STAT6 inhibitory peptide given during RSV infection of neonatal mice reduces exacerbated airway responses upon adult reinfection

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ABSTRACT
Respiratory syncytial virus (RSV)-related hospitalization during infancy is strongly associated with the subsequent development of asthma. Early life RSV infection results in a Th2-biased immune response, which is also typical of asthma. Murine models of neonatal RSV infection have been developed to examine the possible contribution of RSV-driven Th2 responses to the development of airway hyper-responsiveness later in childhood. We have investigated the ability of a cell-penetrating STAT6 inhibitory peptide (STAT6-IP), when delivered selectively during neonatal RSV infection, to modify pathogenesis induced upon secondary RSV reinfection of adults 6 wk later. Neonatal STAT6-IP treatment inhibited the development of airway hyper-responsiveness (AHR) and significantly reduced lung eosinophilia and collagen deposition in adult mice following RSV reinfection. STAT6-IP-treated, RSV-infected neonates had reduced levels of both IL-4 and alternatively activated macrophages (AAMs) in the lungs. Our findings suggest that targeting STAT6 activity at the time of early-life RSV infection may effectively reduce the risk of subsequent asthma development. J. Leukoc. Biol. 101: 000–000; 2017.

Introduction
RSV-related respiratory illness is a leading cause of infant hospitalization. Globally, nearly 34 million respiratory tract infections in infants under 5 yr of age are caused by RSV, with nearly two-thirds occurring within the first year of life [1]. In the United States alone, annual RSV hospitalization rates are estimated to be 48.9/1000 in infants under the age of 3 mo and 28.4/1000 in infants between 3 and 5 mo of age [2].

Recently, a number of epidemiologic studies have supported an association between early-life RSV-related hospitalization and the development of asthma in childhood that lasts into adulthood [3–8]. It is currently unclear whether RSV infection triggers asthma development, if atop individuals have an increased risk of RSV infection requiring hospitalization, and/or whether severe RSV and asthma share common genetic risk factors [9, 10]. It is likely that a combination of these factors, along with the unique characteristics of the neonatal immune system, plays a role in the development of asthma.

The most widely used model of RSV infection is the adult mouse, despite shortcomings that arise from decreased susceptibility of the mouse to RSV infection. The infection of adult mice requires high titers of RSV strain A2 (107–108 TCID50/ml), administered directly into the lower respiratory tract. In addition, RSV replication in mice is relatively poor and results in a strong Th1-type response with efficient CD8+ T cell-mediated viral clearance [11]. In this model, the role of RSV-specific Th2 responses may be restricted to the prevention of exaggerated, Th1-mediated lung pathology [12]. More recently, a neonatal mouse model of sequential RSV infection has been described in which neonatal RSV infection (<7 d), followed by secondary adult reinfection (~6–8 wk of age), results in a Th2-dependent, "allergic asthma-like" phenotype characterized by airway hyper-responsiveness, airway eosinophil influx, and airway remodeling (reviewed in ref. [13]). This worsened response to reinfection is dependent on the age at initial infection and IL-13 production [14] and is enhanced by the presence of anti-RSV IgE antibodies [15]. A central role of Th2 signaling in this model is strongly...

Abbreviations: AAM = alternatively activated macrophage (M2), AHR = airway hyperresponsiveness, AM = alveolar macrophage, ASO = antisense oligonucleotide, ATCC = American Type Culture Collection, BALF = bronchoalveolar lavage fluid, CAM = classically activated macrophage (M1), CP = control peptide, CPR = CP-treated, RSV infected as neonates, CPR-IP = IP-treated, RSV infected as neonates, RSV infected as adults, DC = dendritic cell, DLN = draining lymph node, Fl = formalin inactivated, Hl = heat-inactivated, IM = interstitial macrophages, IP = inhibitory peptide, M1 = Th1-type response with efficient CD8+ T cell-mediated viral clearance, M2 = alternatively activated macrophage (AAM), QTR = quantified total respiratory tract fluid, RSV = respiratory syncytial virus, TCID50 = tissue culture infective dose [50].

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suggested by the up-regulation of IL-4Ra on pulmonary CD4+ T cells in the lungs of reinfected adults, as depletion of this receptor by ASO delivery abolishes enhanced disease upon RSV reinfection [16, 17]. Cells of the innate and adaptive immune system have been implicated in enhanced disease: depletion of NK cells during reinfection attenuated weight loss but not T cell responses, whereas depletion of AMs reduced both NK cells and T cell responses [18]. In the neonate, the elimination of CD8+ T cells decreased enhanced disease, and the reduction of CD4+ and CD8+ T cells in the adult also resulted in attenuated disease [19]. The interplay between these cells in the pathogenesis of diseases is currently unknown.

The STAT6 transcription factor plays a crucial role in signaling downstream of IL-4Ra, activated by IL-4 and IL-13, to promote Th2-type responses in general [20]. We have previously described a cell-penetrating chimeric STAT6-IP comprised of the phosphorylated tyrosine Y641 (of murine STAT6) and the a cell-penetrating chimeric STAT6-IP dependent changes initiated by neonatal RSV infection. STAT6-IP reduces Th2-biased airway inflammatory responses upon adult RSV reinfection. We now report that STAT6-IP treatment during neonatal RSV infection prevents the development of maladaptive Th2-biased airway inflammatory responses upon adult RSV reinfection. Although overall levels of lung inflammation were not altered, BALF eosinophil frequencies were diminished, recovery from weight loss was expedited, and airway hyper-responsiveness was abolished. Airway collagen deposition was also reduced in RSV-reinfected adults treated with STAT6-IP as neonates. To understand better the mechanism(s) of action of STAT6-IP, innate immune responses in RSV-infected neonates were assessed. Although levels of the innate cytokines, TSLP, IL-33, and IL-25, were unaffected by STAT6-IP treatment in the lung, IL-4 levels were reduced, and AAMs also decreased in STAT6-IP-treated mice. Our data suggest that STAT6-IP treatment modulates the Th2-biased and profibrotic microenvironment of the lung initiated by neonatal RSV infection. STAT6-IP-dependent changes appear to be long lasting, leading to diminished pulmonary dysfunction even upon repeat RSV infection many weeks later.

**MATERIALS AND METHODS**

**Cells and virus culture**

RSV A2 (ATCC #VR-1540; ATCC, Manassas, VA, USA) strain was propagated (MOI of 0.5) in human epithelial type 2 cells (ATCC #OCL223) in RSV media (DMEM supplemented with 3% FBS, 10 mM Hepes, 2 mM glutamine, and 50 μg/ml gentamicin; all from Wisent, St-Bruno, QC, Canada) at 37°C. When an extensive cytopathic effect was observed (4–5 d postinfection), culture media and cells were scraped and centrifuged at 3000 g for 15 min at 4°C. A small volume of supernatant was retained with the cell pellets, whereas the rest of the supernatant was maintained on ice. Cell pellets were subjected to 3 quick freeze/thaw cycles and centrifuged again. Supernatants obtained postfreeze-thaw were pooled with the previously collected supernatants; concentrated with polyethylene glycol (PEG) 6000 (EMD Millipore, Billerica, MA, USA), as described previously [24]; and stored at −80°C. RSV was quantified by serial dilution on Vero cell monolayers, plated in 96-well plates, incubated for 7 d, and fixed in 1% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Plates were detected by immunofluorescence, using goat anti-RSV FITC-conjugated antibody (Abcam, Cambridge, MA, USA). Wells were considered positive when ≥1 fluorescent syncytium was detected. Viral titers (TCID50/μl) were calculated by the Reed-Muench method [25]. Titers of up to 107 TCID50/μl were obtained by this method.

**Peptides**

STAT6-IP and STAT6-CP were synthesized by the University of Calgary Integrated Peptide Services (Calgary, AB, Canada), as described previously [21]. The sequence of these peptides is identical, except the phospho-Tyr residue in STAT6-IP is replaced by a Phe residue in STAT6-CP. Peptides were diluted to a final concentration of 10 μg/1 μl in normal SAL and stored at −80°C until used. Normal SAL was used as control.

**Animals, peptide treatment, and infections**

Male and female BALB/c mice (6–8 wk) were obtained from Charles Rivers Laboratories (Saint-Constant, QC, Canada). Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care. In conducting research using animals, the investigators adhere to the laws of the United States and regulations of the U.S. Department of Agriculture. Female mice were grouped together for 2–3 wk to synchronize estrus. Breeders were time mated, and pups were used for experiments within 5–4 d of birth. Pups were randomly assigned to the peptide treatment and euthanasia groups.

**STAT6-IP, STAT6-CP (10 μg/μl in 5 μl, 2.5 μl/nare), and SAL treatments were performed 1 d before and 1 d after RSV infection (106 TCID50/g body weight; ~2 g body weight) of neonates, starting at d 3–4 postbirth. In the double-infection model (see Fig. 1A), weights were followed for 6 d, after which mice were left untreated until reaching ~6 wk of age, at which time, they were reinfected with RSV (106 TCID50/g body weight; 19–22 g body weight). Select animals were euthanized at d 4 and 6 postinfection by CO2 asphyxiation. BALF was obtained after instilling 800 μl 2% FBS in PBS into the airways. The left lung was removed and homogenized for cytokine quantification. The lower lobe of the right lung was removed and placed in formalin for histopathology, and the upper lobes were stored temporarily at 4°C in RSV media for flow cytometry analysis. DLNs (axillary and brachial) from mice in each group were pooled in RSV media for ex vivo restimulation.

Exposure to the neonatal infection model (see Fig. 1B), weights were followed for 7 d post-RSV infection, and groups of mice were euthanized at d 2, 4, and 7 postinfection by CO2 asphyxiation. The left lung was stored temporarily at 4°C in RSV media for flow cytometry analysis (see below). The right lung was removed and homogenized for cytokine quantification.

**Analysis of airway hyper-responsiveness**

To assess airway hyper-responsiveness, mice were anesthetized with xylazine, followed by sodium pentobarbital, after which, they were injected with the paralyzing agent, pancuronium bromide. Measurements were determined using the flexiVent small animal ventilator (Scireq, Montreal, QC, Canada) by exposing mice to increasing concentrations of aerosolized methacholine, as described previously [26].

**BALF leukocyte differentiation counts**

After counting total BALF cell numbers, 20,000 cells/sample were cytospun onto glass slides (Thermo Fisher Scientific, Nepean, ON, Canada) and air dried overnight. Slides were stained with Diff-Quick (Siemens Healthcare...
Isolation and stimulation of lymph nodes

To assess RSV-specific cytokine responses in mice following adult RSV reinfection, lymph node cells were stimulated ex vivo with an RSV antigen preparation derived from HI-RSV. In brief, single-cell suspensions were obtained by passing pooled lymph nodes through cell strainers (BD Falcon; BD Biosciences, Bedford, MA, USA). Cells were collected by centrifugation at 400 g for 5 min, plated in 96-well plates at 1 × 10^5 cells/well in a volume of 200 µl, and stimulated for 72 h with HI-RSV (MOI of 10) or as a positive control, PMA (1 µg/ml) and ionomycin (2 µg/ml). For inactivation, RSV was incubated at 56°C for 45–60 min. Each sample was plated in at least 4 wells as independent replicates. Poststimulation (72 h), plates were centrifuged at 400 g for 5 min and supernatants stored at −80°C until cytokine quantification was performed.

Quantification of cytokines

IL-4, IL-13, IFN-γ, TSLP, IL-25, and IL-33 levels in lungs, BALF, and ex vivo-stimulated lymph node supernatants were quantified using Ready-SET-Go! ELISA kits (eBioscience), following the manufacturer’s instructions.

Histopathology

Lungs were fixed in formalin and processed at the Meakins-Christie Laboratories Histopathology Core Facility (Montreal, QC, Canada), where they were embedded in paraffin blocks. Afterward, 5-µM sections were stained with H&E or MT. The H&E histological inflammatory response was scored, as described by Ponnuraj et al. [27]. In brief, 3–4 independent sections of each lung were scored in a blinded manner, according to the degree of inflammation in the interstitium, alveoli, perivascular, and peribronchial regions. A score of 0 (none), 1 (minimal), 2 (mild), 3 (moderate), or 4 (severe) was assigned to each section. Sums of these scores provided the total histopathological score/lung/animal. MT staining of collagen deposition was scored as described by Yoo et al. [28]. In brief, 3–4 independent sections of each lung were scored in a blinded manner, according to the degree of collagen deposition in the perivascular and peribronchial regions, and each was recorded on a scale of 0–3. Sums of these scores provided the total histopathological score/lung/animal.

Statistical analysis

Outcomes were assessed by Kruskal-Wallis test, followed by post hoc Dunn’s multiple comparison tests against STAT6-IP-treated groups (IPR or IPR-R) using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). In double-infection experiments, SAL-RM and SALM-R groups were included as controls and thus, were not included in statistical analyses. Values of P < 0.05 were considered significant; individual P-value comparisons among groups are reported in the figure legends.

RESULTS

Adult mice, treated with STAT6-IP at the time of neonatal RSV infection, recover from weight loss faster and do not develop AHR upon reinfection

To examine how inhibition of STAT6 activity in the RSV-infected neonate modulated inflammatory responses in adults re-exposed to RSV, neonatal mice were treated with STAT6-IP/C/P/SAL at d −1 and 1 of the initial RSV infection and then reinected with RSV as adults 6 wk later (Fig. 1A). Neither peptide treatment nor RSV infection induced significant changes in weight during the first week of life in neonatal mice (Fig. 2A). However, upon reinfection, 6 wk later (~7 wk of life), all animals lost weight initially, including those treated with STAT6-IP. From d 2 onward, however, only the STAT6-IP-treated animals (IPR-R) and not those treated with SAL (SAL-R) or STAT6-CP (CPR-R), regained weight, nearly reaching their original weights by d 6.

Figure 1. RSV double (neonate/adult)- and single (neonate)-infection models. (A) Double-infection model: neonatal mice (3–4 d after birth) were treated intranasally with STAT6-IP/CP or SAL, 1 d before infection and 1 d postinfection. Mice were infected with RSV on d 0, and weights were followed for 6 d. Animals were reinjected as “adults,” 6 wk after the initial infection. Mice were euthanized d 4 and d 6 after reinfection. (B) Single-infection model: neonatal mice (3–4 d after birth) were treated intranasally with STAT6-IP/CP or SAL, 1 d before infection and 1 d postinfection. Mice were infected with RSV on d 0, and weights were followed for 7 d. Mice were euthanized on d 2, 4, or 7 after infection.

Lungs stored in RSV media at 4°C were minced and then incubated with Collagenase D (Roche Diagnostics, Indianapolis, IN, USA; 1 mg/ml) for 5 h, at 37°C, 5% CO2. Control samples were treated with H&E or MT. The H&E histological inflammatory response was scored, as described by Ponnuraj et al. [27]. In brief, 3–4 independent sections of each lung were scored in a blinded manner, according to the degree of inflammation in the interstitium, alveoli, perivascular, and peribronchial regions. A score of 0 (none), 1 (minimal), 2 (mild), 3 (moderate), or 4 (severe) was assigned to each section. Sums of these scores provided the total histopathological score/lung/animal. MT staining of collagen deposition was scored as described by Yoo et al. [28]. In brief, 3–4 independent sections of each lung were scored in a blinded manner, according to the degree of collagen deposition in the perivascular and peribronchial regions, and each was recorded on a scale of 0–3. Sums of these scores provided the total histopathological score/lung/animal.

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Airway inflammation in reinfected adult mice is modified by STAT6-IP treatment at the time of neonatal RSV infection

The total number of inflammatory cells in the BALF was similar in all mice that had been infected with RSV, both as neonates and as adults, whether or not the mice had been treated (as neonates) with STAT6-IP, STAT6-CP, or SAL (Fig. 3A). However, the proportion of eosinophils recovered in the BALF was significantly lower in mice treated with STAT6-IP (IPR-R) as neonates compared with those treated with STAT6-CP (CPR-R) or SAL (SALR-R; Fig. 3B). The relative frequencies of macrophages, neutrophils, and lymphocytes did not differ in STAT6-IP-treated mice (data not shown). Consistent with the detection of similar numbers of inflammatory cells in the BALF, abundant inflammation was present in lung sections of all mice following RSV reinfection of adults (Fig. 3C and D), whether they were treated with STAT-IP, STAT6-CP, or SAL at the time of neonatal RSV infection. Similar to data from others, airway inflammation, including frequencies of eosinophils, was more modest in the single-infected mice, whether they were infected as neonates (SALR-M) or adults (SALR-R; Fig. 3B–D) [14, 29]. It is interesting that BALF cell numbers were much higher in mice infected with RSV for the first time (SALM-R group) compared with those exposed as neonates (Fig. 3A), yet the histopathology scores were uniformly higher in the latter groups (Fig. 3C).

These observations suggest that inflammation in these lung compartments can be functionally separate.

Cytokine responses in the lungs and DLNs following RSV reinfection of adult mice are modified by STAT6-IP exposure at the time of neonatal RSV infection

Similar levels of Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines were detected in lung homogenates and BALF of adult animals after RSV reinfection, regardless of the neonatal treatment group (Fig. 4A, and data not shown). Ex vivo PMA/ionomycin stimulation of lung cell suspensions confirmed an essentially equivalent potential for the production of these cytokines by all reinfected mice, whether they were treated with STAT-IP, STAT6-CP, or SAL at the time of neonatal RSV infection (Supplemental Fig. 1). Differences among groups became apparent only when we examined cytokine production from DLNs stimulated ex vivo with HI-RSV. Upon stimulation with HI-RSV, IL-4, IL-13, and IFN-γ were all produced by DLN cells harvested from adult reinfected mice, originally treated with SAL at the time of neonatal RSV infection. IL-4/IL-13 production, but not that of IFN-γ, was moderately reduced in DLN cells harvested from mice treated with STAT6-CP as neonates, whereas DLNs from STAT6-IP-treated mice produced small amounts of all of these cytokines (Fig. 4B). Together, these data suggest that STAT6-IP treatment of RSV-infected neonates does not restrict the development of an effective immune response to RSV infection many weeks later; however, it does limit the induction of a detrimental, Th2-biased inflammatory response.

Figure 2. Double-infection model: faster weight recovery and lower AHR in mice treated with STAT6-IP as neonates. Neonatal mice were treated with STAT6-IP on d −1 and 1 and infected with RSV on d 0. Mice were rested for 6 wk and reinfected as adults. (A) Neonate weights are presented as means ± sem/group from 7 independent experiments. (B) Adult weights are presented as means ± sem from 4 independent experiments, with 3–5 mice/group in each experiment. Outcomes for double-infected animals were assessed by Kruskal-Wallistest, followed by post hoc Dunn test, P<0.05, **P<0.01. Groups: IPR: IP treated, RSV infected; IPR-R: IP treated, RSV infected as neonates, RSV infected as adults; CPR: CP treated, RSV infected; CPR-R: CP treated, RSV infected as neonates, RSV infected as adults; SALR: SAL treated, RSV infected as neonates, RSV infected as adults; SALR-M: SAL treated, RSV infected as neonates, mock infected as adults; SALM: SAL treated, mock infected; SALM-R: SAL treated, mock infected as neonates, RSV infected as adults.

Figure 3. Stat6-IP treatment of RSV-infected neonates does not restrict the development of an effective immune response to RSV infection many weeks later; however, it does limit the induction of a detrimental, Th2-biased inflammatory response.
STAT6-IP treatment during neonatal RSV infection decreases IL-4 levels in the neonatal lung but has no impact on TSLP, IL-25, or IL-33

To understand better how inflammatory responses were modulated in adult mice following RSV reinfection, we examined innate responses in the lungs of neonatal mice infected with RSV STAT6-IP, STAT6-CP, and SAL (Fig. 1B). STAT6-IP treatment generally decreased the levels of the Th2 cytokines, IL-4 and IL-13, at d 4 and 7 postinfection. However, only IL-4 levels reached significance on d 4 postinfection (Fig. 5A). Consistent with data from others, demonstrating that infection of respiratory epithelial cells by RSV induces "innate Th2 cytokines," including TSLP [30] and IL-25 [31], our data show trends for increased levels of IL-25 (d 4) and IL-33 (d 2) in the lungs of RSV-infected neonatal mice compared with mock-infected controls (Fig. 5B). Levels of TSLP, on the other hand, were not induced upon RSV infection of neonatal mice. Production of these innate cytokines was not modulated by exposure to STAT6-IP, suggesting that STAT6 does not regulate production of these cytokines in RSV-infected neonates.

STAT6-IP reduces AAM levels in RSV-infected neonates

Shirey et al. [12] have recently demonstrated that RSV infection of adult mice induces AAMs, or M2 macrophages, that appear to reduce RSV-specific, Th1-driven lung pathology. As M2 macrophage differentiation is reduced in STAT6 knockout mice [32], we reasoned that RSV infection of neonates might similarly induce differentiation of M2 macrophages and that these cells would be reduced in STAT6-IP-treated mice. Interstitial and AMs were classified based on F4/80 and CD11c expression (Supplemental Fig. 2) [33]. No differences were found in frequencies of AMs (F4/80+ CD11c+) and IMs (F4/80+ CD11c+) in the lungs of RSV-infected neonates. Within these populations, we also assessed expression of CD206 and CCR7 as markers of M2 and M1 macrophage polarization, respectively. We detected CD206- and CCR7-expressing AMs in RSV and mock-infected neonates (Fig. 6C and D). STAT6-IP treatment abrogated the increase in CD206-expressing M2 AMs (Fig. 6C). Nevertheless, CCR7-expressing M1 macrophages were unaffected in RSV-infected neonates, whether treated with STAT6-IP/STAT6-CP or not (Fig. 6D). Expression of CD206 or CCR7 on IMs was not detected (data not shown). These data provide evidence that STAT6-IP treatment of RSV-infected neonates interferes with the differentiation/expansion of M2 macrophages.

STAT6-IP treatment during neonatal RSV infection decreases collagen deposition in the airways of RSV-reinfected adults

M2 macrophages have been implicated in mucus production, airway hyper-responsiveness, collagen deposition, and airway remodeling in murine models of asthma and viral infection [34–37]. Thus, we examined how neonatal RSV infection ± STAT6-IP treatment affected collagen deposition in adult mice, following RSV reinfection. Consistent with data from You et al. [28], using a similar model, perivascular and peribronchial collagen deposition was increased in the lungs of all animals infected with RSV as neonates, whether they were reinfe...
adults or not (SALR-M; Fig. 7). Moreover, collagen deposition in adult mice was significantly reduced in mice treated with STAT6-IP at the time of neonatal RSV infection (IPR-R; Fig. 7B). Only low levels of collagen were present in the lungs of mice infected with RSV only as adults (SALM-R).

**DISCUSSION**

The prevalence of allergic airways disease has increased dramatically over the past 25 yr in virtually all parts of the world but particularly in resource-rich settings [38]. There is no single cause for the development of airway hyper-responsiveness, but there is little doubt that environmental factors are major contributors to asthma etiology. Whereas the role of respiratory viral infections in asthma exacerbations is well characterized [39], the role these pathogens play in the initiation of asthma has been the subject of much debate [40]. Among the respiratory viruses, RSV and rhinovirus have been most strongly linked with the induction of asthma, whereas influenza viruses have been associated primarily with asthma exacerbations [41]. The global prevalence of RSV [42], its proclivity to infect the young [8], and the induction of strong, Th2-biased responses upon early-life RSV infection [43–45] have all contributed to the interest in possible mechanisms by which RSV triggers asthma development.

To model RSV infection in humans, we and others [13] have exploited the murine neonatal-adult model of sequential RSV infection. Neonatal infection, followed by adult reinfection, reproduces several key features of human asthma, including airway hyper-responsiveness, airway remodeling, and lung eosinophilia. These responses in the adult mouse have been linked to the promotion/maintenance of a Th2-biased pulmonary microenvironment initiated by neonatal infection [14]. In these studies, we used a peptide inhibitor of STAT6 (STAT6-IP) to examine the contribution of STAT6 in early-life RSV infection to the induction of Th2-biased airway inflammatory responses in the adult upon reinfection. Our findings suggest that targeted suppression of STAT6 during neonatal RSV infection markedly diminished both structural and functional changes to the airways in response to subsequent RSV reinfection.

Consistent with the published literature [13], neonatal RSV infection in our model did not cause weight loss or any obvious behavioral changes. A similar lack of overt clinical symptoms and signs is also observed in the majority of human infants infected with RSV. Despite the absence of obvious clinical manifestations in the neonatal mice, however, early-life RSV infection still...
resulted in significant structural and functional changes in the airways of the adults.

The IL-4Rα/STAT6 pathway is strongly implicated in AHR in murine models of allergic airway disease [46, 47]. Therefore, it may not be surprising that STAT6-IP intervention during neonatal RSV infection influenced AHR in adults following RSV reinfection. What was unexpected was the magnitude of this effect (i.e., normalized AHR; Fig. 2C) and the fact that the effect of STAT6-IP treatment persisted many weeks after neonatal treatment. Whereas the precise molecular mechanism of this inhibition of AHR is not yet clear, these data are consistent with the findings of other groups. In particular, Ripple et al. [16] showed that treatment with an ASO, targeting IL-4Rα during neonatal infection, prevented pulmonary dysfunction following adult reinfection. Their ASO and our STAT6-IP target different molecules in the same pathway, yet these treatments resulted in similar declines in AHR upon reinfection, highlighting the importance of IL-4Rα/STAT6 signaling in RSV-related asthma induction [16, 17].

Although we had shown that neonatal STAT6-IP exposure had beneficial effects in allergen-driven AHR, one of our concerns at the outset of the current experiments was that the STAT6-IP might inhibit the development of an effective immune response to RSV. Whereas we found similar viral titers in the lungs of all RSV-infected neonates, treated with peptide or not, RSV was undetectable in all adults previously exposed to RSV as neonates, similar to our findings in the FI-RSV vaccine model [23] (data not shown). Moreover, the IPR-R group also gained weight more rapidly after reinfection and produced equivalent cytokine profiles in lung homogenates and BALF, as well as intracellular staining of CD3+ T cells upon adult reinfection compared with the CPR-R and SALR-R groups. Likewise, inflammatory cell influx into the airways upon adult reinfection was also comparable in all of the neonatally infected groups. Together, these data suggest that neonatal STAT6-IP treatment during the initial RSV infection did not interfere with the ability to mount an effective response to RSV. The significant decreases in eosinophils and M2 macrophages, as well as reduced AHR in STAT6-IP-treated animals, suggest that the (detrimental) Th2-biased quality, rather than the quantity, of the immune response was altered in these animals.

Whereas the adaptive immune response to RSV reinfection in the adult mice treated with STAT6-IP at the time of initial infection was effective, it was not identical to that seen in the
CPR-R and SALR-R groups. In particular, the antigen-induced recall responses in the DLNs harvested from mice post-RSV reinfection were different among the groups. Cytokine production in response to RSV antigen restimulation ex vivo was markedly reduced in the mice that had been treated with STAT6-IP as neonates. These data raised the possibility that STAT6-IP treatment at the time of initial RSV infection had reduced the induction of RSV-specific lymphocyte differentiation, possibly by targeting APCs. This observation led us to examine STAT6-IP effects on lung macrophage populations; in particular, we focused on AAMs, which have been implicated in the induction and maintenance of airway pathology [34] and are directly induced by RSV both in vitro and in vivo (in adult mice) in an IL-4Rα-dependent manner [12].

Similar to the (original) Th1–Th2 dichotomy of T cells, macrophages have also been classified into CAMs (or M1) or AAMs (or M2), based on activating cytokine (IFN-γ and IL-4, respectively) and functional characteristics [48]. M1 macrophages, upon activation, produce IFN-γ and IL-12 and play an important role in inflammation. M2 macrophages produce IL-4 and IL-13 upon activation and promote tissue repair, airway remodeling [35], and asthma development [49]. Recently, Shirey et al. [12] have demonstrated that RSV-induced M2 macrophages reduce lung pathology induced by RSV infection in adult mice. We hypothesized that in the context of an already Th2-biased neonatal lung microenvironment [50], the induction of M2 macrophages by RSV might promote collagen deposition and airway remodeling following RSV-induced tissue damage.

Figure 6. Single-infection model: STAT6-IP treatment during neonatal infection decreases total number of M2 macrophages. Neonatal mice were infected with RSV ± IP/CP/SAL and were euthanized d2, 4, or 7 after infection. Single-cell suspensions were generated by collagenase digestion of lungs and stained for flow cytometry. (A) Total percentage of AMs (F4/80+CD11c+). (B) Total percentage of IMs (F4/80+CD11c−). (C) Expression of CD206, an M2 macrophage marker within the AM population. (D) Expression of CCR7, an M1 macrophage marker within the AM population. Data are presented as mean ± SEM from 3–5 mice/group at each time point from 3 independent experiments. Outcomes were measured by Kruskal-Wallis test, followed by post hoc Dunn’s multiple comparison test against IPR for group comparisons. **P < 0.01.

Figure 7. Double-infection model: STAT6-IP treatment during neonatal infection decreases airway collagen. Neonatal mice were infected with RSV ± IP/CP/SAL and reinfection 6 wk later. (A) Collagen deposition was measured by MT stain, 4 d after adult reinfection. Representative sections presented at 10× original magnification. Black arrows indicate collagen deposition surrounding blood vessels. Red arrows indicate collagen deposition surrounding bronchioles. (B) Sections were evaluated in a blinded manner and assigned a histopathological score. Data are presented as means ± SEM from 2 independent experiments, with 3–5 mice/group in each experiment. Outcomes for double-infected animals were assessed by Kruskal-Wallis test, followed by post hoc Dunn’s multiple comparisons against IPR-R for group comparisons. **P < 0.01.
Although the overall frequency of AMs and IMs did not differ in the lungs of neonatal mice, whether or not they were infected with RSV and/or treated with STAT6-IP/CP, we found significantly fewer CD206+ AAMs in the STAT6-IP treated, RSV-infected group at d 4 postinfection compared with all other groups. Consistent with the ability of M2 macrophages to contribute to fibrosis and airway remodeling [35, 51], a decrease in frequency of these cells during neonatal infection correlated with reductions in collagen deposition in the lungs of adult mice. Harker and colleagues [18] previously observed that depletion of AMs during adult RSV reinfection reduced weight loss and T cell responses, thereby diminishing enhanced disease in this model. Our work, along with that of Harker et al. [18] suggest that macrophages play a crucial role in the pathogenesis of enhanced disease upon RSV reinfection.

It is interesting that in the neonate, the level of CD206 expression in AMs was similar in all animals (except those treated with STAT6-IP) on d 4, independent of RSV infection. This observation is similar to the findings of Empey and colleagues [52], who identified an age-dependent increase in CD206 expression, resulting in an increase in the frequency of "resting M2 macrophages" in the neonatal mouse lung. These observations suggest that the presence of M2 macrophages in the lungs is not sufficient for later collagen deposition and fibrosis. Rather, it is likely that airway remodeling requires M2 macrophages in combination with another signal. The nature of this "second signal" (cytokine, chemokine, or M2 macrophage-related activation of lung collagen) is still undetermined but is likely induced by RSV infection of the neonate, as collagen deposition increased in all of these animals, whether they were reinfeected with RSV as adults or not.

Lung levels of the Th2 cytokines (IL-4 and IL-13), as well as the bronchial epithelium-derived innate cytokines, TSLP, IL-25, and IL-33, have all been implicated in airway remodeling [53–57]. Our data suggest trends for increased levels of IL-25 and IL-33 (but not TSLP) in the lung after RSV infection of neonates, in a manner that was not affected by STAT6-IP/CP treatment. However, STAT6-IP treatment did decrease the concentrations of IL-4 and IL-13 in neonates, although only the former change reached statistical significance, suggesting that STAT6-IP acts upstream of these cytokines. It is possible that one or both of these cytokines (IL-4/IL-13) is/are the second signal required for the activity of M2 macrophages in relation to collagen deposition.

TSLP [30] and IL-25 [31] levels have been reported to increase after RSV infection of cultured airway epithelial cells, and IL-33 has recently been shown to be increased in RSV-infected neonatal mice [58]. In our studies, although we saw no increase in TSLP, we observed a trend in the increase of lung homogenate IL-25 and IL-33 levels after neonatal RSV infection. To our knowledge, the studies that have examined TSLP levels after RSV infection were performed on in vitro airway epithelial cells [30, 59, 60]. Therefore, the extent to which TSLP is induced after infection of neonatal mice still needs to be clarified. IL-25 and IL-33 can both induce and maintain M2 macrophages [61]. The precise mechanism(s) regulating production of IL-25 and IL-33 from infected airway epithelial cells/during RSV infection are unknown. Our data suggest that is not likely a result of STAT6 signaling in epithelial cells, as equivalent levels of these cytokines were present in STAT6-IP and STAT6-CP/SAL control mice after RSV infection. IL-33, but not IL-25, induces M2 macrophage differentiation via STAT6 [61]. Therefore, it seems most likely that STAT6-IP inhibited IL-33-dependent M2 macrophage polarization in the lungs of the RSV-infected neonates. The identity of the IL-4 and IL-13-producing cells in our model is currently unknown but is a central target of our continued efforts. A number of innate cells, including mast cells [62, 63], eosinophils [64], and type 2 cytokine-producing innate lymphoid cells [65], are capable of producing these Th2-deviating cytokines. Whether STAT6-IP prevents the induction of IL-4 and IL-13 from specific cells and/or influences the activity of these cytokines on target cells, such as macrophages or DCs, is unknown.

Although our data strongly implicate M2 macrophages in the enhanced responses to repeated RSV infection, others have focused their efforts on DCs in similar models. TSLP [66], IL-25 [67], and IL-33 [68] can also induce Th2-type responses in DCs with the subsequent development of airway pathology. Moreover, AMs have been shown to activate DC-dependent allergic airway inflammation [69]. Thus, the role of DCs in RSV-induced asthma-like pathology in this model should not be discounted. Recently, Han et al. [66] have shown that neonatal RSV infection induces expression of OX40L on the surface of DCs in the lung and that neutralizing OX40L at the time of neonatal RSV infection inhibits exacerbated responses upon reinfection. In our model, we did not find any changes in OX40L on the surface of neonatal DCs after RSV infection (data not shown). One possible explanation for the differences between these models may be the age of neonatal infection. In the Han et al. study [66], neonates were infected at 7 d after birth, whereas we infected the pups 4–5 d after birth. As the numbers and relative proportions of macrophages and DCs change very rapidly in the lungs of neonatal mice, it is possible that we missed a critical window of DC maturation [70].

With the growing appreciation of the impact of RSV infection on asthma development, it is important to understand the mechanism that underlies this relationship. The only prophylactic treatment for RSV is a mAb (palivizumab) that is currently restricted to infants at highest risk of RSV hospitalization as a result of cost [71]. Furthermore, this expensive prophylaxis reduces hospitalization by only 55% [72]. There is a critical need to develop new approaches to prevent and treat RSV infection across the age range. This imperative may be particularly true for RSV infections in the youngest children who suffer the greatest mortality and who are also the most likely to develop long-lasting, RSV-related airway disease. Our data suggest that inhibitors of the IL-4/IL-STAT6 signaling pathways hold considerable promise to decrease the risk of RSV-induced asthma. The exploitation of preclinical models to define the molecular mechanisms by which STAT6-IP-dependent modulation of innate immunity in early life leads to long-term protection from aberrant Th2-biased airway responses will likely guide further development of new therapies, such as STAT6-IP, to reduce asthma induced or exacerbated by viral infection in humans.
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KEY WORDS: asthma – eosiinophils – alternatively activated macrophages – hyper-responsiveness – collagen deposition