Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses

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Although tissue-resident memory T cells (T_{RM} cells) are critical in fighting infection, their fate after local pathogen re-encounter is unknown. Here we found that skin T_{RM} cells engaged virus-infected cells, proliferated in situ in response to local antigen encounter and did not migrate out of the epidermis, where they exclusively reside. As a consequence, secondary T_{RM} cells formed from pre-existing T_{RM} cells, as well as from precursors recruited from the circulation. Newly recruited antigen-specific or bystander T_{RM} cells were generated in the skin without displacement of the pre-existing T_{RM} cell pool. Thus, pre-existing skin T_{RM} cell populations are not displaced after subsequent infections, which enables multiple T_{RM} cell specificities to be stably maintained within the tissue.

T_{RM} cells are noncirculating lymphocytes that preferentially localize to sites of pathogen entry. T_{RM} cells are critical mediators of antipathogen immunity and are becoming recognized as key players in cancer, autoimmune and allergic pathologies^{1,2}. These cells exist in most organs and tissues in both humans and mice, and are found at high densities at sites of previous infection or inflammation^{3–5}. T_{RM} cells are generated from precursors that are transiently present in the circulation, and they are phenotypically distinct from circulating memory T cells (T_{CIRC} cells), with a unique transcriptional profile that is acquired during differentiation in the tissue^{6–9}.

Their restricted anatomical localization and unique gene expression program enable T_{RM} cells to mediate local immunosurveillance and rapid protection against reinfection. Local antigen-sensing by T_{RM} cells can also result in the enhanced recruitment of both adaptive and innate circulating cells to the site of T_{RM} cell activation^{10–12}. However, surprisingly little is known regarding the fate of T_{RM} cells after secondary pathogen encounter. It is not known whether T_{RM} cells persist into secondary memory after recall responses, or whether pre-existing T_{RM} cells are displaced by ‘new’ T_{RM} cell populations generated after subsequent infections. It is also unclear whether reactivation of T_{RM} cells can lead to re-entry into the recirculating pool.

Skin T_{RM} cells persist in the epidermis, where they remain at the site of initial lodgment without diffusing through the tissue^{13,14}. Skin T_{RM} cells show minimal evidence of turnover in the steady state¹⁵, depend uniquely on fatty acid metabolism¹⁶ and express molecules associated with inhibitory T cell function. Therefore, it might be expected that T_{RM} cells are terminally differentiated and unable to expand and survive after recall. Here we examined the T_{RM} cell response to secondary viral infection, using experimental approaches that enabled us to compare responses by T_{RM} cells and

T_{CIRC} cells. We used a model of herpes simplex virus type 1 (HSV) infection that infects the skin before entering the sensory ganglia, where it emerges via zosteriform spread as a band of lesions over the dermatome that is innervated by a single nerve¹⁷. T_{RM} cells in the tissue can block peripheral HSV replication indirectly by affecting neuronal infection¹⁸. To determine whether skin-lodged T_{RM} cells can control HSV directly at the skin surface, we reasoned that protection would be confined to regions of skin that contained an area, or ‘patch’, of embedded T_{RM} cells. We show that in response to secondary viral infection, T_{RM} cells mediated such local protection, engaged virus-infected cells, remained constrained to their epidermal niche and proliferated in situ. Skin T_{RM} cells were maintained as a stable population after recall. Furthermore, we found that pre-existing T_{RM} cells were not displaced by newly recruited T_{RM} cell populations, but instead remained as a numerically stable population in the tissue.

Results

Skin T_{RM} cells mediate local immune protection. To understand how T_{RM} cells directly control viral infection at the point of pathogen entry, we activated congenically marked CD45.1⁺CD8⁺ gBT-I transgenic mouse T cells specific for the immunodominant determinant from HSV (gB_{498–505}) in vitro to generate effector T cells (T_{EFF} cells). We transferred the gBT-I T_{EFF} cells intravenously (i.v.) into C57BL/6 mice and used the contact sensitizer dinitrofluorobenzene (DNFB) to recruit them into a patch of lower flank skin in a non-specific manner¹⁹. This approach generates skin T_{RM} cells at frequencies similar to that of the gB_{498–505}-specific endogenous response induced by HSV infection but, importantly, leaves the sensory ganglia unpopulated by T cells. CD45.1⁺ gBT-I T_{RM} cells expressing the surface markers CD69 and CD103 were present in DNFB-treated skin, but not in untreated contralateral skin (Supplementary Fig. 1a,b).

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We inoculated the upper flank skin with HSV >1 cm above the DNFB-treated patch containing gBT-I T_{RM} cells to induce HSV replication and infection of the sensory ganglia (Supplementary Fig. 1c). Six days after HSV infection, a band of herpetic lesions spread across the dermatome from the point of inoculation, but the DNFB-treated skin patch remained clear of disease (Supplementary Fig. 1d). Viral titers 4 and 6 d after infection were reduced in DNFB-treated skin that contained gBT-I T_{RM} cells compared with those in contralateral skin or in skin of mice treated with DNFB but not those given transfer of gBT-I T cells (Fig. 1a). Thus, CD8⁺ T_{RM} cells can control HSV infection in the skin in a discrete and localized manner.

The density of skin T_{RM} cells influences local immune protection. After restimulation, T_{RM} cells may facilitate enhanced recruitment of memory T cells from the circulation¹². We examined whether this was the case after recall of DNFB-lodged skin T_{RM} cells via HSV infection on the DNFB-untreated upper flank. We observed reduced recruitment of CD103⁺CD45.1⁺ gBT-I T cells into DNFB-treated skin containing CD103⁺CD45.1⁺ gBT-I T_{RM} cells 3–6 d after HSV infection compared with that in untreated skin that did not contain gBT-I T_{RM} cells (Fig. 1b). This indicated that skin T_{RM} cells were dominating local virus protection, in agreement with previous findings showing that T_{RM} cells can protect in the absence of T_{CIRC} cells^{20–23}. To assess whether this protective response requires an appropriate density of T_{RM} cells to mediate protection, we transferred increasing numbers of Thy1.1⁺ gBT-I T_{EFF} cells into mice and then subjected them to DNFB-mediated skin lodgment. We allowed the mice to rest for >30 d and then injected them intraperitoneally (i.p.) with Thy1.1-specific antibody to deplete Thy1.1⁺ gBT-I T_{CIRC} cells without affecting skin T_{RM} cells²⁰. Increasing the number of input Thy1.1⁺ gBT-I T cells resulted in a concomitant increase in the number of Thy1.1⁺ gBT-I T_{RM} cells in DNFB-treated skin (Fig. 1c) and induced a dose-dependent reduction in viral load 6 d after HSV infection above the DNFB-treated skin patch (Fig. 1d). Thus, skin T_{RM} cells mediate protection from infection in a manner dependent on their local density in the tissue and independent of circulating T cells.

Antigen-specific T_{RM} cells exhibit dynamic responses to skin infection. To examine how skin T_{RM} cells mediate protection after infection, we transferred in vitro-activated EGFP⁺ gBT-I T cells into mice and then applied DNFB to induce skin lodgment. More than 30 d after EGFP⁺ gBT-I T cell transfer, we imaged the DNFB-treated skin patch by intravital two-photon microscopy (IV-2PM)²⁴. EGFP⁺ gBT-I T cells had a dendritic morphology, showed slow migration, and were evenly distributed across the epidermal layer but absent from the dermis in a manner characteristic of skin T_{RM} cells¹³ (Fig. 2a). To determine whether skin T_{RM} cells can respond to antigen in situ, >30 d after EGFP⁺ gBT-I T cell transfer and DNFB treatment, we delivered antigen directly into the epidermis by mixing HSV-derived gB peptide with aqueous sorbolene cream and applying it topically to depilated skin²⁵. Starting at 3 h after gB peptide application, we observed changes in the dendritic morphology of skin EGFP⁺ gBT-I T_{RM} cells, which became more rounded with shorter dendrites and displayed reduced motility (Fig. 2b,c and Supplementary Video 1). In contrast, application of nonspecific ovalbumin (OVA) peptide did not alter the appearance or motility of skin EGFP⁺ gBT-I T_{RM} cells (Fig. 2b,c and Supplementary Video 1), which indicates that epidermal T_{RM} cells responded to cognate antigen in situ.

We next examined the localization and migration of T_{RM} cells in DNFB-treated lower flank skin 3–4 d after infection of the DNFB-untreated upper flank skin with HSV-mCherry²⁶. After zosteriform spread, we observed small epidermal foci of HSV-mCherry⁺ cells in DNFB-treated skin by 3.5 d after infection, and slow but noticeable spreading of mCherry⁺ HSV over the 4-h imaging

Fig. 1 | Antiviral protection by skin T_{RM} cells correlates with T_{RM} cell density. **a**, Skin HSV titers 4 and 6 d after HSV infection of the upper flank in mice that had previously received in vitro-activated gBT-I T cells and been treated with DNFB on the lower left flank. We analyzed DNFB-treated skin (DNFB (+ T_{RM})) or untreated skin from the right (contralateral) flank, as well as skin from DNFB-treated mice that did not receive gBT-I T cells (DNFB). ** P = 0.005, *** P = 0.0004, **** P < 0.0001, one-way ANOVA with Tukey's multiple comparisons test. **b**, Enumeration of CD103⁺ gBT-I T cells in DNFB-treated or contralateral skin after HSV infection. **c**, The number of CD103⁺ T_{RM} cells in DNFB-treated skin of mice >7 d after treatment with anti-Thy1.1. Mice received 0–20 × 10⁶ Thy1.1⁺ gBT-I T_{EFF} cells, were treated with DNFB, and were depleted of Thy1.1⁺ T_{CIRC} cells >30 d later. *** P = 0.005, Kruskal–Wallis test with Dunn's post-test. **d**, DNFB-treated skin HSV titers in the cohorts described in (c) 6 d after HSV infection. **** P < 0.0001, Kruskal–Wallis test with Dunn's post-test. In all plots, bars represent mean values, and circles represent individual data points. Data are representative of two experiments with n = 4 or 6 (a) or n = 3 or 5 (b) mice per group per experiment, or are pooled from two experiments with n = 5 or 6 (c) or n = 8 or 10 (d) mice per group. PFU, plaque-forming units.

period (Supplementary Fig. 2a). EGFP⁺ gBT-I T cells clustered near HSV-mCherry⁺ infected cells, whereas epidermal cells distal to virus foci remained evenly distributed (Supplementary Fig. 2b). This suggested local accumulation of EGFP⁺ gBT-I T cells in response to the virus, but without the possibility of distinguishing between T_{RM} cells and T cells recruited from the circulation. We also observed highly motile EGFP⁺ gBT-I T cells in the underlying dermis (Fig. 2d and Supplementary Video 2). EGFP⁺ gBT-I T cells that engaged mCherry⁺ HSV-infected cells showed reduced motility and more restricted migration around the virus foci compared with that of EGFP⁺ gBT-I T cells not recruited to foci of HSV-infected cells (Fig. 2e,f). Nonspecific EGFP⁺ OT-I T cells transferred to a second group of DNFB-treated mice as a control did not cluster in this manner after mCherry⁺ HSV challenge (Fig. 2d and Supplementary Video 3). EGFP⁺ OT-I T_{RM} cells also retained their dendritic morphology, motility and epidermal localization after mCherry⁺ HSV challenge (Fig. 2d and Supplementary Video 3), which suggested that the changes in T_{RM} cell behavior that we observed during viral recall depended on recognition of cognate antigen. To confirm this, we cotransferred equal numbers of activated EGFP⁺ gBT-I T cells and lymphocytic choriomeningitis-virus-specific DsRed⁺ P14 T cells (from mice with transgenic expression of the P14 T cell antigen receptor specific for an epitope of lymphocytic choriomeningitis virus glycoprotein) into recipient mice and treated these mice with DNFB to generate skin T_{RM} cells of alternative specificities on the lower flank. Thirty days later, we challenged the mice with CFP⁺ HSV on the upper flank and imaged the DNFB-treated skin by IV-2PM.

Fig. 2 | T_{RM} cells respond in an antigen-specific manner after HSV challenge. **a**, An IV-2PM image of skin EGFP⁺ gBT-I T_{RM} cells >30 d after DNFB treatment. **b**, IV-2PM images of EGFP⁺ skin T_{RM} cells 3 h after gB or OVA peptide application. Scale bars, 20 μ m (**a,b**). **c**, Sphericity and velocity of skin EGFP⁺ gBT-I T_{RM} cells 3 h after gB or OVA peptide application. **** P < 0.0001, two-tailed Mann-Whitney U -test. **d**, Maximum-intensity projection images acquired 92–114 h after HSV-mCherry infection across x , y and z dimensions of skin containing DNFB-lodged EGFP⁺ gBT-I or OT-I T cells. Scale bar, 60 μ m (left) or 75 μ m (right). **e**, Tracks of EGFP⁺ gBT-I T cell migration in the skin 94 h after HSV-mCherry infection in cells that did or did not make contact with HSV-mCherry⁺ cells. **f**, Velocity and track displacement of EGFP⁺ gBT-I T cells that did or did not come into contact with mCherry⁺ virus-infected cells. * P = 0.0137, **** P < 0.0001, two-tailed Mann-Whitney U -test. Data are representative of two experiments with n = 5 or 6 mice per experiment (**a,b**) or four experiments (**d**) or are pooled from two experiments with n = 5 or 6 mice per experiment (**c**) or from 5 mice from one of four representative experiments (**e,f**). SHG, second harmonic generation (dermal collagen). In **c** and **f**, horizontal lines represent the mean, and circles represent individual cells.

EGFP⁺ gBT-I T_{RM} cells clustered and dynamically engaged CFP⁺ HSV-infected cells 3.5 d after infection, whereas DsRed⁺ P14 T cells did not (Supplementary Fig. 2c). Thus, local HSV infection drove dynamic responses by skin T_{RM} cells that were dependent on antigen-specific signals.

Skin T_{RM} cell responses are epidermally constrained during recall. We next asked whether epidermal T_{RM} cells can migrate toward foci of infected keratinocytes as well as into the underlying dermis. To distinguish between gBT-I T_{RM} cells and gBT-I T cells recruited from the circulation, we used mice with DNFB-lodged Thy1.1⁺EGFP⁺ gBT-I T_{RM} cells and, >30 d after DNFB treatment, treated them with anti-Thy1.1 to selectively remove Thy1.1⁺ gBT-I T_{CIRC} cells. Ten days after antibody treatment, we infected the mice with HSV-mCherry above the DNFB-treated skin, and 4 d after that we imaged them by IV-2PM. EGFP⁺ gBT-I T_{RM} cells in the mCherry⁺ virus foci adopted a rounded morphology and were more sessile than nearby EGFP⁺ T_{RM} cells that were not in contact with virus-infected cells (Fig. 3a,b and Supplementary Video 4). Although we observed highly motile dermal EGFP⁺ gBT-I T cells in undepleted mice (Fig. 2d and Supplementary Video 2), we did not observe EGFP⁺ gBT-I T cells in the dermis of anti-Thy1.1-treated mice (Fig. 3c), which indicates that T_{RM} cells responding to challenge within the epidermis did not transit into the dermis.

Next, we directly compared the recall responses of DNFB-lodged T_{RM} cells with those mediated by memory T cells recruited from the circulation. To do this, we generated DNFB-recruited skin T_{RM} cells by using gBT-I T cells expressing the photoconvertible protein Kaede, which changes from green fluorescent

(Kaede-green) to red fluorescent (Kaede-red) upon exposure to violet light²⁷. We infected mice with HSV-CFP above DNFB-treated skin, and 2 d later we exposed the DNFB-treated skin patch to violet light to create Kaede-red⁺ epidermal T_{RM} cells, while unconverted gBT-I T cells in the circulation remained Kaede-green⁺. After the spread of HSV to the lower skin flank, on day 3.5, both Kaede-red⁺ and Kaede-green⁺ gBT-I T cells accumulated around CFP⁺ HSV foci (Fig. 3d). Kaede-red⁺ gBT-I T cells that were distal to virus remained evenly distributed and localized to the epidermis, with a lower average velocity compared with that of the predominantly Kaede-green⁺ gBT-I T cells in the dermis (Fig. 3e). To assess whether T_{RM} cells migrate from skin to lymph nodes during the recall response, we next examined the distribution of photoconverted and unconverted gBT-I T cells in the skin and draining axillary lymph node 4–5 d after infection with HSV. The majority (91% $\pm$ 4.6%) of gBT-I T cells in the skin 4 d after HSV infection were Kaede-red⁺ (Fig. 3f,g), which indicated that these cells were resident in the skin before viral spread. On day 5 after infection, we observed a greater proportion (34% $\pm$ 25%) of Kaede-green⁺ gBT-I T cells in the skin (Fig. 3g), which indicated recruitment of gBT-I T cells from the circulation. Notably, we could not detect Kaede-red⁺ gBT-I T cells in the draining lymph node at either time point after infection (Fig. 3g). Moreover, the Kaede-red⁺ gBT-I T cells retained a CD103⁺CD69⁺ T_{RM} cell phenotype, distinct from that of Kaede-green⁺ gBT-I T cells, which were CD103⁺CD69⁻ (Fig. 3h,i). Together, these data indicate that skin T_{RM} cells that responded to recall virus infection remained constrained to the epidermis and did not migrate into the dermis or local draining lymph nodes.

T_{RM} cells proliferate locally in response to antigenic challenge. Phenotypic flow cytometry analysis of gBT-I T_{RM} cells isolated from previously challenged skin >30 d after HSV infection or DNFB treatment showed increased surface expression of multiple coinhibitory molecules, including PD-1, Tim-3, LAG3, CD101, CD244, CTLA4 and ICOS, compared with that in gBT-I T_{CIRC} cells (Supplementary Fig. 3a,b). Because these inhibitory receptors are involved in the inhibition of T cell proliferation and effector functions²⁸, we tested whether skin T_{RM} cell populations could undergo substantial numerical expansion after challenge. To this end, we generated mice with DNFB-lodged Thy1.1⁺ gBT-I T_{RM} cells and treated them after >30 d either with anti-Thy1.1 to deplete gBT-I T_{CIRC} cells or with PBS as a control. After infecting mice with HSV above DNFB-treated skin, we treated them i.p. with bromodeoxyuridine (BrdU) to assess the proliferative capacity of lodged T_{RM} cells responding to HSV spread (Supplementary Fig. 4a). Seven days after HSV infection, we detected a population of circulating CD103⁺ gBT-I T cells

in the DNFB-treated skin of non-antibody-treated control mice, whereas we detected only CD103⁺ T_{RM} cells in DNFB-treated skin of anti-Thy1.1-treated mice (Fig. 4a). Notably, a substantial proportion (36% ± 5.4%) of CD103⁺ gBT-I T_{RM} cells from the skin of Thy1.1-depleted mice had incorporated BrdU 7 d after infection (Fig. 4b,c). The proliferation marker Ki67 was also upregulated by skin gBT-I T_{RM} cells after infection (Fig. 4b and Supplementary Fig. 4b), which indicates that skin T_{RM} cells proliferated in situ after rechallenge. Ki67 was also upregulated by gBT-I T cells in the spleens of PBS-treated controls after HSV infection (Supplementary Fig. 4c). Divided BrdU⁺ gBT-I T_{RM} cells in DNFB-treated skin of Thy1.1-depleted mice remained CD103⁺ 7 d after HSV infection (Supplementary Fig. 4d), which suggests that T_{RM} cell proliferation expanded or maintained the epidermal T_{RM} cell pool, and that T_{RM} cells did not give rise to CD103[−] T cells or lose CD103 expression after stimulation. In a separate approach, we induced skin gBT-I T_{RM} cells from adoptively transferred Thy1.1⁺ gBT-I naive T cells

Fig. 3 | Skin T_{RM} cells remain localized to the epidermis after virus rechallenge. **a**, An IV-2PM image of EGFP⁺Thy1.1⁺ T_{RM} cells entering HSV-mCherry⁺ virus foci in DNFB-treated skin of T_{CIRC} cell-depleted mice 4 d after HSV infection. Scale bar, 30 μm. **b**, Sphericity and velocity of EGFP⁺Thy1.1⁺ gBT-I T_{RM} cells in cells in contact (or not) with mCherry⁺ cells 4 d after HSV-mCherry infection. ****P < 0.0001, two-tailed Mann-Whitney U-test. **c**, The percentage of EGFP⁺Thy1.1⁺ gBT-I T_{RM} cells localizing to the epidermis of DNFB-treated skin 4 d after HSV-mCherry infection. pi, post-infection. **d**, IV-2PM images of Kaede⁺ gBT-I T_{RM} cells in DNFB-treated skin 3.5 d after HSV-CFP infection above the DNFB patch and 2 d after exposure to violet light. White dashed lines represent foci of virus-infected cells. Scale bars, 100 μm (left) or 20 μm (right). **e**, The velocity of Kaede-green⁺ (unconverted) and Kaede-red⁺ (converted) gBT-I T cells in DNFB-treated skin 3.5 d after HSV-CFP infection and 2 d after exposure to violet light. ****P < 0.0001, two-tailed Mann-Whitney U-test. **f–i**, Results of flow cytometry showing Kaede⁺ gBT-I T cell fluorescence phenotypes in DNFB-treated skin and draining axillary lymph node (dLN) 4–5 d after HSV infection and 2 d after exposure to violet light. **f,g**, The proportion of converted or unconverted Kaede⁺ gBT-I T cells localizing to skin or dLN. **h,i**, Expression of CD69 and CD103 among converted and unconverted Kaede⁺ gBT-I T cells. In **f** and **h**, numbers adjacent to outlines indicate the percentage of cells in the gate. SHG, second harmonic generation. Data shown are representative of 7 mice from two experiments (**a**), three experiments with n = 3 mice per experiment (**d,e**), or two experiments with n = 6 mice per experiment (**f–i**) or are pooled from two experiments with n = 14 movies from 7 mice (**b,c**). (**c,g,i**) Data are shown as the mean ± s.e.m. (**b,e**) Horizontal lines represent the mean, and circles represent individual cells.

Fig. 4 | T_{RM} cells proliferate after antigenic challenge. **a**, CD69 and CD103 expression by skin Thy1.1⁺ gBT-I T_{RM} cells 7 d after infection in anti-Thy1.1-treated and PBS-treated (control) cohorts. Numbers adjacent to outlines indicate the percentage of cells in the gate. **b**, Representative BrdU and Ki67 staining on CD103⁺Thy1.1⁺ gBT-I T_{RM} cells 7 d after HSV challenge. **c**, The proportion of DNFB-lodged skin CD103⁺ gBT-I T_{RM} cells with incorporated BrdU in mice >7 d after HSV infection and in noninfected mice, determined by the Overton method of population comparison. **d**, The proportion of VV-gB-infected lodged skin CD103⁺Thy1.1⁺ gBT-I T_{RM} cells with incorporated BrdU in mice >7 d after HSV infection and in noninfected mice. **e**, Representative BrdU and Ki67 staining on CD103⁺Thy1.1⁺ gBT-I T_{RM} cells 2 d after HSV-gB peptide application. In **b** and **e**, numbers in corners indicate the percentage of cells in each quadrant. **f**, The proportion of skin CD103⁺Thy1.1⁺ gBT-I T_{RM} cells with incorporated BrdU 2 d after topical HSV-gB or OVA peptide application, as determined by the Overton method of population comparison. **c**, **d**, **f** Bars represent the mean, and symbols represent individual mice. Data are representative of two experiments with $n = 3$ or 4 mice per group (**a**, **b**, **e**) or are pooled from two experiments with $n = 6$ or 8 mice per group (**c**), $n = 6$ mice per group (**d**) or $n = 5$ –7 mice per group (**f**).

(T_N cells) by epicutaneous skin infection on the lower flank with recombinant vaccinia virus (VV) expressing the HSV gB epitope (VV-gB). After 30 d we treated the mice with anti-Thy1.1 to deplete Thy1.1⁺ gBT-I T_{CIRC} cells, and >7 d after that we infected them with HSV above the primary VV infection site and subsequently treated them with BrdU (Supplementary Fig. 4a). We observed increased BrdU incorporation by skin Thy1.1⁺ gBT-I T_{RM} cells ($33.3\% \pm 4.8\%$) compared with that in uninfected mice ($7.3\% \pm 2.7\%$), which indicates that virally primed T_{RM} cells also divide after rechallenge (Fig. 4d). We also assessed the uptake of BrdU by gBT-I T_{RM} cells 2 d after transcutaneous application of HSV-gB peptide (Fig. 4e). T_{RM} cells induced by DNFB recruitment or local HSV infection showed similar proliferative responses ($45\% \pm 15\%$ or $42\% \pm 6.6\%$ BrdU⁺, respectively) in response to HSV-gB peptide application (Supplementary Fig. 4e). Finally, we tested whether skin gBT-I T_{RM} cell proliferation was antigen specific by applying HSV-gB peptide or a nonspecific OVA peptide to the skin of DNFB-treated mice that had also been treated with BrdU, and we observed that division of skin Thy1.1⁺ gBT-I T_{RM} cells occurred only in response to cognate antigen (Fig. 4f). Together, these observations indicated that skin T_{RM} cells were not terminally differentiated and divided in situ in response to viral challenge.

However, compared with numbers in uninfected mice, we observed only a marginal increase in the number of Thy1.1⁺ gBT-I T_{RM} cells in DNFB-treated skin between 3 and 14 d after HSV infection of skin above the DNFB patch (Supplementary Fig. 4f). Among the rechallenged gBT-I T_{RM} cell population, we observed an increase in the proportions of annexin V⁺ (from $8.5\% \pm 0.7\%$ to $18\% \pm 2.2\%$) and annexin V⁺PI⁺ cells (from $7\% \pm 0.7\%$ to $11\% \pm 0.9\%$) 7 d after HSV infection compared with that of DNFB-lodged gBT-I T_{RM} cells

at steady state (Supplementary Fig. 4f,g), indicative of increased apoptosis of T_{RM} cells after recall in addition to heightened proliferation. In all, these data indicate that T_{RM} cells can be maintained by local proliferation during secondary infections, without the requirement for replenishment from the circulating T cell pool.

Circulating T cells form new T_{RM} cells without displacing pre-existing T_{RM} cells. Next, we examined whether antigen-specific memory T cells in the circulation are recruited to inflamed skin and develop into T_{RM} cells after secondary challenge. We also asked how these incoming cells might affect populations of pre-existing T_{RM} cells in the skin. To this end, we carried out antibody-mediated enrichment of CD44⁺CD45.1⁺ gBT-I T_{CIRC} cells isolated from spleens of mice that were previously infected with HSV, and transferred them into anti-Thy1.1-treated recipient mice with DNFB-lodged Thy1.1⁺ gBT-I skin T_{RM} cells. Two days after CD45.1⁺ gBT-I T_{CIRC} cell transfer, we infected the recipient mice with HSV above DNFB-treated skin to restimulate both skin T_{RM} cells and adoptively transferred T_{CIRC} cells (Supplementary Fig. 5a). We detected CD45.1⁺ gBT-I T_{CIRC} cells in DNFB-treated skin alongside Thy1.1⁺ gBT-I T_{RM} cells and observed that these cells persisted for at least 30 d after HSV infection (Fig. 5a,b). CD45.1⁺ gBT-I T_{CIRC} cells that infiltrated the skin upregulated both CD69 and CD103 (Fig. 5c), indicative of de novo T_{RM} cell formation from circulating memory T cell precursors. Stable numbers of Thy1.1⁺ gBT-I T_{RM} cells were maintained in the DNFB-treated skin before and after HSV challenge, irrespective of CD45.1⁺ gBT-I T_{CIRC} transfer (Fig. 5b), which indicates that the pre-existing skin T_{RM} cell population was not displaced by subsequent T_{RM} cell generation. Together, these findings indicate that both local maintenance of pre-existing T_{RM} cells and de

Fig. 5 | T_{CIRCUM} cells and bystander T_{EFF} cells generate de novo T_{RM} cells after viral challenge. **a**, The frequency of Thy1.1⁺ gBT-I (DNFB-lodged) and CD45.1⁺ gBT-I (T_{CIRCUM}-cell-derived) T cells in DNFB-treated skin >30 d after HSV infection. **b,c**, The number of Thy1.1⁺ gBT-I and CD45.1⁺ gBT-I T_{RM} cells (**b**) and their CD69 and CD103 expression (**c**) in DNFB-treated skin >30 d after HSV infection. ****P* = 0.0002, two-tailed Mann-Whitney test. n.s., not significant. **d**, The frequency of Thy1.1⁺ P14 (DNFB-lodged) and CD45.1⁺ OT-I (T_{EFF}-cell-derived) T cells in DNFB-treated skin >30 d after HSV infection. **e,f**, The number of Thy1.1⁺ P14 and CD45.1⁺ OT-I T_{RM} cells (**e**) and their CD69 and CD103 expression (**f**) in DNFB-treated skin >30 d after HSV infection. *****P* < 0.0001. **g**, The frequency of Thy1.1⁺ gBT-I (VV-gB, lodged) and CD45.1⁺ OT-I (T_{EFF}-cell-derived) T cells in VV-gB-challenged skin >30 d after HSV infection. **h,i**, The number of Thy1.1⁺ gBT-I and CD45.1⁺ OT-I T_{RM} cells (**i**) and their CD69 and CD103 expression (**h**) in VV-gB-challenged skin >30 d after HSV infection. ****P* = 0.0002, two-tailed Mann-Whitney *U*-test. (**b,c,e,f,h,i**) In bar plots, bars represent the mean, and circles represent individual mice. (**a,d,g**) Numbers adjacent to outlines indicate the percentage of cells in the gate. (**c,f,i**) Numbers in corners indicate the percentage of cells in each quadrant. Data are representative of two experiments with *n* = 3 or 5 (**a**), 4 or 5 (**d**), or 4 (**g**) mice per group, or are pooled from two experiments with *n* = 6 or 10 (**b,c**), 9 (**e,f**), or 8 (**h,i**) mice per group.

Fig. 6 | De novo-generated T_{RM} cells do not displace pre-existing T_{RM} cells in skin. **a**, Mice were seeded with 1×10^6 to 100×10^6 CD45.1⁺ gBT-I T_{EFF} cells and treated with DNFB. Shown are the numbers of gBT-I T cells in the spleen and CD103⁺ gBT-I T_{RM} cells in DNFB-treated skin >30 d after treatment. * $P=0.0262$; n.s., not significant; two-tailed Mann-Whitney test. **b**, The number of ‘old’ (DNFB-lodged) EGFP⁺ OT-I T_{RM} cells (generated from 50×10^6 input EGFP⁺ OT-I T_{EFF} cells) and ‘new’ prime-boosted CD45.1⁺ gBT-I T cells in DNFB-treated skin of flu-immunized mice >30 d after the final immunization, or in non-immunized mice. n.s., not significant; Kruskal-Wallis test with Dunn’s multiple comparisons. **c**, The number of DNFB-lodged CD45.1⁺ gBT-I T_{RM} cells and VV-OVA-primed EGFP⁺ OT-I T_{RM} cells in DNFB-treated skin >25 d after the final contralateral VV-OVA immunization, or in non-immunized mice. n.s., not significant; ** $P=0.0098$; Kruskal-Wallis test with Dunn’s multiple comparisons. In all panels, bars represent the mean, and circles represent individual mice. Data are pooled from two experiments with $n=6$ or 7 mice per group (**a**), $n=18$ –25 mice per group (**b**) or $n=9$ or 10 mice (**c**).

novo differentiation of restimulated T_{CIRC} cells into T_{RM} cells contribute independently to the maintenance or expansion of the T_{RM} cell pool after secondary infection.

We then sought to determine whether CD8⁺ T_{EFF} cells of an unrelated specificity would give rise to T_{RM} cells in HSV-infected skin that contained pre-existing T_{RM} cells. We depleted Thy1.1⁺ P14 T_{CIRC} cells in mice with DNFB-recruited skin-lodged non-HSV-specific Thy1.1⁺ P14 T_{RM} cells by administering anti-Thy1.1, and then infected them with HSV above the DNFB patch >7 d later. Mice received adoptive transfers of in vitro-activated CD45.1⁺ OT-I T_{EFF} cells and were examined after 30 d (Supplementary Fig. 5b). HSV infection resulted in recruitment of non-HSV-specific CD45.1⁺ OT-I T_{EFF} cells into DNFB-treated skin (Fig. 5d,e), and these bystander CD45.1⁺ OT-I T_{EFF} cells had converted to a CD69⁺CD103⁺ T_{RM} cell phenotype (Fig. 5f). The number of pre-existing Thy1.1⁺ P14 T_{RM} cells in the skin was not affected by CD45.1⁺ OT-I T_{EFF} cell transfer, despite de novo OT-I T_{RM} cell formation (Fig. 5e).

Because the presence of local antigen in tissue can influence the formation of T_{RM} cells^{29–31}, we next examined the recall of T_{RM} cells induced by direct skin infection with a virus rather than by DNFB recruitment. We transferred Thy1.1⁺ gBT-I T_N cells into mice, infected the mice on the lower flank skin with VV-gB to generate a population of Thy1.1⁺ gBT-I T_{RM} cells, and subsequently administered anti-Thy1.1 to deplete Thy1.1⁺ gBT-I T_{CIRC} cells. More than 30 d after VV-gB infection and >7 d after T_{CIRC} depletion, we challenged the mice with HSV above the initial VV-gB scarification site

and transferred in vitro-activated CD45.1⁺ nonspecific OT-I T_{EFF} cells into them (Supplementary Fig. 5c). We detected both Thy1.1⁺ gBT-I T_{RM} cells generated in response to VV-gB infection and bystander CD45.1⁺ OT-I T_{RM} cells in skin at the previously infected VV-gB site >30 d after HSV challenge (Fig. 5g–i). The number of Thy1.1⁺ gBT-I T_{RM} cells in VV-gB-challenged skin was not changed after HSV infection or by the transfer of bystander OT-I T_{EFF} cells, despite the induction of substantial OT-I T_{RM} cells after HSV infection (Fig. 5h), which suggests that virally induced T_{RM} cells are also stably maintained after reinfection. Further, to examine recall responses to HSV in the absence of antibody-mediated protection, we infected B-cell-deficient (μ MT) *Ighm*^{−/−} mice given adoptive transfer of Thy1.1⁺ gBT-I T_N cells with HSV on the lower flank and then treated them with anti-Thy1.1 >30 d after infection to induce T_{CIRC} cell depletion. Ten days after T_{CIRC} cell depletion, we rechallenged the mice with HSV on the upper flank and administered in vitro-activated CD45.1⁺ OT-I T_{EFF} cells (Supplementary Fig. 5d). We detected Thy1.1⁺ gBT-I T_{RM} cells generated by the primary HSV infection in post-lesional skin >30 d after HSV rechallenge (Supplementary Fig. 5e). Previously infected skin also contained CD45.1⁺ OT-I T_{EFF} cells that had converted into CD69⁺CD103⁺ T_{RM} cells, without any effect on the numbers of Thy1.1⁺ gBT-I T_{RM} cells (Supplementary Fig. 5e). Combined, these results show that pre-existing and incoming T_{RM} cell populations of identical or different specificities can be maintained and established concomitantly, and do not outcompete one another in the context of local infection.

New T_{RM} cells minimally dislodge pre-existing T_{RM} cells. To determine whether there is a maximal capacity for T_{RM} cell generation in the skin, we transferred increasing numbers of CD45.1⁺ gBT-I T_{EFF} cells into mice and induced T_{RM} cell lodgment in the skin with DNFB. Although T cell input in the range of 1×10^6 to 100×10^6 gBT-I T_{EFF} cells resulted in increasing numbers of splenic gBT-I T cells (Fig. 6a), the number of skin gBT-I T_{RM} cells plateaued after input of 50×10^6 to 100×10^6 gBT-I T cells (Fig. 6a), which indicated that a limited number of T_{RM} cells could be generated in the skin, at least during a single inflammatory response. To test whether subsequent responses could add to the T_{RM} cell pool, we transferred CD45.1⁺ gBT-I T_N cells into mice carrying DNFB-induced skin EGFP⁺ OT-I T_{RM} cells. After >30 d, we subjected the mice to prime-boost immunization with i.v.-transferred gB-pulsed dendritic cells followed by subcutaneous and intranasal infection with recombinant influenza viruses, which results in widespread generation of skin T_{RM} cells³¹. We left a separate cohort of mice untreated (Supplementary Fig. 6a). More than 30 d after influenza virus infection, we detected a pool of newly generated (‘new’) CD45.1⁺ gBT-I T_{RM} cells of a different T cell receptor specificity in DNFB-treated skin that also contained ‘old’ EGFP⁺ OT-I T_{RM} cells (Fig. 6b). The generation of new T_{RM} cells by multiple-route prime-boost immunization allowed us to assess whether dislodgment of pre-existing T_{RM} cells occurred in the absence of perturbation to the skin tissue itself. We detected minimal numerical decay of DNFB-induced ‘old’ skin EGFP⁺ OT-I T_{RM} cells in prime-boost-immunized mice compared with that in control mice that were not subjected to prime-boost immunization (Fig. 6b), which indicated that de novo formation of T_{RM} cells with different specificities occurred without displacement of pre-existing T_{RM} cells in the tissue. Moreover, when DNFB-treated mice carrying ‘old’ CD45.1⁺ gBT-I T_{RM} cells received adoptively transferred EGFP⁺ OT-I T_N cells and ‘new’ T_{RM} cell formation was induced via multiple skin immunizations (epicutaneous infection with VV-OVA contralateral to DNFB-treated skin followed by secondary VV-OVA infection at an unrelated skin site) (Supplementary Fig. 6b), the number of pre-existing CD45.1⁺ gBT-I T_{RM} cells in the skin remained constant compared with that in non-immunized mice (Fig. 6c). Collectively, these findings highlight the exceptional stability of skin T_{RM} cell populations after rechallenge.

Discussion

Here we found that T_{RM} cells respond in an antigen-specific manner, proliferate and survive after local restimulation, and are not dislodged by newly generated T_{RM} cells. This has important implications for understanding how immune memory is maintained in peripheral tissues. We show that T_{RM} -cell-mediated protection is directly linked to the density of T_{RM} cells in the skin. This finding was predicted by mathematical models that showed an inverse correlation between the number of $CD8^+$ T cells in the genital mucosa after HSV reactivation and the severity of disease³². We observed that T_{RM} cells that interacted with virus-infected cells decelerated and were restimulated, whereas T_{RM} cells in nearby uninfected epidermis continued to exhibit random migration. Our imaging did not reveal any obvious attraction of T_{RM} cells to virally infected cells, which suggests that T_{RM} cells need to be in the right place at the right time to protect against infection. Consequently, it may take hours to days for epidermal T_{RM} cells to chance upon infected cells, which highlights the importance of T_{RM} cell density for protection. We found that T_{RM} cells were able to protect against local infection independently of T_{CIRC} cells, although the mechanism through which T_{RM} cells execute protection is unclear. It has been shown that T_{RM} cells can directly kill infected cell targets³³. Although we observed skin T_{RM} cells engaging virally infected cells, we were unable to regularly document killing of infected targets by $CD8^+$ T cells in the skin (data not shown). It is possible that the density of T_{RM} cells inversely regulates the recruitment of T cells from the circulation, as near-sterile immunity induced by local T_{RM} cells might negate the need to recruit large numbers of T_{CIRC} cells and reduce inflammation.

T_{RM} cells proliferated in response to antigen, yet numbers of secondary T_{RM} cells in the absence of T_{CIRC} cells did not markedly increase. Skin T_{RM} cell populations seemed to maintain their stability by balancing cell death with proliferation, as we observed increased annexin V staining of skin T_{RM} cells after viral recall. Restimulated T_{RM} cells did not exit the epidermis even after proliferation, at least within the first 5 d after reinfection. T_{RM} cells survive poorly when removed from tissues³³ (data not shown), which potentially limits their relocation to other tissues. Collectively, our results indicate that T_{RM} cells, at least in the skin, are a durable and autonomous population. It will, however, be important to ascertain whether T_{RM} cells in other organs are similarly tissue restricted after reactivation, particularly in tissues such as the lungs, where local T_{RM} cell maintenance relies on constant replenishment from circulating cells³⁴.

We show that T_{CIRC} cells and T_{EFF} cells are recruited into the skin after pathogen re-encounter; there, some convert into T_{RM} cells, thereby adding to the T_{RM} cell pool. Our findings suggest that T_{RM} cells can be maintained in tissues subject to repeated infections, with unrelated infections unlikely to substantially dislodge T_{RM} cells already in the tissue. Such knowledge is of clinical relevance for vaccine strategies that might induce diverse T_{RM} cells to protect against multiple diseases. Nonetheless, under conditions where T_{RM} cells of certain specificities are undesirable, such as psoriasis in the skin^{35,36}, replacing unwelcome T_{RM} cells with desired T_{RM} cell populations may be a challenge.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at <https://doi.org/10.1038/s41590-017-0027-5>.

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Author contributions

S.L.P., A.Z., J.L.H., S.N.C., J.E.P., B.D., Y.O.A., J.L.G., S.N.M. and L.K.M. performed experiments and analyzed data. T.A.R. and D.C.T. provided reagents. S.L.P., A.Z., T.G., F.R.C., W.R.H., S.N.M. and L.K.M. contributed to experimental design. S.L.P., S.N.M. and L.K.M. prepared the manuscript. S.N.M. and L.K.M. led the research program.

Competing interests

The authors declare no competing financial interests.

Additional information

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Methods

Mice and infections. C57BL/6, gBT-I, gBT-I.xB6.SJL-PtprcaPep3b/BoyJ (gBT-I.CD45.1), gBT-I.Thy1.1, gBT-I.ubiquitin-EGFP (gBT-I.EGFP), gBT-I.EGFP.Thy1.1, gBT-I.Kaede, OT-I.EGFP, P14.DsRed, P14.Thy1.1, OT-I.CD45.1 and μ MT *Igmh*^{-/-} mice were bred in the Department of Microbiology and Immunology, The University of Melbourne. Animal experiments were approved by The University of Melbourne Animal Ethics Committee. gBT-I mice encode a transgene for a T cell receptor that recognizes the HSV-1 glycoprotein B (gB)-derived epitope gB₄₉₈₋₅₀₅³⁷. Epicutaneous infection by scarification was done with 1×10^6 plaque-forming units (PFU) of HSV-1 KOS, HSV-1 pCmC (HSV-mCherry)³⁶ or HSVgDUL47Δ YFP (HSV-CFP)³⁸ as described³⁹, or with recombinant vaccinia viruses expressing gB₄₉₈₋₅₀₅ (VV-gB)⁴⁰ or ovalbumin (VV-OVA)⁴¹. The strains of recombinant influenza virus expressing gB used were WSN-gB and $\times 31$ -gB (H1N1) and were administered subcutaneously or intranasally as described³¹. Viral titers were determined in homogenized skin by PFU assay as described¹⁵.

Adoptive T cell transfer and DNFB treatment. Transgenic gBT-I, OT-I or P14 CD8⁺ T cells were activated in vitro by incubation for 4–5 d with gB₄₉₈₋₅₀₅ (SSIEFARL), OVA (SIINFEKL) or gp33 (KAVYNFATM) peptide-pulsed splenocytes in the presence of recombinant human IL-2 (25 U/mL; PeproTech) as described¹⁵. Except where indicated, 2×10^6 to 10×10^6 effector gBT-I or OT-I T cells were adoptively transferred i.v. Mice were shaved and depilated before the application of 15 μ l of DNFB in acetone and oil (4:1) to a 1.5-cm² area of skin on the day of T cell transfer. Alternatively, 5×10^4 to 1×10^6 naive gBT-I or OT-I T cells were transferred i.v. before infection. For adoptive transfer of CD8⁺ memory T cells, we enriched cells from spleens of HSV-immune mice >30 d after infection by staining cell suspensions with anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (TER-119), and anti-I-A/I-E (M5114) monoclonal antibodies (all generated in-house) and then incubating cells with goat anti-rat IgG-coupled magnetic beads (Qiagen) before removing bead-bound cells.

Flow cytometry. We isolated cells from spleen and lymph nodes by grinding organs through a metal mesh to create single-cell suspensions. T cells were isolated from the skin as described¹⁹. Briefly, skin was incubated in dispase solution (2.5 mg/mL; Roche) at 37 °C for 90 min, and the epidermis was separated from the dermis. The dermis was chopped and incubated in collagenase type 3 (3 mg/mL; Worthington) at 37 °C for 30 min, and the epidermis was placed in trypsin–EDTA (Sigma) and incubated at 37 °C for 30 min. Cells were stained with antibodies, and their expression of phenotypic markers was determined with a BD Fortessa (BD Biosciences) or BD FACSCanto II (BD Biosciences) and analyzed with FlowJo (Treestar). We used the following antibodies: anti-mouse CD45.1 (A20), CD8 α (53-6.7), V α 2 (B20.1), CD69 (H1.2F3), CD116/CD32 (2.4G2), and anti-BrdU (B24) from BD Biosciences; anti-mouse CD45.1 (A20), CD3e (eBio500A2), CD8 α (53-6.7), CD69 (H1.2F3), CD103 (2E7), Ki67 (20Raj1), CD101 (Moushi101), CD244 (eBio244F4), CTLA4 (UC10-4B9), LAG3 (e9B7W), PD-1 (J43) and TIM-3 (8B.2C12) from eBioscience; and anti-mouse CD45.2 (104), CD103 (2E7), CD8 α (53-6.7), CD103 (2E7), Thy1.1 (OX-7) and ICOS (C398.4A) from Biolegend. Live cells were discriminated with a fixable LIVE/DEAD stain (Life Technologies or Biolegend) or by propidium iodide staining. Annexin V staining was carried out with a BD annexin V FITC staining kit according to the manufacturer's instructions. We enumerated cells by adding a known number of calibration particles (BD Biosciences) to each sample before analysis.

Intravital two-photon microscopy. Mice were anesthetized with isoflurane (Cenvet; 2.5% for induction, 1–1.5% for maintenance, vaporized at an 80:20 mixture of O₂ and air) and then shaved on the left flank and depilated for flank skin imaging as described⁴². Briefly, we made two incisions (~15 mm apart) longitudinally along the left flank, cutting through the dermis, and separated the peritoneum by cutting away the connective tissues underneath the skin. A 18-mm-wide $\times$ 1-mm-thick stainless steel platform was inserted under the exposed dermis, which was glued to the platform with Vetbond tissue adhesive (3M). The edges of the skin were lined with vacuum grease (Dow Corning), upon which a glass coverslip was placed. Imaging was done with an upright LSM710 NLO multiphoton microscope (Carl Zeiss) with a $20 \times /1.0$ numerical aperture (NA) water-immersion objective enclosed in an environmental chamber that was maintained at 35 °C with heated air. Fluorescence excitation was provided by a Chameleon Vision II Ti:sapphire laser (Coherent) with dispersion correction and fluorescence emission detected by external non-descanned photomultiplier tubes. We excited EGFP and second-harmonic generation at 920 nm, mCherry at 960–980 nm, Kaede-green at 800 nm, and Kaede-red at 900 nm. For four-dimensional datasets, three-dimensional stacks were captured every 1 min for at least 30 min. Raw imaging data were then processed with Imaris 8 (Bitplane), and movies were generated in Imaris and composed in After Effects (Adobe).

Intravital imaging analysis. To distinguish gBT-I T cells on the basis of contact with virus-infected keratinocytes, we generated tracks with the Imaris Spot function and rendered mCherry⁺ virus-infected cells as a three-dimensional surface. Tracks were computed with a custom-modified version of the script “Surface to Spot Distance” through an XT/Matlab interface (Mathworks). To segregate T cells in contact with virus, we used the center of each gBT-I T cell

and defined contacting cells as cells remaining within 10 μ m of rendered virus (mCherry⁺ or CFP⁺) for at least 5 min. Statistical values for mean track speed and mean track displacement length were extracted from Imaris software. Statistical significance was determined by Mann–Whitney *U*-test.

Depletion of circulating Thy1.1⁺ gBT-I T cells. More than 20 d after transfer of activated Thy1.1⁺ gBT-I cells and DNFB treatment, mice were injected i.p. 1–3 times with 0.5–4 μ g anti-mouse/rat Thy1.1 mAb (α -Thy1.1; clone HIS57; eBioscience) in PBS. Control mice received PBS alone.

In vivo photoconversion of Kaede⁺ gBT-I T_{RM} cells. Mice containing Kaede⁺ gBT-I T_{RM} cells were anesthetized, shaved and depilated, and infected with HSV-mCherry. At 2 d post-infection, we used a 410-nm spot lamp (Dymax Bluewave LED; UV Pacific) to expose mice to 100 mJ/cm² violet light for 6 min. Light emitted through an 8-mm-diameter fiber optic guide was mounted at a distance of 4.17 cm directly above the DNFB patch, and areas around the patch were covered with a double layer of aluminum foil to prevent distal photoconversion. Mice were then imaged by IV-2PM from 94 h post-infection.

Transcutaneous delivery of gB and OVA peptide. We added 50 μ g of MHC-I-restricted HSV gB₄₉₈₋₅₀₅ (SSIEFARL) or OVA (SIINFEKL) peptides (Genscript) to 100 mg of sorbolene cream and mixed it thoroughly by successive vortexing and short-pulse centrifugation. Mice containing DNFB-recruited lodged T_{RM} cells were anesthetized, shaved and depilated, and the peptide–sorbolene mixture was applied directly atop the DNFB patch. The peptide–cream mixture was contained above the skin region by a bandage consisting of an adhesive film (OpSite Flexigrid; Smith & Nephew) and surgical tape (Micropore tape; 3M). For intravital imaging experiments, the mixture was allowed to absorb through the skin for 3 h before removal of the bandage. For flow cytometry experiments, the peptide–sorbolene mixture was applied once per day for 2 d, and mice were banded for 24 h after each application.

In vivo bromodeoxyuridine treatment. Mice were injected i.p. with 2 mg of BrdU per day, with treatment commencing on the day of transcutaneous peptide application or 0–2 d after infection. BrdU incorporation was measured with a BrdU flow kit (BD Biosciences) or FoxP3 staining kit (eBioscience), and where indicated the proportion of BrdU⁺ cells was determined by the Overton method of population comparison⁴² (FlowJo; Treestar), with skin gBT-I T cells from an untreated mouse used as the control population.

Generation and transfer of bone-marrow-derived dendritic cells. We differentiated dendritic cells by culturing C57BL/6 bone marrow cells in the presence of 20 ng/mL GM-CSF and IL-4 for 7 d. Dendritic cells were matured overnight in the presence of 150 ng/mL LPS, then pulsed with 1 μ g/mL gB₄₉₈₋₅₀₅ (SSIEFARL) peptide for 45 min before transfer of 2.5×10^5 cells i.v. to recipients.

Statistics and reproducibility. Two-tailed Mann–Whitney *U*-tests, one-way ANOVA with Tukey's multiple comparisons test or Kruskal–Wallis tests with Dunn's multiple comparison test were used where indicated. All proportional numerical values provided in the text are written as the mean $\pm$ s.e.m. All statistical analyses were done in Prism 7.0 (Graphpad). All experiments were performed at least two times, with similar results obtained each time.

Life Sciences Reporting Summary. Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

Data availability. Publicly available source data were not used in this study. Data supporting the findings of this study are available from the corresponding authors upon request.

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► Experimental design

1. Sample size

Describe how sample size was determined.

No statistical test was used to pre-determine sample size.

2. Data exclusions

Describe any data exclusions.

For Fig. 2b, The anterior prostate was excluded from the analysis due to cystic lesions after 12-week time point.

3. Replication

Describe whether the experimental findings were reliably reproduced.

The number of repeats for experiments was described in corresponding figure legends. Only successfully replicated/reproduced experiments were reported.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Genotypes and ages of mice are known to investigators. No pre-established selection criteria for mice were used. All mice with desired genotypes at appropriate ages in corresponding cages were all used and randomized to either the control or experimental group.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

The investigators were not blinded to allocation during experiments and outcome assessment.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☐ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☐ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☐ ☒ The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

GraphPad and R were used to perform general statistical analysis.

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* [guidance for providing algorithms and software for publication](#) may be useful for any submission.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

A detailed description of antibodies used, including vendors and catalogue numbers, was provide in Methods, under "Plasmids, reagents and antibodies" section. Antibodies were validated by gene silencing and/or overexpression, and by other published studies.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

All cell lines were obtained from ATCC.

b. Describe the method of cell line authentication used.

All cell lines were purchased directly from ATCC

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines were checked for mycoplasma by MycoAlert Mycoplasma Detection Kit (Lonza).

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

None of cell lines belongs to misidentified cell lines.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Methods/Murine models

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Methods/TMA analysis and Supplementary Table 1