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Angus T. Stock, Claerwen M. Jones, William R. Heath and Francis R. Carbone

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Cutting Edge: Central Memory T Cells Do Not Show Accelerated Proliferation or Tissue Infiltration in Response to Localized Herpes Simplex Virus-1 Infection¹

Angus T. Stock,* Claerwen M. Jones,* William R. Heath,[†] and Francis R. Carbone^{2*}

Memory T cells mount an enhanced response to secondary infections. Such an enhancement has been attributed in part to the ability of memory cells to more rapidly respond to cognate stimulation. In this study we have examined the rapidity with which murine CD8⁺ memory T cells respond to a localized infection with HSV. Although central memory T cells (TcM), but not the effector memory T cells, mounted a strong recall response to secondary infection, the kinetics of TcM proliferation, the magnitude of their expansion, and their infiltration into infected nonlymphoid tissues were not advanced compared with that observed for naive T cells. These findings imply that it is the lack of accelerated proliferation kinetics and the subsequent delayed dissemination into the periphery that limits the ability of TcM to rapidly control localized virus replication. The Journal of Immunology, 2006, 177: 1411–1415.

Following infection, naive T cells are driven into a program of division and differentiation, forming a numerically large pool of effector CTL capable of migrating into infected peripheral tissue and secreting cytolytic and cytokine effector molecules (1, 2). This response usually resolves through the activation-induced cell death of terminally differentiated effector T cells and the emergence of a self-renewing population of memory T cells (3). Although the memory pool remains numerically stable, the population is both heterogeneous and dynamic, classically being divided between two subsets consisting of central memory T cells (TcM)³ and effector memory T cells (TeM) based upon the expression of lymph node (LN) homing molecules (4, 5). TcM cells express high levels of the LN homing ligand CD62L and the chemokine receptor CCR7 (4), dominate late stages of T cell memory, and are found to transit between secondary lymphoid organs (5). TeM cells expressing low levels of these molecules are located within the spleen, liver, and nonlymphoid tissues (4–6) and are at their numerical peak during the early stage of memory (5).

Although the TeM subset appears to retain most effector functions, TcM cells have been claimed to provide superior protection in a number of viral, bacterial, and parasitic systems (5, 7). This protective value is generally attributed to the heightened proliferative capacity of the TcM subset (5), suggesting that control of a secondary infection requires some level of re-expansion by the memory pool. To this end it has been reported that CD4 and CD8 memory T cells have accelerated proliferation kinetics compared with naive T cells (8, 9). Although this claim has been challenged by others (10), it is the rapidity of the proliferative response that is generally assumed to contribute to protection from secondary infections (11). Although the responding populations in these earlier studies (8, 9) were not strictly examined in light of the subset demarcation, it might be assumed that it was the TcM that originally contributed to the observed hyperproliferation. However, as this possibility was not directly examined, we have compared the response of the different memory cell subsets to a localized infection. Surprisingly, we have found that despite a more rapid induction of effector function, TcM show little in the way of enhanced proliferation kinetics or subsequent tissue infiltration when compared with naive precursors.

Materials and Methods

Mice and viruses

The HSV-1 used in these studies was the KOS strain. C57BL/6 (B6), B6.SJL-Ptprc^cPep3^b/BoyJ (B6Ly5.1), gBT-1.1, and gBT-1.1 × B6.SJL-Ptprc^cPep3^b/BoyJ (gBT-1 × B6Ly5.1) were from the Department of Microbiology and Immunology, University of Melbourne (Melbourne, Australia). The gBT-1 TCR transgenic mice are specific for the immunodominant HSV glycoprotein B (gB) peptide gB_{498–505} (SSIEFARL) (12).

Generation of gBT-1 memory T cells

LN cells (1×10^5) from gBT-1 or gBT-1 × B6Ly5.1 transgenic mice were transferred into B6Ly5.1 or B6 recipients respectively by i.v. tail vein injection. Recipients were inoculated 1 day later with either 4×10^3 PFU of HSV injected s.c. into each hind footlock or with 1×10^6 PFU of HSV after flank scarification (13, 14). Mice were allowed to convalesce for at least 50 days after infection, by which time the CD8⁺ donor gBT-1 T cell population had contracted to ~1% of CD8⁺ T cells.

*Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia.; and [†]Immunology Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

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² Address correspondence and reprint requests to Dr. Francis R Carbone, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, 3010, Australia. E-mail address: fcarbone@unimelb.edu.au

³ Abbreviations used in this paper: TcM, central memory T cell; DRG, dorsal root ganglion; gB, glycoprotein B; LN, lymph node; TeM, effector memory T cell.

Abs, flow cytometry, and sorting

Anti-CD8 α -allophycocyanin (53-6.7), anti-CD8 α -PerCP (53-6.7), anti-CD8 α -PE (53-6.7), anti-CD45.1-PE (A20), anti-CD45.1-biotin (A20), anti-CD45.2-FITC (104), anti-CD62L-FITC (MEL-14), streptavidin-allophycocyanin, and anti-IFN- γ -PE were obtained from BD Pharmingen. Anti-CD4 (GK1.5) was obtained from National Cell Culture Center (Minneapolis, MN). Anti-class II (M5114) and anti-B220 (RA36B2) were culture supernatants. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). For cell sorting, single-cell suspensions were treated with anti-CD4, anti-MHC class II, and anti-B220 and magnetically depleted using goat anti-rat IgG BioMag beads (Qiagen). The CD8⁺ enriched cell suspension was stained, and the CD8⁺ gBT-I T cells were selected and separated into CD62^{+/−} fractions on a FACSaria (BD Biosciences).

IFN- γ , ⁵¹Cr release, and PFU assays

IFN- γ production was measured by culturing 1.5×10^6 lymphocytes with 200 μ M gB peptide in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich) at 37°C in 6.5% CO₂. For assays with gBT-I T cells (Fig. 1), 30 μ g/ml TAP-I (Calbiochem) was added at the initiation of culture and, at the indicated times, cells were stained with anti-CD8-PerCP, anti-CD45.1-biotin, streptavidin-allophycocyanin, and anti-CD62L-FITC before 1% formaldehyde treatment (20 min at room temperature). For assays on endogenous CTL (Fig. 3), cells were cultured for 4 h before staining with CD8-allophycocyanin and CD45.2-FITC. Cells were then stained overnight with anti-IFN- γ -PE diluted in 0.2% saponin (Sigma-Aldrich) for flow cytometric analysis. To measure cytolytic activity, sorted naive or memory gBT-I \times B6Ly5.1 T cells were tested in a standard 4-h ⁵¹Cr release assay. For PFU assays, the secondary skin infection site (14) was removed, freeze-thawed, and homogenized, and the amount of infectious virus was determined using a standard PFU assay on confluent Vero cell monolayers.

In vitro proliferation assay

Sorted naive or memory gBT-I \times B6Ly5.1 T cells were labeled with CFSE (2.5 μ M) at 37°C for 10 min. Labeled cells (1×10^4) were cultured in a 96-well, round-bottom plate with 1×10^5 B6 splenocytes that had been pulsed with 1 μ g/ml gB peptide (37°C for 45 min). After the described times in culture (37°C with 6.5% CO₂), cells were stained with anti-CD8-allophycocyanin and anti-CD45.1-PE and analyzed by flow cytometry.

In vivo expansion and infiltration assays

Sorted naive or memory gBT-I \times B6Ly5.1 T cells (5×10^4) were transferred into B6 recipients by tail vein injections 1 day before HSV flank infection. The dorsal root ganglia (DRG) innervating the infected flank (thoracic DRG levels T8–T13) were digested in type III collagenase (Worthington Biochemical) for 90 min at 37°C. Splenic or DRG-derived cell suspension was stained using anti-CD8-allophycocyanin and anti-CD45.1-PE, and the number of gBT-I T cells within the DRG was calculated by seeding known numbers of Calibrite beads (BD Biosciences) into DRG samples before flow cytometric analysis.

Cotransfer and endogenous memory T cell transfer experiments

CD8⁺CD62L⁺ transgenic T cells were sorted from naive or memory mice and mixed at a 1:1 ratio, combining naive gBT-I T cells (CD45.1[−]CD45.2⁺) with memory gBT-I \times B6Ly5.1 T cells (CD45.1⁺CD45.2⁺) or naive gBT-I \times B6Ly5.1 T cells (CD45.1[−]CD45.2⁺) with memory gBT-I T cells (CD45.1[−]CD45.2⁺). Cell suspensions (1×10^4 cells of each donor type per recipient) were transferred into B6Ly5.1 (CD45.1⁺CD45.2[−]) mice by tail vein injection 1 day before HSV flank infection. Six days later, brachial LN were removed and stained with anti-CD8-PE, anti-CD45.1-biotin, streptavidin-allophycocyanin, and anti-CD45.2-FITC for flow cytometric analysis. For endogenous memory cell transfer, spleens were removed from B6 mice infected 500 days earlier with HSV, and the frequency of gB-specific CD8⁺ T cells was enumerated by both tetramer analysis (staining with K^b/gB peptide-PE tetramer, CD8-allophycocyanin, and CD62L-FITC) and IFN- γ assays. Total splenocytes containing 1×10^4 gB-specific CD8⁺ memory T cells were transferred into B6Ly5.1 recipients that were infected 3 days later with HSV by flank scarification. Six days after infection the magnitude of gB-specific T cells was enumerated in the brachial LN using an IFN- γ assay and staining cells with CD8-allophycocyanin, CD45.2-FITC, and IFN- γ -PE.

Results

HSV-specific memory cells show enhanced effector response

The ability to rapidly initiate effector functions is an important attribute of memory T cells (9, 10). We therefore examined the

functionality of the memory gBT-I population elicited following HSV infection. The level of ex vivo cytotoxicity by memory T cells was initially examined, generating HSV-specific memory T cells by transferring gBT-I \times B6Ly5.1 T cells into B6 mice 1 day before HSV infection and allowing mice to rest for at least 50 days. Donor gBT-I T cells were then sorted by flow cytometry from memory mice and divided into TeM and TcM subsets on the basis of differential CD62L staining (Fig. 1*a*) and tested in an ex vivo ⁵¹Cr release assay (Fig. 1*b*). The CD62L[−] TeM population showed strong CTL activity (42% lysis), whereas the CD62L⁺ TcM population elicited an intermediate level of killing (17% lysis). In comparison, naive gBT-I T cells sorted directly from gBT-I transgenic animals showed little lysis over background killing.

The kinetics of effector cytokine production was also examined by measuring the speed with which the HSV-specific memory and naive T cells initiated IFN- γ synthesis upon stimulation. Memory T cells were examined from HSV-infected B6 recipients of gBT-I T cells. Differential production of IFN- γ between the memory subsets could be visualized by adding a metalloproteinase inhibitor (TAP-I), which prevents the activation-induced cleavage of CD62L (15). Control naive T cell recipients were generated by transferring 1×10^6 naive gBT-I \times B6Ly5.1 CD8⁺ T cells into B6 mice, thereby approximating the frequency of the gBT-I population within memory mice (~1% of CD8⁺ T cells). As seen in Fig. 1*c*, a large fraction of the naive gBT-I T cells responded rapidly upon peptide stimulation, synthesizing some levels of IFN- γ within hours of stimulation. However, both CD62L[−] (TeM) and CD62L⁺ (TcM) memory gBT-I T cells were clearly superior in their speed and in the amount of IFN- γ produced, with nearly all memory T cells

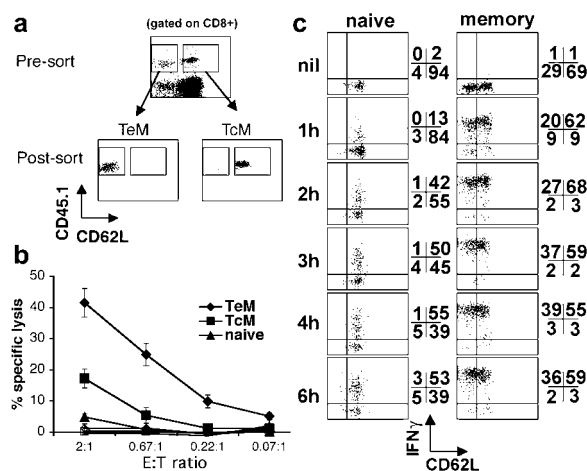


FIGURE 1. Memory T cells have superior effector functions vs naive T cells. *a*, Memory CD8⁺CD45.1⁺ gBT-I T cells were isolated from the LN and spleens of B6 recipient mice infected at least 50 days earlier with HSV and sorted into CD62L[−] (TcM) and CD62L⁺ (TeM) fractions. *b*, Sorted memory T cells or CD62L⁺ naive gBT-I T cells were assayed for SSIEFARL peptide-specific cytolytic activity in a 4-h ⁵¹Cr release assay. Filled symbols represent peptide-pulsed EL4 targets, whereas open symbols are EL4 cells alone. Shown is the average lysis from two independent experiments and the SEM. *c*, Kinetics of IFN- γ synthesis by naive and memory gBT-I T cells. CD8⁺ naive gBT-I \times B6Ly5.1 T cells (1×10^6) were injected into a B6 recipient and, 24 h later, splenocytes from either naive recipient mice or memory mice were stimulated for the indicated times with gB peptide. Plots are gated on CD8⁺CD45.1⁺ gBT-I T cells showing IFN- γ and CD62L expression. A representative plot from three experiments is shown with values indicating the percentage of gBT-I T cells in each quadrant, averaged from six mice.

responding within 1 h of stimulation and subsequently accumulating at least 5-fold higher concentrations of IFN- γ than naive gBT-I T cells at all times tested. Thus, as seen in other systems (9, 10), HSV infection generates memory T cells that have enhanced effector function, both immediate and induced.

TcM show the same proliferation kinetics as naive T cells

As well as showing stronger effector responses, it has been reported that memory cells also exhibit superior proliferation kinetics (8, 9). We examined this issue by comparing the response of the two memory subsets with naive T cells. To do this, CD62L⁺CD8⁺ T cells were isolated directly from naive gBT-I transgenic animals, whereas memory gBT-I T cells were taken from B6 recipients infected at least 50 days prior with HSV and sorted into CD62L⁺ (TcM) or CD62L⁻ (TeM) populations. Naive and memory T cells were labeled by CFSE and cultured with gB peptide-pulsed splenocytes, and at the indicated time points gBT-I division was analyzed by measuring the dilution of CFSE fluorescence (Fig. 2). Neither the subset of memory T cells nor that of the naive gBT-I T cells had divided within 24 h. By 42 h, both the naive and the TcM cells had undergone mostly two rounds of division. In comparison, a considerably smaller proportion of TeM had entered division after 42 h in culture (percentage of dividing: 98% naive, 96% TcM, and 46% TeM), and those cells that had divided lagged behind the TcM and naive populations. The TeM lag was accentuated after 60 h in culture, whereas the naive and TcM T cells again showed identical proliferation profiles. From these results it appears that TcM cells have a lag period between stimulation and division equivalent to that of naive T cells, and once they have entered division they progress through the cell cycle with similar speed.

TcM do not show an accelerated level of T cell expansion in vivo

We next compared the proliferative capabilities of the memory and naive T cell populations in vivo. Purified memory T cell subsets and naive T cells were transferred into B6 recipients, and their expansion was measured following HSV infection (Fig. 3a). TcM are superior to the TeM over this period, consistent with the published findings (5, 16, 17). However, TcM are no better than naive T cells in line with their equivalent rate of in vitro proliferation described above (Fig. 2). To confirm that these two populations have the same expansion potential, we cotransferred equal numbers of naive T cells and TcM into the same animals and examined their relative expansion after challenge. Transfer of this number of T cells is below the level of detection in the absence of infection. Fig. 3b shows that the

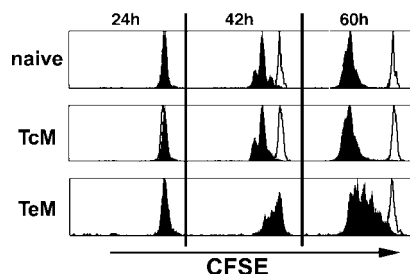


FIGURE 2. Proliferation kinetics of naive and memory T cells. CFSE-labeled CD62L⁺ (TcM) or CD62L⁻ (TeM) memory gBT-I T cells and CD62L⁺ naive gBT-I T cells were cultured with gB peptide-pulsed splenocytes (filled histograms) or nonpulsed splenocytes (open histograms). Histograms are gated on CD8⁺CD45.1⁺ live lymphocytes showing a representative experiment from two to three experiments for each time point.

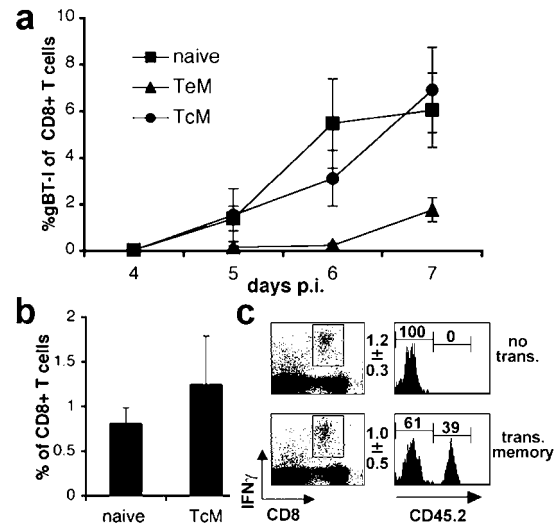


FIGURE 3. Relative in vivo expansion of naive and memory T cells. *a*, Purified CD62L⁺ (TcM) or CD62L⁻ (TeM) memory gBT-I T cells or CD62L⁺ naive gBT-I T cells were transferred into B6 recipients before HSV flank inoculation. At the indicated times, the expansion of the donor T cell population was measured in the spleen of recipient mice on the basis of Ly5.1 and CD8 expression. Shown is the percentage of splenic CD8⁺ T cells that are of donor gBT-I origin, with averages from 4 to 17 mice per time point and the SEM. *b*, Purified CD62L⁺ memory gBT-I, T cells (TcM) were mixed at a 1:1 ratio with naive gBT-I, T cells and transferred into a congenic B6Ly5.1 recipient 1 day before HSV inoculation by flank scarification. Memory and naive gBT-I, T cells were combined, allowing the discrimination between two donor populations and the host on the basis of CD45.1 and CD45.2 protein expression. Six days after inoculation, the expansion of the two donor populations was measured in the draining brachial LN. Shown is the percentage of CD8⁺ T cells that are of naive or TcM donor origin. Averages are from 20 mice, with bars representing SEM. *c*, Splenocytes containing 1×10^4 gB-specific memory CD8⁺ T cells isolated from B6 mice infected 500 days earlier with HSV were transferred into B6Ly5.1 mice. Recipient mice (trans. memory) and nontransfer controls (no trans.) were challenged with HSV, and 6 days later the expansion of host (naive-CD45.2⁻) and donor (memory-CD45.2⁺) gB-specific T cells was enumerated in the brachial LN using IFN- γ assays. Values adjacent to dot plots show the percentage of IFN- γ ⁺ from CD8⁺ T cells with SD. Histograms are gated on CD8⁺IFN- γ ⁺ cells and show the percentage within each marker. A representative plot and average is taken from three to five mice.

combined populations expand to roughly the same level over the first 6 days after infection, each making up $\sim 1\%$ of CD8⁺ T cells in the LN. To test whether nontransgenic naive T cells would also compete efficiently with memory T cells, we transferred a low number (1×10^4) of polyclonal gB-specific memory T cells into B6Ly5.1 mice before infection and measured whether a primary endogenous response would occur in the presence of the donor memory cells. Memory cells were taken from long term infected B6 mice (day 500 postinfection) and were mostly (>90%) CD62L⁺ (data not shown). Following HSV infection, we observed a concurrent and approximately equivalent primary (host-CD45.2⁻) and secondary (donor-CD45.2⁺) gB-specific T cell response in recipient mice (Fig. 3c), confirming that endogenous naive T cells do compete efficiently against and expand in the presence of memory T cells. Combined, these results show that the proliferative equivalence for the TcM and naive T cells seen in the in vitro studies translates to equivalent expansion in vivo.

TcM do not show advanced tissue infiltration kinetics

HSV-specific T cell priming is initiated in skin-draining LN, followed by their release into the circulation and their entry into

infected tissues (14, 18, 19). It has recently been shown that the recall response also requires such LN-based stimulation (20), highlighting the fact that memory T cells must first traverse the draining LN before infiltrating infected tissue. Although the TcM were found to have proliferation and expansion kinetics identical to that of naive T cells, it remains possible that memory T cells progress through the LN faster than naive T cells, infiltrating peripheral tissue at an earlier point. To examine this possibility, we transferred equal numbers of either naive or TcM into B6 recipients 1 day before HSV inoculation via flank scarification and compared the onset of donor T cell infiltration into infected sensory DRG. Fig. 4 shows equivalent rates of DRG infiltration in the TcM and naive T cell recipients. Although the virus reaches the sensory ganglia 36–48 h after infection (14), Ag-specific T cells arrive within the DRG no earlier than day 5 postinfection for both recipient groups. Furthermore, TcM recipients did not show advance in viral clearance from the skin (Fig. 4, *b* and *c*), consistent with a failure of reactivated memory T cells to more rapidly traffic to this site. Thus, in addition to the equivalent proliferation and expansion kinetics, TcM do not show an accelerated point of entry into infected peripheral tissues.

Discussion

The ability of the immune system to more rapidly control subsequent infections has been attributed in part to the accelerated proliferation of the memory T cells (8, 9). However, based on a combination of in vitro and in vivo parameters, we have found that TcM do not show enhanced proliferation or expansion kinetics. We are unable to determine the differences between our results and those showing accelerated proliferative memory T cell responses (8, 9), although our studies are consistent with in vitro studies examining human memory cell subsets (21). Our results may also explain why naive T cells are recruited into secondary immune responses (22, 23) by highlighting that these cells compete efficiently with memory T cells in vivo. Indeed, although there is considerable evidence to suggest that memory T cells generate a superior effector response (9, 10), we show

here that the kinetics of their recall and their infiltration into infected nonlymphoid tissues is no faster than that of their Ag-inexperienced precursors.

Although there is evidence for TcM control of peripheral infections (5), recent reports by Bachman et al. (16, 24) showed that such protection becomes apparent at relatively late stages of infection, whereas TeM could rapidly limit viral replication. These findings are consistent with reports by Woodland and colleagues (25) showing that early clearance of respiratory infections requires a lung resident memory population, arguing that it is the speed with which CTL localize to the site of infection that is critical in dictating disease outcome. However, TcM expansion and peripheral infiltration is largely dependent on dendritic cell activation, an interaction that occurs within LN for most peripheral infections (20, 26). In the absence of accelerated proliferation kinetics, this requirement for lymph node re-stimulation delays the arrival of TcM into infected peripheral tissues. Given this lag in tissue infiltration, we predict that TcM-biased memory can only offer limited localized protection and is unlikely to stop the establishment of new localized infections such as that examined here with HSV.

Disclosures

The authors have no financial conflict of interest.

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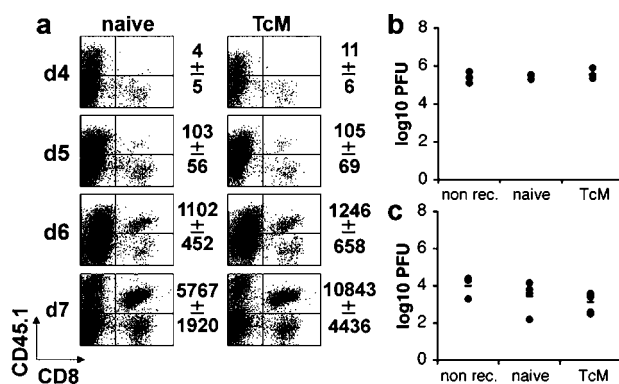


FIGURE 4. Rate of peripheral infiltration by naive and memory T cells. Purified CD62L⁺ memory (TcM) or naive gBT-I T cells were transferred into congenic B6 recipients 1 day before HSV inoculation by flank scarification. *a*, At the indicated times the appearance of the donor gBT-I T cell population was measured in the DRG of recipient mice. Representative plots are shown with values indicating the total number of live CD8⁺CD45.1⁺ gBT-I T cells per DRG sample (T8–T13). Averages were taken from 4 to 17 mice, with error values showing the SEM. *b* and *c*, Viral titers in the secondary site skin of recipient and nontransfer control mice (non rec.) were determined at days 5 (*b*) and 6 (*c*) after HSV infection. Dots represent individual mice, with bars showing the average.

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