

Cross-priming of CD8⁺ T cells stimulated by virus-induced type I interferon

Agnes Le Bon^{1,5}, Nathalie Etchart^{1,4,5}, Cornelia Rossmann², Miranda Ashton¹, Sam Hou¹, Dirk Gewert³, Persephone Borrow¹ & David F Tough¹

CD8⁺ T cell responses can be generated against antigens that are not expressed directly within antigen-presenting cells (APCs), through a process known as cross-priming. To initiate cross-priming, APCs must both capture extracellular antigen and receive specific activation signals. We have investigated the nature of APC activation signals associated with virus infection that stimulate cross-priming. We show that infection with lymphocytic choriomeningitis virus induces cross-priming by a mechanism dependent on type I interferon (IFN- α/β). Activation of cross-priming by IFN- α/β was independent of CD4⁺ T cell help or interaction of CD40 and CD40 ligand, and involved direct stimulation of dendritic cells. These data identify expression of IFN- α/β as a mechanism for the induction of cross-priming during virus infections.

Priming of CD8⁺ T cells requires recognition through the T cell receptor (TCR) of peptide–major histocompatibility complex (MHC) class I complexes on the surface of appropriate APCs. In most cell types, MHC class I–associated peptides are derived solely from proteins synthesized within the cell. However, bone marrow–derived APCs can also translocate antigens from the endocytic to the cytosolic compartment of the cell and thereby direct endocytosed proteins into the MHC class I presentation pathway, a process known as cross-presentation^{1,2}. Although several different bone marrow–derived cells can cross-present antigen *in vitro*, the main APCs able to do so *in vivo* are dendritic cells (DCs)^{3–5}.

Cross-presentation may occur after APC uptake of soluble molecules or particulate matter, including other cells, and is necessary for the priming of CD8⁺ T cell responses against exogenous antigens (cross-priming)^{2,6–9}. However, cross-presentation of antigen by APCs does not necessarily result in cross-priming^{2,10}; DCs also need to receive appropriate activation signals to become competent to induce cross-priming, a process called ‘licensing’ of APCs^{11–14}. Cross-presentation of antigen by nonlicensed DCs stimulates an abortive CD8⁺ T cell response that culminates in tolerance rather than induction of effector activity (cross-tolerance)^{4,15}.

DCs can become licensed for cross-priming by contact with bacteria or certain bacterial products^{16–19}. Therefore, cross-priming may allow for the generation of CD8⁺ T cell responses against bacteria that do not infect DCs directly because endocytosed infected cells will carry both specific antigen and the licensing signal. In contrast, viral-associated signals that can stimulate cross-priming have not been identified, although CD8⁺ T cell responses can be generated against

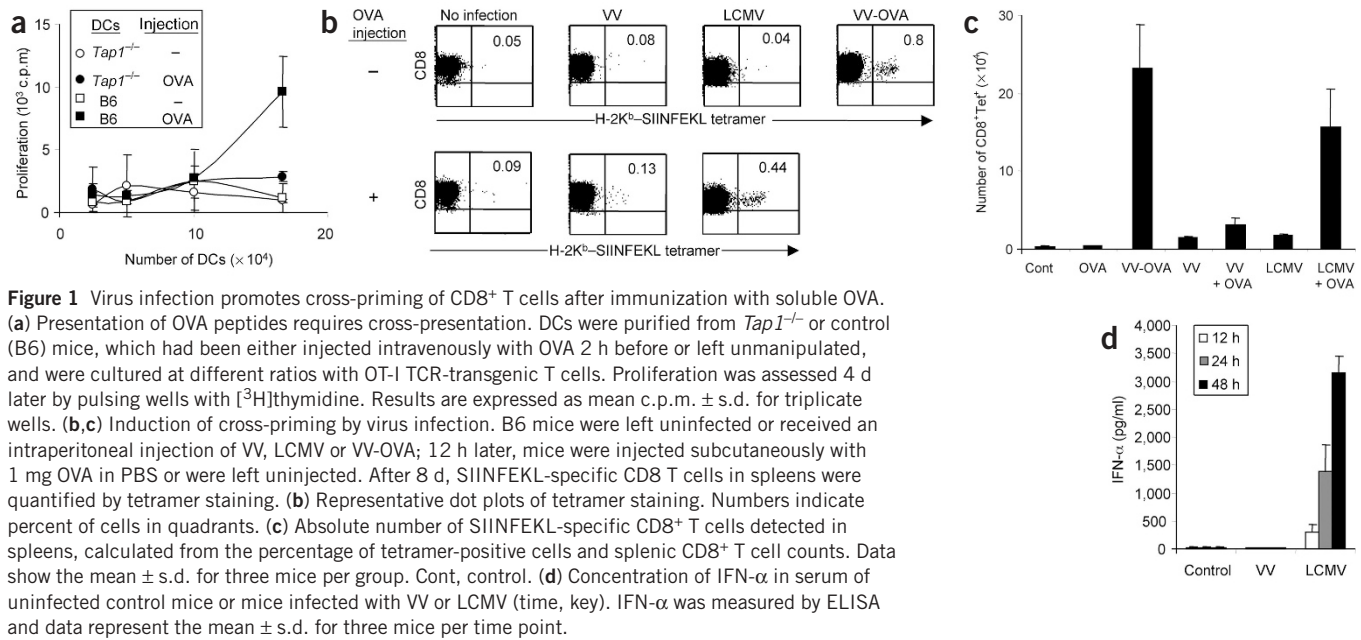
viruses in the apparent absence of direct APC infection^{20,21}, indicating that cross-priming can occur. Two categories of virus-associated stimuli could be involved in licensing APCs: viral components, which would be present in infected cells, and host molecules, expressed by cells in response to virus infection.

One candidate for a host cell–derived stimulus for cross-priming against virally-infected cells is type I interferon (IFN- α/β). IFN- α/β is expressed by most if not all cells in response to virus infection²², and can be secreted in large amounts by plasmacytoid pre-DCs after contact with virus²³. Hence, production of IFN- α/β is a sensitive indicator of virus infection and well suited for a function in linking innate and adaptive immunity. In addition, IFN- α/β both stimulates DC maturation and acts as a strong adjuvant for the humoral immune response^{24–28}.

Some published data indicate that IFN- α/β can enhance CD8⁺ T cell responses. Initial evidence for this came from experiments on tumor cells treated with virus or transfected with genes encoding IFN- α/β , which in both cases provoked stronger responses than did control cells^{29,30}. In another study, injection of poly I:C, a synthetic double-stranded RNA that induces IFN- α/β , enhanced the response of transgenic CD8⁺ T cells to antigen-expressing APCs *in vivo*³¹. This finding indicated that IFN- α/β could augment the CD8⁺ T response to directly presented antigen, although the function of other factors induced by poly I:C was not assessed. Finally, enhancement of cross-priming by oligonucleotides containing immunostimulatory CpG motifs, which mimic bacterial DNA, was reduced in IFN- α/β R-deficient mice^{32,33}, indicating that IFN- α/β contributes to the adjuvant characteristics of this compound. At present,

¹The Edward Jenner Institute for Vaccine Research, Compton, Newbury, Berkshire RG20 7NN, England. ²Acambis, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT, England. ³BioLauncher, 50 Gilbert Road, Cambridge CB4 3PE, England. ⁴Present address: Institut National de la Santé et de la Recherche Médicale U404, IFR 74-CERVI, 21 Avenue Tony Garnier, 69365 LYON Cedex 07, France. ⁵These authors contributed equally to this work. Correspondence should be addressed to D.F.T. (david.tough@jenner.ac.uk).





however, it is unknown if IFN-α/β itself can stimulate cross-priming of CD8⁺ T cells.

Here, we have investigated the ability of viruses to stimulate cross-priming, focusing on a possible function for IFN-α/β in this process. Our results demonstrate that CD8⁺ T cells respond strongly to cross-presented antigen *in vivo* when mice are infected with virus, and that this response is dependent on IFN-α/β. Injection of IFN-α induced functional cross-priming, as shown by the differentiation of CD8⁺ T cells into effector cells that provided protection against virus infection. Induction of cross-priming by IFN-α/β involved direct stimulation of DCs, and was independent of CD4⁺ T cell help or interaction of CD40 and CD40 ligand (CD40L).

RESULTS

Virus infection promotes cross-priming of CD8⁺ T cells

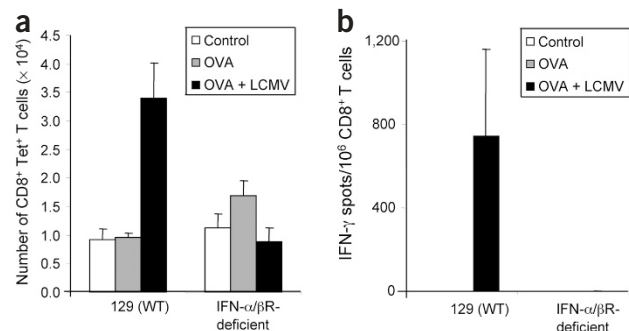
Studying cross-priming during virus infections is complicated by difficulties in excluding direct expression of viral genes in APCs. Therefore, to investigate whether virus-associated signals can promote cross-priming, we chose to examine how virus infection affected the generation of CD8⁺ T cell responses against an independently administered exogenous antigen, ovalbumin (OVA). To confirm that presentation of OVA peptides in association with MHC class I required cross-presentation, we injected OVA into wild-type mice or mice deficient

for the transporter associated with antigen presentation 1 (TAP1). We isolated DCs from the spleens of these mice 2 h later, and assessed them directly (without the addition of antigen *in vitro*) for their ability to stimulate the proliferation of OT-I CD8⁺ T cells; the latter express a transgenic TCR specific for the SIINFEKL peptide of OVA in association with H-2K^b (ref. 34). Only TAP1⁺ DCs were able to activate the OT-I cells, indicating that peptide presentation after injection of OVA protein was dependent on TAP and was therefore the result of cross-presentation (Fig. 1a).

To assess the effect of virus infection on cross-priming, we injected OVA into control B6 mice or mice that had been infected 12 h earlier with a virus, and measured the anti-OVA CD8⁺ T cell response with H-2K^b-SIINFEKL tetramers. We administered vaccinia virus (VV) or lymphocytic choriomeningitis virus (LCMV) intraperitoneally, and injected OVA subcutaneously. As a positive control, we infected mice with a recombinant VV expressing OVA (VV-OVA), which should allow for direct expression of OVA in APCs and hence MHC class I-restricted presentation of OVA epitopes by the endogenous presentation pathway.

After injection of OVA alone into uninfected mice, we found few if any SIINFEKL-specific CD8⁺ T cells (Fig. 1b,c). In contrast, we noted an OVA-specific CD8 response in virus-infected mice after injection of soluble OVA. The response was relatively low in VV-infected mice, but

Figure 2 IFN-α/β is required for LCMV-induced cross-priming against OVA. Wild-type (WT; 129) or IFN-α/βR-deficient mice were left uninfected or received an intraperitoneal injection of LCMV; 12 h later, mice were injected subcutaneously with 1 mg OVA in PBS or were left uninjected. After 8 d, SIINFEKL-specific CD8⁺ T cells in spleens were quantified by tetramer staining (a) or IFN-γ ELISPOT assay (b). Data in a represent the absolute number of SIINFEKL-specific CD8⁺ T cells detected in spleens, calculated from the percentage of tetramer-positive (Tet⁺) cells and splenic CD8⁺ T cell counts. Results are expressed as mean ± s.e.m. for three mice.



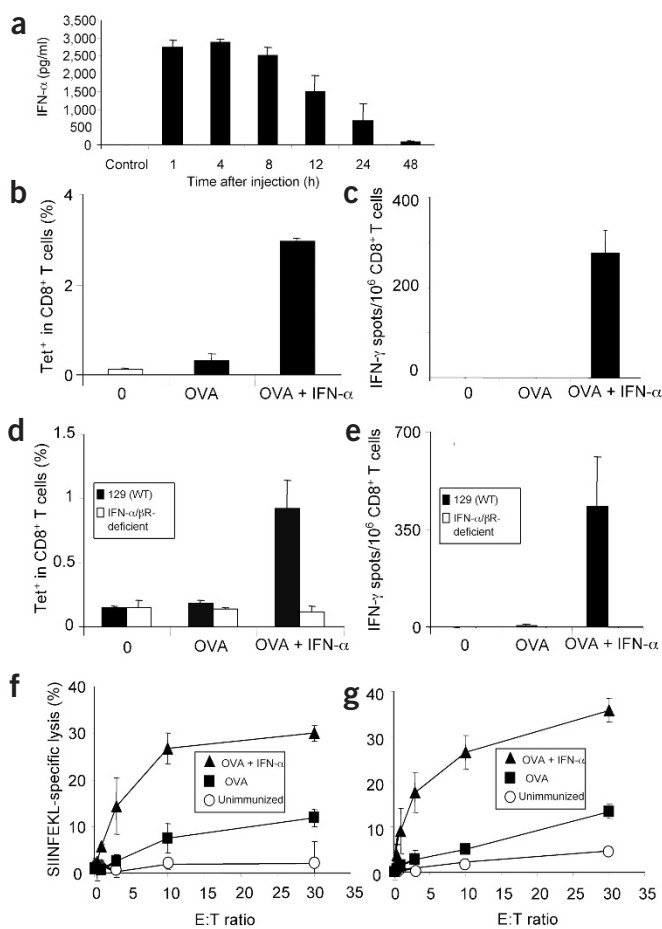


Figure 3 IFN- α promotes cross-priming of CD8 effector cells.

(a) Concentration of IFN- α in serum measured by ELISA after subcutaneous injection of 1×10^5 U IFN- α (time, below graph). (b–g) Mice were left untreated or were immunized by subcutaneous injection of OVA or OVA and IFN- α . After 8 d, SIINFEKL-specific CD8 $^+$ T cells in spleens were quantified by tetramer staining (b,d) or by IFN- γ ELISPOT assay (c,e). b and c, B6 mice; d and e, 129 wild-type (WT) and IFN- α/β R-deficient mice. CTL responses were measured 8 d (f) and 30 d (g) after immunization of B6 mice. Splenocytes from control mice or mice immunized with OVA alone or with OVA and IFN- α were re-stimulated *in vitro* and assessed for their ability to lyse peptide-coated target cells. All results are expressed as mean \pm s.d. for three mice and are representative of at least two separate experiments. Tet $^+$, tetramer-positive; E:T, effector/target ratio.

measured IFN- α in the serum at different times after infection (Fig. 1d). IFN- α was present at a concentration of >300 pg/ml 12 h after LCMV infection (at the time of injection of OVA) and reached concentrations in excess of 3,000 pg/ml by 48 h after infection. Conversely, after VV infection, the serum IFN- α concentration was only slightly above that in control mice (< 30 pg/ml) at all time points studied.

To determine whether IFN- α/β did contribute to virus-induced cross-priming, we examined the effect of LCMV infection on the CD8 response to OVA in mice deficient for the IFN- α/β receptor (IFN- α/β R-deficient mice); we compared this response to that in wild-type mice on the same (129) background. As with B6 mice, we found few if any SIINFEKL-specific CD8 $^+$ T cells after injection of OVA into uninfected mice, whereas we noted cross-priming when we injected OVA into wild-type 129 mice infected 12 h earlier with LCMV (although absolute numbers of tetramer-positive cells were substantially lower than in B6 mice, for unknown reasons; Fig. 2). This was true whether we detected SIINFEKL-specific CD8 $^+$ T cells with tetramers (Fig. 2a) or as IFN- γ -secreting cells by enzyme-linked immunospot (ELISPOT) assay (Fig. 2b). However, LCMV-induced cross-priming against OVA was completely absent in IFN- α/β R-deficient mice. These results indicate that cross-priming stimulated by LCMV is highly dependent on IFN- α/β .

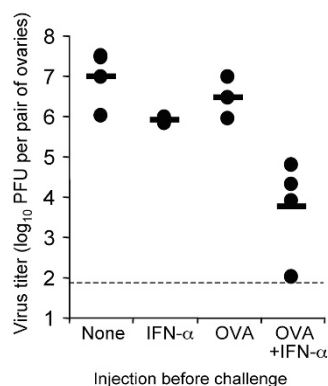
To assess directly whether IFN- α/β is a virus-induced signal capable of promoting cross-priming, we examined the CD8 $^+$ T cell response in mice immunized by injection of soluble OVA and IFN- α . We followed a protocol that has been shown to enhance antibody responses²⁷, in which mice receive antigen and IFN- α subcutaneously on day 0, and IFN- α alone on days 1 and 2 (at the site of antigen injection). With this protocol, IFN- α concentrations peaked at approximately 2,900 pg/ml in the serum 4 h after injection and thus approximated the peak concentration found after LCMV infection (Fig. 3a). As in the previous experiments, tetramer-positive CD8 $^+$ T cells were barely detectable after injection of OVA alone (Fig. 3b). However, there was a strong

was strong in LCMV-infected mice, with the number of SIINFEKL tetramer-positive cells in LCMV-infected mice reaching about two thirds that in mice given VV-OVA (Fig. 1b,c). These data show that cross-priming of CD8 $^+$ T cells against a soluble protein antigen can occur in the context of a virus infection.

IFN- α induces cross-priming of CD8 $^+$ T cells

Although many different factors may have contributed to the greater induction of cross-priming by LCMV than by VV, these viruses seem to differ substantially in the amount of IFN- α/β they elicit after infection. LCMV infection induces large amounts of IFN- α/β ³⁵, whereas VV infection induces IFN- α/β poorly³⁶. This was confirmed when we

Figure 4 Priming by injection of soluble OVA and IFN- α confers protection against OVA-expressing vaccinia virus. B6 mice were left untreated or were injected subcutaneously with IFN- α alone, OVA alone or OVA and IFN- α . After 10 d, mice were infected with VV-OVA (2×10^6 PFU, intraperitoneally). Data show virus titers (\log_{10} PFU) in ovaries of individual mice at day 5 after infection. $P = 0.022$, untreated versus IFN- α ; $P = 0.023$, IFN- α versus IFN- α and OVA; $P = 0.017$, untreated versus IFN- α and OVA. Bars indicate the mean for each group (four mice per group); horizontal dashed line represents the limit of detection in the assay (100 PFU). Data represent one of two experiments with similar results.



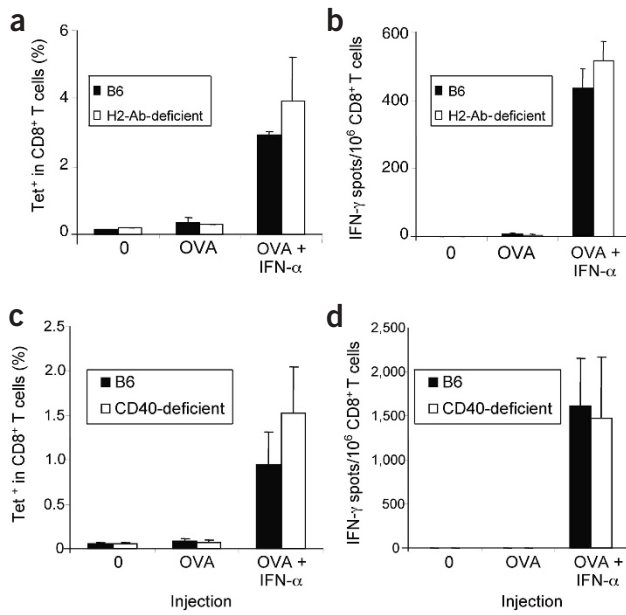


Figure 5 IFN- α enhances cross-priming to soluble OVA by a CD4- and CD40-independent mechanism. Control B6 mice, H2-Ab-deficient mice or CD40-deficient mice were left untreated or were immunized by subcutaneous injection of OVA or OVA and IFN- α . At 8 d after immunization, SIINFEKL-specific CD8⁺ spleen T cells were detected with H-2K^b-SIINFEKL tetramers (a,c) or by IFN- γ ELISPOT assay (b,d). All results are expressed as mean \pm s.d. for three mice. Tet⁺, tetramer-positive.

response in mice injected with OVA and IFN- α , with nearly 3% of all CD8⁺ T cells being specific for SIINFEKL. In addition, induction of cross-priming by IFN- α was also evident when we assayed the response by IFN- γ ELISPOT (Fig. 3c). In this case, the absolute number of SIINFEKL-specific T cells detected was considerably lower than that seen with tetramers, probably reflecting the more stringent requirements for a cell to be considered positive in the ELISPOT assay and/or the effects of the *in vitro* treatments (purification and so forth) involved. Injection of IFN- α did not augment the CD8 response in IFN- α / β R-deficient mice (Fig. 3d,e), confirming that signaling through the IFN- α / β R is required for the adjuvant effect.

Recognition of cross-presented antigen by CD8⁺ T cells can result in either priming or tolerance, with the latter being preceded by a brief period of proliferation¹⁰. Therefore, to establish whether IFN- α had promoted cross-priming, we needed to ascertain if the cells generated after immunization were functional. Although production of IFN- γ by responding cells indicated that this was the case, we further assessed the functional competence of the cells by measuring priming for cytotoxic T lymphocyte (CTL) activity. Here, we obtained spleen cells from mice 8 or 30 d after immunization, restimulated them *in vitro* and assessed them for their ability to lyse ⁵¹Cr-labeled, SIINFEKL-pulsed target cells (Fig. 3f,g). As expected, there was little priming for CTL activity after injection of OVA alone^{37,38}. In contrast, spleen cells from mice injected with OVA plus IFN- α showed substantial cytotoxic activity against SIINFEKL-loaded targets. This occurred at both 8 and 30 d after immunization, providing strong evidence that injection of IFN- α

promoted functional cross-priming rather than tolerance. Thus, OVA-specific CD8⁺ T cells generated in response to injection of soluble OVA and IFN- α show effector function after re-stimulation *in vitro*.

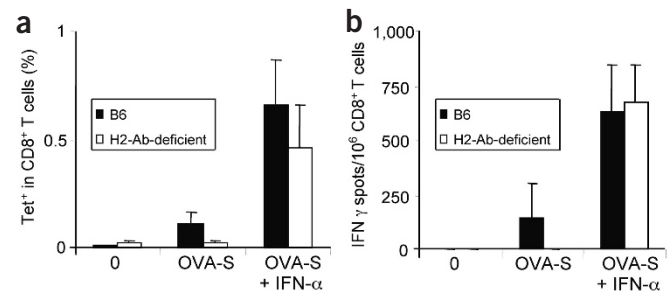
We further investigated whether these cells also showed effector function *in vivo*. We challenged mice with VV-OVA 10 d after immunization, and measured virus titers in the ovaries 5 d later to assess protection (Fig. 4). Consistent with the lack of priming measured *in vitro*, injection of OVA alone did not provide any protection against VV-OVA. We noted a small but statistically significant reduction in virus titer in mice injected with IFN- α alone, indicating that the some antiviral effects of IFN- α that were unrelated to priming of an antigen-specific immune response can last for at least 8 d. However, priming with OVA and IFN- α conferred strong protection against VV-OVA, with virus titers in the ovaries reduced by >3 logs compared with those of untreated mice, and more than 2 logs compared with those of mice injected with IFN- α alone. These results show that IFN- α induces functional cross-priming of CD8⁺ T cells after immunization with a soluble protein antigen.

Independence from CD4⁺ T cells and CD40

APCs become competent for cross-priming after presentation of antigen to CD4 T cells; licensing of APCs by CD4 cells can be mediated by CD40L-CD40 interaction^{12,13,38} and also by CD40-independent mechanisms^{19,39}. Because IFN- α / β can enhance the CD4 T cell response to immunization²⁷, it is possible that IFN- α promoted cross-priming through the generation of CD4 T cell help. To examine the CD4 dependence of IFN- α -induced cross-priming, we compared the CD8 response to OVA in wild-type mice and MHC class II-deficient (H2-Ab-deficient) mice. We quantified SIINFEKL-specific tetramer binding and IFN- γ -secreting cells 8 d after immunization of mice with OVA or OVA and IFN- α (Fig. 5a,b). As noted before, injection of OVA alone did not induce a significant CD8⁺ T cell response; this was true for both wild-type and H2-Ab-deficient mice. Injection of OVA and IFN- α , however, stimulated a strong CD8 response that was of similar magnitude in wild-type and H2-Ab-deficient mice. Therefore, IFN- α was able to promote cross-priming of CD8 cells in a CD4-independent way.

Although these data excluded the possibility of an absolute requirement for CD4 T cells in IFN- α -stimulated cross-priming, CD40L expressed by activated CD8⁺ T cells can contribute to the generation of functional responses⁴⁰, leaving open the possibility that licensing of

Figure 6 IFN- α overcomes the need for CD4 help in cross-priming against cell associated antigen. Control B6 mice or H2-Ab-deficient mice were left untreated or were immunized intraperitoneally with OVA loaded splenocytes (OVA-S) or OVA-S and IFN- α . At 8 d after immunization, CD8⁺ spleen T cells were assessed for staining with H-2K^b-SIINFEKL tetramers (a) or assayed for SIINFEKL-specific IFN- γ secretion by ELISPOT assay (b). All results are expressed as mean \pm s.d. for three mice. Tet⁺, tetramer-positive.



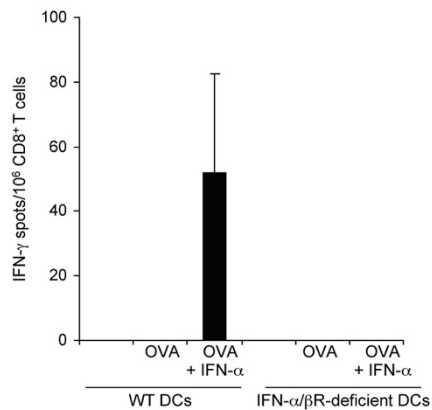


Figure 7 IFN- α induces cross-priming by direct stimulation of DCs. DCs were purified from the spleens of wild-type (WT; 129) or IFN- α/β -deficient mice that were either untreated or had been injected with OVA (in PBS) 2 h before, then were resuspended in PBS or PBS containing IFN- α and injected into IFN- α/β -deficient mice. At 8 d after immunization, CD8 $^+$ spleen T cells were assayed for SIINFEKL-specific IFN- γ secretion by ELISPOT assay. Results are expressed as mean \pm s.e.m. for three mice.

APCs through CD40 could still be involved. To directly investigate the requirement for CD40-CD40L interaction, we assessed the ability of IFN- α to promote cross-priming after injection of OVA into CD40-deficient mice. IFN- α enhanced CD8 cross-priming equally well in wild-type and CD40-deficient mice (Fig. 5c,d), indicating that it did so by a CD40-independent mechanism.

To determine whether IFN- α could completely replace CD4 help in promoting cross-priming, we used a model of cellular cross-priming that has been shown to be absolutely dependent on CD4 help¹¹. In this protocol, soluble protein is loaded into the cytoplasm of irradiated syngeneic cells in hyperosmotic conditions, a process that also induces cell death¹⁵. We injected OVA-loaded cells with or without IFN- α into wild-type and H2-Ab-deficient mice and quantified SIINFEKL-specific tetramer-binding and IFN- γ -secreting cells in the spleen 8 d later. There was a low CD8 response in wild-type but not H2-Ab-deficient mice after injection of OVA-loaded spleen cells alone (Fig. 6), confirming the CD4 dependence of this response. This was notable, given reports that some 'CD4-dependent' CD8 responses in fact are defective only in terms of their ability to make a secondary response to antigen⁴¹⁻⁴⁴. Injection of IFN- α both augmented the CD8 $^+$ T cell response to OVA in wild-type mice and allowed for a strong response in H2-Ab-deficient mice. When mice were immunized with OVA-loaded spleen cells and treated with IFN- α , very similar numbers of SIINFEKL-specific CD8 cells were generated in wild-type and H2-Ab-deficient mice. These data show that IFN- α can convert a CD8 $^+$ T cell response from being CD4 dependent to being CD4 independent.

IFN- α activates DCs for cross-priming

The observation that induction of cross-priming by IFN- α was independent of CD4 $^+$ T cells and CD40 indicated that IFN- α could license DCs by an alternative mechanism. To determine whether direct stimulation of DCs by IFN- α could elicit cross-priming, we used an adoptive transfer system in which we injected wild-type (129) DCs into IFN- α/β -deficient mice; in the recipients, only DCs were able to respond directly to IFN- α/β . To obtain antigen-presenting DCs, we injected soluble OVA into wild-type mice (or IFN- α/β -deficient mice as a control), and isolated DCs from the spleen 2 h later. We then resuspended these DCs in PBS alone or PBS containing IFN- α and

injected them intravenously into IFN- α/β -deficient mice; recipients of IFN- α -exposed DCs received two subsequent injections of IFN- α intraperitoneally.

When we assessed the CD8 response 8 d after immunization, SIINFEKL tetramer-binding cells were not reliably detectable above background in any of the mice (data not shown). However, we noted a SIINFEKL-specific response when assaying by IFN- γ ELISPOT; notably, we found cross-priming only in recipients of wild-type DCs treated with IFN- α (Fig. 7). This response was relatively low, approximately 10% of what typically occurred after immunization of wild-type mice with OVA and IFN- α , indicating why tetramer staining was below the level of detection. The low response probably reflects the small percentage of DCs that migrate to lymphoid organs after adoptive transfer⁴⁵. Nevertheless, these results showed that IFN- α could induce cross-priming through direct stimulation of DCs.

DISCUSSION

Cross-presentation allows APCs to initiate CD8 T cell responses against antigens that are not synthesized within the presenting cells themselves, and has been proposed as a mechanism for the immune system to respond to viruses that do not infect APCs directly⁴⁶. Although the idea that CD8 responses to viruses can be elicited by cross-priming has been supported by studies in mice with restricted expression of virus receptors²⁰, the nature of the signal provided by a virally infected cell that instructs an APC to activate cross-priming is unknown. The data presented here demonstrate that one mechanism by which virus infections can stimulate cross-priming is through inducing expression of IFN- α/β . Because expression of IFN- α/β is a common response to viral infection²², IFN- α/β may be a particularly important link between infection and cross-priming.

Our results demonstrate that IFN- α is able to induce cross-priming through direct stimulation of DCs. As with CD40 cross-linking, the nature of IFN- α -triggered changes in DCs that are crucial for cross-priming remain to be determined. IFN- α stimulates increased expression of TAP1 (refs. 33,47) and upregulation of cell surface MHC class I (ref. 22), indicating that augmented cross-presentation could contribute to the promotion of cross-priming by IFN- α . However, we have found that DCs from mice injected with OVA and IFN- α stimulate proliferation of OT-I cells (*ex vivo*) only marginally better than DCs from mice injected with OVA alone (Supplementary Fig. 1 online). Although this approach does not measure directly the number of H-2K^b-SIINFEKL complexes on the cell surface, these data indicate that increased presentation of antigen is unlikely to be the main mechanism involved in the licensing of DCs by IFN- α/β . Instead, alterations associated with IFN- α/β -induced DC maturation^{24-26,28} are more likely to be important.

LCMV-induced cross-priming against OVA was greatly reduced in IFN- α/β -deficient mice, indicating that IFN- α/β was the main signal responsible for stimulating cross-priming in this model. Nevertheless, these findings do not rule out the possibility that other stimuli associated with virus infection also have this capacity. In particular, an ability of viral components to trigger cross-priming directly may not have been found in this system because these molecules would presumably license only those APCs that have come into direct contact with virus. The chance that the same DCs would have taken up independently both a virally infected cell (or virus itself) and OVA would probably be low, as the virus and OVA were administered separately and at different sites. In contrast, IFN- α/β can act systemically to license APCs for cross-priming.

A conspicuous feature of the cross-priming promoted by IFN- α was its complete independence of CD4 T cells, which was true for

immunization with either soluble or cell-associated antigen. Similarly, CpG oligonucleotides have been shown to stimulate cross-priming in a CD4-independent way¹⁸ and bacterial infection can obviate the requirement for CD40-CD40L interactions during cross-priming against cell-associated antigen¹⁹. Therefore, infection (viral or bacterial) and CD4 help seem to represent alternative signals that can license APCs for cross-priming. Whether these signals activate DCs in an equivalent way, however, is unclear. Memory CD8 T cells generated in the absence of CD4 help are defective in their ability to make secondary responses to antigen^{41–44}. This has been noted in many different models, including bacterial and viral infections, in which the primary CD8 response was only slightly reduced in the absence of CD4 cells. Hence, infection-associated and T helper-mediated signals may normally act in synergy in the induction of cross-priming to stimulate the generation of functional memory cells. Therefore, it would be of interest to compare the production of memory CD8 cells during IFN- α/β -stimulated cross-priming in CD4-deficient versus control mice.

In summary, the data show that virus infection can stimulate cross-priming of CD8⁺ T cells through induction of IFN- α/β expression. Together with previous work showing that IFN- α/β enhances antibody and CD4⁺ T cell responses²⁷, these results demonstrate that IFN- α/β is an important signal that links the innate response to infection with the induction of a broad-based immune response.

METHODS

Mice. Mice were purchased from Charles River-UK, the specific pathogen-free unit at the Institute for Animal Health (Compton, UK) or the pathogen-free unit at the Pasteur Institute (Paris, France). All animal experimentation was done with the approval of the Home Office and the Ethical Review Committee of the Institute for Animal Health.

IFN- α . The mouse gene encoding IFN- $\alpha 4$ was cloned into the pEE12 plasmid (Celltech). After amplification in *Escherichia coli*, pEE12 encoding IFN- α DNA was used to transfect Nso mouse myeloma cells. Colonies were screened after growth in selective medium and a single colony of transfected Nso cells with high expression of IFN- $\alpha 4$ was used for IFN- α production. Cells were cultured for 10–15 d at a density of 0.5×10^6 cells/ml in serum-free medium supplemented with Cholesterol Lipid Concentrate (1 ; Life Technologies). The supernatant was collected and IFN- $\alpha 4$ content was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus on L cells grown in monolayer culture in Falcon microplates (Becton Dickinson). These IFN- $\alpha 4$ preparations had an activity of 2×10^6 U/ml. IFN- α concentrations in serum were measured with a mouse IFN- α enzyme-linked immunosorbent assay (ELISA) kit (PBL Biomedical Laboratories).

Isolation of DCs. Spleens were disrupted by injection of 1 ml RPMI medium containing 5% FCS, 1 mg Collagenase III (Lorne Laboratories) and 0.5 mg DNase I (Sigma) and incubation for 30 min at 37 °C. Subsequently, DC-enriched cell populations were obtained by centrifugation of cell suspensions on Nycoprep 1.077A (Nycomed Pharma AS) gradients. DCs were then purified by positive selection with anti-CD11c MACS beads (Miltenyi), with the resulting population being >98% CD11c⁺.

Immunizations. Soluble OVA (Sigma) was injected subcutaneously either in PBS alone or mixed with 1×10^5 U IFN- α . OVA-loaded splenocytes were prepared as described¹¹, and 5×10^7 irradiated cells were injected intraperitoneally with or without IFN- α . In IFN- α -treated mice, IFN- α was always given with the antigen on day 0 and alone on days 1 and 2 at the site of primary injection. In adoptive transfer experiments, splenic DCs were isolated from control mice or mice that had been injected 2 h before with 10 mg OVA, resuspended in PBS or PBS and IFN- α (1×10^5 U) and injected (2×10^6 cells/mouse) intravenously into recipients. IFN- α -treated mice received further injections of 1×10^5 U IFN- α intraperitoneally 1 and 2 d after injection of DCs.

In vitro cross-presentation. Splenic DCs were isolated from B6 or TAP1-deficient mice 2 h after intravenous injection of 3 mg OVA. DCs were then cultured in the presence of 5×10^4 purified OT-I CD8 T cells. After 4 d, cell proliferation was assessed by pulsing wells with [³H]thymidine.

Flow cytometric analysis. The following reagents were used for flow cytometric analysis: antibody to CD8-Cy5 (anti-CD8-Cy5; clone YTS.169), fluorescein isothiocyanate-conjugated anti-CD11c (HL3; Becton Dickinson) and phycoerythrin-conjugated H-2K^b-SIINFEKL tetramer (ProImmune). Labeled cells were analyzed on a FACScalibur flow cytometer with the use of CellQuest software (Becton Dickinson).

CTL and IFN- γ ELISPOT assays. For CTL assays, spleen cells from immune mice were cultured *in vitro* for 5 d with equal numbers of irradiated (3,000 rad) syngeneic splenocytes from naive mice, in the presence of 1×10^5 SIINFEKL. Viable lymphoblastoid cells were counted by trypan blue exclusion and used as effectors in a standard 6-h ⁵¹Cr-release assay. Targets consisted of radiolabeled, SIINFEKL-pulsed (1×10^5) MC57G cells and radiolabeled, non-peptide-pulsed MC57G cells. Results are expressed as the percentage of SIINFEKL-specific lysis, representing the percentage of specific lysis of peptide-pulsed MC57G cells minus that of non-pulsed MC57G cells.

For IFN- γ ELISPOT assays, cellulose-ester membrane microplates (Millipore) were coated with monoclonal antibody R4-6A2 (BD Pharmingen). Purified CD8⁺ T cells from immune mice (1×10^5 cells/well) were incubated on these plates for 36 h with stimulators consisting of irradiated (3,000 rad) syngeneic splenocytes from naive mice (5×10^5 cells/well), in the presence or absence of 1×10^5 SIINFEKL. Plates were washed extensively and spots were visualized with biotin-conjugated monoclonal antibody XMG1.2 (BD Pharmingen), alkaline phosphatase-conjugated goat anti-biotin (Vector Laboratories) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma), sequentially. No spots were noted in wells containing stimulators alone, or in wells in which CD8⁺ T cells were cultured in the absence of SIINFEKL.

Viruses and infections. Viruses used were LCMV Armstrong, a clone 'triple plaque purified' from Arm CA 1371 (ref. 48); vSC8 (called VV here), which expresses only *E. coli* β -galactosidase⁴⁹, originally obtained from B. Moss (National Institutes of Health); and VV-OVA, which expresses both ovalbumin and *E. coli* β -galactosidase⁵⁰, originally obtained from J. Yewdell (National Institutes of Health). For assessment of the effects of virus infection on cross-priming, mice were injected intraperitoneally with 2×10^5 plaque-forming units (PFU) LCMV Armstrong, 2×10^6 PFU VV or 2×10^6 PFU VV-OVA. Where indicated, 1 mg OVA in PBS was injected subcutaneously 12 h after infection. For challenge experiments, B6 mice were left untreated or were injected subcutaneously with IFN- α alone, OVA alone or OVA and IFN- α . After 10 d, mice were infected with VV-OVA (2×10^6 PFU, intraperitoneally). Viral titers in ovaries of individual mice were determined at day 5 after infection by plaque assay on 143TK⁻ cells.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank R. Lo-Man (Pasteur Institute, Paris) for CD40-deficient mice; C. Tanchot and A. Sarkhan (Necker Institute, Paris) for help with experiments on CD40-deficient mice; and J. Dyson and M. Skinner for discussions. This work was supported by The Edward Jenner Institute for Vaccine Research and the European Community (contract QLK2-CT-2001-02103). This is publication number 63 from the Edward Jenner Institute for Vaccine Research.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 11 June; accepted 18 August 2003

Published online at <http://www.nature.com/natureimmunology/>

- Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. & Amigorena, S. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell. Biol.* **1**, 362–368 (1999).

2. Heath, W.R. & Carbone, F.R. Cross-presentation, dendritic cells, tolerance and immunity. *Annu. Rev. Immunol.* **19**, 47–64 (2001).
3. Kurts, C., Cannarile, M., Klebba, I. & Brocker, T. Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells *in vivo*. *J. Immunol.* **166**, 1439–1442 (2001).
4. Belz, G.T. *et al.* The CD8a⁺ dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J. Exp. Med.* **196**, 1099–1104 (2002).
5. Jung, S. *et al.* *In vivo* depletion of CD11c⁺ dendritic cells abrogates priming of CD8 T cells by exogenous cell-associated antigens. *Immunity* **17**, 211–220 (2002).
6. Bevan, M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* **143**, 1283–1288 (1976).
7. Gordon, R.D., Mathieson, B.J., Samelson, L.E., Boyse, E.A. & Simpson, E. The effect of allogeneic sensitization on H-Y graft survival and *in vitro* cell-mediated responses to H-Y antigen. *J. Exp. Med.* **144**, 810–820 (1976).
8. Gooding, L.R. & Edwards, C.B. H-2 antigen requirements in the *in vitro* induction of SV40-specific cytotoxic lymphocytes. *J. Immunol.* **124**, 1258–1262 (1980).
9. Carbone, F.R. & Bevan, M.J. Class I-restricted processing and presentation of exogenous cell-associated antigen *in vivo*. *J. Exp. Med.* **171**, 377–387 (1990).
10. Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F.A.P. & Heath, W.R. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8⁺ T cells. *J. Exp. Med.* **186**, 239–245 (1997).
11. Bennett, S.R.M., Carbone, F.R., Karamalis, F., Miller, J.F.A.P. & Heath, W.R. Induction of a CD8⁺ cytotoxic T lymphocyte response by cross-priming requires cognate CD4⁺ T cell help. *J. Exp. Med.* **186**, 65–70 (1997).
12. Bennet, S.R.M. *et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393**, 478–480 (1998).
13. Schoenberger, S.P., Toes, R.E.M., van der Voort, E.I.H., Offringa, R. & Melief, C.J.M. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480–483 (1998).
14. Ridge, J.P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and a T-killer cell. *Nature* **393**, 474–478 (1998).
15. Liu, K. *et al.* Immune tolerance after delivery of dying cells to dendritic cells *in situ*. *J. Exp. Med.* **196**, 1091–1097 (2002).
16. Mazzaccaro, R.J. *et al.* Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* **93**, 11786–11791 (1996).
17. Simmons, C.P. *et al.* MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvants. *J. Immunol.* **163**, 6502–6510 (1999).
18. Cho, H.J. *et al.* Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514 (2000).
19. Hamilton, S.E., Tivnneireim, A.R. & Harty, J.T. *Listeria monocytogenes* infection overcomes the requirement for CD40 ligand in exogenous antigen presentation to CD8⁺ T cells. *J. Immunol.* **167**, 5603–5609 (2001).
20. Sigal, L.J., Crotty, S., Andino, R. & Rock, K.L. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* **398**, 77–80, (1999).
21. Mueller, S.N., Jones, C.M., Smith, C.M., Heath, W.R. & Carbone, F.R. Rapid cytotoxic T lymphocyte activation occurs in the draining lymph nodes after cutaneous herpes simplex virus infection as a result of early antigen presentation and not the presence of virus. *J. Exp. Med.* **195**, 651–656 (2002).
22. Goodbourn, S., Didcock, L. & Randall, R.E. Interferons: cell signalling, immune modulation, antiviral responses and virus countermeasures. *J. Gen. Virol.* **81**, 2341–2364 (2000).
23. Siegal, F.P. *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835–1837 (1999).
24. Luft, T. *et al.* Type I IFNs enhance the terminal differentiation of dendritic cells. *J. Immunol.* **161**, 1947–1953 (1998).
25. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: Endogenous activators of dendritic cells. *Nat. Med.* **5**, 1249–1255 (1999).
26. Ito, T. *et al.* Differential regulation of human blood dendritic cell subsets by IFNs. *J. Immunol.* **166**, 2961–2969 (2001).
27. Le Bon, A. *et al.* Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells *in vivo*. *Immunity* **14**, 461–470 (2001).
28. Montoya, M. *et al.* Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99**, 3263–3271 (2002).
29. von Hoegen, P., Zawatzky, R. & Schirmacher, V. Modification of tumor cells by a low dose of Newcastle disease virus. III. Potentiation of tumor-specific cytolytic T cell activity via induction of interferon- α/β . *Cell. Immunol.* **126**, 80–90. (1990).
30. Ferrantini, M. *et al.* IFN- α 1 gene expression into a metastatic murine adenocarcinoma (TS/A) results in CD8⁺ T cell mediated tumor rejection and development of antitumor immunity. Comparative studies with IFN- γ -producing TS/A cells. *J. Immunol.* **153**, 4604–4615 (1994).
31. Tough, D.F., Borrow, P. & Sprent, J. Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. *Science* **272**, 1947–1950 (1996).
32. van Uden, J.H., Tran, C.H., Carson, D.A. & Raz, E. Type I interferon is required to mount an adaptive response to immunostimulatory DNA. *Eur. J. Immunol.* **31**, 3281–3290 (2001).
33. Cho, H.J. *et al.* IFN- α/β promote priming of antigen-specific CD8⁺ and CD4⁺ T lymphocytes by immunostimulatory DNA-based vaccines. *J. Immunol.* **168**, 4907–4913 (2002).
34. Hogquist, K.A. *et al.* T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17–27 (1994).
35. Merigan, T.C., Oldstone, M.B. & Welsh, R.M. Interferon production during lymphocytic choriomeningitis virus infection of nude and normal mice. *Nature* **268**, 67–68 (1977).
36. Korngold, R., Blank, K.J. & Murasko, D.M. Effect of interferon on thoracic duct lymphocyte output: Induction with either poly I:poly C or vaccinia virus. *J. Immunol.* **130**, 2236–2240 (1983).
37. Moore, M.W., Carbone, F.R. & Bevan, M.J. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **54**, 777–785 (1988).
38. Lefrancois, L., Altman, J.D., Williams, K. & Olson, S. Soluble antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. *J. Immunol.* **164**, 725–732 (2000).
39. Lu, Z. *et al.* CD40-independent pathways of T cell help for priming of CD8⁺ cytotoxic T lymphocytes. *J. Exp. Med.* **191**, 541–550 (2000).
40. Zhan, Y., Corbett, A.J., Brady, J.L., Sutherland, R.M. & Lew, A.M. CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. *J. Immunol.* **165**, 3612–3619 (2000).
41. Bourgeois, C., Rocha, B. & Tanchot, C. A role for CD40 expression on CD8⁺ T cells in the generation of CD8⁺ T cell memory. *Science* **297**, 2060–2063 (2002).
42. Janssen, E.M. *et al.* CD4⁺ T cells are required for secondary expansion and memory in CD8⁺ T lymphocytes. *Nature* **421**, 852–856 (2003).
43. Sun, J.C. & Bevan, M.J. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**, 339–342 (2003).
44. Shedlock, D.J. & Shen, H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**, 337–339 (2003).
45. Josien, R. *et al.* TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells *in vivo*. *J. Exp. Med.* **191**, 495–502 (2000).
46. Bevan, M.J. Immunology. Stimulating killer cells. *Nature* **342**, 478–479 (1989).
47. Jamaluddin, M. *et al.* IFN- β mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280**, L248–L257 (2001).
48. Dutko, F.J. & Oldstone, M.B. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J. Gen. Virol.* **64**, 1689–1698 (1983).
49. Chakrabarti, S., Brechling, K. & Moss, B. Vaccinia virus expression vector: coexpression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5**, 3403–3409 (1985).
50. Restifo, N.P. *et al.* Antigen processing *in vivo* and the elicitation of primary CTL responses. *J. Immunol.* **154**, 4414–4422 (1995).