

Antigen-dependent competition shapes the local repertoire of tissue-resident memory CD8⁺ T cells

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Tissue-resident memory CD8⁺ T cells (T_{RM}) constitute a major component of the immune-surveillance system in nonlymphoid organs. Local, noncognate factors are both necessary and sufficient to support the programming of T_{RM} cell fate in tissue-infiltrating T cells. Recent evidence suggests that TCR signals received in infected nonlymphoid tissues additionally contribute to T_{RM} cell formation. Here, we asked how antigen-dependent pathways influence the generation of skin-resident memory T cells that arise from a polyclonal repertoire of cells induced by infection with an antigenically complex virus and recombinant vaccine vector. We found that CD8⁺ T cells of different specificities underwent antigen-dependent competition in the infected tissue, which shaped the composition of the local pool of T_{RM} cells. This local cross-competition was active for T cells recognizing antigens that are coexpressed by infected cells. In contrast, T_{RM} cell development remained largely undisturbed by the presence of potential competitors when antigens expressed in the same tissue were segregated through infection with antigenically distinct viral quasispecies. Functionally, local cross-competition might serve as a gatekeeping mechanism to regulate access to the resident memory niche and to fine-tune the local repertoire of antiviral T_{RM} cells.

INTRODUCTION

Tissue-resident memory CD8⁺ T cells (T_{RM}) reside within epithelial barriers of nonlymphoid tissues and provide accelerated protection from local reinfection (Schenkel and Masopust, 2014; Park and Kupper, 2015; Mueller and Mackay, 2016). T_{RM} cells from various tissues share a common core transcriptional program that distinguishes them from their central memory T cell (T_{CM}) and effector memory T cell (T_{EM}) counterparts (Mackay et al., 2013, 2015b, 2016). The coordinated down-regulation of transcription factors T-bet, Eomes, and KLF2 (a positive regulator of tissue egress receptors sphingosine 1-phosphate receptor 1 [S1P1] and CCR7), and the up-regulation of the Blimp1 homologue Hobit, determine T_{RM} cell fate and long-term retention and survival in nonlymphoid tissues (Mackay et al., 2013, 2015b, 2016; Skon et al., 2013). Interestingly, T_{RM} cells in the skin were found to share clonal origin with T_{CM} cells, indicating that these cells may develop from common naive T cell precursors

(Gaide et al., 2015). KLRG1^{lo} effector cells initially activated in secondary lymphoid organs can seed nonlymphoid tissues during the early stages of immune responses and develop locally into T_{RM} cells (Mackay et al., 2013). Recent studies have established that various barrier tissues, including the gut, the skin, and the female reproductive tract, can autonomously provide the environmental conditions that enable T_{RM} cell development independently of local cognate signals (Casey et al., 2012; Mackay et al., 2012; Shin and Iwasaki, 2012; Skon et al., 2013). Together, these studies raised the possibility that precursor cells primed in lymphoid organs are recruited to nonlymphoid tissues, where local cues guide T_{RM} cell development irrespective of additional cognate signals and, hence, independent of additional selection for specificity. Challenging this view, local cognate signals have been proposed to facilitate the induction of T_{RM} cells in the CNS, the sensory ganglia, the lung (Wakim et al., 2010; Lee et al., 2011; Mackay et al., 2012), and potentially the skin (Gebhardt et al., 2009; Mackay et al., 2015a). Khan et al. (2016) have di-

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Abbreviations used: MVA, modified VACV Ankara; pi, post infection; S1P1, sphingosine 1-phosphate receptor 1; T_{RM}, tissue-resident memory T cell; VACV, vaccinia virus.

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rectly tested this possibility and found that local expression of antigen dramatically amplified the generation of T_{RM} cells in vaccinia virus (VACV)-infected skin. VACV encodes a large array of different antigenic CD8⁺ T cell epitopes and, consequently, VACV infection results in the induction of a broad CD8⁺ T cell response directed against many different viral antigens (Moutaftsi et al., 2006; Kastenmuller et al., 2007). The notion that local antigen expression contributes to T_{RM} cell generation (Khan et al., 2016) thus raises the question how local antigen-dependent signals influence the formation of tissue-resident memory cells from a pool of polyclonal endogenous precursor cells during infection with antigenically complex pathogens.

To directly address this question, we have simultaneously tracked the establishment of T_{RM} cells specific for different viral epitopes in skin infected with the nonreplicating modified vaccinia Ankara (MVA) strain of VACV. MVA is a recombinant vaccine vector being evaluated in clinical studies against infectious diseases and tumors, and a licensed first-line vaccine against smallpox (Drexler et al., 2004). Importantly, MVA has been considered for vaccination approaches at mucosal barrier tissues (Neutra and Kozlowski, 2006; Kastenmuller et al., 2009; Manrique et al., 2009), where vaccine-induced T_{RM} cells may provide superior protection against invading pathogens. Expanding previous observations based on replicating virus (Khan et al., 2016), our work suggests that transient expression of antigens by a nonreplicating vaccine vector is sufficient to dramatically improve T_{RM} cell formation at barrier sites. Importantly, we observed that the presence of multiple antigens in virus-infected skin triggered competition between T_{RM} cell precursors of different specificity, which profoundly shaped the clonal composition of the resulting antiviral T_{RM} cell pool. From a comparably broad repertoire of antiviral T cells initially primed in secondary lymphoid organs, those cells able to receive cognate stimulation at the site of infection were preferentially selected to establish the local pool of T_{RM} cells. Importantly, T cell cross-competition did not restrain the recruitment of effector cells to the site of infection during the acute phase of infection, but specifically regulated the generation of T_{RM} cells. Our data suggest that cross-competition is orchestrated in the defined context of antigen-presenting cells in the tissue, as competition strongly affected the T_{RM} cell generation of T cells recognizing antigens coexpressed by infected cells. In contrast, T_{RM} cell development remained undisturbed by the presence of potential competitors when antigens expressed in the same tissue environment were segregated through infection with antigenically distinct viruses, mimicking the presence of viral quasispecies during infection. Our work identifies local antigen-dependent cross-competition in nonlymphoid tissues as a mechanism to optimally stock a peripheral cellular niche with the most useful T cell receptor specificities to ensure a highly efficient barrier to local reinfection.

RESULTS

Localized epidermal MVA infection promotes the formation of T_{RM} cells

To study the formation of T_{RM} cells after acute viral infection of the skin, we adopted a model of intraepidermal vaccine delivery (Bins et al., 2005) to locally infect with nonreplicating vaccinia virus MVA. Intraepidermal delivery of MVA into the dorsal ear pinnae of mice generates a highly localized infection that remains largely confined to the epidermal layer of the skin, as determined by histological analysis of viral antigen expression in ear skin 16 h post infection (pi) with *lacZ*-encoding MVA (Fig. 1 A). As a result of the lack of replication of MVA, viral gene expression in the skin was transient, with peak expression around 12 h pi and a strong decline >24 h pi, as determined with luciferase expressing MVA (unpublished data). Intraepidermally infected mice mounted a robust systemic antiviral CD8⁺ T cell response, which was dominated by CD8⁺ T cells recognizing the H2-K^b-restricted viral epitope B8R₂₀. B8R-specific CD8⁺ T cells were readily detectable in the spleen 7 d pi (the peak of effector cell expansion) and were maintained in immunized animals as a small but stable population for at least 70 d pi (Fig. 1 B). During the acute phase of the response (7 d pi), B8R-specific effector CD8⁺ T cells also markedly accumulated in MVA-infected ears, as indicated by a strong increase in both the frequency and absolute number of B8R-specific CD8⁺ T cells in infected versus noninfected contralateral ears (Fig. 1 C, D). Importantly, significant numbers of B8R-specific CD8⁺ T cells remained detectable in previously MVA-infected ears even long after clearance of the localized infection (70 d pi; Fig. 1, C and D). These cells exhibited increased expression of CD69 and CD103 (Fig. 1 E) and primarily localized to the epidermal compartment (Fig. 1 F), consistent with previous reports of T_{RM} cell formation upon cutaneous infection or immunization (Gebhardt et al., 2009, 2011; Ariotti et al., 2012; Jiang et al., 2012; Khan et al., 2016). Therefore, epidermal infection with MVA resulted in the generation and accumulation of bona fide T_{RM} cells at the site of prior skin infection.

Local cognate antigen is dispensable for effector CD8⁺ T cell recruitment to infected skin

The aforementioned results suggested that virus-specific skin T_{RM} cells were predominantly generated at the site of prior local MVA infection. To address the extent to which noncognate versus cognate antigen-dependent signals contributed to this localized T_{RM} cell formation in previously infected skin, we devised an experimental strategy in which activated circulating T cells with a defined specificity (OVA) could be recruited into skin epidermally inoculated with PBS (mock), MVA-WT (noncognate), or MVA-OVA (cognate). To generate a pool of activated and circulating OVA-specific effector T cells, congenically marked CD45.1⁺ naive TCR-transgenic CD8⁺ OT-I cells were adoptively transferred into CD45.2⁺ WT recipient mice and primed in vivo with MVA-OVA (i.v.). Immedi-

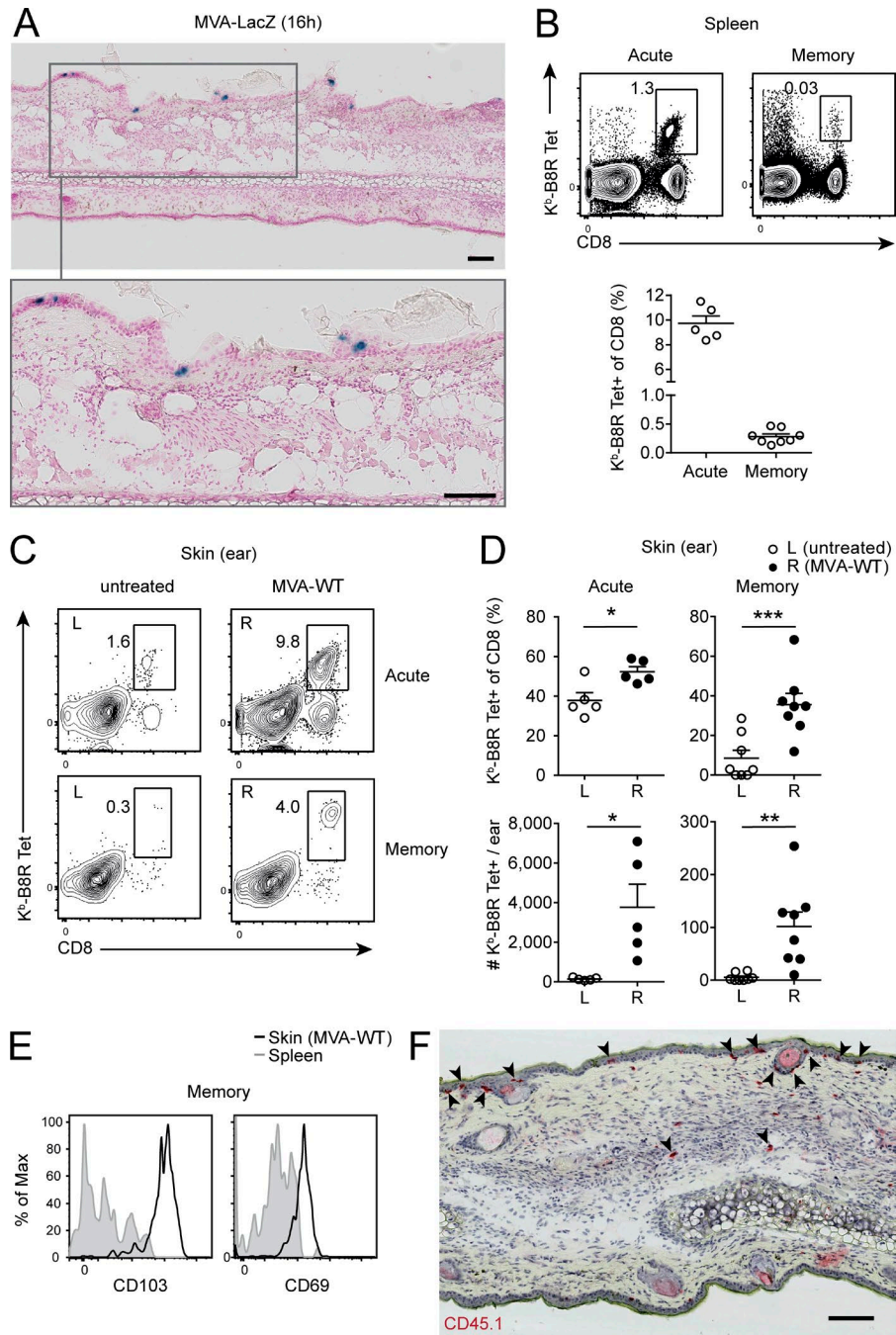


Figure 1. Localized MVA skin infection induces bona fide T_{RM} cells. (A) Localization of virus-infected cells in ear skin 16 h after intraepidermal infection with MVA-LacZ (10⁷ IU), as visualized by X-Gal staining. Bar, 100 μm. Shown is a representative staining of at least three independent experiments. (B) Representative plots (top) and percentage (bottom) of B8R-specific CD8⁺ T cells, in the spleen 7 d (Acute) or >70 d (Memory) pi of ear skin with MVA-WT, as determined by tetramer staining. (C) Representative plots showing the B8R-specific CD8⁺ T cell fraction among CD45⁺ cells in untreated left (L) or MVA-WT-infected right (R) ears during the acute or memory phase. (D) Frequency (top) and absolute number (bottom) of B8R-specific CD8⁺ T cells in the indicated ears during the acute and memory phase. (E) CD103 and CD69 expression on B8R-specific memory CD8⁺ T cells isolated >70 d pi from previously MVA-WT-infected ears (black histogram) or the spleen (gray shaded histogram). (F) Distribution of adoptively transferred OVA-specific CD45.1⁺ OT-I cells (arrows) in ear skin 50 d after local infection with MVA-OVA, as detected by immunohistochemical staining of the CD45.1 (arrowheads). Bar, 100 μm. Shown is a representative staining of at least three independent experiments. Data are derived from one experiment (Acute, *n* = 5; representative of two independent experiments) or were pooled from three independent experiments (Memory, day 70–90, *n* = 8). Bars show mean + SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (paired Student's *t* test).

ately thereafter, these mice were split into three groups that received different epidermal inoculations on their left and right ears (Fig. 2 A). Importantly, the priming of a systemic OT-I response and local skin inoculations occurred at the same time (with a maximum delay <1 h) to synchronize the kinetics of primary effector cell expansion and localized skin infection. The first group was mock infected (PBS mock) on one ear pinna only to study the impact of non-specific inflammation induced through the intraepidermal delivery procedure alone. The second group was mock in-

ected on the left ear pinna and infected with MVA-WT on the right ear pinna to directly compare the impact of a nonspecific inflammation in the context of epidermal injury versus a local noncognate virus infection. To assess the relative contribution of in situ cognate antigen expression to the local CD8⁺ T cell response, a third group was locally infected with MVA-WT versus MVA-OVA on the left versus right ear pinnae, respectively. We observed only minimal migration of systemically primed OT-I cells into untreated ear skin on 7 d pi, suggesting that circulating antiviral ef-

factor CD8⁺ T cells did not readily enter the skin in the absence of local tissue injury or infection (Fig. 2 B, group 1). In contrast, we found a sizeable population of OT-I cells in previously PBS-treated contralateral ears, indicating that the mechanical skin irritation was sufficient to promote a local accumulation of effector cells (Fig. 2 B, group 1). Interestingly, the accumulation of OT-I cells was increased in MVA-WT-infected ears, which harbored around 10 times more OT-I cells than PBS controls (Fig. 2 B; group 2). Consistent with recent reports (Hickman et al., 2015; Khan et al., 2016), expression of cognate antigen in infected skin seemed dispensable for entry of OT-I cells into the infected tissue, because the numbers of OT-I cells were comparable between both MVA-WT- and MVA-OVA-infected ears on 7 d pi (Fig. 2 B, group 3). Together, these data confirm that the entry of circulating antiviral effector CD8⁺ T cells into the infected skin is guided by inflammatory signals regardless of the expression of their cognate antigen *in situ* (Hickman et al., 2015; Khan et al., 2016).

Local antigen strongly amplifies the generation of T_{RM} cells in virus-infected skin

Next, we analyzed the formation of OT-I T_{RM} cells in ears treated differentially as described above (Fig. 2 A). OT-I T_{RM} cells were nearly absent from untreated ears 40 d pi (Fig. 2 C, group 1), suggesting that systemic viral infection alone did not efficiently promote the lodgment of T_{RM} cells to healthy skin. Although mock infection triggered the recruitment of effector OT-I cells during the acute phase of infection, we failed to detect a significant enrichment in OT-I T_{RM} cells in mock-infected ears when compared with untreated contralateral ears during the memory phase (Fig. 2 C, group 1). Surprisingly, infection with MVA-WT was inefficient in mediating the long-term retention of OT-I cells (Fig. 2 C, group 2 and 3) despite the pronounced recruitment of effector T cells during acute infection (Fig. 2 B). A sizeable population of OT-I T_{RM} cells was only observed in ears previously infected with MVA-OVA (Fig. 2 C, group 3). Compared with local infection with MVA-WT, delivery of cognate antigen to the skin by MVA-OVA infection led to a >10-fold increase in T_{RM} cell numbers (Fig. 2 C, group 3). These data thus suggest that *in situ* recognition of cognate antigen plays a critical role in generating T_{RM} cells in the infected skin, consistent with recent data obtained in a model of local infection with replicating VACV (Khan et al., 2016). Despite major differences in the local abundance of memory OT-I cells in MVA-OVA- versus MVA-WT-infected skin, the majority of memory OT-I cells isolated from either skin location showed characteristic expression of the skin T_{RM} markers CD69 and CD103 (unpublished data), confirming that the presence of cognate antigen *in situ* is dispensable for the acquisition of the prototypic T_{RM} cell phenotype (Mackay et al., 2012; Skon et al., 2013). Importantly, however, our findings suggest that local antigen-dependent signals critically regulate the quantity of T_{RM} cells at the site of prior infection.

Local antigen-dependent competition shapes the pool of skin-resident memory T cells

We noticed that OT-I T_{RM} cells represented a large fraction of the pool of local CD8⁺ T_{RM} cells in previously MVA-OVA-infected skin and that only a small proportion of CD8⁺ T_{RM} cells were of endogenous (CD45.1⁻) origin (Fig. 2 C, group 3). In contrast, MVA-WT-infected contralateral skin was populated by a small number of OT-I T_{RM} cells, but instead harbored a sizeable population of endogenous CD8⁺ T_{RM} cells (Fig. 2 C, group 2 and 3). This suggested that the marked antigen-dependent local accumulation of OT-I T_{RM} cells in MVA-OVA-infected ears restrained the generation of endogenous virus-specific T_{RM} cells, raising the possibility that antigen-dependent T_{RM} cell formation in MVA-infected skin could be a competitive process that selects preferentially for those CD8⁺ T cell specificities that are able to recognize cognate antigen *in situ*. To investigate the role of competition between T cells recognizing different viral epitopes (cross-competition), we performed a side-by-side analysis of adoptively transferred OT-I cells and endogenous B8R-specific CD8⁺ T cells in MVA-WT versus MVA-OVA-infected skin. Consistent with our previous findings, we observed a robust influx of OT-I cells and B8R-specific effector cells into infected skin during the acute phase of infection (Fig. 3 A). Importantly, the relative proportions of OT-I cells and B8R-specific T cells within the skin-infiltrating effector CD8⁺ T cell population were comparable between MVA-WT- and MVA-OVA-infected skin (Fig. 3 A). However, after establishment of memory, previously MVA-OVA-infected skin was almost exclusively populated by OT-I T_{RM} cells (up to 90% of all skin T_{RM} cells), whereas harboring only a minor fraction (<5%) of skin T_{RM} cells specific for B8R (Fig. 3 A). In contrast, B8R-specific T_{RM} cells were present in significantly higher number and frequency (representing ~30% of the local T_{RM} pool) in previously MVA-WT-infected contralateral skin, which was occupied only by a small number of OT-I T_{RM} cells (Fig. 3 A). Given that B8R-specific CD8⁺ T cells readily accumulated in MVA-OVA-infected skin during the acute response, the aforementioned results argue that their local transition into T_{RM} cells was efficiently impaired in the presence of cross-competing OT-I cells. Our data suggest that cross-competition observed between OT-I cells and B8R-specific T cells in skin harboring both OVA and B8 antigens was antigen dependent, as OT-I effector cells infiltrating the MVA-WT-infected ears (containing only B8 antigen) were unable to curtail the local formation of B8R T_{RM} cells. Notably, we consistently found that the generation of B8R-specific T_{RM} cells was comparable between MVA-OVA and MVA-WT-infected skin in the absence of competitor OT-I cells, and therefore was unaffected by the co-delivery of OVA alone, or the local co-establishment of endogenous OVA-specific T_{RM} cells (Fig. 3 B). However, the amount of B8R-specific T_{RM} cells in skin sites previously infected with MVA-OVA could be titrated by transferring different amounts of naive OT-I cells (unpublished data), suggesting that precursor frequency affects cross-competition, and raising the possibility that immuno-

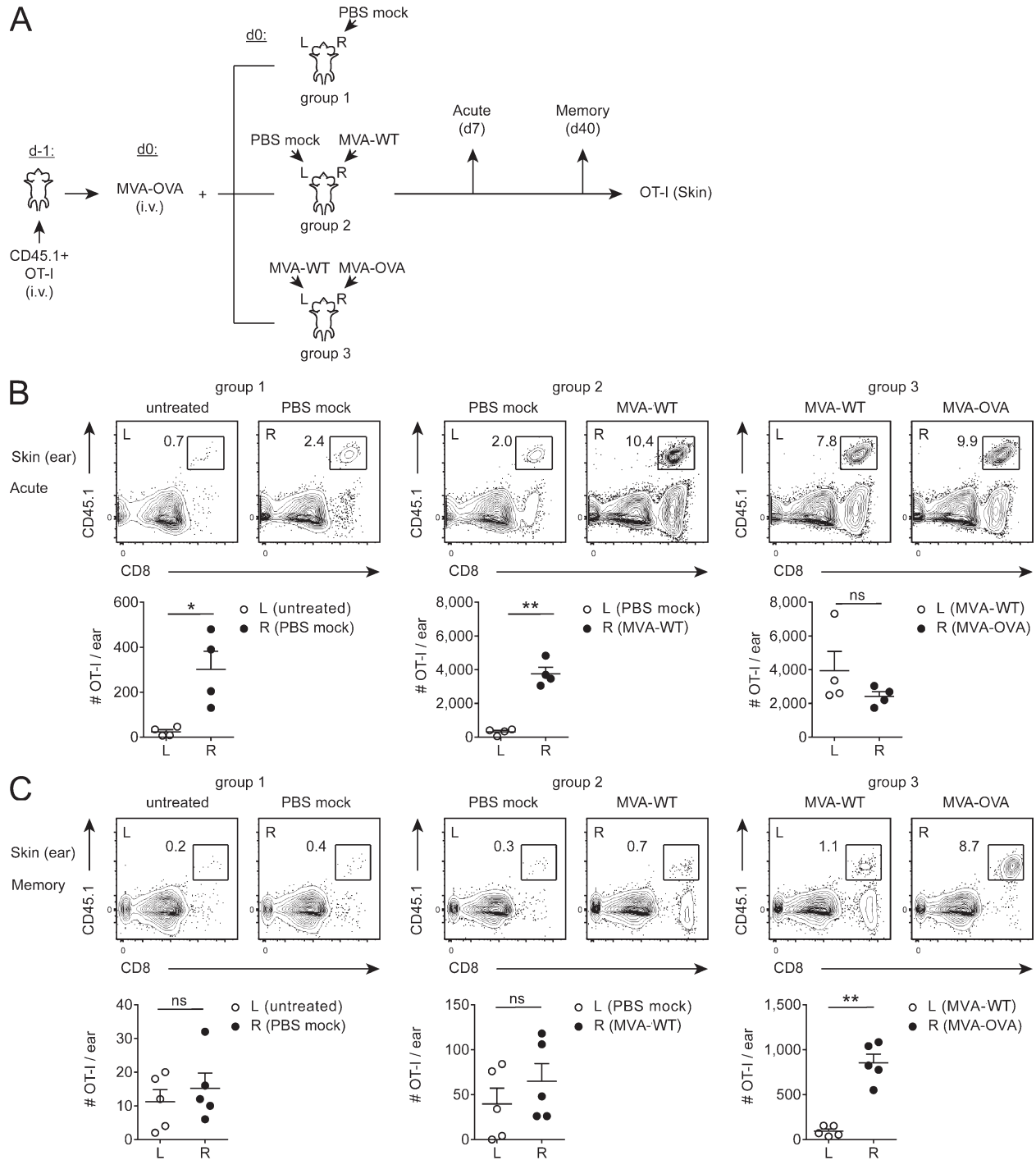


Figure 2. Local cognate antigen expression dramatically amplifies generation of antiviral skin T_{RM} cells. (A) Mice were adoptively transferred with 5×10^4 OVA-specific CD45.1⁺ OT-I cells 1 d before infection (i.v.) with MVA-OVA. Immediately after i.v. infection, mice were divided into three groups. Group 1 was mock infected (PBS mock) on the right ear (R). Group 2 was mock infected (PBS mock) on the left ear (L) and infected with MVA-WT on the right ear (R). Group 3 was infected on the left (L) and right ear (R) with MVA-WT and MVA-OVA, respectively. 7 d (Acute) and 40 d (Memory) pi, total numbers of OT-I cells were determined in differentially inoculated ears. (B and C) Representative plots depicting the frequency of OT-I cells (among CD45⁺ cells) in the indicated skin region 7 d (B) or 40 d (C) pi (top). Total number of OT-I cells in each ear 7 d (B) and 40 d (C) after inoculation (bottom). Data are derived from one experiment representative of at least two independent experiments (Acute, $n = 4$; Memory, $n = 5$). Dots in graphs show individual mice and bars show mean + SEM. *, $P < 0.05$; **, $P < 0.01$ (paired Student's t test).

dominant responses preferentially outcompete subdominant ones. To address whether local cross-competition of endogenous polyclonal T cells was physiologically relevant for shaping the pool of T_{RM} cells, we asked whether B8R-specific T cells, which dominate the endogenous pool of T_{RM} cells in previously MVA-OVA-infected skin (Fig. 3 B), would restrain T_{RM} cell formation of the subdominant endogenous OVA-specific T cells. To this end, we compared the composition of the T_{RM} cell pool in skin previously infected with either MVA-OVA or the *B8R* deletion mutant MVA-OVA Δ B8R, respectively (Fig. 3 C). Importantly, *B8R* is a fragmented nonfunctional gene of MVA, and its deletion does not compromise the infectivity of MVA (Kastenmuller et al., 2007). Consistent with our previous data, we found few B8R-specific T_{RM} cells on day 30 pi in ears previously infected with MVA-OVA Δ B8R. In contrast, B8R-specific T_{RM} cells readily formed in contralateral ears that had undergone infection with B8R-sufficient MVA-OVA (Fig. 3 C). Importantly, OVA-specific T_{RM} cells were increased in both frequency and absolute number in MVA-OVA Δ B8R-infected ears compared with MVA-OVA-infected contralateral ears (Fig. 3 C). Thus, in MVA-OVA Δ B8R-infected skin (i.e., in the absence of B8 antigen), B8R-specific T cells were unable to receive local cognate stimulation and therefore failed to enter into competition with endogenous OVA-specific T cells, allowing them to become the dominant population of local T_{RM} cells. In contrast, in MVA-OVA-infected skin, concomitant local expression of OVA and B8 antigen favored the access of B8R-specific T cells to the local T_{RM} cell pool, while limiting the formation of OVA-specific T_{RM} cells. Note that the total pool of $CD69^+ CD8^+ T_{RM}$ cells was similar in size in the respective left and right ears of experimental groups depicted in Fig. 3 (A–C; and not depicted). Together, these data indicate that antigen-dependent cross-competition serves as a mechanism that shapes the repertoire of polyclonal antiviral T_{RM} cells persisting in previously infected skin.

Local cross-competition in response to coexpressed viral antigens

Our data provide evidence that the generation of T_{RM} cells at the site of viral skin infection is a competitive process and that local antigen-dependent signals facilitate the successful competition of T_{RM} precursors. It remained unclear whether T cells compete directly at the level of APCs (APC-proximal competition model), or whether local antigen presentation enables T cells to compete for factors that may act independently of a dedicated APC (APC-distal competition model). In the APC-proximal model, competition occurs between $CD8^+$ T cells recognizing antigens that are presented by a shared APCs, as previously suggested (Kedl et al., 2000). The APC-distal model, in contrast, is consistent with antigens being presented to competing T cells on different APCs. To discriminate between these possibilities, we established a local co-infection approach in which OVA and B8 antigen were either co-delivered by a single virus, or delivered separately by two individual viruses encoding either OVA or B8. For separate delivery of

these antigens, we infected the left ears of MVA-OVA-primed mice with a 1:1 mixture of MVA-OVA Δ B8R (encoding OVA, but not B8) and MVA-WT (encoding B8, but not OVA). For co-delivery, contralateral right ears were infected with a 1:1 mix of MVA-OVA (encoding both B8 and OVA) and MVA Δ B8R (encoding neither antigen). The empty MVA Δ B8R vector in the latter mix served to obtain a comparable local infectious inoculum, as well as comparable expression levels of the respective antigens (Fig. 4 A). These experiments allowed us to track the generation of T_{RM} cells in response to infection with an antigenically homogenous viral population (coexpressed antigens) versus an antigenically heterogeneous viral population (segregated antigen expression), potentially reflecting the presence of viral quasispecies during infection. Consistent with our previous data, we found no difference in the percentages of OT-I cells and B8R-specific $CD8^+$ T cells between the two differentially infected ears on day 7 pi (Fig. 4 B). On day 30 pi, OT-I cells heavily dominated the local T_{RM} pool at the site of antigen co-delivery (prior MVA-OVA + MVA Δ B8R co-infection) with OT-I T_{RM} cells and B8R-specific T_{RM} cells taking up \sim 80% and 5% of all skin $CD8^+$ T cells, respectively (Fig. 4 B). In contrast, when OVA and B8 were delivered separately by two different viruses (co-infection with MVA-OVA Δ B8R and MVA-WT), B8R-specific T_{RM} cells were significantly increased to 20–30% of the local T_{RM} cell pool, despite the presence of potential competitor OT-I cells (Fig. 4 B). Notably, this relative increase also correlated with a marked rise in the absolute number of B8R-specific T_{RM} cells in this skin region (unpublished data).

These data suggest that T cell cross-competition is regulated at the level of local antigen-presenting APCs in the skin. APC-proximal cross-competition may therefore serve as a gatekeeping mechanism to locally fine-tune the repertoire of skin T_{RM} cells in response to antigenically complex pathogens, while preserving the ability to respond to the local emergence of antigenically distinct quasispecies.

DISCUSSION

Many studies support the idea that T_{RM} cell development requires local instruction in nonlymphoid tissues. Major open questions pertain to the local pathways and cellular interactions that regulate the establishment of the T_{RM} cell niche (Schenkel and Masopust, 2014; Iijima and Iwasaki, 2015; Mueller and Mackay, 2016). Our work identifies the local antigen-dependent competition of T cells with different specificities as a key mechanism regulating the diversity and composition of local pools of T_{RM} cells. Our data highlight how T_{RM} cells are preferentially selected in the infected skin from a comparably broad repertoire of antiviral T cells infiltrating the inflamed tissue. Those cells that are able to detect cognate antigen at the site of infection can preferentially enter the local pool of T_{RM} cells. Antigen-dependent local cross-competition may hence serve as a means to optimally stock a peripheral cellular niche with the most useful T cell receptor specificities to ensure a highly efficient barrier to local reinfection.

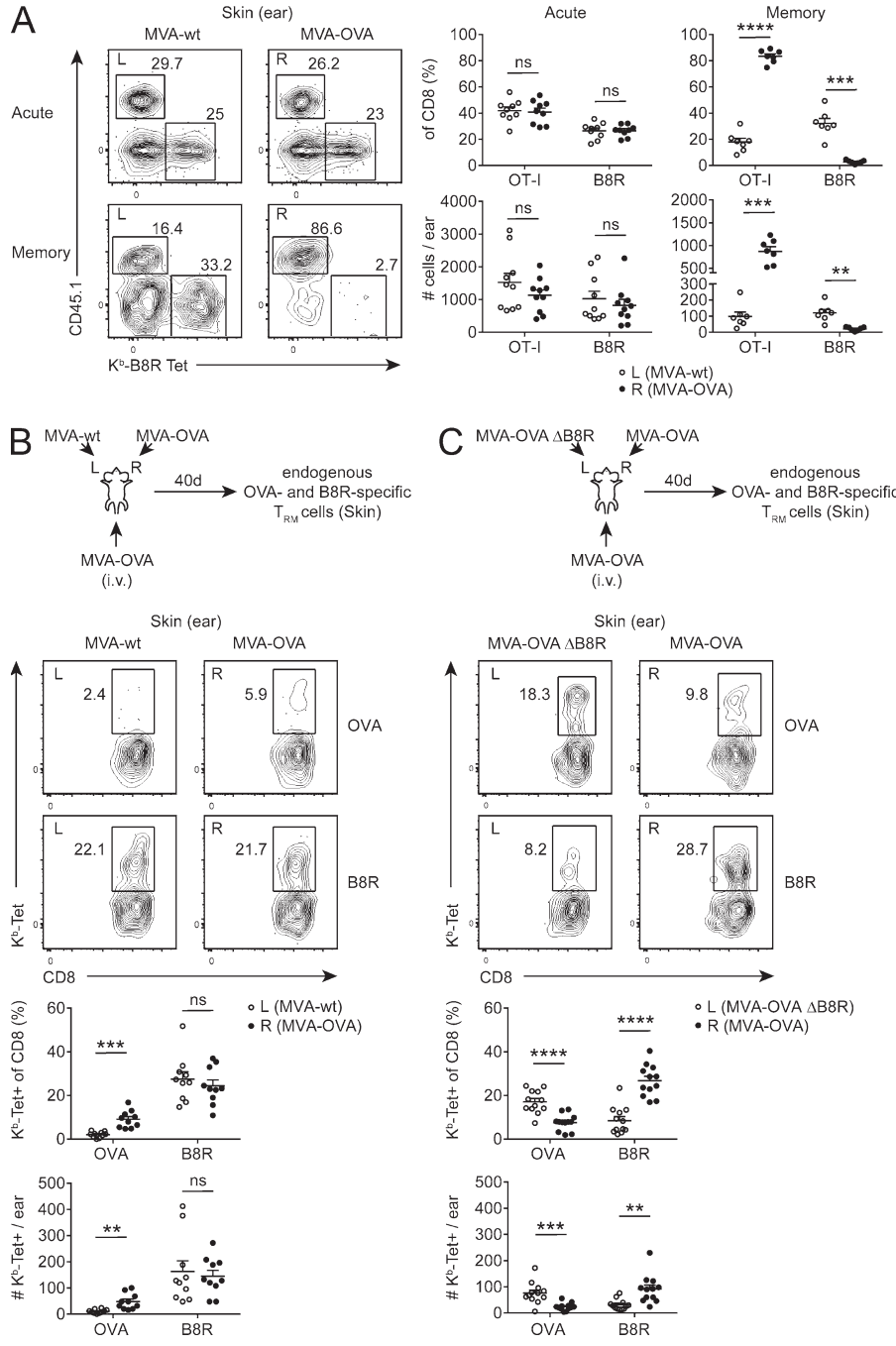


Figure 3. Local antigen-dependent competition shapes the pool of skin-resident memory T cells. (A) OT-I-recipient mice were primed i.v. with MVA-OVA and immediately thereafter infected on the left (L) or right (R) ear pinna with MVA-WT or MVA-OVA, respectively (Fig. 2 A, group 3). Percentage (representative plots and top graphs, right) and absolute number (bottom graphs, right) of CD45.1⁺ OT-I cells and endogenous B8R-specific T cells among CD8⁺ T cells in the differentially infected skin 7 d (Acute) and 40 d (Memory) pi. Data were pooled from two to three independent experiments (Acute, *n* = 9; Memory, *n* = 7). (B) Percentage (representative blots and top graph) and absolute number (bottom graph) of endogenous OVA- and B8R-specific T cells among CD8⁺ T cells in MVA-WT and MVA-OVA-infected ears 40 d pi of mice as in A, but without prior transfer of OT-I. Data were pooled from two independent experiments (*n* = 10). (C) Fraction (representative blots and top graph) and absolute number (bottom graph) of OVA- and B8R-specific T cells among CD8⁺ T cells in MVA-OVA ΔB8R-infected versus MVA-OVA-infected skin 40 d pi. Data were pooled from two independent experiments (*n* = 12). Dots in graphs depict individual mice and bars show mean + SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.001 (paired Student's *t* test).

Consistent with a recent study by Khan et al., our data suggest that local antigen in the skin is critical for the efficient generation of T_{RM} cells (Khan et al., 2016). This is intriguing, considering that during infection with the nonreplicating vaccine vector MVA, active gene expression is expected to cease well before the entry of primed effector T cells into the skin, which we observed between days 2.5 and 3.5 pi (unpublished data). Despite a limited period of viral gene expression, however, we found that MVA-derived antigen remained detectable in the skin at least until 4 d pi (unpublished data).

Thus, the presence of local viral antigen coincides with effector T cell entry into the skin, providing a time window for cognate stimulation in situ.

Cognate signals may directly favor T_{RM} cell generation at multiple stages. Local interactions with antigen-bearing cells may extend local proliferation (Kang et al., 2011), and may reduce the mean velocities of skin-infiltrating CD8⁺ T cells (Macleod et al., 2014), thereby prolonging their dwell time within the tissue and their exposure to local factors critical for instructing T_{RM} cell fate. TCR engagement in infected

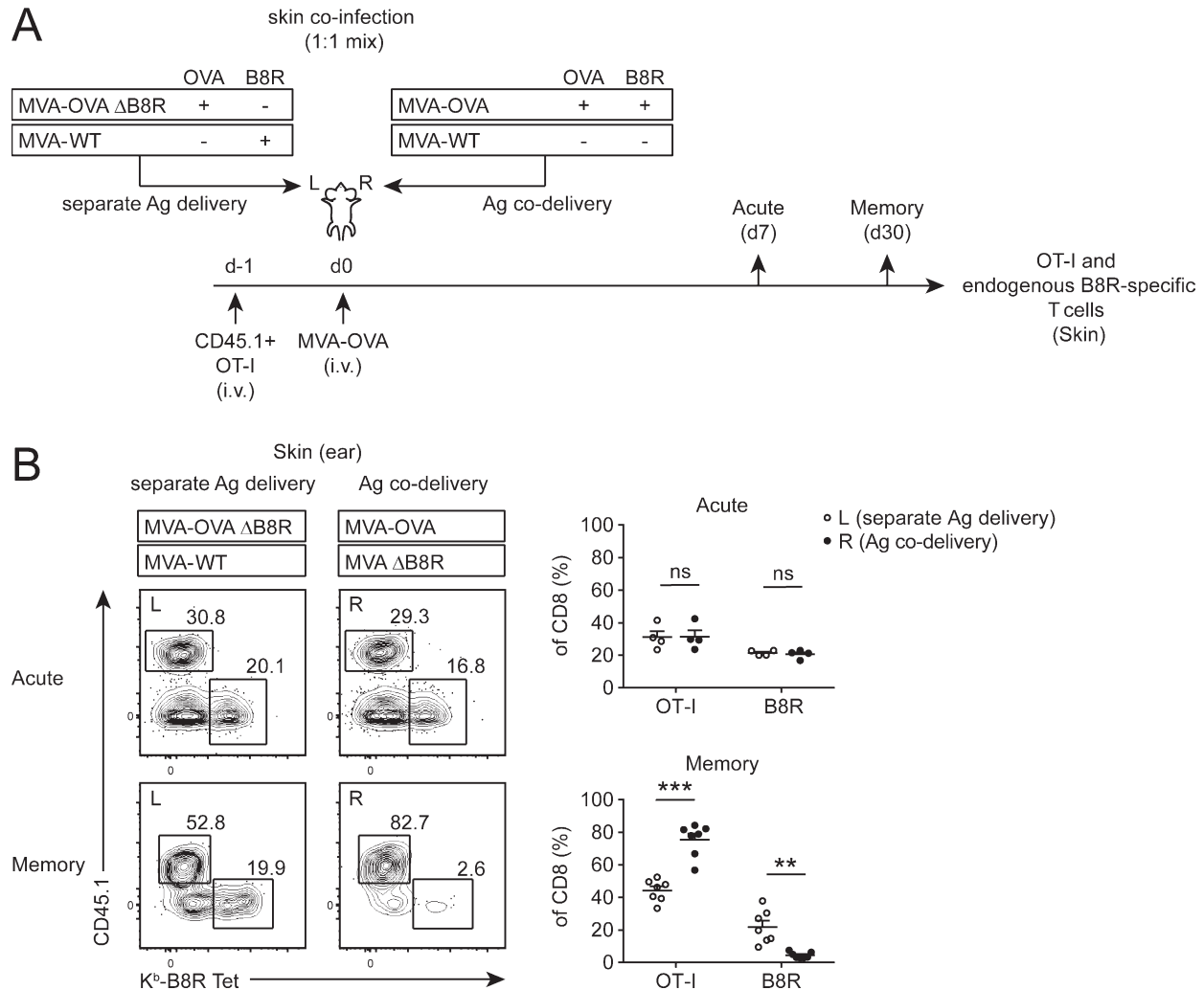


Figure 4. Local cross-competition in response to coexpressed viral antigens. (A) OT-I-recipient mice were primed i.v. with MVA-OVA and co-infected on the left ear (L) with a 1:1 mixture of mutant MVA-OVA Δ B8R (expressing OVA but not B8) and MVA-WT (lacking OVA but expressing B8) for separate delivery of OVA and B8 into the skin. For antigen (Ag) co-delivery, contralateral (right) ears (R) were co-infected with a 1:1 mix of MVA-OVA (expressing both OVA and B8) and MVA Δ B8R (lacking both OVA and B8). (B) Percentages of OT-I and endogenous B8R-specific CD8⁺ T cells in the indicated skin regions 7 d (Acute) and 40 d (Memory) pi. Data are derived from one experiment (Acute, $n = 4$, representative of two independent experiments) or were pooled from two independent experiments (Memory, $n = 7$). Dots in graphs show individual mice and horizontal bars show mean + SEM. **, $P < 0.01$; ***, $P < 0.001$ (paired Student's *t* test).

tissues may directly inhibit egress through afferent lymphatics by reinforcing the cytokine-mediated down-regulation of KLF2 and its downstream targets S1P1 and CCR7 (Kuo et al., 1997; Schober et al., 1999; Bromley et al., 2005; Debes et al., 2005; Mackay et al., 2013; Skon et al., 2013), and by increasing CD69-mediated tissue retention of skin-infiltrating CD8⁺ T cells (Shiow et al., 2006; Mackay et al., 2015a; Khan et al., 2016). Although all of these factors could independently act on individual cells and regulate the size of their individual T_{RM} cell progeny, our work reveals that the antigen-dependent establishment of a polyclonal pool of T_{RM} cells is a competitive process between T cells of different specificities and identifies T cell cross-competition in nonlymphoid tis-

sue as a regulatory mechanism of T_{RM} cell development. Our data suggest that cross-competition is enforced in the context of antigen-presenting cells in the tissue, as competition was functional for T cells recognizing antigens that are coexpressed by infected cells. In contrast, T_{RM} cell development remained undisturbed by the presence of potential competitors when antigen expression was segregated to distinct cells. Consistently, even large numbers of effector OT-I cells infiltrating MVA-WT-infected ears during the acute phase failed to restrain B8R-specific T_{RM} cell development (Fig. 3 A and not depicted). These data support an APC-proximal model of cross-competition in nonlymphoid tissues, in which factors that may be provided or regulated by shared APCs influence

antigen-specific T cells accumulating in their direct vicinity. These data are consistent with the idea that antigen-dependent competitive selection of the T_{RM} cell pool would favor cells that can recognize antigen being presented directly by infected tissue cells, as they would potentially have the highest chance to act as immediate antigen-specific sentinels during reinfection (Schenkel et al., 2013). Interestingly, Macleod et al. have recently reported that HSV-infected epidermal cells, such as keratinocytes and dendritic epidermal $\gamma\delta$ T cells (DETCs), directly present viral antigen to effector $CD8^+$ T cells and thereby stimulate their production of IFN- γ in situ (Macleod et al., 2014). Although in our model, direct antigen presentation by infected nonprofessional APCs (keratinocytes and DETCs) could be relevant for mediating cross-competition among infiltrating T_{RM} cell precursors, our data do not exclude the possibility that uninfected cross-presenting cells can orchestrate cross-competition after phagocytosis of coupled viral antigens coexpressed in infected cells.

T_{RM} cell competition for an epidermal niche has previously been suggested through the almost complete loss of DETCs from the T_{RM} -enriched previously HSV-infected epidermis (Zaid et al., 2014). Although the replacement of DETCs could be the consequence of an altered microenvironment in naive skin versus post-herpetic lesions, our findings identify cross-competition between antiviral $CD8^+$ T cells of different specificities exposed to the same tissue environment in virus-infected skin. In contrast to the local competition in the skin, cross-competition was not observed in secondary lymphoid organs during primary systemic infection with MVA over a broad range of viral doses (Kastennmuller et al., 2007), raising the possibility that T cells may compete for a limited niche size in the skin. Notably, local infection with replicating VACV gave rise to an even larger pool of $CD103^+ CD8^+ T_{RM}$ cells in the ear as compared with infection with MVA (Khan et al., 2016; and unpublished data), suggesting that a restricted niche size alone is unlikely to limit T_{RM} generation in our experiments. Instead, our data are consistent with the idea that antigen-dependent interactions in situ enable access to signals that regulate the size of the respective T_{RM} cell population and that T cells of different specificities compete for these signals, which are possibly delivered by a shared APC.

These observations have implications for the design of vectored vaccines targeting local T_{RM} compartments. As demonstrated in this study, the genetic deletion of potentially interfering vector backbone-encoded $CD8^+$ T cell epitopes, or the local delivery of relevant antigens by a mix of single-antigen-expressing vectors, are strategies to mitigate local T cell cross-competition within the tissue and to improve the generation of T_{RM} cells with the desired antigen specificities. The data obtained by Khan et al. (2016) using viruses that replicate >10 d pi raised the possibility that prolonged antigen expression is required to amplify T_{RM} cell generation. Our work suggests, however, that transient expression of antigens achieved through the local delivery of a nonreplicating

vaccine vector is sufficient to dramatically improve T_{RM} cell formation at barrier sites. In this context, our findings raise the possibility that a promising prime/pull vaccination strategy may be further improved through the local co-delivery of peptide or protein antigens (Shin and Iwasaki, 2012).

Our findings highlight the regionalization of T cell memory within the skin. From an evolutionary perspective, it is tempting to speculate that the antigen-dependent selection of regional T_{RM} cells may have evolved to optimize immune control of locally persisting pathogens. For example, herpes viruses, which represent major human pathogens, can locally persist in root ganglia and reactivate within local dermatomes, requiring immediate T cell-dependent control to prevent potentially lethal systemic spread. Antigen-dependent mechanisms shaping the composition of regional pools of T_{RM} cells may also be critical to prevent invasive infection of commensal microbes. Epidermal IL-17-producing $CD8^+ T_{RM}$ cells recognizing bacterial antigens have recently been identified and shown to become activated during skin injury (Naik et al., 2012). Because bacterial communities exhibit strong regional heterogeneity depending on the area of the skin, one would predict that the pool of T_{RM} cells in different skin areas exhibits regional differences according to the local composition of the microbiota and, hence, the locally differing antigens that may be encountered during skin injury. In light of these considerations, it will be important to test whether the pools of T_{RM} cells that have been identified in many organs are similarly shaped through local antigen-dependent competition, or whether our observations identify an evolutionary adaptation to the requirements of the skin.

In summary, our work highlights how a broad repertoire of T cells initially activated in secondary lymphoid organs is locally fine-tuned to generate a pool of tissue-resident memory cells best suited to recognize viral antigens presented in the tissue, and, therefore, to potentially protect against local infection.

MATERIALS AND METHODS

Animals

WT C57BL/6N mice were purchased from Charles River. $CD45.1^+$ OT-I T cell receptor transgenic mice (bred on a C57BL/6 background) were provided by D.H. Busch (Institute of Microbiology, Immunology and Hygiene, Technical University Munich, Munich, Germany). All mice were kept under specific pathogen-free conditions following institutional guidelines. Animal experiments have been conducted according to the German Animal Welfare Act (Tierschutzgesetz) and have been approved by the regional authorities (District Government of Upper Bavaria, Germany and the North Rhine-Westphalia State Environment Agency [LUA NRW], Germany).

Viruses

MVA expressing the full-length OVA gene under the control of the modified early/late promoter PH5 (MVA-OVA)

was derived from WT cloned isolate IInew, as previously described (Staib et al., 2004). MVA-OVA and the B8R deletion MVA mutant (MVA-OVA Δ B8R), both expressing full-length OVA under control of the early/late promoter P7.5, as well as MVA Δ B8R and MVA-LacZ (expressing β -galactosidase under the early vaccinia viral promoter PK1L), were derived from WT MVA cloned isolate F6 and generated as previously described (Kastenmuller et al., 2007). Corresponding WT isolates were used as MVA-WT control virus. All viruses were routinely propagated and titrated according to standard methodology (Staib et al., 2004).

Immunizations

Only female mice between 8 and 12 wk of age were used for immunizations. Unless otherwise stated, all mice received a standard dose of 2×10^6 infectious units MVA. For intraepidermal immunization, MVA (diluted in 5 μ l sterile PBS) was tattooed into the dorsal ear pinnae for 20 s using a rapidly oscillating sterile 15-needle bar (Magnum15) mounted on a rotary tattoo device (Cold skin; B&A Trading). Needle depth was adjusted to 0.5 mm. Before tattooing, mice were anesthetized by intraperitoneal injection of 1 ml/kg ketamine/xylazine (Sigma-Aldrich). Intravenous MVA immunizations were performed via tail vein injection of MVA (diluted in 200 μ l sterile PBS).

Adoptive transfer

For adoptive transfer experiments, CD8⁺ OT-I cells were isolated from the spleens of naive female CD45.1⁺ OT-I mice. 1 d before immunizations, 5×10^4 cells were injected into the tail vein of recipient mice.

Histological analysis

Skin tissue was excised, embedded in TissueTek Medium, and immediately snap-frozen in liquid nitrogen. 10- μ m sections were prepared on a CM1950 cryostat (Leica). For detection of LacZ expression, 10- μ m cryosections were fixed for 10 min in 4% paraformaldehyde, washed with PBS, and then incubated for 24 h in X-Gal staining solution. After X-Gal staining, sections were washed with PBS and counterstained for 5 min in nuclear fast red. Immunohistochemical staining was performed on an automated BOND-MAX instrument using Bond Polymer Refine Red Detection kit (Leica). For detection of CD45.1, acetone-fixed skin sections were incubated with FITC-conjugated anti-CD45.1 (A20; eBioscience; 1:200) and secondary polyclonal rabbit anti-FITC antibodies (Invitrogen; 1:500). Images were acquired using the Leica SCN400 slide scanner analysis software.

Preparation of single-cell suspensions from skin and lymphoid tissue

For cell isolation from skin, mice were perfused with 20 ml PBS through the left cardiac ventricle and skin tissue was excised, cut into small pieces, and incubated for 90 min at 37°C with 3 mg/ml collagenase type III (Worthington) and

5 μ g/ml DNase I (Sigma-Aldrich) in RPMI medium supplemented with 10% FCS and 1 mM CaCl₂ and MgCl₂, followed by addition of 10 mM EDTA and 5 min incubation at room temperature. Skin suspensions were filtered twice through a 70- μ m cell-strainer before antibody staining and filtered again through a 30- μ m cell strainer before flow cytometric analysis.

Splenic cell suspensions were obtained by mashing the perfused spleens over metal wire grid, followed by filtering through a 100- μ m cell-strainer and lysis of red blood cells using Tris-NH₄Cl buffer.

Flow cytometry

The following antibodies were used for flow cytometric analysis: purified anti-CD16/CD32 (2.4G2) was obtained from BD. Fluorochrome-conjugated antibodies anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD8a (53.6.7), anti-CD103 (2E7), and anti-CD69 (H1.2F3) were purchased from eBioscience. PE-conjugated H-2K^b/B8R₂₀ and H-2K^b/OVA₂₅₇ tetramers were provided by D.H. Busch (Technical University Munich). All antibody incubation steps were performed on ice for 30 min. Dead cells were excluded by staining with propidium iodide (Molecular Probes) immediately before analysis. Samples were acquired on a LSR II flow cytometer (BD) and FlowJo software (Tree Star) was used for data analysis.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 6.0 software. Results are expressed as mean + SEM. Statistical significant differences in cell numbers or percentages between left and right ear skin of individual mice were calculated by a nonparametric two-tailed paired Student's *t* test.

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