The delivery of cytokines by recombinant virus in early life alters the
immune response to adult lung infection

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Running title: Early life immunomodulation by recombinant virus
Respiratory syncytial virus (RSV) is the main cause of bronchiolitis, the major cause of hospitalization in infancy. An ideal RSV vaccine would be effective in neonates, but the immune responses of infants differ markedly from those in adults, often showing a bias towards T helper 2 (Th2) responses and reduced interferon gamma (IFN-γ) production. We have previously developed recombinant RSV vectors expressing the IFN-γ and interleukin-4 (IL-4) that allow us to explore the role of these key Th1 and Th2 cytokines during infection. The aim of the current study was to explore whether immunomodulation of infant responses could enhance protection. Expression of IFN-γ by a recombinant RSV (rRSV/IFN-γ) vector attenuated primary viral replication in newborn mice without affecting the development of specific antibody or T cell responses. On challenge rRSV/IFN-γ mice were protected from the exacerbated disease observed in mice primed with wild type RSV; however, anti-viral immunity was not enhanced. Conversely, expression of IL-4 by recombinant RSV did not affect virus replication in neonates but greatly enhanced Th2 immune responses on challenge without affecting weight loss. These studies demonstrate that it is possible to manipulate the infant immune responses using cytokine-expressing recombinant viruses and that neonatal deficiency in IFN-γ responses may lead to enhanced disease during secondary infection.
Introduction

Respiratory syncytial virus (RSV) is the most important cause of viral lung infection in infants (31). The hospitalization rate in the USA is 17 per 1,000 children with outpatient rates much higher (15) and re-infection in infancy is common (14). The mortality caused by RSV in developing countries and the high rates of hospitalization in industrialized countries means that the development of an RSV vaccine is an urgent health priority. A major problem in the development of an RSV vaccine is the requirement for efficacy in very early life; this is problematic because of the immaturity of the immune system in infancy (38). This immune immaturity decreases vaccine efficacy (29) and increases susceptibility to all respiratory viruses (34).

In general, protection against viral infection is mediated by a balance of neutralizing antibody responses and long lasting virus specific CD8 T cell memory. Both of these compartments are severely impaired in infants. There are several aspects that contribute to the reduced immune response in early life. Total lymphocyte and dendritic cell numbers are significantly lower in neonates than adults (3). Microarchitectural structures thought to be crucial for antibody development (i.e. lymphoid follicles, follicular dendritic cell networks and germinal centers) are absent at birth and only form between a few days and a few weeks of age (13). The full repertoire of antigen presenting cells is only achieved several weeks after birth (32). Finally, the responses primed by infantile exposure to pathogens, or other inflammatory stimuli, are significantly altered compared to
adult responses, with memory responses dominated by the T helper 2 (Th2) arm of the immune system (2,3).

Increased levels of IL-4, the archetypal Th2 cytokine, are associated with increased RSV disease severity in infancy (1,18,25). In addition, severe RSV infection is linked with the development of wheeze and asthma in later life (30), diseases that are classically linked to aberrant Th2 responses. Infection of neonatal mice with RSV results in the development of Th2 skewed immunity and increased disease severity when mice are re-infected with RSV as adults (8). In comparison RSV infection of adult mice results in a Th1 and CD8 T cell response and the subsequent development of protective immunity (28,35). It is therefore hypothesized that increased Th2 responses are detrimental to the outcome of neonatal RSV infection and reduction of Th2 responses and/or promotion of Th1 responses would be beneficial.

We have previously demonstrated that recombinant RSV expressing IFN-γ (RSV/IFN-γ) can be used to promote Th1 immunity in adult mice, attenuating viral replication without affecting immunogenicity (5). In contrast co-expression of IL-4 by RSV (RSV/IL-4) causes defective T cell responses but does not affect viral clearance (6). On challenge RSV/IFN-γ primed adult mice show enhanced CD8 T cell recruitment, leading to immunopathology, whilst RSV/IL-4 primed mice show enhanced eosinophilia (16). The aim of the current study was to investigate the effects of cytokine modulation on the neonatal immune response to RSV. We found that RSV/IFN-γ was protective against the disease enhancing effects of neonatal RSV infection, whilst RSV/IL-4 significantly increased the Th2 skewing of the immune response without worsening disease.
Materials and Methods

Virus stocks & Mouse Infection.

RSV (strain A2) was obtained from the ATCC and recombinant RSV expressing mouse IFN-γ (RSV/IFN-γ) or IL-4 (RSV/IL-4) or wildtype recombinant virus (RSV/wt) were made as described in (5,6). Time mated pregnant BALB/c mice (Harlan, Iscoed, UK) were purchased at <14d gestation and pups were weaned at 3wk old. Mice were infected intranasally (i.n.) with 4x10^4 FFU/g body weight virus at 4 days (neonatal ~ 10^5) under isoflurane anesthesia. Secondary RSV challenge was given i.n. at 8 weeks post priming, with 10^6 FFU in 100µl. Weight was measured daily to monitor disease severity. All work was approved and licensed by the UK Home Office. Experiments were performed n≥2 times with n≥4 mice per experiment.

Quantification of Viral RNA.

RNA was extracted from the lung using RNA stat-60 (AMS Biotech Ltd.) and cDNA was generated with random hexamers using Omniscript RT (Qiagen). Real time PCR was carried out with for a sequence in the RSV L gene using 900 nM forward primer (5'-GAACTCAGTGAGGAGATGTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTTTCTCTGCAAT-3') and 100 nM probe (5'-FAM-TTTGAACCTGTCTGAACAT-TAMRA-3') on an ABI Prism 7000 Sequence Detection System. This detects viral genomic RNA, viral anti-genomic RNA and intracellular RSV L mRNA, referred to here as RSV L RNA.
Cell Recovery.

Collection of bronchoalveolar lavage (BAL) for cells and supernatants, the harvesting of lung tissues was carried out as previously described (7). For the preparation of lung mash supernatants, lungs were homogenized through 100 μm cell strainers (BD Pharmingen) and washed through with a 1ml volume of RPMI five times, following centrifugation this supernatant was retained for ELISA analysis. After the removal of the supernatants from all tissues, cells were treated with red blood cell lysis buffer for 5 minutes, then DNAse I (40μg/ml)/Collagenase (50μg/ml) in RPMI 1640 (10% FCS) for 5 minutes at room temperature to remove clumps, finally they were resuspended in RPMI. Cell viability was assessed by trypan blue exclusion, and total cell numbers were counted by disposable multiwell haemocytometer (Immunesystems, UK). Airway cells were differentiated by H&E staining of BAL cell samples.

Flow Cytometry.

Prior to staining cells were blocked with CD16/32 (Fc Block, BD). For surface staining antibodies against the surface markers CD4, CD8, CD3, CD69, CD11b,CD11c, CD80, CD86, MHCII, CD62L, CD44 and DX5 (BD) were added in 1:100 dilution for 30 minutes on ice. RSV specific CD8 cells were characterized using the RSV M2 MHC class I pentamer (SYIGSINNI; Proimmune). Gating for lymphocytes was determined by back gating on CD3/CD8 double positive cells. For the detection of intracellular cytokines, cells were incubated with 50ng/ml PMA, 500ng/ml ionomycin and 10μg/ml Brefeldin A for 4 hours at 37°C. Samples
were permeabilised with 0.5% saponin in PBS for 10 minutes. Anti-cytokine antibodies (anti-IFN-γ, anti-IL-4:BD) or isotype controls were added for a further 20 minutes at room temperature. Cells were analyzed on a CyAn ADP (Dako) flow cytometer collecting data on at least 50,000 events.

Cytokine ELISA.
Cytokine levels were assessed from BAL and lung mash supernatants. ELISA plates (Nunc) were coated with capture antibody (anti IL-4, IL-5 or IFN-γ: BD) overnight at 4°C. Wells were washed and blocked with 1% BSA for 1h at room temperature. 100ul of sample or standard was added to blocked wells for 2h. Bound cytokine was detected by using biotinylated anti-cytokine antibody, avidin horseradish-peroxidase and tetramethylbenzidine. Color development was terminated with 2N H₂SO₄, and OD read at 490nm. The concentration of cytokine was determined from the standard curve.

RSV-specific antibody ELISA.
Serum antibody was assessed by ELISA. Antigen was prepared by infecting HEp-2 cells with RSV at 1 FFU/cell. Microtiter plates were coated overnight with 100μl of a 1:500 dilution of either RSV or HEp-2 antigen. After blocking with 1% BSA for 1h, dilutions of test samples were added for a further 1h. Bound antibody was detected using peroxidase-conjugated rabbit anti-mouse Ig (Dako) and o-phenylenediamine as a substrate. Color development was blocked with 2M H₂SO₄, and OD read at 490nm. RSV-specific antibody was
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determined by subtracting the RSV absorbance from the HEp-2 absorbance for the same sample. Specific isotypes were measured following the same protocol, changing the primary antibody. Total, non specific, IgE was measured according to manufacturer’s instructions (BD).

Statistical analysis.

Results are expressed as mean ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey tests when there were greater than 3 groups and t tests for the comparison of 2 groups using GraphPad Prism software.
Results

Primary neonatal infection with recombinant RSV expressing either IFN-γ or IL-4. We have previously described the construction and characterization of recombinant RSVs expressing either IFN-γ or IL-4 (16). These viruses grow with similar kinetics to wild type viruses in both human and mouse cell lines (data not shown). In adult BALB/c mice RSV/IL-4 grows at wildtype levels whilst RSV/IFN-γ is attenuated approximately 10-fold (5,6,16). To assess the effect of these viruses on neonates, mice less than 1 week of age were infected with $8 \times 10^4$ FFU RSV/wt, RSV/IFN-γ or RSV/IL-4 intranasally (i.n.). Weight was monitored daily as a measure of disease severity (Figure 1a). Neonates gained weight rapidly from day 0 of infection, doubling their weight by day 8 post infection; there was no significant change in weight gain following infection.

RNA was extracted from the lungs on days 2, 4, 7 and 14 post infection and levels of RSV L RNA assessed by real time PCR (Figure 1b). Following infection with RSV/wt or RSV/IL-4 there was a peak of viral RNA detected on days 2 and 4, which declined on d7 and was cleared by d14. There was no significant difference between RSV/wt and RSV/IL-4 infection. On day 2, the level of total RSV L RNA from RSV/IFN-γ infected mice were approximately half those seen in the other groups and on day 4 post infection they were 3-fold lower than RSV/wt ($p<0.05$). There was no RSV L RNA detectable in the lungs of RSV/IFN-γ infected mice from day 7 onwards, indicating that viral clearance was quicker than after RSV/wt or RSV/IL-4 infection.
As has previously been seen in adult mice, infection with RSV/IFN-γ caused a significant increase in lung IFN-γ concentration on day 2 post infection compared to infection with RSV/wt, RSV/IL-4 or naïve mice (Figure 1c, p<0.01). Likewise RSV/IL-4 caused a significant peak of IL-4 on day 2 post infection compared to the other groups (Figure 1d, p<0.05). Therefore, the recombinant RSV are infectious in neonates inducing local cytokine production which alter the viral clearance kinetics.

Effect of the co-expression of IL-4 and IFN-γ on cellular recruitment during primary neonatal RSV infection. We were interested in the effect of recombinant viral vectors on neonatal immune responses. Lung cells were collected on days 4, 7 and 14 post infection from neonatal mice infected with RSV/wt, RSV/IFN-γ, RSV/IL-4 or left uninfected. The total number of cells in the lung increased with age and RSV infection did not significantly alter this at any timepoint (Figure 2a). Infection did not affect lymphocyte percentages in the lungs, which went from approximately 30% on day 4 post infection to approximately 60% at day 14 post infection in all cases (Figure 2b).

The effect of cytokine on antigen presenting cells in the neonatal lung was also assessed. There were no differences in the numbers of CD11c+CD11b+ dendritic cells recruited to the lung following infection (data not depicted). However there were significantly more CD11c+CD11b- macrophages in the lungs on day 4 post infection in the RSV/IFN-γ infected mice than RSV/wt or RSV/IL-4 infection (Figure 2c, p<0.05). The activation status of these cells was also significantly increased by RSV/IFN-γ infection, measured by MHCII (Figure...
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2d), CD80 (Figure 2e) and CD86 (Figure 2f). NK cell numbers (CD3\textsuperscript{+}DX5\textsuperscript{+}) increased with age, and RSV infection had little effect on this (Figure 2g). The cell surface protein CD69, is a marker of activation on both NK and T cells (39). On day 4 post infection only mice infected with RSV/IFN-\gamma had an increased percentage of CD69\textsuperscript{+} NK cells in their lungs (p<0.05, Figure 2h). On subsequent days there was no difference between RSV infected animals and naïve animals irrelevant of the virus used.

The number of T cells in the lungs also increased from day 4 to 14 post infection (day 18 of life). On day 4 post infection, naïve mice had approximately 1.2 x 10\textsuperscript{4} CD4 T cells (Figure 3a) and approximately 4.8 x 10\textsuperscript{3} CD8 T cells (Figure 3b). By day 14 after infection, day 18 of age, the number of CD4 T cells in the naïve lung had risen to approximately 5.3 x 10\textsuperscript{4} cells per lung, whilst the number of CD8 T cells was 1 x 10\textsuperscript{4} cells per lung. As with total cell and lymphocyte recruitment infection with RSV/wt or either of the recombinant viruses seemed to have little effect on the pattern of T cell recruitment.

In order to determine whether infection affected T cell activity rather than affecting total recruitment, we analyzed the expression of a number of cell surface markers. CD62L and CD44 can be used to differentiate effector T cells (CD44\textsuperscript{hi}CD62L\textsuperscript{lo}) and memory T cells (CD44\textsuperscript{hi}CD62L\textsuperscript{hi}) (37). Very few memory cells were seen in the lungs at any time point but there were observable changes in the percentage of effector T cells with infection. The co-expression by RSV of either IFN-\gamma or IL-4 caused an increase in the proportion of CD4 effector cells on day 4 p.i. compared to RSV/wt infected and naïve mice (Figure 3c). By day 7 p.i. this difference was not observable but on day 14 p.i. all infected mice showed a
significant increase in the percentage of effector CD4 T cells compared to naïve mice and at this timepoint there was no difference between the different viral infections (p<0.05). Expression of CD69 by CD4 T cells followed a similar pattern to CD44. On day 4 post infection RSV/IL-4 and RSV/IFN-γ infected mice had higher percentage of CD69+ CD4 T cells than RSV/wt (Figure 3d). On day 14 there were slightly elevated percentages in infected mice compared to naïve mice.

There was no significant difference in CD44 expression by CD8 cells on d4 post infection. On d7 and d14 post infection, RSV/IFN-γ had significantly fewer CD8 effector cells than RSV/wt or RSV-IL-4 (Figure 3e, p<0.05). Infection with RSV/IFN-γ or RSV/IL-4 caused significant upregulation of CD69 on CD8 T cells compared to infection with RSV/wt on day 4 post infection (p<0.05). On subsequent days RSV/wt infected neonates had increased CD69+ CD8 T cells, on day 14 post infection there were significantly more active CD8 T cells than any other group (p<0.05). At this timepoint the percentages of activated CD8 T cells in mice infected with RSV/IFN-γ or RSV/IL-4 were similar to those found in naïve mice (Figure 3f).

On day 14 post infection, after viral clearance from the lungs, CD4 T cells were stimulated with PMA and ionomycin and stained for intracellular IL-4 and IFN-γ. The number of IFN-γ+ CD4 T cells was low in all groups, however both RSV/wt and RSV/IFN-γ had significantly more than naïve mice (p<0.05, Figure 4a). RSV/IL-4 infection did not result in an increased number IFN-γ+ CD4 T cells. RSV/wt and RSV/IL-4 infection resulted in significantly increased numbers of IL-4+ CD4 T cells in the lungs compared to RSV/IFN-γ infection (Figure 4b, p<0.05).
An MHC class I tetramer containing the immunodominant RSV specific CD8 T cell epitope (SYIGSINNI) located in the M2-1 protein (23) was also used on d14 to determine the number of RSV-specific CD8 T cells. RSV/wt and RSV/IFN-γ infection resulted in the recruitment of more RSV specific CD8 T cells than RSV/IL-4 (Figure 4c). By contrast to the cellular responses, similar serum levels of RSV specific antibody were detected in mice infected with all three viruses at day 14 post infection (Figure 4d). From this we observe that cytokine co-expression subtly alters the profile of cells recruited during neonatal infection.

IFN-γ co-expression during primary infection prevents the development of enhanced disease on challenge as adults. As both recombinant viruses altered the immune response to neonatal RSV infection we hypothesized that secondary immune responses and disease outcome would also be affected. Neonatal mice were primed and then re-challenged 8 weeks after priming with 10^6 FFU of wildtype RSV. As we have previously reported (8) neonatal infection with RSV/wt resulted in weight loss on secondary infection, peaking on day 4 with recovery around day 7 (Figure 5a). Mice primed with RSV/IL-4 also lost weight on challenge, with a similar profile to RSV/wt primed mice but RSV/IFN-γ primed lost significantly less weight than the RSV/IL-4 primed mice on days 2-5 post infection (p<0.05).

In spite of the change in weight loss, viral loads were similar in all three groups on day 4 post infection and no viral RNA was detectable on days 7 or 14 post challenge (Figure 5b). RSV/IFN-γ primed animals had significantly reduced anti-RSV antibody titer (Figure 5c, p<0.05). Interestingly neonatal priming with
RSV/IFN-γ resulted in reduced concentrations of both IFN-γ (Figure 5d) and IL-4 (Figure 5e) in the BAL on day 4 post infection compared to and RSV/IL-4 (p<0.05).

Cellular responses during secondary infection. The numbers of CD4, CD8 and NK cells in the lungs were assessed on days 4 and 7. There were significantly more CD4 T cells in the lungs of RSV/wt primed mice on day 7 post challenge compared to mice primed with RSV/IFN-γ or RSV/IL-4 (Figure 6a, p<0.01). The CD4 cells were significantly more activated (CD69+, Figure 6b, p<0.05) and produced more IFN-γ (Figure 6c, p<0.01). There were no differences in the number, activation status or IFN-γ production of the CD4 T cells from RSV/IL-4 or RSV/IFN-γ primed mice. However, there were significantly more IL-4+ CD4 T cells in the lungs of mice primed with RSV/IL-4 on day 4 post challenge than in either RSV/IFN-γ or RSV/wt primed mice (Figure 6d, p<0.01).

CD8 T cell recruitment into the lungs peaked on day 7 post challenge in all groups with recruitment being greatest after priming with RSV/wt compared to RSV/IFN-γ or RSV/IL-4 (Figure 6e, p<0.01). There was no significant difference in the activation of these cells as measured using CD69 (Figure 6f), but there were significantly more RSV specific (Figure 6g, p<0.01) and IFN-γ producing (Figure 6h, p<0.01) CD8 T cells following RSV/wt priming on day 7 post challenge. On day 4 post challenge RSV/IL-4 and RSV/wt primed mice had significantly more lung NK cells than RSV/IFN-γ primed mice (Figure 6i, p<0.01).

On day 7 post infection NK cells numbers had greatly reduced and were similar in all three primed groups.
IL-4 priming results in significant enhancement of Th2 responses on re-challenge. The disease seen on re-challenge of mice primed with RSV in the neonatal period has been described as the result of a Th2 skewed immune response (8). Eosinophil (Figure 7a) and neutrophil (Figure 7b) recruitment was mainly observed in mice primed neonatally with RSV/wt or RSV/IL-4, but not RSV/IFN-γ. Co-expression of IL-4 during neonatal infection caused an approximately 4 fold increase in airway eosinophilia on days 4 and 2.5 fold increase on day 7 post challenge compared to RSV/wt infection (p<0.01). IL-5 is a key cytokine in the recruitment and survival of eosinophils and on day 4 post challenge and levels of IL-5 were significantly lower in RSV/IFN-γ treated animals compared to the other two groups (Figure 7c, p<0.05).

As an additional readout of the Th1/Th2 balance of re-challenged mice, RSV specific antibody subtypes were determined on day 7 post challenge. IgG2a responses were slightly, but not significantly, higher in RSV/wt primed mice compared to RSV/IL-4 primed animals (Figure 7d). RSV- specific IgG1 (Th2 associated subtype) responses were significantly higher in mice primed with RSV/IL-4 than those seen with either RSV/wt (p<0.05) or RSV/IFN-γ infections (Figure 7e, p<0.001). Serum was also tested for the presence of total IgE on day 7 post challenge. Neonatal priming with RSV/IL-4 resulted in significantly elevated levels of total IgE compared to priming with RSV/wt or RSV/IFN-γ (Figure 7f, p<0.05). Interestingly all three neonatally primed groups had significantly elevated levels of serum IgE compared to naïve mice or mice primed as adults, in which the development of serum IgE is not normally observed (data
not depicted). Therefore the co-expression of IL-4 by RSV during primary neonatal enhances the Th2 nature of the secondary response.

**Discussion**

The aim of the current study was to investigate the effects of cytokine modulation on the neonatal immune response to RSV, in the context of improving neonatal vaccine efficacy. As seen previously (5,16), reduced viral titers were observed following infection with the RSV/IFN-γ virus compared to RSV/wt and RSV/IL-4. This could be due to the insertion of the IFN-γ cDNA within the RSV genome, affecting replication. However, it has been shown that RSV can accept inserted genes with little effect on genome transcription or replication (5). A similar phenotype of reduced viral load is observed with a recombinant RSV that expresses IL-18; in this case, replication occurs normally in vitro but is reduced in vivo (17). It is possible that both RSV recombinants have impaired growth in vivo but that IL-4 expression leads to impaired antiviral defense and enhanced viral replication that matches the impaired growth caused by gene insertion (6).

However, the simplest explanation is that IFN-γ expression decreased viral load because it augments anti-viral immunity.

It has been reported that the enhanced disease seen after re-challenge of RSV-primed neonates is characterized as Th2 skewed with increased IL-4 secreting CD4 T cells, eosinophilia and IL-13 production (8,10). Many of the features of the immune response that were increased by RSV/IL-4 priming, for example IgE and eosinophilia have been postulated to be important in severe
RSV disease. In infants, IgE levels correlate with the severity of disease (36) and IgE is required for enhanced airway hyperresponsiveness in neonatally primed mice (9). In a similar fashion eosinophilia has been associated with enhanced lung disease following both RSV infection (20) and during asthma and allergy (19).

Whilst over-expression of IL-4 during neonatal priming resulted in increased IgE and eosinophilia on challenge it did not result in increased disease. The role of eosinophils in allergic airway disease is currently being re-evaluated with the demonstration that they have a beneficial role in innate anti-viral immunity and are dispensable in the development of asthma (12,22,27). Furthermore, depletion of CD4 cells during neonatal murine RSV infection only marginally reduces weight loss, despite eliminating eosinophilia (35). We have also observed that CD8 T cells are a critical determinant of disease, but there may be a balance between protective and pathogenic CD8 T cells (33). These data indicate that whilst Th2 responses are often seen during enhanced RSV disease, their presence does not directly correlate with severity.

Despite a lack of disease enhancement with increased IL-4 expression, priming neonates with RSV/IFN-γ abrogated weight loss after re-challenge. Recently Lee, et al. demonstrated that the administration of recombinant IFN-γ during neonatal infection prevented the development of airway hyperresponsiveness or eosinophilia on re-challenge (24). The proposed mechanism for this was an increase in Th1 memory responses and a reduction in Th2 memory responses. RSV/IFN-γ, however, did not increase recruitment of IFN-γ secreting CD4 cells or the production of IFN-γ during re-challenge;
suggesting that it did not enhance Th1 cell development. Therefore the data presented here suggest there may be an alternative mechanism of protection. Viral infection in adults is known to cause long term changes in lung resident APCs including macrophages (11). Interestingly infection of neonates with RSV/wt did not result in significant maturation and activation of these cells in vivo. However, priming with IFN-γ caused rapid and significant maturation of macrophages in the neonatal lung. Therefore, one possible mechanism of protection for RSV/IFN-γ could be through maturation of the innate components of lung immunity. On re-challenge, RSV/IFN-γ priming resulted in significantly reduced neutrophil and NK cell recruitment at the peak of weight loss. This suggests that, along with T cells, cells of the innate immune system contribute to weight loss. In infantile bronchiolitis, neutrophils have been observed to be the major cell type in the airways of RSV infected infants (26). The activation of NK cells by over expression of IL-18 enhances disease and weight loss during primary RSV infection (17) and NK cells are the major source of early IFN-γ production during RSV infection (21). Both NK cells and neutrophils are activated and recruited to the lungs by a similar range of mediators, produced primarily by resident lung macrophages in RSV infection, supporting the idea that macrophages may be involved in neonatally primed disease (4,28).

We conclude that exogenous cytokine expression can modulate the neonatal immune response and alter the immunological memory generated during early childhood. Importantly the ability of IFN-γ to protect in this neonatal model contrasts to the results in adult mice, where RSV/IFN-γ enhances disease.
during re-challenge (16). It is therefore necessary to consider the age of first priming in the development of vaccines, since the age at which the vaccine is administered is critical in determining the pattern of immune response that results.

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Figure legends

Figure 1: Recombinant virus replication and cytokine production in primary neonatal infection. 4 day old BALB/c mice were infected with $8 \times 10^4$ FFU of RSV/wt (■, black bars), RSV/IFN-γ (▲, hatched bars), RSV/IL-4 (○, lined bars) i.n. or left naive (◊, open bars). Weight was monitored daily and weight change compared to weight prior to infection was plotted (A). TaqMan PCR for RSV L RNA was carried out on RNA from lungs were collected on days 2, 4, 7 and 14 post infection (B). Lung supernatants were taken on days 2 and 4 post infection and the concentrations for IFN-γ (C) and IL-4 (D) determined by ELISA. Data representative of 3 experiments, points represent n = 5 ± SEM mice per group, *p<0.05, **p<0.01.

Figure 2: The recruitment and activation of innate immune cells is altered by viral cytokine expression.
4 day old BALB/c mice were infected with $8 \times 10^4$ FFU of RSV/wt (■), RSV/IFN-γ (▲), RSV/IL-4 (○) i.n. or left naive (◊). Cells were taken from lungs and total lung cell number after homogenization (A) and percentage lymphocytes (B) determined on days 4, 7 and 14 post infection. Lung macrophages (CD11c+CD11b- cells) were characterized by flow cytometry, measuring number (C), MHCII (D), CD80 (E) and CD86 (F) expression. Total NK cells (CD3-DX5+) (G) and activation of NK cells/CD69+ (H) were also measured. Data representative of 2 experiments, points represent n = 5 ± SEM mice per group.
Figure 3: Neonatal T cell responses are altered by viral cytokine expression. 4 day old BALB/c mice were infected with $8 \times 10^4$ FFU of RSV/wt (■), RSV/IFN-γ (▲), RSV/IL-4 (○) i.n. or left naive (◊). Cells were taken from lungs and total CD4 (A), CD8 (B) T cells were measured on days 4, 7 and 14 post infection by flow cytometry. CD62L-CD44+ memory (C, E) and CD69+ activated (D, F) CD4 and CD8 cells respectively were also measured at the same timepoints. Data representative of 2 experiments, points represent n = 5 ± SEM mice per group.

Figure 4: T cell cytokine production and RSV specific antibody responses following recombinant RSV infection. 4 day old BALB/c mice were infected with $8 \times 10^4$ FFU of RSV/wt (■, black bars), RSV/IFN-γ (▲, hatched bars), RSV/IL-4 (○, lined bars) i.n. or left naive (◊, open bars). On day 14 post infection PMA/ionomycin stimulated CD4 T cells were stained for the intracellular presence of IFN-γ (A) and IL-4 (B). At the same timepoint CD8 T cells were stained with a RSV-M2 specific pentamer (C). Serum was taken at 14 days post infection and the level of RSV specific Ig determined by ELISA (D). Data representative of 2 experiments, points represent n = 5 ± SEM mice per group, *p<0.05, **p<0.01.

Figure 5: IFN-γ expression in the neonatal period protects against exacerbated weight loss. 4 day old BALB/c mice were infected i.n. with $8 \times 10^4$ FFU of RSV/wt (■, black bars), RSV/IFN-γ (▲, hatched bars), RSV/IL-4 (○, lined bars). They were challenged with $10^6$ FFU of wildtype RSV A2 i.n. at 8 weeks of
age. After challenge, weight was monitored daily and plotted against their starting weight (A). On day 4 post challenge RNA was extracted from the lungs and the amount of RSV L RNA determined by real time PCR (B). Total RSV-specific Ig was measured in serum at d7 (C). BAL supernatants were taken on days 4 and 7 to determine the levels of IFN-γ (D) and IL-4 (E) by ELISA. Data representative of 2 repeats, points represent n = 5 ± SEM mice per group, **p<0.01, *** p<0.001.

**Figure 6: T cell responses in the lungs on challenge.** 4 day old BALB/c mice were infected i.n. with $8 \times 10^4$ FFU of RSV/wt (solid bars), RSV/IFN-γ (hatched bars) or RSV/IL-4 (lined bars). They were challenged with $10^6$ FFU of wildtype RSV A2 i.n. at 8 weeks of age. Lung CD4 T cell numbers (A) and activation (B) and the intracellular expression of IFN-γ (C) and IL-4 (D) were measured on days 4 and 7 post infection. Lung CD8 T cell number (E), activation (F), M2 specificity (G) and intracellular IFN-γ expression following stimulation (H) and NK cell numbers (I) were measured at the same timepoints. Data representative of 2 repeats, points represent n = 5 ± SEM mice per group, * p<0.05, **p<0.01.

**Figure 7: Th1/Th2 balance of the recall response.** 4 day old BALB/c mice were infected i.n. with $8 \times 10^4$ FFU of RSV/wt (◼, black bars), RSV/IFN-γ (▲, hatched bars), RSV/IL-4 (○, lined bars). They were challenged with $10^6$ FFU of wildtype RSV A2 i.n. at 8 weeks of age. BAL was carried out on days 4 and 7 post infection and the number of airway eosinophils (A) and neutrophils (B) recruited to the lung determined by H&E staining. IL-5 concentration in BAL
supernatants was determined by ELISA (C). Serum was taken on day 7 post challenge and the levels of RSV specific IgG2a (D), IgG1 (E) and total serum IgE (F) were quantified by ELISA. Data representative of 2 repeats, points represent n = 5 ± SEM mice per group, * p<0.05, **p<0.01, ***p<0.001.
A

% of original weight

days post infection

B

RSV L RNA copy number in lung

days post infection

C

Lung IFN-γ (ng/ml)

day 2 day 4

D

Lung IL-4 (ng/ml)

day 2 day 4

JVI, Harker, Figure 1
RSV/wt
RSV/IFN-γ
RSV/IL-4
naive

days post infection

viable lung cell count x 10^6

days post infection

% of total events that fall in the lymphocyte gate

days post infection

total CD11c+CD11b- cells x 10^5

days post infection

% MHCII+ macrophages

% CD80+ macrophages

days post infection

% CD86+ macrophages

days post infection

NK cells x 10^5

days post infection

% of NK cells expressing CD69

JVI, Harker, Figure 2
RSV/wt  
RSV/IFN-γ  
RSV/IL-4  
naive  
days post infection  
CD4 T cells x 10^5  

RSV/IFN-γ  
RSV/IL-4  
naive  
days post infection  
CD8 T cells x 10^5  

% of CD4 T cells that are CD44hiCD62Llo  
days post infection  

% of CD8 T cells that are CD44hiCD62Llo  
days post infection  

% of CD4 T cells expressing CD69  
days post infection  

% of CD8 T cells expressing CD69  
days post infection  

JVI, Harker, Figure 3
JVI, Harker, Figure 4
JVI, Harker, Figure 5