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Localization of the T-Cell Response to RSV Infection is Altered in Infant Mice

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Abstract

Objectives—Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infections worldwide, causing disproportionate morbidity and mortality in infants and children. Infants with stronger Th1 responses have less severe disease, yet little is known about the infant T-cell response within the air space. Thus, we tested the hypothesis that RSV infected infant mice would have quantitative and qualitative deficiencies in CD4⁺ and CD8⁺ T-cell populations isolated from the bronchoalveolar lavage when compared to adults and that local delivery of IFN- γ would increase airway CD4⁺ Tbet⁺ and CD8⁺ Tbet⁺ T-cell responses.

Methods—We compared the localization of T-cell responses in RSV-infected infant and adult mice and investigated the effects of local IFN- γ administration on infant cellular immunity.

Results—Adult CD8⁺ CD44^{HI} and CD4⁺ CD44^{HI} Tbet⁺ T-cells accumulated in the alveolar space whereas CD4⁺ CD44^{HI} Tbet⁺ T-cells were evenly distributed between the infant lung tissue and airway and infant lungs contained higher frequencies of CD8⁺ T-cells. Delivery of IFN- γ to the infant airway failed to increase the accumulation of T-cells from the airspace and unexpectedly reduced CD4⁺ CD44^{HI} Tbet⁺ T-cells. However, intranasal IFN- γ increased RSV F protein-specific CD8⁺ T cells in the alveolar space.

Conclusion—Together, these data suggest that quantitative and qualitative defects exist in the infant T-cell response to RSV but early, local IFN- γ exposure can increase the CD8⁺ RSV-specific T-cell response.

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Disclosures

The authors declare no conflict of interest.

Keywords

T-cell response; infant mice; RSV; airway; IFN- γ

Introduction

Respiratory tract infections represent the single largest disease burden worldwide¹ and are especially prominent in infants and young children. In 2015, lower respiratory tract infections (LRTI) were responsible for 15.3% of mortality in children < 5 years old². Globally, respiratory syncytial virus (RSV) is the most common cause of acute LRTI in children resulting in a significant number of hospital admissions³. Elderly adults are also at increased risk for severe RSV infection compared to younger adults, further emphasizing the importance of age in host immune responses and disease pathology⁴. Despite RSV's immense contribution to the global burden of lung disease, there are no effective treatments or licensed vaccines. The anti-RSV antibody, palivizumab, reduces the number of RSV-associated hospitalizations in *high-risk* infants when given prophylactically⁵, yet 80% of RSV-related hospitalizations consist of previously healthy infants and children who do not receive prophylaxis^{6,7}. A universal measure aimed at preventing primary RSV-related morbidity and mortality is a healthcare priority. Therefore, a more complete understanding of the infant immune response to RSV is necessary to guide the development of future vaccines and therapeutic interventions.

The importance of T lymphocytes in clearing RSV has been well established in murine^{8–11} and human studies¹². However, debate still exists regarding the effectiveness of T-cell recruitment to the air space of RSV-infected infants^{13,14}. Autopsy samples from infants infected with RSV revealed that T-cell infiltration into the lung was meager but importantly, the small number of T-cells located within the alveolar space were highly activated, suggesting close proximity to RSV-infected airway epithelium increases activation¹³. Similarly, bronchoalveolar lavage (BAL) samples from RSV-infected infants showed massive neutrophil recruitment but relatively small increases in the frequency of T-cells^{15,16}. The CD8⁺ T-cells recovered from the BAL of these infants had a highly activated, effector phenotype, were proliferating, and producing granzyme B¹⁶. However, in an infant murine model of influenza, recovery of T-cells from the alveolar space was deficient when compared to adults¹⁷. Collectively these studies indicate that T-cells exert their anti-viral effects proximal to RSV-infected airway epithelial cells within the bronchoalveolar space but the accumulation of infant T-cells may be insufficient. Murine studies investigating the role of T lymphocytes in RSV disease have relied heavily on adult models and may be missing important differences in infant T-cell accumulation and activation in the airway.

Numerous studies have demonstrated infants' Th2 biased responses to RSV, however those with higher Th1 responses are associated with improved disease outcomes^{14,18–20}, expedited viral RSV clearance, and reduced Th2-driven pathogenesis^{10,21}. Neonatal mice have demonstrated the ability to mount effective Th1 responses given the appropriate co-stimulation, such as IL-12 and/or IFN- γ ^{22,23}. We previously showed that RSV-infected infant mice treated with intra-nasal (i.n.) IFN- γ reduced RSV burden and increased the

expression of markers associated with classically-activated alveolar macrophages, including CD86, MHCII, and CCR7²⁴. However, the ability of IFN- γ to alter the T-cell phenotype of RSV-infected infants has not been investigated. Furthermore, there is a scarcity of data regarding the age-dependent differences that may exist in T-cell accumulation throughout the pulmonary architecture of RSV-infected adult and infant mice. Therefore, we hypothesized that RSV infected infant mice would have quantitative and qualitative deficiencies in CD4⁺ and CD8⁺ T-cell populations isolated from the BAL when compared to adults and that local delivery of IFN- γ would increase airway CD4⁺ Tbet⁺ and CD8⁺ Tbet⁺ responses.

Materials and Methods

Mice and Virus

Balb/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age and bred in-house, as previously described²⁴. All mice were maintained in a pathogen-free facility at the University of Pittsburgh, Division of Laboratory Animal Resources (Pittsburgh, PA) and handled according to protocols approved by The University of Pittsburgh Institutional Animal Care and Use Committee. Mice were infected intranasally (i.n.) with 5×10^5 plaque forming units (pfu) of RSV Line 19 (RSV L19, a gift from Martin Moore, Emory University) per gram of body weight at 4-5 PND (infants, $\sim 1.5 \times 10^6$ pfu in 10 μ l) or 6-8 weeks of age (adults, $\sim 10^7$ pfu in 100 μ l) under isoflurane anesthesia. The RSV L19 was propagated and viral titers were quantified as previously described²⁵. Recombinant murine IFN- γ (16ng/g) (Peprotech, Rocky Hill, NJ) or vehicle (PBS) were administered i.n. to infants on 1, 3 and 5 days post-infection (DPI) under isoflurane anesthesia.

Cell Preparation and Flow Cytometry

Bronchoalveolar lavage (BAL) was collected using HBSS + 30 μ M EDTA; the cellular component was isolated and resuspended in HBSS for enumeration. The superior/upper right lobe (URL) was harvested and processed into a single cell suspension, as previously described²⁶. Each BAL and URL sample represents a single adult animal; infant BAL samples (2-4 infant mice) were pooled to acquire sufficient cell numbers for flow cytometry. To maintain consistency with the BAL, the same infant URL samples were pooled prior to enumeration. Cells ($0.5 - 1 \times 10^6$) were surface stained with antibodies against TCR β -H57-597, CD62L-MEL-14, and CD19-6D5 (Biolegend, San Diego, CA), CD4-GK1.5, CD8a-53-6.7, CD44-IM7 (BD Biosciences, San Jose, CA). Cells were fixed and permeabilized for transcription factor staining using the BD Pharmingen Transcription Factor Buffer Set, according to manufacturer recommendations (BD Biosciences). Intracellular staining of GATA3-16E10A23 and Tbet-4B10 (Biolegend) was performed following overnight incubation in BD Fix/Perm solution. Where indicated, BAL samples were incubated with RSV F-protein MHC I pentamer (H-2kd KYKNAVTEL, ProImmune, Sarasota, FL). Samples were run on a BD LSRFortessa (BD Biosciences) managed by the University of Pittsburgh United Flow Core and analyzed using FlowJo V10 software (FLOWJO, Ashland, OR).

Statistical Analysis

Results are displayed as the mean $\pm$ SEM for individual samples. Statistical significance was calculated using 2-way ANOVA or ANOVA with Dunnett's or Bonferroni post-tests or a *t* test using GraphPad Prism software (La Jolla, CA) as indicated in the figure legends.

Results

Pulmonary T-cell recruitment peaks 10 days after RSV infection in adult and infant mice

To determine peak pulmonary T-cell recruitment following RSV infection, we analyzed total CD4⁺ and CD8⁺ T-cells recovered from the lungs of infant and adult BALB/cJ mice at multiple time points following RSV infection. Total CD4⁺ and CD8⁺ T-cells peaked at 10 DPI in RSV+ adults (Fig. 1A & B) and infants (Fig. 1C & D), indicating that infants and adults have similar T-cell recruitment kinetics following RSV infection. Subsequent experiments characterized age-dependent, phenotypic T-cell responses at this 10-day time point, which represented peak T-cell responses for both age groups.

T-cell accumulation in the airway is reduced in RSV-infected infant mice

To better understand the role of age in T-cell localization during RSV infection, flow cytometry was used to determine the frequency of CD4⁺ and CD8⁺ T-cells in the lung tissue and BAL of infant and adult mice. At 10 DPI, the frequencies of CD4⁺ and CD8⁺ T-cells were significantly higher in adult compared to infant BAL (Fig. 2A, B, E and F). However, both adults and infants had significantly higher proportions of CD4⁺ T-cells in the URL compared to BAL (Fig. 2A-E). Importantly, higher frequencies of adult CD8⁺ T-cells were present in the BAL when compared to the URL (Fig. 2A, 2C, and 2F), whereas CD8⁺ T-cells were greater in the URL compared to BAL in RSV-infected infant mice (Fig. 2B, D, and F). Although the proportions of CD8⁺ T-cells in adult BAL were significantly higher than that of infants (Fig. 2F), the ratio of CD8⁺ to CD4⁺ T-cells was greater in the infant BAL compared to URL. These data indicate that CD4⁺ and CD8⁺ T-cells both accumulate in the adult airway, but aggregation of CD8⁺ T-cells is more prominent than CD4⁺ T-cells in the BAL. Moreover, frequencies of infant T-cells in BAL were significantly diminished compared to adults, which may contribute to delayed RSV clearance among infants.

T-cells within the BAL display an activated phenotype compared to the URL

To determine the effect of age and location on T-cell activation, CD44^{HI} expression was compared on CD4⁺ and CD8⁺ T-cells in the BAL and URL (Fig. 3). Adults and infants had significantly higher CD44^{HI} expression with an increased median fluorescence intensity (MFI) on CD4⁺ T-cells in the BAL when compared to the URL (Fig. 3A and B). Age-based comparisons of CD44^{HI} expression on CD4⁺ T-cells in the BAL demonstrated that adults had significantly higher proportions of CD4⁺ CD44^{HI} T-cells and higher CD44 MFI than infants (Fig. 3A and B). Similarly, frequency of CD8⁺ CD44^{HI} T-cells and the MFI of CD44 on CD8⁺ T-cells was higher in adult compared to infant BAL (Fig. 3C and D). As with the CD4⁺ T-cells, the BAL consistently contained a higher percentage of CD8⁺ CD44^{HI} T-cells in both adults and infants when compared to the URL (Fig. 3C). Age-dependent comparisons revealed a higher MFI of CD44 on CD8⁺ T-cells in adult BAL compared to

URL. Infants showed a similar trend, but was not statistically significant. These results suggest that following RSV infection, T-cells isolated from the alveolar space (BAL) are more highly activated compared to those isolated from lung tissue (URL).

Localization of Tbet⁺ CD4⁺ T-cells within the alveolar space is diminished in infant mice

To elucidate the phenotype of activated CD4⁺ T-cells responding to RSV infection at the peak of T cell recruitment (10dpi), intracellular transcription factor staining of CD4⁺ CD44^{HI} T-cells in the BAL and URL of RSV-infected adults and infants was performed. The ratio of CD4⁺ CD44^{HI} Tbet⁺:GATA3⁺ T-cells (using total cell counts), was near zero in adult URL samples but rose significantly in the BAL (Fig.4A). The frequency of CD4⁺GATA3⁺ T-cells remained stable in both URL and BAL (Fig. 4B), indicating that increases in adult CD4⁺ Tbet⁺ T-cells drove the increased Tbet⁺:GATA3⁺ ratio in the BAL (Fig.4A & B). Strikingly, infants had elevated Tbet⁺:GATA3⁺ ratios in their URL and BAL that were comparable to adult ratios in the BAL (Fig. 4A). Analysis of individual Tbet⁺ and GATA⁺ cells demonstrated that the majority of Tbet⁺ cells were migrating to the BAL whereas in the infants, more than half of Tbet⁺ T-cells remained in the URL (Fig. 4B), suggesting an incomplete localization of Tbet⁺ T-cells to the URL of infants. GATA3⁺ T-cell frequency was higher than that of Tbet⁺ T-cells regardless of age or sample type (Fig. 4B). These data suggest that infants do not localize CD4⁺ CD44^{HI} Tbet⁺ T-cells to the BAL as efficiently as adults following RSV infection.

IFN- γ reduces CD4⁺ CD44^{HI} Tbet⁺:GATA3⁺ T-cell ratios compared to RSV infection alone

To determine how T-cell phenotype changes in infants following the local delivery of a Th1-type cytokine, i.n. IFN- γ was administered to RSV-infected infant mice. Intracellular Tbet⁺ and GATA3⁺ expression in CD4⁺ CD44^{HI} T-cells in the URL and BAL was subsequently analyzed. Although frequencies of CD4⁺ and CD4⁺ CD44^{HI} T-cells were unaltered following i.n. IFN- γ (Supplementary Fig. 1), Tbet⁺:GATA3⁺ CD44^{HI} CD4⁺ T-cell ratios were significantly reduced in the URL and BAL compared to untreated controls (Fig. 5A). Importantly, reduced CD4⁺ CD44^{HI} Tbet⁺:GATA3⁺ T-cell ratios in the BAL were driven by reductions in CD4⁺ CD44^{HI} Tbet⁺ T-cell frequency rather than increases in the frequency of CD4⁺ CD44^{HI} GATA3⁺ T-cells (Fig.5B). These data suggest that local IFN- γ delivery to the infant airway during early RSV infection reduced the frequency of CD4⁺ Tbet⁺ T-cells without altering CD4⁺ GATA3⁺ frequencies at 10 DPI.

Local IFN- γ increases RSV F-protein specific CD8⁺ T-cells in infant BAL

We next sought to determine if IFN- γ altered the CD8⁺ T-cell response in the RSV-infected infant airway. Following i.n. delivery of IFN- γ , total CD8⁺ T-cells were reduced in the infant airway compared to untreated controls (Fig. 6A). The overall reduction in the CD8⁺ T-cells was accompanied by significant reductions in CD8⁺ CD44^{HI} T-cells (data not shown) and reductions in CD8⁺ CD44^{HI} T-cells expressing Tbet (Fig. 6B). These data were surprising given our previous findings, which showed that i.n. IFN- γ expedited viral clearance compared to age-matched controls²⁴. To resolve these seemingly incongruous results, we sought to determine the effect of i.n. IFN- γ on CD8⁺ T-cell specificity using an RSV F protein-specific pentamer. Intriguingly, local IFN- γ administration significantly increased the frequency of RSV F protein-specific CD8⁺ T-cells in the BAL of RSV-infected infant

mice compared to untreated controls (Fig. 6C). Together, these results suggest that IFN- γ contracts the CD8⁺ T-cell population while enhancing CD8⁺ T-cell RSV F protein specificity in the BAL of RSV-infected infant mice.

Discussion

The results presented here describe important age-based differences in T-cell localization and activation following RSV infection. Collectively, these data highlight three important findings. First, following RSV infection, infant mice have a dramatically reduced T-cell response compared to adult mice. However, the kinetics of pulmonary T-cell recruitment in response to RSV infection were similar between age-groups with both adult and infant mice peaking at 10 DPI. Although infant mice had lower T-cell frequencies in lung tissue compared to adult mice, the differences were most striking in the BAL, where average CD4⁺ and CD8⁺ T cell frequencies were reduced by 95% and 86% (Fig. 2), respectively.

Previous reports have demonstrated reduced and delayed infant murine T-cell responses when compared to adults in models of influenza, methicillin-resistant *Staphylococcus aureus* pneumonia, and *Pneumocystis carinii* pneumonia^{17,26,27}. A similar observation was made when infant and adult lung tissues from mice were compared following primary RSV infection whereby the frequencies of infant CD44^{HI} CD62L^{LO} CD4⁺ and CD8⁺ T-cells were significantly lower than that of adults²⁸. Consistent with work published by Tregoning et al., the current study demonstrated reduced CD8⁺ T-cell frequencies in infant mouse lung tissue when compared to adults.²⁸ More importantly, data presented here showed the most dramatic age-dependent differences in the T-cell response following RSV infection occurred in the BAL. Lines et al showed similar findings in a neonatal murine model of influenza¹⁷, whereby delayed viral clearance was associated with poor T-cell accumulation in the BAL despite a vigorous T-cell response in the interstitium¹⁷. Moreover, the reduced T-cell frequency in infant compared to adult murine airways is consistent with our previous findings demonstrating prolonged viral detection in RSV-infected infant mice compared to adults²⁴. In addition to quantitative T-cell defects, these data suggest environmental or cellular differences are also important in regulating the activation phenotypes of T-cells in the infant alveolar space.

A second critical finding generated from these studies was the higher proportion of activated T-cells in the BAL compared to lung tissue (Fig. 3). Specifically, a greater proportion of T-cells isolated from the alveolar space of both adult and infant mice were effector/memory T-cells (CD4⁺/CD8⁺ CD44^{HI} CD62L⁻) compared to T-cells from lung homogenate (Fig.3). These findings are consistent with previous studies demonstrating that RSV infects the superficial cells of the upper and lower airways. In-vitro studies of RSV infection in primary human cartilaginous airway epithelium, as well as histologic studies in RSV-infected patients, have consistently demonstrated the virus' tropism for ciliated epithelium while sparing goblet cells and underlying basal epithelial cells^{13,29–31}. Moreover, Lukens et al. found larger proportions of activated and RSV-specific CD8⁺ T-cells in the airways of adult mice compared to lung tissue³². Contrary to reports highlighting the functional inactivation of RSV-specific CD8⁺ T-cells in the lung parenchyma^{32,33}, they also showed that RSV-specific CD8⁺ T-cells isolated from the BAL remained functionally active through 21 DPI³².

This was in sharp contrast to RSV-specific CD8⁺ T-cells isolated from murine lung homogenate whose production of IFN- γ dramatically decreased by ~43% between 8 and 21 DPI³². Collectively, these results suggest that localization of T-cells within the airway is important when considering their activation and functional status in response to RSV infection.

The importance of CD8⁺ T-cells in the clearance of RSV is widely appreciated^{8–10,34} but what may be less recognized is the location of these cells within the pulmonary architecture in RSV-infected infants versus adults. In our study, adult murine CD8⁺ T-cells were found in high frequencies in the air space where these cells made up a larger proportion of the lymphocytes (mean 48%) than in lung homogenate (mean 34%). Infant mice displayed a starkly different distribution, with higher frequencies of CD8⁺ T-cells in lung homogenate (mean 22.9%) compared to BAL (mean 6.6%). The accumulation of CD8⁺ T-cells in the adult, murine airway suggests that high frequencies of CD8⁺ T-cells within the alveolar space is an important factor in effective RSV clearance, yet a similar CD8⁺ T-cell response is remarkably absent in infant mice.

Another important age-dependent distinction was the location of CD4⁺ Tbet⁺ T-cells within the lung architecture. CD4⁺ CD44^{HI} Tbet⁺ T-cell responses accumulated in the airways of adult mice but were split between the lungs and airways of infants (Fig. 4). The frequency of effector memory CD4⁺ T-cells was higher in the BAL of RSV-infected infant mice than the lung tissue, yet infants still had significantly lower percentages than adult mice. Phenotypic analysis of CD4⁺ effector memory cells revealed that adult mice had a higher Tbet⁺ expression compared to infants. Although GATA3⁺ CD4⁺ T-cells were found with high frequency throughout sample types and across age-groups, Tbet⁺ CD4⁺ T-cells were virtually undetectable in adult murine lung homogenate and instead were found predominately in the BAL. In infant mice, CD4⁺ Tbet⁺ T-cells were distributed throughout the lung tissue and airway with equal frequency, suggesting that while adult mice mount a pointed CD4⁺ Tbet⁺ T-cell response at the site of RSV infection, the infant CD4⁺ Tbet⁺ T-cell response was less focused, possibly leading to more diffuse immune-mediated pathology. Based on kinetic studies demonstrating T-cell numbers peaked at 10 dpi, differences in infant and adult T-cell phenotypes were interrogated at this time point. While it is possible that Tbet⁺ T-cell localization to infant BAL may increase at later time points, the data presented here suggests that age contributes to incomplete or inefficient accumulation of T-cells in infant compared to adult BAL.

The small proportions of T-cells found in the BAL of RSV-infected infant mice may indicate a lack of effective recruitment. However, delivery (i.n.) of either IFN- γ , CXCL9, or CCL2 did not increase T-cells in the BAL in an infant murine model of influenza¹⁷. Although these agents were administered late in infection (7, 8, and 9 DPI), our studies also showed that early delivery of i.n. IFN- γ during RSV infection (1, 3, and 5 DPI) did not increase the frequencies of T-cells recovered from the alveolar space at 10 DPI (Supplemental Figure S1). Moreover, T-cell chemokines (CCL2 and CCL5) and IL-2 were significantly increased in adult compared to infant BAL early in infection (Data not shown), suggesting that infant mice may have poor integrin/chemokine signaling or the BAL may lack factors essential to T-cell survival. Both scenarios require further investigation.

Third, local delivery of IFN- γ to the infant, murine airway unexpectedly lowered the frequency of CD4⁺ Tbet⁺ T-cells compared to untreated controls, while frequencies of CD4⁺ GATA3⁺ T-cells remained stable (Fig. 5). Surprisingly, CD8⁺ T-cell populations were also reduced in IFN- γ -treated infant mice yet, i.n. IFN- γ increased the RSV F protein-specific CD8⁺ T-cell response compared to untreated controls (Fig. 6).

Multiple clinical studies have demonstrated correlations between Th2 biased immune responses and severe RSV disease^{14,18–20}. Our lab and others have shown that local delivery of the Th1-type cytokine, IFN- γ , mitigates disease pathology in RSV-infected infant mice^{24,35–37}. The studies presented here advance the current understanding of IFN- γ 's effect on T-cell expression patterns in the RSV-infected infant airway. Specifically, IFN- γ unexpectedly reduced Tbet⁺ CD4⁺ T-cell frequencies in infant mouse BAL at 10 DPI while the frequencies of GATA3⁺ CD4⁺ T-cells remained unchanged (Fig. 4). Initially, we suspected this decrease in infant murine CD4⁺ Tbet⁺ T-cells may be reflective of a rebound effect given the strong inflammatory pressure of IFN- γ , but analysis of an earlier seven-day time point showed similar reductions in Tbet⁺ CD4⁺ T-cells with no differences in overall CD4⁺ T-cell numbers or GATA3⁺ expression (Supplemental Figure S2). Given that CD4⁺ Tbet⁺ frequencies decreased but GATA3⁺ CD4⁺ frequencies remained unchanged, further studies will be required to understand what phenotype these CD4⁺ T-cells became, including investigating frequencies of Th17 CD4⁺ T-cells.

Finally, IFN- γ -treated infant mice demonstrated a reduction in the BAL effector memory CD8⁺ T-cell pool compared to untreated controls but increased RSV F-protein-specific CD8⁺ T-cells (Fig. 6), suggesting that IFN- γ may regulate CD8⁺ T-cell contraction while enriching the pool of RSV-specific effector memory CD8⁺ T-cells by 10 DPI. The 10 DPI time point was chosen based on the kinetic data demonstrating that both infant and adult murine T-cell responses peak at 10 DPI (Fig. 1). However, additional studies will assess both M2- and F-specific CD8 T cell specificity and whether these age-based differences in T-cell phenotype and RSV-specificity are consistently deficient in infant mice.

The current findings are consistent with previously published reports showing that IFN- γ is associated with CD8⁺ effector/memory contraction and enhanced specificity^{38–40}. Moreover, Sercan and colleagues demonstrated the importance of IFN- γ receptor signaling in CD11b⁺ innate cells in the control of CD8⁺ effector proliferation^{38,39}. This is consistent with our previous reports showing the upregulation of CD11b⁺ on CD11c⁺ cells in the BAL of infant mice primed with i.n. IFN- γ ²⁴ and provides indirect evidence that IFN- γ may contribute to the observed decrease in CD8⁺ effector/memory cells. Furthermore, IFN- γ signaling blocked the formation of memory precursor CD8⁺ T-cells that responded to weak TCR agonists and instead promoted the accrual of high-affinity memory CD8⁺ T-cells⁴⁰. Although the F protein is highly antigenic, it does not contain the primary, immunodominant epitope, thus future studies will determine the effect of i.n. IFN- γ on effector/memory CD8⁺ T-cell development targeting the immunodominant M2 protein.

In summary, local delivery of IFN- γ to the infant airway reduced CD8⁺ effector memory T-cell responses in the BAL but improved CD8⁺ RSV F-protein specificity, suggesting that

targeted T-cell responses in infants following RSV infection may be improved through early IFN- γ exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Pulmonary T-cell recruitment peaks 10 days after RSV infection in adult and infant mice

Adult (6-8 weeks) and infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g). Right lungs were collected at 3, 7, 10, and 15 DPI and processed for flow cytometry. Lymphocytes were gated from FSC vs. SSC, then CD4⁺ and CD8⁺ T-cells were determined from TCR β ⁺ cells in the lymphocyte gate. Total T-cells were compared between naïve and RSV⁺ adult mice (A & B) using ANOVA with a Dunnett's post-test. Infant T-cell totals (C & D) were compared using 2-way ANOVA with a Bonferroni post-test. Naïve adult lungs (n = 1) were processed simultaneously with RSV⁺ samples at each time point to account for day to day variations in analysis via flow cytometry. However, the data from all naïve adults were pooled and represented graphically as 0 DPI. Naïve, infant samples (n = 3-4) were analyzed at each time point to account for developmental changes over the course of the study. Data points represent individual mice (naïve adults) or the mean of n = 3-4 samples per group $\pm$ SEM; ** p -value < 0.01. Data in A-B & C-D are representative of 2 independent experiments.

Figure 2. Infant T-cell accumulation in the BAL is reduced compared to adults

Adult (6-8 weeks) and infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g). BAL and URL were collected at 10 DPI and processed for flow cytometry. The lymphocytes were gated according to typical FSC vs. SSC characteristics. CD4⁺ and CD8⁺ T-cells were determined from TCR β ⁺ cells in the lymphocyte gate. The dot plots represent adult and infant BAL (A & B) and URL (C & D), respectively. The frequencies of CD4⁺ (E) and CD8⁺ (F) T-cells were compared between URL and BAL by 2-way ANOVA with a Bonferroni post-test. The frequencies of CD4⁺ (E) and CD8⁺ (F) T-cells in the BAL were compared between age groups using 2-tailed unpaired *t* test. Points represent individual mice (adults) or individual samples from 2 pooled infants, lines represent the mean of *n* 4 samples per group $\pm$ SEM; ** *p*-value < 0.01. Data in E & F are representative of 2 independent experiments.

10 Days Post-Infection

Figure 3. T-cells isolated from the BAL express an activated phenotype compared to the URL
 Adult (6-8 weeks) and infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g). BAL and URL were collected at 10 DPI and processed for flow cytometry. The lymphocytes were gated according to typical FSC vs. SSC characteristics. % CD44^{HI} and median fluorescence intensity (MFI) were determined from TCRβ⁺ CD4⁺ or CD8⁺ T-cells in the lymphocyte gate. The CD4⁺ T-cell frequencies and MFI of CD44^{HI} CD4⁺ (A and B, respectively) and CD8⁺ T-cell frequencies and MFI of CD44^{HI} CD8⁺ T-cells (C and D, respectively) were compared between the BAL and URL using 2-way ANOVA with a Bonferroni post-test. CD4⁺ T-cell frequencies and MFI of CD44^{HI} CD4⁺ (A and B, respectively) and CD8⁺ T-cell frequencies and MFI of CD44^{HI} CD8⁺ T-cells (C and D, respectively) from the BAL were compared between age groups using 2-tailed unpaired t test. Points represent individual mice (adults) or individual samples from 2 pooled infants, lines represent the mean of n = 4 samples per group ± SEM; ** p-value < 0.01. Data in A-D are representative of 2 independent experiments. All CD44^{HI} cells were also CD62L⁻.

10 Days Post-Infection

Figure 4. Localization of Tbet⁺ CD4⁺ T-cells in the alveolar space is more efficient in RSV-infected adult compared to infant mice

Adult (6-8 weeks) and infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g). BAL and URL were collected at 10 DPI and processed for flow cytometry. Lymphocytes were gated according to typical FSC vs. SSC characteristics. A ratio of TCR β ⁺ CD4⁺ CD44^{HI} Tbet⁺ cells to TCR β ⁺ CD4⁺ CD44^{HI} GATA3⁺ cells was calculated from total cell counts of the URL and BAL (A). The frequency of CD4⁺ CD44^{HI} GATA3⁺ and Tbet⁺ T-cells were compared in URL and BAL for adults and infants, respectively (B). A 2-way ANOVA was used to compare the Tbet⁺:GATA3⁺ CD4⁺ T-cell ratios between sample type and age groups with a Bonferroni post-test (A). The frequencies of CD4⁺ CD44^{HI} GATA3⁺ and Tbet⁺ T-cells were compared in URL and BAL using 2-way ANOVA with a Bonferroni post-test (B). Points represent individual mice (adults) or individual samples from 2 pooled infants, lines represent the mean of $n = 4$ samples per group $\pm$ SEM; ** $p < 0.01$. Data in A & B are representative of 2 independent experiments. All CD44^{HI} cells were also CD62L⁻.

Figure 5. Intranasal IFN- γ reduces Tbet⁺:GATA3⁺ CD4⁺ T-cell ratios at 10 DPI compared to RSV infection alone

Infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g) and administered i.n. IFN γ (16ng/g) or vehicle on 1, 3, and 5 DPI. BAL and URL were collected at 10 DPI and processed for flow cytometry. Lymphocytes were gated according to typical FSC vs. SSC characteristics. Ratios of TCR β ⁺ CD4⁺ CD44^{HI} Tbet⁺ cells to TCR β ⁺ CD4⁺ CD44^{HI} GATA3⁺ cells were calculated from total cell counts of the URL and BAL (A). The frequency of CD4⁺ CD44^{HI} GATA3⁺ and Tbet⁺ T-cells were compared in BAL for RSV and RSV + IFN γ infants (B). A 2-way ANOVA was used to compare the Tbet⁺:GATA3⁺ CD4⁺ T-cell ratios between URL, BAL, and treatment groups with a Bonferroni post-test (A). The frequencies of CD4⁺ CD44^{HI} GATA3⁺ and Tbet⁺ T-cells in the BAL were compared between RSV and RSV + IFN γ infants using 2-way ANOVA with a Bonferroni post-test (B). Points represent individual samples of 2 pooled infants, lines represent the mean of $n = 4$ samples per group $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$. Data is representative of a single experiment. All CD44^{HI} cells were also CD62L⁻.

Figure 6. IFN- γ priming of RSV-infected infant mice improves RSV F-protein specific CD8⁺ T cell accumulation within the BAL compared to RSV alone, despite overall reductions in CD8⁺ T-cells

Infant (PND 4-5) Balb/cJ mice were infected with i.n. RSV L19 (5×10^5 pfu/g) and administered i.n. IFN γ (16ng/g) or vehicle on 1, 3 and 5 DPI. BAL was collected at 10 DPI and processed for flow cytometry. The lymphocytes were gated according to typical FSC vs. SSC characteristics. Total CD8⁺ T-cells (A), total CD8⁺ CD44^{HI} Tbet⁺ T-cells (B), and the percentage of CD19⁻ RSV F protein-specific CD8⁺ T-cells (C) were compared between groups using an unpaired, 2-tailed t test. For the quantification of RSV F protein-specific CD8⁺ T-cells, we first subtracted the percentage of background obtained from an LPS negative control BAL sample stained with RSV F protein-specific pentamer. Points represent individual samples of 2 pooled infants, lines represent the mean of $n = 9$ samples per group $\pm$ SEM; ** p -value < 0.01 . Data in A - C are representative of 2 independent experiments. All CD44^{HI} cells were also CD62L⁻.