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_J Immunol_ 2015; 194:2059-2063; Prepublished online 26 January 2015;
doi: 10.4049/jimmunol.1402256
http://www.jimmunol.org/content/194/5/2059

Supplementary Material  http://www.jimmunol.org/content/suppl/2015/01/23/jimmunol.1402256.DCSupplemental

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Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention

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Tissue-resident memory T cells provide local immune protection in barrier tissues, such as skin and mucosa. However, the molecular mechanisms controlling effector T cell retention and subsequent memory formation in those locations are not fully understood. In this study, we analyzed the role of CD69, an early leukocyte activation marker, in regulating effector T cell egress from peripheral tissues. We provide evidence that CD69 surface expression by skin-infiltrating CD8 T cells can be regulated at multiple levels, including local Ag stimulation and signaling through type I IFNRs, and it coincides with the transcriptional downregulation of the sphingosine-1-phosphate receptor S1P1. Importantly, we demonstrate that expression of CD69, by interfering with sphingosine-1-phosphate receptor function, is a critical determinant of prolonged T cell retention and local memory formation. Our results define an important step in the generation of long-lived adaptive immune memory at body surfaces. The Journal of Immunology, 2015, 194: 2059–2063.

Protective immunity relies on the rapid accumulation of effector T cells within infected tissues that act to control invading pathogens (1). Upon resolution of infection, there remains a population of CD8 T cells that differentiates in situ from KLRG1− effectors to form a long-lived population of tissue-resident memory T (T_{RM}) cells (2–4). These T_{RM} cells provide immediate local immunity (2, 5, 6) and, therefore, their generation holds promise for novel vaccines against pathogens that target barrier tissues, such as skin and mucosa. Although some aspects of peripheral memory formation were determined recently, such as the contributions of the cytokines TGF-β and IL-15 (3, 4, 7), the molecular mechanisms regulating early effector T cell retention as a prerequisite for subsequent T_{RM} cell differentiation are not fully understood.

Peripheral T cell accumulation is regulated at multiple levels, including tissue-specific recruitment, active retention, and local survival (1). In addition, the rate of tissue egress via afferent lymphatics can impact on peripheral T cell numbers, with the chemokine receptor CCR7 playing a key role in T cell exit from normal and acutely infected tissues (8–11). In line with this, Ccr7−/− effector CD8 T cells introduced into uninfected skin fail to exit the injection site and, consequently, show enhanced local conversion into CD69⁺CD103⁺ T_{RM} cells (3). Other migration molecules, such as the sphingosine-1-phosphate (S1P) receptor S1P1, have been implicated in the regulation of peripheral T cell accumulation and long-term retention, although its precise function in this process remains to be determined (10, 12, 13). Effector T cells use S1P1 to sense S1P gradients among blood, tissues, and lymph, thereby guiding entry into efferent lymphatics during egress from lymphoid tissues (14). Expression of S1P1 is driven by the transcription factor KLF2 (15), and down-regulation of KLF2 and S1P1 coincides with establishment of T_{RM} cells in a variety of tissues (12). Accordingly, loss of S1P1 expression forms part of a common T_{RM} cell–specific transcriptional signature recently defined for CD103⁺ T_{RM} cells from skin, lung, and gut (3).

In addition to KLF2-dependent transcriptional regulation of S1P1, the early leukocyte activation marker CD69 can physically interact with S1P1, resulting in mutual inhibition of surface expression and S1P1 degradation (16). As a consequence, CD69 induction can block S1P-mediated T cell egress from lymphoid tissues (17). Despite this, following virus infection, peak numbers of circulating Cd69−/− effector CD8 T cells appear normal (3, 18, 19). Although such results indicate that CD69-dependent regulation of S1P1-mediated egress from lymph nodes (LNs) may have surprisingly little impact on the expansion of virus-specific CD8 T cells, we showed recently that CD69 expression is required for optimal T_{RM} cell formation in nonlymphoid tissues, such as skin and dorsal root ganglia, following infection with HSV (3). Simi-
larly, Cd69−/− CD4 Th cells fail to establish bone marrow–resident populations of memory cells required for the generation of long-lived Ab responses (20). In light of the important role of CD69 in T<sub>RM</sub> cell formation, we analyzed the spatiotemporal kinetics of CD69 expression by CD8 effector T cells in skin. We provide evidence that multiple signals are involved in CD69 regulation and, importantly, define CD69-mediated interference with S1P<sub>1</sub> function as an important checkpoint in prolonged tissue retention and local formation of T<sub>RM</sub> cells.

**Materials and Methods**

**Mice**
Female C57BL/6, B6.SJL-Ptprc<P>Gay3b/BoyJ (B6.CD45.1), gBT-1 × B6. CD45.1, gBT-1-GFP, gBT-1-Cd69<sup>−/−</sup>, gBT-1-I<sup>fluc</sup>/2<sup>−/−</sup> and OT-I × B6. CD45.1 mice (6–10 wk) were bred in the Department of Microbiology and Immunology, The University of Melbourne. All animal experiments were approved by The University of Melbourne Animal Ethics Committee.

**Virus infection and treatment with immunomodulatory compounds**
Mice were infected by skin scarification with 1 × 10<sup>6</sup> PFU HSV-1 KOS, as described (2). For 1-fluoro-2,4-dinitrobenzene (DNFB) treatment, 15 µl 0.3% DNFB (Sigma-Aldrich) in acetone/oil (4:1) was applied to a 1-cm<sup>2</sup> area of skin 2 d post-HSV infection. FTY720 (1 mg/kg) was dissolved in 2% cyclodextrin (both from Sigma-Aldrich) in PBS and administered to mice by i.p. injection from 6–8 d postinfection. Cells were treated with pertussis toxin (100 µg/ml; Sigma-Aldrich) for 90 min prior to transfer.

**T cell transfers and in vitro activation**
Naive gBT-1 cells isolated from LNs were transferred i.v. at 5 × 10<sup>6</sup> cells/population or at 2.5 × 10<sup>5</sup> cells/population in cotransfer experiments (cells transferred at 1:1). In vitro–generated effector gBT-1 and OT-I cells were activated with gB<sub>498</sub>–505 or OVA<sub>257</sub>–264-pulsed targets for 4–5 d in the presence of IL-2 (25 U/ml; PeproTech), as described (2), and 0.5–1 × 10<sup>6</sup> cells were injected intradermally, as described (3).

**Dendritic cell–T cell cocultures**
Dermal dendritic cells (DCs) were isolated from infected skin 5 d postinfection and sorted into CD11c<sup>+</sup>CD11b<sup>+</sup> cells by flow cytometry, as described (21). DCs (5 × 10<sup>5</sup>) were cultured with in vitro–activated T cells (1.25 × 10<sup>5</sup>) for 18 h prior to flow cytometric analysis. Control conditions contained 1 µg/ml gB<sub>498</sub>–505 or OVA<sub>257</sub>–264 peptide.

**Flow cytometry and Abs**
T cells were recovered from skin, as described (2, 6). Briefly, skin tissue was incubated for 90 min at 37°C in dispase (Roche; 2.5 mg/ml), followed by the separation of epidermis and dermis. Epidermal sheets were incubated for 30 min in trypsin/EDTA (0.25%/0.1%), and the remaining skin tissue was chopped into small fragments and incubated for 30 min at 37°C in collagenase III (Worthington; 3 mg/ml). Cells suspensions were stained with the following Abs for flow cytometry: anti-CD45.1 (A20), anti-CD45.2 (B20.1), anti-CD8α (53-6.7), anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD69 (H1.2F3), anti-TCRβ (H57-597), and anti-CD103 (2E7) (BD Pharminogen or ebBioscience).

**Histology**
Flank skin was harvested and processed, as described (3). Briefly, tissue sections were fixed in PLP (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.2 M L-lysine, and 0.1 M sodium periodate with 2% paraformaldehyde), snap frozen, and stained with rabbit anti-keratin 14 (AF64; Covance), Alexa Fluor 647–conjugated donkey anti-rabbit (A31573; Invitrogen), or PE-conjugated anti-CD69 (H1.2F3) Ab. Images were acquired with a Zeiss LSM710 microscope and processed using Imaris 7.1 software (Bitplane).

**Quantitative RT-PCR**
gBT-1 cells were isolated from skin and LNs and sorted by flow cytometry, using a FACSCanto III (BD Biosciences), as FITC<sub>Vα2</sub>CD45.2<sup>+</sup>CD<sup>+<sub>CD4</sub> T<sub>CD<sub>11c</sub>−</sub>CD11b−</sub> events, as indicated. RNA extraction, cDNA synthesis, preamplification, and quantitative RT-PCR (Q-PCR) were performed using TaqMan Gene Expression Cells-To-Ct Kit, TaqMan Fast Preamp MasterMix, and TaqMan assays (Mm00437762_m1, Mm0046073_m1, Mm00460968_m1, Mm01244979_g1, and Mm00514644_m1) with TaqMan Fast Advanced MasterMix (Life Technologies). The threshold cycle of respective gene for each cell population was normalized to the mean of Hprt, B2m, and Tbp (ΔCt). Normalized gene expression was compared with that of naive gBT-I cells according to the 2<sup>−ΔΔCt</sup> method.

**Statistical analysis**
All statistically significant differences were determined using the Mann-Whitney U test and GraphPad Prism software (GraphPad).

**Results and Discussion**

**Early induction of CD69 expression by skin-infiltrating effector T cells**
We showed previously that expression of CD69 is required for efficient effector T cell retention in infected skin and consequently, for the formation of T<sub>RM</sub> cells (3). Because CD69 appeared to function early after skin entry, we sought to determine its expression kinetics in skin-infiltrating effector T cells. To track virus-specific CD8 T cell responses, we used skin infection with HSV in combination with adoptive transfer of naive TCR-transgenic T cells (gBT-I) specific for a natural determinant derived from the viral glycoprotein B. Flow cytometric analysis revealed the presence of CD69 expression from the earliest detection of gBT-I cells in skin 5 d postinfection, with an enrichment in the epidermal layer (Fig. 1A, Supplemental Fig. 1A). Although the percentage of CD69<sup>+</sup> cells decreased on day 6, most likely as a result of increased infiltration by CD69<sup>−</sup> cells at this time (21), by 11 d postinfection the vast majority of gBT-I cells in the skin expressed high levels of CD69. In contrast, their counterparts in the spleen were largely CD69<sup>−</sup> at all times, and only few cells in LNs draining the site of infection were CD69<sup>+</sup> (Fig. 1A, Supplemental Fig. 1B). Microscopy to track transferred GFP<sup>+</sup> gBT-I cells in sections stained with Abs against gBT-I confirmed the presence of CD69<sup>+</sup> gBT-I cells in the epidermis, hair follicle epithelium, and dermis 6 and 14 d postinfection (Supplemental Fig. 1C). Of note, by 14 d the majority of gBT-I cells were CD69<sup>+</sup> and localized to the epidermis or were associated with hair follicles (78 ± 5% on day 14 versus 21 ± 3% on day 6), with only few cells found in the dermis proper. Q-PCR analysis of purified gBT-I populations further revealed that CD69 surface expression by skin-infiltrating effector gBT-I cells was associated with an increase in the abundance of CD69 RNA transcripts compared with their CD69<sup>−</sup> naive counterparts (Fig. 1B). Together, these results demonstrated an early induction of CD69 surface expression in skin-infiltrating effector CD8 T cells that was associated with transcriptional upregulation.

**DCs can provide Ag and type I IFN for CD69 induction in T cells in vitro**
TCR-dependent activation and exposure to various combinations of cytokines, including type I IFN, IL-33, TNF-α, and TGF-β, induce CD69 expression in T cells (7, 22). Given that we observed CD69 expression by T cells in both skin epithelium and dermis, we contemplated whether DCs accumulating in these tissue compartments during infection (21) could provide the relevant Ag and/or noncognate activation signals to T cells. To investigate this, we sorted CD11c<sup>+</sup>CD11b<sup>+</sup> DCs from infected skin, which include dermal and monocyte-derived inflammatory DC subsets (21), and cultured them with in vitro–activated CD8 T cells. Culture for 18 h resulted in induction of CD69 surface ex-
expression by roughly half of gBT-I cells (Fig. 2A, left panel). Of note, expression levels on CD69+ gBT-I cells were similar to those from cultures containing cognate peptide. In contrast, CD69+ gBT-I cells were largely absent from control cultures without DCs or peptide Ag. Interestingly, DCs from HSV-infected skin also induced CD69 expression in a considerable portion of effector OT-I cells with an irrelevant specificity, although expression levels on CD69+ cells from these cultures were marginally lower than those from peptide-control conditions (Fig. 2A, middle panel). These results indicated that, although Ag recognition may promote CD69 induction in vitro, other noncognate stimuli appeared to have a more pronounced effect in those assays, at least in the absence of high concentrations of exogenous Ag. In line with this, gBT-I cells deficient in expression of type I IFNRs (gBT-I,

Ag and type I IFN are dispensable for CD69 expression in vivo

To assess the extent to which Ag recognition was required for CD69 induction in vivo, we infected gBT-I recipient mice with HSV and induced skin inflammation on contralateral flanks by treatment with DNFB 2 d later (6). Skin lesions on both flanks were resolved by day 10. Effector gBT-I cells primed by HSV infection infiltrated both infected and non-infected skin regions and, subsequently, formed a population of CD103+ TRM cells (data not shown). Interestingly, until 2 wk postinfection, significantly higher fractions of gBT-I cells expressed CD69 in previously HSV-infected skin compared with nontreated skin, although high proportions of CD69+ gBT-I cells were found in both locations at later times (Fig. 2B). This result was consistent with a role for local Ag recognition in early CD69 upregulation or, alternatively, it could have reflected differences between the inflammatory environments in virus-infected and chemically inflamed skin. Nevertheless, in situ Ag recognition was not strictly required for CD69 induction in T cells that persisted well beyond resolution of infection or inflammation. Likewise, type I IFN signals were not required, because gBT-I,

Late CD69 expression coincides with Klf2 and S1pr1 downregulation

It was shown recently that CD69 surface expression is associated with transcriptional downregulation of Klf2 and S1pr1 expression in effector T cells upon entry into peripheral tissues (12). To correlate the kinetics of CD69 expression with the regulation of these genes during skin infection, we analyzed the abundance of Klf2 and S1pr1 RNA transcripts in gBT-I cells purified from infected skin using Q-PCR, as in Fig. 1B. Although an ~2-4-fold downregulation was evident 8 d postinfection, we observed up to a 100-fold reduction in mRNA transcripts encoding both Klf2 and S1pr1 in CD103+ gBT-I cells isolated 14 d postinfection (Fig. 2D). Thus, transcriptional regulation of Klf2 and S1pr1 expression correlated with CD69 surface expression by gBT-I cells at later times, whereas this correlation was less evident for early infiltrating T cells.

CD69 regulates T cell retention after intradermal transfer

Our results were in line with previous studies showing that CD69 expression could be regulated by multiple factors, including Ag-independent signals (3, 7, 22, 23). To test whether CD69 expression was functionally involved in T cell retention in the absence of local Ag recognition, we coinjected in vitro–activated wt and CD69−/− gBT-I cells into flank skin of naive mice. Although roughly equal frequencies of wt and CD69−/− gBT-I cells were recovered from skin 4 d postin-
CD69 interference with S1P receptor function regulates T cell retention

Our results suggested that CD69 controlled early T cell retention and, consequently, the size of the local T<sub>RM</sub> cell population. However, the mechanism by which CD69 regulated retention remained unclear. Based on previous reports on the functional interaction of CD69 with S1P<sub>1</sub> for the regulation of T cell egress from lymphoid tissues (17), we hypothesized that the defective retention that we observed for Cd69<sup>−/−</sup> T cells was related to their enhanced responsiveness toward S1P. To address this, we compared skin retention of wt and Cd69<sup>−/−</sup> gBT-I cells in HSV-infected mice that were treated with FTY720, an agonist of S1P receptors rendering T cells unresponsive toward S1P signals (24). In line with our previously published data (3), although both types of gBT-I cells infiltrated infected skin in equal numbers at 6 d postinfection, there were roughly 5-fold more wt cells than Cd69<sup>−/−</sup> gBT-I cells by day 9 in untreated mice (Fig. 4, Supplemental Fig. 2D). Conversely, because FTY720 treatment did not affect the number of wt effector cells, CD69 expression alone appeared to interfere effectively with S1P receptor function in regulating tissue egress. In addition, the lack of FTY720 effects on wt cells argued against relevant T cell–extrinsic effects of FTY720 in those experiments. As such, our combined results support the view that CD69-mediated regulation of S1P-dependent egress can impact strongly on local effector T cell accumulation.

In summary, our results describe a complex and redundant regulation of CD69 expression by effector T cells that directly impacts on effector cell retention and subsequent memory formation in skin. Although we cannot rule out other functions of CD69, our results are consistent with a scenario whereby induction of CD69 expression is critical for early effector T cell retention by blocking S1P-regulated tissue egress prior to complete downregulation of S1P<sub>1</sub> expression coupled with T<sub>RM</sub> cell differentiation (3, 12). In contrast, once S1P<sub>1</sub> expression is extinguished through K<sub>J2</k><sub>2</sub> downregulation in T<sub>RM</sub> cells, CD69 surface expression may become functionally redundant in regulating S1P responsiveness. Interestingly, Ag recognition and exposure to type I IFN do not appear to be strictly required for CD69 induction in T cells, consistent with the observation that T<sub>RM</sub> cells can form in the absence of local Ag recognition or overt inflammation (3, 5–7). Nevertheless, given the strong induction of CD69 expression by Ag stimulation and exposure to inflammatory cytokines, it appears likely that such signals cooperate to support effector T cell retention and T<sub>RM</sub> cell generation (2, 6, 12, 19). Overall, our results define the early induction of CD69 expression as an important checkpoint in peripheral effector T cell retention and the generation of long-lived immune memory at body surfaces.

Disclosures

The authors have no financial conflicts of interest.

References


