due to RSV infection and not exposure to GM-CSF. Moreover, intranasal GM-CSF failed to reduce viral lung titers (Fig. 8D and H), suggesting that IFN γ may be more beneficial for viral clearance in the virally-infected infant airway.

Despite the observed increases in cytokine and innate immune cell marker expression, weight gain was not delayed in RSV-infected infant mice receiving intranasal cytokine compared to PBS-treated controls (Fig. 9A–B). These data may reflect the minimal cytokine concentrations achieved in the blood following intranasal cytokine delivery.

4. Discussion

In this study the cytokine kinetics and immune stimulating effects of IFN γ and GM-CSF were examined in adult and infant BALB/c mice following intranasal administration. Long-term protection against RSV by vaccination is a global health priority, yet concerns over vaccine-enhanced RSV disease and limited infant host immunity continue to pose a significant challenge. Moreover, repeated infections with RSV throughout life suggests that induction of sterilizing immunity may not be feasible and vaccinations that induce sufficient immunity to protect against serious RSV infection may be a more realistic goal [31]. Stimulating the innate immune response to prevent or treat pulmonary infections is of growing interest in the areas of antimicrobial resistance, impaired host immunity, and for viral infections lacking an effective vaccine [32–35]. Many of these strategies target pattern recognition receptors to activate transcription factors such as NF- κ B and IRF3, resulting in the expression of inflammatory cytokines and other facets of cellular activation [36]. Vaccine strategies combine non-infectious antigen with immune potentiating adjuvants in an

effort to condition the adaptive immune response in preparation for subsequent exposure to infectious organisms. However, infants often do not reap the same protective benefits from vaccination as older children and adults due to inefficient or suppressive innate and adaptive host immunity [37, 38]. A growing number of studies have reported safe and effective antiviral activity with vaccine-mediated enhancement of pro-inflammatory cytokines [10, 11, 14, 39–43], yet less is known about direct immune stimulation. IFN γ and GM-CSF have been identified as critical cytokines for the protection against such organisms as RSV, influenza, rhinovirus, and tuberculosis [44–48], yet there remains little information describing direct innate immune stimulation in the virally-infected infant airway.

In the present investigation, analysis of cytokine delivery was focused on pulmonary innate immune cell exposure and activation following intranasal delivery of IFN γ and GM-CSF in infant versus adult murine lungs. Following identical weight-based dosing, infant compared to adult mice had markedly reduced AUCs and C_{pk}s, indicating reduced cytokine concentrations were achieved in infant compared to adult murine lungs. It is reasonable to consider that the narrow infant airways compared to adults' limited delivery of these 15kDa proteins in an age-dependent manner. However, examination of lung and body weights over time indicated that the rate of postnatal infant lung growth exceeded that of body weight through 10 days of age, suggesting that pulmonary doses of weight-based drugs were underestimated when scaling from adult doses. These findings are consistent with previous studies demonstrating that pulmonary deposition of aerosolized drugs depend primarily on differences in ventilation per gram of body weight and may account, in part, for lower AUCs and C_{pk}s in infant compared to adult mice treated with IFN γ or GM-CSF [49]. Moreover, reduced cytokine clearance rates, which are calculated based on dose and AUC, were also lower in infant compared to adult murine lungs and blood, suggesting that bioavailability rather than increased proteolytic activity was responsible for the reduced infant concentrations. Together, these data showed reduced AUCs and clearance rates in infant compared to adult mice treated with a single intranasal dose of IFN γ or GM-CSF.

Discrete weight-based doses of IFN γ and GM-CSF were used based on previous studies in infant and adult mice designed to optimize immune activation [21, 22, 50]. As such, the expression frequency of CD11b, MHCII, and CD86 on CD11c⁺ innate immune cells as well as cytokine production were evaluated. To determine the broad activation potential for each of these cytokines, no attempts were made to characterize pulmonary macrophages or dendritic cells, rather our goal was to detect general innate immune cell activation. Infant and adult mice showed increased CD11b expression following intranasal GM-CSF which likely reflects its well-appreciated monocyte-recruitment activity and may also reflect the higher levels achieved in blood compared to IFN γ . Despite their relatively low AUC and C_{pk} levels, infants showed an increased frequency of MHCII and CD86 following intranasal IFN γ compared to GM-CSF. Conversely, adult mice showed enhanced expression of MHCII and CD86 following GM-CSF as compared to IFN γ . Although cytokine exposure between infant and adult mice was disparate, exposure to each cytokine within age groups was similar, emphasizing the importance of understanding how age affects the response to different cytokines.

Prominent age-dependent differences in the frequency of activation marker expression and response time were observed, which are largely attributed to greater cytokine AUC and Cpk in adult compared to infant lungs. Additionally, the immunosuppressive infant compared to adult lung environment is known to play an important role in altering the infant's threshold for innate immune cell activation [51]. To this point, innate immune cells, including macrophages and DCs are largely regulated by the local cytokine milieu. Elevated baseline cytokine levels in adult compared to infant lungs may be responsible for lowering the threshold of activation following infection. Overall, adult animals expressed higher cytokine concentrations throughout the two-hour timeframe analyzed, with the notable exception of KC and MIP-1 α following IFN γ delivery as well as KC, IL-1 β and TNF α following intranasal GM-CSF. KC, the mouse homologue of human IL-8, is responsible for robust neutrophil recruitment and is associated with RSV-mediated pathology within the infant airway [52, 53]. Despite increases in KC, overall cellular recruitment to the lung was not observed. Similarly, RANTES which is largely responsible for eosinophil recruitment, was markedly greater in adults compared to infants following both IFN γ and GM-CSF with no discernable increase in cell recruitment to the lung. The lack of increased cell numbers in infant and adult lungs despite increased chemokine levels suggests that chemokine levels achieved in the blood were not sufficient to increase cellular recruitment within the 24-hour time frame evaluated in these studies.

Classically activated macrophages are induced by the combination of dual signals, IFN γ and TNF α , resulting in enhanced anti-microbial activity [54, 55]. Typically, production of these cytokines are mediated through TLR-ligation by pattern associated molecular patterns [56] and a growing number of studies report cytokine-eliciting adjuvants as promising vaccine strategies [10, 11, 13, 42]. To determine if immune activation was altered following direct delivery of IFN γ or GM-CSF during RSV infection in the infant lung, infant mice were infected with RSV and treated with intranasal IFN γ or GM-CSF as described in the methods section.

The current work demonstrated that during RSV infection, the frequency of activation marker expression following intranasal IFN γ in RSV-infected infant mice is blunted at 4 dpi and does not surpass that of uninfected animals until 7dpi. At 4 dpi, viral clearance did not correlate with elevated frequencies of CD86 and MHCII observed in the PBS treated groups. Yet, RSV lung titers were reduced at 4 and 7dpi in infant mice treated with intranasal IFN γ , suggesting an indirect mechanism of cytokine-mediated viral clearance or early activation of innate cells that do not express CD11c such as airway epithelial cells [57]. Consistent with activation marker expression, increased cytokine production was not observed until 7dpi in IFN γ -treated infant mice, except for IL-10 (data not shown) which was increased at 4dpi, but was then undetectable by 7dpi (Fig. S3). Alternatively, treatment of infant mice with intranasal GM-CSF failed to induce increased expression of activation markers at any time points tested and was, in fact suppressed compared to PBS-treated controls at the 4dpi time point. In concert with these findings, RSV lung titers were not reduced in GM-CSF-treated mice compared to PBS-treated control animals. The findings reported here in infant mice, contradict previous studies in adult mice demonstrating that GM-CSF increases alveolar macrophage activation and reduces RSV lung titers in adult mice [24, 27].

5. Conclusion

In conclusion, the findings reported here showed that intranasal cytokine delivery was well-tolerated in infant mice, causing no significant weight loss in RSV-infected versus naïve animals. Differential kinetics with reduced cytokine concentrations and reduced immune responses in infant compared to adult mice further emphasize that study findings in adults may not readily translate to infants. Moreover, delivery of exogenous IFN γ but not GM-CSF, reduced viral burden compared to untreated controls, consistent with improved IFN γ -mediated innate immune activation. Lastly, these data highlight the importance of age-based studies in vaccine and immune therapy and emphasize the importance of age as a consideration when targeting enhanced cytokine production for viral pulmonary infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Adult mice clear pulmonary cytokines faster than infant mice.
- IFN γ , but not GM-CSF, enhances RSV clearance from the infant murine airway.
- Intranasal IFN γ and GM-CSF achieve higher levels in adult vs infant airways.
- Low levels of IFN γ and GM-CSF are detected in the blood after intranasal delivery.
- Cytokine blood levels are delayed in infant vs. adult mice after pulmonary delivery.

Figure 1. The pharmacokinetic profile of intranasal IFN- γ depends largely on age at the time of administration

Adult and infant BALB/c mice were treated with a single intranasal dose of IFN γ (16ng/g). IFN γ concentrations were subsequently measured in homogenized lung tissue and serum using a multi-plex Luminex platform at the indicated times. (A) Adult and (B) infant IFN γ concentrations in the lung (y-axis) and blood (z-axis) were reported on discriminate scales to accommodate differential concentration ranges; insets provide a detailed depiction of the kinetic changes during the early time points from 0–4 hours. Data was transformed on a log scale to identify the terminal elimination phase of IFN γ in adult and infant (C) lungs and (D) blood. Each time point represents the mean and SEM of three separate mice.

Figure 2. The pharmacokinetic profile of intranasal GM-CSF is largely dependent on age
 Adult and infant BALB/c mice were treated with a single intranasal dose of GM-CSF (50ng/g). GM-CSF concentrations were subsequently measured in homogenized lung tissue and serum using a multi-plex Luminex platform at the indicated times. (A) Adult and (B) infant GM-CSF concentrations in the lung (y-axis) and blood (z-axis) were reported on discriminate scales to accommodate differential concentration ranges; insets provide a detailed depiction of the kinetic changes during the early time points from 0–4 hours. Data was transformed on a log scale to identify the terminal elimination phase of IFN γ in adult and infant (C) lungs and (D) blood. Each time point represents the mean and SEM of three separate mice.

Figure 3. GM-CSF and IFN γ differentially induce expression of activation markers in adults and infants

Adult and infant BALB/c mice were given a single intranasal dose of IFN γ (16ng/g) (shaded), GM-CSF (50ng/g) (dashed line), or PBS only (solid line). At the indicated time points lungs were harvested for flow cytometry to compare the frequency of CD11b (A and D), MHC class II (B and E), and CD86 (C and F) on CD11c⁺ large cells between the two different intranasal cytokines in both infants and adults.

Figure 4. Adult cells are more responsive than infant cells to IFN γ and GM-CSF

Adult and infant BALB/c mice were given a single intranasal dose of (A–C) IFN γ (16ng/g) or (D–F) GM-CSF (50ng/g). At the indicated time points lungs were harvested for flow cytometry to determine the frequency of CD11b (A and D), MHC class II (B and E), and CD86 (C and F) on CD11c+ large cells. A Kruskal-Wallis, 1-way ANOVA with Dunn's correction for multiple comparisons was used to identify differences within groups over time; dashed (infants) and solid (adults) lines indicate significant differences; $p < 0.05$. A 2-way ANOVA with Bonferroni correction for multiple comparisons was used to determine differences between groups over time; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 5. IFN γ differentially induced cytokine production in adult and infant lungs

Adult and infant BALB/c mice were given a single intranasal dose of IFN γ (16ng/g). At the indicated time points lungs were harvested and processed for a multi-panel Luminex cytokine array (A–F). A Kruskal-Wallis, 1-way ANOVA with Dunn's correction for multiple comparisons was used to identify differences within groups over time; asterisks indicate significant differences from the respective infant or adult time "0" control. Lines indicate differences between Infants and adults at the same time point; * $p < 0.05$. ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. GM-CSF differentially induced cytokine production in adult and infant lungs

Adult and infant BALB/c mice were given a single intranasal dose of GM-CSF (50ng/g). At the indicated time points lungs were harvested and processed for a multi-panel Luminex cytokine array following protein normalization by BCA (A–F). A Kruskal-Wallis, 1-way ANOVA with Dunn's correction for multiple comparisons was used to identify differences within groups over time; asterisks indicate significant differences from the respective infant or adult time "0" control. Lines indicate differences between Infants and adults at the same time point; * $p < 0.05$. ** $p < 0.01$; *** $p < 0.001$.

Figure 7. IFN γ -induced activation of innate immune cells correlate with reduced viral clearance Infant BALB/c mice were infected with 5×10^5 PFU/g of body weight, then intranasal IFN γ (16ng/g) or PBS was delivered on 1, 3, and 5 dpi. At 4 or 7 dpi, lungs were harvested for flow cytometry to determine the frequency of CD11b (A and E), MHC class II (B and F), and CD86 (C and G) on CD11c+ large cells. RSV plaque assays were performed on homogenized lung to determine differences in plaque forming units (PFU)/g of lung weight at (D) 4 dpi and (H) 7dpi. A Kruskal-Wallis, 1-way ANOVA with Dunn's correction for multiple comparisons was used to identify differences in marker expression between groups

at 4 or 7 dpi. $p < 0.001$. An unpaired t-test was used to determine differences in RSV (PFU/g of lung) at 4dpi or 7 dpi. * $p < 0.05$; ** $p < 0.01$; ***

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Figure 8. GM-CSF fails to increase activation markers or reduce viral titers in infant lungs

Infant BALB/c mice were infected with 5×10^5 PFU/g of body weight. The mice were then given intranasal IFN γ (16ng/g) or PBS on 1, 3, and 5 dpi or intranasal GM-CSF (50ng/g) or PBS daily. At 4 or 7 dpi, lungs were harvested for flow cytometry to determine the frequency of CD11b (A and E), MHC class II (B and F), and CD86 (C and G) on CD11c+ large cells. RSV plaque assays were performed to determine differences in plaque forming units (PFU)/g of lung weight at (D) 4 dpi and (H) 7dpi. A Kruskal-Wallis, 1-way ANOVA with Dunn's correction for multiple comparisons was used to identify differences in marker

expression between groups at 4 or 7 dpi. $p < 0.001$. An unpaired t-test was used to determine differences in RSV (PFU/g of lung) at 4dpi or 7 dpi. * $p < 0.05$; ** $p < 0.01$; ***

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Figure 9. No delay in weight gain was observed following intranasal delivery of IFN γ or GM-CSF in RSV-infected infant mice

Infant BALB/c mice were infected with 5×10^5 PFU/g of body weight. The mice were then given intranasal IFN γ (16ng/g) or PBS on 1, 3, and 5 dpi (A) or intranasal GM-CSF (50ng/g) or PBS daily (B). Weights were recorded daily through 10 dpi for all groups. A 2-way ANOVA with Bonferroni correction for multiple comparisons was used to identify differences in marker expression between cytokine-treated groups and their respective controls over time. * $p < 0.05$.

Table 1

	IFN γ dose per total body wt	Avg total wt	Total IFN γ delivered	Avg lung wt	IFN γ delivered per lung wt
Infants (4d)	16ng/g	5.83g	93ng/10ul	61mg	1.52ng/mg lung
Adults (8–10wks)	16ng/g	21g	320ng/100ul	298mg	1.07ng/mg lung

IFN γ (interferon gamma)

Table 2

	IFN γ	AUC (ng $\cdot$ hr/ml)	Cl (ml/hr)	Cpk (ng/ml)	Time of Pk Conc (hrs)	MRT (hrs)	% cleared in 1, 4, 12hrs
Lung	Infants	79.19	0.55	10.07	1	14.62	51%, 81%, 99%
	Adults	285.95	1.12	29.05	1	20	43%, 82%, 98%
Blood	Infants	1.61	26.86	0.13	6	102	5%, 31%, 93%
	Adults	14.82	21.60	1.2	2	3.73	9%, 78%, 99%

IFN γ (interferon gamma); AUC (area under the curve); Cpk (peak concentration); MRT (mean residence time)

Table 3

	GM-CSF	AUC (ng*hr/ml)	Cl (ml/hr)	Cpk (ng/ml)	Time of Pk Conc (hrs)	MRT (hrs)	% cleared in 1 and 4hrs
Lung	Infants	407.13	0.33	141.82	0.5	2.72	67%, 99%
	Adults	1391.5	0.72	424.17	0.5	3.5	71%, 97%
Blood	Infants	63.73	2.12	4.6	4	6.8	39%, 72%
	Adults	40	25	14.79	1	3.88	71%, 96%

GM-CSF (granulocyte macrophage-colony stimulating factor); AUC (area under the curve); Cpk (peak concentration); MRT (mean residence time)