Increasing antigen presentation on HSV-1-infected cells increases lesion size but does not alter neural infection or latency

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Abstract

CD8+ T cells have a role in the control of acute herpes simplex virus (HSV) infection and may also be important in the maintenance of latency. In this study we have explored the consequences of boosting the efficacy of CD8+ T cells against HSV by increasing the amount of an MHC I-presented epitope on the surface of infected cells. To do this we used HSVs engineered to express an extra copy of the immunodominant CD8+ T cell epitope in C57Bl/6 mice, namely gB498 (SSIEFARL). Despite greater presentation of gB498 on infected cells, CD8+ T cell responses to these viruses in mice were similar to those elicited by a control virus. Further, the expression of extra gB498 did not significantly alter the extent or stability of latency in our mouse model, and virus loads in skin and sensory ganglia of infected mice were not affected. Surprisingly, mice infected with these viruses developed significantly larger skin lesions than those infected with control viruses and notably, this phenotype was dependent on MHC haplotype. Therefore increasing the visibility of HSV-infected cells to CD8+ T cell attack did not impact neural infection or latency, but rather enhanced pathology in the skin.

INTRODUCTION

Herpes simplex virus (HSV) is a successful pathogen with worldwide distribution that causes a lifelong, though frequently asymptomatic, infection. HSV infection is characterised by a lytic phase that precedes the establishment of latency within the host. During latency, HSV DNA persists in neurons in the absence of a detectable infectious virus, but the virus can periodically reactivate from this state to cause renewed lytic infection that may lead to virus shedding and disease. Adaptive immune responses, including CD8+ T cells, are required for the control of acute HSV infection allowing the host to survive and harbour latent infection [1–5].

The role of CD8+ T cells in HSV infection is further highlighted by the expression of the immunomodulator ICP47 by HSV. ICP47 reduces the presentation of viral epitopes to CD8+ T cells by inhibiting the transporter associated with antigen processing (TAP) [6–8]. ICP47 has poor affinity for mouse TAP and was initially considered to be ineffective in this species, however deletion of ICP47 from HSV reduces the ability of the virus to invade the brain in mice [9–11]. These conflicting reports leave the extent of ICP47 activity in mouse models compared with human infection an open question.

Beyond the acute infection, a role for CD8+ T cells in latency has been suggested both in mouse models and in human studies [12–14]. This role has led to proposals that immunotherapy might be an effective treatment for HSV and so the consequences of increasing any parameter associated with CD8+ T cell immunity to HSV is of interest [14]. Therefore, we wanted to explore the consequences of making HSV-infected cells more visible to CD8+ T cells by increasing the amount of viral antigen presented on MHC class I.

One potential method of increasing the amount of a particular CD8+ T cell epitope that is presented on infected cells is to express it as a minimal epitope construct (minigene). An epitope minigene comprises the sequence that encodes a minimal immunogenic peptide, preceded by a start codon. The short polypeptides produced are efficiently presented on MHC I, presumably because they do not require any processing or trimming [15–17]. A variation on this method is to add an endoplasmic reticulum (ER)-targeting sequence to the front of the minimal epitope, such that presentation...
no longer requires processing by proteases, or transport by TAP [18]. In the case of HSV, such a construct would be expected to circumvent any inhibition by ICP47.

Here we employed these methods to make HSV-1s that present more of the immunodominant gB498 peptide on MHC I and tested the impact of this increased expression on the pathogenesis of infection.

RESULTS

Generation of recombinant HSV-1s with an additional copy of gB498 as a cytosolic or ER-targeted minigene

Initially, a recombinant HSV-1 was constructed with an additional copy of the gB498 epitope as a minigene with an ER-targeting motif, and named HSV-1 ESminigB_Cre, Fig. 1(b). An eGFP/cre fusion gene under the control of the cytomegalovirus immediate early (CMV IE) promoter was also introduced into this virus simultaneously. HSV-1 ESminigB_Cre was then modified to remove the ER-targeting motif, so this second new virus contains a cytosolic gB498 epitope (named HSV-1 minigB_Cre), Fig. 1(c). These viruses were compared to HSV-1 pC_eGC, Fig. 1(d), which contains the eGFP/cre fusion gene under the control of the CMV IE promoter, but contains only the native gB498 epitope located within the gB protein.

Adding a gB498 minigene to HSV-1 enhances presentation on infected cells

The short peptides expressed as minigenes are too small and short in half life to allow direct detection. Therefore to confirm that the gB498 minigenes are expressed and able to enhance presentation on MHC-I, an indirect in vitro antigen presentation assay was established. This assay uses a T cell hybridoma (HSV-2.3.2E2) that recognises the gB498 epitope presented on the MHC I allomorph H-2Kb and responds by expressing LacZ (under control of an IL-2 promoter) [19]. The hybridoma was co-cultured with HSV-infected 293KbC2 cells and activation, quantified by the amount of gB498 epitope being presented. In these assays, HSV-1 ESminigB_Cre and HSV-1 minigB_Cre-infected cells stimulated more β-gal activity from the gB498 hybridoma than the control HSV-1 pC_eGC, Fig. 1(e). However, there was no significant difference observed between the two viruses that expressed additional copies of the gB498 epitope. Therefore we concluded that regardless of whether gB498 was targeted to the ER or not, an additional copy resulted in enhanced presentation of this epitope on infected cells.

The magnitude of CD8+ T cell responses are not changed by addition of a gB498 minigene to HSV-1

Next we wanted to examine whether enhanced presentation on HSV-infected cells might lead to a change in the magnitude of gB498-specific CD8+ T cell responses in mice. To do this, mice were infected with the viruses by tattoo on the flank and gB498-specific CD8+ T cell responses measured in the spleen and infected dorsal root ganglia (DRG) seven days later, Fig. 2(a, b). There were no significant differences in the size of the gB498-specific CD8+ T cell responses at either site across the three viruses. We also examined the fraction of splenic gB498-specific CD8+ T cells that were positive for granzyme B to infer their functional capacity and found no differences across viruses (not shown). Finally, we used staining for granzyme B and CD62L as a measure of the total CD8+ T cell response (irrespective of specificity) in the spleen [20] and also found no difference between the control and viruses expressing additional gB498, Fig. 2(c).

The extent and stability of latency is unaltered in mice infected with HSV-1 expressing a gB498 minigene

CD8+ T cell responses have been shown to be important in control of acute HSV infection in the DRG and have been implicated in the control of latency [21-27]. For this reason we were interested to determine whether latency establishment or stability might be altered by increasing expression of gB498. All the viruses used here express Cre recombinase under the cytomegalovirus immediate early (CMV-IE) promoter enabling us to use the ROSA26 Cre reporter mouse system [26, 28]. In this model, Cre under the CMV-IE is expressed in all virus-infected cells, regardless of whether the outcome is lytic or latent infection, leading to β-gal expression from the ROSA26 genome. The result is an indelible marking of all surviving neurons with a history of HSV infection that can then be counted by appropriate staining and microscopy. In ROSA26 mice infected with HSV-1 ESminigB_Cre, or minigB_Cre, Fig. 3(a, c), latency is established in a similar number of cells as that previously reported using the control virus HSV-1 pC_eGC [26, 27]. In addition, the spread of viruses also appears similar, as determined by the number of DRG that contain at least one β-gal+ cell, Fig. 3(b, d). Finally, the total number of β-gal+ cells is similar across days 20, 40 and 100 p.i., in mice infected with these viruses, indicating that HSVs expressing additional gB498 maintain stable latency. The one parameter we noted was different in these experiments, compared with our previous published work using HSV-1 pC_eGC, was that the number of β-gal+ cells did not rise significantly between days 5 and 10. For this reason we examined the acute infection with these viruses more closely.

The impact of expressing a gB498 minigene on the acute HSV-1 infection in vivo

We next decided to investigate the impact of enhanced gB498 antigen presentation on acute herpes simplex in mice after flank infection more broadly. To do this we examined (i) skin lesions, (ii) numbers of neurons marked in ROSA26 mice and (iii) amounts of infectious virus in the skin and DRG. (i) The most striking finding was that infection with HSV-1 ESminigB_Cre or HSV-1 minigB_Cre resulted in the formation of significantly larger skin lesions, Fig. 4(a). However, the larger lesions were not associated with an increase in other clinical signs of infection (posture, activity and fur
Fig. 1. Design of recombinant viruses containing an additional copy of the gB epitope. (a) Schematic representation of the HSV-1 genome with the location of U₃ and U₄ indicated, to scale. (b) Schematic representation of the CMV IE promoter eGFP/Cre expression cassette inserted into the intergenic space between U₃ and U₄ in HSV-1 pC_eGC, with an ER-targeting motif (shown in orange) and the minimal gB₄₉₉ minigene (shown in purple) under the control of the gB promoter. (c) As in (b) but with the minimal gB₄₉₉ minigene only (shown in purple) under the control of the gB promoter. (d) Schematic representation of the control virus HSV-1 pC_eGC. (e) 293KbC2 cells were infected with HSV-1 pC_eGC (shown in grey), HSV-1 ESminigB_Cre (shown in blue) or HSV-1 minigB_Cre (shown in green) for 6 h, or were mock infected with PBS (shown in black) and were then cocultured with HSV-2.3.2E2 cells. Cells were lysed and assayed for β-gal expression using ONPG, and percent stimulation calculated relative to maximal gB₄₉₉ peptide stimulation. Each stimulation was performed in triplicate and results presentation are mean±SEM. The results shown are representative of three independent experiments.
(ii) There were significantly fewer β-gal⁺ cells in the mice infected with HSV-1 ESminigB_Cre or HSV-1 minigB_Cre relative to HSV-1 pC_eGC at days 7, 10 and 13 p.i., Fig. 4(b). However, the spread of the virus up and down the spinal column, as determined by the number of DRG that contain at least one β-gal⁺ cell, was similar for all viruses on all days, Fig. 4(c). (iii) Despite the changes noted above, at five days after infection there were no significant differences in virus titre amongst the viruses, either in the skin or in DRG, Fig. 4(d, e). Having stated the lack of statistical significance, we note that the mean titres in DRG were lower for the viruses expressing the gB₄₉₈ minigenes and this might be construed to be consistent with less marking of neurons at days 7 and 10 after infection.
The impact of expressing a gB<sub>498</sub> minigene from HSV-1 on skin lesions depends on the MHC anchor residue and strain of mice

To improve these findings and to confirm they were linked to enhanced presentation of the gB<sub>498</sub> peptide and not the general construction of the particular recombinant viruses used, we repeated the experiments with a more ideal control virus. This virus, named HSV-1 L8A_Cre, contains the eGFP/Cre fusion gene under the control of the CMV IE promoter, along with a modified copy of the gB<sub>498</sub> minigene, Fig. 5(a). This modified gB<sub>498</sub> minigene encodes an alanine rather than leucine as the last residue of the peptide, abolishing binding to MHC-I K<sup>b</sup> and therefore presentation to CD8<sup>+</sup> T cells [29].

With this new control in hand we repeated the experiments described above. (i) Confirming previous results, lesion size was found to be significantly larger on mice infected with the gB<sub>498</sub> minigene viruses compared with the L8A control, Fig. 5(b). Other clinical signs were not different amongst the viruses (data not shown). (ii) Marking of neurons in ROSA26 mice by HSV-1 L8A_Cre over the acute infection showed a similar general pattern as published previously and shown in Fig. 4 for the other viruses used here Fig. 5(c, d). However, a direct comparison of this control virus and HSV-1 ESminigB_Cre or HSV-1 minigB_Cre found that equal numbers of neurons were marked on days 7 p.i. This is in contrast to findings with the original control virus. (iii) The virus loads were also similar across these three viruses, both in the skin and in the DRG. Therefore only the skin lesion phenotype remained as a consistent finding associated with expression of gB<sub>498</sub> minigenes when the L8A virus was used as a control.

In the experiment using L8A as a control, the difference in lesion size was less striking and only significant at day 6. To...
ensure that this result was robust, we repeated the comparison of HSV-1 minigB_Cre and HSV-1 L8A_Cre observing lesions until day 6 when virus titres were determined in skin and DRG. In this experiment, the difference in lesion size was significant both on day 5 and on day 6. At day 6, when the difference in lesions was largest, Fig. 6(a, b), we also examined virus loads in the skin and found no difference between the viruses, Fig. 6(c). The loss of the skin phenotype with a single amino acid change that is known to disrupt binding of the gB498 peptide to MHC class I strongly implicates antigen presentation and CD8+ T cells in the mechanism. However, to test this further we examined the lesions caused by our viruses on BALB/c mice that have the H-2d MHC haplotype that does not present the gB498 peptide, Fig. 6(d). Unlike the experiments with C57Bl/6 mice, no difference in lesions was found across viruses that express the additional gB498 peptide and the L8A control in BALB/c mice.

**DISCUSSION**

The overall aim of this study was to examine the consequences of enhancing the visibility HSV-1-infected cells to CD8+ T cells in vivo. We did this by expressing two minigene versions of gB498, one that would be expected to require transport by TAP and another that would be directly inserted into the ER. The decision to include the ER-targeted form was because the extent to which ICP47 might be active in blocking murine TAP remains unknown. On the one hand, biochemical studies suggest that the affinity of ICP47 binding to mouse TAP is very poor, on the other deletion of this gene reduced spread of HSV-1 to the central nervous system [9–11]. Here we found no difference in presentation of gB498 in vitro using a T cell hybridoma-based assay, irrespective of whether we used an ER targeting motif or not, which was particularly surprising because we used human cells (derived from 293A). We have not tested otherwise for the TAP-independence of the ER targeted peptide, so it remains possible that in HSV-ESminigB_Cre this was not functioning as designed. Alternatively it might be that exceptionally high levels of peptide are generated by the cytoplasmic construct and enough is transported even in the presence of ICP47 to allow high levels of activation of the T cell hybridoma. We have mass spectrometry data to show that antigen presentation is not entirely blocked by ICP47 in the 293KbC2 cells used here and the extent of the block is somewhat epitope dependent (TV, DCT unpublished). It has also been noted by others that upregulation of TAP as a result of interferon-γ exposure can temper the effect of ICP47 [30]. Never-the-less, both minigene viruses presented more gB498 than the parent HSV and they behaved identically in mice in vivo, acting as independently derived control viruses.

HSV-1 ESminigB_Cre and HSV-1 minigB_Cre activated similar levels of gB498-specific CD8+ T cells in spleen and DRG, compared with the control virus. This is despite greater presentation of gB498 on infected cells. Rather than being contradictory, this was expected because of two observations in the literature: first, CD8+ T cell responses to cutaneous HSV-1 infection of mice are thought to be largely cross-primed, which means that non-infected dendritic cells pick up exogenous antigen for processing and presentation...
on MHC I [19, 31–33]. Second, epitope minigenes are poorly presented by cross presentation [34–36]. In support of this interpretation, rather than there being any unique properties of gB498 minigenes, expression of minigene gB498 from vaccinia virus, which is expected to directly prime CD8+ T cells, leads to higher responses to this peptide than the full gB protein [37].

Studies going back more than two decades have suggested that the main role for CD8+ T cells in mouse models of HSV is protection of sensory neurons, so we expected that any impact of increasing presentation of gB498 would be seen in DRG [21]. This included potential effects on the stability of latency, because CD8+ T cells have been suggested to play a role in maintaining the latent state [24, 38, 39]. Despite the historic view that the nervous system is immunoprivileged, a role for adaptive immunity is reasonable because the presence of activated CD8+ T cells and evidence of lytic gene expression suggest that antigen presentation is possible even during latency [25–27, 40, 41]. However, taking all of our results together, we found no difference in any parameter of infection of DRG or latency in mice infected with viruses that had gB498 minigenes. These findings echo those of a study that had the potential to improve antigen presentation by expression of an MHC I heavy chain from HSV-1 [42]. There are several caveats that should be acknowledged in our experiment. First, we cannot be entirely sure that the minigenes were expressed in neurons. As noted above, these short polypeptides are too small and evanescent to allow detection. However, using the same promoter driving Cre from the same locus in the HSV-1 genome we have recently shown that many neurons are marked in ROSA26 mice, both during the acute infection and during latency [27]. Second, even if the genes were expressed, they might not lead to more presentation of gB498 on neurons, or the amount presented from the endogenous copy of the epitope may already be adequate to ensure a maximum response in DRG. Third, if the action of CD8+ T cells is non-lytic suppression of virus or reactivation as has been suggested [21, 24], this would be undetectable in our model. Finally, the model used is one that mimics stable latency so impacts on reactivation were not assessed beyond noting that virus could be produced at typical rates after explant culture of latently-infected DRG (not shown). A more physiologically relevant model of reactivation that occurs in the context of the CD8+ T cell response [43, 44] may demonstrate some effect of enhanced gB498 presentation. Taking these into consideration our experiment cannot be interpreted as bearing significantly on the

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**Fig. 5.** Increased lesion size is the only consistent finding associated with addition of gB498 minigenes to HSV-1. (a) Schematic representation of the CMV IE promoter eGFP/Cre expression cassette inserted the intergenic space between U3 and U4 in HSV-1 pC_eGC, with the alternate L8A substituted gB498 minigene (shown in red) under the control of the gB promoter. Groups of ROSA26 mice were tattoo infected with the indicated viruses, and lesion size measured daily (b). At the days indicated, mice were culled and the innervating DRG were removed and processed for determination of β-gal expression. Both (c) the total number of β-gal+ cells per mouse and (d) the number of DRG per mouse containing at least one β-gal+ cell are shown, with each point indicating a single mouse, and the column indicating the mean value (nd=not determined). Groups of four C57Bl/6 mice were infected with the indicated virus and at 6 days p.i. were culled and the virus titres in the skin (e) and DRG (f) were determined. Results are pooled from two independent experiments (n=8–10 per time point). Statistical significance was determined by a one-way ANOVA with Bonferroni’s post-test to make pairwise comparisons, with key statistical differences indicated (P<0.05).
question of the role of CD8$^+$ T cells in HSV-1 latency and reactivation.

Where we failed to find a phenotype for our viruses in DRG, there was a striking increase in the size of lesions caused by those viruses incorporating a gB$_{498}$ minigene. Our method of measuring size incorporates an estimate of the total area where there are lesions as well as the fraction of skin in this area that is affected. This is important because in some mice infected with control viruses, the lesion does not become confluent across the whole dermatome. In the case of mice infected with the minigene viruses, the lesions covered a larger area as well as being more confluent. Of note, the amounts of infectious virus in the skin of these mice were always the same, even at day 6 when there was a difference in lesion size in all experiments, suggesting the cause of the larger lesions was immunopathology. This is supported by the L8A control, which differed from the cytoplasmic minigene by only a single amino acid and the lack of a difference in lesion size in BALB/c mice. This implicates presentation on MHC class I and therefore the action of CD8$^+$ T cells, at least locally in the skin, in the pathology of the larger lesions. The role of proinflammatory processes in modulating the progression and resolution of HSV skin lesions has been previously noted, with a clinical trial showing an increased efficacy of the commonly used HSV topical antiviral acyclovir when combined with hydrocortisone cream [45]. Any mechanism remains highly speculative, but we suggest that increasing the amount of antigen may increase (a) the recruitment/retention of CD8$^+$ T cells, (b) their range of targets, perhaps including uninfected cells by ‘cross-dressing’, and/or (c) the amount of proinflammatory mediators they release in infected skin.

In conclusion we find that our strategy for making HSV-infected cells more easily targeted by CD8$^+$ T cells has not impacted any parameter of neural infection, but instead was associated with increased pathology in the skin. We suggest that more consideration of potential unexpected immunopathology may be needed when pursuing CD8$^+$ T cell-based immunotherapies for HSV-1.

**METHODS**

**Viruses and cell lines**

All viruses were grown and titrated on Vero cells (ATCC CCL-81). The immortalised Vero cell line, and HSV-2.3.2E2 hybridoma [19] used for the antigen presentation assay, were maintained in Minimal Essential Medium (Life Technologies) supplemented with 2 or 10 % heat inactivated fetal calf serum (FCS; Serana), 5 mM HEPES, 4 mM L-glutamine and 50 µM 2-mercaptoethanol (Life Technologies). All transfections were carried out in 293A cells using Lipofectamine 2000 (Life Technologies). Cells of the 293KbC2 line [46] were used for the antigen presentation assay and were maintained in Dulbecco’s Modified Eagles Medium (DMEM; Life Technologies) supplemented with 2 or 10 % heat inactivated FCS (Serana) and 2 mM L-glutamine (Life Technologies).

HSV-1 pC_eGC contains an eGFP/Cre fusion gene under the control of the (CMV IE) promoter located in the intergenic region between U$_L$3 and U$_L$4 of HSV-1 KOS [47].

**Plasmid construction**

All sequence references below are to the HSV-1 KOS genome accession JQ673480. Briefly, the plasmids used to...
construct the recombinant viruses all contained the eGFP/Cre fusion gene (amplified from pIGCN21 [48]) under the control of the CMV IE promoter (amplified from pTracer CMV/bsd; Life Technologies), with the bovine growth hormone (BGH) polyA termination sequence (amplified from pTracer CMV/bsd; Life Technologies), inserted into the SpeI site of pU3.2kbF [47]. To construct pESminigB_Cre, an ER-targeted gB minigene [49] under the control of the gB promoter sequence (HSV-1 KOS 55985-56282) followed by the SV40 polyA sequence was inserted into the NotI site of pU3.2kbF by In-Fusion cloning (Clontech). pminigB_Cre contains the gB minigene under the control of the gB promoter, but lacking the ER-targeting motif. Finally, pL8A_Cre contains the gB minigene with the L8A substitution under the control of the gB promoter, lacking the ER-targeting motif.

The plasmid pX330 (Addgene plasmid 42230) has been previously described [50]. The plasmid pX330-minigB was constructed by annealing two complimentary oligodeoxynucleotides (CACCGGCAGGTGACTGGCGCA and AAACCTGCGGGACTCTGCGG) and ligating the resulting dsDNA fragment into the BbsI site of pX330.

**Construction of recombinant viruses**

To construct HSV-1 ESminigB_Cre, linearised plasmid DNA from pESminigB_Cre was transfected into Vero cells, followed by infection with HSV-1 KOS as previously described [47]. Plaques containing the desired recombinant virus were identified based on eGFP expression and PCR screening, and at least three rounds of plaque purification were carried out.

To construct HSV-1 minigB_Cre and HSV-1 L8A_Cre, transfection of linearised pminigB_Cre or pL8A_Cre with pX330-minigB was performed, followed by infection with HSV-1 ESminigB_Cre as previously described [47]. Desired recombinant virus was identified based on PCR screening, and at least three rounds of plaque purification were carried out. PCR screening and sequencing were used to ensure that the viruses contained the desired modification and were free from parental virus. All viruses grew with normal kinetics after low multiplicity infection of cells in culture and were able to be reactivated from the DRG of latently-infected mice (not shown).

**Antigen presentation assay**

Single cell suspensions of 293KbC2 cells were infected with the appropriate virus at a MOI of 5 as previously described [51]. After 6 h, infected cells (stimulators) were mixed with $5 \times 10^4$ HSV-2.3.2E2 hybridoma cells (effectors) at a stimulator to effector ratio of 1 : 5, 1 : 10, 1 : 50 or 1 : 100, in triplicate. Background LacZ levels were determined using hybridoma cells alone, while uninfected cells served as stimulators for a negative control, and 293KbC2 cells stimulated with 0.125 $\mu$M synthetic gB$_{98}$ (sequence SSIEFARL; GenScript) served as a positive control. After 12 h at 37°C, 5 % CO$_2$, β-gal activity was measured using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate as previously described [52], reading on a Tecan Infinite M1000 Pro plate reader.

**Mice and infections**

Female specific pathogen free C57Bl/6, BALB/c or B6.129S4-Gt(Rosa26)tm1Sor/J (referred to as ROSA26) mice [53] greater than 8 weeks of age were obtained from the Australian Phenomics Facility (Canberra, Australia). Mice were housed and experiments carried out under the oversight of the Animal Ethics and Experimentation Committee of The Australian National University and according to Protocol Numbers A2011.015, A2014.025 or A2017-039. Mice were infected using a flank infection model where $1 \times 10^8$ p.f.u. ml$^{-1}$ virus was introduced into the flank by tattoo as previously described, mice were anaesthetised with 1,1,1-tribromoethanol for the infection process [47]. The same virus dose and route of infection were used for all experiments.

**CD8$^+$ T cell assays**

Mice were culled 7 days p.i. and single cell suspensions of spleens were prepared. To prepare single cell suspensions of DRG, DRG were collected from mice from spinal levels T5 to L1, trimmed of excess tissue, and placed directly into 1 ml of 1 mg ml$^{-1}$ Type IV Collagenase (at least 160 units ml$^{-1}$; Worthington) and 0.03 mg ml$^{-1}$ DNase (at least 600 units ml$^{-1}$; Roche) in DMEM supplemented with 2 % FCS. The DRG were incubated at 37°C, 200 r.p.m. for 60 min, and gently ground through a 70 µM cell strainer and washed with excess cold PBS containing 2 % FCS.

Cells were stained with one or more of the following mAbs (Biolegend): anti-CD8α-APC-Cy7 (clone 53–6.7), anti-CD62L-FITC (clone MEL-14), anti-CD45.2-BV481 (clone GK1.5) and anti-GzmB-AF647 (clone GB11). This was combined with H-2K$^b$/gB-PE dextramer staining as previously described [54]. Cells from the spleen or DRG were fixed with 1 % paraformaldehyde, and data was acquired on a LSRII flow cytometer (BD biosciences). Analysis was performed using Flowjo software (Tree Star), with events gated for live lymphocytes on FSC-A × SSC-A plots, with appropriate parameters examined after doublet discrimination.

**Titration of virus from skin and DRG**

A 1 cm$^2$ portion of skin located over the inoculation site and the DRG found on the ipsilateral side corresponding to spinal levels L1–T5 were collected 5 days post-infection (p.i.). Skin or DRG were homogenised in MEM supplemented with 2 % FCS. Homogenates were subjected to three cycles of freeze/thawing and infectious virus quantified by plaque assay on Vero cells.

**Histochemical detection of β-gal expression and detection of fluorescence in whole DRG**

Mice were culled by CO$_2$ asphyxiation and the innervating whole DRG were removed as soon as possible and fixed in 2 % paraformaldehyde/0.5 % glutaraldehyde for 1 h on ice, permeabilised and stained with 1 mg ml$^{-1}$ X-gal for the
detection of β-gal expression as previously described [27]. The DRG were then visualised and photographed using an Olympus CKX41 light microscope and Olympus DP20 camera. The number of β-gal+ cells was determined with the aid of ImageJ software [55].

**Statistical analysis**

Statistical comparisons were performed with the aid of Prism software (version 7.01; GraphPad).

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

Mice were housed and experiments carried out under the oversight of the Animal Ethics and Experimentation Committee of The Australian National University. According to Protocol Numbers A2011.015, A2014.025 or A2017.039.

**References**

1. Simmons A, Tscharke D, Speck P. The role of immune mechanisms in control of herpes simplex virus infection of the peripheral nervous system. Curr Top Microbiol Immunol 1992;179:31–56.
2. Bedoui S, Greyer M. The role of dendritic cells in immunity against primary herpes simplex virus infections. Front Microbiol 2014;5:533.
3. Egan KP, Wu S, Wiegahls B, Jennings SR. Immunological control of herpes simplex virus infections. J Neurovirol 2013;19:328–345.
4. Ouwendijk KJ, Bertram K, Cunningham AL. Understanding natural herpes simplex virus immunity to inform next-generation vaccine design. Clin Transl Immunology 2016;5:e94.
5. Sandgren KJ, Bertram K, Cunningham AL. Understanding natural herpes simplex virus immunity to inform next-generation vaccine design. Curr Opin Virol 2013;3:452–460.
6. York IA, Roop C, Andrews DW, Riddell SR, Graham FL et al. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell 1994;77:525–535.
7. Hill A, Jugovic P, York I, Russ G, Bennink J et al. Herpes simplex virus turns off the TAP to evade host immunity. Nature 1995;375:411–415.
8. Früh K, Ahn K, Djaballah H, Sempé P, van Endert PM et al. A viral inhibitor of peptide transporters for antigen presentation. Nature 1995;375:415–418.
9. Tomazin R, van Schoot NE, Goldsmith K, Jugovic P, Sempé P et al. Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP. J Virol 1998;72:2560–2563.
10. Goldsmith K, Chen W, Johnson DC, Hendricks RL. Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the CD8+ T cell response. J Exp Med 1998;178:341–348.
11. Ahn K, Meyer TH, Uebel S, Sempé P, Djaballah H et al. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. Embo J 1996;15:3247–3255.
12. Divito S, Cherpes TL, Hendricks RL. A triple entente: virus, neurons, and CD8+ T cells maintain HSV-1 latency. Immunol Res 2006;36:119–126.
13. Orr MT, Mathis MA, Lagunoff M, Sacks JA, Wilson CB. CD8+ T cell control of HSV reactivation from latency is abrogated by viral inhibition of MHCI class I. Cell Host Microbe 2007;2:172–180.
14. van Velzen M, Jing L, Osterhaus AD, Sette A, Koelle DM et al. Local CD4 and CD8 T-cell reactivity to HSV-1 antigens documents broad viral protein expression and immune competence in latently infected human trigeminal ganglia. PLoS Pathog 2013;9:e1003547.
15. Porgador A, Yewdell JW, Deng Y, Bennink JR, Germain RN. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. Immunity 1997;6:715–726.
16. Princiotta MF, Finzi D, Qian SB, Gibbs J, Schuchmann S et al. Quantitating protein synthesis, degradation, and endogenous antigen processing. Immunity 2003;18:343–354.
17. Anton LC, Yewdell JW, Bennink JR. MHC class I-associated peptides produced from endogenous gene products with vastly different efficiencies. J Immunol 1997;158:2535–2542.
18. Back I, Cox JH, Anderson R, Yewdell JW, Bennink JR. TAP (transporter associated with antigen processing)-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino- but not carboxyl-terminus of the peptide. J Immunol 1994;152:381–387.
19. Mueller SN, Jones CM, Smith CM, Heath WR, Carbone FR. 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 2002;195:651–656.
20. Yuen TJ, Fliesh IE, Hollett NA, Dobson BM, Russell TA et al. Analysis of A47, an immunoprevalent protein of vaccinia virus, leads to a reevaluation of the total antiviral CD8+ T cell response. J Virol 2010;84:10220–10229.
21. Simmons A, Tscharke DC. Anti-CD8 impairs clearance of herpes simplex virus from the nervous system: implications for the fate of virally infected neurons. J Exp Med 1992;175:1337–1344.
22. Decman V, Kinchington PR, Harvey SA, Hendricks RL. Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. J Virol 2005;79:10339–10347.
23. Sheridan BS, Khanna KM, Frank GM, Hendricks RL. Latent virus influences the generation and maintenance of CD8+ T cell memory. J Immunol 2006;177:8356–8364.
24. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR et al. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. Science 2008;322:268–271.
25. Mackay LK, Wakim L, van Vliet CJ, Jones CM, Mueller SN et al. Maintenance of T cell function in the face of chronic antigen stimulation and repeated reactivation for a latent virus infection. J Immunol 2012;188:2173–2178.
26. Ma JZ, Russell TA, Spielman T, Carbone FR, Tscharke DC. Lytic gene expression is frequent in HSV-1 latent infection and correlates with the engagement of a cell-intrinsic transcriptional response. PLoS Pathog 2014;10:e1004237.
27. Russell TA, Tscharke DC. Lytic promoters express protein during herpes simplex virus latency. PLoS Pathog 2016;12:e1005729.
28. Proença JT, Coleman HM,Connor V, Winton DJ, Efstathiou S. A historical analysis of herpes simplex virus promoter activation in vivo reveals distinct populations of latently infected neurons. J Gen Virol 2008;89:2965–2974.
29. Stock AT, Jones CM, Heath WR, Carbone FR. CTL response compensation for the loss of an immunodominant class I-restricted HSV-1 determinant. Immunol Cell Biol 2006;84:543–550.
30. Tigges MA, Leng S, Johnson DC, Burke RL. Human herpes simplex virus (HSV)-specific CD8+ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN-gamma or when virus host shutoff functions are disabled. J Immunol 1996;156:3901–3910.
31. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA et al. Migratory dendritic cells transfer antigen to a lymph node-
resistant dendritic cell population for efficient CTL priming. *Immunity* 2006;25:153–162.

32. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol* 2009;10:488–495.

33. Macleod BL, Bedoui S, Hor JL, Mueller SN, Russell TA et al. Distinct APC subtypes drive spatially segregated CD4+ and CD8+ T-cell effector activity during skin infection with HSV-1. *PLoS Pathog* 2014;10:e1004303.

34. Wolkers MC, Brouwenstijn N, Bakker AH, Toebes M, Schumacher et al. Antigen presentation and retention in latently infected sensory ganglia. *Curr Opin Immunol* 2003;15:603–608.

35. Khanna KM, Lepisto AJ, Decman V, Hendricks RL. Antigen expression and epitope presentation during virus infection with herpes simplex virus type 2 confers protective immunity. *J Virol* 1998;72:9567–9574.

36. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

37. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

38. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

39. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

40. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

41. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

42. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

43. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

44. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

45. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

46. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

47. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

48. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

49. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

50. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

51. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

52. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

53. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

54. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

55. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

56. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

57. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

58. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

59. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

60. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

61. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

62. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

63. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

64. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

65. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.

66. Liu T, Khanna KM, Chen X, Fink DJ, Hendricks RL. CD8+ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. *Exp Mol Med* 2001;33:819–823.