Deletion of gene A41L enhances vaccinia virus immunogenicity and vaccine efficacy

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INTRODUCTION

Vaccinia virus (VACV) is the vaccine that was used to eradicate smallpox and is being developed as a recombinant vaccine for other pathogens. Removal of genes encoding immunomodulatory proteins expressed by VACV may enhance virus immunogenicity and improve its potential as a vaccine. Protein A41 is a candidate for removal, having sequence similarity to the VACV chemokine-binding protein, vCKBP, and an association with reduced inflammation during dermal infection. Here, it is shown that, at low doses, VACV strain Western Reserve (WR) lacking A41L (vA41L) was slightly more virulent than wild-type and revertant controls after intranasal infection of BALB/c mice. The primary immune response to vA41L was marked by an increase in the percentage of VACV-specific gamma interferon-producing CD8+ T cells and enhancement of cytotoxic T-cell responses in the spleen. However, this augmentation of cellular response was not seen in lung infiltrates. Splenic CD8+ T-cell responses were also enhanced when VACV strain modified vaccinia virus Ankara (MVA) lacking A41L was used to immunize mice. Lastly, immunization with VACV MVA lacking A41L provided better protection than control viruses to subsequent challenge with a 300 LD50 dose of VACV WR. This study provides insight into the immunomodulatory role of A41 and suggests that MVA lacking A41 may represent a more efficacious vaccine.
mind, vaccination strategies utilizing MVA to generate strong CD8+ T-cell responses are being developed (Sutter & Moss, 1995). Preliminary data suggest that DNA prime–MVA boost is an effective way of generating a strong CD8+ T-cell response (Hanke et al., 1998; Schneider et al., 1998) with high-avidity CD8+ T cells (Estcourt et al., 2002).

Although MVA is already a useful vector, its immunogenicity may be enhanced by the removal of other genes encoding immunomodulatory proteins that remain in the genome. MVA gene 184 encodes an interleukin 1β (IL-1β)-binding protein that is secreted from infected cells (Antoine et al., 1998; Blanchard et al., 1998), analogous to the soluble IL-1β receptor (IL-1βR) encoded by other VACV strains and cowpox virus (Alcamí & Smith, 1992, 1996; Spriggs et al., 1992). Recently, it was shown that removal of this gene enhanced the CD8+ T-cell response generated against MVA (Staub et al., 2005). Another immunomodulatory protein expressed by MVA is A41.

Gene A41L was studied previously in VACV strain WR and is conserved in all 16 strains of VACV tested (Ng et al., 2001). A41 has amino acid similarity to a family of poxvirus proteins that are secreted from the infected cell and bind CC chemokines or other ligands. These include the T1 protein from Orf virus, the T2 protein from cowpox virus (Alcamí & Smith, 1992, 1996; Spriggs et al., 1992), and a related protein from Orf virus, celled GIF, that binds IL-2 and granulocyte–macrophage colony-stimulating factor (Deane et al., 2000). The ligand for A41 remains unknown, but deletion of the gene from VACV strain WR suggested an immunomodulatory role for this protein. In a mouse intradermal model of infection (Tschark & Smith, 1999, 2002), the mutant lacking the A41L gene induced larger lesions with a greater influx of inflammatory cells compared with control viruses, and the rate of virus clearance was accelerated (Ng et al., 2001).

In this study, we have examined the immunogenicity of VACV strains WR and MVA lacking gene A41L. By using VACV strain WR vA41L, we show that the primary immune response following intranasal infection exhibits an increase in the relative number of virus-specific splenic CD8+ T cells that display cytolytic activity or produce gamma interferon (IFN-γ). A similar increase was seen in the CD8+ T-cell memory response against MVA recombinants lacking A41L compared with controls. This increase in virus-specific memory CD8+ T cells was accompanied by increased protection in mice challenged intranasally with VACV WR. Therefore, not only was a particular arm of the immune response affected by A41, but removal of this immunomodulator created a more potent vaccine.

**METHODS**

**Cells and viruses.** VACV strain WR was grown in BSC-1 cells in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and was titrated as described previously (Mackett et al., 1985). VACV WR lacking gene A41L (vA41L) and wild-type (vA41L) and revertant (vA41L-rev) control viruses were constructed previously (Ng et al., 2001). VACV strain MVA was grown in CEFs in DMEM with 10% FBS and was titrated on CEFs. To enumerate foci of infection, cell monolayers were stained with rabbit polyclonal antibody to VACV followed by biotinated donkey anti-rabbit IgG antibody (Amersham Biosciences) and streptavidin–alkaline phosphatase (Sigma).

**Construction of VACV strain MVA A41L deletion and revertant viruses.** To construct an MVA lacking gene A41L, oligonucleotide primers were used to amplify the regions flanking A41L from MVA genomic DNA, as was done for VACV WR (Ng et al., 2001). These PCR-generated fragments were cloned into a pGEM-based plasmid, pK1L, which also contained the VACV K1L host-range gene (Tschark & Smith, 2002), and were sequenced to confirm fidelity. The same primers were used because the primer-binding sites are identical in MVA and VACV WR. The resultant plasmid, pdeleteA41L, was transfected into cells infected with a recombinant MVA virus expressing the Plasmodium berghei circumsporozoite protein (Pbscp) (Schneider et al., 1998) and parental (MVA-A41L) and deletion mutant (MVA-AA41L) viruses were constructed by transient dominant selection (Falkner & Moss, 1990), using the K1L host-range gene as a selectable marker (Tschark & Smith, 2002). Recombinant viruses expressing the K1L gene replicated on RK13 cells, whereas those lacking this gene did not, but would grow on CEFs. The genotype of individual plaque isolates was screened by PCR. A revertant virus in which A41L was reinserted into its natural locus in MVA-AA41L was constructed using plasmid pA41Lrev and was called MVA-A41L-rev. pA41Lrev was similar to pdeleteA41L except that it was based on pBluescript rather than pGEM and the full-length MVA A41L gene was ligated into the plasmid, rather than the A41L-flanking regions.

**Immunization strategies.** For infection with VACV WR or derivative viruses, groups of female BALB/c mice (6–8 weeks old) were anaesthetized and infected intranasally with 5 × 105 p.f.u. virus in 20 μl PBS. Mice were weighed individually and monitored for signs of illness (Alcamí & Smith, 1992). Any mouse that had lost >30% of its weight compared with the weight on day 0 was sacrificed. Virus present in the lung and spleen was released by homogenization, freeze–thawing three times and sonication and was titrated by plaque assay on BSC-1 cells. For immunizations with MVA or derivative viruses, mice were infected subcutaneously with 106 p.f.u. virus in 200 μl PBS. After 3 weeks, this procedure was repeated. Three weeks later, the animals were sacrificed and their spleens were processed for immunological analyses (see below). For challenge experiments, mice were immunized subcutaneously with either 106 or 107 p.f.u. MVA. Four weeks later, animals were infected intranasally with 3 × 106 p.f.u. VACV WR and their body weight and signs of illness were monitored. A sample of the inoculum used to infect each group of mice was titrated to ensure that the correct dosage had been administered.

**Recovery of immune cells from infected mice.** For VACV WR infections, mice were sacrificed and lavaged as described by Russell et al. (1997). Bronchial alveolar lavage (BAL) samples were centrifuged at 800 g to obtain BAL cells. Leukocytes were obtained from lung homogenates by enzymic digestion, lysis of erythrocytes and centrifugation through 20% Percoll (Sigma-Aldrich) as described by Lindell et al. (2001). Leukocytes were then resuspended in RPMI/5% FBS. Splenocytes were obtained by homogenization of spleens, lysis of erythrocytes, centrifugation and resuspension in RPMI/5% FBS. Live cells in all single-cell suspensions were enumerated using a haemocytometer and trypan blue exclusion.

**Cell phenotyping, intracellular cytokine staining (ICS) and flow cytometry.** Single-cell suspensions of BAL, lung or spleen cells
were blocked with 10% normal rat serum and 0.5 μg Fc block [BD Biosciences (Pharmingen)] in fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.1% BSA and 0.1% sodium azide) on ice for 30 min. Cells were stained with appropriate combinations of fluorescein isothiocyanate-, phycoerythrin (PE)- or tricolour-labelled anti-CD3, anti-CD8, anti-CD4, anti-F4/80 (macrophage), anti-Ly-6G (neutrophil) or anti-pan NK (DX5) and the relevant isotype antibody controls (Pharmingen). The distribution of cell-surface markers was determined on a FACScan flow cytometer with CellQUEST software (BD Biosciences). A lymphocyte gate was used to select at least 20,000 events.

For ICS, single-cell suspensions of spleen or lung cells (10^6) were stimulated with specific peptides or VACV-infected cells or were mock-stimulated. For peptide stimulation, P815 cells were incubated with the relevant peptide at 10 μg ml^-1 [P99 epitope, SYIPSAEKI; VACV-9 epitope, VGPSNSPTF (D. C. Tscharke, unpublished results)] for 2 h and then washed three times. Mock stimulations were P815 cells incubated with an irrelevant peptide (HIV-10 epitope, RGPGRAGFVTI).

For stimulation with VACV, P815 cells were infected with VACV WR at 10 p.f.u. per cell for 2 h in RPMI and then for a further 2 h after addition of 10 ml RPMI/10% FBS. Cells were then washed three times. Effector and stimulator cells were incubated in RPMI/10% FBS (ratio 5:1) for 5 h at 37°C. After 90 min, brefeldin A (10 μg ml^-1; Sigma) was added. Cells were stained for cell-surface markers (as above) and then fixed with 2% paraformaldehyde in PBS for 30 min at room temperature (RT). Cells were permeabilized with 0.5% saponin in FACS buffer for 10 min. PE-conjugated anti-mouse IFN-γ or tumour necrosis factor alpha (TNF-α) (Pharmingen) was added for a further 30 min at RT and the cells were washed once with 0.5% saponin in FACS buffer and twice in FACS buffer alone. Cells were analysed on a BD Biosciences flow cytometer, collecting data on at least 100,000 lymphocytes.

ELISPOT. The IFN-γ ELISPOT assay using peptide stimulation has been described by Miyahira et al. (1995). Briefly, assay plates (Millipore Multiscreen HA, MAHA54510) were coated overnight with 50 μl rat anti-mouse IFN-γ mAb (4 μg ml^-1; Pharmingen) at 4°C. Plates were washed extensively with PBS and blocked for 2 h at RT with RPMI/10% FBS. Splenocytes resuspended in RPMI/2-5% FBS were added and diluted twofold to yield at least three concentrations. For experimental wells, 2 μl ml^-1 (final concentration) of peptides P99 or VACV-9 or 50,000 WR-infected P815 cells were added to each well and the volume was adjusted to 100 μl with RPMI/2-5% FBS. Samples were assayed in triplicate. Negative-control wells contained splenocytes and 50,000 uninfected P815 cells or an irrelevant peptide (HIV-10) at 2 μg ml^-1.

Plates were incubated at 37°C for 12 h, washed with PBS and incubated for 2 h at RT with biotinylated rat anti-mouse IFN-γ mAb (4 μg ml^-1; Pharmingen). Plates were washed again with PBS and incubated with streptavidin–alkaline phosphatase (Sigma, diluted 1:1000) for 2 h at RT. Plates were washed with PBS again and developed with BCIP/NBT (Sigma). Spots were counted by using an AID ELISPOT reader with version 3.0 software (Autoimmun Diagnostika).

Cytotoxic T-lymphocyte (CTL) cytolyis assay. VACV-specific CTL activity in single-cell suspensions from lung and spleen was assayed by 51Cr-release assay on VACV-infected P815 cells. P815 cells were mock-infected or infected with VACV WR at 10 p.f.u. per cell for 2 h at 37°C in 250 μl RPMI (~FBS) in the presence of Na2CrO4 (2 MBq in 1×10^6 cells). These cells were washed twice, left for 2 h in 10 ml RPMI/10% FBS and washed again. Serial dilutions of effector cells were incubated in duplicate cultures with either mock-infected or VACV-infected target cells in 96-well V-bottomed plates. Cells were collected by centrifugation after 6 h and 50 μl supernatant was transferred to a LumaPlate-96 (Packard Instrument Company, Inc.) 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 <5% of total lysis.

Statistical analysis. Student’s t-test (two-tailed) was used to test the significance of the results (P<0.05).

RESULTS

Deletion of A41L from VACV WR enhances virulence slightly in a murine intranasal-infection model

Previously, it was reported that deletion of the A41L gene did not affect the outcome of intranasal infection of BALB/c mice with doses of 10^4 p.f.u. or greater (Ng et al., 2001). To investigate whether there were differences at lower doses of virus, mice were infected with 5×10^3 p.f.u. vA41L, vAA41L or vA41L-rev and weight loss and signs of illness were monitored (Fig. 1a). Mice infected with vAA41L lost more weight than those infected with either control virus (P<0.05 on day 7) and also showed greater signs of illness.
Deletion of A41L from VACV WR does not alter immunity in the infected lung

Next, the infiltration of cells into the lungs and BAL fluids of mice infected intranasally with vA41L, vΔA41L or vA41L-rev was analysed by flow cytometry. No significant differences in the numbers of infiltrating macrophages, neutrophils, natural killer (NK) cells, CD8\(^+\) or CD4\(^+\) lymphocytes were found in the lung tissue or BAL fluids at days 3, 6 and 9 p.i. (data not shown). This was the case when either the absolute number of cells or the percentage of each cell type was compared.

The CD8\(^+\) T-cell response in the lung was examined more closely by using ICS to determine the percentage of lung CD8\(^+\) cells that produced IFN-\(\gamma\) in response to stimulation with the VACV-9 peptide (a newly identified H-2D\(^d\)-restricted epitope from VACV; D. C. Tscharke, unpublished results). In the gated lung-lymphocyte population, the percentage of CD8\(^+\) cells that produced IFN-\(\gamma\) at either day 6 or 9 p.i. (Fig. 2a, b) was similar following infection with viruses that did or did not express A41. Likewise, after stimulation with VACV WR-infected P815 cells, no difference in the percentage (or absolute number) of IFN-\(\gamma\)-producing CD8\(^+\) cells in the lung was detected on day 6 or 9 p.i. (data not shown).

To determine whether lung lymphocytes from mice infected with vΔA41L showed altered killing of VACV-infected autologous target cells compared with controls, chromium-release assays were performed. No significant difference in cytolytic activity was detected between groups on day 6 or 9 p.i. in the lungs (Fig. 2c, d).

Deletion of A41L increases CD8\(^+\) T-cell responses in the spleen

In contrast to results with lung cells, ICS of splenocytes at days 6 and 9 p.i. revealed an increase in the percentage of CD8\(^+\) cells that produced IFN-\(\gamma\) following intranasal infection with vΔA41L compared with infection with control viruses (Fig. 3a, b). This difference was significant on day 9 (\(P<0.05\)). Similarly, when cells that produced IFN-\(\gamma\) in response to stimulation with VACV WR-infected cells were studied by ELISPOT, there was an increased number of IFN-\(\gamma\)-producing splenocytes (per 1000000 splenocytes) from vΔA41L-infected mice compared with control viruses (Fig. 3c, d) and this difference was significant (\(P<0.05\)) on day 9 p.i. The numbers of spot-forming units for mock-stimulated splenocytes from all mice and stimulated splenocytes from mock-infected mice were negligible (data not shown).

Cytotoxic T-cell killing assays showed that splenocytes from mice immunized with vΔA41L lysed a significantly greater percentage of autologous VACV-infected target cells compared with control groups (Fig. 3e, f) (\(P<0.05\)). Thus, although A41 had little effect on CTL activity at the primary
site of infection, the number of CTLs in the spleen, or their cytolytic effector function, was enhanced when A41 was absent.

Taken together, these data indicate that A41 interferes with the generation of optimal CD8\(^+\) T-cell responses to VACV in the spleen.

**Deletion of A41L from MVA enhances CD8\(^+\) T-cell memory**

VACV strain WR is not a vaccine strain. Therefore, to determine whether the enhanced immune response deriving from loss of the A41L gene from VACV WR was also true for vaccine strains of VACV, the A41L gene was deleted from VACV strain MVA and the immunogenicity was compared with wild-type and revertant controls. The parent virus selected was an MVA strain expressing the *P. berghei* circumsporozoite antigen (Schneider *et al.*, 1998), because this enabled the CD8\(^+\) T-cell response to a foreign antigen to be evaluated in addition to the anti-VACV response. Mice were immunized subcutaneously with 10\(^8\) p.f.u. parental (MVA-Pb), deletion (MVA-Pb-ΔA41L) or revertant (MVA-Pb-A41L-rev) virus on days 0 and 21. Mice were sacrificed on day 42 and the splenic CD8\(^+\) memory immune response was assessed.

Splenocytes were stimulated with VACV WR-infected P815 cells (Fig. 4a) or *P. berghei* peptide (Schneider *et al.*, 1998) (Fig. 4b) and the number of IFN-γ-producing splenocytes was quantified by ELISPOT. Mice immunized with MVA-Pb-ΔA41L virus produced a significantly greater number of IFN-γ-producing cells than mice immunized with either control virus, irrespective of whether the splenocytes were stimulated with VACV WR and the IFN-γ-producing cells were counted. At days 6 (c) and 9 (d) p.i., the cytolytic activity of CTLs derived from spleen was determined by \(^{51}\)Cr-release assay. Cytolytic activity is presented as the mean percentage ± SEM. Significant differences (*P* < 0.05) between vΔA41L and both wild-type VACV and vA41L-rev are indicated.

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**Fig. 3.** CD8\(^+\) T-cell response in the spleen of mice mock-infected (●) or infected intranasally with VACV WR viruses [vA41L (△), vΔA41L (○) or vA41L-rev (■)] as described in the legend to Fig. 1. At 6 (a, c, e) and 9 (b, d, f) days p.i., mice were sacrificed, spleens were excised and single-cell suspensions were prepared. (a, b) ICS analysis of lung cells revealed the percentage of CD8\(^+\) T cells making IFN-γ after stimulation with P815 cells loaded with VACV-9 peptide. (c, d) ELISPOT analysis of IFN-γ-producing splenocytes at days 6 (c) and 9 (d) p.i. Splenocytes were stimulated with P815 cells that had been infected with VACV WR and the IFN-γ-producing cells were counted. At days 6 (e) and 9 (f) p.i., the cytolytic activity of CTLs derived from spleen was determined by \(^{51}\)Cr-release assay. Cytolytic activity is presented as the mean percentage ± SEM. Significant differences (*P* < 0.05) between vΔA41L and both wild-type VACV and vA41L-rev are indicated.
pulsed with the Pb9 peptide (Fig. 5b) or VACV-9 peptide (Fig. 5c).

ICS was also used to measure TNF-α production in the splenocytes of mice immunized with different recombinant MVA viruses (Fig. 6). Although the overall number of splenocytes producing TNF-α was lower than those producing IFN-γ, the number of cells producing TNF-α was higher following infection with MVA-Pb-DΔA41L than in control groups. This result was true when cells were stimulated with VACV-infected cells (Fig. 6a) or cells pulsed with Pb9 (Fig. 6b) or VACV-9 (Fig. 6c) peptides and, in each case, the difference was statistically significant. In conclusion, all of the methods used suggest that removal of A41 from MVA-Pb enhances the size of the antiviral CD8+ T-cell memory pool.

Deletion of A41L from MVA improves vaccine efficacy

To examine whether deletion of A41L from MVA created a more effective vaccine, mice were vaccinated with 10^6 p.f.u. of each virus or were mock-infected and, 28 days later, were challenged with 3 × 10^6 p.f.u. VACV WR. This challenge dose represents approximately 300 LD_{50} for BALB/c mice of this age and, consequently, even vaccinated mice began to lose weight after infection (Fig. 7a). During the first 5 days p.i., a similar trend in weight loss was observed for the mice vaccinated with each virus. However, by day 6 or 7 p.i., animals infected with wild-type and revertant viruses had lost >30% of their original body weight and were sacrificed (humane end point). In contrast, mice vaccinated with MVA-Pb-DΔA41L started recovering weight by day 6 and survived the challenge (Fig. 7a). In addition, by day 7, mice vaccinated with MVA-Pb-DΔA41L showed significantly fewer signs of illness than mice immunized with wild-type and revertant controls and were starting to recover (Fig. 7b).

In another experiment, animals were vaccinated with a higher dose (10^7 p.f.u.), which induced better protection against the same challenge dose. At this immunization dose, all three groups of mice survived the challenge, but, as seen in the first experiment, animals immunized with the virus lacking A41 were protected better and had reduced weight loss (Fig. 7c) and signs of illness (Fig. 7d) than controls. Thus, vaccination with MVA-Pb-DΔA41L not only generated
an enhanced memory CD8\(^+\) T-cell response against VACV and foreign antigens, but it also conferred better protection against challenge with VACV WR.

**DISCUSSION**

Data are presented showing that deletion of gene A4IL from VACV strains WR and MVA increased virus immunogenicity. VACV WR lacking A41 was slightly more virulent in an intranasal-infection model, but induced a stronger CD8\(^+\) T-cell response in the spleen. The upregulation of VACV-specific CD8\(^+\) T cells in the spleen was observed not only in the primary adaptive immune response to intranasal infection with VACV WR, but also in the memory response after subcutaneous immunization with MVA. Moreover, immunization with MVA lacking A41 induced better protection to challenge with a lethal dose of VACV WR. Collectively, these data show that deletion of A4IL from VACV may result in a more immunogenic and efficacious vaccine, particularly if enhanced CD8\(^+\) T-cell responses are important for vaccine efficacy.

The slight increase in virulence of vAA41L in the intranasal model compared with control viruses was seen after infection with low virus doses only and not at the higher doses used previously (Ng et al., 2001). This can be explained by higher doses of virus inducing a more severe infection and so masking the subtle difference induced by loss of gene A4IL. Previously, it was observed that VACV WR strains lacking the IL-1\(\beta\)R (Alcamí & Smith, 1992, 1996) or CC chemokine-binding protein (Reading et al., 2003a) were more virulent than controls expressing these proteins in this model. In the intradermal model, infection with vAA4IL caused a larger lesion size than controls, but the virus was cleared more rapidly than viruses expressing A41 (Ng et al., 2001). In contrast, the slightly increased virulence in the intranasal model was not accompanied by an alteration in virus titres in the lungs or spleen. The altered virulence following intranasal infection might have been attributable to a difference in the cells infiltrating into the infected lung. However, analysis showed that removal of A4IL had no significant influence on either the number of macrophages,

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**Fig. 6.** TNF-\(\alpha\)-producing splenocytes after prime–boost immunization with MVA. Experimental details were as described in the legend to Fig. 5 except that ICS was used to measure production of TNF-\(\alpha\) rather than IFN-\(\gamma\).

**Fig. 7.** Challenge of mice immunized with MVA viruses. Groups of five mice were mock-infected (•) or immunized subcutaneously with 10\(^6\) (a, b) or 10\(^7\) (c, d) p.f.u. of the indicated viruses (■, vA41L; ○, vAA41L; ▲, vA41L-rev) and challenged intranasally 28 days later with 3 \(\times\) 10\(^6\) p.f.u. VACV WR. Weight change (a, c) and signs of illness (b, d) were monitored daily and are presented as described in the legend to Fig. 1. Significant differences between vAA41L and both control viruses are indicated (*\(P<0.05\)).
neutrophils, NK cells, CD8+ and CD4+ T cells migrating into the lung or their activation state (data not shown). So, if increased activation of cells or their infiltration into the lungs was the cause of enhanced virus virulence, these changes may have been in cells not analysed in our study.

Investigations into the CD8+ T-cell response at days 6 and 9 p.i. by using three methods provided strong evidence that A41 influences the adaptive immune response. Firstly, the percentage of CD8+ T cells in the spleen that produced IFN-γ in response to VACV-infected cells or a VACV epitope in ICS assays was increased after infection with vAA41L. This trend was seen early in the adaptive immune response (day 6) and then, more profoundly, at the height of the adaptive response (day 9). Surprisingly, statistically significant upregulation of the IFN-γ response was not observed for the CD8+ T lymphocytes infiltrating into the lung. Secondly, the ICS data were supported by the results of ELISPOT assays. Finally, increased CTL activity, the hallmark of the ICS data were supported by the results of ELISPOT assays was increased after infection with vAA41L. This trend was seen early in the adaptive immune response (day 6) and then, more profoundly, at the height of the adaptive response (day 9).

A possible role for A41 in subverting cellular migration is also suggested by its sequence similarity to known poxvirus chemokine-binding proteins. However, only weak binding to the CXC chemokines IP-10 (CXCL10), ITAC and MIG (CXCL9) was identified and A41 did not inhibit the chemotactic activity of these CXC chemokines in biological assays. Interestingly, IP-10 and MIG are active as chemokine factors for stimulated, but not for resting, T cells and have been shown to have a role in lymphocyte migration to the lung (Loetscher et al., 1996). Additionally, VACV expressing either IP-10 (crg-2) or MIG downregulated IFN-γ production and NK cytolytic activity in the spleen in mouse infections (Mahalingam et al., 1999).

Several immunomodulatory proteins expressed by VACV have an inhibitory influence on the host cellular immunity. A 3β-hydroxysteroid dehydrogenase encoded by gene A44L inhibits the percentage of IFN-γ-producing CD8+ cells in the lung and the cytolytic activity after intranasal infection of mice (Reading et al., 2003b). This enzyme synthesizes steroid hormones, such as glucocorticoids, that have a general immunosuppressive effect. Similarly, the IL-18-binding protein (gene C12L) expressed by VACV WR targets the Th1 response (Symons et al., 2002; Reading & Smith, 2003). In mice infected intranasally with a virus lacking C12L, there was an enhanced CTL activity in the lung and spleen and a dramatic increase in the percentage of IFN-γ-producing CD8+ T cells in the lung. In neither case was the memory CD8+ T-cell response to VACV measured. Here, we show that removal of A41L from MVA enhances the antiviral CD8+ T-cell memory response, as has also been shown for the VACV IL-1βR (Staib et al., 2005).

Lastly, the efficacy of MVA lacking A41L as a vaccine was tested by immunizing mice with MVA with or without A41L and subsequently challenging the animals with a lethal dose of VACV WR. Viruses lacking A41L induced better protection than controls, demonstrating a biological relevance that extends beyond the T-cell assays. While we have shown that CD8+ T-cell responses are increased when A41L is deleted, we cannot assume that the increased protection against a VACV challenge is due only to CD8+ T cells. Measurement of the antibody titres after infection with VACV WR (or MVA) with or without A41 by ELISA (using extracts of VACV-infected cells and purified recombinant B5 protein) and intracellular mature virus neutralization assay showed no significant differences between the groups (data not shown). However, it remains possible that other parameters might contribute to the enhanced protection.

In summary, this study has revealed a novel property of A41 immunobiology and demonstrated that the immunogenicity of VACV-based vaccines can be increased. The influence of A41 on the CD8+ splenocytes gives insight into the immunomodulatory role of this secreted protein and its deletion illustrates the potential benefit of removing other immunomodulators from the MVA genome to improve vaccine potency.

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