IL-9 Regulates Pathology during Primary and Memory Responses to Respiratory Syncytial Virus Infection

Jonathan S. Dodd, Eda Lum, John Goulding, Roshell Muir, Jacques Van Snick and Peter J. M. Openshaw

*J Immunol* 2009; 183:7006-7013; Prepublished online 13 November 2009;
doi: 10.4049/jimmunol.0900085

http://www.jimmunol.org/content/183/11/7006

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/11/13/jimmunol.0900085.DC1

References
This article cites 27 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/183/11/7006.full#ref-list-1

Why *The JI*? Submit online.

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-9 Regulates Pathology during Primary and Memory Responses to Respiratory Syncytial Virus Infection1

Jonathan S. Dodd,* Eda Lum,* John Goulding,† Roshell Muir,* Jacques Van Snick,‡ and Peter J. M. Openshaw2*2

IL-9 is a cytokine of great current interest associated with allergic/Th2 responses. High levels of IL-9 are present in bronchial secretions from infants with respiratory syncytial virus (RSV) bronchiolitis. To test its effects in RSV disease with a Th2 profile, BALB/c mice were vaccinated with recombinant vaccinia virus expressing the RSV G protein. On RSV challenge, immunized mice developed augmented disease characterized by enhanced pulmonary Th2 and local IL-9 production peaking on days 7–10 of RSV infection. Depletion with anti-IL-9 Ab at vaccination or RSV challenge enhanced viral clearance. Depletion only at challenge had no effect on disease severity, whereas depletion at immunization and challenge enhanced Th1 responses, inhibited virus-specific IgG1 production, and enhanced disease severity. By contrast, depletion of IL-9 at immunization boosted IgG2a and inhibited the Th2 response and disease during subsequent infection without a concomitant increase in type 1 cytokines. Adoptive transfer of secondary memory CD4 T cells from the spleens of IL-9-depleted mice into naive recipients replicated many of the effects of depletion, indicating that IL-9 acts via CD4 T cells. Therefore, IL-9 is a previously unknown but key modulator of antiviral immunity, regulating T and B cell responses and having potent and specific effects on viral lung disease. The Journal of Immunology, 2009, 183: 7006–7013.

Viral bronchiolitis is the commonest cause of infant hospitalization in the Western world, and is estimated to cause 91,000 hospital admissions with associated costs of $300,000,000 per year in the United States alone (1). Approximately 70% of children hospitalized with bronchiolitis are infected with respiratory syncytial virus (RSV) (2), and children who recover from severe bronchiolitis tend to develop asthma and recurrent wheeze in later life (3). In addition, RSV has recently been shown to cause significant disease in elderly persons, particularly those with chronic pulmonary insufficiency (4, 5). Many studies have highlighted the role that T cells play in promoting and sustaining inflammation during RSV disease (6). In animal models, CD8 T cells are the dominant T cell subtype at the time of virus elimination (7), and depletion of T cells or chemokines (such as CCL11 or CCL5) can reduce disease severity (8, 9). In the absence of safe and effective vaccines or clinically useful antiviral drugs, there is an urgent need to develop new therapies that target the excessive immune response that is characteristic of bronchiolitis.

While measuring the cytokines present in bronchial secretions from full-term infants with severe bronchiolitis, McNamara et al. found unexpectedly high levels of IL-9 (10, 11), at that time a relatively little studied member of the γc cytokine family. IL-9 is made by CD4 T cells, primarily those of the Th2 subset (12, 13), but is also produced by eosinophils, mast cells, and neutrophils (14, 15). It acts as a T cell growth factor, enhances IL-4-mediated IgE production by B cells, promotes the survival and activation of eosinophils and mast cells, increases expression of IgERα, and thereby affects IgE-mediated immunity (12). Increased levels of IL-9 mRNA and IL-9R have been reported in allergic asthma (16, 17), suggesting that IL-9 plays an important role in airway inflammation. Because severe RSV bronchiolitis is associated with strong Th2 (i.e., increased IL-4) and weak Th1 (i.e., reduced IFN-γ) responses to RSV infection (18), IL-9 is an attractive candidate for interventional therapy.

Because IL-9 seems to be an important mediator in human bronchiolitis, we used a well-characterized mouse model of RSV disease to study its pathogenic role in detail. In this model, immunization with vaccinia virus expressing RSV G protein results in the development of Th2-driven immunopathology during subsequent RSV challenge that is characterized by lung eosinophilia and increased IL-4 production (6). We found that IL-9 depletion enhanced viral clearance regardless of timing, but caused remarkably different effects on the immunopathogenesis of disease at different phases of the response. Depletion only at the time of RSV challenge had little effect on disease severity, but depletion at both immunization and challenge enhanced disease, increased lymphocytosis, and enhanced IFN-γ and TNF secretion by CD4 T cells while abrogating Th2 cytokine production. Paradoxically, IL-9 depletion at immunization alone alleviated disease by reducing subsequent Th2 cytokine production without a concomitant increase

1Address correspondence and reprint requests to Prof. Peter Openshaw, Centre for Respiratory Infections and Department of Respiratory Medicine, National Heart and Lung Institute, Faculty of Medicine, Imperial College of Science Technology and Medicine, St. Mary’s Campus, London W2 1PG, U.K. E-mail address: p.openshaw@imperial.ac.uk.
2Abbreviations used in this paper: RSV, respiratory syncytial virus; BAL, bronchoalveolar lavage; MOI, multiplicity of infection; rVV-G, recombinant vaccinia virus expressing the RSV G protein.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00
in IFN-γ or TNF production. Therefore, adjuvants or immune modulators aimed at reducing IL-9 production might modulate virus-specific Th2 responses and antiviral immunity, depending on the stage of immune priming or recall.

Materials and Methods

Virus and mice

The HEp-2 cell-derived A2 strain of RSV was snap frozen and assayed for infectivity (19). Recombinant vaccinia virus expressing the RSV G protein (rVV-G) (a gift from G. Wertz, University of Alabama, Birmingham, AL) was stored at −80°C. All virus preparations were free of mycoplasma (Gen-Probe).

Eight-week-old female BALB/c mice were purchased from Harlan Olac and kept under specific pathogen-free conditions. All protocols used in this study were reviewed and approved by ethics, safety, and regulatory committees.

Mouse infection and treatment

For skin infection, rumps were shaved, decortined, and infected with 10^6 PFU (10 μl) of RVV-G. For challenge, mice were anesthetized and infected intranasally with 7.5 × 10^5 PFU (75 μl) of RSV. Various groups were treated i.p. with 0.5 mg (0.5 ml) of marine anti-IL-9 monoclonal (MM9Cl) or isotype control (C1405F9) Ab 1 day before and 2 days after immunization and/or challenge (see supplemental Fig. 1).4

Quantification of viral replication

Total RNA was extracted from lungs stored in TRIzol. One microgram of total RNA (20 μl) was used to synthesize cDNA using random hexanucleotides. Viral L gene copies were measured against standard plasmids (10^-10^ copies) and a nontemplate control. Two micrograms of cDNA was used per reaction with forward primer (900 nM), reverse primer (300 nM), and probe (100 nM) in a 25-μl total volume using one cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s.

Cell recovery

After sacrifice by i.p injection of 3 mg of pentobarbital, bronchoalveolar lavage (BAL) fluid, lung tissue, and serum were harvested (20). In brief, the lungs were inflated six times with 1 ml of 12 mM lidocaine in Earle’s balanced salt solution and kept on ice. One hundred microliters of BAL fluid from each mouse was cytocentrifuged onto glass slides, and cells were enumerated by light microscopy. The remainder of the BAL fluid was centrifuged and the supernatant was removed and stored at −80°C. Pelleted cells were resuspended in RPMI 1640 with 10% FCS, 2 mM/l glucose, 50 U/ml penicillin, and 50 mg/ml streptomycin (R10F). The cell suspension was passed through a 100-μm filter (Sartorius) and RBCs were lysed with ACK (ammonium chloride-potassium) buffer. The cells were washed, resuspended in R10F medium, and live cells were counted using trypan blue.

Flow cytometric analysis of intracellular and surface Ags

Lung cells (2 × 10^6) were left unstimulated or stimulated for 6 h with plate-bound anti-CD3 (5 μg/ml) with brefeldin A (10 μg/ml) for the final 4 h. Intracellular IFN-γ (AN18-allophycocyanin or AN18-FITC), IL-4 (11B11-PE), IL-5 (TRFK5-PE), and TNF (MP6-XT22-FITC) were assessed in CD4 (RM4-5-Quantum Red) or CD8 (53-6.7-Quantum Red) T cells by flow cytometry as described (20). All Abs were purchased from BD Pharmingen unless otherwise stated. Unstimulated cells from individual mice were also stained for CD4 (RM4-5-allophycocyanin) and CD8 (53-6.7-Quantum Red). At least 50,000 cells from each sample were analyzed on an LSR BD Pharmingen flow cytometer.

In vitro cytokine production from lung cells

Cells (4 × 10^6 cells/well) were cultured for 72 h in the presence of medium alone, RSV (multiplicity of infection (MOI), 2.0), or a plate-bound anti-CD3/TCRβ (5 μg/ml) Ab in 2 ml of R10F medium in 24-well plates. After 72 h the supernatants were harvested and stored at −80°C for later cytokine analysis.

IFN-γ, RANTES (R&D Systems), IL-4, IL-5, IL-9, and TNF-α were quantified using paired Abs from BD Pharmingen unless otherwise stated. In brief, microtiter plates were coated with 100 μl of capture Ab overnight at 4°C. After three washes with PBS containing 0.5% Tween 20, plates were blocked with 200 μl of PBS with 1% BSA and left for 2 h at room temperature. Samples and standards (diluted in PBS with 1% BSA and 0.05% Tween 20) were incubated overnight at 4°C. After four washes, bound cytokine was detected (read at 490 nm) using biotinylated Abs and then avidin-HRP, followed by O-phenylenediamine dihydrochloride. Standard curves were used to calculate concentrations; for IL-4, this covered the range 7.8–2000 pg/ml; for IL-5, 15.6–2000 pg/ml; for IL-9, 30–4000 pg/ml; for TNF, 15.6–3000 pg/ml; and for IFN-γ, 31.3–10000 pg/ml.

RSV-specific Ab ELISA

RSV-specific IgA, IgE, IgG1, and IgG2a were detected by coating 96-well plates with RSV Ag or control materials, incubating with dilutions of serum, and detecting Ig with conjugated anti-mouse Ig Abs (BD Biosciences).

MACS sorting and adoptive transfer of splenic CD4 T cells

Mice were immunized, challenged, and IL-9 depleted as described above for the “immunization” and “immunization and challenge” groups. The mice were left for 28 days and spleens were harvested. CD4 T cells were extracted using a Dynal CD4 T cell negative isolation kit according to the manufacturer’s instructions (Invitrogen). CD4 T cell purity of >90% was obtained. Cells (2.5 × 10^6) were processed i.p into each recipient mouse 24 h before RSV challenge. Mice were monitored daily and BAL and lungs were harvested 7 days postchallenge.

Data analysis

Student’s t test was used to analyze differences between two groups. One-way ANOVA was used to analyze differences between more than two groups, and significance was assumed at p < 0.05. Tukey’s post-test was used to identify differences between specific groups with a significance threshold of p < 0.05.

Results

IL-9 production is enhanced during vaccine-augmented RSV disease

In RSV-infected mice the severity of lung pathology correlates with weight loss, cell recruitment, and cytokine production, and immunized mice have greater disease; we therefore assayed IL-9 levels at several time points postchallenge. Pathology upon primary infection peaks at days 7 or 8 post challenge, whereas in mice immunized against the RSV G protein it peaks at days 5 or 6. To determine whether raised IL-9 levels are present in the lungs of mice with normal or augmented RSV disease, IL-9 was measured in BAL and lung cell supernatants after RSV infection. IL-9 was found at a low level in the BAL fluid (Fig. 1a), and at higher levels in the supernatant from lung cells isolated from mice after RSV challenge stimulated in vitro with RSV Ag (Fig. 1b). In either case, IL-9 levels were significantly increased by prior rVV-G sensitization. In the BAL, levels peaked at days 7–10 postchallenge; in the stimulated lung cell supernatants it peaked when the cells were taken at day 7 (postchallenge). Because IL-9 levels were boosted by prior rVV-G sensitization, subsequent studies were performed in this setting.

IL-9 depletion at immunization reduces cytokine secretion by peptide-specific T cells

To test the effect of in vivo IL-9 depletion on the development of T cell responses, mice were immunized by scarification with rVV-G; 14 days later, isolated spleen cells were stimulated with anti-TCRβ Ab, triggering secretion of IFN-γ (Fig. 2a), TNF (Fig. 2b), IL-4 (Fig. 2c), and IL-5 (Fig. 2d). Stimulation with G184–198 peptide induced production of IFN-γ, TNF and IL-4, but not IL-5. IL-9 depletion during vaccinia infection had no effect on these responses to anti-TCRβ Ab stimulation but significantly reduced secretion of IFN-γ (Fig. 2a) and IL-4 (Fig. 2c) from the peptide-stimulated cultures. IL-9 depletion had no effect on TNF release (Fig. 2b).

4 The online version of this article contains supplemental material.
Depletion of IL-9 during challenge alone had no significant effect on CD4 T cells (Fig. 3c). Thus, whereas IL-9 depletion at immunization reduced cell influx into the airways, depletion at immunization and challenge enhanced and prolonged T cell efflux.

Because eosinophils are a hallmark of a Th2 response, we determined eosinophil influx into the BAL. In control RSV-challenged immunized mice, eosinophil influx began on day 4 and peaked on day 7; this was not affected by IL-9 depletion during RSV challenge alone, but depletion during immunization and challenge caused a blunting of the eosinophilic response at days 4 and 7 (Fig. 3d; p < 0.001). By contrast, in mice depleted only during immunization eosinophil influx was greatly attenuated on days 4, 7 (p < 0.001), and 10. Therefore, eosinophil influx during RSV challenge was dependent on IL-9 during immunization, but not during recall responses.

**IL-9 depletion enhances viral clearance from the lungs**

In primary RSV infection, quantitative TaqMan PCR typically detects ~10^6 L gene copies per 2 μg of lung RNA (Fig. 3e). Prior immunization with the G protein greatly reduces viral load but does not fully prevent viral replication. IL-9 depletion resulted in further reductions in viral genome levels on day 4 regardless of the time of administration of Ab (Fig. 3e).

**Effects of IL-9 depletion on pulmonary cytokine production during RSV challenge**

After RSV challenge, IFN-γ secretion peaked on day 4 and fell to baseline by day 7. IL-9 depletion at either challenge or immunization alone had no effect on this profile, but IL-9 depletion at immunization and challenge prolonged and enhanced IFN-γ secretion at days 7–10 (day 7 is shown in Fig. 4a). IL-9 depletion had...
IL-9 depletion during immunization inhibits IgG1 but boosts RSV-specific serum IgG2a responses to RSV challenge

To determine the effect of IL-9 depletion on Ab production during primary and secondary responses to RSV, mice were vaccinated with or without IL-9 depletion. Sera from immunized mice contained low levels of RSV-specific IgG1 (Fig. 6a) and IgG2a (Fig. 6c) Abs before RSV challenge. After RSV challenge, Ab levels rose dramatically (Figs. 6c and d).

IL-9 depletion during immunization reduced RSV-specific IgG1 responses before RSV challenge (Fig. 6a). IL-9 depletion at immunization and/or challenge had similar but even more dramatic effects on IgG1, and combined depletion almost abolished IgG1 responses (Fig. 6b). However, the IgG2a booster responses to RSV challenge were greatly enhanced by depletion during immunization, whereas additional or separate IL-9 depletion during RSV challenge had little effect on IgG2a (Fig. 6d). No RSV-specific IgA or IgE was detected (data not shown).

Adoptive transfer of splenic CD4 T cells from IL-9-depleted mice enhances disease in RSV-infected recipient mice

Having shown that IL-9 depletion at both immunization and challenge exacerbates disease, we wished to determine whether passive transfer of CD4 T cells from mice that had recovered from immunization and challenge, with depletion at immunization or double IL-9 depletion, could cause comparable effects in naive, RSV-infected, syngeneic recipients. Therefore, spleen cells containing secondary memory CD4 T cells were isolated 28 days postchallenge and injected into naive recipients 24 h before intranasal challenge with RSV. CD4 T cells from naive mice were used as a control. To determine the phenotype of the cells being transferred, we assessed the expression of the three major TCR Vβ clonotypes.
(8.2, 8.3, and 14) expressed by RSV-G-specific CD4 T cells (supplementary Fig. 2). Only mice primed with rVV-G and challenged with RSV had increased numbers of CD4 T cells expressing all three clonotypes. There were no differences in the numbers of Vβ1/H9252 8.2/H11001 and 8.3/H11001 CD4 T cells between the primed and challenged groups. There was a small but significant increase in the number of splenic Vβ1/H9252 14/H11001 CD4 T cells in mice depleted at immunization and challenged compared with those depleted at immunization only (13 × 10^4 and 8 × 10^4, respectively). We also measured IFN-γ and IL-5 production from RSV-G-specific CD4 T cells (supplementary Fig. 3). No IL-5 was detected in any group (data not shown). Cells from naive mice did not respond to RSV-G-peptide, in contrast to those from primed and challenged mice. RSV-G-specific CD4 T cells from mice depleted at immunization and challenged produced more IFN-γ than cells from mice depleted at immunization only (56 vs 36%, respectively).

All mice lost weight after challenge, but those given cells from mice depleted during both immunization and challenge lost significantly more weight than other mice (Fig. 7a). This was associated with increased total cell efflux into the BAL (Fig. 7d), increased CD4 and CD8 T cell efflux (Fig. 7f and e), significantly higher levels of IFN-γ (Fig. 7b) and TNF (Fig. 7c) in the BAL fluid, and increased IFN-γ (Fig. 7g and i) and TNF production (Fig. 7h) by CD4 T cells, with an increased efflux of CD8 T cells into the BAL.

**Discussion**

We found that regardless of timing, IL-9 depletion enhanced clearance of RSV and reduced eosinophil influx into the BAL. Depletion of IL-9 at immunization lessened weight loss after challenge RSV infection with reduced cell influx to the airway and reduced IL-4 and IL-5 production, but without increases in IFN-γ, RANTES, and TNF production. By contrast, depletion at challenge had fewer effects whereas depletion at both immunization and challenge ablated Th2 immunity, boosted Th1 responses, and exacerbated disease (as measured by weight loss). Many of these features were transferred into naïve recipients upon administration of memory CD4 T cells from recovered mice. Therefore, IL-9 is crucial for...
preventing the induction of exacerbating Th1 memory responses and has detrimental effects on antiviral immunity by promoting disease-enhancing Th2 responses that are poorly antiviral. However, IL-9 during memory recall phase is critical for inhibiting exacerbating Th1 immunity.

IL-9 is present in the respiratory secretions of infants with RSV bronchiolitis (13). Children suffering from RSV bronchiolitis tend to produce stronger Th2 (i.e., increased IL-4) and weaker Th1 (i.e., reduced IFN-\( \gamma \)) responses to viral infection than children who exhibit mild disease (18). McNamara et al. have previously highlighted the potential importance of IL-9 in promoting this detrimental response (13). Our finding that IL-9 depletion during the primary response to the RSV G protein (the only protein expressed by the virus known to promote Th2 responses) decreases both IFN-\( \gamma \) and IL-4 secretion by G-specific T cells, as well as IL-4 and IL-5 secretion by memory T cells, and alleviates Th2-mediated phenomena (e.g., eosinophil influx) upon viral challenge further supports the concept that early IL-9 production is critical for setting the pattern of T cell cytokine secretion.

In the mouse model of RSV disease, T cells play a crucial role in both clearing virus and causing disease (6). CD8 T cells depletion slows viral clearance (7), but depletion of CD4 T cells or chemokines (such as CCL11) that causes a reduction in CD4 T cell influx to the lung also alleviates disease (8). Furthermore, depletion of Th2 effector cytokines (IL-4 and/or IL-13) also alleviates Th2-augmented disease (21, 22). These Th2 cytokines play an

**FIGURE 5.** Timing of IL-9 depletion modulates type 1 and 2 cytokine secretion by CD4 T cells. Mice were treated as described in Fig. 3. Lung cells from individual mice were stimulated with anti-CD3 (\( \alpha \)CD3) Ab (5 \( \mu \)g/ml) for 2 h and then in the presence of brefeldin A (10 \( \mu \)g/ml) for an additional 4 h. Cells were then stained for surface CD4 and intracellular IFN-\( \gamma \) (a), TNF (b), IL-4 (c), and IL-5 (d). Cells were analyzed on a BD LSR I flow cytometer. At least 50,000 cells were analyzed from each sample. Results are expressed as percentage of CD4 T cells, deducting the percentage observed with the isotype control Ab. Error bars represent SEM. The graph is representative of three independent experiments. ANOVA (Tukey’s post-test) result: *, \( p < 0.05; **, p < 0.01; ***, p < 0.001. \( \alpha \)IL-9, Anti-IL-9.

**FIGURE 6.** IL-9 depletion alters the RSV-specific serum Ab isotype levels. Mice were treated as in Fig. 3. Sera from individual vaccinated mice were taken on day 14 before RSV challenge (a and c) or 14 days post-RSV challenge (b and d) and the levels of RSV-specific IgG1 and IgG2a were determined by ELISA. The results are representative of three independent experiments. Student t test results: *, \( p < 0.05; **, p < 0.01. \) ANOVA (Tukey’s post-test) results: *, \( p < 0.05; **, p < 0.01; ***, p < 0.001. \( \alpha \)IL-9, Anti-IL-9.
important role in defense against other pathogens (e.g., Trichinella spiralis (23), and IL-9 exerts its effects through effector cytokines such as IL-4 (24). In our studies, adoptive transfer of CD4 T cells from IL-9-depleted mice conferred many of the characteristics of IL-9 depletion on naive recipients, showing that it is the programmed CD4 T cells that mediate the effects of IL-9.

The γc cytokines are essential for naive T cell survival but are not essential for memory T cell survival or proliferation (25). Studying mixed lymphocyte reactions, Poulin et al. showed that IL-9 depletion impaired IL-5 secretion by responding naive CD4 T cells and that optimal IL-5 production required functional IL-9 receptor expression on the CD4 T cell surface (26). In our studies, the effect of IL-9 depletion was critically dependent on timing; depletion during RSV challenge had no effect on disease severity (as measured by weight loss), cellular influx to the airways, or in the cytokine profiles of both the airways and lung cells. By contrast, depletion of IL-9 at immunization significantly reduced disease severity and cellular influx to the airways with a concomitant reduction in Th2 cytokine production by CD4 T cells. Therefore, IL-9 has critical effects on the establishment of the cytokine environment and responses by naive CD4 T cells but little influence on T cells that have already established patterns of cytokine production.

In our model, control mice show a strong CD4 T cell-driven Th2 memory response after viral challenge that induces significant pathology. Our finding that IL-9 depletion during initial vaccination decreases IL-4 and IL-5 secretion and alleviates lung eosinophilia and Th2-associated weight loss suggests that early IL-9 production during the initial stages of an immune response is critical for setting the cytokine production pattern and activity of antiviral T cells, which are later recruited to the lungs. Conversely, the enhanced pathology observed with IL-9 depletion at immunization and challenge is consistent with the robust Th1 responses observed in these mice. Therefore, IL-9 depletion at different times alters pathology by dictating the phenotype and magnitude of the immune response. Recent studies of skin graft rejection show that IL-9 is a critical factor for regulating mast cell-dependent tolerance (27). Therefore, altering cutaneous mast cell activity by IL-9 depletion at the time of the initiation of T cell responses could have significant effects on immune sensitization in our experimental model.

Our data also show that depletion of IL-9 enhances viral clearance from the lungs regardless of the time of administration of the depleting Ab. This is likely to be partly due to increases in the antiviral Ab, because we observed an increase in RSV-specific IgG2a Abs in the mice depleted during immunization alone. We believe that the enhanced clearance caused by IL-9 depletion reflects a more antiviral environment associated with weakened Th2 responses. The ability of CD4 T cells from mice depleted of IL-9 during immunization and challenge to produce IFN-γ and TNF, as well as recruit CD8 T cells (as observed in our cell transfer studies), lends support to this idea. Even in mice depleted only during challenge there was a reduction in IL-5 production associated with enhanced viral clearance. Thus, IL-9 depletion at different times...
may promote antiviral immunity via different mechanisms; IL-9 depletion at immunization boosts the virus-specific IgG2a Ab, whereas IL-9 depletion at immunization and challenge enhances Th1 and CD8 T cell responses (as demonstrated by the adoptive transfer experiment). IL-9 depletion at challenge may also boost Th1 responses by decreasing IL-5 levels.

By adoptive transfer we found that CD4 T cells from IL-9-depleted mice conferred many of the characteristics of IL-9 depletion on naïve recipients. The transferred CD4 T cells comprised a similar number of the three major Vβ clonotypes known to be RSV-G protein-specific (supplemental Fig. 2), but those from mice depleted at immunization and challenged produced more IFN-γ upon stimulation (supplemental Fig. 3), consistent with differences in the cytokine profile during the acute response. After RSV challenge, the recruitment of CD8 T cells to the BAL when CD4 T cells from mice depleted at both immunization and challenge were transferred, compared with those depleted at immunization alone, demonstrate the shift to a Th1 profile in memory CD4 T cells upon the abrogation of IL-9 vs depletion at immunization only. CD4 T cells from the mice depleted only at immunization had no significant effect on disease severity or phenotype. This apparent difference from the initial observation (Fig. 2a) may be explained by the lack of IL-9 and IL-5 in the lung environment of the recipient mice, allowing primed CD4 T cells to elicit Th1 responses upon RSV challenge.

In conclusion, our findings show that IL-9 plays a key role in the development of virus-specific Th2 responses and in modulating antiviral immunity. These new insights extend our understanding of the role of IL-9, which has not previously been shown to be involved in antiviral defense. Moreover, they open the field for further studies of the role of IL-9 and mast cells in the regulation of anti-infective immune responses in animal models and human infections and suggest that studies of the association of bronchiolitis and later childhood wheeze should include elucidation of the role of IL-9.

Acknowledgments
We thank Sarah Wythe and Prof Brigitta Askonas for critical reading of this manuscript and Siti Affendi for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References
Supplemental Figure 1. Study design. Mice were treated as described in Figure 3. Anti-IL-9 antibody was administered one day before, or two days after scarification with rVV-G or RSV challenge. Mice were weighed for 14 days and organs harvested at the indicated time points post challenge.

Supplemental Figure 2. TCR Vβ expression by splenic CD4 T cells. Mice were treated as in Figure 3. Groups were naïve (‘N’), Immunisation only (‘I’), and Immunisation & challenge (‘I&C’) as previously described. Mice were left for 28 days after RSV challenge, the spleens removed cell prepared. Cell counts were determined by trypan blue exclusion and counted on a haemocytometer. TCR Vβ expression (Vβ8.2, 8.3, and 14) was determined on CD4 T cells from individual mice by flow cytometry. Results are expressed as number of CD4 T cells expressing each clonotype. ANOVA (Tukey post test) result *: p<0.05, **: p<0.01, ***: p<0.001.

Supplemental Figure 3. IFN-γ and IL-5 expression by RSV-G-specific CD4 T cells. Mice were treated as in Figure 3. Spleen cells (2×10⁶/ml) from each group were cultured with control media, RSV G peptide (10μg/ml), or αCD3 (5μg/ml) in a final volume of 2ml overnight. IFN-γ production was detected using the cytokine capture (Miltenyi Biotech) and cells analysed on a Dako Cyan 9 flow cytometer. At least 50 000 cells were analysed from each sample. Representative dotplots are shown for each group are shown. Results are expressed as percentage of CD4 T cells expressing each cytokine.
Harvest points

Immunization With rVV-G

RSV Challenge

Depletions with anti-IL9 antibody

0    2     4          7         10         14

'd' group

'C' group

'IC' group

Dodd et al. Supplemental Fig.1.
CD4 T cell Vβ expression

Dodd et al. Supplemental Fig.2.
Dodd et al. Supplemental Fig.3.