The role of T cells in the enhancement of RSV infection severity
during adult re-infection of neonatally sensitized mice

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Abstract

Respiratory syncytial virus (RSV) is the major cause of infantile bronchiolitis, and hospitalization. Severe RSV disease is associated with the development of wheezing in later life. In a mouse model of the delayed effects of RSV, the age at primary infection determines responses to re-infection in adulthood. During primary RSV infection, neonatal BALB/c mice developed only mild disease and recruited CD8 cells that were defective in interferon gamma production. Secondary re-infection of neonatally primed mice caused enhanced inflammation and profuse lung T cell recruitment. CD4 cell depletion during secondary RSV challenge attenuated disease (measured by weight loss); depletion of CD8 cells also markedly attenuated disease severity but enhanced lung eosinophilia and depleting both CD4 and CD8 cells together completely abrogated weight loss. Depletion of CD8 (but not CD4) cells during primary neonatal infection was protective against weight loss during adult challenge. Therefore, T cells, in particular CD8 cells, play a central role in the outcome of neonatal infection by enhancing disease during secondary challenge. These findings demonstrate a crucial role for T cells in the regulation of immune responses after neonatal infection.
Introduction

Newborn children are highly susceptible to infectious diseases such as measles, mumps, pertussis and diphtheria. In non-vaccinated infants, case fatality rates for these infections are typically 10 to 100 times greater than those in children aged 10 or above. Similarly, cytomegalovirus, *chlamydia spp.*, group B streptococci and *H. influenzae* are important causes of neonatal pneumonia, but do not normally cause pneumonia in adults. Despite the great need for vaccines effective in neonates, responses to vaccination are weak and usually ineffective in this age group (1,4).

The characteristics of neonatal immune responses not only influence disease susceptibility and vaccine efficacy, but also the outcome of later infections. Childhood infection can lead to life-long protection against some viruses (e.g. measles), while giving poor protection against re-infection with other agents (e.g. many bacterial infections, helminths and respiratory syncytial virus (RSV)). Childhood exposure to infection may also contribute non-specifically to immune maturation. According to the hygiene hypothesis, exposure to environmental microbiota and episodes of infection help normal immune maturation and protect against later development of allergy (18) and autoimmunity (26). However, infantile wheezy colds (16), viral bronchiolitis (24) and bacterial colonization (3) are associated with wheezing and asthma diagnosis in later childhood. This association is particularly well established for RSV (23), a pneumovirus that is the principal cause of childhood hospitalization in the developed world (22).

Although RSV infections tend to be severe in infancy, repeated re-infections occur throughout life. The fact that severe RSV disease is associated with over exuberant immune responses (20) has held back vaccine development. We have previously shown that the age at which primary infection occurs critically influences
the outcome of secondary challenge in mice. RSV infection in the first week of life
causes enhanced disease during secondary challenge, characterized by increased
weight loss and enhanced lung inflammation (8).

In the present studies, we found that primary RSV infection of neonatal mice
causd only mild disease and led to the recruitment of RSV specific T cells, very few
of which made IFNγ. Re-infection of neonatally primed mice during adulthood lead to
enhanced disease characterized by lung inflammation and weight loss, and CD8 or
CD4 cell depletion during secondary challenge greatly reduced disease severity. If
CD8 cells (but not CD4) cells were depleted during primary infection, no weight loss
was seen during later re-infection. Therefore, CD8 cells play a key role in both
programming for enhanced disease in the neonatal period, and in the pathogenesis of
the enhanced disease seen in adulthood.
Materials and Methods

Mice and virus stocks. Time mated pregnant BALB/c mice (Harlan, Berkhamsted, UK) were purchased at <14d gestation and pups were weaned at 3wk old. BALB/c mice were infected intranasally (i.n.) with 4x10^4 PFU/g RSV A2 at 4 days (neonatal ~ 10^5) or 4-6 weeks of age (immature adults ~ 5 x 10^5) under isoflurane anesthesia. Secondary RSV challenge was given i.n. at 8wk, with 10^6 PFU in 100μl (i.e. the same PFU RSV/g as primary infection). RSV A2 strain was grown in HEp-2 cells and viral titer determined by plaque assay. Following infection, sickness was monitored by measuring weight daily. Lung function was assessed using whole-body plethysmography (Buxco, UK) to record the Penh (enhanced pause), described previously (25).

For cell depletion, mice were treated with 500μl (adults) or 50μl (neonates) of 1mg/ml antibody i.p. on d-1, d+2 and d+5 p.i. CD4 cells were depleted using clones YTA 191 and YTA 3, CD8 cells were depleted with clone YTS 156 and control treatment used an irrelevant matched isotype control, all antibodies were IgG2b (all antibodies were a kind gift of S. Cobbold, Oxford University). All work was approved and licensed by the UK Home Office. Experiments were performed n≥2 times with n≥4 mice per experiment.

Cell Preparation and histology. After infection, animals were sacrificed by i.p. pentobarbitone injection and tissues collected as described (7). Cells were processed to single cell suspension and live cells counted by trypan blue exclusion. Histology samples were processed from 4% formalin fixed lungs as described (25) and stained with haematoxylin and eosin (H & E) and mucus-producing goblet cells were detected using the periodic acid-Schiff (PAS) method.
Flow Cytometry. Cell staining was performed as described (7). For surface staining antibodies against the surface markers CD4, CD8, CD44, CD62L, MHCII and CD11c (BD) were used or using the RSV M2 MHC class I pentamer (SYIGSINNI; Proimmune) were added in 1:100 dilution for 30 mins on ice. Gating for lymphocytes was determined by back gating on CD3/CD8 double positive cells. Stimulation for intracellular staining was performed using the SYIGSINNI peptide, (1 µg ml⁻¹ for 4h in the presence of IL-2 (50 U ml⁻¹) and Brefeldin as described (7). Vβ screening was performed by flow cytometry (Pharmingen). Samples were run on an LSR (BD) and analyzed using Winlist (Verity).

Cytokine ELISA. Cytokine levels were assessed in bronchoalveolar lavage (BAL) or lung mash supernatants by ELISA using a pair of capture and biotinylated detection antibodies (Cytokine: BD or Chemokine: R&D systems) following the manufacturer’s instructions. Mediator concentrations were quantified by comparison to recombinant cytokine standards. For neonatal BAL where sample volume was low, cytokines were quantified using Luminex (Applied cytometry systems) following manufacturer’s instructions.

Quantification of Viral RNA. Total RNA was extracted from snap frozen lungs using RNA STAT-60 (AMS Biotech Ltd.) and cDNA was generated with random hexamers using Omniscript RT (Qiagen). Real time PCR was carried out for the RSV L gene using 900 nM forward primer (5’-GAACTCAGTGTAGGTAGAATGTTTGCA-3’), 300 nM reverse primer (5’-TTCAGCTATCATTTCTCTGCAAT-3’) and 100 nM probe (5’-FAM-
TTTGAACCTGTCTGAACAT-TAMRA-3’) on an ABI Prism 7000 Sequence Detection System as described (8).

**Statistical analysis.** Results are expressed as mean ± S.E.M.; statistical significance was calculated by ANOVA followed by Tukey tests using GraphPad Prism software.
Results

Primary RSV infection in neonatal and adult mice

In neonatal mice, RSV infection had no effect on normal growth (Fig.1A; the slow down in growth seen in neonates on days 12-14 post infection (p.i.) is normal just before weaning). In adult mice, illness and weight loss appeared from d5 of RSV infection (Fig.1B). There was a small and non-significant increase in the cellularity of the lungs of neonates (Fig.1C), less marked than that seen in adult mice after infection (Fig.1D). There was no inflammation seen in control treated lungs of either age (Fig.1E, F). Neonatal infection resulted in a minor perivascular and peribronchiolar infiltrate of mononuclear cells, (Fig.1G), in contrast to the marked histopathological effects seen after adult infection (Fig.1H).

Accompanying the significant differences in disease, weight loss, cell recovery and histological change, adults showed enhanced recruitment of lymphocytes to the lungs (time course of recruitment shown in Fig.2, with sample data in online supplemental Fig.1). In control mice undergoing mock infection, there was no time-dependent increase in activated CD4 or CD8 T cells and no accumulation of pentamer positive or IFNγ producing cells (data not depicted). However, after live RSV infection of adults, the number of activated CD4 T cells increased rapidly to a peak on d11, declining thereafter (Fig.2A). A similar pattern of accumulation of activated CD8 T cells was seen after a slight delay (Fig.2B) which was mirrored by the appearance of CD8 T cells specific to the immunodominant, H-2Kd restricted peptide epitope of the RSV M2 protein (amino acids 82 to 90; Fig.2C). The proportion of cells responding to this peptide by IFNγ production peaked on d7 (Fig.2D), suggesting the possibility of activation of bystander cells at this early stage.
of infection. There was no significant difference in B cell recruitment to the lungs of infected animals (data not depicted).

The response of neonates was similar to that seen in adults, but with significantly reduced magnitude. Interestingly, at d11 there were similar proportions of RSV-specific CD8 T cells in adults and neonates, but only the adult cells responded to peptide stimulation by abundant IFNγ production (Fig.2D). Peptide-specific tumor necrosis factor (TNF) production by CD8 T cells followed the same pattern in adults and neonates, as did IFNγ production following PMA/ionomycin stimulation of T cells (data not depicted). As previously demonstrated, infection of adult mice results in CCL5 (RANTES) release (7). Measuring CCL5 levels in the supernatant of lung homogenates supernatant showed a rapid increase between d4 and d11, peaking at approximately 8,000 pg/ml (Fig.2E). The pattern of CCL5 levels mirrored CD4 T cell recruitment. A small increase in CCL5 was also in neonatal mice (reaching only 2,000 pg/ml). Similar profiles were observed in bronchoalveolar lavage (BAL) supernatants, but levels were lower. On d9 post infection neonatal mice had significantly reduced TNF responses compared with adult mice (Table I; p<0.05). There were no differences in the levels of IL-4 and IL-15 between adult and neonatal mice. There was more detectable IL-5 in the BAL of neonatal mice (not significant) and significantly more IL-9 (p<0.001). Adult mice had significantly greater viral load (3.1 x 10^5 ± 10^5 L gene copies per μg lung RNA) than neonatal mice (7.5 x 10^4 ± 10^4 L gene copies per μg lung RNA) on d4 p.i. (p<0.001). This difference in viral load was seen when adult and neonatal mice were infected with the same dose per gram body weight or identical actual doses.
Secondary infection of adult or neonatal primed mice

To study the effect of primary neonatal RSV infection on the outcome of a secondary infection, mice were challenged at 8 weeks of age with $10^6$ PFU of RSV - the same PFU/g body weight as was used in the primary infection (Fig.3A). Neonatally primed mice showed significant weight loss during secondary RSV challenge, while mice first infected at 4 weeks lost no weight (Fig.3B). Weight loss in neonatally primed mice peaked on d6 and was preceded by a decrease in baseline lung function inferred from enhanced pauses (Penh) on whole body plethysmography (Buxco Technologies, UK, Fig.3C) which peaked on d3 and d4. Compared to a primary adult infection (Fig.1B) the onset of illness (measured by weight loss) in neonatal primed mice was accelerated. The weight loss during adult challenge following neonatal priming was not dependent upon the priming dose, weight loss in neonatal mice was also seen when both adult and neonatal mice were primed with identical doses ($5 \times 10^5$ PFU RSV; data not depicted).

Accompanying this enhanced disease, neonatally primed mice showed significantly increased lung cell numbers on d7 p.i. when compared to adults (p<0.001; Fig.3D), this trend was reflected in BAL (data not depicted). Significant eosinophilia and neutrophilia were seen in the BAL of neonatally primed mice (Fig.3E). This was associated with significantly enhanced levels of CCL11 (eosinophil chemoattractant) and KC (a functional homologue of IL-8 in mice and a neutrophil chemoattractant) in the BAL (Fig.3F). More CD8 than CD4 T cells were recruited to the lungs of neonatally primed mice (Fig.3G). By contrast, mice first infected during adulthood and then re-challenged showed a dominance of CD4 T cells at d7 p.i. (Fig.3G). During re-challenge, CD8 T cells recovered from the lungs of adult or neonatally primed mice were approximately 70% RSV pentamer positive
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(data not depicted). There was no significant difference in TCR Vβ usage between adults and neonates and the response was polyclonal (data not depicted).

As previously demonstrated (8) the CD4 T cell response was Th2 skewed with more IL-4 secreting Th2 CD4 T cells (Fig.3H). There were more TNF and IFNγ positive CD8 T cells (Fig.3I), indicating a stronger inflammatory response to secondary infection. Inflammatory mediators in neonatally primed mice were also greater during secondary challenge, there was significantly more (p<0.05) lung IL-2 and CCL3 (MIP1α). Similar patterns (although with lower absolute levels) were seen in the BAL fluid.

Neonatally primed mice also showed an increase in lung inflammation during adult re-challenge, judged using histology. At d4 p.i. this was characterized by peribronchiolar and perivascular infiltrates similar that seen in adult mice (Fig.4A and 4B), but at d7 p.i. there was more evidence of alveolar infiltrate (Fig.4C) compared to adult mice (Fig.4D). At d4 p.i. there were also more goblet cells in the neonatally primed mice (Fig.4E and 4I), not seen adult primed mice (Fig.4F and 4J). At d7 p.i. there were detectable goblet cells in both adult and neonatally primed mice (Fig.4G and 4H).

**T cell depletion**

Since enhanced disease was associated with rapid recruitment of activated CD4 and CD8 T cells during secondary challenge, we selectively depleted CD4 cells, CD8 cells, or both, on days -1, +2 and +5 of re-infection (Fig.5A). Whilst CD4 cell depletion reduced the severity of weight loss, CD8 cell depletion reduced weight loss significantly more than CD4 cell depletion (p<0.05 on d4 and d5, Fig.5B). Combined CD4/8 cell depletion virtually abolished disease in neonatally primed mice during adult re-infection (Fig.5B).
CD4 or CD8 depletion both increased viral load, and combined depletion significantly increased it further (Fig. 5C). The reduction in disease severity was accompanied by a significant reduction in lung cellularity (Fig. 5D). Antibody treatment was highly effective at depleting T cells in the lung (online supplemental Fig. 2A-D), reducing each respective cell type to ≤1% (Fig. 5E). While CD4 depletion reduced eosinophilia, CD8 cell depletion during secondary challenge dramatically increased airway eosinophilia during challenge (Fig. 5F). This was associated with enhanced CCL11 (Fig. 5G) and IL-5 (Table III) levels in the BAL. CD8 depletion also increased BAL neutrophilia (Fig. 5H) and KC levels (Fig. 5I). The pattern of reduced disease following T cell depletion (Fig. 5B) was reflected in inflammatory mediator production. IL-2, IL-4, IL-13 and CCL5 levels all decreased in proportion to the decrease in inflammation (Table III).

To determine whether T cell depletion in primary infection had similar effects to those seen during secondary challenge, we selectively depleted CD4 cells, CD8 cells, or both, on days -1, +2 and +5 of primary infection (Fig. 6A). Depletion in the neonatal period was very effective (online supplemental Fig. 2E-H), reducing the respective cell type to ≤2% of the lung lymphocytes, but had no apparent effect on the course of primary infection. However, the effect on weight loss during secondary re-challenge was striking. While CD4 depletion had no effect, CD8 depletion or CD4/8 dual depletion inhibited weight loss (Fig. 6B). After RSV challenge, T cell depletion had no significant effect on viral load at d4 p.i. of secondary infection (Fig. 6C), or in primary infection (data not shown). CD8 cell depletion during primary infection reduced the cellularity of the BAL during secondary challenge (Fig. 6D), but did not significantly alter the lung cell number (data not depicted). Neonatal CD8 depletion increased the percentage of CD4 T cells (Fig. 6G) and decreased the percentage of CD8 T cells in the lung during secondary challenge (Fig. 6H). This change was RSV-
specific because mice that had been treated with both anti-CD4 and anti-CD8 as neonates (in the absence of RSV infection) had normal T cell numbers and responded normally to a primary RSV infection as adults (data not depicted). As seen in secondary depletion CD8 depletion increased BAL eosinophilia (Fig.6F) and neutrophilia (Fig.6H), with changes seen in the related chemokines CCL11 (Fig.6G) and KC (Fig.6I). No distinct pattern of cytokine was seen following primary neonatal depletion, CD8 depletion increased IFNγ production and IL-2 production, but reduced CCL3 production, however CD4/8 dual depletion which caused a similar phenotype of protection against rechallenge made no changes to these mediators (data not depicted).
Discussion

As show previously age at primary infection is critical to the outcome of secondary RSV infection (8). The current study takes this finding further linking the marked differences between adult and neonatal mice during primary RSV infection and the outcome of secondary infection, in particular identifying a critical role for T cells. During primary infection both adult and neonatal mice recruited RSV specific T cells, but neonatal RSV specific CD8 cells were IFNγ deficient. T cells generated during primary infection were shown to be of critical importance because the detrimental effects of neonatal priming were largely prevented by their depletion. T cells were critical both as effectors during secondary challenge when either CD4 or CD8 depletion reduced weight loss and cell recruitment and during primary infection when notably only CD8 depletion was effective at preventing ‘imprinting’ for disease enhancement.

In mice, it has previously been shown that RSV replication, disease and pathology increases with age (11). This seems paradoxical, in that natural RSV disease is most severe in very young infants (age <6 months) and rarely leads to hospitalization in normal or older children. However, it is notable that elderly persons are highly susceptible to re-infection and can experience life-threatening effects of RSV disease (10). We saw evidence of this neonatal RSV infection was associated with less inflammation and disease than adults. Neonatal immune responses have been shown to be ‘Th2’ skewed (1); in our study neonates made higher levels of IL-5 and IL-9 than adults, indicating Th2 skewing. Enhanced levels of the cytokine IL-9 is interesting because it has been previously shown to be elevated in babies with severe bronchiolitis (19) and has an association with asthma. It may be that IL-9 produced during early life infection changes the response to future infections and that it can act as a master switch regulating other cytokines.
The difference in IFNγ production by RSV-specific CD8 T cells recruited during primary infection was also of interest. These results compare with those of Chang et al (5), who show that pulmonary RSV-specific CD8 cells are deficient in IFNγ production and that this defect was reversed by the addition of IL-2 to ex vivo cultured cells. During stimulation with peptide for intracellular staining, we routinely add IL-2. This explains the high levels of peptide-specific IFNγ production that we observed in adult cells, but suggests either neonatal cells are refractory to IL-2 or are defective in IFNγ production.

These IFNγ-low RSV-specific CD8 T cells are of critical importance in causing weight loss during secondary challenge, because CD8 depletion during primary infection reduced weight loss. It has been demonstrated that the addition of recombinant IFNγ during neonatal RSV infection leads to protective immunity (17) and here we demonstrate that neonatal CD8 T cells unable to make this cytokine skew the outcome of future exposure to RSV. We have previously shown that cytokine delivery using recombinant virus can shape future infections (12). It is possible that the CD8 cells are acting as Tc2 cells which have been show to lead to enhanced eosinophilia in some models (21). Alternatively the CD8 cells may affect other cells for example macrophages leading to rapid recruitment of other cells to during secondary infection.

Secondary infection after neonatal priming showed some characteristics of ‘Th2’ disease, e.g. increased IL-4 producing CD4 T cells and eosinophilia. It has been shown previously that the effects of neonatal RSV infection are IL-13 dependent (9). However, the enhanced disease is not only accounted for by Th2 CD4 cells, since CD4 depletion only partially prevented the effects and CD8 depletion had more
impact than CD4 depletion. Furthermore the depletion of CD8 cells during challenge increased eosinophilia without increasing pathology.

We have previously shown the inhibitory effect of CD8 cells on eosinophilia, in that mice primed with recombinant vaccinia expressing the RSV F protein develop eosinophilia only after CD8 cell depletion (15). Passive transfer of CD8 T cells also attenuates eosinophilia caused by passive transfer of CD4 T cells with a Th2 cytokine secretion profile (2). We have seen that enhanced eosinophilia following RSV infection is not always associated with weight loss (12). CD8 depletion also had a marked impact on neutrophilia, suggesting that CD8 cells can exert the same inhibitory effect on neutrophil recruitment as they do on eosinophil recruitment. It has recently been shown that adaptive immune cells can profoundly influence the vigor of innate responses (10), possibly by delivering an inhibitory signal to infected cells or by taking up cytokines released by infected cells that would otherwise activate non-infected cells and sustain a cycle of enhanced inflammation. That CD8 depletion lead to increased granulocyte recruitment without enhanced disease further supports the central role of T cells.

During secondary infection, T cell depletion has an effect as early as d2 p.i. (Fig.5B), prior to detectable T cell recruitment. Woodland et al (13,14) have described a set of resident airway cells able to control initial viral infections; these are capable of producing large amounts of IFNγ, thus recruiting or activating other cells in the airways (particularly macrophages and NK cells) which in turn can recruit more T cells. When T cells were depleted during secondary infection we saw decreased levels of IL-2, CCL5 and TNF, all of which can contribute to recruitment and proliferation of cells in the airway. The effects of T cells in enhanced disease have been demonstrated before, transferring RSV specific CD4 T cells into naïve mice prior to RSV infection leads to weight loss and enhanced pathology (2). If CD4 cells
are depleted after priming with formalin inactivated RSV, the severity of pulmonary histopathological change is reduced (6). Of note was the different roles of T cells in the effector (secondary infection) stage when both CD4 and CD8 T cells contribute to disease and the priming (primary infection) stage when only CD8 T cells play a role and the difference between these two stages. During the effector stage, both cell types contribute to inflammation and disease, most probably by the direct release of cytokines (Table III), for example CCL5 which we have previously shown to be of importance in inflammation following RSV infection (7). During the priming stage CD8 cells skew the response of the rest of the immune system. This is an important novel finding because CD4 T cells are often described as being the driving factor of RSV disease enhancement, but here we show that in early life CD8 cells are critical.

In conclusion, we find that T cells, particularly CD8 T cells, play a pivotal role in controlling disease enhancement following neonatal RSV infection. The effects we observe may be due to direct actions of T cells on infected cells in the lung, or may be indirect. The complex interplay between different components of the immune system are still not fully understood, and are impossible to predict without studying in vivo models of disease. Although the mouse model has limitations, it remains the best animal model available for studying the complexities of the immunopathogenesis of viral lung disease.
Online Supplemental Material

Supplemental Figure 1. Representative flow cytometry plots of lung cells following primary infection. Mice were intranasally infected at 4 days or 4 weeks of age with 4x10^4 PFU RSV per gram body weight as described in Fig.2. Lungs were harvested after infection and cell types assessed by flow cytometry. M2 specific pentamer at d11 (A, B). RSV peptide specific IFNγ production measured by intracellular staining at d7 (C, D) and d 11 (E, F). Numbers are the % of gated lymphocytes.

Supplemental Figure 2. Representative flow cytometry plots of lung cell depletion by antibody treatment. T cell subsets were depleted using antibody during RSV infection. Example dot-plots from lungs of control depleted mice (A, E), anti-CD4 and anti-CD8 (B, F); anti-CD4 (C, G), anti-CD8 (D, H) treated mice are shown. Secondary RSV challenge (A-D). Primary RSV infection (E-H).
Acknowledgments

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Reference List


**Figure legends**

**Figure 1. Primary RSV infection is more severe in adult mice than neonates.** Mice were intranasally (i.n.) infected neonatally (age 4 days; ▼) or as immature adults (age 4-6 weeks; ■) with 4x10^4 PFU RSV per gram body weight. Graphs show change in body weight post primary RSV (closed symbols) or mock (open symbols) infection in neonatal (A) or adult (B) mice. Lung cell counts post infection in neonatal (C) or adult (D) mice after RSV (filled bars) or control (open bars). H&E stained formalin fixed lung histology sections at d7 post treatment in neonatal (E, G) or adult mice (F, H). n≥4 mice per group ± SEM, * p<0.05, ** p<0.01, *** p<0.001. Scale bar represents 25μm.

**Figure 2. Dynamic changes in lung after primary RSV infection.** Neonatal (▼) or adult (■) mice were infected as in Fig.1, and lungs were harvested at days 4, 7, 11 and 14 post primary infection. Cell types in the lung were assessed by flow cytometry, CD4 T cell effector memory (CD44 hi, CD62L lo; A); CD8 T cell effector memory (CD44 hi, CD62L lo; B); M2 pentamer-positive CD8 T cells (C); M2 peptide specific IFNγ producing CD8 T cells measured by intracellular staining (D). CCL5 in lung digests measured by ELISA (E). n≥4 mice per group ± SEM. * p<0.05, *** p<0.001. See online supplemental figure 1 for sample FACS plots.

**Figure 3. Timing of primary RSV infection determines effect of adult rechallenge.** Mice were infected with RSV at age 4 days; (▼ and white bars) or as adults (■ age 4 weeks; and black bars) and challenged with 5x10^5 PFU RSV at 8 weeks old (A). Change in body weight (B), baseline Penh (C) after secondary
infection. Lung cell counts (D), BAL granulocytes (E) and chemokines (F), lung CD4 and CD8 T cells (G), and percentage of CD4 (H) and CD8 (I) cells positive for IFNγ, TNF and IL-4 at d7 post infection. n≥4 mice per group ± SEM, * p<0.05, ** p<0.01, *** p<0.001.

**Figure 4. Neonatal RSV infection induces inflammation and goblet cell hyperplasia following secondary RSV challenge.** Mice were infected as adults or neonates and rechallenged 8 weeks later as in Fig.3C. Representative H&E (A-D) and PAS (E-H) stained formalin fixed lung sections taken on d4 (A, B, E, F) and d7 (C, D, G, H) after adult challenge, scale bar represents 20μm. 5x enlargements of regions with goblet cells are shown (I-L).

**Figure 5. Depleting T lymphocytes during secondary RSV challenge reduces lung inflammation.** Mice were infected at 4 days of age and re-challenged at 8 weeks. During secondary RSV challenge, mice were treated i.p. with T cell depleting antibodies according to the treatment schedule (A). Weight change after secondary RSV challenge (B). Lung viral load 4 days after secondary challenge (C). Lung cell number (D) and T cells (E) on d7 after RSV challenge. Eosinophilia (F), CCL11 (G), neutrophilia (H) and KC (I) measured in BAL 7 days after secondary RSV challenge. n≥4 mice per group ± SEM, * p<0.05, ** p<0.01, *** p<0.001. See online supplemental figure 2A-D for sample FACS plots.

**Figure 6. Depleting CD8 cells during primary neonatal RSV infection affects the outcome of adult rechallenge.** Mice were infected with RSV as neonates and challenged 8 weeks later with RSV. During primary neonatal RSV infection, mice were treated i.p. with T cell depleting antibodies according to the treatment schedule
(A). Weight change after secondary RSV challenge (B). Lung viral load at d4 p.i. (C). BAL cell number (D), lung T cells (E) and eosinophilia (F), CCL11 (G), neutrophilia (H) and KC (I) measured in BAL 7 days after secondary RSV challenge. n≥4 mice per group ± SEM. * p<0.05, ** p<0.01, *** p<0.001. See online supplemental figure 2E-H for sample FACS plots.
Table I. Lung inflammatory mediators following primary infection.

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<th>Mediator $^a$ (ng/ml)</th>
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<th>Neonatal $^c$</th>
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<tr>
<td></td>
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<td>IL-4</td>
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<td>TNF</td>
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$^a$ Inflammatory mediators were measured in BAL 9 days post secondary challenge by Luminex. Shown as mean of $n \geq 3$ mice in ng/ml ± SEM.

$^b$ Primary infection at 4 weeks old.

$^c$ Primary infection at 4 days old.
Table II. Lung inflammatory mediators following secondary challenge.

<table>
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<td>IL-15</td>
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<td>0.99 ± 0.00</td>
<td>ns</td>
</tr>
<tr>
<td>CCL3</td>
<td>8.40 ± 1.50</td>
<td>39.60 ± 14.00</td>
<td>*</td>
</tr>
<tr>
<td>CCL5</td>
<td>9.81 ± 1.68</td>
<td>20.90 ± 6.16</td>
<td>ns</td>
</tr>
<tr>
<td>TNF</td>
<td>5.17 ± 2.30</td>
<td>3.22 ± 0.51</td>
<td>ns</td>
</tr>
</tbody>
</table>

a Inflammatory mediators were measured in BAL seven days post secondary challenge by Luminex or ELISA. Shown as mean of n≥4 mice in ng/ml ± SEM.

b Primary infection at 4 weeks old.

c Primary infection at 4 days old.
Table III. Lung inflammatory mediators following depletion during secondary challenge.

\(^a\) Inflammatory mediators were measured in BAL seven days post secondary challenge by Luminex or ELISA. Shown as mean of \(n \geq 4\) mice in ng/ml ± SEM.

<table>
<thead>
<tr>
<th>Mediator (^a) (ng/ml)</th>
<th>Neonatal</th>
<th>Anti-CD4</th>
<th>Anti-CD8</th>
<th>Anti-CD4/8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>± SEM</td>
<td>Mean</td>
<td>± SEM</td>
</tr>
<tr>
<td>IFN(\gamma)</td>
<td>2.10</td>
<td>0.39</td>
<td>0.99</td>
<td>0.40</td>
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<td></td>
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<tr>
<td>IL-2</td>
<td>7.35</td>
<td>0.75</td>
<td>5.12</td>
<td>0.94</td>
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<tr>
<td>IL-4</td>
<td>0.34</td>
<td>0.01</td>
<td>0.27</td>
<td>0.02</td>
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<tr>
<td>IL-5</td>
<td>13.72</td>
<td>9.82</td>
<td>1.41</td>
<td>0.13</td>
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<tr>
<td>IL-13</td>
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<td>0.32</td>
<td>1.64</td>
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</tr>
<tr>
<td>CCL5</td>
<td>29.67</td>
<td>7.38</td>
<td>8.21</td>
<td>1.84</td>
</tr>
<tr>
<td>TNF</td>
<td>4.14</td>
<td>0.35</td>
<td>1.94</td>
<td>0.18</td>
</tr>
</tbody>
</table>
**Figure 1**

(A) Neonatal

(B) Adult

Days after primary infection

% Original Weight

Days after primary infection

Lung Cell Number (x10^-6)

Days after primary infection

[Graphs showing changes in % Original Weight and Lung Cell Number over time for neonatal and adult models after primary infection.]
A

1º RSV  Ab  2º RSV  Ab  Ab Harvest

-8 wk

B

% Original Weight

Anti-CD4

Anti-CD8

Anti-CD4/8

Days after secondary infection

Copies of RSV L gene per μg lung RNA

C

***

***

*

D

Lung Cell Number (x10^6)

CD4

CD8

E

Lung T cells (x10^-6)

F

BAL Eosinophils (x10^-5)

G

BAL CCL11 pg/ml

H

BAL Neutrophils (x10^-5)

I

BAL KC pg/ml

Figure 4

Tregoning et al J Virol
**A**

Percentage Original Weight

- RSV
- Anti-CD4
- Anti-CD8
- Anti-CD4/8

Days after infection: 0, 3, 5, 7

---

**B**

Lung Cell Number (x10⁶)

- RSV
- Anti-CD4
- Anti-CD8
- Anti-CD4/8

Days after infection: 0, 3, 5, 7

***

---

**C**

Lung T cells (x10⁶)

- CD4
- CD8

---

**D**

BAL Macrophage (x10⁶)

- RSV
- Anti-CD4
- Anti-CD8
- Anti-CD4/8

---

**E**

BAL MC (x10⁶)

- RSV
- Anti-CD4
- Anti-CD8
- Anti-CD4/8
Figure 6

1º RSV

Ab 1º RSV Ab

2º RSV

Harvest

8 wk

% Original Weight

Days after secondary infection

RSV L gene copies
per μg Lung RNA

Airway Cell Number (x10^-6)

Lung T cells (x10^-5)

BAL Eosinophils

BAL Neutrophils

BAL CCL11 pg/ml

BAL KC pg/ml