Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 345 (2006) 231 – 243

VIROLOGY

www.elsevier.com/locate/yviro

Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host

Min Fang [a], Hong Cheng [b], Zhongping Dai [b], Zimei Bu [b], Luis J. Sigal [a,*]

[a] *Program of Viral Pathogenesis, Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA*

[b] *Program of Biomolecular Structure and Function, Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA*

Received 22 July 2005; returned to author for revision 23 August 2005; accepted 26 September 2005
Available online 25 October 2005

Abstract

Subunit vaccines that use the vaccinia virus extracellular envelope protein A33R alone or combined with other structural proteins are excellent candidates for a new smallpox vaccine. Since a new smallpox vaccine would be used in humans, who are the natural hosts for the *Orthopoxvirus variola*, the agent of smallpox, it would be important to determine whether a prospective smallpox vaccine can protect from a lethal Orthopoxvirus infection in a natural host. We addressed this question using the mouse-specific Orthopoxvirus ectromelia virus. We demonstrate that immunization with recombinant ectromelia virus envelope protein EVM135 or its ortholog vaccinia virus A33R produced in *E. coli* protects susceptible mice from a lethal ectromelia virus infection. This is the first report that a subunit vaccine can provide protection to a lethal Orthopoxvirus infection in its natural host.

Keywords: Rodent; Viral; Immunization; Poxvirus; Vaccinia virus; Ectromelia virus; Smallpox; Subunit vaccine

Introduction

Smallpox, an epidemic disease that decimated human populations periodically throughout history, was caused by variola virus (VARV), an Orthopoxvirus (OPV). Fortunately, smallpox was eradicated worldwide through immunization (Fenner et al., 1988). However, there is great fear that VARV, monkeypox virus (MPXV) or engineered forms of these pathogens could be used as biological weapons (Henderson, 1999). The effect of such an attack would be devastating because massive immunization against smallpox was discontinued in 1978 and most of the human population is presently not immune. Furthermore, in recent years, epidemics of human MPXV have occurred sporadically in west and central Africa, and a recent outbreak occurred in the Midwestern United States (Cunha, 2004; DiGiulio and Eckburg, 2004; Hutin et al., 2001) indicating that preparedness for vaccination against MPXV may be necessary. In addition, there is a theoretical risk that OPVs prevalent in animal populations could cross the species barrier and become human pathogens.

The vaccine used by the World Health Organization to eradicate naturally occurring smallpox disease is live vaccinia virus (VACV), another well-known OPV (Fenner et al., 1988). All OPV are highly homologous at the DNA and protein level, accounting for the cross-protection that infection with these viruses induces (Goebel et al., 1990; Shchelkunov et al., 2001, 2002).

While immunization with live VACV provides excellent protection against smallpox, it routinely causes a pustular skin lesion, frequently induces lymphadenopathy and fever, and occasionally results in life-threatening disease (Cono et al., 2003; Fulginiti et al., 2003). Indeed, immunization with VACV is not recommended for millions of people with immune deficiencies, eczema, atopic dermatitis and heart disease, all of whom are at an increased risk of severe complications. Therefore, there is a need to develop a safer vaccine that,

* Corresponding author. Fax: +1 215 728 2412.
E-mail address: Luis.Sigal@fccc.edu (L.J. Sigal).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.09.056

ideally, should be non-infectious but able to afford full protection.

There are two forms of infectious OPV. The intracellular mature virion (IMV) is infectious when released from disrupted cells. It is resistant to the environment and thought to be important in host-to-host transmission of the virus. However, it induces antibodies that by themselves are not protective. The extracellular enveloped virion (EEV), is released from the infected cells. It is labile in the environment and has an important role in dissemination of the virus within the host. Importantly, it is the target of protective antibodies (Appleyard et al., 1971; Blasco and Moss, 1992; Moss, 1996; Payne, 1980; Smith et al., 2002). Unfortunately, killed VACV induces antibodies but is not effective at preventing disease because the killed virions are mostly IMV (Appleyard et al., 1971; Galmiche et al., 1999; Payne, 1980; Payne and Kristensson, 1985). Therefore, it has been proposed that a new vaccine may include subunit vaccines based on recombinant viral proteins important for virus spread and pathogenesis. In support of this, Galmiche et al. (1999) demonstrated that immunization with recombinant VACV EEV proteins A33R or B5R produced in insect cells protected mice from a lethal intra-nasal challenge with high-dose VACV virus (10^7 plaque forming units, PFU). More recently, Fogg et al. (2004) reported results of immunization with the VACV EEV proteins A33R and B5R and the IMV L1R produced in insect cells. They found that, when used individually, A33R was more protective but that the combination of all three proteins was the most effective. In related experiments, Hooper et al. (2004) found that DNA vaccines consisting of cloned MPXV EEV genes A33R, B5R and IMV L1R and A27L, protected rhesus macaques from high dose intravenous (i.v.) challenge with MPXV. While these are important developments towards a new vaccine, an important caveat is that mice are not the natural hosts of VACV nor are rhesus monkeys a natural host of MPXV, requiring high doses of virus i.v. to initiate infection (Hooper et al., 2004) and bypassing the natural mechanism of virus spread by producing instant viremia (Earl et al., 2004). There are several reasons why a prospective smallpox vaccine (a natural human disease) should be tested in a natural host. First, viruses typically replicate better in natural hosts and the immune mechanism that may protect against a poorly replicating virus in non-natural hosts may not be adequate to clear a virus that replicates and spreads rapidly in a natural host. Second, vaccines must protect individuals from transmission from host to host and experiments of this type can only be performed in natural hosts. Third, only with natural hosts can we evaluate protection to a virus entering through the natural route and spreading as during the normal course of the infection. Fourth, while the ultimate goal of these studies is to develop a novel smallpox vaccine, for obvious ethical reasons, such a vaccine cannot be tested in humans; thus, experiments with similar viruses in natural hosts combined with results of experiments in non-human primates with MPXV (which are not natural pathogens of monkeys but of wild rodents) may collectively provide the best possible surrogates to predict protection from smallpox.

While its evolutionary host is not known, the OPV ectromelia virus (ECTV) can be considered a natural pathogen of the laboratory mouse (*Mus musculus*) because in this species it can complete its life cycle and be perpetuated. In fact, the mouse is the only known natural host of ECTV (Fenner, 1994). While all outbred and inbred strains of laboratory mice can be infected with ECTV by any route, footpad infection of susceptible strains causes mousepox and can be lethal at very low doses only in susceptible strains such as A/J and Balb/c. For example, as few as 3 (plaque forming units (PFU) of the Moscow strain of ECTV injected in the footpad was 100% lethal to A/J male mice (Chen et al., 1992). In our own experiments, 80–100% of Balb/c mice succumbed and 100% developed mousepox following footpad inoculation with 3×10^3 PFU ECTV Moscow. Furthermore, introducing an infected mouse into a cage of uninfected susceptible mice results in natural transmission with 100% incidence of mousepox with high lethality (Fenner, 1994; Niemialtowski et al., 1994).

Because ECTV infection of the mouse is a good model for VARV or MPVX infection in humans, we tested whether mice can be protected from mousepox and death by immunization with a single recombinant EEV protein. Our interest was to test a protein produced in bacteria because it is a very efficient and economical method to generate recombinant proteins. Since immunization with insect-produced A33R is the most effective in protecting mice from lethal VACV infection (Fogg et al., 2004; Galmiche et al., 1999), we produced and tested ECTV EVM135 (the ECTV ortholog of VACV A33R, 90.27% amino acid identity) expressed in *E. coli* as a vaccine against ECTV. In addition, to test whether well-conserved OPV orthologs can be cross-protective, we also produced and tested as a mousepox vaccine recombinant VACV A33R. The results presented here demonstrate that both proteins provide a high degree of protection against death, but not against disease. Our work provides insight for the development of a safer recombinant vaccine to OPV infections in their natural host, which should be useful for the development of new and safer vaccines against smallpox and MPXV.

Results

Cloning, expression and purification of recombinant ECTV EVM135

The coding sequence of the extraviral domains of ECTV EVM135 with a His-Tag at the N terminus was cloned into the expression vector pET-28a(+) to generate pET/EVM135 (Fig. 1A) which was used to produce recombinant EVM135 in *E. coli*. As shown in Fig. 1B, SDS-PAGE and Coomassie brilliant blue revealed a strong band with the expected molecular size for recombinant EVM135 in inclusion bodies but not in the supernatants of *E. coli* induced with IPTG. The band was excised and partially sequenced by Edman degradation, confirming its identity. The inclusion bodies containing EVM

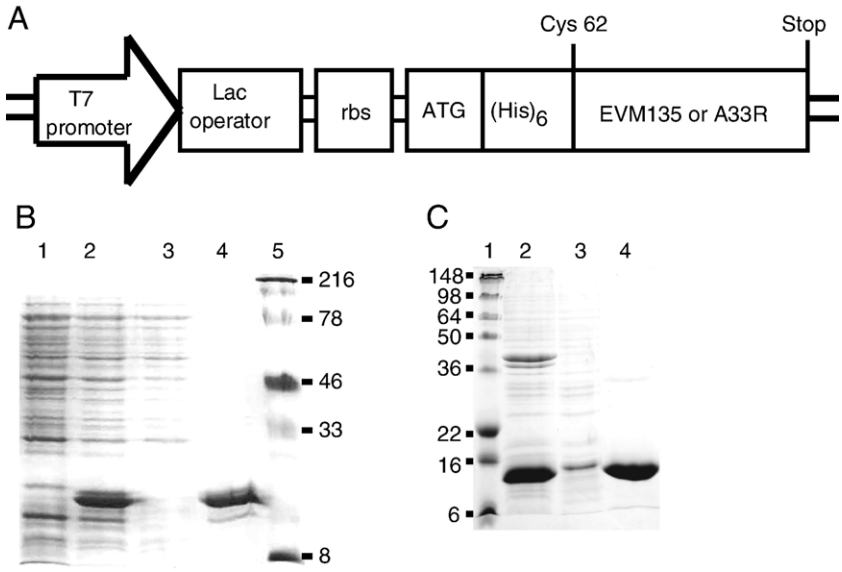

Fig. 1. Cloning, expression and purification of ECTV EVM135. (A) Scheme of the expression vector. The extraviral domains of ECTV EVM135 (Moscow) was amplified by PCR and cloned into pET-28a(+) to yield constructs pET/EVM135. An N-terminal histidine tail was added to facilitate protein purification. (B) Expression of EVM135 in BL21(DE3) cells induced with IPTG. Lane 1: lysate of cells transformed with pET-28a(+). Lane 2: lysates of cells transformed with pET/EVM135. Lane 3: as lane 2 but only the supernatant. Lane 4: as Lane 2 but only the pellet. Lane 5: molecular weight standards. (C) Purification of ECTV EVM135. Lane 1: molecular weight standards. Lane 2: the inclusion bodies before purification. Lane 3: column flow through. Lane 4: purified ECTV EVM135.

135 were washed and solubilized in 8M urea. EVM135 was purified under denaturing conditions by chromatography using Ni-NTA agarose columns (Fig. 1C) and refolded by dialysis over PBS.

Characterization of recombinant EVM135

The production of recombinant proteins in bacteria is simpler and more economical than in insect cells. However, there is the caveat that normally glycosylated proteins may not fold properly when expressed in bacteria. Because EVM135 is normally glycosylated, we determined whether our recombinant EVM135 produced in *E. coli* was properly folded. We recorded far-UV circular dichroism (CD) spectra in the absence and presence of DTT and guanidinium chloride (GuCl) as denaturants. As shown in Fig. 2A, the spectrum of the recombinant EVM135 in PBS exhibited a strong negative band around 200 nm and a minor shoulder around 222 nm. Assuming a molar mean-residue ellipticity of $-34{,}100$ mdeg cm^{-2} $dmol^{-1}$ for a fully α-helical peptide (Scholtz and Baldwin, 1992), the data indicate that recombinant EVM135 has secondary structure with at least 10% α-helix content. Few changes were observed in the spectra following addition of 1 mM DTT indicating that EVM135 has a compact structure that is very stable in solution. When 5.9 M GuCl, was added, the spectra with or without DTT showed that all the features of the spectra compatible with a secondary structure were lost indicating that under these harsh conditions, recombinant EVM135 unfolds. Together, the CD data indicates that recombinant EVM135 is folded in solution.

VACV A33R protein and most likely its orthologs (including EVM135) are glycosylated transmembrane proteins that form dimers joined by disulfide-bonds (Roper et al., 1996). It was therefore important to determine the oligomeric state of recombinant EVM135. Size exclusion gel filtration analysis of the thawed protein stock revealed a major peak with an apparent molecular weight corresponding to that of dimers (peak 2 in Fig. 2B). An additional minor peak detected as a faster eluting shoulder was also apparent (peak 1, Fig. 2B). This seemed to indicate the presence of some contaminating aggregates in the protein stock. Peak 2 was purified by gel filtration and analyzed by light scattering, which allows for the precise determination of the hydrodynamic radius and size of a molecule in solution. As shown in the lower panel of Fig. 2C, the scattering intensity distribution of the purified peak 2 gave a single peak, suggesting the absence of aggregation. The hydrodynamic radius of the protein was 31.9 Å and the molecular weight was 27.7 kDa as determined by dynamic light scattering (DLS) and static light scattering (SLS), respectively. This confirmed the gel filtration data indicating that the major component of EVM135 in our stock forms dimers in solution.

EVM135 and A33R are natural targets of the anti-OPV antibody response

To determine whether ECTV EVM135 and its VACV ortholog A33R are natural targets of the anti-OPV antibody response and whether they are cross-reactive, we produced VACV A33R exactly as described for EVM135. ELISA plates were coated with the recombinant proteins and tested for recognition by anti-ECTV or anti-VACV antisera obtained from mice that had been infected 8 weeks earlier. As shown in Figs. 3A and B, anti-ECTV and anti-VACV antisera recognized both recombinant proteins. However, the cross-reactivity was not

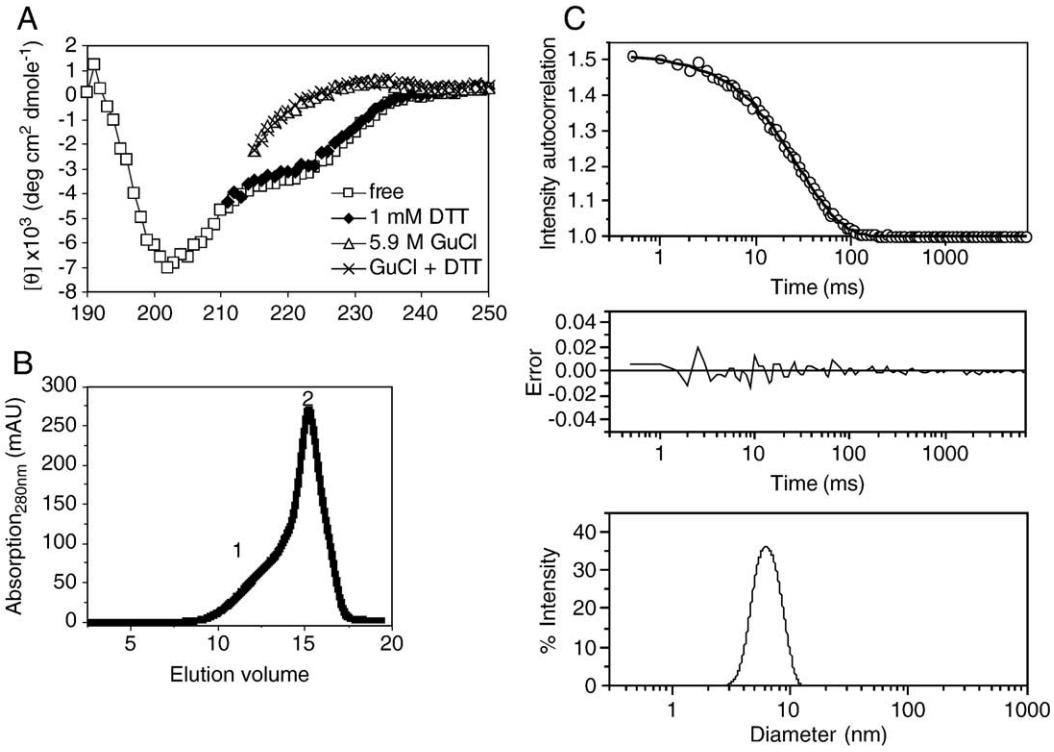

Fig. 2. Recombinant EVM135 is folded and forms dimers in solution. (A) Far-UV circular dichroism spectra was recorded in the absence and presence of DTT and GuCl as denaturants as indicated. (B) Gel filtration separation and analysis of EVM135. The protein shows a major peak at elution volume of about 15 ml consistent with the size of dimers. (C) Dynamic light scattering spectra of peak fraction (2). The hydrodynamic radius of the protein measured by dynamic light scattering (DLS) was 31.9 Å and the molecular weight was 27.7 kDa as determined by static light scattering (SLS) confirming that the protein forms dimers in solution. The upper panel is the measured correlation function (O). The solid line is the regularization fit to the correlation function. Middle panel is the sum-of-squares differences between the experimental measured autocorrelation function and the fit. Lower panel is Laplace transformation of the regularization fit. A single peak indicates the absence of aggregation.

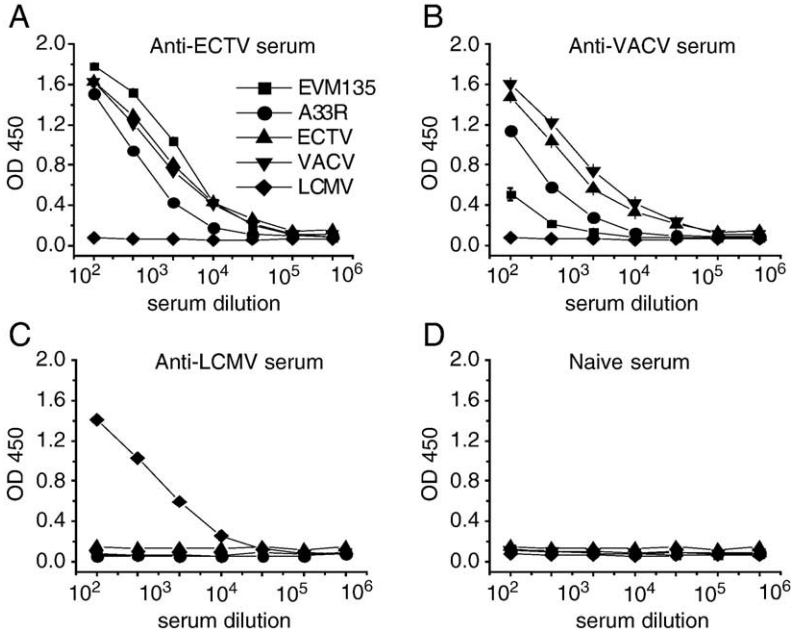

Fig. 3. EVM135 and A33R are natural targets of the anti-OPV antibody response. 96-well bottom ELISA plates were coated with purified recombinant EVM135, A33R or lysates of ECTV, VACV or LCMV-infected cells as indicated. The indicated antisera were collected from ECTV, VACV or LCMV infected mice 8 weeks pi. (A) Reactivity of ECTV-immunized serum with different antigens. (B) Reactivity of VACV-immunized serum with different antigens. (C) Reactivity of LCMV-immunized serum with different antigens. (D) Reactivity of naive serum with different antigens.

complete because each antisera recognized the homologous protein better. As expected, both antisera recognized control VACV or ECTV-coated plates (Figs. 3A and B). Additional controls confirmed that the ELISA assays were specific because the anti-ECTV or anti-VACV antisera did not react with ELISA plates coated with lysates of LCMV-infected cells (Figs. 3A and B) while anti-LCMV antisera reacted with plates covered with LCMV lysates but not with any of the OPV antigens (Fig. 3C). Moreover, sera from naive mice did not react with any of the antigens (Fig. 3D). Together, these data indicate that EVM135 and A33R are natural targets of the anti-ECTV and anti-VACV antibody responses and that the elicited antibodies are cross-reactive with the heterologous protein. In addition, the data show that the recombinant EVM135 and A33R maintain at least some of the epitopes of the natural proteins produced during infection.

Immunization with EVM135 or A33R elicit antibody responses in mice

To determine whether recombinant EVM135 or A33R can elicit antibody responses in mice, groups of 10 mice were immunized twice in the base of the tail with incomplete Freund adjuvant (IFA) admixed with 10 μg EVM135 (IFA-EVM135) or A33R (IFA-A33R) in PBS or only with PBS (IFA-PBS) as negative control. The presence of specific antibodies before immunization and 3 weeks after the second immunization was determined in sera by ELISA. The results using pooled sera from 10 mice shows that immunization with EVM135 and A33R elicited strong IgG responses , which seemed to be partially cross-reactive as detected by ELISA in which plated EVM-135 was recognized ≈3-fold better by anti-EVM135 antisera (Fig. 4A) and A33R was recognized ≈3-

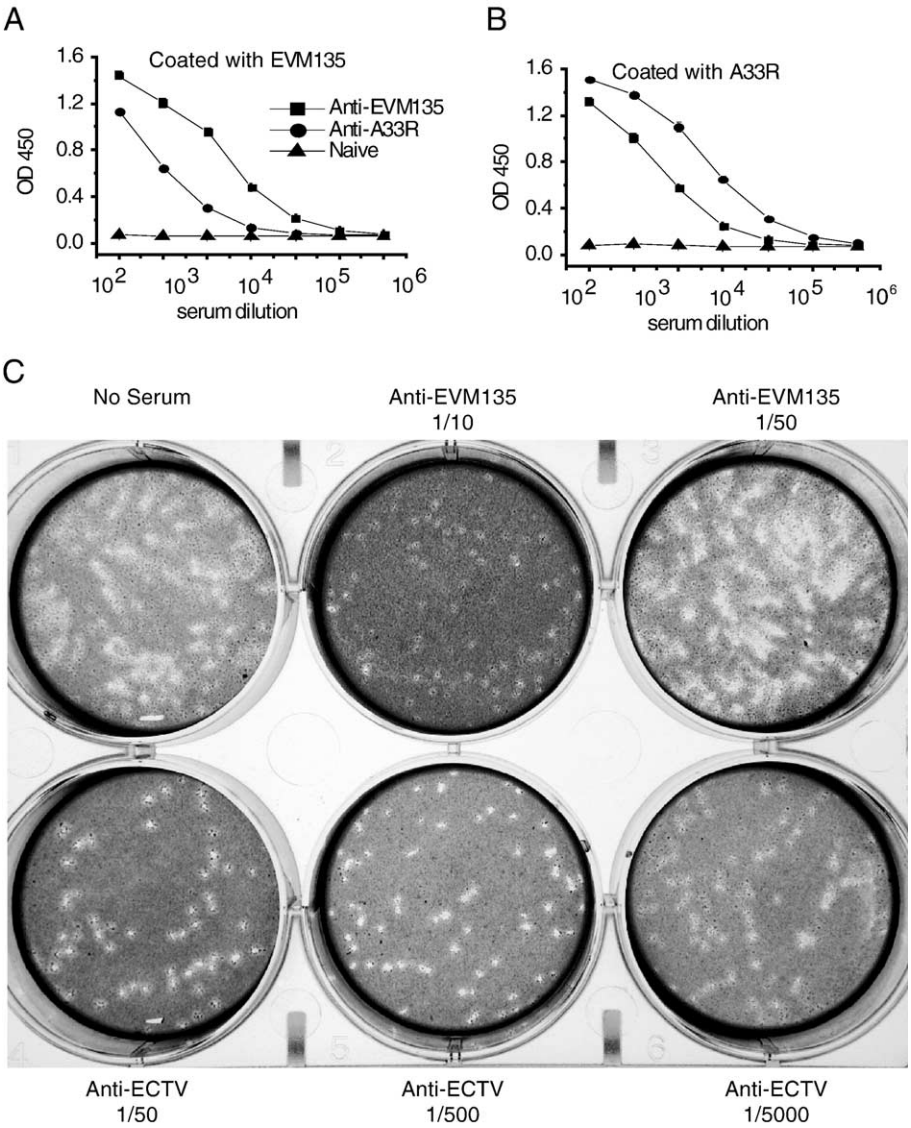

Fig. 4. Immunization with recombinant EVM135 or A33R elicits strong antibody responses that are functional in vitro. (A and B) 96-well bottom ELISA plates were coated with purified recombinant EVM135 (A) or A33R (B) 5 both at 5 μg/well. Serum was collected from EVM135- or A33R-immunized mice. Specific IgG antibodies were detected using anti-mouse immunoglobulin G (IgG) (γ chain specific) peroxidase-conjugated antibody. (C). BSC-1 cell monolayers in 6 well plates were infected with 60 PFU ECTV. After 1 h, the media was replaced with fresh media containing the indicated dilutions of the anti-EVM135 or ECTV antisera. Five days later, the supernatant was aspirated and the monolayers were fixed and stained with crystal violet.

fold better by anti-A33R antisera. This partial cross-reactivity was further confirmed in limiting-dilution experiments where titers were determined by ELISA using sera from three individual mice immunized with EVM135. In this case the antibody titers expressed in Log_{10} were 5.66 ± 0.16 when EVM135 was on the plate, and 4.39 ± 0 when A33R was the coating antigen. Conversely, sera from three individual mice immunized with A33R reacted somewhat better with A33R than with EVM135 (end-point titers of 5.5 ± 0.16 and 4. 5 ± 0.16, respectively). In both cases the *P* value in two-tailed *t* test was <0.00002. This demonstrates that the cross-reactivity between the two proteins was not complete, probably due to the presence of public and private epitopes. As mentioned above, EEVs are responsible for the in vivo long-range dissemination of OPVs. For OPV isolates that produce large numbers of EEV such as the VACV strain IHD-J, this is mimicked by the appearance of comet-shaped plaques in infected cultured cell monolayers with liquid overlays. These comets are formed by a single primary plaque that represents the head of the comet, and many secondary plaques that represent the tail of the comet and result from the release of EEVs from the primary plaque to the extracellular milieu and the subsequent infection of other cells (Appleyard et al., 1971; Vanderplasschen et al., 1997). It has been shown that when antibodies to EEV or the EEV proteins B5R or A33R are added to the liquid overlay of recently infected cell monolayers, the release of EEV to the media is blocked, the formation of comets is inhibited and the plaques take a rounded appearance. Therefore, inhibition of comet formation is indicative of biological activity of the antibody (Appleyard et al., 1971; Fogg et al., 2004; Galmiche et al., 1999; Vanderplasschen et al., 1997). In our experience, the Moscow strain of ECTV also forms comets. Therefore, we determined whether comet formation by ECTV could be inhibited with antisera to EVM135 or, as a positive control, to ECTV. Fig. 4C shows that, anti ECTV antisera could inhibit comet formation even at a dilution of 1/5000. Anti-EVM135 antisera was also able to completely inhibit comet formation although, as reported for anti-A33R (Galmiche et al., 1999), only at high concentrations. Therefore, anti-EVM135 antisera produced by immunization with a recombinant protein produced in bacteria shows functional activity in vitro.

Recombinant EVM135 and A33R protect susceptible mice from lethal ECTV infection

To test whether immunization can protect susceptible mice from footpad ECTV infection, we immunized groups of mousepox-sensitive Balb/c mice twice with IFA-EVM135, IFA-A33R or IFA-PBS and 3 weeks later we challenged them with 3,000 PFU ECTV in the footpad. As a positive control for protection, we included a group of mice that had been infected with VACV 3 weeks earlier. To evaluate protection from disease, we observed the mice for the characteristic signs of mousepox and determined their weight over a period of 6 weeks pi. Moribund mice (unresponsiveness to touch, lack of voluntary movements) were euthanized and counted as dead. Three independent experiments, each with 10 mice per group, were performed. The data for the combined experiments are shown in Fig. 5. Only 2 (less than 7%) mice in the IFA-PBS group survived past 2 weeks pi (Fig. 5A) and the two surviving mice developed rash and experienced a severe weight loss that peaked 13 days pi (Fig. 5B). As expected, all mice immunized with VACV survived and did not experience any weight loss. Most important for this work, mice immunized with any of the recombinant proteins were equally and significantly protected from death (P = 0.0001, Fig. 5A) with 70% of the mice immunized with EVM135 (21 mice) and 73% of those immunized with A33R (22 mice) surviving. Still, immunization with the recombinant proteins was not as effective in protecting mice as VACV immunization because mice immunized with either EVM135 or A33R were significantly less protected from death, and developed mousepox and experienced substantial weight loss (Fig. 5B). However, the signs of mousepox and weight loss were not as pronounced as those of the two surviving IFA-PBS controls and their recovery was faster. Therefore, immunization with recombinant EVM135 or A33R can protect against death but not against disease following infection with an OPV in a natural host under our experimental conditions.

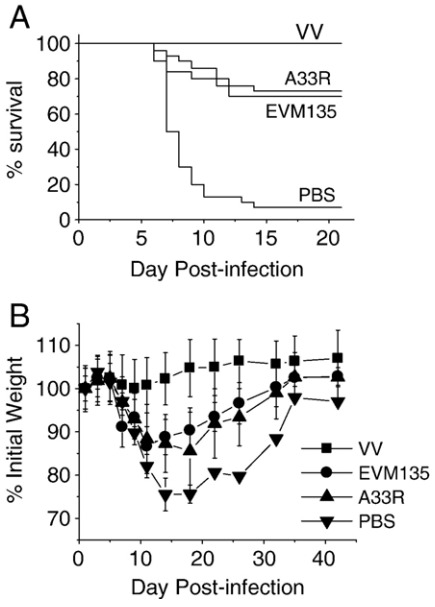

Fig. 5. Immunization with recombinant EVM135 or VACV A33R protects susceptible mice from lethal ECTV infection. BALB/c mice were immunized twice with IFA-EVM135, IFA-A33R or IFA-PBS or once with VACV. Three weeks after the last immunization, mice were infected with 3×10^3 PFU ECTV in the footpad. (A) Kaplan–Meier survival curves following ECTV challenge of immunized and mock-immunized mice. Log-rank test demonstrated a significant difference between the IFA-EVM 135 and IFA-PBS groups (P = 0.0001) and also between the IFA-A33R and IFA-PBS (P = 0.0001). There was no significant difference between the IFA-EVM-135 and IFA-A33R groups (P = 0.7401). The difference between both groups immunized with recombinant protein compared with mice immunized with VACV was significant (P = 0.012). (B) Weight change following ECTV challenge. Accumulated data for three experiments with a total of 30 mice/treatment. Data points indicate means ± SE.

Immunization with EVM135 or A33R partially protect against naturally transmitted ECTV infection

The results above demonstrate that immunization with recombinant EVM135 or A33R can protect mice from lethal experimental inoculation with ECTV. However, it is also of interest to determine to what extent immunization can protect from a naturally transmitted infection and independent of the experimental virus dose. Therefore, naive mice were infected with ECTV and immediately introduced into cages containing uninfected mice that had been immunized with IFA-EVM135, IFA-A33R or IFA-PBS. As shown in Table 1, all mice immunized with IFA-EVM135 or IFA-A33R developed mousepox but survived the infection. On the other hand, all the mice in the IFA-PBS group developed severe mousepox and 50% died 14–20 days after introducing the infected mice. Therefore, immunization with EVM135 or A33R can also protect from death but not disease when ECTV is transmitted naturally.

Reduced virus loads in organs of EVM135- and A33R-immunized mice after ECTV infection

The results above suggested that mice immunized with IFA-EVM135 or IFA-A33R controlled the virus better than mice inoculated with IFA-PBS but less well than mice that received VACV. To confirm that this was the case, we compared virus titers in spleen and liver of the different groups 7 days pi. The results in Fig. 6A show that immunization with EVM135 or A33R resulted in a ≈20-fold reduction of virus titers in spleen and $\approx 10^4$ reduction in liver as compared with the PBS-immunized group. These differences in virus titers between immunized and mock-immunized mice were statistically significant. Because death from mousepox is due to massive liver necrosis as a consequence of uncontrolled virus replication, the reduced virus loads in liver of mice immunized with EVM135 or A33R may account for the decreased mortality. Consistent with the absence of morbidity, immunization with VACV resulted in even lower virus titers in spleens and livers (i.e., more than 10^5 lower than in mice immunized with IFA-PBS).

Table 1
Immunization with EVM135 or A33R partially protect against natural ECTV infection

Immunization	PBS	EVM135 or A33R
Mousepox/total	10/10	10/10
Death/total	5/10	0/10
Day of death[a]	14–20	NA

Naive mice were infected with ECTV and immediately introduced into cages of uninfected mice that had been vaccinated with IFA-EVM135, IFA-A33R or IFA-PBS.

[a] After introduction of infected mice. All groups consisted of 10 mice. NA: not applicable.

Mice immunized with EVM135 and A33R have higher number of lymphocytes and antigen-specific $CD8^+$ T cells in their spleens following ECTV infection

The reports mentioned above where subunit vaccines were tested for protection against VACV in mice or MPXV in monkeys looked at antibody responses but not at T cell responses (Fogg et al., 2004; Galmiche et al., 1999; Hooper et al., 2003). However, the $CD8^+$ T cell response is known to be important for protection against primary OPV infections including VACV and ECTV (Blanden, 1970; Blanden, 1971b; Buller et al., 1987; Fenner et al., 1988; Karupiah et al., 1996). Therefore, we thought it was important to determine whether immunization with EVM135 could have any affect on the T cell response after virus challenge. Mice immunized with recombinant EVM135, A33R, mock-immunized mice and mice immunized with VACV were challenged with ECTV in the footpad and 7 days later, their spleens were collected. The total number of splenocytes of IFA-EVM135 ($2.2 \pm 0.3 \times 10^8$) and IFA-A33R mice ($2.1 \pm 0.5 \times 10^8$) increased, but not significantly in respect to uninfected controls ($1.5 \pm 0.05 \times 10^8$) while IFA-PBS mice experienced a sharp three-fold decrease ($0.58 \pm 0.2 \times 10^8$) which was statistically significant (Fig. 7A). Moreover, while IFA-EVM135, IFA-A33R and IFA-PBS mice had a decrease in the percentages of $CD8^+$ T cells (Fig. 7B), this reduction was much more severe in IFA-PBS mice. This resulted in almost 10-fold reduction in the absolute number of $CD8^+$ T cells in the spleens of IFA-PBS mice (Fig. 7D). Therefore, while the relative numbers of virus-specific $CD8^+$ T cells in immunized and mock-immunized mice were similar as determined by intracellular granzyme B (GzB) and IFN-γ staining following 6 h in vitro restimulation with VACV-infected cells (Fig. 7C), the absolute numbers of virus-specific $CD8^+$ T cells in the spleens of IFA-EVM135 ($1.83 \pm 0.2 \times 10^6$) and IFA-A33R ($1.85 \pm 0.5 \times 10^6$) were much higher than in IFA-PBS mice ($0.24 \pm 0.1 \times 10^6$) (Fig. 7E). This decrease in the cell content in the spleens of mock-immunized mice is due to necrosis as a result of the infection (Blanden, 1971a; Fenner, 1994). Therefore, the reduced virus burden in mice immunized with the recombinant proteins seems to prevent lymphocyte depopulation of the spleens, resulting in very increased number of antigen-specific $CD8^+$ T cells that may substantially contribute to their survival. Of note, proportionally fewer IFN-γ or GzB producing cells were found in the spleens of VACV-immunized mice. This is consistent with our finding that most of the T cell response against footpad ECTV in VACV-immunized mice occurs in the draining lymph node and not in the spleen (unpublished observations).

Discussion

Little is known about the mechanism by which immunization with VACV virus confers protection against OPV infections. Several lines of evidence, primarily natural experiments involving smallpox patients with underlying defects of the immune system, indicate that both the humoral and cell-mediated arms of the immune system play important roles in

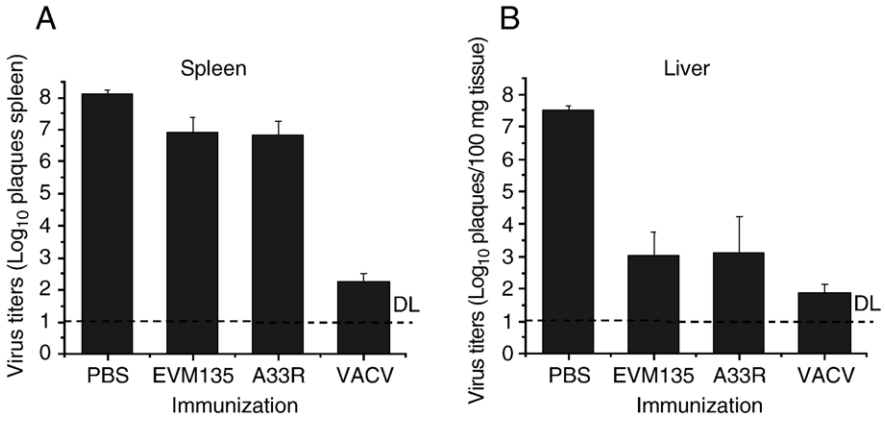

FIG. 6. Immunization with recombinant EVM135 and A33R decreases virus loads in spleen and liver after ECTV challenge. Groups of five Balb/c mice were immunized twice with IFA-EVM135, IFA-A33R or IFA-PBS or once with VACV virus. Three weeks following the last immunization, mice were infected with 3,000 PFU ECTV in the footpad. Seven days pi, mice were euthanized and virus titers were determined. (A) Virus titers in spleens. Two tailed *t* tests demonstrated significant differences in virus titers between mice immunized with IFA-PBS and IFA-A33R ($P = 0.003$), IFA-EVM135 ($P = 0.01$) or VACV ($P = 0.0004$). (B) Virus titers in liver. Two tailed *t* test demonstrated significant differences between mice immunized with IFA-PBS and IFA-A33R ($P = 0.007$), IFA-EVM135 ($P = 0.001$) or VACV ($P = 0.002$). DL, detection limit.

resistance to primary infection (Kempe, 1960). Belyakov et al. (2003) reported that antibodies were necessary for protection against VACV induced disease in vaccinated mice whereas $CD4^+$ and $CD8^+$ T cells were neither necessary nor sufficient. In addition, they showed that the $CD4^+$ and $CD8^+$ T cell responses could protect from VACV death in the absence of antibody (Belyakov et al., 2003). In our own studies, many immunodeficient mice such as TAP, B cell or CD40-deficient mice can control VACV infection (5×10^6 PFU/mouse, i.p.) with no adverse effects (unpublished results). However, survival from primary ECTV infection requires antibodies, $CD4^+$ and $CD8^+$ T cells (Fang and Sigal, in press). Furthermore, immunization with VACV virus cannot protect mice from ECTV infection in the absence of antibodies (Fang and Sigal, unpublished observations). This indicates that the requirements for the control of ECTV are much more stringent even though VACV and ECTV are very related. Therefore, it is important to confirm results of protective immunization against VACV in an animal model with a natural pathogen such as ECTV.

In this paper, we produced recombinant ECTV EVM135 and its ortholog VACV A33R in *E. coli*. Characterization of the recombinant proteins showed that they fold in stable structures and form dimers, which is the dominant form of the natural proteins (Roper et al., 1996). When we used the recombinant proteins as probes, we found that both were recognized by anti-ECTV or anti-VACV antisera but each was better recognized by antisera to the homologous virus. This indicates that EVM135 and A33R are natural targets of the antibody responses during ECTV and VACV infections and, as expected from their sequence homology, that they are partially cross-reactive. Importantly, these data also indicate that antibodies produced during natural ECTV and VACV infections recognize recombinant EVM135 and A33R produced in *E. coli*. Therefore, while we do not know whether all natural epitopes of the EVM135 and A33R are conserved in the recombinant proteins, it is clear that at least some are retained and this may be important for protection.

Immunization of mice with recombinant EVM135 or A33R elicited strong IgG antibody responses that were also partially cross-reactive. Despite the incomplete cross-reactivity, immunization with either protein protected 70% of susceptible BALB/c mice from death following ECTV footpad infection. Moreover, 100% of the immunized mice survived infection by natural transmission, an experiment that can only be accomplished with a natural pathogen such as ECTV in mice. The reason why the level of protection achieved with both proteins was similar while the antibody cross-reactivity was only partial is not resolved by our data. However, a lack of correlation between antibody titer and degree of protection was also observed by others that looked for protection to VACV infection (Galmiche et al., 1999). Among others, possible reasons to account for similar protection without complete cross-reactivity may be that protection is dependent on specific epitopes shared between the two proteins or that protection is mediated by T cells cross-reactive between the two proteins.

All mice immunized with recombinant EVM135 or A33R developed mousepox after ECTV inoculation or natural transmission. Therefore, immunization with the recombinant proteins provided significant protection from death but not from disease. This was different from mice immunized with VACV, which remained healthy. The outcome of the different immunizations in terms of protection is consistent with the virus titers after challenge. Thus, mock-immunized mice had 10^7–10^8 PFU of virus in spleen and liver, mice immunized with the recombinant proteins had a ≈20-fold reduction in virus loads in spleen and $\approx 10^4$-fold drop in liver, and VACV-immunized mice had more than 10^5 decrease in both organs.

Recently, Fogg et al. (2004) reported that among several recombinant proteins produced in insect cells, immunization with A33R was the most effective in protecting from intranasal inoculation with 10^6 PFU VACV. However, protection was not complete and the animals experienced weight loss similar to our results. Therefore, production of OPV proteins in *E. coli* may be a good alternative to insect cells to produce

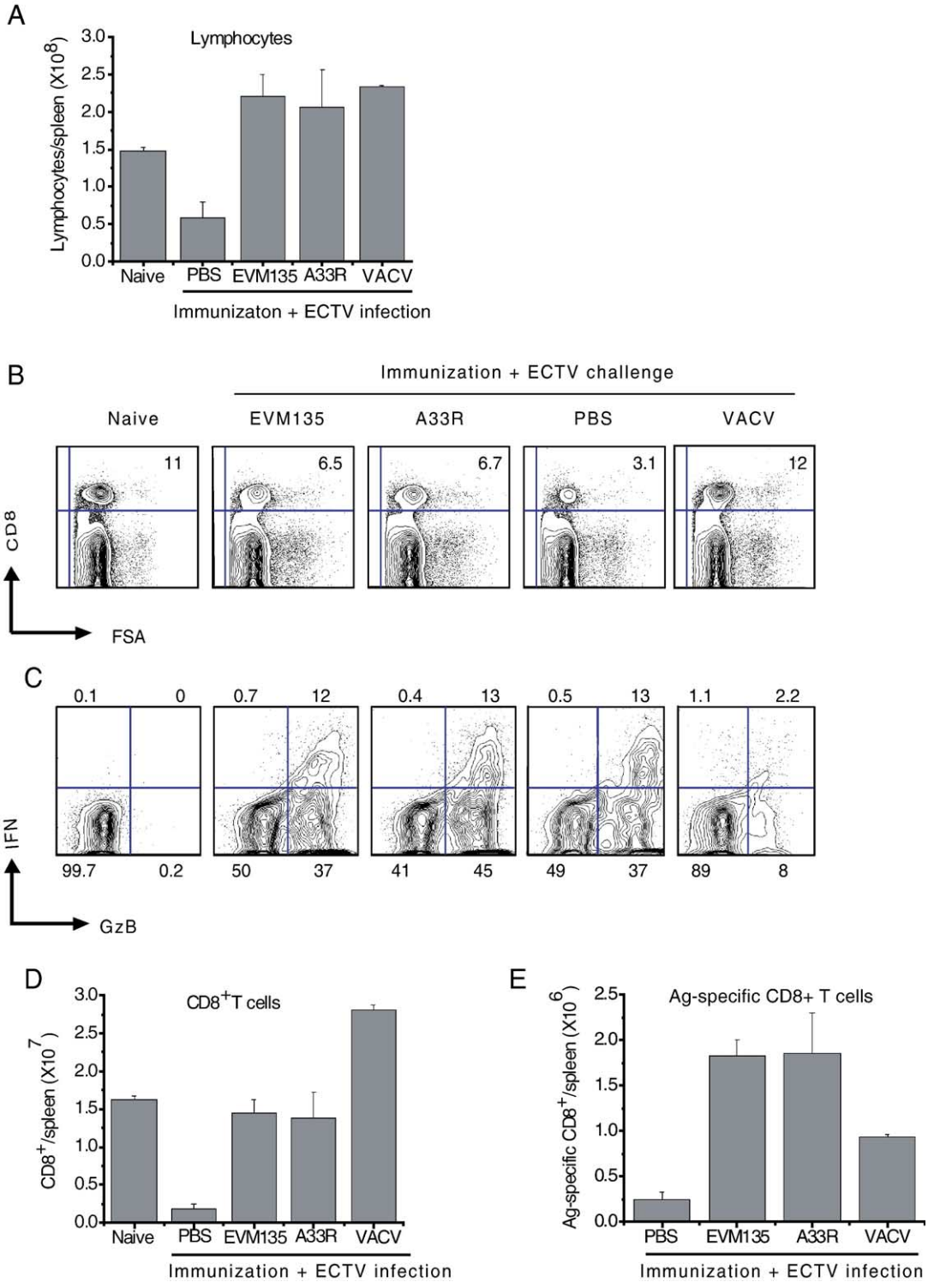

Fig. 7. Immunization with recombinant EVM135 or A33R protect the spleen from ECTV-induced lymphocyte depletion resulting in a larger number of antigen-specific T cells. Groups of five mice were immunized with IFA-EVM135, IFA-A33R or IFA-PBS twice or VACV once. Three weeks after the last immunization, mice were challenged with ECTV. One week pi mice were euthanized and spleen cells analyzed by flow cytometry. (A) Total lymphocytes per spleen in different groups as determined by counting live cells at the microscope by trypan blue exclusion. The difference in total lymphocytes between IFA-PBS and the naive control was statistically significant ($P = 0.008$). (B) Flow cytometry analysis showing the proportion of $CD8^+$ T cells in the spleen. FSA: forward scatter amplitude. Stains as indicated. (C) Flow cytometry analysis showing antigen-specific $CD8^+$ T cell responses. Graphs are gated on $CD8^+$ T cells. Numbers indicate the proportion of $CD8^+$ T cells in the nearest quadrant. Stains as indicated. (D) Total number of $CD8^+$ T cells per spleen in different groups calculated from A and B. The difference in total $CD8^+$ T cells between IFA-PBS and the naive control was statistically significant in two tailed t tests ($P = 0.0001$). (E) Antigen-specific $CD8^+$ T cells per spleen in different groups calculated from D and C. Two tailed t tests demonstrated significant differences in the absolute numbers of antigen-specific $CD8^+$ T cells between mice immunized with IFA-PBS and IFA-A33R ($P = 0.01$), IFA-EVM135 ($P = 0.001$) or VACV ($P = 0.002$).

recombinant proteins for OPV vaccines. Fogg et al. also reported improved protection by combining A33R together with B5R and/or the IMV protein L1 also produced in insect cell. It will therefore be interesting to determine whether these or other combinations of recombinant proteins produced in *E. coli* can afford complete protection from mousepox and compare with those produced in insect cells.

None of the previous reports that tested subunit vaccines in different models of OPV infections analyzed the effect of immunization on the T cell responses (Fogg et al., 2004; Galmiche et al., 1999; Hooper et al., 2004). However, it has been reported by Galmiche et al. (1999) that mice immunized with recombinant B5R were protected against a lethal VACV challenge, and the protection was most likely mediated by neutralizing antibodies. A33R was also protective in active and passive immunization, but in contrast to B5R, protection with A33R did not correlate with antibody titers in serum (Galmiche et al., 1999). In addition, because anti-A33R antibodies did not neutralize EEV in vitro (Galmiche et al., 1999), it was proposed that the protection mediated by A33R might partly involve a mechanism different from antibody neutralization such as complement-mediated lysis (Lustig et al., 2004) or T cell responses. Because T cell responses are known to be important in protection against primary ECTV infections, we tested whether EVM135 or A33R-immunized mice generated stronger T cell responses than mock-immunized mice following ECTV challenge. Interestingly, we found that the proportion of $CD8^+$ T cells that produced IFN-γ and GzB was not markedly different between the immunized and mock-immunized group. However, the total number of antigen-specific $CD8^+$ T cells in the spleen of mice immunized with EVM135 or A33R was increased 10-fold due to a slight increase as opposed to a sharp decrease in the total number of lymphocyte in the spleen after infection. The depletion of lymphocytes in mock-immunized mice is likely due to the necrosis of the spleen due to the infection (Fenner, 1994). The mechanism of protection form lymphocyte depletion in the immunized groups is still unknown. In our view, the most likely explanation is that the presence of anti-EVM135/A33R antibodies delays the spread and/or replication of ECTV sufficiently to allow for the development of a strong cellular response, which is essential for protection even in the presence of antibodies (submitted). Alternatively, vaccination may induce EVM135/A33R-specific $CD8^+$ T cell responses through cross-priming (Norbury and Sigal, 2003), a mechanism whereby professional antigen presenting cells can present exogenous antigens with their own MHC class I molecules. In this regard, we have found that naive Balb/c mice can be protected from disease and death following ECTV challenge when adoptively transferred with memory $CD8^+$ T cells from VACV-immunized mice (submitted). Adoptive transfer experiments with $CD8^+$ T cells from mice immunized with EVM135/A33R are unlikely to succeed because the number of memory cells should be much lower than following VACV immunization. While for technical reasons, we have been unable to directly determine whether $CD8^+$ T cells specific for EVM135/A33R are induced by vaccination, based on our own experience immunizing with other non-replicating antigens such as chicken ovalbumin, we think it is improbable that all the virus-specific $CD8^+$ T cells that we detect 7 days pi are directed to EVM135/A33R. More likely, a secondary response of a low number of memory $CD8^+$ T cells resulting from the vaccination could aid the primary response of $CD8^+$ T cells of other specificities (submitted). Unfortunately, this hypothesis cannot be tested at the present time. While $CD8^+$ T cell epitopes shared between ECTV and VACV have been found for the C57BL/6 mouse ($H\text{-}2^b$) (Tscharke et al., 2005), none have yet been described for the Balb/c background ($H\text{-}2^d$). In any case, the overall increase in antigen-specific $CD8^+$ T cells resulting from the immunization with EVM135 or A33R is likely to be crucial for the improved survival of the immunized mice.

In summary, our work demonstrates that immunization with recombinant EVM135 or its VACV ortholog can protect a natural host from a lethal OPV infection following experimental inoculation or natural transmission. While this protection is partial and much less effective than that induced by VACV immunization, our results are promising because they showed for the first time that a single recombinant EEV protein produced in *E. coli* can be used to protect against a lethal OPV infection in its natural host. To achieve complete protection, EVM135 could be combined with other EEV or IMV proteins or secreted proteins involved in pathogenesis such as mimics of cytokines and chemokines receptors that are used by the virus to evade the immune response (Alcami and Koszinowski, 2000; Alcami and Smith, 1996; Smith et al., 1999). Testing these and other proteins in protection from mousepox will provide a very valuable model for a safer subunit vaccine to smallpox and other OPV infections in humans.

Materials and methods

Cells

HeLa S3, A20 and BSC-1 cells were obtained from ATCC. As standard tissue culture media, we used RPMI 10 that consisted of RPMI 1640 tissue culture media (Invitrogen) supplemented with 10% fetal calf serum (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 10 mM Hepes buffer (Invitrogen) and 0.05 mM 2-mercaptoethanol (Sigma). When indicated, RPMI 2.5 (as above but with 2.5% FCS) was used instead. When required, 10 U/ml interleukin 2 (IL-2) was added to RPMI 10 (RPMI 10-IL2). All cells were grown at 37 °C and 5% CO_2.

Viruses

Initial stocks of VACV Western Reserve were obtained from Dr. Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and amplified in HeLa S3 cells as described (Elroy-Stein and Moss, 1992). Briefly, HeLa S3 cells in T150 flasks were infected with 0.1 PFU/cell VACV. After 3 or 4 days cells were collected, resuspended in PBS, frozen and thawed three times, and stored in aliquots at −80 °C as virus stock. Virus titers in VACV stocks were determined by plaque assays on confluent BSC-1 cells using 10-fold serial dilutions

of the stocks in 0.5 ml RPMI 2.5 in six well plates (2 wells/dilution) for 1 h. 2 ml fresh RPMI 2.5 was added and the cells incubated at 37 °C for 3 days (VACV). Next, the media was aspirated and the cells fixed and stained for 10 min with 0.1% crystal violet in 20% ethanol. The fix/stain solution was subsequently aspirated, the cells air-dried, the plaques counted, and the PFU/ml in stocks were calculated accordingly.

Initial stocks of ECTV Moscow (Chen et al., 1992; Fenner, 1949) were obtained from ATCC (#VR-1374). New stocks were expanded by infecting BALB/c mice with 3,000 PFU of ECTV in the footpad. Seven days post-infection, the spleen and liver were removed and homogenized using a tissue homogenizer. The solid material was pelleted by centrifugation and the supernatant was stored in aliquots at −80 °C as virus stock. Titers in stocks were determined as for VACV but the plates were incubated for 5 days. For the determination of virus titers in spleens, the organs were removed from experimental mice 7 days after footpad infection, made into a single cell suspension between two frosted slides and resuspended in 10 ml complete RPMI medium. 1 ml of the cell suspensions were frozen and thawed three times and titers determined in 10-fold serial dilutions of the cell lysates as above. Virus titers were calculated as PFU/spleen.

To determine the virus titers in liver, a portion of the liver was weighed and homogenized in medium using a tissue homogenizer. The virus titers were calculated as PFU/100 mg liver. Stocks of lymphocytic choriomeningitis virus strain Armstrong were a kind gift of Dr. Glenn Rall (Fox Chase Cancer Center).

Production and characterization of recombinant EVM135 and A33R

The coding sequences for the extraviral domains of ECTV Moscow EVM135 and VACV Western Reserve A33R (Galmiche et al., 1999; Roper et al., 1996) were amplified by PCR from genomic DNA. To facilitate purification, the recombinant proteins were designed to contain a terminal 6 × His tag. Because VACV A33R and its orthologs such as ECTV EVM135 are type II proteins (Roper et al., 1996), their C-termini are exposed at the surface of EEV. Therefore, the 6 × His tag was fused to the N-terminus. For EVM135, we used 5′AAACCATGGGCCATCACCATCACCATCACTGCAAACCATGGGCCATCACCATCACCATCACTGCATGTCTGCTAACGAGGTTG and 5′ AAACTCGAGTTAGTTCATTATTTTAACACAAAAATACTTTC as the forward and reverse primers respectively. For A33R the forward and reverse primers used were 5′AAACCATGGGCCATCACCATCACCATCACTGCATGTCTGCTAACGAGGCTG and AAACTCGAGTTAGTTCATTGTTTTAACACAAAAATACTTTC respectivelyThe amplified products were cloned into pET-28a(+) vector and transformed into DH5α competent cells. The expression vector was verified by DNA sequencing and then transformed into BL21(DE3) competent cells for expression. The transformed BL21(DE3) cells were grown overnight at 37 °C and inoculated at 5% into LB medium. The culture was grown at 37 °C until the A_{600} was achieved. IPTG (final concentration 0.4 mM) was added to induce protein expression and cells were harvested 4h later and lysed by sonication. The inclusion bodies were pelleted by centrifugation for 20 min at 8000 × *g*, washed with washing buffer (20 mM Tris–HCl pH 8.0, containing 1% Triton-X100) followed by distilled water to remove contaminating salts and detergents.

Recombinant EVM135 and A33R were purified under denaturing conditions by Ni-NTA metal affinity chromatography. The inclusion bodies were solubilized with 8M urea and loaded onto Ni-NTA agarose (Qiagen), according to the manufacturer's recommendations. After washing out the unbound proteins, the target protein was eluted by 0.5M imidazole in 8M Urea lysis buffer. The purified proteins were refolded by dialysis against PBS (phosphate-buffered saline). Protein concentrations were determined using a bicinchoninic acid assay (Pierce) with bovine serum albumin as a standard. The purity of each protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The same proteins were used for characterization, immunization and ELISA.

Circular dichroism (CD) spectra were acquired at 20 °C on an Aviv 62A spectropolarimeter (Aviv, Lakewood, NJ), in 1 mm quartz cuvettes using 9.6 μM protein in PBS. Each CD spectrum is an average of five scans recorded in the far-UV region (190–250 nm) with a band pass of 2 nm. The spectra in the presence of DTT were obtained by adding concentrated 1 M DTT stock solution to a final concentration of 1 mM. The spectra in the presence of guanidium chloride (GuCl) were obtained by dissolving the protein in 5.9 M GuCl solution. The spectra in the presence of DTT and GuCl were only recorded from 210–250 and 215–250 nm, respectively because at other wavelengths DTT and GuCl contribute heavily to the signal.

To determine the tertiary structure and the oligomeric state, the recombinant EVM135 was size-analyzed by gel filtration FPLC using a Superdex 200 10/30 column. The major peak observed by gel filtration, which corresponded to the expected size of EVM135 dimers, was collected and further analyzed by light scattering at 10 °C on a DynaPro Molecular Sizing Instrument with Dynamics V6 data analysis software (Protein Solutions, Inc, NJ). This instrument has the capability of conducting both static light scattering (SLS) and dynamic light scattering (DLS) experiments simultaneously. SLS measures the absolute molecular weight of the protein, while DLS measures the hydrodynamic radius R_h and estimates the monodispersity of the measured proteins (van Holde et al., 1998). Two concentrations at 0.5 and 1.05 mg/ml were measured.

Production of antisera

Anti-virus antisera was produced by infecting C57BL/6 mice with ECTV (3,000 PFU in the footpad), VACV (5×10^6 PFU ip) or LCMV (3000 PFU ip). Eight weeks pi, mice were anesthetized and exsanguinated from the orbital sinus. The blood was allowed to clot, and the sera separated by centrifugation. Antisera was stored in aliquots at −80 °C.

Immunization protocol

6- to 8-week-old female BALB/c mice bred at Fox Chase were immunized subcutaneously (base of tail) two times with

EVM135 or A33R proteins (10 μg/injection) or PBS emulsified in Freund's incomplete adjuvant (IFA). Preliminary experiments showed that immunization of mice one or 2 weeks apart induced similar levels of antibodies when measured 2 weeks after the booster immunization (not shown). The 1-week interval was chosen for all the experiments. For immunization with VACV, 6- to 8-week-old female BALB/c mice were inoculated once intraperitoneally with 0.5 ml PBS containing 5×10^6 PFU VACV. Four weeks after the first injection or VACV infection, the mice were challenged in the hind footpad with 3000 PFU of ECTV. The mice were observed daily for disease and imminent signs of death, and their body weights were monitored. The amount of specific antibody contained in a blood sample taken before challenge or at different time after challenge was determined by ELISA. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

ELISA

96-well bottom ELISA plates were coated 4 °C overnight with 0.1 ml of recombinant protein (50 μg/ml) or lysates of VACV virus-infected cell (2×10^7 PFU/ml), ECTV virus (2×10^7 PFU/ml) or LCMV infected cell lysates, all in Na_2HPO_4–$NaHPO_4$ buffer, pH 9.0. Plates were blocked for 2 h at 37 °C with PBS 1% BSA. Mouse sera were serially diluted in PBS containing 0.5% BSA, 0.05% Tween-20, and 0.1 ml was added to each well. The plates were then incubated for 1 h at 37 °C and washed three times with PBS containing 0.05% Tween-20. 0.1 ml of peroxidase-conjugated affinity purified goat anti-mouse IgG (γ chain specific) or IgM (μ chain specific) (both from KPL) were added to each well at a dilution of 1:2,000, incubated for 1 h at 37 °C, and then washed five times with PBS containing 0.05% Tween-20. SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL) was added to each well (50 μl) and the plates were incubated at room temperature for 5–20 min. The reactions were stopped by addition of 100 μl 0.12M HCl. The optical density (OD) at 450 nm was determined using a microplate spectrophotometer (μQuant, Bio-Tek).

Comet inhibition assay

Confluent BSC-1 cells in 6 well plates were infected with 60 pfu ECTV in 0.5 ml RPMI 2.5. After 1-h incubation at 37 °C, the media containing virus was aspirated and the cells overlaid with 1.5 ml RPMI 2.5 containing the indicated dilutions of antisera. Cells were incubated for 5 days at 37 °C and stained with crystal violet as described for virus titers.

Flow cytometry

Seven days after ECTV infection, mice were euthanized and single-cell suspensions of each spleen were prepared in 10 ml complete RPMI medium. Following osmotic lysis of red blood cells with 0.84% NH_4Cl, the spleen cells were washed, and 10^6 cells were stimulated for 6 h at 37 °C with 2×10^5 VACV-infected or uninfected A20 cells in 96-well plates. Brefeldin A (BFA, Sigma) was added after 5 h to block the secretory pathway and allow for the accumulation of cytokines inside the cells. Following stimulation, antibody 2.4G2 (anti-Fcγ II/III receptor, ATCC) was added to block non-specific binding of labelled antibodies to Fc receptors. The cells were then stained for cell surface molecules, fixed, permeabilized, and stained for intracellular molecules using the Cytofix/Cytoperm kit (Becton Dickinson, BD) according to the manufacturer's instructions. The following antibodies were used: anti-CD8a (53–6.7, BD), anti-IFN-γ (clone XMG1.2, BD), an isotype control (clone A95-1, BD) and anti-human granzyme B (GzB, Caltag) that is cross-reactive with mouse GzB (Wolint et al., 2004). 100,000–200,000 cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility using an LSR II system (BD) and analyzed with FlowJo software.

Acknowledgments

This work was supported by NIH grants AI-058179, AI-048449 to L.S. and CA-06927 to Fox Chase Cancer Center as well as an appropriation from the Commonwealth of Pennsylvania. We would like to thank Dr. Glenn Rall for helpful comments, Dr. Samuel Litwin and Ms Tianyu Li from Fox Chase Cancer Center's Biostatistics Facility for help with statistical analysis and Fox Chase Cancer center's Flow Cytometry, DNA Sequencing and Laboratory Animal Facilities for their invaluable help.

References

Alcami, A., Koszinowski, U.H., 2000. Viral mechanisms of immune evasion. Trends Microbiol. 8 (9), 410–418.

Alcami, A., Smith, G.L., 1996. Receptors for gamma-interferon encoded by poxviruses: implications for the unknown origin of vaccinia virus. Trends Microbiol. 4 (8), 321–326.

Appleyard, G., Hapel, A.J., Boulter, E.A., 1971. An antigenic difference between intracellular and extracellular rabbitpox virus. J. Gen. Virol. 13 (1), 9–17.

Belyakov, I.M., Earl, P., Dzutsev, A., Kuznetsov, V.A., Lemon, M., Wyatt, L.S., Snyder, J.T., Ahlers, J.D., Franchini, G., Moss, B., Berzofsky, J.A., 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc. Natl. Acad. Sci. U.S.A. 100 (16), 9458–9463.

Blanden, R.V., 1970. Mechanisms of recovery from a generalized viral infection: mousepox: I. The effects of anti-thymocyte serum. J. Exp. Med. 132 (5), 1035–1054.

Blanden, R.V., 1971a. Mechanisms of recovery from a generalized viral infection: mousepox. 3. Regression infectious foci. J. Exp. Med. 133 (5), 1090–1104.

Blanden, R.V., 1971b. Mechanisms of recovery from a generalized viral infection: mousepox: II. Passive transfer of recovery mechanisms with immune lymphoid cells. J. Exp. Med. 133 (5), 1074–1089.

Blasco, R., Moss, B., 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol. 66 (7), 4170–4179.

Buller, R.M., Holmes, K.L., Hugin, A., Frederickson, T.N., Morse III, H.C., 1987. Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. Nature 328 (6125), 77–79.

Chen, W., Drillien, R., Spehner, D., Buller, R.M., 1992. Restricted replication of ectromelia virus in cell culture correlates with mutations in virus-encoded host range gene. Virology 187 (2), 433–442.

Cono, J., Casey, C.G., Bell, D.M., 2003. Smallpox vaccination and adverse

reactions. Guidance for clinicians. Morb. Mort. Wkly. Rep., Recomm. Rep. 52 (RR-4), 1–28.
Cunha, B.E., 2004. Monkeypox in the United States: an occupational health look at the first cases. AAOHN J. 52 (4), 164–168.
DiGiulio, D.B., Eckburg, P.B., 2004. Monkeypox in the Western hemisphere. N. Engl. J. Med. 350 (17), 1790–1791 (author reply 1790-1).
Earl, P.L., Americo, J.L., Wyatt, L.S., Eller, L.A., Whitbeck, J.C., Cohen, G.H., Eisenberg, R.J., Hartmann, C.J., Jackson, D.L., Kulesh, D.A., Martinez, M.J., Miller, D.M., Mucker, E.M., Shamblin, J.D., Zwiers, S.H., Huggins, J.W., Jahrling, P.B., Moss, B., 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature 428 (6979), 182–185.
Elroy-Stein, O., Moss, B. (Eds.), (1992). Gene Expression using the Vaccinia Virus/T7 RNA Polymerase, Current Protocols in Molecular Biology, vol. 2. Wiley Interscience, New York. Edited by F. Ausubel, R. Brent, R. Kingston, J. Moore, J. Seidman, and K. Struhl. 1 vols.
Fang, M., Sigal, L.J., in press. Antibodies and CD8+ T cells are complementary and essential for natural resistance to highly lethal cytopathic virus J. Immunol.
Fenner, F., 1949. Mouse-pox; infectious ectromelia of mice; a review. J. Immunol. 63 (4), 341–373.
Fenner, F., 1994. Mousepox (ectromelia). In: Osterhaus, A. (Ed.). Virus infections of rodents and lagomorphs vol. 5. Elsevier Science, Amsterdam, p. 412.
Fenner, F., Henderson, D.A., Arita, I., Jezek, Z., Ladnyi, D., Organization, W.H., 1988. Smallpox and its eradication. History of International Public Health, vol. 6. World Health Organization, Geneva.
Fogg, C., Lustig, S., Whitbeck, J.C., Eisenberg, R.J., Cohen, G.H., Moss, B., 2004. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. J. Virol. 78 (19), 10230–10237.
Fulginiti, V.A., Papier, A., Lane, J.M., Neff, J.M., Henderson, D.A., 2003. Smallpox vaccination: a review, part II. Adverse events. Clin. Infect. Dis. 37 (2), 251–271.
Galmiche, M.C., Goenaga, J., Wittek, R., Rindisbacher, L., 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology 254 (1), 71–80.
Goebel, S.J., Johnson, G.P., Perkus, M.E., Davis, S.W., Winslow, J.P., Paoletti, E., 1990. The complete DNA sequence of vaccinia virus. Virology 179 (1), 247–266, 517–563.
Henderson, D.A., 1999. The looming threat of bioterrorism. Science 283 (5406), 1279–1282.
Hooper, J.W., Custer, D.M., Thompson, E., 2003. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. Virology 306 (1), 181–195.
Hooper, J.W., Thompson, E., Wilhelmsen, C., Zimmerman, M., Ichou, M.A., Steffen, S.E., Schmaljohn, C.S., Schmaljohn, A.L., Jahrling, P.B., 2004. Smallpox DNA Vaccine Protects Nonhuman Primates against Lethal Monkeypox. J. Virol. 78 (9), 4433–4443.
Hutin, Y.J., Williams, R.J., Malfait, P., Pebody, R., Loparev, V.N., Ropp, S.L., Rodriguez, M., Knight, J.C., Tshioko, F.K., Khan, A.S., Szczeniowski, M.V., Esposito, J.J., 2001. Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. Emerg. Infect. Dis. 7 (3), 434–438.
Karupiah, G., Buller, R.M., Van Rooijen, N., Duarte, C.J., Chen, J., 1996. Different roles for CD4+ and CD8+ T lymphocytes and macrophage subsets in the control of a generalized virus infection. J. Virol. 70 (12), 8301–8309.
Kempe, C.H., 1960. Studies smallpox and complications of smallpox vaccination. Pediatrics 26, 176–189.
Lustig, S., Fogg, C., Whitbeck, J.C., Moss, B., 2004. Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement. Virology 328 (1), 30–35.
Moss, B., 1996. Poxviridae: the viruses and their replication, 3rd ed. In: Fields, B.N., Knipe, D.M., Howley, P.M. Fields Virology, vol. 2. Raven Press, Philadelphia, p. 2950. 2 vols.
Niemialtowski, M.G., Spohr de Faundez, I., Gierynska, M., Malicka, E., Toka, F.N., Schollenberger, A., Popis, A., 1994. The inflammatory and immune response to mousepox (infectious ectromelia) virus. Acta Virol. 38 (5), 299–307.
Norbury, C.C., Sigal, L.J., 2003. Cross priming or direct priming: is that really the question? Curr. Opin. Immunol. 15 (1), 82–88.
Payne, L.G., 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J. Gen. Virol. 50 (1), 89–100.
Payne, L.G., Kristensson, K., 1985. Extracellular release of enveloped vaccinia virus from mouse nasal epithelial cells in vivo. J. Gen. Virol. 66 (Pt. 3), 643–646.
Roper, R.L., Payne, L.G., Moss, B., 1996. Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. J. Virol. 70 (6), 3753–3762.
Scholtz, J.M., Baldwin, R.L., 1992. The mechanism of alpha–helix formation by peptides. Annu. Rev. Biophys. Biomol. Struct. 21, 95–118.
Shchelkunov, S.N., Totmenin, A.V., Babkin, I.V., Safronov, P.F., Ryazankina, O.I., Petrov, N.A., Gutorov, V.V., Uvarova, E.A., Mikheev, M.V., Sisler, J.R., Esposito, J.J., Jahrling, P.B., Moss, B., Sandakhchiev, L.S., 2001. Human monkeypox and smallpox viruses: genomic comparison. FEBS Lett. 509 (1), 66–70.
Shchelkunov, S.N., Totmenin, A.V., Safronov, P.F., Mikheev, M.V., Gutorov, V.V., Ryazankina, O.I., Petrov, N.A., Babkin, I.V., Uvarova, E.A., Sandakhchiev, L.S., Sisler, J.R., Esposito, J.J., Damon, I.K., Jahrling, P.B., Moss, B., 2002. Analysis of the monkeypox virus genome. Virology 297 (2), 172–194.
Smith, G.L., Symons, J.A., Alcami, A., 1999. Immune modulation by proteins secreted from cells infected by vaccinia virus. Arch. Virol., Suppl. 15, 111–129.
Smith, G.L., Vanderplasschen, A., Law, M., 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. 83 (12), 2915–2931.
Tscharke, D.C., Karupiah, G., Zhou, J., Palmore, T., Irvine, K.R., Haeryfar, S.M., Williams, S., Sidney, J., Sette, A., Bennink, J.R., Yewdell, J.W., 2005. Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. J. Exp. Med. 201 (1), 95–104.
van Holde, K.E., Johnson, W.C., Ho, P.S., 1998. Principles of Physical Biochemistry. Prentice Hall, Upper Saddle River, NJ.
Vanderplasschen, A., Hollinshead, M., Smith, G.L., 1997. Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. J. Gen. Virol. 78 (Pt. 8), 2041–2048.
Wolint, P., Betts, M.R., Koup, R.A., Oxenius, A., 2004. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J. Exp. Med. 199 (7), 925–936.