Long-lived epithelial immunity by tissue-resident memory T (T_{RM}) cells in the absence of persisting local antigen presentation

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Although circulating memory T cells provide enhanced protection against pathogen challenge, they often fail to do so if infection is localized to peripheral or extralymphoid compartments. In those cases, it is T cells already resident at the site of virus challenge that offer superior immune protection. These tissue-resident memory T (T_{RM}) cells are identified by their expression of the α-chain from the integrin α_E(CD103)β_7, and can exist in disequilibrium with the blood, remaining in the local environment long after peripheral infections subside. In this study, we demonstrate that long-lived intraepithelial CD103$^+$CD8$^+$ T_{RM} cells can be generated in the absence of in situ antigen recognition. Local inflammation in skin and mucosa alone resulted in enhanced recruitment of effector populations and their conversion to the T_{RM} phenotype. The CD8$^+$ T_{RM} cells lodged in these barrier tissues provided long-lived protection against local challenge with herpes simplex virus in skin and vagina challenge models, and were clearly superior to the circulating memory T-cell cohort. The results demonstrate that peripheral T_{RM} cells can be generated and survive in the absence of local antigen presentation and provide a powerful means of achieving immune protection against peripheral infection.

Microbial infection gives rise to immunity against reexposure to the same pathogen. This anamnestic immune memory relies on distinct subsets of adaptive cells such as B cells and CD4$^+$ and CD8$^+$ T cells that orchestrate the accelerated and enhanced immune response seen after secondary antigenic encounter (1). CD8$^+$ memory T cells are specialized in the generation of large quantities of proinflammatory, immunoregulatory, and microbicidal mediators upon activation and are further able to rapidly eliminate infected target cells (2). In line with this, numerous studies have demonstrated their protective role, often in models of systemic bacterial and viral infections (3–7). In these cases, circulating memory T cells in the blood directly encounter and eliminate microbes in lymphoid filter tissues such as lymph nodes and spleen (8).

In contrast to their potency in dealing with systemic infection, memory T cells have often shown surprisingly limited ability to control infections localized to peripheral tissues (3, 4, 7, 9). It has been argued that the circulating memory cells are intrinsically unable to enter peripheral tissues or lose this ability over time without recent stimulation (8). Nonetheless, a large proportion of the body's memory T cells are sequestered in nonlymphoid tissues (10, 11), where they can provide first-line defense against peripheral infection (12). Although some of these are probably recently stimulated effector or effector-memory T (T_{EM}) cells in transit through the peripheral compartments (13, 14), there also exist populations of T cells in disequilibrium with the circulating T-cell pool (15, 16). These sequestered cells are seeded during the early or effector phase of the response and thereafter remain lodged in the periphery without further input from the blood (17, 18), forming a distinct tissue-resident memory (T_{RM}) cell subset (19). Given the limited ability of circulating memory T cells to successfully control peripheral infection in many settings, we sought to determine whether we could embed memory T cells in peripheral sites in the absence of ongoing antigen stimulation, and in so doing, exploit T_{RM} cells to provide effective barrier immune protection.

Results

Circulating CD8$^+$ Memory T Cells Do Not Control Skin Infection with HSV. Certain peripheral infections are efficiently eliminated by effector CD8$^+$ T cells but are less well controlled by their memory counterparts (3, 4, 9, 20). We wanted to address whether skin infection with herpes simplex virus (HSV) exhibited a similar pattern of circulating memory cell resistance. To this end, we vaccinated C57BL/6 mice with a recombinant influenza virus that contains the immunodominant determinant from the HSV glycoprotein B (gB) molecule (flu.gB) to generate cohorts with effector (day 10 after vaccination) or memory (day 30 after vaccination) T-cell populations in the circulation. When challenged by HSV skin infection, mice recently immunized with the recombinant flu.gB showed marked protection, with strongly reduced viral loads in the inoculation site compared with nonimmunized controls (Fig. 1*A*). This protection was also associated with the absence of zosteriform skin lesions that developed in nonimmunized mice as a result of viral replication in sensory neurons, followed by viral recrudescence into a large area of flank skin (21) (Fig. 1*B*). Despite this robust control during the effector phase of the response, CD8$^+$ T cell-mediated immunity was very short-lived, as memory mice inoculated 30 d after immunization did not show control of HSV replication (Fig. 1*A*), and also developed herpetic skin disease (Fig. 1*B*). This lack of protection by circulating memory T cells was also observed even when we inoculated with 10,000-fold less infectious viral particles for challenge infection (Fig. S1*A*).

One possible reason for the disparity in protection between the effector and memory time points could have been a decrease in virus-specific T cells, as their numbers are reduced to approximately 10% to 15% of those present at the day-10 peak of the primary response (Fig. 1*C*), which is consistent with the contraction seen for other antiviral responses (22). We therefore used a prime–boost regimen to increase memory T-cell numbers in the circulation. After transfer of gBT-I T cells, recipient C57BL/6 mice were primed with dendritic cells (DCs) coated with the immunodominant gB peptide and then boosted with the flu.gB recombinant. This regimen promotes rapid expansion of the specific T cells on boosting, which thereafter show only a slow contraction (23)

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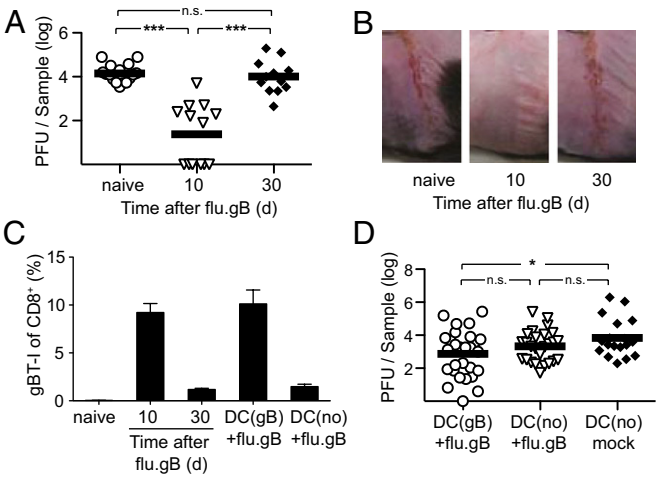

Fig. 1. Circulating memory CD8$^+$ T cells fail to provide efficient protection against HSV-1 skin infection. (*A* and *B*) Mice were immunized by intranasal flu.gB and infected with HSV during the effector (day 10) or memory (day 30) phase of the flu.gB response. A control cohort was not immunized (i.e., naive). (*A*) Viral titres in the skin 3 d after HSV infection and (*B*) representative photographs show lesion formation 6 d following HSV infection. (*C* and *D*) Mice were seeded with naive gBT-I T cells, and T cells were primed with gB peptide-pulsed DCs, followed by flu.gB intranasal booster infection 1 wk (days 7–8, $n = 7$ per group), 2 wk (days 12–19, $n = 9$–12 per group), or 9 to 13 wk (days 63–89, $n = 9$–10 per group) after immunization. (*C*) Frequency of gBT-I memory cells in peripheral blood during the effector and memory phases of flu.gB infection (days 10 and 30, respectively) or following DC priming and subsequent flu.gB infection [DC(gB)+flu.gB] or control immunization [DC(no)+flu.gB], 1 d before HSV challenge infection. Bars represent mean ± SEM. (*D*) Viral titers in the skin 6 d following HSV infection. Symbols represent individual mice; bars represent the mean. Data are pooled from five independent experiments.

such that, at 6 wk after infection, secondary memory gBT-I T-cell numbers were approximately equivalent to those found during the primary effector response (Fig. 1*C*). In addition, consistent with previous reports (23), the majority of these secondary-memory T cells were of the T_{EM} phenotype, expressing low levels of CD62L (Fig. S1*B*). Despite containing elevated numbers of T_{EM} cells in the circulation, mice subjected to the prime–boost treatment showed only minimal protection against HSV-1 skin infection (Fig. 1*D*). Six days after HSV skin challenge, virus loads were comparable between the two cohorts of immunized mice (Flu.gB alone or DC.gB plus Flu.gB), and prime-boosted mice showed a marginal, albeit statistically significant, reduction in viral titers compared with naive controls [i.e., non-pulsed DC (DC-no) plus intranasal mock challenge; Fig. 1*D*]. Of note, this lack of overt protection was reflected by a similar course of viral skin disease with the development of characteristic skin lesions in all groups of mice. Thus, in the case of HSV skin infection, despite the ability of CD8$^+$ effector T cells to mediate control of replicating virus, there was minimal protection by the circulating T cells during the memory phase of the immune response.

Inflammation Enhances T_{RM} Lodgement in Skin in Absence of Local Antigen Stimulation. Given that circulating memory CD8$^+$ T cells proved ineffective in protecting against peripheral HSV infection, we wanted to test whether T_{RM} cells would be superior in this respect. Although primed T cells intrinsically infiltrate a wide variety of extralymphoid tissues in the absence of local infection (10, 24) and thereafter leave behind a residual resident population (17), we reasoned that targeted skin inflammation would enhance memory T-cell lodgement. To demonstrate such inflammation-enhanced T_{RM} formation, we transferred in vitro activated gBT-I T cells into C57BL/6 mice and then treated one flank (left) with the contact-sensitizing agent 2,4-dinitrofluorobenzene (DNFB), while leaving the other (right) as an untreated control. In this approach, DNFB acted as a nonspecific inflammatory stimulus to recruit the virus-specific T cells into the skin in the absence of transient or ongoing local antigen presentation. As shown in Fig. 2 *A* and *B*, memory T cells could be found in far greater numbers within the left flank of mice that had undergone DNFB treatment compared with the nonsensitized right flanks. The lack of T_{RM} cells in the nontreated flanks was not a consequence of a wider absence of memory cells, as they were easily detected in the spleen (Fig. 2*B*). Intravital microscopy showed that the T cells localized to the outermost epithelial layers of the skin, similar to what is seen after natural HSV infection (18) (Fig. S2). Overall, the results demonstrate that nonspecific inflammation alone resulted in the efficient recruitment and lodgement of T_{RM} cells in flank skin, which then persisted for at least 1 y after lodgement in the absence of ongoing antigen stimulation.

CD103 Is Selectively Up-Regulated on CD8$^+$ T Cells in Skin in Absence of Local Antigen Stimulation. CD103, part of the α_E(CD103)β_7 integrin, is expressed by CD8$^+$ T_{RM} cells in different peripheral tissues (19, 25, 26), and has been shown to be important for survival of T cells in these compartments (26, 27). Accordingly, T cells embedded in skin by DNFB treatment expressed high levels of CD103 (Fig. 3*A*), and the proportion of CD103$^+$ CD8$^+$ T cells in DNFB-treated skin increased with time in a similar manner to that seen after HSV skin infection (Fig. S3). The expression of CD103 by the T cells recruited by using a nonspecific inflammatory signal was surprising given that Wakim et al. (26) showed that up-regulation of this molecule in brain appeared to require local antigen recognition. It was possible that epithelial tissues, such as skin and mucosa (described later), uniquely permitted CD103 up-regulation in the absence of local antigen recognition. To show this, we infected left and right flanks of mice with WT HSV or a recombinant virus [K.L8A (28)], respectively, with the latter having a mutated immunodominant gB determinant. HSV enters free nerve endings in the skin and then travels to the distinct sensory ganglia (the dorsal root ganglia) that innervate the regions of skin regions involved in initial infection (29). Thus, in doubly infected mice, WT virus primes cytotoxic T cells specific for the natural gB determinant. These cytotoxic T lymphocytes are recruited into inflamed skin and ganglia infected with either the WT virus (left side) or mutant K.L8A virus (right side). Examination of the recruited T cells showed that,

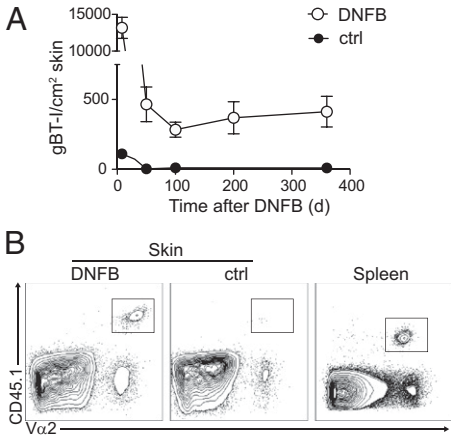

Fig. 2. T-cell persistence in skin sites subject to nonspecific inflammation. Mice were seeded with in vitro activated gBT-I T cells and treated with DNFB on the left flank. (*A*) Number of gBT-I T cells in DNFB-treated or nontreated control (ctrl) skin sites at the indicated times after treatment. Bars represent mean ± SEM ($n = 4$–8 per group). (*B*) Representative flow cytometry plots showing gBT-I T cells in DNFB-treated or control skin sites and in the spleen 360 d after DNFB treatment ($n = 5$–6).

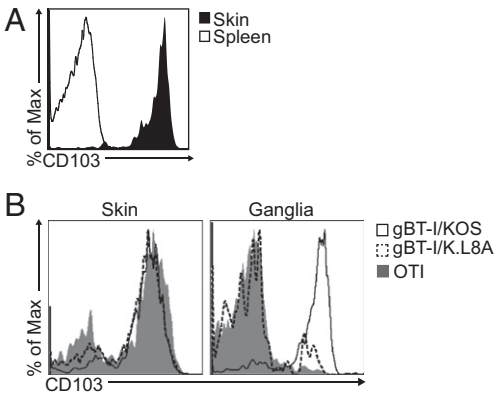

Fig. 3. CD103 is selectively up-regulated on T cells in skin in the absence of local antigen stimulation. (*A*) Representative flow cytometry plot showing CD103 expression by gBT-I T cells in DNFB-treated skin and the spleen 360 d after DNFB treatment. (*B*) Mice were seeded with naive gBT-I T cells and 1×10^7 in vitro activated OT-I T cells and infected with HSV KOS (WT virus) on the left flank and the HSV variant K.L8A on the right flank of the same mouse. Shown is CD103 expression by gBT-I T cells in the skin and ganglia of HSV KOS-infected flanks (gBT-I/KOS; black line), gBT-I T cells in the skin and ganglia of flanks infected with the HSV variant K.L8A (gBT-I/K.L8A; dotted line), and OT-I T cells in the skin and ganglia of HSV KOS-infected flanks (OT-I; gray filled line), at days 28 to 35 after infection. All data are representative of three independent experiments.

whereas antigen recognition was critical to CD103 up-regulation in the sensory ganglia, it was dispensable in the case of those memory cells resident in the skin (Fig. 3*B*). Note that CD103 is up-regulated after T cells enter the ganglia, as the early recruits do not express this marker (Fig. S4). The antigen-independent CD103 up-regulation in skin, but not sensory ganglia, was also seen when activated OT-I T cells of irrelevant (i.e., ovalbumin) specificity were transferred before flank infection (Fig. 3*B*). The CD103 up-regulation on the OT-I cells and their survival in skin also argued that these events were not caused by aberrant cross-reactivity between the gBT-I transgenic cells and DNFB. Overall, the results suggest that skin has an inherent capability of supporting the formation of $CD103^+$ T_{RM} cells.

Skin T_{RM} Cells Provide Local Protection Against Infection in Absence of Ongoing T-Cell Stimulation. We next wanted to determine whether the virus-specific T_{RM} cells lodged by DNFB treatment could control local HSV infection. To this end, we transferred activated T cells to C57BL/6 mice that were treated on the left flank with DNFB and then infected on the left and right flanks with HSV. Fig. 4*A* shows that, although herpetic zosteriform lesions appeared on control flanks deficient in T_{RM} cells, skin containing this population was protected from disease. Consistent with this protection, virus replication was severely suppressed in skin previously treated with DNFB (Fig. 4*B*). Virus control was dependent on the presence of $CD8^+$ memory T cells, as mice that had undergone DNFB treatment but had not received transferred gBT-I cells showed little signs of protection in terms of alleviated disease (Fig. 4*A*) or reduced virus load (Fig. 4*B*). Such protection was also evident for at least 100 d after T_{RM} lodgement (Fig. S5). Protection was antigen-specific, as skin containing ovalbumin-specific memory OT-I $CD8^+$ T cells showed a lack of protection (Fig. 4*B*). As surface replication feeds virus into the sensory ganglia that persists thereafter (30), we reasoned that inhibition of skin infection should also limit the extent of latent infection. The latency set-point was indeed reduced, as shown by using a recombinant virus (KOS.LβA) that expresses the reporter β-gal gene under the latency promoter (31) (Fig. 4*C*) and, separately, by estimation of the average genome copy number persisting after active virus replication had subsided (32) (Fig. 4*D*).

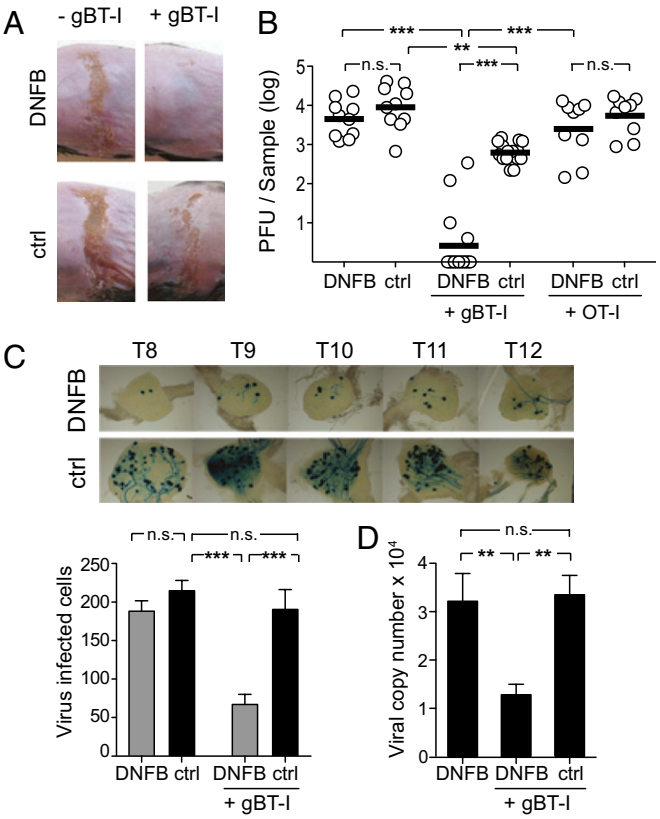

Fig. 4. Virus-specific T cells artificially lodged in the skin are protective against infection with HSV. Mice were seeded with in vitro-activated gBT-I T cells and treated with DNFB on the left flank. A control cohort did not receive gBT-I T cells or received in vitro-activated OT-I T cells. Mice were infected with HSV on both left (DNFB) and right (ctrl) skin flanks 30 to 38 d after DNFB treatment. (*A*) Representative photographs showing lesion formation and (*B*) viral titers in the skin 6 d after HSV infection in the cohorts described. Symbols represent individual mice; bars represent the mean. Data are pooled from three experiments. (*C*) Mice were treated as described and infected on the left and right flanks with HSV KOS.LβA virus at day 35 after DNFB treatment. At day 20 after infection, the innervating ganglia (T8–12) were harvested, fixed, and stained to detect β-gal expression in infected cells. Shown are representative photomicrographs of whole mounted ganglia from a single mouse (*Left*), and pooled data are shown in the bar graph. Bars represent mean ± SEM of viral-infected cells in pooled ganglia/mouse ($n = 5$ mice per group). (*D*) Viral copy number in the innervating ganglia at day 20 after HSV infection. Bars represent mean ± SEM. Data are representative of two independent experiments.

T_{RM} Cells Offer Superior Protection Against Peripheral Infection Compared with Circulating Memory T Cells. Although the preferential protection on DNFB-treated flanks suggested that local T_{RM} cells offered superior immunity compared with the circulating memory populations, one could have argued that the effect actually involved some DNFB-induced perturbation of the skin architecture that then enhanced recruitment of the circulating memory population. It has been shown that T_{RM} precursors are only present in the circulation for a short time after antigen stimulation (17), so we progressively delayed DNFB treatment after transfer of activated T cells. Fig. 5*A* shows that a delay of 15 d resulted in dramatic reduction of T_{RM} lodgement in the skin despite equal numbers of virus-specific memory T cells present in the spleen (Fig. 5*B*). As a consequence, this approach allowed the generation of two cohorts with equivalent numbers of circulating memory T cells, but different densities of T_{RM} cells in the skin. After flank infection by scarification, only mice with T_{RM} cells were protected against skin disease and

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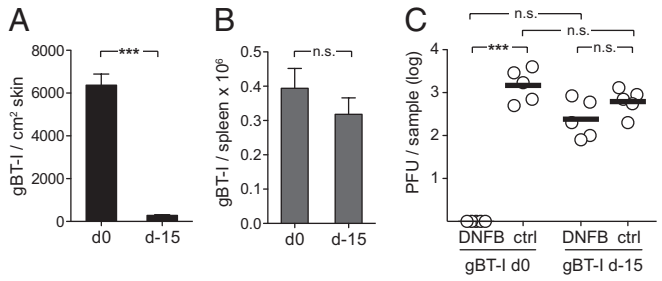

Fig. 5. T_{RM} cells offer superior protection against HSV compared with circulating memory T cells. Mice were seeded with in vitro-activated gBT-I T cells at the time of DNFB treatment or 15 d before treatment. Shown is the number of gBT-I T cells in (*A*) DNFB-treated skin sites and (*B*) the spleen 10 d after treatment. Bars represent mean ± SEM. (*C*) Cohorts of mice were infected with HSV on both left and right flanks 30 d following DNFB treatment. Shown are viral titers in the skin 6 d after HSV infection. Symbols represent individual mice; bars represent the mean. Data are representative of two experiments.

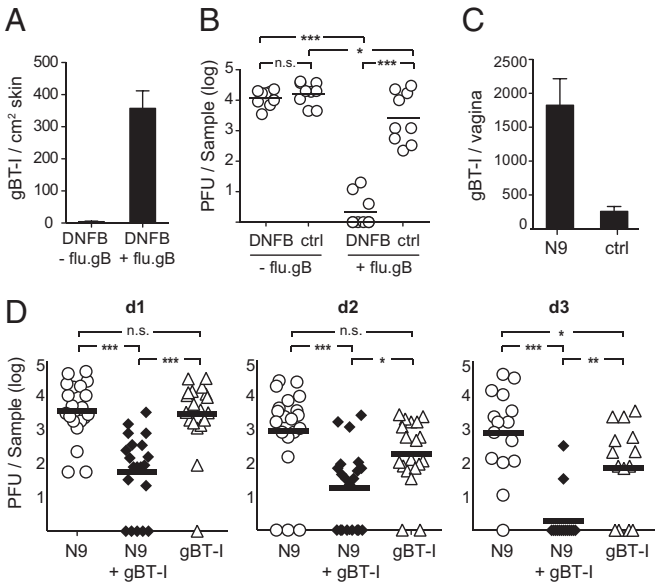

Fig. 6. T_{RM} cells can protect against HSV challenge in different tissues and after different lodgement modalities. (*A* and *B*) Mice were seeded with naive gBT-I T cells and immunized with flu.gB intranasally. A control cohort was nonimmunized. Mice were treated with DNFB on the left flank 10 d after infection. (*A*) Number of gBT-I T cells in DNFB-treated skin in cohorts with and without flu.gB infection 30 d after DNFB treatment. Bars represent mean ± SEM. (*B*) Mice were infected with HSV on left (DNFB) and right (ctrl) skin flanks 30 d after DNFB treatment. Shown are viral titers in the skin at day 6 after infection. Symbols represent individual mice; bars represent the mean. Data are pooled from two experiments. (*C* and *D*) Mice were treated intravaginally with N9 and transferred with in vitro-activated gBT-I T cells at day 2 of treatment. (*C*) Number of gBT-I T cells in the vagina of N9-treated or nontreated mice 30 d following treatment. Bars represent mean ± SEM (n = 3–4 per group) and are representative of three experiments. (*D*) Mice were infected intravaginally with HSV 30 d after N9 treatment (N9 + gBT-I). Control cohorts were N9-treated mice without gBT-I T cells transferred (N9) and mice with gBT-I T-cell transferred that were not N9-treated (gBT-I). Shown are viral titres from vaginal swabs at the indicated days after infection. Symbols represent individual mice; bars represent the mean. Data are pooled from three experiments.

showed reduced levels of local virus replication (Fig. 5*C*). These data formally demonstrate that T_{RM} cells afford superior protection against localized skin infection with HSV compared with their circulating counterparts.

T_{RM} Cells Can Protect Against HSV Challenge in Different Tissues and After Different Lodgement Modalities. Although the transferred activated T cells were useful in demonstrating that T_{RM} cells could protect against a localized infection, we wanted to show that these results could be translated to a variety of different settings. To this end, we repeated the DNFB-mediated lodgement and protection experiments, this time vaccinating mice with the recombinant influenza virus, flu.gB, used in the experiments shown in Fig. 1. Fig. 6*A* shows that the in vivo-primed virus-specific $CD8^+$ memory T cells lodged and survived in skin treated with DNFB but were undetectable in untreated control regions of skin. Areas treated in this fashion showed superior protection after HSV challenge compared with control flank skin in the same animals (Fig. 6*B*). Thus, effective peripheral resident memory could be generated with a recombinant vaccine that otherwise offered poor control of peripheral infection.

Finally, to show wider application, we extended this approach to a different barrier tissue and used a different inflammatory stimulus to achieve lodgement of the T_{RM} cells. For this, we used the surfactant nonoxynol-9 (N9), the active ingredient in a number of commercially available spermicides, to generate nonspecific inflammation within the female reproductive tract (33). Mice received activated gBT-I T cells and were then treated with N9 for 6 d by intravaginal application. Fig. 6*C* shows that this treatment resulted in efficient recruitment and retention of virus-specific T cells in the vagina compared with nontreated controls. These T cells expressed high levels of CD103 (Fig. S6), consistent with their localization to the epithelium (27). Note that vaginal T cells in control mice also had some CD103 expression. However, when combined with the results in Fig. 6*C*, we estimate that N9 increased $CD103^+$ T-cell numbers approximately 10-fold. Critically, the combination of N9 treatment and transfer of activated gBT-I cells gave superior protection against HSV challenge compared with N9 or transferred gBT-I cells alone (Fig. 6*D*). Importantly, this suggests that regional protection by embedded T_{RM} cells can be extended to whole organs, in this case the lower female reproductive tract.

Discussion

Circulating $CD8^+$ memory T cells provide enhanced immune protection in many settings, especially against disseminating pathogens (3–7). However, these same memory cells often prove little better than their naive counterparts if infection is localized to peripheral compartments (3, 4, 7, 9). Access to extralymphoid tissues is critical for T-cell control of peripheral infection, yet memory T cells can exhibit poor peripheral recirculation in the absence of recent stimulation (34, 35). On transition into memory and in the absence of persisting antigen, T cells lose expression of homing molecules and chemokine receptors that drive extravasation and tissue infiltration (17, 18, 36). This loss appears restricted to the $CD8^+$ T cells, resulting in the selective recirculation of only $CD4^+$ T cells through peripheral compartments such as skin and mucosal tissues (18, 36). Consistent with this, lymphatic cannulation studies show a deficit in $CD8^+$ T cells returning from peripheral tissues (37, 38), and $CD8^+$ memory T cells provide inferior peripheral immunity in the absence of renewed or ongoing stimulation (7, 9).

Despite this deficiency in steady-state peripheral immunosurveillance, virus-specific $CD8^+$ memory T cells can be recruited from the blood by localized infection (39). However, there is an inherent lag associated with such entry (4), meaning that T cells already resident in a peripheral compartment have a decided advantage in localized pathogen control (4, 39–41). As a consequence, memory cells that permanently reside in extralymphoid tissues should, in principle, provide superior T cell-based peripheral immunity. Here we show this to be the case. In our hands, circulating memory T cells, even of the effector-memory type, failed to stop progression of HSV infection. In sharp contrast,

permanent lodgement of memory T cells within the epithelium provided strong and long-lived protection, inhibiting HSV skin disease and reducing the extent of latent infection. We propose that the presence of virus-specific T_{RM} cells at high density proximal to the point of first contact is the key to their effectiveness in HSV control, rather than any inherent superiority in their responsiveness.

We found that skin and mucosal epithelia can intrinsically support CD103 up-regulation, an event thought important for optimal T_{RM} formation (26, 27). Consistent with this, T_{RM} lodgement has been demonstrated in mucosa and skin in apparent absence of local infection (17, 19). However, these numbers are less than those achievable when the tissues are subjected to infection or inflammation processes; so much so that, here, they were below our level of detection. Thus, the ability to support efficient T_{RM} lodgement by local inflammation and their long-term persistence in the absence of ongoing antigen-presentation expands the potential use of this population in infection control. It means that T_{RM} cells can be used to protect the epithelial surfaces that form key pathogen entry points in the body without the use of vectors that drive ongoing presentation, as is otherwise necessary for effective peripheral immunity based on circulating memory T cells (9, 34, 42).

As early virus replication in the skin is crucial for the establishment of high-copy HSV latency (30, 43), we were able to use skin-embedded T_{RM} cells to reduce the latency set-point for ongoing HSV infection. Indeed, the rapidity with which virus otherwise moves from skin when surface replication has been initiated (43), to the sensory ganglia that serves as the source of persisting infection (29), is the likely reason why HSV has proven to be such a difficult virus to control by traditional means of vaccination (44). As a consequence, T_{RM} cells embedded in barrier epithelia could potentially be used to control other viruses that are similarly reliant on a wave of surface replication to feed downstream reservoirs of persisting infection. HIV, which initially establishes foci of infection at the point of first contact (45), serves as a prominent example of this type of pathogen. Such translational application, however, will require further preclinical studies to extend this concept to other infections by using homologous systems in which pathogens are studied in their natural host species. At a minimum, our demonstration of the permanent lodgement of an immunologically potent population of $CD8^+$ memory T cells at pathogen entry points shows a level of the flexibility and feasibility that warrants their wider exploration as a means of providing effective barrier protection against infection.

Materials and Methods

Mice. C57BL/6, gBT × B6.CD45.1 (gBT-I.CD45.1), gBT-I.GFP, and OTI × B6.CD45.1 (OTI.CD45.1) were bred in the Department of Microbiology and Immunology at the University of Melbourne. The gBT-I and OT-I mice are $CD8^+$ TCR transgenic mice that recognize the H-$2K^b$-restricted HSV-1 gB epitope of aa 498 to 505 ($gB_{498–505}$) and the ovalbumin-derived epitope of aa 257 to 264 ($OVA_{257–264}$), respectively. Animal experiments were approved by the University of Melbourne Animal Ethics Committee.

Virus Infection and DNFB and N9 Treatment. Viruses used were the KOS strain of HSV, K.L8A (28), and KOS.LβA, as well as WSN/NA/gB (i.e., flu.gB) (46). KOS.LβA was generated by recombining SC16LβA (31) with the KOS strain by transfecting vero cells with a mixture of SC16.LβA and KOS DNA and selecting plaque-purified nonneurovirulent recombinants. Epicutaneous infection by scarification was carried out by using 1×10^6 pfu HSV (KOS, K.L8A, or KOS.LβA) as described (32). Intravaginal infection was performed on progesterone-treated mice (Depo Provera, 2 mg per mouse). For infection, mice were intravaginally swabbed with calcium alginate swabs and inoculated with 1×10^6 pfu HSV. For flu.gB infections, 50 pfu was administered intranasally and mice were bled at peak of response to measure gBT-I T-cell responses. Mice that showed high gBT-I T-cell responses were used for HSV rechallenge experiments (>15% gBT-I of total $CD8^+$ T cells). For DNFB treatment, mice were shaved and depilated before the application of 15 μL of 0.5% DNFB in acetone/oil (4:1) to a 1 cm^2 area of skin. For N9 treatment, Gynol II vaginal contraceptive jelly [3% (wt/vol) N9; Ortho Options] was diluted 3:1 in PBS solution and administered intravaginally in a 60 μL volume by using a blunt-ended pipette, following swabbing the vaginal vault with a moist calcium alginate swab.

Determination of Viral Titer, Copy Number, and β-Gal Detection. The level of infectious virus was determined within homogenized skin or in vaginal fluids by pfu assays as described (32). Vaginal fluids were collected by using calcium alginate swabs. For determination of the number of latently infected ganglia cells, X-gal staining for β-gal expression was performed on whole mounted ganglia as described (43). Average viral copy number was determined by real-time PCR as previously described (43).

In Vitro Activation and Adoptive Transfer of Transgenic $CD8^+$ T Cells. All adoptive transfers of gBT-I and OT-I cells were carried out intravenously with lymph node suspensions (5×10^4) or in vitro-generated effector splenocytes (5×10^6 gBT-I cells, 1×10^7 OT-I cells), which were activated by peptide-pulsed targets as described (19).

Generation and Transfer of Bone Marrow-Derived DCs. Bone marrow-derived DCs were generated according to a standard protocol described previously (5). Briefly, bone marrow cells were cultured for 7 to 10 d in the presence of 20 ng/mL GM-CSF and IL-4 to allow for DC differentiation. DCs were then matured overnight in the presence of 150 ng/mL LPS, and half these cells were further pulsed with $gB_{498–505}$ peptide (1 μg/mL, 45 min) or left untreated. A total of 1 to 2.5×10^5 DCs were transferred intravenously into recipients.

Flow Cytometry and mAbs. Skin tissue was incubated for 90 min at 37 °C in Dispase (2.5 mg/mL) followed by the separation of epidermis and dermis. Epidermal sheets were subsequently incubated for 30 min in trypsin/EDTA (0.25%/0.1%), and the remaining tissue was chopped into fragments and incubated for 30 min in collagenase type 3 (3 mg/mL) and DNase. Ganglia were digested for 90 min in collagenase type 3 (3 mg/mL) as described (19). Vaginal tissues were chopped into fragments and incubated in 1.3 mM EDTA for 30 min at 37 °C, followed by digestion for 90 min in collagenase type 3 (1 mg/mL) as described (18). Skin, vagina, or ganglia cell suspensions were stained with antibodies for flow cytometry. The following antibodies were purchased from BD Pharmingen: anti-CD45.1, anti-Vα2, anti-CD8α, and anti-Vβ8. Anti-CD45.2 and anti-CD103 were purchased from eBioscience. A FACSCanto II system and FlowJo software (TreeStar) were used for analysis.

Intravital Two-Photon Microscopy. Mice were anesthetized and depilated, and the skin was separated from the peritoneum and adhered to a stable raised platform, attached to a imaging platform maintained at 35 °C. Images were acquired by using an upright LSM710 NLO multiphoton microscope as previously described (18).

Statistics. Comparison of data sets was performed by one-way ANOVA followed by Tukey posttest comparison.

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