

Reduction of vector gene expression increases foreign antigen-specific CD8⁺ T-cell priming

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Viral vectors have been shown to induce protective CD8⁺ T-cell populations in animal models, but significant obstacles remain to their widespread use for human vaccination. One such obstacle is immunodominance, where the CD8⁺ T-cell response to a vector can suppress the desired CD8⁺ T-cell response to a recombinantly encoded antigen. To overcome this hurdle, we broadly reduced vector-specific gene expression. We treated a recombinant vaccinia virus, encoding antigen as a minimal peptide determinant (8–10 aa), with psoralen and short-wave UV light. The resulting virus induced 66 % fewer vector-specific immunodominant CD8⁺ T cells, allowing the *in vivo* induction of an increased number of CD8⁺ T cells specific for the recombinant antigen.

INTRODUCTION

CD8⁺ T cells (T_{CD8+}) can mediate protective immunity against many viruses, intracellular bacteria and parasites and can alleviate tumour burden. As such, the initiation of protective T_{CD8+} responses represents an attractive target for vaccination. Many strategies have been proposed to generate protective T_{CD8+} in animal models. The most reproducible success for generating T_{CD8+}-mediated vaccines has emerged from the use of recombinant viral vectors containing transgenes encoding antigen(s) to which a T_{CD8+} response is desired. Immunization using recombinant viral vectors induces a strong and long-lived T_{CD8+} response that is rarely matched in efficacy by other modes of immunization. This strong response to viral vectors may be a fortuitous by-product of the evolution of the innate immune system to combat viral infection and thus to recognize viral vectors as 'dangerous' (Truckenmiller & Norbury, 2004). Nonetheless, significant challenges remain prior to the successful use of recombinant viral vectors to induce protective T_{CD8+} in humans.

One of the major obstacles to the use of recombinant viruses to produce protective T_{CD8+} is the phenomenon known as immunodominance. This occurs when T_{CD8+}, responding to an 'immunodominant' determinant, suppress the expansion and effector function of T_{CD8+} specific for other so-called 'subdominant' determinants via a variety of mechanisms (Yewdell & Bennink, 1999). When a recombinant viral vector is used, immunodominance is manifested by a strong T_{CD8+} response, targeted to determinants expressed naturally by the vector, that reduces or prevents a T_{CD8+} response to the recombinantly encoded foreign antigen. For example, recombinant vaccinia virus (rVACV) shows a reduced efficacy at inducing T_{CD8+} to transgene-encoded antigens compared with the pathogens that encode the antigen naturally (Harrington *et al.*, 2002). In a recent study using a VACV-based cancer vaccine in humans, the dominant T_{CD8+} response was directed against VACV proteins, rather than against the recombinantly encoded tumour antigen (Smith *et al.*, 2005). Although it is possible in experimental systems to remove immunodominant determinants in order to enhance the response to subdominant antigens (Tanaka *et al.*, 1989; Webby *et al.*, 2003; Weidt *et al.*, 1998), this approach is not practical in a therapeutic setting, as there are no means to predict the immunodominance of antigens in an outbred population.

In this study, we have successfully employed a strategy that reduces immunodominance following immunization with VACV vectors. This was accomplished by inhibiting vector gene expression by using psoralen and UV irradiation. UV/psoralen has been suggested as an inactivating agent for vaccines (Brockstedt *et al.*, 2005) and its use is well established as an experimental tool for studying vaccinia virus (Tsong *et al.*, 1996). DNA damage induced by UV/psoralen is random and smaller genes are therefore inactivated less frequently. Here, we exploit this property of psoralen/UV by using recombinant VACV vaccines that express minimal antigenic-peptide 'minigenes' (8–10 aa). Minigenes are small targets for irradiation-induced DNA damage, allowing broad ablation of VACV gene expression while minigene expression remains relatively intact. We show that the resulting VACV vector is non-replicating and elicits less inflammation, but induces more foreign epitope-specific T_{CD8+}, than untreated virus.

METHODS

Mice.

C57BL/6 mice were purchased from Charles River Laboratories and housed in the specific-pathogen-free animal facility of the Hershey Medical Center. All studies were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee.

Viruses and cells.

rVACVs were made by homologous recombination (Chakrabarti *et al.*, 1985) and have been described previously (Anton *et al.*, 1999; Gould *et al.*, 1989; Restifo *et al.*, 1995; Yewdell *et al.*, 1985, 1986). The control rVACV used expressed β -galactosidase (β -gal) driven by the p11 promoter, as do the other rVACVs used in this study (Chakrabarti *et al.*, 1985). WT3 cells (Pretell *et al.*, 1979) were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 5 % fetal bovine serum (FBS). B3Z 1.G7D7 T-cell hybridoma cells (Sanderson & Shastri, 1994) were subcloned and grown in RPMI 1640 medium (Invitrogen) with 5 % FBS.

Peptides.

Antigenic peptides for vesicular stomatitis virus N_{52–59} (RGYVYQGL), influenza A virus (IAV) PR8 NP_{366–374} (ASNENMETM), VACV B8R_{20–27} (TSYKFESV), VACV A47L_{138–146} (AAFEFINSL), VACV K3L_{6–15} (YSLPNAGDVI), VACV A42R_{88–96} (YAPVSPIVI), VACV A19L_{47–55} (VSLDYINTM) and chicken egg ovalbumin (OVA)_{257–264} (SIINFEKL) were synthesized by the Macromolecule Core Facility at the Hershey Medical Center or purchased from Mimotopes (Fisher Research).

Intracellular cytokine staining (ICS).

Female C57BL/6 mice, 6–8 weeks of age, were immunized intraperitoneally (i.p.) or intravenously (i.v.) with 0.5×10^6 p.f.u. virus (using pre-treatment values). After 6 days, spleens were harvested and homogenized. Lymphocytes were isolated by centrifugation over lymphocyte separation medium (Cambrex) and then stimulated for 6 h at 37 °C with WT3 cells that were either virus-infected for 4 h or pulsed at 25 °C for 40 min with 1 μ M peptide (Ljunggren *et al.*, 1990). Two hours into the stimulation, brefeldin A (Sigma) was added to a concentration of 5 μ g ml⁻¹. Lymphocytes were then incubated on ice for 15 min in supernatant from the 2.4G2 hybridoma containing 10 % normal mouse serum (Sigma). Cells were stained with phycoerythrin–Cy5-conjugated anti-CD8 α antibody (clone 53-6.7; BD Pharmingen) for 40 min on ice, then fixed with 1 % paraformaldehyde for 10 min at 25 °C. Lymphocytes were stained with fluorescein isothiocyanate-conjugated anti-mouse gamma interferon (IFN- γ) antibody (clone XMG1.2; BD Pharmingen) in Fc block/0.5 % saponin (Sigma) for 40 min on ice. Flow cytometry was used to determine the percentage of CD8⁺ lymphocytes producing IFN- γ . Background levels of staining in the absence of stimulation were subtracted to obtain final values. In all experiments involving ICS, immunization with rVACV treated with psoralen and no UV induced levels of specific T_{CD8+} comparable to those with untreated rVACV. Data shown are representative of several repeats of each experiment.

UV/psoralen treatment of viruses.

4,5',8-Trimethylpsoralen (Sigma) was added to 1 ml rVACV stock (1×10^8 p.f.u. ml^{-1}) to give a final concentration of $10 \mu\text{g ml}^{-1}$. The stock was then exposed to UV-C (254 nm) for various times by placing a portable UV lamp approximately 1 cm above the liquid.

Quantification of cell-surface peptide–major histocompatibility complex (MHC) class I complexes.

WT3 cells were infected with treated or untreated rVACV for 4 h. Cells were washed twice in Iscove's modified Dulbecco's medium (Invitrogen) with 10 % FBS and incubated for 15 min in Fc block. Cells were then stained with Alexa Fluor 647-conjugated antibody specific for the SIINFEKL–K^b complex (25D1.16) (Porgador *et al.*, 1997). Fluorescence was analysed by flow cytometry.

Activation of OVA-specific T-cell hybridoma.

WT3 cells were infected with treated or untreated rVACV in the presence of $40 \mu\text{g}$ cytosine arabinoside (Sigma) ml^{-1} and co-incubated at $37^\circ\text{C}/5\% \text{CO}_2$ with B3Z T-cell hybridomas at a ratio of 1 : 1. After 24 h, a β -gal substrate that contained 0.125 % Igepal and chlorophenol red- β -D-galactopyranoside (Sigma) was added to the cells (Malarkannan *et al.*, 2001). Plates were incubated at 37°C until a colour change was apparent (30–45 min); the reaction was then halted by adding 300 mM glycine and 15 mM EDTA at pH 12.0 (Malarkannan *et al.*, 2001). A_{570} was determined with an MRX plate reader.

Measurement of inflammation.

Mice were immunized intradermally (i.d.) in each ear pinna with $10 \mu\text{l}$ 1.0×10^6 p.f.u. VACV ml^{-1} suspended in Hanks' balanced salt solution (HBSS)/0.1 % BSA. We used wild-type VACV Western Reserve rather than the recombinant viruses used elsewhere in the study. The insertion of genes by homologous recombination causes deletion of the thymidine kinase gene, which reduces the *in vivo* replication of viruses (Buller *et al.*, 1985) and dramatically reduces the induction of inflammation induced *in vivo*. Ear thickness was measured at 24 h intervals by using a micrometer (Mitutoyo America Corp.). Swelling in the ears of mice immunized with untreated VACV was comparable to that of mice immunized with VACV that were treated with psoralen and no UV.

Analysis of late gene expression.

WT3 cells were infected with either VSC8 (β -gal driven by the p11 promoter) or VLW (β -gal driven by the p7.5 promoter) that had been treated with psoralen and exposed to UV-C for varying lengths of time. After 7 h, β -gal expression was quantified with chlorophenol red- β -D-galactopyranoside as outlined above.

RESULTS

rVACVs are inefficient at inducing T_{CD8+} responses specific for an encoded foreign antigen

The T_{CD8+} response induced by vesicular stomatitis virus (VSV) in an H-2^b mouse is directed almost exclusively towards aa 52–59 of the nucleoprotein (N) presented by the MHC class I molecule H2 K^b (Fremont *et al.*, 1992). C57BL/6 mice were immunized i.p. with VSV, rVACV encoding the full-length VSV N, rVACV encoding the VSV N_{52–59} minimal antigenic determinant or a control rVACV. After 6 days, splenocytes were isolated and stimulated with VSV-infected cells (which are able to present the entire repertoire of H-2^b-restricted VSV determinants), VSV N_{52–59}-pulsed cells (which present only the VSV N_{52–59} determinant) or control rVACV-infected cells (which are able to present the entire repertoire of H-2^b VACV determinants). IFN- γ production in response to peptide-pulsed or virus-infected cells was determined by using an ICS assay.

Immunization with VSV itself was significantly more efficient (8–9-fold higher percentage of T_{CD8+}) at producing both VSV-specific and VSV N_{52–59}-specific T_{CD8+} than immunization with rVACV (Fig. 1a, b). In contrast, 2–3-fold greater numbers of VACV-specific T_{CD8+} were induced compared with VSV-specific or N_{52–59}-specific T_{CD8+} following immunization with rVACV encoding the full-length VSV N (Fig. 1c). Thus, rVACV immunization remains markedly less efficient at inducing a specific T_{CD8+} response than immunization with a virus expressing antigen in its natural context.

We also examined the T_{CD8+} response directed against IAV following immunization with IAV or an rVACV expressing an antigen derived from IAV. We examined the induction of T_{CD8+} specific for an immunodominant peptide derived from influenza nucleoprotein (NP_{366–374}) 6 days after immunization with IAV or rVACV encoding either full-length NP or the NP_{366–374} minigene.

Similar to the results seen with VSV, i.p. immunization with IAV was more efficient at producing NP_{366–374}-specific T_{CD8+} than immunization with rVACV expressing either full-length NP or the NP_{366–374} minigene (Fig. 1d, e). Similar to previous reports (Harrington *et al.*, 2002), the numbers of T_{CD8+} specific for VACV determinants were up to 30-fold higher than the numbers of T_{CD8+} specific for the recombinant antigen (Fig. 1f). Similar results were observed for T_{CD8+} cells isolated by peritoneal lavage 6 days post-immunization and for splenic memory T_{CD8+} responses measured 4 weeks post-immunization (data not shown). These results indicate that the induction of a T_{CD8+} response to the same antigen is markedly less efficient when it is expressed during rVACV infection than when expressed in its natural context.

Ablation of viral transcription by using UV/psoralen treatment

We reasoned that rVACV vectors in which viral gene expression is inhibited while foreign antigen expression is maintained will have an advantage as a vaccine, as they will induce fewer immunodominant T_{CD8+}. To achieve such a vaccine, we used an rVACV expressing a foreign antigen as a minigene and inactivated viral gene expression by using UV/psoralen treatment. This method will be effective only if there is a dose of treatment that will broadly reduce VACV

gene expression while leaving expression of the minigene (which represents a much smaller target for DNA damage) intact.

Long-wave UV/psoralen treatment has previously been shown to ablate VACV genes in a size-dependent manner (Tsung *et al.*, 1996). To test whether UV-C/psoralen-mediated inhibition of gene expression is similarly dependent on the size of a gene, we used two rVACVs that express the green fluorescent protein (GFP). The first rVACV expresses the chimeric protein NP–GFP (Anton *et al.*, 1999) (744 aa, >2.2 kb) and the second expresses the shorter GFP alone (237 aa, 0.7 kb). Virus stocks were subjected to UV/psoralen at a range of doses and GFP fluorescence was measured by flow cytometry after 4 h infection of WT3 cells. Expression of the larger NP–GFP was almost undetectable in infected cells when the virus was treated with 10 µg psoralen ml⁻¹ and irradiated for 20 s with UV-C (Fig. 2a). In contrast, GFP expression was readily detectable, although significantly reduced, after 60 s exposure to UV-C (Fig. 2a). These results demonstrate that, by choosing dose carefully, UV/psoralen can be used to inhibit the expression of large VACV-encoded genes selectively, while leaving the expression of shorter genes relatively intact.

To show that presentation of T_{CD8+} epitopes is similarly sensitive to UV/psoralen treatment, we used rVACV encoding the H-2K^b-restricted SIINFEKL epitope from OVA as a minigene (rVACV-OVA_{257–264}) or as part of full-length ovalbumin (rVACV-OVA). Virus stocks were treated with varying doses of UV/psoralen and used to infect WT3 cells. Levels of cell-surface peptide–MHC complexes were quantified by using the antibody 25D1.16, which detects SIINFEKL–K^b complexes (Porgador *et al.*, 1997; Princiotta *et al.*, 2003). As expected, much higher levels of surface SIINFEKL–K^b complexes were generated after infection with rVACV-OVA_{257–264} than with rVACV-OVA, due to the larger quantities of relevant peptide produced after infection with the former, in addition to the requirement for proteasomal processing of the latter antigen. As little as 40 s UV-C treatment of rVACV-OVA reduced the levels of SIINFEKL–K^b complexes to background levels (Fig. 2b). In contrast, after infection with rVACV-OVA_{257–264} treated with 40 s UV-C, surface SIINFEKL–K^b levels were still greater than those generated from untreated rVACV-OVA. Indeed, SIINFEKL–K^b complexes could be detected on the surface of cells infected with rVACV-OVA_{257–264} treated with psoralen and UV-C for 120 s (data not shown).

Although 25D1.16 staining is a direct measurement of peptide–MHC presentation, T-cell activation may occur at levels of surface peptide–MHC that are undetectable using this antibody (Porgador *et al.*, 1997). To assess antigen presentation to T cells, we examined the ability of virus-infected cells to trigger the SIINFEKL-specific *lacZ* T-cell hybridoma B3Z. The B3Z hybridoma expresses the *lacZ* gene driven by the interleukin-2 response element NFAT promoter, so β-gal is produced when the cells are stimulated through the T-cell receptor (Sanderson & Shastri, 1994). WT3 cells were infected with rVACV-OVA or rVACV-OVA_{257–264} that had been treated with psoralen and varying doses of UV-C. Infected WT3 cells were incubated overnight with the B3Z T-cell hybridoma, and antigen presentation was measured the following day by using a colorimetric substrate of β-gal.

B3Z T-cell hybridomas detected SIINFEKL–K^b complexes with kinetics similar to those for 25D1.16 staining, but presentation could be detected after treatment of virus with UV-C and psoralen for up to 5 min (data not shown). T-cell activation by WT3 cells infected with rVACV-OVA is detectable at low levels after pre-treatment of virus with psoralen and 40 s exposure to UV-C, but is ablated completely after 60 s UV-C exposure (Fig. 2c). Thus, MHC class I-restricted presentation of minigene is preserved under conditions that ablate presentation of peptides derived from longer proteins.

UV/psoralen treatment enhances *in vivo* T_{CD8+} responses to rVACV-encoded minigenes, but inhibits responses to native VACV epitopes

To test whether UV/psoralen-treated rVACV could induce primary T_{CD8+} to an encoded minigene, mice were immunized i.v. with rVACV-OVA_{257–264} treated with psoralen and increasing doses of UV-C. Six days later, splenic responses to VACV-infected cells and SIINFEKL peptide were measured by using ICS for IFN- γ . The percentage of VACV-specific T_{CD8+} decreased with increasing doses of UV-C, with a >66 % decrease when rVACV-OVA_{257–264} was exposed to UV-C for 1 min (Fig. 3a). In contrast, the percentage of SIINFEKL-specific T_{CD8+} increased with UV/psoralen treatment of rVACV-OVA_{257–264}, reaching a >8-fold increase with 1 min treatment (Fig. 3b). Immunization with UV/psoralen-treated VACV results in a lesser expansion in the cellularity of the spleen than immunization with VACV treated with psoralen alone (Fig. 3c). However, when the reduced splenic cellularity was taken into account, the increase in the percentage of SIINFEKL-specific T_{CD8+} represented a 3-fold increase in the total number of these cells (Fig. 3d). To investigate whether the increase in SIINFEKL-specific T_{CD8+} was related to the size of a target antigen gene for psoralen incorporation, we also immunized mice with UV/psoralen-treated VACV expressing full-length OVA. When SIINFEKL was encoded in the context of the full-length OVA gene, as little as 1 min UV-C/psoralen treatment was able to ablate the specific T_{CD8+} response to this determinant (Fig. 3e), indicating that the enhanced response was due to the small size of the SIINFEKL minigene.

Recently, five MHC class I-restricted determinants for VACV in H-2^b mice were identified (Tscharke *et al.*, 2005), allowing us to test the effect of UV/psoralen treatment on priming of T_{CD8+} to individual vector epitopes. These epitopes are located at various distances from the amino terminus of their protein, ranging from as few as 6 to 138 aa. Four of these determinants, namely B8R_{20–27}, K3L_{6–15}, A47L_{138–146} and A19L_{47–55}, are derived from the products of genes classified as early. Only one, A42R_{88–96}, is derived from the product of a gene classified as late. In the experiment described above, lymphocytes were also stimulated with peptide-pulsed cells and the response to each of the VACV epitopes was quantified similarly by using ICS for IFN- γ . The response to four of the five VACV determinants decreased or remained stable at low levels upon UV/psoralen treatment, with the exception of K3L_{6–15} (Fig. 4a). Similar to a minigene, the response to this peptide increased with UV/psoralen treatment, with a maximum response occurring after 1 min exposure to UV-C. This result is not unexpected, because the epitope is only 6 aa from the amino terminus of the K3L protein and so will probably act in a manner resembling the similarly sized minigene. The sum of the number of T_{CD8+} responding to the five

known VACV determinants was compared with the number of T_{CD8+} responding to VACV-infected cells. The T_{CD8+} response to the VACV determinants did not decrease as rapidly as that to VACV-infected cells (Fig. 4b), indicating that the entire VACV response may act differently from that of the mapped determinants.

UV/psoralen treatment ablates viral replication

By treating VACV with psoralen and UV to reduce vector gene expression, we anticipated that we would reduce virus replication and the resulting inflammation that can result following immunization. Long-wave UV (UV-A and -B) and psoralen treatment can ablate the replication of viruses (Lane *et al.*, 1969) and bacteria (Brockstedt *et al.*, 2005), and has been used to attenuate rVACV vaccine vectors in other studies (Oertli *et al.*, 1996; Zajac *et al.*, 1997) by blocking late gene transcription. To verify that our UV-C/psoralen treatment also inhibits late gene expression, we used rVACVs that express β -gal driven either by the strict late p11 promoter or the early/late p7.5 promoter. Virus stocks were treated with psoralen and increasing doses of UV-C, and functional β -gal production was measured after 7 h infection of WT3 cells. UVC treatment for 10 s reduced β -gal expression from the strict late p11 promoter to background levels, whilst reducing expression from the early/late promoter by only 25 % (Fig. 5a). Ablation of β -gal production driven by p7.5 required 30 s UV treatment. We next assessed the ability of UV/psoralen-treated virus to generate plaques in a standard titration assay. The replicative competence of the rVACV in our studies was compromised severely after 30 s UV treatment (Fig. 5b) and there were no observable plaques following as little as 1 min UV/psoralen treatment (data not shown).

UV/psoralen treatment ablates the inflammatory response following VACV immunization

The inflammatory response induced following VACV immunization can cause severe complications that would preclude the use of VACV as a vaccine vector in the general population (Lane *et al.*, 1969). Reduction or ablation of replication has been linked to a significant reduction in the inflammatory response induced *in vivo* (Sutter *et al.*, 1994). To assess whether this was the case following UV/psoralen treatment, we infected mice i.d. in each ear pinna (Tschärke *et al.*, 2002) with VACV that was treated with psoralen and 0 or 1 min UV-C. Ear thickness was then measured daily to gauge the severity of the resulting inflammatory response.

Following challenge with 10⁴ p.f.u. VACV, ear thickness increased rapidly in mice infected with VACV that had been treated with psoralen and no UV-C. Ear thickness continued to increase until 5 days post-injection, when a mean increase of 0.7 mm was measured (Fig. 6). After 6 days, the swelling did not subside, but the injected ears displayed extensive scabbing at the inoculation site and substantial necrosis was apparent. In contrast, mice inoculated with VACV treated with psoralen and 1 min UV-C treatment displayed no measurable increase in ear thickness (Fig. 6) and were comparable to mice injected with HBSS only (data not shown).

Upon i.v. infection with rVACV, there is typically a large increase in the cellularity of the spleen, reaching a maximum at 6 days post-infection, that results from the expansion of many lymphoid

and myeloid cell types (C. C. Norbury, unpublished results). The number of splenocytes immunized with rVACV treated with no UV-C was greater than twice that of mice immunized with rVACV treated for 5 min with UV-C, and 74 % greater than that following immunization with rVACV treated with UV-C for 1 min (Fig. 3c). Thus, UV/psoralen-treated VACV induces a markedly reduced inflammatory response *in vivo* following either i.d. or i.v. immunization compared with untreated VACV.

DISCUSSION

VACV, variants of VACV and other poxviruses are presently among the most widely used vaccine vectors. However, such viruses are inefficient at inducing T_{CD8+} specific for recombinantly expressed antigen, due to a large T_{CD8+} response that targets the vector itself. In this study, we have successfully employed a strategy to reduce the undesirable features of VACV vectors, namely immunodominance and inflammation, following immunization with VACV vaccines. At the same time, the presentation of physiologically relevant levels of peptide–MHC complexes derived from recombinant minigenes remains intact, resulting in a viral vector that is more efficient at inducing T_{CD8+} to the desired recombinantly expressed antigen.

Previously, treatment with psoralen and long-wave UV-A has been used to ablate the replication (Hanson *et al.*, 1978) and cytopathic effects (Tsung *et al.*, 1996) of VACV. This treatment blocks transcription of late genes, but not early genes, and produces a non-replicating virus that is significantly attenuated and safer to use as a vaccine vector (Oertli *et al.*, 1996; Zajac *et al.*, 1997), as it can neither spread from the site of infection nor initiate a severe inflammatory response. Inflammatory complications and unrestrained growth of the virus following immunization are one of the leading obstacles to the use of VACV as a vector, particularly in immunocompromised populations. Here, we exploit the cross-linking efficacy of short-wave UV-C to ablate production of a large number of intact VACV proteins encoded by both early and late genes. By encoding recombinant antigen as a minigene, the resulting drop in the efficiency of antigen presentation is functionally irrelevant, as the minigene produces much higher levels of peptide–MHC complexes in infected cells than those generated from rVACV encoding full-length antigen.

The increase in T_{CD8+} response to a minigene after broad inhibition of VACV gene expression is probably due to two mechanisms acting in a cooperative manner. First, ablation of VACV gene expression reduced the induction of VACV-specific T_{CD8+}, freeing minigene-specific T_{CD8+} from immunodomination by VACV-specific T_{CD8+}. In other systems, removal of an immunodominant determinant leads to an increase in the frequency of T_{CD8+} specific for subdominant determinants (Tanaka *et al.*, 1989; Webby *et al.*, 2003; Weidt *et al.*, 1998). By treating rVACV with UV/psoralen, we have decreased the production of viral proteins, the source of all immunodominant determinants, regardless of the genetic background of the organism being immunized. The resulting increase in the percentage of T_{CD8+} specific for the OVA_{257–264} determinant can be reproduced after immunization with rVACV encoding minigenes derived from both VSV N protein and Sendai virus nucleoprotein (data not shown). Reduced

immunodominance probably acts in concert with the ablation of the production of VACV immunomodulators by UV/psoralen treatment. Genetic removal of two such genes from VACV has been shown to increase the magnitude of the VACV-specific T_{CD8+} response (Clark *et al.*, 2006; Staib *et al.*, 2005) and would probably increase the response to an encoded minigene.

As little as 1 min UV/psoralen treatment reduced the entire VACV-specific T_{CD8+} response by as much as 66 %, a much greater decrease than that seen in the sum of the five VACV determinants mapped to date (Fig. 4b). In fact, these five determinants comprise only about 40 % of the entire response in H-2^b mice (Tscharke *et al.*, 2005). Thus, the decrease in the T_{CD8+} response to VACV-infected cells probably results from UV/psoralen treatment-mediated ablation of hitherto-undiscovered VACV encoded determinant(s).

UV/psoralen treatment for 1 min that produces a 66 % reduction of the VACV-specific T_{CD8+} response probably results from the targeting of two groups of determinants. The primary targets of UV/psoralen treatment are those determinants encoded within late genes. One of the determinants to which the T_{CD8+} response was relatively stable after 5 min UV/psoralen treatment, A42R_{88–96}, is a product of a gene that has been classified as late (Blasco *et al.*, 1991). Late gene transcription was blocked under conditions (10 µg psoralen ml⁻¹, 1 min UV-C) that were still able to generate a T_{CD8+} response to the A42R determinant (Fig. 5a). Thus, either this determinant is generated from protein in the viral stocks, an unlikely scenario as increasing UV treatment to longer than 20 min ablates the response (data not shown), or the A42R gene is not a strict late gene. In the initial characterization of this VACV gene, low levels of protein were generated in the presence of cytosine arabinoside, an analogue of deoxycytidine that blocks DNA replication and thus late gene production (Blasco *et al.*, 1991). Mapping of VACV-encoded determinants in late genes has typically been disregarded, as recombinant antigens driven by late promoters often fail to induce antigen-specific T_{CD8+} following immunization (Bronte *et al.*, 1997; Coupar *et al.*, 1986). However, our data indicate that viral genes may not always fall into easily distinguishable categories, and disregarding groups of genes as non-immunogenic may produce misleading results.

In addition to a direct effect upon responses to late VACV genes, UV/psoralen treatment will also probably target antigenic determinants present at sites further from the initiation of genes in which they are encoded. The T_{CD8+} response to a determinant encoded by the K3L gene (K3L_{6–15}) increased significantly upon UV/psoralen treatment, with the number of responding cells peaking at 1 min UV-C exposure and declining thereafter (Fig. 4a). The kinetics of this increase were similar to those observed with the response to the encoded OVA_{257–264} minigene (Fig. 3b). K3L_{6–15} is very close to the amino terminus of the protein, making psoralen incorporation between the start codon and the coding sequence of the determinant very unlikely. Thus, expression of this determinant following UV/psoralen treatment is probably preserved in a similar way to the recombinant minigene determinant. In contrast, the A47L_{138–146} determinant is located furthest from the start codon and the T_{CD8+} response to this determinant was ablated completely upon 1 min UV/psoralen treatment.

Decreased inflammation following immunization with UV/psoralen-treated rVACV is an attractive characteristic from a vaccine perspective. Attenuated VACV strains, such as modified virus Ankara (MVA), are commonly used as vaccine vectors and have been shown to induce a milder inflammatory response than wild-type VACV (Stickl & Hochstein-Mintzel, 1971). However, these attenuated strains are still limited by many of the other drawbacks of wild-type VACV when used as a vaccine vector. MVA elicits a robust humoral response (Jones-Trower *et al.*, 2005) that can prevent reimmunization and, despite deletions in large regions of the genome, MVA still encodes many immunomodulatory genes that can blunt a T_{CD8+} response to a recombinant antigen. Following immunization with UV/psoralen-treated rVACV, we observed a dramatic (16-fold) reduction in the titres of neutralizing antibody (data not shown). In addition, UV/psoralen treatment of VACV can ablate production of virus-encoded immunomodulatory molecules. Finally, MVA still elicits large T_{CD8+} responses that can be immunodominant and suppress the desired T_{CD8+} response to a recombinant antigen (Smith *et al.*, 2005). It is possible that the reduction of vector gene expression in attenuated VACV strains such as MVA could improve the efficacy of these vectors as vaccines.

The strategy outlined here of selectively targeting VACV gene expression reduces vector immunodominance and expression of immunomodulators, leading to an increased frequency of T_{CD8+} specific for the recombinantly encoded minigene. The minigene-specific memory T_{CD8+} response under these circumstances is similar in magnitude to that observed after immunization with replicating VACV (data not shown). Thus, production of a potential vector of equal or greater efficacy that induces significantly reduced levels of anti-VACV neutralizing antibody and dramatically inflammatory responses points a way to a safe and effective vaccination strategy. Overall, we have demonstrated that the reduction of VACV vector expression allows the more effective generation of antigen-specific T_{CD8+} following immunization.

ACKNOWLEDGEMENTS

We thank Irene Reider, Eric Tewalt and Amanda Schell for excellent technical assistance and Drs Jon Yewdell and Jack Bennink for many of the rVACVs used. We also acknowledge the contributions of Nate Sheaffer of the Flow Cytometry Core Facility and Anne Stanley of the Macromolecular Core Facility of the Penn State College of Medicine. This work was supported by National Institutes of Health grants AI056094 and AI070537 to C. C. N. and an NHMRC (Australia) Howard Florey Centenary Fellowship #224273 to D. C. T. All animal experiments were conducted in a facility constructed with support from Research Facilities Improvement grant no. C06 RR-15428-01 from the National Center for Research Resources, National Institutes of Health.

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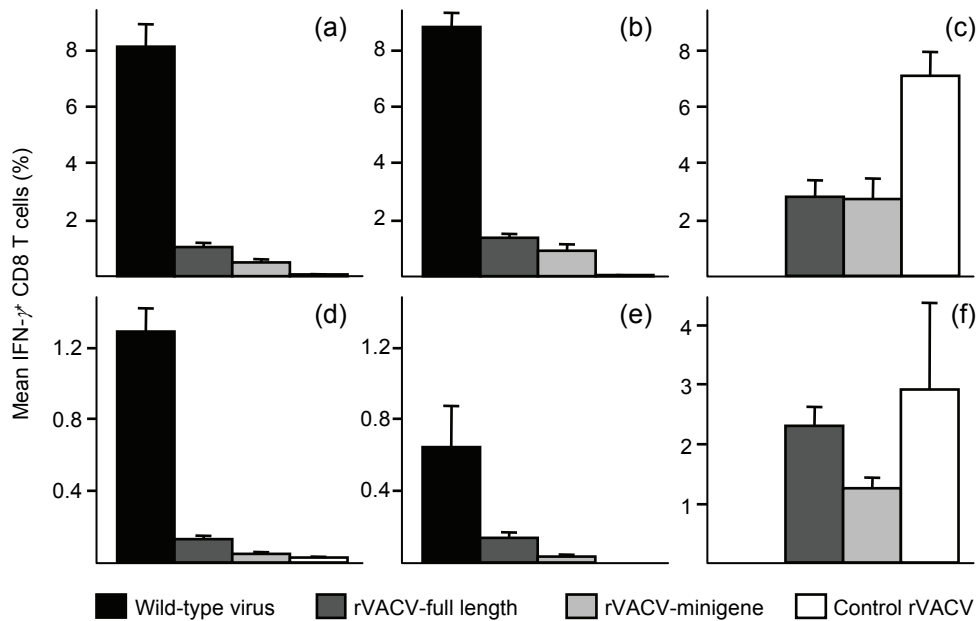


Fig. 1. Inefficient induction of a T_{CD8+} response by rVACV. Mice were immunized i.p. with VSV, rVACV-VSV N, rVACV-VSV N₅₂₋₅₉ (a-c) or IAV, rVACV-NP, rVACV-NP₃₆₆₋₃₇₄ (d-f). After 6 days, lymphocytes were isolated and stimulated with VSV-infected cells (a), VSV N₅₂₋₅₉ peptide-pulsed cells (b), IAV-infected cells (d), IAV NP₃₆₆₋₃₇₄ peptide-pulsed cells (e) or rVACV-infected cells (c, f). IFN- γ production was measured by using ICS.

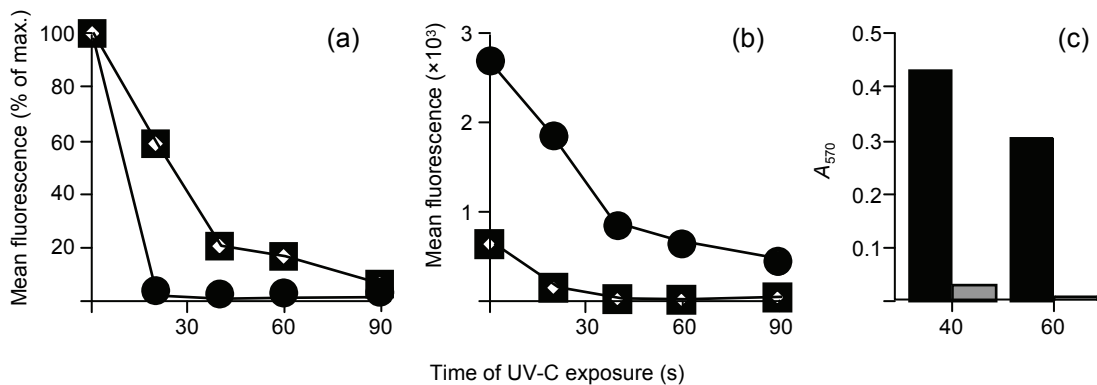


Fig. 2. UV/psoralen treatment ablates the transcription of ORFs and subsequent presentation of encoded peptides. (a) rVACVs encoding either NP-GFP (●) or GFP (■) were treated with psoralen and irradiated with UV-C for the periods of time shown. Fluorescent gene production was measured by flow cytometry following infection of WT3 cells. (b, c) WT3 cells were infected *in vitro* with either rVACV-OVA₂₅₇₋₂₆₄ (●, black bars) or rVACV-OVA (■, grey bars) that had been treated with psoralen and UV-C as shown. Cell-surface presentation of SIINFEKL-K^b complexes was then measured directly by using 25D1.16 antibody staining (b) or indirectly by culturing infected WT3 cells with the B3Z *lacZ* T-cell hybridoma and then measuring β -gal activity (c).

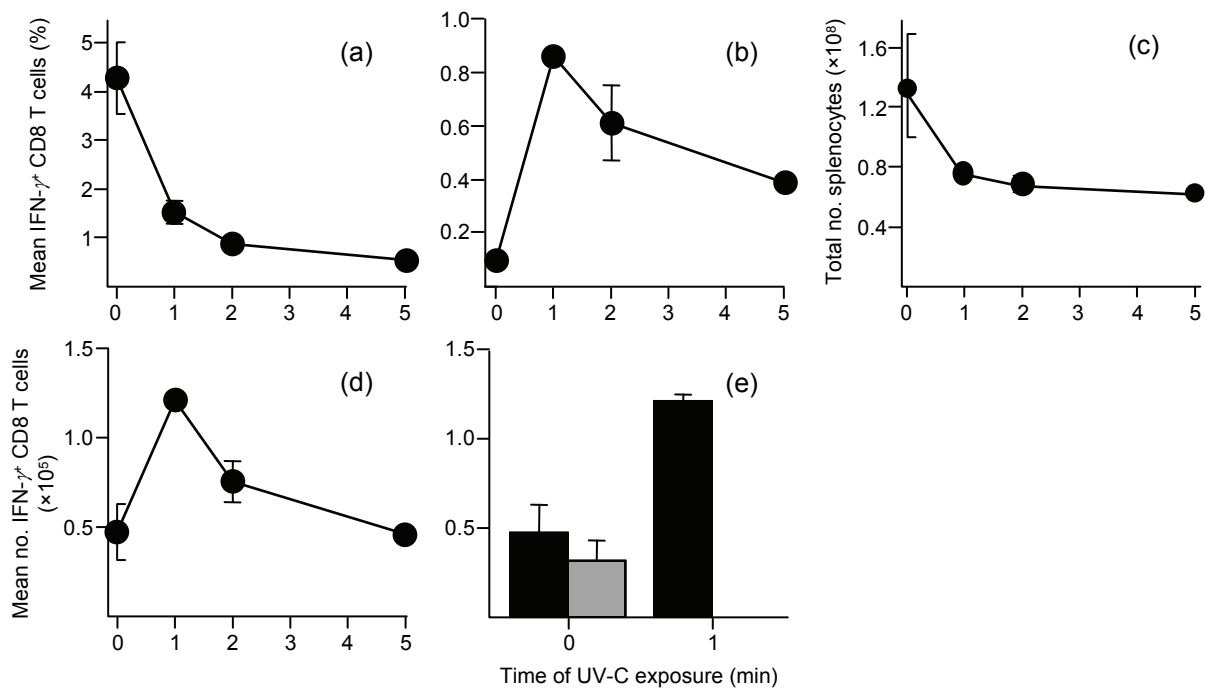


Fig. 3. Immunization with UV/psoralen-treated rVACV-OVA₂₅₇₋₂₆₄ decreased the total VACV-specific T_{CD8+} response, but enhanced the T_{CD8+} response to the recombinantly encoded determinant. Mice were immunized with rVACV-OVA₂₅₇₋₂₆₄ that had been treated with psoralen and UV-C as shown. After 6 days, lymphocytes were isolated and stimulated with WT3 cells that were infected with a control rVACV (a) or pulsed with the SIINFEKL peptide (b, d). IFN- γ production was measured by using ICS. Total splenocyte counts are also shown (c). (e) SIINFEKL-specific T_{CD8+} response following immunization with rVACV-OVA₂₅₇₋₂₆₄ (black bars) and rVACV-OVA (grey bars) that was untreated, or treated with UV/psoralen for 1 min.

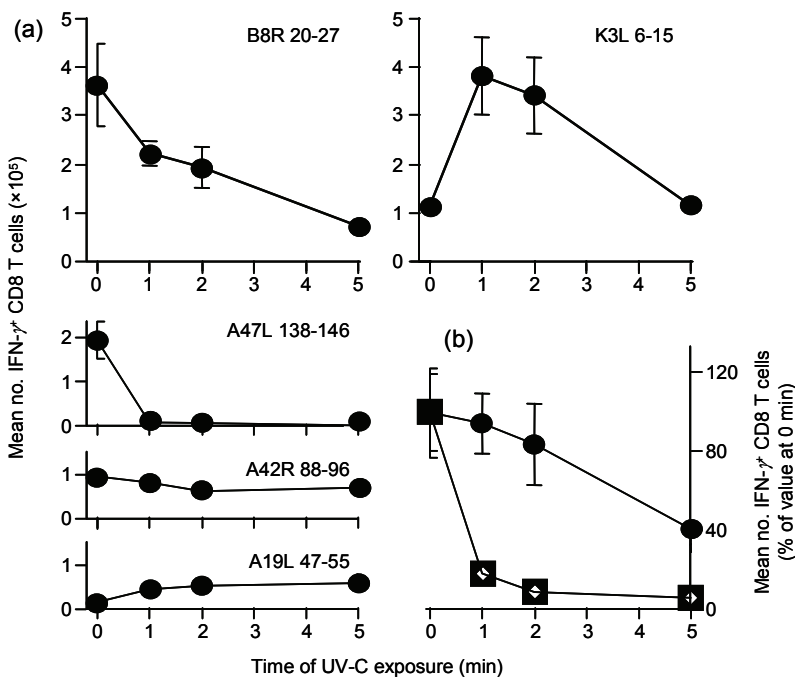


Fig. 4. UV/psoralen treatment of rVACV-OVA₂₅₇₋₂₆₄ altered the VACV-specific T_{CD8+} response in a determinant-specific manner. Mice were immunized with rVACV-OVA₂₅₇₋₂₆₄ that had been treated with psoralen and UV-C as shown. (a) After 6 days, lymphocytes were isolated and stimulated with WT3 cells pulsed with the peptides that correspond to each of five known VACV determinants. IFN- γ production was measured by using ICS. (b) The change in the sum of these responses (●) was then compared with the change in the total VACV specific T_{CD8+} response (■) as shown in Fig. 3(a).

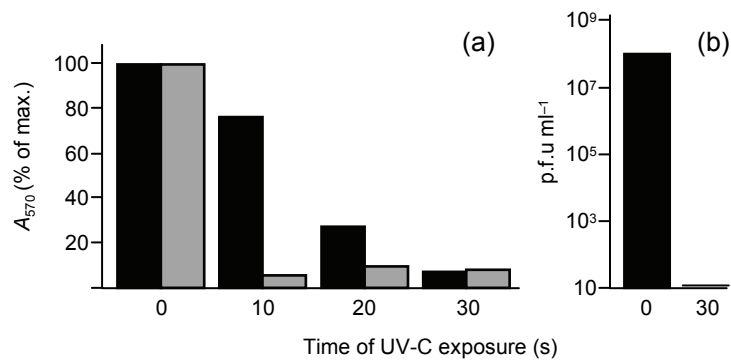


Fig. 5. UV/psoralen treatment ablates VACV replication and late gene expression. (a) WT3 cells were infected with rVACV that had been treated with psoralen and UV-C as shown. β -Gal was either driven by the early/late promoter p7.5 (black bars) or the late promoter p11 (grey bars). β -Gal production was assayed by using a colorimetric assay. (b) Psoralen and UV-C as shown were used to treat rVACV-OVA₂₅₇₋₂₆₄. Treated stocks were then used to infect a monolayer of cells *in vitro* and replication was determined by counting the number of plaques formed.

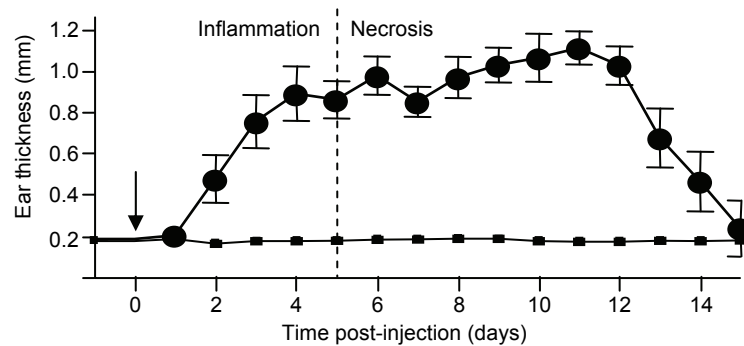


Fig. 6. UV/psoralen treatment of VACV reduces the inflammatory response induced after immunization. C57BL/6 mice were immunized i.d. in each ear with VACV Western Reserve treated with psoralen and no UV-C (●) or treated with psoralen and 1 min UV-C (■). Ear thickness was measured at 24 h intervals.