Local antigen in nonlymphoid tissue promotes resident memory CD8\(^+\) T cell formation during viral infection

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Tissue-resident memory (Trm) CD8\(^+\) T cells are functionally distinct from their circulating counterparts and are potent mediators of host protection against reinfection. Whether local recognition of antigen in nonlymphoid tissues during infection can impact the formation of Trm populations remains unresolved. Using skin infections with vaccinia virus (VacV)–expressing model antigens, we found that local antigen recognition had a profound impact on Trm formation. Activated CD8\(^+\) T cells trafficked to VacV–infected skin in an inflammation-dependent, but antigen-independent, manner. However, after viral clearance, there was a subsequent ~50-fold increase in Trm formation when antigen was present in the tissue microenvironment. Secondary antigen stimulation in nonlymphoid tissue caused CD8\(^+\) T cells to rapidly express CD69 and be retained at the site of infection. Finally, Trm CD8\(^+\) T cells that formed during VacV infection in an antigen-dependent manner became potent stimulators of localized antigen-specific inflammatory responses in the skin. Thus, our studies indicate that the presence of antigen in the nonlymphoid tissue microenvironment plays a critical role in the formation of functional Trm CD8\(^+\) T cell populations, a finding with relevance for both vaccine design and prevention of inflammatory disorders.

Trm CD8\(^+\) T cells are often identified in nonlymphoid tissue by expression of CD103, the \(\alpha_\text{E}\) integrin chain, which pairs with \(\beta_7\) to become a ligand for E-cadherin (Cepek et al., 1994). After localizing to the skin, Trm memory precursors express CD103 upon entering the epithelium, and this is thought to either retain Trm at this site or promote their survival. In fact, this integrin is essential for Trm CD8\(^+\) T cell formation after HSV infection of the skin (Mackay et al., 2013). Trm CD8\(^+\) T cells also express CD69, which functions as an antagonist to sphingosine-1 phosphate receptor (S1PR1) signaling (Shio et al., 2006). S1PR1 is a specialized G protein–coupled receptor that regulates T cell egress out of lymph nodes by directing their migration to higher concentrations of S1P in the efferent lymph (Schwab et al., 2005; Cyster and Schwab, 2012). It has also been suggested that S1PR1 controls the migration of T cells out of nonlymphoid tissues and into the draining lymphatic vessels (Ledgerwood et al., 2008). Expression of S1PR1 is lower on Trm compared with circulating memory CD8\(^+\) T cells and forced overexpression of S1PR1 impairs formation of Trm populations (Mackay et al., 2013; Skon et al., 2013). Furthermore, CD69 has also been shown to impact Trm formation after HSV infection of the skin (Mackay et al., 2015a), suggesting that CD69 could function as an additional retention signal on CD8\(^+\) T cells after their recruitment into nonlymphoid tissues. Although it has been demonstrated that expression of both CD103 and CD69 are critical for generating Trm in nonlymphoid tissues,
the mechanisms regulating their expression in vivo have yet to be fully characterized.

After their activation in lymphoid organs, antigen-specific CD8+ T cells enter the circulation and migrate into nonlymphoid tissues. Once within these tissue microenvironments, exposure to cytokines, including TGF-β, TNF, type I IFN, and IL-33 orchestrate the differentiation into Trm, and this is believed to occur independently of secondary antigenic stimulation. In fact, continual exposure to antigen during chronic LCMV infection results in diminished CD103 expression on CD8+ T cells in nonlymphoid tissues, suggesting that antigen recognition may play a negative role in generating Trm CD8+ T cells (Zhang and Bevan, 2013; Beura et al., 2015). In contrast, in vitro stimulation with TGF-β in combination with either IL-33 or type I IFN is sufficient to induce expression of CD103 and CD69 on effector CD8+ T cells (Casey et al., 2012; Skon et al., 2013). Similar lines of evidence demonstrating that differentiation of Trm can occur independently of local antigen recognition have been found in vivo. After acute infection with LCMV, effector CD8+ T cells migrate to several nonlymphoid tissues, including the gut, female reproductive tract, lung, and liver, and are believed to differentiate into Trm independently of local antigen stimulation (Masopust et al., 2010; Casey et al., 2012; Schenkel et al., 2013, 2014). In addition, recruitment of effector CD8+ T cells into artificially inflamed skin or directly injecting in vitro–activated CD8+ T cells into normal skin both result in the generation of Trm (Mackay et al., 2012, 2013, 2015a; Skon et al., 2013). Finally, Trm can also form in the female reproductive tract after subcutaneous HSV vaccination, independent of local antigen recognition (Shin and Iwasaki, 2012). Based on these observations, it has generally been accepted that CD8+ T cells differentiate into Trm in nonlymphoid tissues in an antigen-independent manner. However, whether local antigenic stimulation in nonlymphoid tissue enhances, inhibits, or has no impact on Trm formation has not been directly addressed.

Because of the importance for understanding how Trm formation occurs with regards to host protection, vaccine design, and their potential to contribute to inflammatory and autoimmune disorders, we explored whether antigen in nonlymphoid tissue plays a role in shaping the formation of the Trm population after infection. To directly address this, we used VacV infections, in which expression of specific model antigens could be restricted to individual tissue microenvironments. Using this strategy, we found that local antigen recognition significantly enhances the formation of Trm after VacV infection of the skin. Enrichment of antigen-specific CD8+ Trm in the skin after VacV infection resulted in heightened inflammatory responses when previously infected skin was later challenged with antigen locally. This suggests that Trm CD8+ T cells initiate antigen-specific inflammatory responses more effectively than the memory CD8+ T cells in the circulation and that local antigen encounters are required to seed these types of memory CD8+ T cells in the skin.

Overall, these findings suggest that the precise biological and molecular mechanisms that regulate Trm CD8+ T cell formation are still being defined and that both inflammation-mediated recruitment and subsequent local antigenic stimulation are both required for optimal Trm generation to occur.

RESULTS

Trm CD8+ T cells are generated in the skin after infection with VacV–GP33

Because of its success as a vaccine agent, epitucaneous infection with VacV has emerged as an attractive approach to investigate the mechanisms regulating the activation and functional capacity of the adaptive immune system (Hickman et al., 2013, 2015). In this study, we used a recombinant version of VacV that expresses the GP33-41 epitope from lymphocytic choriomeningitis virus (LCMV) as a model antigen. After infection of the skin on one ear by scarification, VacV-GP33 replicated rapidly and viral load peaked on day 3 and was not cleared from the skin until ~2 wk after infection (Fig. 1 A). VacV-GP33 remained localized and did not spread to other tissues, such as the contralateral ear, draining lymph node, or ovary (Fig. 1 B). We next tested whether endogenous antigen-specific CD8+ T cells were activated after infection of the skin using ex vivo peptide stimulation, followed by intracellular stain for IFN-γ. On day 10 after infection, we detected significant expansion of GP33-specific CD8+ T cells in the draining lymph node of the VacV-GP33–infected ear (Fig. 1, C and D). This was not a result of aberrant overexpression of the GP33-41 epitope by recombinant VacV, as stimulation with B8R20-27 (the immunodominant epitope in VacV; Tscharke et al., 2005) stimulated similar IFN-γ production as GP33 (Fig. 1, C and D). Therefore, these data demonstrate that a localized VacV-GP33 infection primes a CD8+ T cell response specific to both endogenous VacV antigens and model antigen B8R20-27 in the draining lymph node.

Infection of the skin with VacV has been shown to generate Trm CD8+ T cells, although this analysis has been largely restricted to transfers of OT-I TCR–transgenic CD8+ T cells (Jiang et al., 2012). To determine whether endogenous CD8+ T cells specific for GP33-41 differentiate into Trm, we infected naive B6 mice with VacV-GP33 by scarification on the left ear and monitored the subsequent GP33-specific CD8+ T cell response in the ears, draining, and nondraining lymph node by H2-Dβ–GP33 tetramer. After infection, expansion of GP33-specific CD8+ T cells could be detected in the draining lymph node, but not the contralateral nondraining lymph node (Fig. 2 A, top row). This antigen-specific CD8+ T cell response peaked on day 10 after infection in both the draining lymph node and in the spleen, contracted, and formed a memory population that could be detected in the circulation (Fig. 2, A–D). GP33-specific CD8+ T cells also trafficked to the VacV-GP33–infected ear, but not the contralateral uninfected ear, and these cells also underwent substantial contraction after day 15 after infection (Fig. 2, A and C). Nevertheless, on day 40 after infection, GP33-specific
CD8+ T cells were highly enriched in the previously infected VacV-GP33–infected ear compared with the uninfected ear (Fig. 2, E and F). In contrast to the GP33-specific CD8+ T cells found in the spleen, GP33-specific CD8+ T cells isolated from the previously infected ear expressed the αE integrin CD103 (Fig. 2, G and H), which identifies Trm CD8+ T cells in the skin (Carbone, 2015). B8R–specific CD8+ T cells were also enriched in the previously infected ear and also expressed CD103 (Fig. 2, I–L). Collectively, these data demonstrate that CD8+ T cells specific for both endogenous VacV antigens and ectopically expressed model antigens become Trm in the skin after resolution of the viral infection.

Local antigen recognition regulates Trm CD8+ formation in VacV-infected skin

To define the mechanisms that regulate the formation of Trm CD8+ T cells during VacV infection, we used P14 TCR-transgenic (TCR-tg) CD8+ T cells, which recognize the GP33-41 antigen expressed by VacV-GP33. We transferred a physiologically relevant number of naive Thy1.1 P14 CD8+ T cells into B6 mice, which were then infected with VacV-GP33 on the left ear by scarification (Fig. 3 A). On day 40 after infection, circulating memory P14 CD8+ T cells were detected in both the lymph nodes and spleen (Fig. 3 B). Similar to our results from Fig. 2, memory P14 CD8+ T cells were also highly enriched in the skin of the VacV-GP33–infected ear compared with the uninfected ear (Fig. 3, B and C). The quantity of P14 CD8+ T cells in the previously draining and nondraining lymph nodes was not significantly different (Fig. 3 D), which shows that memory CD8+ T cells in the circulation had distributed equally at this time point. P14 CD8+ T cells from the skin of the VacV-GP33–infected ear also expressed CD103 and CD69 (Fig. 3, E and F), demonstrating that TCR-tg CD8+ T cells also became Trm, similar to antigen-specific cells in the endogenous repertoire. Therefore, these data further support the conclusion that activated CD8+ T cells subsequently differentiate into Trm CD8+ T cells primarily at the site of infection.

Recent experimental evidence suggests that Trm may exhibit a variety of different phenotypes (including the complete lack of CD69 expression) and that certain subsets may become enriched during the cell isolation process from tissues (Steinert et al., 2015). As shown in Fig. 3 E, memory P14 CD8+ T cells isolated from the previously infected skin also showed a range of phenotypes based on expression of CD69 and CD103. To determine which memory CD8+ T cell subsets could be classified as Trm after VacV infection of the skin, we used a low dose of Thy1.1-depleting antibody, as this strategy has been demonstrated to eliminate cells in the circulation, but not Trm in nonlymphoid tissues (Schenkel et al., 2013; Mackay et al., 2015b). In agreement with those studies, memory P14 CD8+ T cells
Figure 2. **Trm CD8⁺ T cells are generated in the skin after the resolution of VacV infection.** (A) Naive B6 mice were infected on the left ear with 5 × 10⁶ PFU of VacV-GP33 by scarification. Frequencies of GP33-specific CD8⁺ T cells were determined with H2-D⁺-GP33-41 tetramer stain from the indicated tissue on days 10 and 40 after infection. (B–D) Cumulative data over time from A. (E) Mice were infected as in A. On day 40 after infection, GP33-specific CD8⁺ T cells were identified by H2-D⁺-GP33-41 tetramer in the previously infected ear. Number indicates total GP33-specific CD8⁺ T cells. (F) Quantification of GP33-specific CD8⁺ T cells in the previously infected left ear and uninfected right ear. (G) GP33-specific CD8⁺ T cells from E were analyzed for percentage of cells expressing CD103. (H) Quantification of G. (I–L) Same as E–H, except B8R-specific CD8⁺ T cells were identified with H2-K⁺-B8R20-27 tetramer. Data are representative of more than three independent experiments with five mice per group. *, P < 0.05; **, P < 0.001, paired Student’s t test.
were depleted in the spleen of VacV-GP33–infected mice treated with anti–Thy1.1–depleting antibody, but the majority of the memory P14 CD8+ T cells remained in the skin (Fig. 3 G). Interestingly, the quantity of both the CD103+/CD69− and CD103−/CD69+ subsets in the skin was unaffected after treatment with Thy1.1–depleting antibody, whereas the CD103−/CD69− cells were almost completely eliminated (Fig. 3, G–J). Thus, the data from this assay suggest that after VacV infection of the skin, both CD69+/CD103− and CD69−/CD103+ memory CD8+ T cells could be considered tissue resident.

Numerous studies have shown that activated CD8+ T cells are able to differentiate into Trm in nonlymphoid tissues, including the skin, independent of antigen recognition. However, these studies relied on strategies including the transfer of activated CD8+ T cells directly into the skin (Mackay et al., 2013), or on recruiting CD8+ T cells to the antigen-free skin with the chemical hapten dinitrofluorobenzene (Mackay et al., 2012; Skon et al., 2013). To directly test whether the presence of local cognate antigen affected the subsequent formation of Trm CD8+ T cells, we used co-infection with VacV-GP33 and VacV expressing the model antigen OVA (VacV-OVA) on...
the contralateral ear. We predicted that using this co-infection model should result in two VacV-infected tissue micro-environments in opposite ears on the same animal, where the only difference would be the local expression of two different model antigens. Indeed, when mice were simultaneously infected with the two viruses, expansion of GP33-specific CD8+ T cells was restricted to the draining lymph node from the VacV-GP33–infected ear and likewise, OVA-specific CD8+ T cell activation was found only in the draining lymph node from the VacV-OVA–infected ear (Fig. 4 A). In contrast, expansion of B8R-specific CD8+ T cells was found in both draining lymph nodes, demonstrating that the two viral infections were equivalent in their capacity to activate CD8+ T cells (Fig. 4 A, right).

To determine whether local antigen recognition regulated the formation of Trm CD8+ T cells, we transferred equal numbers of naive P14 (Thy1.1/1.1) and OT-I (Thy1.1/1.2) CD8+ T cells into B6 mice (Thy1.2/1.2), which were subsequently infected with VacV-GP33 on the left ear and VacV-OVA on the right ear (Fig. 4 B). On day 40 after infection, we analyzed the Thy1.1+, CD69+ CD8+ T cells in both the left ear previously infected with VacV-GP33 and the right ear that was infected with VacV-OVA for enrichment of either the P14 or OT-I CD8+ T cell populations. Strikingly, the generation of Trm after VacV infection occurred in largely an antigen-specific manner, where most of the Trm in the VacV-GP33–infected ear were P14 CD8+ T cells and most of the Trm in the VacV-OVA–infected ear were OT-I CD8+ T cells (Fig. 4, C and D). The spleen contained similar frequencies of P14 and OT-I CD8+ T cells, demonstrating that the generation of circulating memory CD8+ T cells was nearly equal for the two specificities (Fig. 4 D). Thus, these data demonstrate that local antigen recognition, when present, significantly enhances the subsequent quantity and differentiation of antigen-specific tissue-resident memory CD8+ T cells that are generated after the resolution of viral infection.

Activated CD8+ T cells are recruited to VacV-infected skin in an antigen-independent manner, but do not become Trm without local antigen recognition

Our data in Fig. 4 demonstrate that formation of Trm CD8+ T cells occurs in largely an antigen–dependent manner. To
define the mechanisms that regulate antigen-dependent formation of Trm, we first tested whether antigen-specific CD8^+ T cells were preferentially recruited to infected tissue microenvironments where cognate antigen was present. Naive P14 CD8^+ T cells were transferred into B6 mice and subsequently co-infected with VacV-GP33 on the left ear and VacV-OVA on the right ear. At early time points after infection (days 0–10), P14 CD8^+ T cells trafficked to both ears equally, demonstrating that the inflammatory environment caused by the VacV infection, and not local antigen, controls recruitment of activated CD8^+ T cells into infected skin (Fig. 5, A and B). After the resolution of the VacV-GP33 infection, P14 CD8^+ T cells remained in the skin and subsequently differentiated into Trm, indicated by high expression of both CD103 and CD69. In contrast, the quantity of P14 CD8^+ T cells in the VacV-OVA–infected skin declined rapidly after the resolution of infection, and the cells expressed substantially lower levels of both CD103 and CD69 (Fig. 5, A–D). The quantity of endogenous B8R–specific Trm CD8^+ T cells was equivalent in both the VacV-GP33 and VacV-OVA–infected ears, as was their expression of both CD69 and CD103 (Fig. 5, E–G). Thus, these data demonstrate that recruitment of recently activated CD8^+ T cells is independent of antigen specificity, but that the subsequent formation of Trm CD8^+ T cell populations is strongly influenced by local antigen in nonlymphoid tissues.

**CD8^+ T cells in nonlymphoid tissue express CD69 in an antigen-dependent manner during viral infection**

Expression of the IL-7 receptor (CD127) and KLRG1 are used to identify short-lived effector (CD127Lo/KLRG1Hi) and memory precursor (CD127Hi/KLRG1Lo) CD8^+ T cells (Joshi et al., 2007), and it has also been suggested that Trm are derived from a KLRG1Lo precursor (Mackay et al., 2013). Because prosurvival cytokines such as IL–7 are essential for memory CD8^+ T cell formation (Schluns et al., 2000), we next determined if the expression of CD127 and KLRG1 was influenced by local antigen recognition in nonlymphoid tissue. Between days 7 and 15 after infection, the percentage of CD127Hi/KLRG1Lo memory precursors within the P14 CD8^+ T cell compartment increased in both VacV-infected ears, as well as in the spleen (Fig. 6, A and B). In fact, the percentage of memory precursors was generally higher in the VacV-GP33–infected skin than in the spleen (Fig. 6, A and B), which correlated with clearance of the virus from the infected skin (Fig. 1 A). Interestingly, in contrast to the differential expression of CD69 in the two VacV-infected microenvironments, expression of CD103 was not regulated by local antigen during these early time points (days 10 and 15; Fig. 6, E and F), suggesting that expression of this integrin chain occurs through an antigen-independent mechanism. However, on day 40 after infection, expression of CD103 was reduced on the small number of P14 CD8^+ T cells that were recovered from the VacV-OVA–infected ear, suggesting that expression of CD103 on nearly half of the P14 CD8^+ T cells on day 15 after infection was not sufficient to cause them to become Trm without local antigen stimulation. Therefore, these data demonstrate that recognition of local antigen in nonlymphoid tissues causes CD8^+ T cells to rapidly express CD69, but expression of CD103 occurs through an antigen-independent mechanism.

**CD8^+ T cells rapidly express CD69 and Nur77 after secondary TCR stimulation in nonlymphoid tissue**

Ligation of the TCR is a potent stimulator of cell cycle progression and proliferation. To determine whether antigen in nonlymphoid tissue causes CD8^+ T cells to undergo local proliferation at the site of infection, we treated mice with BrdU for 3-d intervals after co-infection with VacV-GP33 and VacV-OVA. Between days 6–9 and 9–12 after infection, nearly all P14 CD8^+ T cells that had localized to the VacV-GP33–infected skin had incorporated BrdU (Fig. 7 A). However, this proliferation likely occurred during priming of naive CD8^+ T cells in the draining lymph node of the VacV-GP33–infected skin, as similar incorporation of BrdU was detected in the P14 CD8^+ T cells that had localized to the VacV-OVA–infected skin (Fig. 7 A). Furthermore, limited levels of proliferation were detected 12 d after infection, and both proliferating and nonproliferating P14 CD8^+ T cells expressed similar levels of CD69 in the VacV-GP33–infected skin (Fig. 7, B and C). Thus, these data suggest that activated CD8^+ T cells do not undergo significant proliferation in nonlymphoid tissue when antigen is present in the microenvironment and that proliferation of CD8^+ T cells is not directly linked to expression of CD69.

The aforementioned data suggest that if CD8^+ T cells were recognizing antigen locally, it did not result in proliferation at the site of infection. To directly address whether CD8^+ T cells in the skin were receiving TCR stimulation during the VacV infection, we analyzed expression of the orphan nuclear hormone receptor Nur77. Nur77 is a direct target of TCR-mediated gene transcription, is highly expressed in thymocytes undergoing negative selection, and its expression correlates with strength of TCR signaling in
vitro and in vivo (Moran et al., 2011; Au-Yeung et al., 2014). Thus, expression of Nur77 is a more reliable indicator of TCR stimulation than is expression of CD69, which can occur through both antigen-dependent and antigen-independent mechanisms (Shiow et al., 2006; Moran et al., 2011). In agreement with the prediction that CD8+ T cells were receiving additional TCR stimulation in the skin, more P14 CD8+ T cells expressing Nur77 were in the VacV-GP33-infected skin compared with either the VacV-OVA-infected skin or spleen on day 10 after infection (Fig. 7, D and E). Furthermore, P14 CD8+ T cells that were Nur77Hi expressed higher levels of CD69 than did the Nur77Lo subset (Fig. 7 F). This suggests that within the local microenvironment, stimulation of the TCR results in the concurrent expression of both Nur77 and CD69.

Increased expression of Nur77 suggested that recently activated CD8+ T cells were receiving TCR stimulation in non-lymphoid tissue. However, it was clear from the data that there were P14 CD8+ T cells that expressed CD69, but not Nur77 (Fig. 7 F). To define the relationship between TCR signaling and expression of Nur77 and CD69, we stimulated P14 CD8+ T cells from the spleen on day 10 after VacV-GP33 infection with either GP33-41 peptide or PMA/Ionomycin. Without stimulation, P14 CD8+ T cells isolated from the spleen ex-
pressed low levels of both Nur77 and CD69 (Fig. 7, G and H). However, stimulation with either 100 nM GP33-41 peptide or PMA/Ionomycin for 2 h was sufficient to induce expression of both Nur77 and CD69 (Fig. 7, G and H), demonstrating that there is rapid, concurrent expression of the two genes in response to TCR-mediated signal transduction. Furthermore, similar concentrations of GP33-41 peptide were required to induce expression of Nur77 and CD69 on the P14 CD8+ T cells isolated from the spleen (Fig. 7 I). This suggests that the strength of TCR stimulation required for expression of Nur77 is similar to that required to induce expression of CD69, and that circulating effector CD8+ T cells will rapidly express both CD69 and Nur77 after a secondary antigen encounter.

Previous studies have demonstrated that expression of Nur77 occurs transiently when naive CD8+ T cells are stimulated with agonistic peptide (Moran et al., 2011). To determine whether effector CD8+ T cells also transiently express Nur77 after a secondary antigen encounter, we again stimulated P14 CD8+ T cells from the spleen with GP33-41 peptide for an extended time course. In agreement with our data in Fig. 7 G, expression of both Nur77 and CD69 was readily induced after 3 and 5 h of stimulation with 1 nM GP33-41 peptide (Fig. 7 J). However, by 24 h after peptide stimulation, expression of Nur77 had returned to background levels on the P14 CD8+ T cells. In contrast, CD69 continued to be highly expressed through 48 h after peptide stimulation.
Expression of CD69 correlates with increased expression of Nur77, but not local proliferation of CD8+ T cells in nonlymphoid tissues. (A) Naive Thy1.1 P14 CD8+ T cells were transferred into B6 mice and subsequently co-infected with VacV-GP33 on the left ear and VacV-OVA on the right ear. Mice were treated with BrdU, and proliferation of P14 CD8+ T cells was quantified during the indicated time interval. (B) Mice were treated with BrdU on days 12–15 after infection, and percentage of P14 CD8+ T cells expressing CD69 was determined on the BrdU+ and BrdU− P14 CD8+ T cells in the VacV-GP33–infected ear on day 15. (C) Quantification of the data in B. (D) Naive Thy1.1+ P14 CD8+ T cells were transferred into B6 mice and subsequently co-infected with VacV-GP33 on the left ear and VacV-OVA on the right ear. On day 10 after infection, frequency of P14 CD8+ T cells expressing Nur77 was analyzed in the VacV-GP33–infected ear, VacV-OVA–infected ear, and spleen. (E) Quantification of data from D. (F) Expression of CD69 was quantified on the Nur77+ and Nur77− expressing P14 CD8+ T cells in the VacV-GP33–infected ear skin. (G) P14 CD8+ T cells isolated from the spleen on day 10 after infection with VacV-GP33 were stimulated with 100 nM GP33-41 peptide or PMA and ionomycin for 2 h, and frequencies of cells expressing both CD69 and Nur77 were determined. (H) Quantification of G. (I) Same as G, except cells were stimulated with the indicated concentration of GP33-41 peptide for 5 h and expression of CD69 and Nur77 was determined by flow cytometry. (J) Same as G, except cells from the spleen were stimulated with 1 nM GP33-41 peptide and frequencies of cells expressing Nur77 and CD69 were determined at the indicated time after stimulation. (K) Quantification of J. Data in A–C and G–K are representative of two independent experiments with three mice per group. Data in D–F are cumulative results from three independent experiments of three to five mice per group. *, P < 0.01; ** P < 0.0001; n.s., paired Student’s t test.
Tissue-resident memory CD8⁺ T cells in the skin are mediators of antigen-specific inflammatory responses

Trm CD8⁺ T cells have been shown to rapidly produce IFN-γ after antigenic stimulation, which subsequently recruits leukocytes into nonlymphoid tissues, causing significant inflammation (Schenkel et al., 2013). Because our data demonstrate that local antigen stimulation regulated the magnitude of antigen-specific Trm CD8⁺ T cells that formed at the site of VacV infection, we next determined whether this would impact subsequent local antigen-specific inflammatory responses. Mice previously co-infected with VacV-GP33 and VacV-OVA on opposite ears 40 d earlier were challenged with GP33-41 peptide or control peptide (LCMV peptide NP396-404) on both ears. After challenge with GP33-41 peptide, skin previously infected with VacV-GP33 became inflamed and swelling of the ear occurred as early as 20 h after challenge (Fig. 8 A). However, when GP33-41 peptide was administered on the skin previously infected with VacV-OVA at the same time on the same animal, significantly less swelling occurred compared with the VacV-GP33–infected skin, demonstrating that GP33-specific Trm CD8⁺ T cells were the central mediators of the inflammatory response. Limited swelling of either ear was observed after challenge with the NP396-404 control peptide, further demonstrating that the inflammation occurred in an antigen-specific manner. Consistent with the observed pathology, there was an overall increase in the total number of CD45⁺ leukocytes 40 h later in the VacV-GP33–infected skin challenged with GP33-41 peptide compared with the peptide–challenged VacV-OVA–infected skin (Fig. 8 B). Further phenotypic analysis of the CD45⁺ cell infiltrate demonstrated significant increases in leukocytes that were myeloid derived, including neutrophils (Ly6G⁻/Ly6C⁺) and inflammatory monocytes (CD11b⁺/Ly6C⁻/Ly6G⁻; Fig. 8, C–E). However, the inflammatory response was not limited to a myeloid-derived infiltrate, as total CD8⁺ T cell numbers also increased after GP33-41 challenge of the skin previously infected with VacV-GP33 (Fig. 8 F). Thus, these data demonstrate that Trm CD8⁺ T cells that form in an antigen-dependent manner during VacV infections are potent initiators of localized inflammatory responses after restimulation by antigen.

DISCUSSION

Trm CD8⁺ T cell formation has been believed to occur largely independently of antigen recognition in many nonlymphoid tissues including the skin. In fact, after acute infection with LCMV, Trm CD8⁺ T cells can be found in several nonlymphoid tissues, including the gut, female reproductive tract, heart, lung, liver, and stomach (Masopust et al., 2010; Steinert et al., 2015). It has therefore been proposed that both the trafficking of effector CD8⁺ T cells into tissue microenvironments, as well as the subsequent Trm formation, occurs stochastically during systemic viral infections. However, with systemic infections, the mechanisms that regulate the recruitment of effector CD8⁺ T cells into tissues or whether there is any stimulation by antigen locally are difficult to define. Therefore, in this study, we used localized co-infections of the skin with VacV expressing model antigens to directly determine how localized antigen in nonlymphoid tissues regulates the magnitude of Trm generation after a viral infection. In agreement with recent data, we found that recruitment of activated CD8⁺ T cells into skin occurred in an antigen-independent, but inflammation-dependent, manner (Nolz and Harry, 2014; Hickman et al., 2015). However, once in the infected skin, optimal conversion of activated CD8⁺ T cells into Trm requires cognate antigen in the local microenvironment. Overall, these data demonstrate that both inflammation-mediated trafficking, as well as antigen-dependent signaling, cooperate to shape the formation of Trm CD8⁺ T cell populations in the skin, and perhaps other nonlymphoid tissues.

One possible explanation for how local antigen enhances Trm CD8⁺ T cell formation is that a secondary TCR engagement is driving a unique Trm differentiation pathway that has not yet been described in other model systems. This seems unlikely, as Trm CD8⁺ T cells that form after VacV infection do not exhibit any obvious phenotypic or functional differences compared with Trm generated after other viral or bacterial infections. On the other hand, Trm generation may indeed be regulated almost exclusively by cytokine-mediated mechanisms, but antigen recognition may be acting as a stop signal to help retain more activated CD8⁺ T cells at the site of infection as they differentiate into Trm. In fact, studies using intravital microscopy have shown that antigen–specific CD8⁺ T cells will form stable contact with VacV-infected cells in the skin, a process that also appears to require migration mediated by the CXCR3 chemokine receptor (Hickman et al., 2015). Because our data also show that recently activated CD8⁺ T cells will rapidly begin expressing Nur77 and CD69 after a secondary antigen encounter, interactions with infected cells are the most likely source of TCR stimulation that is driving differential expression of CD69. Furthermore, our current study also does not rule out the possibility that there could be residual antigen in the infected skin that could influence Trm maintenance. In fact, VacV antigens have been shown to persist in the draining lymph node for several days after resolution of the primary infection (Tamburini et al., 2014). Future studies exploring how chemokine receptors mediate migration toward virus-infected cells presenting cognate antigen and whether there is antigen persistence will be needed to fully understand how the integration of specific chemokine receptors and TCR signaling cooperate to tune the formation of Trm CD8⁺ T cell populations.
S1PR1-mediated signaling regulates the egress of T cells from lymph nodes and has also been implicated in directing the migration of activated CD8+ T cells out of peripheral tissues and into draining lymphatic vessels (Ledgerwood et al., 2008). In fact, a recent study demonstrated that overexpression of S1PR1, or its transcriptional regulator Kruppel-like Factor 2 (KLF2), causes diminished Trm formation and in vitro stimulation of previously activated CD8+ T cells with TGF-β and IL-33 results in decreased expression of both S1PR1 and KLF2 (Skon et al., 2013). Besides changes in expression of S1PR1, S1PR1-mediated migration can also be antagonized by CD69. CD69 can be expressed by CD8+ T cells in response to antigen, type I IFN, and potentially other cytokines, such as TNF. However, our data demonstrate that activated CD8+ T cells in VacV-infected skin did not express significant levels of CD69 without antigen, suggesting that type I IFN and other cytokines may not play a major role in regulating expression of CD69 in the skin after this viral infection. Thus, the cytokine milieu that is present after VacV infection does not appear to be sufficient for CD8+ T cells to express CD69 or differentiate into Trm in the absence of local antigen stimulation.

In addition to CD69, the generation of Trm CD8+ T cells in the skin also requires expression of the αE integrin CD103. TGF-β is a potent stimulator of CD103 expression on CD8+ T cells both in vitro and in vivo (Casey et al., 2012) and is believed to facilitate retention or survival of Trm populations within tissues. In fact, a dominant-negative version of
TGF-βRII limits Trm formation in the intestinal epithelium after LCMV infection, and TGF-βRII−/− CD8+ T cells do not become Trm after HSV-1 infection of the skin (Casey et al., 2012; Mackay et al., 2013). However, TGF-β–mediated expression of CD103 appears to be dispensable for Trm formation in the lamina propria after infection with Yersinia pseudotuberculosis (Bergsbaken and Bevan, 2015), demonstrating that TGF-β and/or expression of CD103 may not be critical for Trm CD8+ T cells to form in all biological contexts or tissues. Interestingly, our data suggest that expression of CD103 occurs in an antigen-independent manner, as approximately half of the CD8+ T cells that localize to VacV-infected skin express this integrin regardless of antigen specificity. Furthermore, expression of CD103 is not prominent until the later stages of the viral infection (day 15 and after), whereas antigen–dependent changes in expression of CD69 and Nur77 can already be detected as early as day 10 after infection. If expression of CD103 is dependent on TGF-β signaling during VacV infection, this would suggest that expression levels of active TGF-β or the capacity for activated CD8+ T cells to respond to TGF-β–mediated signaling does not occur until the infection is resolving. Overall, our data suggest that within the skin microenvironment, antigen and cytokines (such as TGF-β) may be acting in synergy, but perhaps sequentially, to promote the formation of functional Trm CD8+ T cells populations.

Finally, there now seems to be a disparity in the mechanisms that regulate Trm formation during different viral infections. Studies using the HSV-1 model of infection have demonstrated that CD8+ T cells do not require antigen recognition to express CD103 in the skin, but antigen is required for CD103 to be expressed on HSV-specific CD8+ T cells that localize to the ganglia (Gebhardt et al., 2009; Mackay et al., 2012). In agreement with that finding, HSV-specific CD8+ T cells that are maintained in the trigeminal ganglion after corneal infection also continue to express CD69 during the latent stage of the infection (Khanna et al., 2003). The mechanisms that allow CD103+ Trm to form in the skin independent of antigen after HSV infection, but not VacV infection, are currently unclear, but may relate to the pathogenesis of the individual viral infections, in a direct or indirect manner. HSV-1 initially infects epithelial cells of the skin, and then becomes latent in the associated sensory nerve ganglion. CD8+ T cells and IFN-γ are required to maintain HSV in its latent state in mice (Liu et al., 2000, 2001; Knickelbein et al., 2008), suggesting that active, antigen–specific immune recognition plays a role in maintaining viral latency. In contrast, VacV infects predominantly keratinocytes after intradermal infection, and then is cleared from the skin (Hickman et al., 2013). Thus, the cellular tropism of different viruses or the latency capacity of HSV-1 could potentially influence the mechanisms regulating Trm formation. Alternatively, the collection and timing of cytokines produced during different viral infections or localization to different anatomical compartments may also impact the subsequent differentiation of the ensuing memory CD8+ T cell populations. Altogether, these studies suggest that the molecular mechanisms that regulate Trm CD8+ T cell formation may vary depending on the biological context. In addition, it is likely that CD8+ T cells now collectively referred to as Trm may actually consist of a heterogeneous mixture of distinct cell populations that remains to be fully defined.

In summary, our study identifies a critical role for antigen recognition in the tissue microenvironment in shaping the formation of Trm CD8+ T cell populations after a viral infection. Specifically, our data demonstrate that once CD8+ T cells are activated and traffic to the VacV-infected skin in an antigen-independent fashion, a secondary TCR stimulation significantly enhances CD69 expression, which likely helps retain these antigen–specific CD8+ T cells at the site of infection. Another important aspect of our study is that antigen–specific Trm CD8+ T cells that form in the skin are potent stimulators of localized inflammatory responses after cognate antigen challenge. Thus, our data suggest that the presence of antigen in nonlymphoid tissues during either infection or a vaccination protocol will be critical for optimizing the generation of Trm populations. Alternatively, recognition of either self-antigen or common allergens could drive the accumulation of pathogenic Trm CD8+ T cells in the skin and these antigen–specific T cells could be central mediators of detrimental, recurring inflammatory responses. Overall, these findings provide important insight about the mechanisms that result in the generation of Trm cell populations that are important for host defense, but also contribute to chronic inflammatory disorders, such as allergic contact dermatitis and psoriasis.

**MATERIALS AND METHODS**

**Mice and viruses.** C57BL/6j mice were purchased from The Jackson Laboratory and used for experiments at 6–10 wk of age. P14 and OT-I TCR-transgenic mice have been previously described and were maintained by sibling x sibling mating (Pircher et al., 1989; Hogquist et al., 1994). For adoptive transfers, 2.5–5.0 × 10⁶ Thy1.1+ P14 or OT-I CD8+ T cells from either blood or spleen were injected intravenously in 200 µl of PBS. VacV expressing GP33-41 (VacV-GP33) and OVA257-264 (VacV-OVA) have been previously described and were propagated using BSC-40 cells and standard protocols (Oldstone et al., 1993; Restifo et al., 1995). Infections with VacV were performed on anesthetized mice by placing 5 × 10⁶ PFU of virus in 10 µl of PBS on the ventral side of the ear pinna, and then poking the virus-coated skin 25 times with a 27-gauge needle. Quantification of viral load in the infected skin was determined using standard plaque assays on BSC-40 cells. In brief, infected ears were removed and homogenized in 1 ml of RPMI supplemented with 1% fetal bovine serum. Skin homogenates were then subjected to three rounds of freeze–thaw before serial dilutions were inoculated on BSC-40 cells in a 12-well plate that was then covered with 1% Seakem agarose in Modified Eagle Medium (Lonza). Plaques were visualized three days later after overnight incubation with Neu...
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In vivo peptide challenge. GP\textsubscript{33-41} or NP\textsubscript{396-404} peptides (5 µg) were dissolved in 20 µl of 4:1 acetone/DMSO. Mice were anesthetized with isofluorane, and the dissolved peptide solution was applied to the dorsal side of previously infected ears. The ear skin was then gently poked with a 27-gauge needle 10 times. Thickness of the ear pinna was measured on anesthetized mice with a dial micrometer (Ames).

Leukocyte isolation from skin. Ears from infected mice were removed, and the dorsal and ventral sides of the ear pinna were separated and allowed to incubate for 1–2 h at 37°C with 1–2 ml HBSS (Gibco) containing CaCl\textsubscript{2} and MgCl\textsubscript{2} supplemented with 125 U/ml collagenase (Invitrogen) and 60 U/ml DNase-I (Sigma-Aldrich) at 37°C. Whole-tissue suspensions were then generated by gently forcing the tissue through a wire mesh screen. Leukocytes were then purified from whole-tissue suspensions by resuspending the cells in 10 ml of 35% Percoll (GE Healthcare)/HBSS for 10 min at room temperature.

Flow cytometry and antibodies. The following antibodies and corresponding isotype controls were used in this study: CD8\textsuperscript{a} (53–6.7 Tonbo), CD44 (1M7; BioLegend), CD45.2 (104; Tonbo), CD103 (2E7; BioLegend), CD69 (H1.2F3; BioLegend), IFN-γ (XMG1.2; Tonbo), Thy1.1 (OX-7; BioLegend), Thy1.2 (53–2.1; BioLegend), CD127 (A7R34; BioLegend), KLRG1 (2F1; Tonbo), Nur77 (12.14; eBioscience), CD3ε (17A2; BioLegend), Ly6C (HK1.4; BioLegend), Ly6G (IA8; BioLegend), and CD11b (M1/70; BioLegend). H-2D\textsuperscript{b}-GP\textsubscript{33-41} and H-2K\textsuperscript{b}-OVA\textsubscript{257-264} tetramers were provided by J. Harty (University of Iowa, Iowa City, IA). H-2K\textsuperscript{b}-B8R\textsubscript{20-27} tetramer was obtained from the National Institutes of Health tetramer core facility. Staining for surface antigens was performed in PBS/1% fetal bovine serum for 15 min at 4°C. For tetramer binding, cells were incubated for 45 min at room temperature. Data were acquired using either a Fortessa or LSRII Flow Cytometer (BD) in the OHSU Flow Cytometry Core Facility. Flow cytometry data were analyzed using FlowJo software, version 9.9. For depletion of Thy1.1-expressing CD8\textsuperscript{T} cells, mice were treated with 2–3 µg of control rat IgG (Sigma-Aldrich) or anti-Thy1.1 antibody (clone 19E12; BioXCell) in 200 µl of PBS by intraperitoneal injection.

Ex vivo peptide stimulation and intracellular stain. Lymph nodes and spleens were collected on the indicated days after VacV infection, and single-cell suspensions were generated by gently forcing the tissue through a mesh screen. Cells were then incubated for 5 h with 500 nM of the indicated peptides (Bio-synthesis) in the presence of Brefeldin A (BioLegend). Intracellular cytokine staining was performed using the CytoFix/CytoPerm kit (BD) according to the manufacturer’s protocol. For measurement of expression of Nur77 and CD69, whole splenocytes were stimulated with the indicated concentration of GP\textsubscript{33-41} peptide or 250 ng/ml PMA and 500 ng/ml ionomycin in RPMI supplemented with 10% fetal bovine serum. Cells were stained for CD69 and other surface antigens, washed, and then incubated with Transcription Factor Fix/Perm Buffer (Tonbo) for 30 min at room temperature. Cells were washed twice with Perm/Wash Buffer and then incubated with Nur77 antibody in Perm/Wash Buffer for 30 min at room temperature. Cells were then washed two more times with Perm/Wash Buffer and resuspended in PBS for analysis by flow cytometry.

BrdU incorporation. To measure cellular proliferation in vivo, mice were given 2 mg of BrdU (Sigma-Aldrich) in 200 µl PBS by intraperitoneal injection, and then provided drinking water containing 0.8 mg/ml BrdU for the indicated time period after infection. Staining of cells for BrdU incorporation was performed using the BrdU Flow kit (BD) according to the manufacturer’s protocol. In brief, single-cell suspensions of leukocytes were generated from the skin as described in Leukocyte isolation from skin. After staining of surface antigens, cells were treated with Cytofix/Cytoperm buffer for 30 min. Cells were washed with Perm/Wash Buffer, and then fixed with Cytoperm Permeabilization Buffer Plus for 15 min. Cells were again washed with Perm/Wash buffer and then treated with Cytofix/Cytoperm buffer a second time for 10 min. Cells were then incubated with DNase I (0.33 mg/ml in PBS) for 75 min at 37°C before cells were stained with antibody against BrdU (BD) in Perm/Wash Buffer for 30 min at room temperature. Cells were then washed twice with Perm/Wash Buffer and resuspended in PBS for analysis by flow cytometry.

Statistical analysis. Statistical analyses were performed with Prism software (version 6.0; GraphPad Software) using either the paired or unpaired Student’s t test or ANOVA with Tukey’s post-test for significance.

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