A kinetic analysis of immune mediators in the lungs of mice infected with vaccinia virus and comparison with intradermal infection

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The early inflammatory response to a virus may be critical in restricting infection and in shaping the subsequent adaptive immune response. In this study we have examined the early inflammatory response of mice following infection with vaccinia virus (VV) strain Western Reserve (WR). Respiratory challenge of BALB/c mice with VV led to early virus replication in the lung and upper respiratory tract followed by dissemination of virus to other visceral organs and to the brain. The number of inflammatory cells, largely macrophages and T lymphocytes, recovered from bronchoalveolar lavage (BAL) fluid increased markedly during infection and coincided with the expression of CC chemokine ligands (CCL) 3, 2 and 11 and CXC chemokine ligands (CXCL) 1 and 2/3 in BAL. The peak of the inflammatory response occurred around day 10 and declined thereafter. The antiviral cytokines IFN-γ and TNF-α, and the reactive nitrogen intermediate nitric oxide (NO), were also detected in BAL from VV-infected mice. A markedly different inflammatory response was observed after intradermal inoculation of WR into the ear pinnae of mice. Intradermal challenge was followed by highly localized virus replication and by a cellular influx, consisting largely of neutrophils and T lymphocytes, into the dermal compartment of the infected ear. Together these findings highlight differences in the pathogenesis and in the cellular inflammatory response to WR following intranasal and intradermal inoculation of mice.

INTRODUCTION

*Vaccinia virus* (VV) and other members of the *Poxviridae* are complex DNA viruses that replicate in the cell cytoplasm and encode numerous proteins to interfere with specific components of the host response to infection (Alcamı ¯ & Koszinowski, 2000; Smith, 2000). These proteins provide important information regarding disease pathogenesis and their study is relevant to the development of recombinant VV vaccines. The development and characterization of appropriate animal models is therefore critical to understanding the infection processes and the interactions of VV proteins with the host. One problem in the development of experimental models of VV infection is that the natural host of the virus remains unknown (Baxby, 1981); however, VV displays a broad host range. The mouse model has been used extensively to examine VV infection and pathogenesis, with inoculation described via intracranial, intravenous, intranasal (i.n.) and intradermal (i.d.) routes (Turner, 1967; Buller et al., 1985; Williamson et al., 1990; Lee et al., 1992; Tscharke & Smith, 1999; Tscharke et al., 2002). Natural infections with orthopoxviruses can occur through the respiratory tract (Fenner et al., 1989): thus the i.n. model may provide some important insights into the host response to VV infection.

I.n. administration of VV strain Western Reserve (WR) results in an acute, productive infection of the lung followed by dissemination of the virus to visceral organs and the brain. The virus induces an inflammatory infiltrate in the lungs and is cleared 10 to 15 days after infection. The mechanisms regulating leukocyte trafficking during VV infection are currently poorly understood; however, it seems likely that chemokines and/or cytokines play an important role in the cellular events required for motility and adhesion. Therefore, we wished to characterize the inflammatory response to acute VV infection of the lung to investigate the nature of the leukocytes recruited and the inflammatory mediators present. For comparison, we have also examined the inflammatory response to the highly localized infection that follows i.d. inoculation of WR into the ear pinnae of mice (Tscharke & Smith, 1999; Tscharke et al., 2002) and which more closely mimics dermal vaccination. The analysis of the host response to i.n. and i.d. infection with wild-type VV strain WR establishes a benchmark against which the response to infection with virus mutants lacking specific immunomodulatory proteins can be compared.
METHODS

Cells and viruses. Monkey BS-C-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (DMEM(10)). VV strain WR was grown, titrated and purified as described (Mackett et al., 1985).

Infection of mice. To assess virulence in the i.n. model, groups of female 6- to 8-week-old BALB/c mice were anaesthetized and infected i.n. with 10^5 p.f.u. of VV in 20 μl of PBS. Each day, mice were weighed individually and monitored for signs of illness (Alcamì & Smith, 1992), and those suffering a severe infection or having lost >25% of original body weight were sacrificed. To determine virus titres in organs, mice were sacrificed and their lungs, trachea, brain and spleen were removed, homogenized, frozen and thawed three times and sonicated. For nasopharyngeal washings, mice were anaesthetized and 1 ml of sterile PBS containing 1 mg BSA ml⁻¹ was injected slowly into the external nares, and the outflow was collected from the nasopharynx. The samples were assayed for infectious virus by plaque assay on BS-C-1 cells.

In the i.d. model, groups of female C57BL/6 or BALB/c mice were anaesthetized and 10 μl containing 10^5 p.f.u. of WR was injected into the ear pinnae as described (Tscharke & Smith, 1999; Tscharke et al., 2002). Mice were examined daily and the diameter of lesions at the inoculation site was estimated to the nearest 0.5 mm using a micrometer. To determine virus titres in ears, mice were killed and the entire ear was removed, ground using 1 ml tissue grinders, sonicated and subjected to three rounds of freezing and thawing before the titre of infectious virus was determined by plaque assay on BS-C-1 cells.

Recovery of immune cells for flow cytometry and cytotoxic assays. Bronchoalveolar lavage (BAL) fluid and lungs were obtained from mock-infected and VV-infected mice at various times post-infection (p.i.) as described (Reading et al., 2003).

After i.d. inoculation, the cells in the inflammatory ear dermis were recovered as described previously (Belkaid et al., 1996, 1998). Briefly, at various times after i.d. inoculation the ears were collected, rinsed in 70% ethanol and allowed to dry. The ventral and dorsal dermal sheets were separated using fine forceps and transferred immediately to culture medium (RPMI 1640 supplemented with 2.5 mM HEPES pH 7.4, 10% FBS, 50 IU penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹) and placed in a sterile plastic plate (hydrophobic surface). Serial sheets were separated using fine forceps and transferred immediately to culture medium (RPMI 1640 supplemented with 2.5 mM HEPES pH 7.4, 10% FBS, 50 IU penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹). Dermal side down into a sterile plastic plate (hydrophobic bottom) for 8 h at 37 °C in 5% CO₂. Nonadherent cells that had emigrated out of the dermal layers were pooled and loosely adherent cells were recovered by further incubation for 20 min at 37 °C in PBS without calcium or magnesium, containing 2 mg glucose ml⁻¹. Adherent and nonadherent cells were pooled, filtered through a 70 μm nylon cell strainer (Becton Dickinson) and washed twice in medium. Red blood cells were lysed in Tris/NH₄Cl and the remaining cells were washed in medium and the total number of viable cells was determined by trypan blue exclusion. The remaining cells were used in FACs analysis, cytotoxicity assays and induction of intracellular cytokines.

Flow cytometric analysis of cell-surface antigens. Single cell suspensions of BAL or dermal cells were blocked with 10% normal rat serum and 0.5 μg of Fc block (Pharmingen) in FACs buffer (PBS containing 0.1% BSA and 0.1% sodium azide) on ice for 20 min. Cells were stained with appropriate combinations of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- or quantum red (QR)-labelled antibodies to F4/80 (clone CLA3-1, Caltag), DEC-205 (clone NLDC-145, Serotec), Class II MHC (clone 2G9, Pharmingen), Ly-6G (clone RB6-8C5, Caltag) and CD3 (clone CT-CD3, Caltag). The relevant isotype antibody controls were also used. After staining, the cells were fixed with 2% paraformaldehyde in PBS, washed and 10^6 cells were analysed for each sample. Inflammatory cells were recognized by characteristic size (forward scatter, FSC) and granularity (side scatter, SSC) combined with two- and three-colour analysis as described (Belkaid et al., 1996, 1998). Briefly, macrophages were identified as F4/80-positive and MHC class II-low or -negative. Lymphocytes were identified by their small size and by CD3 staining. Neutrophils were small, Ly-6G-positive and F4/80-negative cells. Dendritic cells (DC) were large cells that were MHC class II- and DEC-205-positive. Each sample was also costained in parallel and stained with Diff Quick (Dade Behring) and the percentages of neutrophils, lymphocytes and mononuclear phagocytes were estimated by microscopic examination; the proportions of cells determined by this method was similar to those obtained by flow cytometry.

Lymphocyte subsets were also analysed by flow cytometry using the following mAbs: anti-αβ T-cell receptor (TCR; clone H57-597, Caltag), anti-γδ TCR (clone GL3, Caltag), anti-CD3 (clone CT-CD3, Caltag), anti-CD4 (clone CT-CD4, Caltag), anti-CD8a (clone 53-67, Sigma), anti-B220 for B lymphocytes (clone RA3-6B2, Caltag) and anti-NK cell (clone DX5, Caltag). FITC- or PE-conjugated isotype-matched antibodies were used as negative controls. Lymphocytes were identified by their characteristic FSC/SSC characteristics, and 10^4 cells were analysed for each sample.

Chemotaxis assays. The chemotactic activity of BAL fluid was evaluated using 10 mm tissue culture inserts (Life Technologies) placed in 24-well plates and single cell suspensions of mediastinal lymph nodes (MLNs) from mice 10 days after i.n. infection with 10^5 p.f.u. of WR (Reading et al., 2003). Briefly, a 0.5 ml aliquot of BAL fluid or PBS was placed in the lower compartment and 0.5 ml of MLN suspension (7.5 × 10^6 cells) was placed in the upper chamber, separated by a polycarbonate filter (8 μm pore size). After incubation at 37 °C for 2 h, the filter was washed, fixed and stained with 4’,6’-diamidino-2-phenylindole. The number of migrating cells in at least five high-powered (400 ×) fields was counted using a fluorescence microscope.

ELISA for cytokine and chemokines in BAL. Cytokines were measured in BAL by specific ELISA. ELISA kits for IFNγ, TNFα and IL-4 were purchased from Pharmingen. Levels of CC chemokine ligands (CCLs) 2, 3 and 11 (formerly MCP-1, MIP-1α and eotaxin, respectively) and CXC chemokine ligands (CXCLs) 1 and 2/3 (formerly KC and MIP-2, respectively) in mouse BAL fluids were measured by commercial ELISA kits for CCL3 (R&D Systems) and CCL2 (Pharmingen), and CCL11. CXCL1 and CXCL2/3 levels were determined by sandwich ELISA using matched Ab pairs from R&D Systems according to the manufacturer’s instructions. Reagents for CCL2, CCL11, CXCL1 and CXCL2/3 ELISAs were kindly supplied by Clare Lloyd and Lynne Murray, Biomedical Sciences Division, Imperial College, London, UK. BAL samples and culture supernatants were centrifuged at 1000 r.p.m. for 10 min before the concentration of chemokines was determined.

Nitrite determination. Measurement of nitrite in BAL fluids provides an indirect indication of the amount of nitric oxide (NO) production in the lung. Nitrite was measured colorimetrically at 540 nm by mixing 100 μl of BAL fluid with freshly prepared Griess reagent as described elsewhere (Ding et al., 1988). Each sample was assayed in duplicate and sodium nitrite was used to generate standard curves.

Cytotoxic assays. VV-specific cytotoxic T lymphocyte (CTL) activity in single cell suspensions from BAL, lungs and ears was assayed in a standard 3HCr-release. When effector cells were prepared from BALB/c mice P815 cells (H2b, mastocytoma) were used as targets and EL4 targets (H2b, thymoma) were used to assay CTL activity from C57BL/6 mice. Prior to labelling with Na2CrO4 (150 μCi per 3 × 10^6 cells), P815 or EL4 cells were mock-infected or infected with VV WR at 10 p.f.u. per cell for 2 h at 37 °C. Serial
dilutions of effector cells were incubated in triplicate cultures with either uninfected or VV-infected target cells in 100 μl of R10 in 96-well V-bottomed plates at 37 °C in 5% CO2. After 6 h cells were collected by centrifugation and 50 μl of supernatant was transferred to a Lumaplate-96 (Packard) and counted. The percentage of specific 51Cr release was calculated as: % specific lysis = ([experimental release − spontaneous release])/(total detergent release − spontaneous release]) × 100%. The spontaneous release values were always <10% of total lysis.

In some experiments, CD8+ cells were depleted from lung cell suspensions by incubation at 37 °C with an anti-CD8 mAb (clone 3.115; Sarmiento et al., 1980) in the presence of human complement. Analysis by flow cytometry revealed selective depletion of the desired cell populations. Depleted cells were added to cytotoxicity assays without adjustment for the depletion in cell number.

Intracellular staining for cytokine expression. To detect intracellular cytokines, 10^6 lung or ear cells ml⁻¹ were stimulated with 50 ng PMA (Sigma) ml⁻¹ and 500 ng ionomycin (Calbiochem) ml⁻¹ in the presence of 10 μg brefeldin A (Sigma) ml⁻¹ for 5 h at 37 °C. Cells were washed with FACS buffer and stained with QR-conjugated CD4 (clone CT-CD4, Caltag Laboratories) and FITC-conjugated CD8α (clone 53-67, Sigma) for 30 min on ice and then fixed for 30 min at room temperature with 2% paraformaldehyde in PBS. Samples were permeabilized with 0.5% saponin in FACS buffer for 10 min. PE-conjugated anti-mouse IFN-γ (clone XMG1.2 Pharmingen) or anti-mouse IL-4 (clone 11B11, Pharmingen) was added for a further 30 min at room temperature and the cells were washed once with 0.5% saponin in FACS buffer and twice in FACS buffer alone. Cells were analysed on a Becton Dickinson flow cytometer collecting data on at least 20 000 lymphocytes.

RESULTS

Intranasal infection of mice with VV

To study the pathogenesis of VV infection, BALB/c mice were infected i.n. with 10^4 p.f.u. of VV strain WR and monitored daily for weight loss and signs of disease. Infection with this dose of WR resulted in severe weight loss, ruffled fur, huddling behaviour and pneumonia (Fig. 1a, b). All mice did, however, recover from infection.

We next examined the ability of VV to grow within the respiratory tract of mice. Mice were inoculated i.n. with 10^4 p.f.u. of VV strain WR under anaesthesia and the titres of virus present in lung and tracheal extracts and in nasopharyngeal washings were determined by plaque assay. As shown in Fig. 1(c), VV was recovered from all three sites by day 1 p.i. and peak virus titres were recorded between days 3 and 7. Virus was completely eliminated from tracheal extracts and nasopharyngeal washings and was detected in only 1/5 lung extracts by day 15.
WR is a neurovirulent strain of VV that was passaged repeatedly in suckling mouse brain (Bronson & Parker, 1941). In the murine i.n. model, VV infection is accompanied by extensive respiratory infection and virus dissemination to multiple organs (Turner, 1967; Williamson et al., 1990). To examine the kinetics of virus dissemination, homogenates of brain, spleen and liver were assayed for infectious virus at various times after i.n. infection (Fig. 1d). Virus titres were below detection limits at 4 h and 1 day p.i.; however, infectious virus was recovered from all organs by day 3. Virus peaked between days 3 and 7 and had been completely cleared by day 15. These results demonstrate that following i.n. inoculation WR replicates in the upper respiratory tract and lung before spreading to secondary sites of infection including the brain, spleen and liver.

Leukocyte recruitment to the lung following i.n. infection with VV

To examine the inflammatory response the numbers and types of cells infiltrating the lungs were assessed at various intervals after infection. Cell numbers in the BAL increased significantly after infection, with a maximum increase of approximately 8-fold noted 10 days p.i. (Fig. 2a). Few cells (approximately 10⁴ cells per mouse) were recovered from BAL of uninfected mice. By using mAbs and flow cytometry to identify specific inflammatory cell types, we determined the nature of the cellular infiltrate present in BAL during the course of VV infection (Fig. 2b). The inflammatory cells comprised mainly lymphocytes and mononuclear phagocytes, with few neutrophils at any time tested. The proportion of lymphocytes rose after day 3 and peaked at nearly 50% of all cells at day 10. Morphological examination

Fig. 2. Characterization of cells recovered from the BAL of VV-infected mice. BALB/c mice were infected i.n. with 10⁴ p.f.u. of WR and cells infiltrating into the lungs were collected at various times via BAL. (a) Total numbers of viable cells were determined by trypan blue exclusion. Data are means ± SEM of cell counts for four or five mice at each time-point and are representative of two independent experiments. (b) Inflammatory cell types, and (c) lymphocyte subsets in BAL from VV-infected mice. BAL cells were stained with PE- or FITC-conjugated mAbs. Cells were analysed by flow cytometry, and classified as described in Methods. The detection limit was less than 1% based on staining with isotype-matched control antibodies. The means ± SEM of data from two separate experiments at each time-point are shown. Groups of four to five mice were used at each time-point. TCR, T-cell receptor.
and quantification by light microscopy confirmed these findings (data not shown).

The proportions of lymphocyte subsets in the BAL was determined by flow cytometric analysis. Fig. 2(c) shows the majority of lymphocytes to be $\alpha/\beta$-T cells at days 7, 10 and 15, with small numbers of $\gamma/\delta$-T cells, B cells and NK cells detected.

**Chemokine levels in BAL fluids from VV-infected mice**

Chemokines are important mediators of leukocyte motility and adhesion (Baggiolini, 1998; Rossi & Zlotnik, 2000). To gain insight into factors controlling leukocyte recruitment during VV infection, we assayed BAL fluids by ELISA for the CC-chemokines CCL2, 3 and 11 (formerly MCP-1, MIP-1$\alpha$ and eotaxin, respectively) and the CXC-chemokines CXCL1 and 2/3 (formerly KC and MIP-2, respectively). Baseline levels of chemokine expression were measured in BAL fluids from mock-infected animals. Despite considerable dilution during the lavage process, each chemokine could be detected in BAL fluids from infected, but not mock-infected animals (Fig. 3a). For all chemokines tested, only low levels of protein could be detected in BAL fluids early in infection between days 1 and 3. Chemokine levels peaked around days 7 to 10 p.i., coinciding with the period of maximal cellular infiltration into the lung (Fig. 2a). The chemotactic activity of BAL fluid could also be demonstrated in vitro; day 7 BAL fluid chemoattracted MLN cells of VV-infected mice in a microchemotaxis assay more efficiently than did BAL fluid from days 1 or 15 (Fig. 3b). These findings are consistent with chemokines in the lung playing an important role in leukocyte trafficking during VV infection.

**IFN-$\gamma$, TNF-$\alpha/\beta$ and nitrite levels in BAL fluids from VV-infected mice**

A number of immune mediators have been shown to play important roles in the resolution of acute infection by poxviruses such as VV. Because VV is sensitive to IFN-$\gamma$ in vitro (Mellkova & Esteban, 1994) and IFN-$\gamma$ production is critical for recovery of mice after VV infection (Karupiah et al., 1990; Ruby & Ramshaw, 1991; Dalton et al., 1993), we investigated if this cytokine could be detected in BAL following VV infection. As shown in Fig. 4(a), IFN-$\gamma$ was detected in BAL fluids, peaking between days 7 and 10, corresponding to the maximal influx of leukocytes into the lung (Fig. 2).

TNF is another important cytokine associated with inflammation and antiviral defence, and several strains of VV (but not WR) encode soluble and cell-surface TNF receptors (vTNFRs) (Alcamì et al., 1999; Reading et al., 2002). TNF-$\alpha$ was also detected in BAL fluids 7 and 10 days after infection with WR (Fig. 4b). Whilst both IFN-$\gamma$ and TNF-$\alpha$ were detected in BAL, we were unable to detect IL-4, a Th2-type cytokine, in BAL fluid from mock- or VV-infected mice at any time-point tested ($< 10 \text{ pg ml}^{-1}$) (data not shown).

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**Fig. 3.** Chemokine levels and chemotactic activity in BAL fluid from VV-infected mice. (a) BAL was performed on BALB/c mice at various times after infection with $10^4$ p.f.u. of WR and chemokine concentrations were determined by ELISA in cell-free supernatants. BAL fluids from four or five mice were pooled for each time-point. The detection limits for the assays were 8 pg ml$^{-1}$ for CCL3, 20 pg ml$^{-1}$ for CCL2, 5 pg ml$^{-1}$ for CCL11 and 10 pg ml$^{-1}$ for CXCL1 and CXCL2/3. Data are expressed as mean concentrations $\pm$ SEM for two independent experiments. (b) Chemotactic activity of BAL fluid from VV-infected mice for leukocytes. Cell-free BAL supernatants were collected from mock-infected mice, or from mice 1, 7 and 15 days p.i. with $10^4$ p.f.u. of WR, and their ability to induce leukocyte migration was determined in a chemotaxis assay. Each BAL tested represents the pool from groups of three to five mice. Single cell suspensions were prepared from MLNs of mice infected 10 days previously with $10^4$ p.f.u. of WR. The mean number of migrating MLN cells per field $\pm$ SEM under high power ($n=5$) is shown.
Reactive nitrogen intermediates such as nitric oxide (NO) have been shown to inhibit VV replication in vitro (Karupiah & Harris, 1995) and in vivo (Rolph et al., 1996). The inducible nitric oxide synthase (iNOS) enzyme produces high levels of NO and is up-regulated by cytokines such as IFN-γ and TNF-α. The NO reaction produces nitrite, molecular oxygen and water, and thus nitrite levels provide an indication of NO production. As seen in Fig. 4(c), nitrite levels peaked 10 days p.i. and declined thereafter.

**I.d. infection of mice with VV**

In contrast to the systemic infection observed following i.n. inoculation of VV, i.d. injection of VV into mouse ear pinnae is characterized by a local infection without signs of systemic illness (Tscharke & Smith, 1999; Tscharke et al., 2002). We therefore wished to examine the cellular inflammatory response elicited following i.d. inoculation and compare this with the i.n. model. Preliminary studies showed that C57/BL6 mice developed larger lesions with a greater number of infiltrating cells than did BALB/c mice (Tscharke et al., 2002) and that the increased cell numbers facilitated a quantitative analysis. For this reason, C57BL/6 mice have been used in the following studies. Lesion size (Fig. 5a) and infectious virus titres (Fig. 5b) were determined at various times after i.d. inoculation with 10^4 p.f.u. of VV WR. Despite the larger lesion size, peak virus titres recovered from ear homogenates were similar to those reported after i.d. inoculation of BALB/c mice with this dose of virus (Tscharke & Smith, 1999), although virus was cleared earlier (day 12) from BALB/c mice. Infectious virus was not found in brains, spleens or livers of infected C57BL/6 mice at any time-point (data not shown), confirming that WR infection is highly localized in this model.

**Leukocyte recruitment to the ear following intradermal infection with VV**

Cells were recovered from the ears of mice at various times after i.d. inoculation with 10^4 p.f.u. of WR (Methods) and

![Fig. 4](image-url)  
*(a) IFN-γ; (b) TNF-α and (c) NO in BAL fluids from VV-infected mice. BAL was performed on BALB/c mice at various times p.i. with 10^4 p.f.u. of WR and cell-free supernatants were assayed for IFN-γ and TNF-α by ELISA, and for nitrite levels as described in Methods. BAL fluids from four or five mice were pooled for each time-point and data represent the mean ± SEM from two individual experiments. Detection limits were 20 pg ml^{-1} for IFN-γ, 15 pg ml^{-1} for TNF-α and 5 μM for nitrite.*

![Fig. 5](image-url)  
*(Virulence of VV strain WR in the i.d. model. Groups of C57BL/6 mice were inoculated i.d. in the left ear pinnae with 10^4 p.f.u. of WR and lesion sizes (a) were estimated daily. Points represent the mean lesion size ± SEM from at least six mice. (b) Infectious virus titres in ear homogenates were also determined. Points represent the mean virus titre ± SEM from groups of four or five mice.)*
viable cells were quantified by trypan blue exclusion (Fig. 6a). Few cells were recovered from the ears of mock-infected animals or from VV-infected animals at days 1 and 3 p.i. Cell numbers increased significantly by day 7 p.i., and peaked at day 15. Analysis became difficult after this time as mice developed scabs that fell off sporadically over a period of a week, and this led to inconsistent results between animals.

Cell populations within the infiltrate were identified and quantified by flow cytometry as described in Methods (Fig. 6b). A striking feature of the cellular infiltrate in the i.d. model is the predominance of neutrophils, which constituted the majority of recruited cells recovered from ears at 7 and 10 days p.i. Compare this to the infiltrate in BAL following i.n. inoculation, in which neutrophils comprised <10% of cells at all time-points tested (Fig. 2b). Macrophages were recruited early (day 3) and low numbers of DCs were detected at all time-points tested. Analysis of cells from mock-infected and VV-infected ears 1 day p.i. were not performed due to limited cell numbers recovered from the dermal compartment. The composition of the cellular infiltrate recovered from the ears of BALB/c mice 10 days after infection with $10^4$ p.f.u. of WR was similar to that from C57BL/6 mice, with neutrophils and lymphocytes the predominant cell type (data not shown). A more detailed analysis of the BALB/c model was difficult due to limited numbers of recruited cells.

Next, the lymphocyte subsets recovered from VV-infected
ears were characterized at days 7, 10 and 15 p.i. (Fig. 6c). The majority of lymphocytes expressed α/β-TCR, with low numbers of γ/δ-TCR, B lymphocytes and NK cells detected.

**Functional analysis of T lymphocytes in the lung during VV infection**

T lymphocytes are important in the resolution of primary virus infection, through cytolysis of infected cells and secretion of cytokines such as IFN-γ. Results presented above illustrate the recruitment of CD4⁺ and CD8⁺ T lymphocytes to the lungs (Fig. 2c) and ears (Fig. 6c) of mice following i.n. and i.d. inoculation, respectively; however, they reveal nothing about the function of these cells. Therefore, CTL activity was assayed in the BAL (Fig. 7a) and lung (Fig. 7b) of mice following i.n. infection, and in cells recovered from the ears of mice after i.d. infection (Fig. 7c). CTL activity against VV-infected, MHC-matched targets was detected at day 7 p.i. and was greatest at day 15 p.i., corresponding with the time of virus clearance in either model. All effector populations showed low cytotoxic activity against uninfected MHC-matched targets (<10% at an effector-to-target ratio of 100:1; data not shown). Furthermore, depletion of CD8⁺ cells using an anti-CD8 mAb and human complement abrogated the anti-viral activity of day 10 lymphocytes recovered from lung, BAL or ear (data not shown).

The elevated IFN-γ production detected in BAL fluids from VV-infected mice suggested that lymphocytes recruited to the lungs were producing IFN-γ; therefore, intracellular IFN-γ staining of lung lymphocytes was performed 7, 10 and 15 days after VV infection (Fig. 7d). Intracellular staining demonstrated (i) both CD4⁺ and CD8⁺ lung lymphocytes produced IFN-γ, and (ii) the percentage of cells producing IFN-γ increased throughout infection, peaking at day 15. We also observed intracellular production of IFN-γ in both CD4⁺ and CD8⁺ lymphocytes recovered from the ears of mice 7, 10 and 15 days after i.d. VV infection (Fig. 7e). IL-4 was not detected (<1% of total lymphocytes) in lung or ear cells by intracellular staining at days 7, 10 or 15 (data not shown).

**DISCUSSION**

The inflammatory responses to VV strain WR in mice following i.n. and i.d. infections is reported. I.n. infection led to early virus replication in the upper respiratory tract and lung followed by dissemination to visceral organs and

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**Fig. 7.** CTL activity (a, b and c) and intracellular production of IFN-γ (d and e) by lymphocytes from BAL, lungs and ears after VV infection of mice. Groups of mice were infected i.n. (a, b, d) or i.d. (c, e) with 10⁴ p.f.u. of WR and on days 3 (●), 7 (●), 10 (■) and 15 (▲) p.i. single cell suspensions were prepared. Specific lysis of WR-infected target cells was assessed in a standard ⁵¹Cr-release assay. Each effector-to-target (E:T) ratio was assayed in triplicate. For intracellular IFN-γ production, lung and ear cells were stimulated with PMA and ionomycin for 4 h and brefeldin A was added to retain cytokines in the cytoplasm. Cells were stained with FITC-labelled anti-CD8, QH-labelled anti-CD4, and after permeabilization using saponin, with PE-labelled anti-IFN-γ before analysis by three-colour flow cytometry. Shown is the percentage of CD4⁺ or CD8⁺ T lymphocytes producing IFN-γ. Values are averaged from two groups (n = 3–5 per group).
Chemokines probably play an important role in regulating leukocyte trafficking to the lungs during VV infection. The profile of chemokines induced in the lung following VV infection closely reflects the nature of the cellular infiltration observed in BAL fluids. The proinflammatory properties of CC chemokines such as CCL2 and CCL3 are well known, and these and other chemokines are chemotactic for monocytes and activated T lymphocytes (Rollins et al., 1990; Schall et al., 1993; Carr et al., 1994) that were prominent in the pulmonary infiltrate. The CXC chemokines CXCL1 and CXCL2/3 were both detected in BAL at low levels throughout VV infection, consistent with the low numbers of neutrophils recovered from the lung. The lack of neutrophils in lavage fluids suggests that other neutrophil-attracting CXC chemokines and/or their appropriate receptors are also expressed at low levels. CCL11 has selective chemotactic activity for eosinophils, but despite a moderate expression of this chemokine we were unable to demonstrate eosinophils in the lung of VV-infected mice (Fig. 2). These findings are consistent with other reports citing the expression of CCL11 (and other eosinophil chemoattractants such as CCL3 and CCL5) in the lung of RSV-infected mice in the absence of eosinophil recruitment (Haeberle et al., 2001) and suggest that other eosinophil growth factors such as IL-5 and GM-CSF might be required to promote eosinophil infiltration. Alternatively, expression of the CCL11/eotaxin receptor (CCR3) might be low in VV-infected lungs or perhaps poxviruses such as VV have evolved other strategies to combat eosinophil recruitment. It is unclear whether the chemokines detected in BAL fluids are produced by cells recruited to the site of infection or by cells resident in VV-infected lungs.

A common feature of cellular recruitment to both the lung and the ear was the influx of T lymphocytes by 7 days p.i. The majority of T cells were αβ-TCR+γδ-, with low levels of γδ-TCR+ cells detected at any time-point. Interestingly, γδ-T cells play a role in protection following intraperitoneal infection of mice with VV through their ability to secrete IFN-γ and lyse VV-infected cells (Selin et al., 2001). MHC class 1-restricted CD8+ CTLs are important in recovery from poxvirus infections through cytolytic action of virus-infected cells and CTL activity was demonstrated in cell preparations from BAL, lungs and ears at days 7, 10 and 15 p.i. (Fig. 7a–c). Both CD4+ and CD8+ T lymphocytes in lungs and ears were also shown to produce intracellular IFN-γ (Fig. 7d, e) and IFN-γ was detected in BAL fluids of mice after i.n. VV infection (Fig. 4a). Numerous studies have shown IFN-γ to be critical in the resolution of acute VV infection (Karupiah et al., 1990; Dalton et al., 1993; Huang et al., 1993), and although VV and other poxviruses encode a number of intracellular and extracellular proteins to inhibit IFN-γ, the VV IFN-γ receptor secreted from infected cells is unable to neutralize mouse IFN-γ (Alcamí & Smith, 1995; Mossman et al., 1995; Symons et al., 2002b). Regulation of IFN-γ might also be influenced by the ability of VV strain WR (and other poxviruses) to express a binding protein for IL-18 (Smith et al., 2000; Symons et al., 2002a), given that this cytokine promotes NK cell and T cell activation. The antiviral activity of IFN-γ is mediated through the activation of leukocytes and the induction of several proteins and enzymes including iNOS. Induction of iNOS in the lung can lead to the production of reactive nitrogen species (Fig. 4c) that have been shown to inhibit VV replication (Karupiah & Harris, 1995; Rolph et al., 1996).

Mouse models of infection have been used to uncover potential roles for a number of VV genes. In a recent report we compared a range of deletion mutants of VV strain WR along with their relevant wild-type and revertant viruses.
in i.n. and i.d. models and showed that of 16 genes tested, half are associated with distinct phenotypes in only one of the models (Tscharke et al., 2002). The differing inflammatory responses observed following i.n. and i.d. are consistent with the ability of certain mutants to express a phenotype in one model but not the other. For example, a secreted viral CC chemokine inhibitor (vCC) expressed by several strains of VV and other orthopoxviruses (cowpox and camelpox) was shown to reduce virulence and regulate leukocyte trafficking in the lungs after i.n. VV infection (Reading et al., 2003); however, no phenotypic differences were associated with expression of vCC in the i.d. model (Tscharke et al., 2002). These findings are not surprising given that neutrophils, the predominant cell type recruited to the dermal compartment after VV infection, are sensitive to modulation by CXC, rather than CC, chemokines. These findings highlight the importance of using more than one model when studying VV virulence.

In summary, the data presented in this report provide a reference characterization against which mutant viruses can be compared and highlight the different characteristics of the host response to infection observed following inoculation of VV by different routes.

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