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Herpes Simplex Virus-Specific CD8+ T Cells Can Clear Established Lytic Infections from Skin and Nerves and Can Partially Limit the Early Spread of Virus after Cutaneous Inoculation

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HSV infects skin or mucosal epithelium as well as entering the sensory nerves and ganglia. We have used TCR-transgenic T cells specific for the immunodominant class I-restricted determinant from HSV glycoprotein B (gB) combined with a flank zosteriform model of infection to examine the ability of CD8+ T cells to deal with infection. During the course of zosteriform disease, virus rapidly spreads from the primary inoculation site in the skin to sensory dorsal root ganglia and subsequently reappears in the distal flank. Virus begins to be cleared from all sites about 5 days after infection when gB-specific CD8+ T cells first appear within infected tissues. Although activated gB-specific effectors can partially limit virus egress from the skin, they do so only at the earliest times after infection and are ineffective at halting the progression of zosteriform disease once virus has left the inoculation site. In contrast, these same T cells can completely clear ongoing lytic replication if transferred into infected immunocompromised RAG-1−/− mice. Therefore, we propose that the role of CD8+ T cells during the normal course of disease is to clear replicating virus after infection is well established rather than limit the initial spread of HSV from the primary site of inoculation. The Journal of Immunology, 2004, 172: 392–397.

Herpes simplex virus of either type 1 or type 2 (HSV-1 or HSV-2, respectively) infects skin and mucosal epithelium where it can undergo rounds of local replication. In addition, the virus enters the sensory nerve endings and travels to the neural ganglia via retroaxonal flow. It is within these secondary sites that virus ultimately persists as a latent infection, although it may periodically re-emerge to form recurrent lesions at or near the initial site of infection. Various immune mediators have been implicated in the control of virus replication. Both CD4+ Th cells and CD8+ effector T cells are capable of limiting the severity of HSV infection, although there is some conflict in the literature as to where, and therefore when, these cells contribute to this effect. Simmons and Tschakke (1) showed that depletion of CD8+ T cells had a marked effect on limiting HSV-1 titer in the sensory ganglia, whereas Manickan and Rouse (2) found that mice lacking these cells showed no defect to challenge with this same virus, arguing instead that CD4+ Th cells play the major role in protection against infection. Both studies found that removal of CD8+ T cells had variable to little effect on the clearance of virus from infected skin. Countering this latter point, Koelle et al. (3) were able to isolate HSV-specific CD8+ T cells present in skin biopsies from individuals undergoing recurrent infection. That CD8+ T cells are likely to play at least some role in HSV infection, can be inferred from the fact that the virus encodes mechanisms by which it can circumvent recognition by these cells (4–6).

Given the complexity of HSV pathogenesis, which involves transitions between different phases of the infection (lytic and latent) as well as the physical movement of virus between different tissues (skin and nerves), it is not surprising that uncertainty exists as to exactly how and when the immune response deals with this virus. Coupled to this is the inherent difficulty in tracking the movement of virus-specific T cells at the various times after infection. Multimeric MHC molecules have been invaluable in this respect, and these have recently been applied to the tracking of HSV-specific CD8+ T cells with some success (7, 8). TCR-transgenic T cells are also a powerful tool, providing one of the few means of examining the earliest stages of the immune response (9, 10) and facilitate the manipulation and transfer of defined virus-specific T cell populations in both primed and resting forms. To this end, we have generated a TCR-transgenic animal having the majority of its T cells specific for a dominant class I-restricted determinant from the HSV glycoprotein B (gB)3 (11). In this study, we use these animals to study the involvement of HSV-specific CD8+ T cells during the various lytic stages of HSV infection. We have coupled these reagents with a mouse zosteriform model of flank skin infection (12). In this system, virus is inoculated in the mouse flank by mechanical scarification whereupon it migrates to a relatively restricted group of thoracic sensory dorsal root ganglia (DRGs). From here, virus re-emerges via retroaxonal flow along the sensory neurons originating within this ganglion, giving rise to a band-like or zosteriform region of vesiculating lesions spreading ventrally from the site of primary inoculation.
toward the anterior midline. These experiments reveal that the major role of CD8$^+$ T cell effectors is likely to be the clearance of replicating virus from both skin and nerves once infection is already well established in these sites.

Materials and Methods

Viruses and mice

The KOS strain of HSV-1 was grown and titrated on Vero cells (CSL, Parkville, Australia). Vero cells were grown in MEM10 (MEM medium supplemented with 10% heat-inactivated FCS, 4 mM l-glutamine, 5 × 10^{-5} M 2-ME, and antibiotics). Stocks were stored at −70°C until required. C57BL/6, RAG-1 knockout (RAG-1$^{-/-}$), gBT-I, and OT-I female mice were purchased from Department of Microbiology and Immunology, University of Melbourne, Animal House, and housed in specific pathogen-free conditions. The gBT-I mice are gB-specific, TCR-transgenic, MHC class I-restricted mice expressing a TCR that recognizes the HSV-1 gB498-505 determinant (SIIFERAIL) complexed with K$^b$ (11). The OT-I mice are OVA-specific, TCR-transgenic, MHC class I-restricted mice expressing a TCR that recognizes the OVA257-264 peptide (SIINFKEKL) complexed with K$^b$ (13).

HSV-1 infections

Female C57BL/6 and RAG-1$^{-/-}$ mice, 6–12 wk old, were anesthetized by i.p. injection (10 μKg of body weight) of a 1:1 ketamine (Parnell Laboratories, Alexandria, Australia)/Ilium Xylazil-20 (Troy Laboratories, Smithfield, Australia) solution in saline. The left flank of each mouse was clipped and depilated with Nair (Carter-Wallace, Frenchs Forest, Australia). A small area of skin, near the top of the spleen, was shaved using a MultiPro power tool (Dremel, Racine, WI) with a grindstone attachment (3.2 mm), held on the skin for 20 s to create a 2- to 4-mm$^2$ area of abraded skin. An 1-cm$^2$ piece of skin from the lower flank, the secondary site, was taken. To remove HSV infection shows that they are first seen in the DRGs at 4 and 5 (7). By this stage of infection, virus can be found throughout the lesion and patches of skin that were excised to measure virus in tissue samples was determined using standard PFU assays on confluent Vero cell monolayers. Samples were thawed and homogenized, and 10-fold serial dilutions were then tested for plaque formation to determine plaque titer in the original tissue sample.

Adoptive transfer of naive CD8$^+$ T cells

Lymph node cells were obtained from naive gBT-I mice. Approximately 10$^6$ CD8$^+$ T cells, as determined by flow cytometry, were transferred i.v. into infected C57BL/6 mice 24 h before infection. Control mice received naive OT-I CD8$^+$ T cells 24 h before infection.

Identifying CD8$^+$ T cells infiltrated in DRG

Mice were sacrificed and perfused with PBS, and ganglia innervating the infected site were removed and collected in 1 ml of collagenase type 1 (Sigma-Aldrich, St. Louis, MO) at 3 mg/ml in RPMI 1640 supplemented with 2% FCS. All ganglia from a single mouse were pooled. The samples were kept at 37°C for 1.5 h and dispersed into single-cell suspensions with trituration at both 1 and 1.5 h. Staining was performed on ice with anti-CD8 (53-6.7; BD PharMingen, San Diego, CA) and then at 37°C with either gB- or OVA-specific tetramer complexes. Both the K$^b$-gB and the K$^b$-OVA tetramer were prepared essentially using the protocol by Arman et al. (14). Propidium iodide was added before analysis of 1 × 10$^5$ live cells/sample using a FACSort and CellQuest software (BD Biosciences, San Jose, CA).

Adoptive transfer of activated CD8$^+$ T cells

A total of 5 × 10$^5$ transgenic splenocytes (from either gBT-I or OT-I mice) were cultured for 4 days with 5 × 10$^5$ irradiated C57BL/6 splenocytes pulsed with either 0.1 μg of gB498-505 peptide or OVA257-264 peptide, in 40 ml of RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 5 × 10^{-5} M 2-ME, and antibiotics. The cultures were diluted 1 in 2 on days 2 and 3 with fresh medium containing 10 U/ml IL-2. On day 4, cells were collected, and 10$^5$ T cells were transferred into recipient mice i.v. injections in a total volume of 200 μl of HBSS.

Detection of HSV-1 by immunoperoxidase staining

Skin samples were fixed at room temperature for 1 h in freshly prepared paraformaldehyde-lysine-periodate fixative (15). HSV Ags were detected in paraffin-embedded sections of tissue using the EnVision System (DAKO, Carpinteria, CA). The primary Ab was rabbit and mouse antisera to HSV-infected cells. Binding was detected using a peroxidase-labeled polymer conjugated to goat anti-rabbit and goat anti-mouse IgG. Non-specific peroxidase activity was blocked with hydrogen peroxide (0.03%) containing sodium azide. Peroxidase activity was detected with the substrate 3,3′-diaminobenzidine chromogen solution which gives rise to a brownish staining in positive cells. Negative control slides were included in each staining run and incubated with diluent in place of primary Ab. All slides were counterstained with hematoxylin.
around 5 days after infection and make up up to 38% of all ganglionic CD8$^{+}$ T cells 6 days after infection (Fig. 2). It should be noted that, even at the relatively low numbers transferred here, transgenic T cells dominate over endogenous gB-specific precursors so that the bulk of the gB-specific CD8$^{+}$ T cell response is comprised of transferred transgenic T cells (data not shown). Control OVA-specific OT-I transgenic T cells are never seen in the DRGs, showing that DRG infiltration is dependent on virus-specific stimulation of the resting T cells.

The CD8$^{+}$ T cells alone are sufficient to control infection and clear replicating virus from nerves and skin

Because the appearance of gB-specific T cells in the DRGs coincides with the cessation of replication (Fig. 1A), we wondered whether these cells could actually clear replicating virus from this site. To show this, and exclude all other elements of the adaptive immune response, we transferred in vitro-activated gBT-I T cells

FIGURE 1. Characterization of the flank zosteriform infection in C57BL/6 mice. A cohort of C57BL/6 mice was infected with HSV-1 KOS using flank scarification. On days 1–7, groups of four mice were sacrificed, and the primary site, secondary site, and infected DRGs were collected. A, Viral titers in these tissues were determined using standard PFU assays. B, The positions of the primary and secondary sites are shown.

FIGURE 2. CD8$^{+}$ T cell infiltration of infected DRG during a flank infection with HSV-1. C57BL/6 mice received 1 $\times$ 10$^6$ resting gBT-I or OT-I CD8$^{+}$ T cells 24 h before infection. Following flank scarification and infection, innervating ganglia were removed on days 4–7 and analyzed for CD8$^{+}$ T cell infiltrates by flow cytometry. Cells were stained with anti-CD8 mAb and either K$^b$-gB tetramer (left panel) or K$^b$-OVA tetramer (right panel) as required. The percentage of CD8$^{+}$ T cells that were tetramer positive is shown next to each plot.

FIGURE 3. The effect of HSV-1-specific CD8$^{+}$ T cells on survival and viral titers in RAG-1$^{-/-}$ mice following HSV-1 flank infection. A, RAG-1$^{-/-}$ mice were infected with HSV-1 KOS using flank scarification. The percentage of animals that remain healthy is shown for untreated control mice (n = 11) and mice that have received 10$^7$ in vitro-activated gBT-I CD8$^{+}$ T cells 4 days after infection (n = 12). B, RAG-1$^{-/-}$ mice were infected with HSV-1 KOS using flank scarification and left for 4 days before half received 10$^7$ in vitro-activated gBT-I CD8$^{+}$ T cells. Virus titers from skin and DRGs were determined at day 4 (pretransfer) and days 5 and 14 posttransfer for mice that received gBT-I T cells. Virus levels in control infected RAG-1$^{-/-}$ mice that did not receive gBT-I cells were determined only for the day 5 posttransfer time, because this cohort succumbed to hindleg paralysis around 13 days after infection.
into RAG-1−/− mice that lack both B and T lymphocytes. Because we know that gB-specific CD8+ T cells are not released from the priming lymph nodes until at least 4–5 days after infection (7), we allowed the infection to establish itself for 4 days before T cell transfer to mimic the natural progression of infection and release of CD8+ effector T cells. The mice not receiving transgenic T cells completely fail to control the infection and succumb to hindleg paralysis by day 13 postinfection (Fig. 3A). In comparison, the animals receiving activated gBT-I T cells were able to significantly reduce replicating virus in the DRG and completely clear virus from skin within 5 days of transfer (Fig. 3B). No replicating virus was detectable in the DRGs and skin by 14 days after transfer of activated CD8+ T cells.

**TCR-transgenic, CD8+ T cells only inhibit the progression of zosteriform lesions early after infection**

The preceding data argue that activated gB-specific CD8+ T cells can clear established HSV infection, but they are probably released too late to deal with the initial spread of virus. However, they do not address whether these same cells can at any time limit the progression of infection from the initial site of inoculation. To address this, gBT-I T cells that had been previously activated by in vitro stimulation with peptide where transferred into normal mice 1 day before infection. Fig. 4A shows that these cells protect recipient mice from developing zosteriform lesions. Adoptive transfer of activated gB-specific T cells, but not irrelevant OT-I control cells, lowered the peak virus load by ~100-fold in both the primary site of skin infection and the DRGs (Fig. 4B), suggesting that the HSV-specific CD8+ T cells affect disease outcome by reducing virus load as infection progresses through the neurodermatome.

Examination of HSV Ag expression in infected skin shows a marked reduction in HSV Ag expression in the epidermis of mice that receive the activated gBT-I T cells before infection. By 48 h after infection, Ag expression is observed extensively within the epidermis surrounding the site of inoculation in unmanipulated mice (Fig. 5). In contrast, transfer of activated gBT-I cells before infection restricted the spread of virus so that Ag expression was only seen immediately adjacent to the point of scarification. These data suggest that the T cells appear to operate within the skin epidermis by limiting the earliest stages of zosteriform virus spread from the initial site of infection.

Finally, to determine whether the activated T cells could also limit zosteriform spread once virus had left the site of inoculation within the skin, we transferred the activated gBT-I cells at progressively longer times after infection. The results presented in Fig. 6 show that the activated T cells were effective at stopping the formation of secondary lesions only if transferred within the first 24 h after inoculation. Some faint lesions appear if transfer is delayed to 36 h postinfection, whereas T cell transfer at 48 h postinfection has almost no effect on the formation of secondary lesions. It should be noted that, at 48 h, the transferred CD8+ T cells fail to limit lesion formation even though virus has yet to re-emerge to the secondary site of skin infection (see Fig. 1A). Thus, CD8+ T cells...
effector T cells appear to be effective at inhibiting the progression of zosteriform disease only at the earliest stages of infection, before virus has left the skin. After this point, they are rendered relatively ineffective at limiting subsequent progression of infection throughout the remainder of the neurodermatome and fail to stop nerve-to-skin transmission of HSV.

Discussion

Although the effectiveness of HSV-specific CD8\(^+\) T cells in the skin has been questioned in the past (1, 2), our results prove that these cells can act directly in this site. This can been seen by the inhibition of virus spread along the skin in mice that receive the transferred T cells as shown in Fig. 5. The inoculation used here relies on localized mechanical scarification and results in an infection initially confined to those epithelial cells immediately adjacent to the site of skin damage. There is a progressive spread of Ag expression to neighboring cells within the epidermis in the first 24–48 h after infection (17). The inhibition of early virus replication within the skin could limit subsequent entry into the DRGs, thus explaining the ability of these transferred T cells to abolish zosteriform disease. Therefore, although HSV replication in peripheral tissues is not always required for ganglionic infection (18, 19), early skin replication may be necessary for optimal infection of the DRGs in this system, possibly because it provides greater access to sensory nerve endings. These experiments do not exclude that T cells act within the ganglia to limit further spread of virus, although we find no infiltration earlier than day 3 postinfection (A. van Lint and F. R. Carbone, unpublished results), which is usually enough time for virus to reach maximal levels within the nerves and already spread to the secondary site of skin infection.

Despite this demonstration that CD8\(^+\) T cells can limit egress of HSV from skin to nerves, it is clear that, during the normal course of infection, they would play little role in this respect. Simply, our data show that, unless these T cells are present within the first 24 h after skin inoculation, they fail to control the spread of virus throughout the remainder of the neurodermatome. This is consistent with previous experiments by Simmons and Nash (12) using adoptive transfer of unfragmented immune lymphocytes, which showed that these cells lose effectiveness after 24-h postinfection. Therefore, despite a very rapid initial activation of CD8\(^+\) T cells shortly after infection (7), the HSV-specific CD8\(^+\) T cells are simply released too late to limit the initial spread of virus from the primary site of inoculation.

Given this, we argue that virus-specific CD8\(^+\) T cells are primarily involved in clearance of established lytic infection rather than limiting the initial spread of virus from the skin. We have shown that gB-specific transgenic T cells can effectively abolish virus replication in the DRGs from RAG-1\(^{-/-}\) mice (Fig. 3), and their arrival in these tissues during the normal course of infection (Fig. 2) coincides with the elimination of lytic replication in C57BL/6 mice (Fig. 1). Combined, the results argue that gB-specific CD8\(^+\) T cells are likely to be at least partly responsible for cessation of the lytic phase of infection within the DRGs, consistent with data showing that elimination of CD8\(^+\) T cells compromises virus elimination from this tissue (1). In addition, the virus-specific CD8\(^+\) T cells probably play a role in eliminating virus from the areas of skin infected as a consequence of zosteriform virus spread. Indeed, HSV-specific CD8\(^+\) T cells can probably eliminate virus from all tissues once they are released into the circulation at around days 4–5 after infection (7). This may partly explain the near-simultaneous clearance of replicating virus at this time during the normal progression of zosteriform disease (Fig. 1A).

Our results do not exclude the involvement of other cells or other facets of the immune system in clearance of virus. Indeed, studies have shown that CD4\(^+\) T cells, γδ-T cells, and NK-T cells can all protect against infection with HSV (2, 20–23). NK cells are also known to be particularly important in limiting the early spread of HSV, and Ab can inhibit direct spread of virus between skin and nerves (24–27). However, regardless of the action of these other immune mechanisms, our results using the RAG-1\(^{-/-}\) mice show that CD8\(^+\) T cell effectors alone can clear replicating virus from all infected tissues.

Finally, we have exclusively focused on the lytic phase of infection and have ignored latency and virus reactivation. Hendricks and colleagues (28) have suggested that CD8\(^+\) T cells play an active role in inhibiting virus reactivation, and gB-specific T cells in particular have been proposed to be critical during latency in the infected neurons. Therefore, despite a very rapid initial activation of CD8\(^+\) T cells at a time when virus has first entered the DRGs, but not yet reached the secondary site of skin infection, we showed that activated CD8\(^+\) T cells appear relatively ineffective at halting the actual progression throughout the neurodermatome once virus has left the primary site of infection. In other words, they fail to halt the nerve-to-skin transition that mimics the progression of infection on virus reactivation. However, once lytic infection is firmly re-established, CD8\(^+\) effector T cells will be recruited into tissues harboring replicating virus as seen here with the primary infection. There they would ensure subsequent elimination of replicating virus and the establishment of the equilibrium associated with stable latency.

References


