Virally delivered cytokines alter the immune response to future lung infections

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Running title: Viral disease after lung cytokine expression

Viral disease after cytokine expression in the lung
Abstract

Respiratory Syncytial Virus (RSV) is an important cause of infant morbidity and mortality worldwide, increasingly recognised to have a role in the development and exacerbation of chronic lung diseases. There is no effective vaccine and we reasoned that it might be possible to skew the immune system towards beneficial non-pathogenic responses by selectively priming protective T cell subsets. We therefore tested recombinant RSV (rRSV) candidates expressing prototypic murine Th1 (Interferon gamma) or Th2 (Interleukin-4) cytokines, with detailed monitoring of responses to subsequent infections with RSV or (as a control) influenza A. Although priming with either recombinant vector reduced viral load during RSV challenge, enhanced weight loss and an enhanced pulmonary influx of RSV-specific CD8+ T cells was observed after challenge in mice primed with rRSV/IFN-γ. By contrast, rRSV/IL-4 primed mice were protected against weight loss during secondary challenge, but showed airway eosinophilia. When rRSV/IL-4 primed mice were challenged with influenza, weight loss was attenuated but was again accompanied by marked airway eosinophilia. Thus, immunization directed towards enhancement of Th1 responses reduces viral load but is not necessarily protective against disease. Counter to expectation, Th2-biased responses were more beneficial but also influenced the pathological effects of heterologous viral challenge.
Introduction

Viral lung infections are an important cause of mortality and morbidity worldwide (18). Respiratory syncytial virus (RSV) is the main cause of infantile bronchiolitis and therefore infant hospitalisation, and is associated with asthma and wheezing in later life (28,29). Studies in humans and animal models emphasise the importance of host responses in the pathogenesis of RSV disease (22).

In humans, severe RSV disease is associated with increased Th2 responses and with mutations associated with increased transcription of the Interleukin-4 (IL-4) gene (6). An increased IL-4/Interferon gamma (IFN-γ) ratio is also seen in the nasal secretion of those infants with RSV lower respiratory tract infection compared to those with milder upper respiratory tract infection (16). Another type 2 cytokine, IL-9, has also been recently been found at high levels in the airways of infants with severe bronchiolitis (17).

A number of studies in murine models of RSV disease have defined roles for both Th1 & Th2 responses. IFN-γ receptor$^{-/-}$ and IFN-γ$^{-/-}$ mice display increased eosinophilia and airway hyperresponsiveness (AHR) following RSV infection (24), whilst IL-4$^{-/-}$ mice clear RSV with minimal pathology (2). Use of anti-IL-5 and anti-IL-4 antibodies during RSV infection also reduces the development of AHR after airway sensitisation (24), and overexpression of IL-4 has also been shown to reduce cytotoxic T lymphocyte activity and reduce viral clearance in some cases (1,11).

The pattern of response to RSV can also be modified by prior exposure to previous infections or vaccination with RSV antigens. Primary infection induces
Th1 responses (14), and vaccination that enhances Th1 responses tend to be associated with reduced disease during infection (30). Expression of RSV G, F, or M2 result in: T-helper 2 cell expansion and eosinophilia (31); T-helper 1 cell expansion and neutralising antibodies (33); or cytotoxic CD8+ T cell expansion respectively (7). Immunization with formalin-inactivated RSV tends to induce Th2-biased responses and lung eosinophilia and enhanced disease during RSV infection but this disease has recently been shown to be abrogated by the reduction of carbonyl groups in the formalin treated vaccine (19).

One possible approach to the development of novel and effective vaccines is to use recombinant vectors to deliver host cytokines to the site of infection, with the aim of programming protective, non-pathogenic host responses. For example, expression of IFN-γ augments Th1 responses and suppress the Th2 responses, whilst IL-4 has the opposite effect (21). However, the effects of cytokine expression in viral vectors can lead to an adverse immune profile. For example, recombinant mousepox (ectromelia) expressing IL-4 causes enhanced disease and widens lethality to normally non-susceptible mouse strains. Such recombinants cause fatal illness even in vaccinated hosts (15).

We have developed live recombinant RSV vectors expressing murine Th1 (IFN-γ) (4) or Th2 (IL-4) (5) cytokines. rRSV/IFN-γ shows many characteristics that make it seem a good vaccine candidate, with attenuated growth in vivo and good protection against subsequent viral replication (4). By contrast, the IL-4 expressing virus has been reported to cause delayed and reduced CD8+ T cell responses without affecting viral clearance (5).
To test and compare these recombinants further, we studied the development of virus-specific immune responses to these two vectors in parallel and examined the effects of secondary challenge with RSV or influenza A in vaccinated mice. The results were unexpected: priming with rRSV/IFN-γ caused marked weight loss during secondary challenge with wild-type RSV (wt RSV), with enhanced lymphocytosis and an increase in virus-specific CD8+ T cells. By contrast, rRSV/IL-4 did not cause any weight loss but did lead to significant lung eosinophilia during secondary challenge with either RSV or, surprisingly, influenza A infection.
Materials and Methods

**Virus stocks & Mouse Infection.** cDNA clones of mIL-4 and mIFN-γ were modified to be flanked by the RSV gene start and gene end transcription signals and Xmal sites. The PCR products were digested with Xmal and inserted into an Xmal site that had been engineered into the G-F intergenic sequence of a cloned cDNA of the RSV antigenome. The recombinant viruses were recovered by cotransfection of the antigenomic plasmid and plasmids expressing the N, P, L, and M2-1 support proteins into HEp-2 cells along with the recombinant MVA vaccinia virus expressing T7 polymerase (4,5).

Wild type A2 strain RSV (wt RSV) and recombinant RSV virus expressing murine IFN-γ (rRSV/IFN-γ) or IL-4 (rRSV/IL-4) were grown in HEp-2 cells. Four to 8 week old female BALB/c mice (Harlan Ltd., Queen’s Park, UK) were maintained in pathogen-free conditions according to institutional and UK Home Office guidelines. Mice were inoculated with 5x10^5 PFU of virus in 100µl intranasally (i.n.). Mice were challenged with 10^6 PFU of wt RSV in 100µl. Influenza A strain X31 was obtained from NIMR. Five haemagglutinin (HA) units of the X31 strain were given i.n. using a volume of 100µl. After challenge infection, individual body weights were measured daily.

**Cell Recovery and analysis.** Collection of bronchoalveolar lavage (BAL) for cells and supernatants, harvesting of lung tissues and staining for flow cytometry were carried out as previously described (8). For visualization of peptide-specific, cytokine-producing CD8+ T cells, 2x10^6 lung cells were
incubated with the RSV peptide M2^{82-90} (SYIGSINNI) for 5h in the presence of IL-2 (50 U/ml) and 10µg/ml Brefeldin A. Cells were analyzed on a BD LSR flow cytometer collecting data on at least 50,000 events. Data was analysed using WinList (Verity).

Quantification of Viral RNA. Total 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 for the RSV L gene using 900 nM forward primer (5'-GAACTCAGTGTAGGTAGAATGTTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTTTCTCTGCAAT-3') and 100 nM probe (5'-FAM- TTGAACCTGTCTGAACAT-TAMRA-3') on an ABI Prism 7000 Sequence Detection System as described (9).

Cytokine ELISA. Cytokine levels were assessed from BAL supernatants as described before (8). Briefly, ELISA plates (Nunc) were coated with capture antibody (anti IL-4 or anti 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. Colour development was terminated with 2N H₂SO₄, and OD read at 490nm. The concentration of cytokine was determined from the standard curve.
Virus-specific antibody ELISA. Serum antibody was assessed by ELISA as described previously (8). RSV antigen was prepared by infecting HEp-2 cells with RSV at 1 PFU/cell. Microtiter plates were coated overnight with 100µl of a 1:500 dilution of either RSV or HEp-2 antigen. Purified influenza X31 antigen was kindly provided by Dr Alan Douglas (NIMR). 400ng/well was coated to Microtiter plates overnight at 4°C in Sodium carbonate buffer (pH9.6). 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. Colour development was blocked with 2M H$_2$SO$_4$, and OD read at 490nm. RSV-specific antibody was 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 peroxidase-conjugated rabbit anti-mouse Ig secondary antibody for antibodies specific for mouse IgG1 and IgG2a.

Statistical analysis. Results are expressed as mean ± S.E.M. Statistical significance was calculated by ANOVA followed by Student's t test for p value as described in the legends using GraphPad Prism software.
Results

Primary infection with recombinant RSV expressing IFN-γ or IL-4

BALB/c mice were infected with wt RSV, rRSV/IFN-γ or rRSV/IL-4. Using TaqMan PCR to monitor viral load, we found that rRSV/IFN-γ was significantly attenuated, showing an approximate 10 fold reduction in peak viral replication on day 4 and day 8 respectively (Fig. 1A), confirming previous findings using viral plaque assay (4). On days 2 and 4, striking increases in the associated cytokines were present in the bronchoalveolar lavage (BAL) fluid, indicating efficient in vivo expression of IFN-γ or IL-4 (Fig. 1B, C).

Secondary infection with wild type RSV of mice primed with recombinant RSV expressing IFN-γ or IL-4

On day 28 after primary infection, mice were challenged with wt RSV. During secondary viral challenge, mice primed with rRSV/IFN-γ showed significantly enhanced and sustained weight loss compared to mice primed with wt RSV or rRSV/IL-4 (Fig. 2A). This weight loss was associated with an early boost in total BAL cell numbers (Fig. 2B, C), particularly lymphocytes (Fig. 2D). Mice primed with rRSV/IL-4 showed significant eosinophilia during secondary infection (Fig. 2E) not seen in the other groups of mice. However, this eosinophilia was not associated with increased weight loss. No virus was detected in any group on d4 post challenge (data not depicted).

Examining the lung lymphocyte subsets in more detail, rRSV/IFN-γ primed for enhanced CD8+ T-cell responses during secondary challenge (Fig. 3A), but
fewer CD4+ cells (Fig. 3B). DX5+ (natural killer - NK) cells tended to be most abundant on day 4, and declined on day 7 and 15 in mice primed with wt RSV or rRSV/IFN-γ. However, mice primed with rRSV/IL-4 showed a reversal of this pattern with a low initial NK response, climbing and remaining sustained to day 15 (Fig. 3C).

To examine the functional specificity of the CD8+ T-cell subset primed with rRSV/IFN-γ, lung cells were re-stimulated in vitro 7 days after challenge with the M2 peptide (SYIGSINNI), the immunodominant RSV MHCI epitope for BALB/c mice (derived from amino acids 82-90 of the RSV M2-1 protein). CD8+ cells expressing IFN-γ in response to this peptide were abundant in mice primed with rRSV/IFN-γ. Expression of IL-4 during primary infection had no significant effect on the abundance of IFN-γ-positive CD8+ cells during challenge (Fig. 4A, B). No IL-4 producing CD8+ cells were detected (data not shown), nor were there significant peptide-specific TNF-α producing cells.

The rate of appearance and levels of RSV-specific immunoglobulin during primary infection was unaffected by virally expressed IL-4 or IFN-γ (Fig. 5A). However, on day 15 of primary infection, expression of IL-4 significantly boosted IgG1 and depressed IgG2a responses to RSV (Fig. 5B). By contrast, expression of IFN-γ depressed IgG1 responses on day 15 of primary infection (Fig. 5B). During secondary challenge with RSV, mice initially infected with rRSV/IFN-γ had depressed levels of total RSV specific immunoglobulin (Fig. 5C) while expression of IL-4 resulted in a marginal increase of the antibodies. Expression of IL-4 during
primary infection tended to boost IgG1 responses during secondary challenge; while expression of IFN-γ led to some reduction in IgG2a responses (Fig. 5D).

The influence of recombinant RSV infection on challenge with influenza A

To determine whether the effects were specific to RSV, we tested the effect of rRSV on challenge with influenza A. Mice were infected with wt RSV, rRSV/IFN-γ, rRSV/IL-4 or left naive; four weeks later mice were challenged i.n. with 5 HA units of the X31 strain of influenza A, and groups of mice harvested on days 3, 8 and 16 after challenge.

Mice that had received wt RSV or rRSV/IL-4 showed a faster recovery from influenza infection induced weight loss than naïve mice or mice that had been primed with rRSV/IFN-γ (Fig. 6A). Influenza titres in all 4 groups were similar on day 3 and day 8 post challenge, and RSV-L gene was not detectable prior to, or during challenge (data not depicted).

BAL was performed on days 3, 8 and 16 post challenge. Total viable cell counts showed that cellular infiltration was greatest in all groups on day 8. Mice primed with rRSV/IFN-γ showed significantly increased BAL cell counts on d3 and d8 compared to mice treated in other ways (data not depicted and Fig. 6B). Conversely, mice primed with rRSV/IL-4 showed reduced cell numbers on day 8 post challenge compared to other mice (Fig. 6B). Primary infection with recombinant RSV had no significant effect on anti-influenza or anti-RSV antibody titres (Fig. 6C).

At d8 p.i., the majority of BAL cells in naïve mice or mice primed with wt RSV or rRSV/IFN-γ were CD8+ T cells (Fig. 6E). CD8+ T cell levels were similar...
in naïve mice or mice infected with rRSV/IL-4 whilst CD4^+ T cell levels were slightly higher in mice primed with RSV/IL-4 (Fig. 6D). However, rRSV/IL-4 priming lead to a significant increase in the number of eosinophils present in the BAL both at day 3 (data not depicted) and day 8 post challenge (Fig. 6F). At day 8 post challenge, elevated levels of BAL IL-4 (Fig. 6G) and IL-5 (Fig. 6H) were seen in mice primed rRSV/IL-4, and of IFN-γ in mice primed with rRSV/IFN-γ (Fig. 6I).
Discussion

Our recombinant RSV vectors were highly effective at producing murine cytokines in vivo. Primary infection with wt RSV or cytokine-expressing viruses induced no appreciable weight loss, but did induce distinct patterns of host immunity which resulted in reduced viral load during subsequent challenge with non-recombinant RSV. An unexpected finding was that priming with rRSV/IFN-γ resulted in weight loss during secondary challenge with native RSV. This altered immunopathology was characterised by an influx of lymphocytes into the lungs during challenge, especially RSV specific CD8+ T cells producing IFN-γ. By contrast, rRSV/IL-4 induced lung eosinophilia during secondary challenge, but without evidence of disease augmentation as characterised by weight loss. Remarkably, the downstream effects of RSV-delivered cytokines were not limited to RSV challenge: when influenza was used as the challenge virus, we again observed enhanced CD8+ T cell influx in rRSV/IFN-γ primed mice, and eosinophilia in mice primed with rRSV/IL-4.

Other studies have shown the potential of IL-4 to increase the severity of viral infections (27). Co-expression of IL-4 in ectromelia overcame genetic resistance to infection, making the recombinant lethal to resistant mouse strains (15). This suggested that IL-4 might lead to an excessive Th2 response and increase viral replication; by contrast, we found no such pathogenic effect with rRSV/IL-4. This virus was essentially non-pathogenic and induced protective immunity to secondary RSV challenge. The rRSV/IL-4 virus also dampened the T cell response during challenge, with fewer CD4 and CD8 cells detectable in the lung. These differences may be due to the different nature of the viruses and viral...
clearance mechanisms. In the case of ectromelia, both NK and CD8+ T cells are important in lysis of infected cells and reducing the viral load, whereas cellular immunity is often pathogenic in RSV disease. This suggestion is supported by the observation that local over-expression of IL-4 in mice infected with influenza A does not affect viral clearance or mortality compared to wild type controls, despite a significantly reduced CD8+ T cell response (3).

Altering the secondary outcome by different priming regimes not only applies to the whole virus but also to the delivery of individual viral proteins. It has been shown that, priming with various RSV proteins causes weight loss, illness and enhanced lung pathology during RSV challenge (22). In some ways, the effects we observed with rRSV/IL-4 mirrors the effect of immunization with vaccinia virus expressing RSV glycoprotein G (which leads to enhanced Th2 responses and lung eosinophilia during challenge). Eosinophilia per se does not appear to cause enhanced weight loss. It may, however, lead to airway remodelling (13) or airway hyperresponsiveness (25), neither of which we studied. The effects of rRSV/IFN-γ parallel those of priming with vaccinia expressing the RSV M2 protein (which generates powerful CD8+ T cell responses and weight loss) (22). These results underline the need for caution when developing vaccines designed to skew T helper responses. In viral infections, there is a general presumption that Th1 responses are safe and antiviral but that Th2 pathogenic, but the present results instead show the opposite. Whilst cytokines can be used as effective adjuvants in some models (12), their use needs to be carefully considered on a case-by-case basis.
Another interesting phenomenon is the non-specific effect of viral cytokine delivery. It has been shown that previous exposure to pathogenic agents can alter the course of secondary respiratory infections. This can either be in the same organ (10) or in different organs (34). Our data on heterologous secondary infection shows that rRSV/IL-4 can induce enhanced levels of IL-4 during influenza challenge and result in eosinophil recruitment. We think this is probably due to the presence of bystander RSV-specific Th2 T cells early during heterologous challenge, thus influencing the phenotype and function of influenza-specific T cells. The effect of rRSV/IFN-γ on the recruitment of CD8+ T cells during influenza A challenge is also of interest. It has been shown that influenza specific CD8+ T cells are recruited during RSV infection, and can dampen eosinophilia (32), and that RSV specific T cells remain in the airways for up to 50 days post infection (23). Thus it is possible that bystander recruitment of IFN-γ primed RSV-specific CD8+ T cells are responsible for this increase. However no difference was observed in the percentage of RSV-specific CD8+ T cells during influenza re-challenge. It is also possible that infection creates inducible lymphoid tissue (iBALT) which may skew future infections (20).

Another possibility is that the recombinant RSV lead to persistent infection (26) and thus to persistent production of the encoded cytokine. However, we were unable to detect RSV L gene at any time later than day 12 after primary infection with the recombinants using TaqMan RT-PCR. Further, RSV-F and NS2 genes (which have a higher transcriptional frequency) were undetectable prior too or during influenza re-challenge (data not depicted). We ascribe the absence of persistence to the use of recombinant RSV vectors instead of the strains used.
in the previous study, and believe that persistence of recombinant virus is not responsible for the altered phenotype observed during secondary challenge. Rather, we propose that innate and acquired immune responses to secondary challenge were modified by local antigenic and cytokine ‘imprinting’ resulting from the primary infection.

This work is of interest because it shows that cytokine delivery by viral vectors has an effect on the target organ, not only during the initial primary infection but also during challenge with the same pathogen. Furthermore, cytokine delivery can skew the immune environment in the lung so that heterologous infections are also affected, and Th1 skewing is not always beneficial to the safe and effective clearance of viral infections.
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Reference List


Viral disease after cytokine expression in the lung


Figure Legends

Figure 1
Primary infection of mice with recombinant RSV expressing murine cytokines. 4 week old BALB/c mice were infected i.n. with wt RSV (grey squares), rRSV/IFN-γ (white circles, broken line) or rRSV/IL-4 (black triangles).

Viral titre during primary infection, measured by Taqman quantification of the RSV L gene (A). Bronchoalveolar (airway) lavage cytokine levels after infection measured by ELISA; IFN-γ (B), IL-4 (C). Points represent mean ± SEM of n≥4 mice. * p<0.05 between outlier group and other groups.

Figure 2
Effects of primary infection with cytokine expressing virus on secondary RSV challenge. Mice infected i.n. with wt RSV (grey squares), rRSV/IFN-γ (white circles) or rRSV/IL-4 (black triangles) were all challenged i.n. with wt RSV four weeks later. Weight loss following 2° challenge (A). Airway cell number four days (B) and seven days (C) after challenge. Airway cell types seven days after challenge after challenge: lymphocytes (D); eosinophils (E). Points and bars represent mean ± SEM of n≥4 mice. * p<0.05 between outlier group and other groups.
Figure 3

Lymphocyte subsets in lung after RSV rechallenge. Mice infected i.n. with wt RSV (grey squares), rRSV/IFN-γ (white circles, broken line) or rRSV/IL-4 (black triangles) were challenged i.n. with wt RSV four weeks later. Lung cells were analysed by flow cytometry CD8⁺ T cells (A), CD4⁺ T cells (B), NK cells (C). Points represent mean of n≥4 mice ± SEM. * p<0.05.

Figure 4

rRSV/IFN-γ primed mice have increased levels of RSV specific IFN-γ secreting CD8⁺ cells in lungs. Mice infected i.n. with RSV (grey squares), rRSV/IFN-γ (white circles) or rRSV/IL-4 (black triangles) were challenged i.n. with wt RSV four weeks later. Lung cells from mice day 7 after challenge were taken and stimulated ex vivo with RSV M2 peptide. Sample FACS plots of individual mice (A). Pooled data (B); dots represent individual mice, lines represent mean of n≥4 mice ± SEM. * p<0.05.

Figure 5

Priming with rRSV/IL-4 alters the subtype of RSV specific serum antibody. RSV-specific antibody was measured by ELISA in sera of mice infected i.n. with wt RSV (grey squares), rRSV/IFN-γ (white circles) or rRSV/IL-4 (black triangles).

Primary infection: Total anti-RSV Ig (A); IgG subtypes (B - IgG2a white, IgG1 Black) measured at 15 days post infection. RSV Challenge: Total anti-RSV Ig (C); IgG subtypes (D - IgG2a white, IgG1 Black), 15 days after RSV rechallenge. Points/Bars represent mean of n≥4 mice ± SEM. * p<0.05. A₄₉₀ at 1:400 dilution.
Figure 6

RSV is partially protective against challenge with influenza A.

Four week old BALB/c mice were infected i.n. with wt RSV (grey squares), rRSV/IFN-γ (white circles, broken line) or rRSV/IL-4 (black diamonds) or left as naïve mice (white squares). 4 weeks later the mice were infected with X31 flu i.n. Weight loss following 2°C challenge (A). Airway cell number (B), Anti-Influenza and Anti- RSV total immunoglobulin (C), CD4+ T cell number (D), CD8+ T cell number (E) and airway eosinophilia (F) on d8 after challenge. BAL cytokines (pg/ml) on d8 after challenge, IL-4 (G), IL-5 (H), IFN-γ (I). Points/Bars represent mean of n≥4 mice ± SEM. * p<0.05, ** p<0.01, *** P<0.001.
Figure 1

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Figure 2

A.

B.

C.

D.

E.
Figure 3

A. Lung CD8+ T cells x 10^6
- wt RSV
- rRSV/IL-4
- rRSV/IFN-γ

B. Lung CD4+ T cells x 10^6

C. Lung NK cells x 10^6

Days after 2nd infection
Figure 4

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Figure 5

A

B

C

D

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Figure 6

A. % Original weight vs. Days post challenge for different groups.

B. Airway cell number x 10^6 for different groups.

C. Flu Specific Ig (A490) for different groups.

D. Airway CD4+ T cell x 10^5 for different groups.

E. Airway CD8+ T cell x 10^5 for different groups.

F. Airway eosinophils x 10^4 for different groups.

G. Airway IL-4 (pg/ml) for different groups.

H. Airway IL-5 (pg/ml) for different groups.

I. Airway IFN-γ (pg/ml) for different groups.

Legend:
- wt RSV
- rRSV/IL-4
- rRSV/IFN-γ
- 1° Flu