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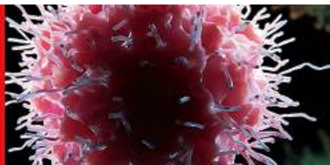

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The Functional Requirement for CD69 in Establishment of Resident Memory $CD8^+$ T Cells Varies with Tissue Location

Daniel A. Walsh,*,† Henrique Borges da Silva,*,† Lalit K. Beura,*,‡ Changwei Peng,*,† Sara E. Hamilton,*,† David Masopust,*,‡ and Stephen C. Jameson*,†

Recent studies have characterized populations of memory $CD8^+$ T cells that do not recirculate through the blood but are, instead, retained in nonlymphoid tissues. Such $CD8^+$ tissue resident memory T cells (T_{RM}) are critical for pathogen control at barrier sites. Identifying T_{RM} and defining the basis for their tissue residency is therefore of considerable importance for understanding protective immunity and improved vaccine design. Expression of the molecule CD69 is widely used as a definitive marker for T_{RM}, yet it is unclear whether CD69 is universally required for producing or retaining T_{RM}. Using multiple mouse models of acute immunization, we found that the functional requirement for CD69 was highly variable, depending on the tissue examined, playing no detectable role in generation of T_{RM} at some sites (such as the small intestine), whereas CD69 was critical for establishing resident cells in the kidney. Likewise, forced expression of CD69 (but not expression of a CD69 mutant unable to bind the egress factor S1PR1) promoted $CD8^+$ T_{RM} generation in the kidney but not in other tissues. Our findings indicate that the functional relevance of CD69 in generation and maintenance of $CD8^+$ T_{RM} varies considerably, chiefly dependent on the specific nonlymphoid tissue studied. Together with previous reports that suggest uncoupling of CD69 expression and tissue residency, these findings prompt caution in reliance on CD69 expression as a consistent marker of $CD8^+$ T_{RM}. ***The Journal of Immunology*, 2019, 203: 946–955.**

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$CD8^+$ tissue resident memory T cells (T_{RM}) play a key role in protecting nonlymphoid tissues (NLT) from reinfection (1). Expression of the C-type lectin CD69 and the integrin chain αE (CD103) are often considered definitive markers for typical $CD8^+$ T_{RM}. Because CD103 is an adhesion receptor for E-cadherin, its contribution to tissue residency in epithelial tissues is predictable. Yet, $CD8^+$ T_{RM} in many nonlymphoid sites do not express CD103, and even in NLT, where $CD103^+$ T_{RM} are abundant, CD103 was not always required for their generation (2), suggesting the functional role for CD103 in establishing residency is limited. CD69, by contrast, is expressed by the vast majority of T_{RM} in diverse NLT, yet its contribution to residency is unclear. Increased cell-surface CD69 can be driven by either TCR stimulation or certain cytokines (3). CD69 binds and antagonizes the cell-surface expression of G-protein–coupled sphingosine-1-phosphate receptor-1 (S1PR1) in a cell-intrinsic manner (3, 4). S1PR1 signaling promotes trafficking toward its lipid ligand, sphingosine-1-phosphate (S1P), which is found in high concentrations in the blood and lymph but much lower concentrations in tissues. In this way, S1PR1 provides a critical mechanism for T cell egress from lymphoid and nonlymphoid sites (5). By inhibiting expression of S1PR1, CD69 can therefore impair egress and promote T cell residency (6, 7). In this way, CD69 expression may promote establishment of resident cells in NLT during the acute phase of the immune response. In addition to regulation of S1PR1, other functions of CD69 have been defined (8, 9), although whether these impact $CD8^+$ T cell residency programs are not known. As a result of the widespread expression of CD69 on $CD8^+$ T_{RM} and its known effect on S1PR1, many consider $CD69^+$ cells (with or without CD103 coexpression) as de facto tissue resident, and this criteria has been adopted in studies of T_{RM} in mice, humans, and nonhuman primates (10–12).

However, the fidelity of CD69 expression as a critical characteristic of $CD8^+$ T_{RM} has been called into question. In the context of lymphocytic choriomeningitis virus (LCMV) infection, some definitively tissue-resident T_{RM} (as defined by parabiosis studies), fail to express CD69 (13). Likewise, several studies in mice and humans showed no increased *Cd69* gene expression in $CD8^+$ T_{RM} compared with recirculating memory cells (even, remarkably, when CD69 protein expression itself was used to separate these populations) (11, 14). It is possible, however, that these situations reflect a transient requirement for strong CD69 expression in seeding resident $CD8^+$ T cells, and that CD69 expression can subsequently decline in established $CD8^+$ T_{RM}. Some studies are consistent with such a model (15). Alternatively, CD69 could be a purely passive marker rather than a functional regulator of tissue residency. This hypothesis is based on the fact that mutual antagonism of CD69 and S1PR1 for cell-surface expression results in CD69's appearance at the plasma membrane of T cells expressing low levels of S1PR1 (16). The transcription factor

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Abbreviations used in this article: CD103, integrin chain αE; FRT, female reproductive tract; IEL, intraepithelial; LCMV, lymphocytic choriomeningitis virus; LP, lamina propria; NLT, nonlymphoid tissue; sg, single-guide; SI, small intestine; S1P, sphingosine-1-phosphate; S1PR1, sphingosine-1-phosphate receptor-1; T_{RM}, resident memory T cell; VSV-OVA, vesicular stomatitis virus–expressing OVA; WT, wild-type.

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KLF2 promotes S1PR1 expression, and both S1PR1 and KLF2 are downregulated in CD8$^+$ T$_{RM}$ (11, 14, 17); this loss of expression is functionally important, because sustained expression of KLF2 or S1PR1 blocked establishment of CD8$^+$ T$_{RM}$ (17). Hence, transcriptional downregulation of S1PR1 could play the key role in establishing residency versus recirculation, with elevated cell-surface CD69 expression on T$_{RM}$ simply serving as a marker of S1PR1 low cells, rather than constituting an active player in driving tissue residency.

Still, CD69-mediated inhibition of S1PR1 and transcriptional regulation of S1PR1 might both play critical roles in generation of T$_{RM}$, perhaps operating sequentially during T$_{RM}$ establishment and maintenance (15). Alternatively, CD69-mediated inhibition of S1PR1 and transcriptional regulation of S1PR1 could be differentially involved in forming T$_{RM}$, depending on the model or specific NLT studied. As a more definitive test of the mechanistic role played by CD69, various groups have examined T$_{RM}$ generation by CD69-deficient (*Cd69*$^{-/-}$) CD8$^+$ T cells. These studies have yielded mixed results. Some studies indicated that *Cd69*$^{-/-}$ CD8$^+$ T cells were severely disadvantaged in generation of CD8$^+$ T$_{RM}$, whereas other reports found a much more moderate effect or a minimal defect in generation of *Cd69*$^{-/-}$ CD8$^+$ T$_{RM}$ (15, 18–20). These studies used a variety of models but focused on a limited set of NLTs, making it difficult to assess the role of CD69 in forming T$_{RM}$ at diverse tissue sites.

To more broadly examine CD69's impact on CD8$^+$ T cell residency, we explored generation of T$_{RM}$ by *Cd69*$^{-/-}$ CD8$^+$ T cells in multiple NLT, following the response to pathogens that establish tissue-specific or systemic infections, and subunit vaccination. Our results indicate that, contrary to current dogma, CD69 expression plays no detectable role in establishment or maintenance of CD8$^+$ T$_{RM}$ in some NLT, such as the small intestine (SI) intraepithelial (IEL) and lamina propria (LP) populations, whereas a substantial requirement for CD69 was observed for CD8$^+$ T$_{RM}$ generation in the kidney. CD69 expression moderately favored CD8$^+$ T$_{RM}$ establishment in the lung following influenza infection and the female reproductive tract (FRT) following LCMV infection. In complementary studies, forced expression of CD69 enhanced generation of T$_{RM}$ in the kidney but had minimal impact on induction of T$_{RM}$ in the other NLT tested. Our studies reveal that, despite the near ubiquity of cell-surface CD69 as a feature of CD8$^+$ T$_{RM,}$ its functional relevance for establishing or maintaining tissue residency is highly variable across distinct nonlymphoid sites. This suggests caution in interpreting the presence or absence of CD69 expression as an indication of CD8$^+$ T cell tissue residency.

Materials and Methods

Mice

C57BL/6 and B6.SJL mice were purchased from the National Cancer Institute. CD69 deficient mice were provided by Dr. Linda Cauley at the University of Connecticut (18) and crossed to P14 and OT1 TCR transgenic animals, as well as onto the C57BL/6 background. These mice were originally derived by Dr. Toshinori Nakayama at the University of Chiba. KLF2/GFP-expressing mice (21) were crossed to P14 TCR transgenic animals. Animals were maintained under specific pathogen-free conditions at the University of Minnesota. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Adoptive transfers

A mixture of splenocytes containing between 5 × 10^2 and 5 × 10^5 TCR transgenic cells at an approximately 1:1 ratio were coadoptively transferred into recipients. When the precursor frequency is not specified, 5 × 10^3 TCR transgenic cells were used. C57BL/6 or B6.SJL recipients were used depending on the congenic markers of the transferred cells.

Pathogens and infections

When applicable, 2 × 10^5 LCMV strain Armstrong was injected i.p. (22). A total of 1 × 10^6 vesicular stomatitis virus–expressing OVA (VSV-OVA) Indiana strain was injected intravascularly. A total of 5 × 10^2 PR8 influenza-gp33 was applied intranasally following anesthesia with ketamine and xylazine. VSV-OVA (23) and PR8 influenza-gp33 (24) were provided by Dr. David Masopust at the University of Minnesota.

TriVax immunization

A mixture containing 250 μg of gp33 peptide, 100 μg of agonist αCD40, and 50 μg of poly(I:C) was injected into the tail veins of recipient mice. Protocol was modified from Cho et al. (25).

In vivo 16-h migration assay

A mixture of splenocytes containing 5 × 10^3 CD69-sufficient and -deficient P14 cells at an approximately 1:1 ratio was coadoptively transferred into congenically distinct recipients. The next day, recipients were infected with LCMV Armstrong. Six days postinfection, CD8$^+$ T cells were purified via MACS negative selection from the spleens of recipients, and 5 × 10^6 CD8$^+$ T cells were adoptively transferred into infection-matched recipient animals. Sixteen hours after the adoptive transfer, the secondary recipients were euthanized, and lymphocytes were isolated from the kidney and other tissues. Protocol was modified from Ma et al. (26).

In vitro activation followed by intradermal cotransfer

Splenocytes were isolated from CD69-sufficient and -deficient P14 TCR transgenics and separately activated in vitro with plate-bound αCD3 and αCD28 as well as 10 ng/ml Il-2 for 72 h at 37°C. At 48 h, media was changed, and cells were moved to new plates without αCD3 and αCD28. After the 72 h incubation, an ∼1:1 mixture of cultured cells containing 1 × 10^6 total cells was made and was intradermally transferred into congenically distinct recipient animals. Nine days after the intradermal transfer, animals were euthanized, and lymphocytes were isolated from the skin and other tissues.

Isolation of lymphocytes from NLT

To differentiate cells in circulation from those in NLT, mice underwent the i.v. labeling procedure as previously described (27), with a 5-min gap between i.v. Ab injection and euthanasia. Lung, kidney, and salivary gland were digested with collagenase I and buffers as previously described (17), with the following alterations. Lung was incubated with collagenase I and digestion buffer for 60 min. Additionally, following enzymatic digestion, but prior to Percoll purification all of these tissues were ground using a gentleMACS Tissue Dissociator, using the program "Spleen1" twice. Cells were isolated from the liver using the gentleMACS Tissue Dissociator, followed by Percoll purification, as in other tissues. Cells were isolated from the IEL, LP, spleen, and inguinal lymph nodes as previously described (17). Lymphocytes were isolated from the skin and FRT as described by Beura et al. (28).

Retroviral transduction of P14 cells

CD8 T cells were purified by magnetic separation and subsequently activated in vitro with plate-bound αCD3 and αCD28 for 24 h at 37°C. Cell cultures were then spinfected for 2 h at 37°C with viral supernatant containing polybrene, βME, and 10 ng/ml mIl-2. After spinfection, cells rested for 48 h. Cells were then adoptively transferred into congenically distinct recipient animals, who were infected with LCMV Armstrong 1 d later. Adoptive transfers contained 1 million total cells, between 3 and 36% of which were transduced with empty vector, CD69, or CD69Δ31 (4), as determined by the transduction marker CD90.1. Plasmids encoding these constructs were kindly provided by Dr. Jason Cyster (University of California, San Francisco). Transduced cells were adoptively transferred either alone or in competition with congenically distinct empty vector-transduced cells. Protocol was modified from Skon et al. (17).

In situ peptide stimulation of FRT T$_{RM}$

At least 30 d after coadoptive transfer, followed by LCMV infection, animals were challenged transcervically with gp33 peptide as in Beura et al. (29). Twelve hours later, lymphocytes were isolated from NLT and analyzed by flow cytometry.

Parabiosis

C57BL/6J mice were cotransferred with CD69-sufficient and -deficient P14 cells at an approximately 1:1 ratio 1 d prior to being infected with LCMV.

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A separate group of mice received no transgenic T cells but were infected with LCMV to create infection-matched mice. At least 30 d postinfection, the P14 transgenic mice were surgically joined along the flank to the infection-matched, non-P14–bearing mice. Parabiosis surgery was performed as described in Beura et al. (29). Equilibration of circulating T cell population was confirmed in the peripheral blood of conjoined pairs 4 wk postsurgery. Parabiotic pairs were euthanized, and lymphocytes were harvested from indicated tissues 29 or 38 d postsurgery. The formula

$$100 \times (1-((\#\text{ in recipient parabiont } \times 2)/(\#\text{ in donor} + \text{recipient parabiont})))$$

was adapted from Steinert et al. (13) and was used to calculate percent residency.

CD69 knockout via CRISPR/Cas9

KLF2/GFP P14 $CD8^+$ T cells were isolated by magnetic separation and activated with plate-bound αCD3 and αCD28 (both 5 μg/ml) and 100 U/ml rhIl-2 for 48 h at 37°C. The next day, a 3:1 mixture of single-guide (sg)–RNA with Cas9 was made and incubated at 20°C for 10 min prior to nucleofection using a Lonza 4D-Nucleofector X Unit (program CM137) per Seki and Rutz (30). Congenically distinct cells received sgRNA targeting either CD69 (5′-CAUUUUGAGAAGCAUCAUGA-3′ and 5′-CAUCACGUCCUUAAUAAUAG-3′) or a scramble control (5′-GCACUACCAGAGCUAACUCA-3′). Cells were allowed to recover for 2 h at 37°C before being transferred to a 24-well plate with 100 U/ml rhIl-2 for 2 d. Approximately 40 h posttransfection, 5×10^4 of each cell type (CD69/scramble sgRNA) were coadoptively transferred into congenically distinct B6 recipient animals, followed by LCMV infection 1 h later. Aliquots of cells were sequenced 7 d postelectroporation and inference of CRISPR edits scores calculated (ice.synthego.com) to determine targeting efficiency. Inference of CRISPR edits scores ranged from 83 to 86. Effective knockout efficiency was determined via protein expression in vivo (Supplemental Fig. 4).

Statistical analysis

Data were analyzed using GraphPad Prism software (7.0a). Where indicated, one-tailed *t* tests were compared against the known transfer ratio. Normalization of results in experiments involving an adoptive cotransfer of unmanipulated cells were performed by dividing each individual data point by the known transfer ratio for that experimental repeat. Normalization of results in experiments involving transduction with retroviral vectors was performed by calculating the displacement of the percent of transduced cells in each individual sample from the average percent of cells transduced in the spleen. Splenic averages were calculated separately for each experimental repeat. The gate defining transduced cells ($CD90.1^+$) was set on congenically distinct populations of either endogenous host cells or cotransferred empty vector-transduced cells when appropriate. Because the gate defining transduced cells varied from tissue to tissue, ANOVA with multiple comparisons was only used to compare within tissues. ANOVA with multiple comparisons was also used for analysis in Figs. 4 and 6 comparing within tissues. Two-tailed, unpaired, *t* test was used in Supplemental Fig. 2. Two-tailed, paired *t* test was used in Fig. 2 and Supplemental Fig. 4. Datasets as prism files are available on request.

Results

CD69 deficiency does not impact $CD8^+$ T cell residency in many NLT

Previous studies suggested a role for CD69 in generating site-specific T_{RM} following local infections (the skin after HSV infection and the lung subsequent to influenza infection) (15, 18–20). Whether there is a requirement for CD69 in establishing T_{RM} in multiple NLT following systemic infections was unclear. Because T_{RM} have been extensively investigated in the context of acute LCMV infection in mice (2, 13), we first used this model. Equal numbers of wild-type (WT) and *Cd69*$^{-/-}$ P14 (LCMV gp33 epitope–specific transgenic T cells) $CD8^+$ T cells were cotransferred into congenically mismatched recipients, which were subsequently infected with LCMV Armstrong, and the ratio of the CD69-deficent and -sufficient cells was determined at various timepoints in lymphoid and nonlymphoid sites (Fig. 1A). Animals underwent i.v. Ab labeling prior to the tissue harvest to distinguish between cells in the vasculature and the tissue parenchyma (27, 31) (Supplemental Fig. 1A). Unexpectedly, analysis at memory timepoints (>day 30) showed minimal differences in the relative proportions of WT and *Cd69*$^{-/-}$ P14 cells in the parenchyma of many tissue sites, although we observed a 3–5-fold disadvantage for *Cd69*$^{-/-}$ P14 in the kidney and a slightly increased proportion of *Cd69*$^{-/-}$ $CD8^+$ T cells in the parenchymal cells of the lung as well as among cells within the circulation (blood and spleen; Fig. 1B, vascular-associated cells in NLT, Supplemental Fig. 1B). Because the magnitude of these changes were relatively mild, data were aggregated from individual experiments (an example is given in Supplemental Fig. 1C), following normalization to the adoptive transfer ratio.

Previous studies suggested that the functional relevance of CD69 may be more substantial during initial establishment of the tissue-resident population (15, 17, 18); therefore, we also examined the relative distribution of WT and *Cd69*$^{-/-}$ P14 at the effector phase (day 7) of the LCMV response. Similar to memory phase, there was minimal impact of CD69 deficiency on representation of P14 cells in the parenchyma of many tissues, although, again, there was a significant underrepresentation of this population in the kidney (Fig. 1C). The magnitude of the kidney residency defect was maintained from this acute timepoint until late memory (Supplemental Fig. 1D). Hence, our data suggest a minimal role for CD69 in generating or retaining tissue-resident $CD8^+$ T cells during the response to acute LCMV infection.

It was important to investigate whether CD69 deficiency impacted $CD8^+$ T cell homeostasis or initial activation, because this could potentially offset a requirement for CD69 in T_{RM} generation. Previous work showed that CD69 deficiency has no discernable effect on $CD8^+$ T cell expansion or cytotoxic function following activation (32–34). We observed that CD69 deficiency slightly altered the phenotype, but not the numbers of naive P14 $CD8^+$ T cells (Supplemental Figs. 1G, 2A), that WT and *Cd69*$^{-/-}$ P14 populations showed similar parking efficiency following adoptive transfer into animals that were sacrificed prior to infection (Supplemental Fig. 2B) and that expression of activation and memory markers on WT and *Cd69*$^{-/-}$ P14 $CD8^+$ T cells was similar over multiple timepoints (Supplemental Fig. 1G). Finally, it was notable that CD69 expression by WT P14 cells in the blood was essentially undetectable on day 4 of the response to LCMV [when the cells are auditioning for tissue entry (24)], consistent with a negligible impact of CD69 deficiency in these studies (Fig. 1D, Supplemental Fig. 1G).

In addition to acting as cytotoxic effectors, T_{RM} serve as immune sentinels at barrier surfaces (23). To test the alarm functions of T_{RM}, we evaluated peptide rechallenge via the FRT (35). Animals received a cotransfer of WT and *Cd69*$^{-/-}$ P14s, followed by LCMV infection. At memory timepoints, *CD69*$^{-/-}$ P14s were present at slightly reduced frequency in the FRT, similar to what was observed in the kidney (Figs. 1B, 2A). To assess the function of the FRT WT and *CD69*$^{-/-}$ P14 T_{RM}, a cohort of these memory-phase animals was transcervically challenged with cognate gp33 peptide (or PBS as a control) to activate FRT T_{RM} (Fig. 2B) and measured granzyme B, IFN-γ, CD44, and CD69 expression on the P14 population 12 h later (Fig. 2C–E). *Cd69*$^{-/-}$ P14s exhibited strong induction of activation markers, IFN-γ production, and upregulation of the cytolytic molecule granzyme B, albeit some of these responses were modestly altered compared with WT P14 cells in the same site. Hence, our data indicate that, despite reduced frequency of *Cd69*$^{-/-}$ P14 T_{RM} in the FRT, these cells exhibit a potent recall response.

Previous studies, using a distinct *Cd69*$^{-/-}$ strain, had reported substantial defects in generation of skin *Cd69*$^{-/-}$ T_{RM} following intradermal injection of in vitro activated $CD8^+$ T cells

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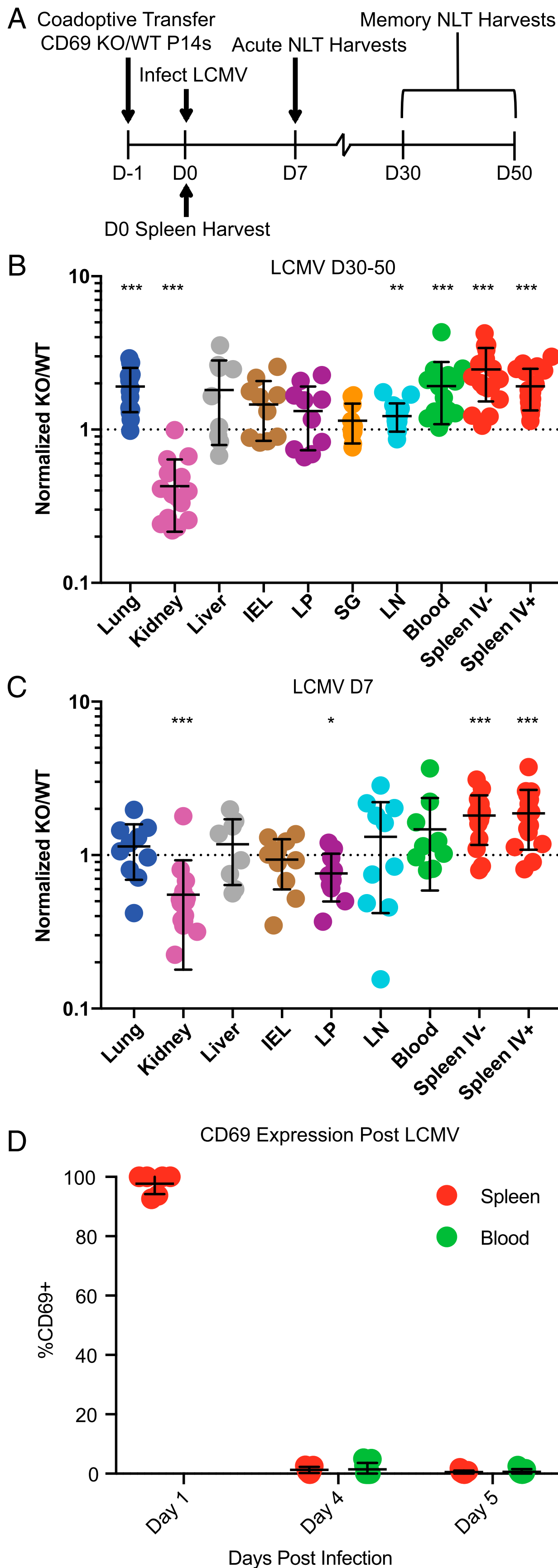

FIGURE 1. CD69 plays a minimal role in promoting tissue residency after an LCMV infection. Animals received a coadoptive transfer of congenically distinct $Cd69^{-/-}$ and WT P14 CD8$^+$ T cells. At both acute and memory timepoints, lymphocytes were isolated from a variety of different NLT (**A**). Graphed is the percentage of i.v.$^-$ transferred cells that were $Cd69^{-/-}$ over the percentage WT, isolated from the indicated tissues days 30–50 (**B**) and 7 (**C**) postinfection (p.i.). The percentage of CD69 WT cotransferred cells expressing CD69 (gates set on $Cd69^{-/-}$ cells) at the indicated timepoints, in the indicated tissues (**D**). The ratio presented on the *y*-axis of (B) and (C) is normalized across experimental repeats by dividing each individual data point by the known transfer ratio for that experimental repeat. Four independent repeats for each of the timepoints are combined in the presented normalization. Error bars show mean ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, one-tailed *t* test against the known ratio of transferred cells on $\log_{10}$ transformed data. SG, salivary gland.

(15). Indeed, we found that CD69 deficiency led to a substantial reduction in the generation of CD103$^+$ skin T_{RM} following this approach (Supplemental Fig. 2C, 2D). Our findings therefore support earlier conclusions on a role for CD69 in controlling CD8$^+$ T cell tissue residency in some assays (15) and, hence, validate the impact of CD69 deficiency in our studies. It is important to note, however, that in this model, CD69 expression by WT cells is clearly detectable at the time of injection (Supplemental Fig. 2E), which contrasts with the low expression of CD69 on blood cells following LCMV infection (Fig. 1D, Supplemental Fig. 1G). Such data suggest that intradermal studies magnify the role of CD69, with respect to cell-surface CD69 at the time of NLT entry.

The site of infection dictates CD69's impact on tissue residency

It was possible that the limited role for CD69 in CD8$^+$ T cell tissue residency we observed following LCMV infection (including the reduction in kidney $Cd69^{-/-}$ T_{RM}), was unique to the tissue tropism of that pathogen. Previous reports showed that CD69 deficiency impairs tissue residency in the lungs after influenza infection (18), and the skin after HSV infection (15, 19), consistent with a role for CD69 being contingent on the site of tissue tropic infection. Hence, we extended our studies to assess the impact of CD69 deficiency on the response to influenza, using a rPR8 strain expressing the gp33 epitope, recognized by P14 CD8$^+$ T cells. In agreement with previous studies (18, 20), we found that $Cd69^{-/-}$ P14 T_{RM} were underrepresented in the lung following influenza infection (Fig. 3A, Supplemental Fig. 2G–K). This contrasts with our findings after LCMV infection, in which $Cd69^{-/-}$ cells were overrepresented among the lung parenchymal pool (Fig. 1B, 1C), although previous studies suggest this population is not truly resident (36). However, similar to our findings with LCMV, influenza infection resulted in reduced accumulation of $CD69^{-/-}$ P14 CD8$^+$ T cells in the kidney (Fig. 3A, Supplemental Fig. 2J).

It was possible that the role for CD69 in producing T_{RM} within the kidney might relate to the P14 TCR specificity; it has been proposed that P14 T cells recognize a self-epitope expressed in the adrenal gland, which is proximate to the kidney (37, 38). Also, chronic infections with LCMV tend to persist in the kidney, potentially implying preferential tropism for this tissue (39). To address this, we extended our data to another TCR transgenic model and another pathogen, testing the response of WT and $CD69^{-/-}$ OT-I TCR transgenic CD8$^+$ T cells following infection with VSV-OVA. As for the studies with P14 T cells, CD69 deficiency resulted in reduced OT-I recruitment to the kidney, but there was no substantial affect in other NLTs tissues, including the skin (Fig. 3B, Supplemental Fig. 2L–O).

Because unexpected viral tropism or immune clearance mechanisms might account for the repeated pattern of CD69 dependency in kidney T_{RM} generation, we substituted a peptide vaccination

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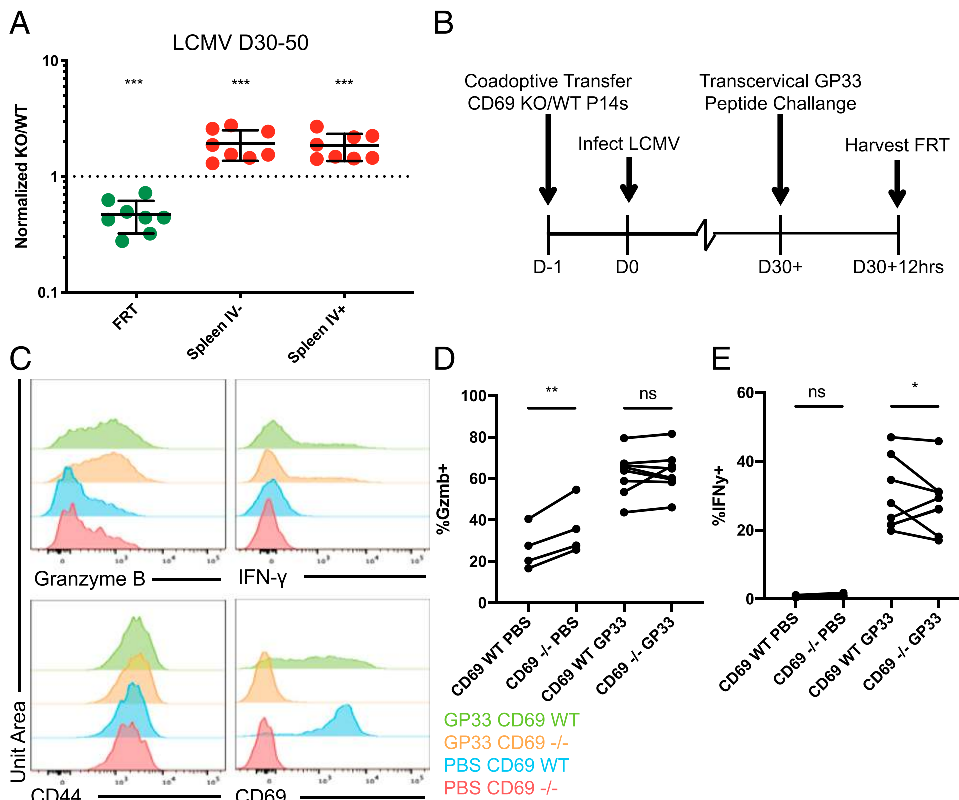

FIGURE 2. Tissue-resident, CD69-deficient cells are functionally competent. Animals received a coadoptive transfer of congenically distinct $Cd69^{-/-}$ and WT P14 CD8+ T cells followed by LCMV infection. At memory timepoints, lymphocytes were isolated from the whole FRT and spleen of mice that did not receive additional treatment (**A**). A similar cohort of animals received transcervical rechallenge with gp33 peptide or PBS at memory timepoints with isolation of lymphocytes from the FRT 12 h posttreatment (**B**). Representative expression of granzyme B, IFN-γ, CD44, and CD69 on $Cd69^{-/-}$ or WT cells isolated from the FRT of LCMV immune animals transcervically challenged with either gp33 peptide or PBS control (**C**). Quantified expression of granzyme B (**D**) and IFN-γ (**E**). Two independent repeats are presented for the normalization in (A) and the combined data in (D) and (E). Data points are normalized to the known ratio of coadoptively transferred cells as in Fig. 1 for (A). Representative histograms shown in (C). Error bars show mean ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, one-tailed *t* test against the known ratio of transferred cells on log10 transformed data for (A), paired *t* test for (D) and (E). ns, not significant.

approach. P14 T cells were primed using TriVax immunization [comprising the LCMV gp33 peptide, poly(I:C), and agonistic anti-CD40 Ab (25)]. Similar to the other immunization methods, $Cd69^{-/-}$ P14 showed impaired accumulation in the kidney, but not the liver and spleen (Fig. 3C, Supplemental Fig. 2P–S).

Together, these results strengthen the conclusion that CD69 promotes recruitment and retention of resident cells at sites of local infection, as well as in the kidney regardless of immunization strategy, but that CD69 deficiency has little consequence for T_{RM} at some other tissue sites, such as the SI-IEL.

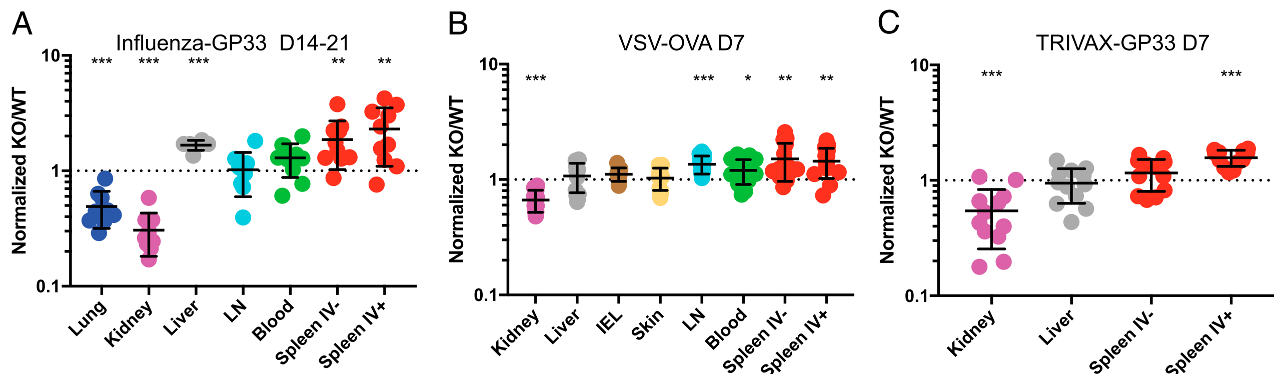

FIGURE 3. CD69 is not required for tissue residency in the majority of tissues following infection with a variety of different models. As in Fig. 1, animals received a coadoptive transfer of $Cd69^{-/-}$ and WT CD8+ T cells. P14 TCR transgenics were used in (**A**) and (**C**), whereas OT1 TCR transgenics were used for (**B**). The ratio of cotransferred, i.v.− cells isolated from a variety of different tissues between 14 and 21 d postinfection with influenza-gp33 (A), 7 d after VSV-OVA (B), or 7 d after TriVax (C). Data points are normalized to the known ratio of coadoptively transferred cells as in Fig. 1. Three independent repeats for each model are combined in each of the presented normalizations. Error bars show mean ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, one-tailed *t* test against the known ratio of transferred cells on log_{10} transformed data.

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Naive CD8+ T cell precursor frequency can alter the defect in residency

We observed a relatively modest impact of CD69 deficiency even in tissues such as the kidney and influenza-infected lung, whereas some previous studies reported much more substantial defects in generation of *Cd69*$^{-/-}$ T_{RM} (15, 18, 19). Notably, a wide range of experimental approaches have been employed in these studies, including substantial differences in the numbers of adoptively transferred CD8+ T cells used to evaluate the role for CD69 (15, 18–20). To test the impact of this variable, we cotransferred *Cd69*$^{-/-}$ and CD69 WT P14 CD8+ T cells of varying precursor number (between 5×10^2 and 5×10^5 P14 cells) into naive recipients followed by LCMV infection. Seven days later, lymphocytes from a variety of different NLT were isolated (Supplemental Fig. 3A). Increasing the naive precursor frequency significantly increased the magnitude of the residency defect observed for *Cd69*$^{-/-}$ P14 CD8+ T cells in the kidney (Fig. 4A, Supplemental Fig. 3B–E). Adoptive transfer doses of 5×10^2 and 5×10^3 that lead to engraftment of P14 in the physiological range of endogenous gp33/D^b-specific T cells (40), however, revealed less-substantial effects of CD69 deficiency. Notably, increasing the precursor frequency did not alter the relative representation of *Cd69*$^{-/-}$ T cells in other tissues (Supplemental Fig. 3F). These data suggest that use of non-physiologically high adoptive transfer studies may artificially exaggerate the significance of CD69 on establishing residency, at least in some tissues.

CD69 enhances early recruitment to the kidney

Although we found the CD69 deficiency reduced the frequency of resident CD8+ T cells in the kidney at effector and memory phases of the immune response, it was not possible to determine whether the role of CD69 was primarily in initial recruitment to the site the result of sustained retention. To explore this, we modified a previously described model of early T cell trafficking to the kidney (26). Mice received coadoptive transfers of Ag-specific WT and *Cd69*$^{-/-}$ P14 CD8+ T cells followed by LCMV infection as in Fig. 1. Six days postinfection, we purified CD8+ T cells from the spleens of infected host mice and retransferred them into infection-matched hosts (Fig. 4B). Of note, neither population was expressing CD69 on the cell surface at the time of transfer (Supplemental Fig. 3G). In secondary hosts, *Cd69*$^{-/-}$ cells were disadvantaged at entering and being retained in the kidney, although the magnitude of the residency defect was less than observed in Fig. 1 (Fig. 4C, Supplemental Fig. 3H, 3I). This indicates that CD69 can act within a 16-h window to promote tissue entry and/or short-term retention in the kidney. Hence, in keeping with previous models, these data suggest that contributions of CD69 to CD8+ T cell tissue residency are primarily reflected in initial recruitment.

Forced CD69 expression enhances residency in some tissues

Similar to previous reports (15, 18–20), our studies focused on defining the role played by CD69 in tissue residency through analysis of *Cd69*$^{-/-}$ CD8+ T cells. However, an important corollary to this approach is to test whether ectopic CD69 expression might promote generation of tissue-resident cells. To address this, we performed retroviral transduction of in vitro activated P14 cells to force expression of CD69, prior to adoptive transfer and in vivo priming with LCMV (Supplemental Fig. 4A–D). This approach does not lead to substantial CD69 overexpression (Supplemental Fig. 4A) but,

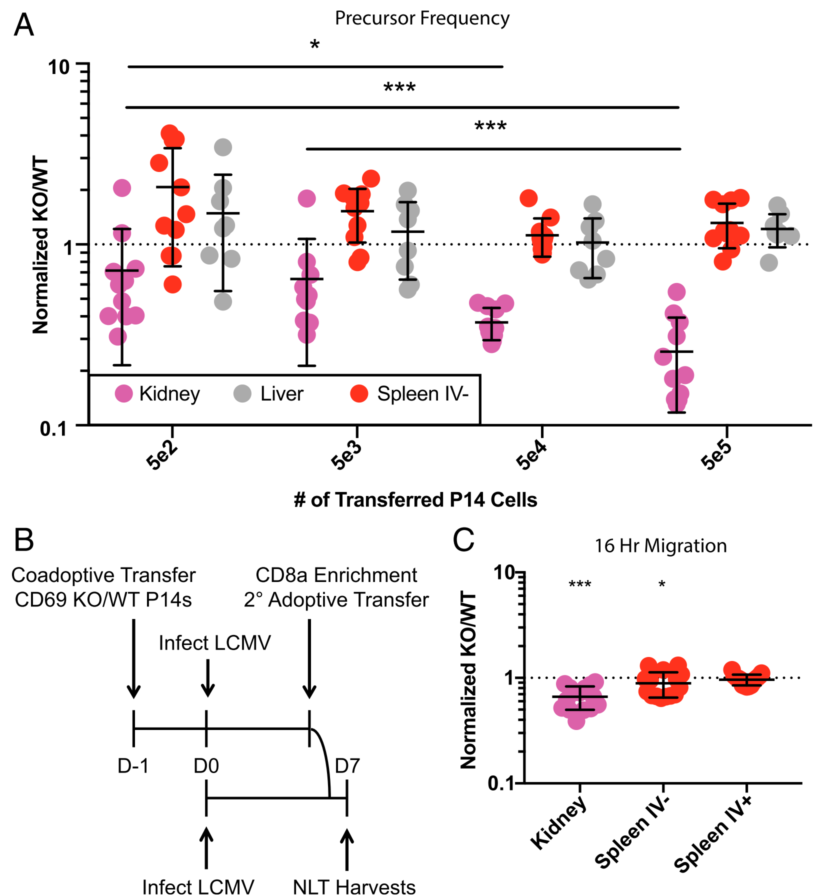

FIGURE 4. The frequency of Ag specific cells present prior to infection correlates with the magnitude of the defect in the kidney, but not elsewhere. A range of precursor cell numbers were coadoptively transferred into recipient animals who were then infected with LCMV as in Fig. 1. The normalized ratio (*Cd69*$^{-/-}$/*Cd69* WT) of i.v.$^-$ transferred cells in each organ 7 d postinfection with LCMV (**A**). CD69 impairs migration into the kidney within a 16 h window. *Cd69*$^{-/-}$ and WT P14s were coadoptively transferred as in Fig. 1, followed by LCMV infection. Six days later, 5 million CD8+ T cells were purified from spleens of infected animals by magnetic enrichment and adoptively transferred into infection-matched recipient animals (**B**). Graphed is the normalized ratio of i.v.$^-$ cells in the indicated tissues 16 h after the secondary cotransfer (**C**). Data points are normalized to the known ratio of coadoptively transferred cells as in Fig. 1. Three independent repeats for each model are combined in each of the presented normalizations. Error bars show mean ± SD. $*p \leq 0.05$, $***p \leq 0.001$, ANOVA with multiple comparisons on $\log_{10}$ transformed data for (A), and one-tailed *t* test against the known ratio of transferred cells on $\log_{10}$ transformed data for (C).

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rather, uncouples *Cd69* gene expression from normal transcriptional control. Compared with empty vector-transduced cells, P14 with forced expression of CD69 were enriched in the kidney (Fig. 5A–F). In other NLTs, there was often a slight trend toward enrichment of CD69 transduced cells, but it was minor and not statistically significant. These data illustrate that for some NLT, establishment of $CD8^+$ T cell residency does not detectably require CD69; yet, enforced CD69 expression can improve generation of the resident population. Although significant, enhanced recruitment of *Cd69*-vector–transduced cells in all tissues was relatively slight, in line with the reciprocal effects of CD69 deficiency. The retroviral transduction approach allowed us to address the functional basis by which CD69 can promote residency in some tissue sites, through use of Cd69 mutants. Although CD69 is a C-type lectin and has been proposed to have several ligands, its best-defined interaction is with S1PR1, leading to mutual inhibition of cell-surface expression. Indeed, earlier studies suggested that the role of CD69 in promoting $CD8^+$ T cell tissue residency could be mimicked by pharmacological blockade of S1PR1 (15). To more specifically define the role of CD69/S1PR1 interactions in controlling $CD8^+$ T cell tissue residency, we tested the effect of forced expression of a CD69 mutant (6N6-Δ31, called CD69-Δ31 in this study), which does not interact with S1PR1 because of a domain swap of the CD69 transmembrane domain, but does not alter the C-type lectin, stalk, or intracellular domains of CD69 (4). In contrast with cells transduced with vectors encoding WT CD69, P14 transduced with retroviruses encoding *Cd69*-Δ31 failed to show enrichment in any NLTs (Fig. 5A–F). This is consistent with CD69-mediated S1PR1 regulation being the key mechanism that mediates enhanced generation of tissue-resident cells, although alterations in interactions with other CD69 ligands by the Δ31 mutation cannot be excluded. Together, these results indicate that, like CD69 deficiency, enforced expression of CD69 has only a limited effect on generation of tissue-resident $CD8^+$ T cells.

CD69 deficiency impairs residency and appearance of $KLF2^{low}$ $CD8^+$ T_{RM} in the kidney

The defining feature of T_{RM} is their maintenance within tissues in the absence of recirculation. Parabiosis has been used to differentiate between resident memory and effector memory cells that transiently traffic through NLT (13, 17, 29). To stringently assess how CD69 functionally impacts tissue residency, we used parabiosis to define the recirculation ability of CD69-sufficient and -deficient cells in various tissues. Animals received a cotransfer of $Cd69^{-/-}$ and WT P14s followed by LCMV infection. At memory timepoints, these parabiotic donor mice were surgically conjoined with infection-matched parabiotic recipients that had not received P14 cells. At least 30 d later, the percentage of P14 cells in the blood (Supplemental Fig. 4E) and spleen (Supplemental Fig. 4F, 4G) were similar in donor and recipient parabionts. We used a formula adapted from Steinert et al. (13) to quantify the percent of $Cd69^{-/-}$ or WT cells that were tissue resident (Fig. 6A). As expected, the frequency of resident cells detected by flow cytometry varied considerably with tissues, ranging from high (in the SI) to very low (in the lung). The impact of CD69 deficiency on residency also differed with tissue site, but in a distinct pattern. Thus, in the SI-IEL and SI-LP, CD69-sufficient and -deficient cells exhibited a similar, very high degree of residency. Likewise, although few liver cells were resident, this was no different for $Cd69^{-/-}$ or WT cells. In the kidney, however, there was a clear and significant residency defect for $Cd69^{-/-}$ cells. Furthermore, $CD69^{-/-}$ P14 showed a trend of reduced residency in the FRT, although this was not statistically significant. Hence, the impact of CD69 deficiency on tissue residency varies considerably across NLT.

Reduced KLF2 expression is a functionally relevant feature of tissue-resident memory $CD8^+$ T cells (11, 14, 17), prompting the question of whether the failure of kidney $CD69^{-/-}$ P14 cells to establish residency would correlate with altered KLF2 expression. To test this, we used the CRISPR/Cas9 system to generate

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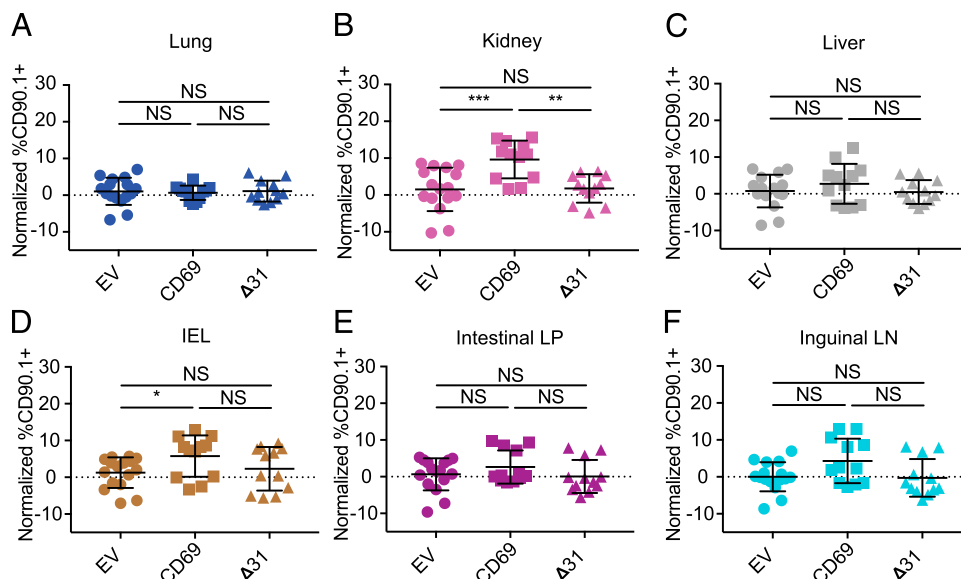

FIGURE 5. CD69 acts primarily via S1PR1 to promote tissue residency in the kidney. In vitro activated P14 cells were transduced retroviral vectors containing either empty vector, CD69, or CD69Δ31, which cannot interact with S1PR1. Cells were transferred into hosts who were infected with LCMV the next day, and NLT were harvested 6 d after LCMV infection. Shown is the percentage of $i.v.^-$ transferred cells expressing the transduction marker CD90.1 on their cell surface, normalized to the percentage expressing it in the average of the spleens for each experimental repeat in the lung (**A**), kidney (**B**), liver, (**C**) intestinal epithelium, (**D**) intestinal LP, (**E**) and inguinal lymph nodes (**F**). Three independent repeats for each model are combined in each of the presented normalizations. Error bars show mean ± SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, ANOVA with multiple comparisons.

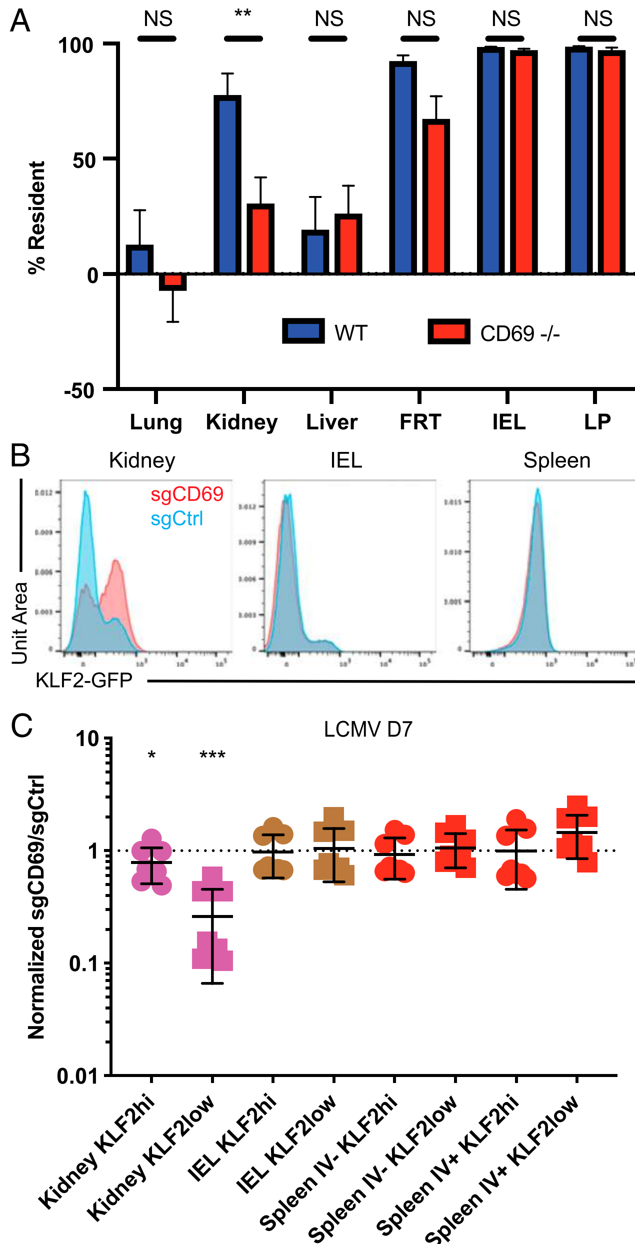

FIGURE 6. CD69 deficiency impairs tissue residency and appearance of KLF2^{low}CD8^{+} T cells in the kidney. Animals received a coadoptive transfer of congenically distinct *Cd69*$^{-/-}$ and WT P14 CD8^{+} T cells followed by LCMV infection. At a memory timepoint, these animals were surgically conjoined to infection-matched animals and left to equilibrate for at least 30 d. At this point, cells were isolated from each of the indicated tissues with i.v.$^{+}$ cells excluded from analysis for the NLT. The number of cells in the donor and recipient parabionts were used to calculate the percentage of cells that were tissue resident using methods from Steinert et al. (**A**). CRISPR/Cas9 was used to knockout CD69 in KLF2/GFP-expressing P14 T cells. Animals received a cotransfer of these cells alongside the congenically distinct KLF2/GFP P14s that used a scramble sgRNA as a control, followed by LCMV infection. Expression of KLF2/GFP on cells that received CD69 sgRNA and cells that received scramble sgRNA in the indicated tissues (**B**). The ratio of recovered CD69 sgRNA/scramble sgRNA receiving cells in KLF2/GFP^{hi} and KLF2/GFP^{low} cells in the i.v.$^{-}$ fraction of the indicated organs 7 d postinfection (d.p.i.) is shown in (**C**). Data points are normalized to the known ratio of coadoptively transferred cells as in Fig. 1 for (C). Two independent repeats are presented for the combined data in (A) and the normalization in (C). Representative histograms shown in (B). Error bars show mean ± SEM for (A) and mean ± SD for (C). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ANOVA with multiple comparisons for (A), one-tailed *t* test against the known ratio of transferred cells on $\log_{10}$ transformed data for (C).

CD69-deficient, KLF2/GFP-expressing P14 cells to explore the impact of CD69 deficiency on KLF2 expression and to further characterize *Cd69*$^{-/-}$ cells present in the kidney. Activated KLF2/GFP-expressing P14s were treated with Cas9 and either CD69 sgRNA or scramble controls (30), cotransferred into recipient mice, and infected with LCMV infection the same day. Seven days postinfection, lymphocytes were isolated from NLT. The absence of CD69 was confirmed on the surface of cells in NLT (Supplemental Fig. 4H). Independent of KLF2/GFP expression, the ratio sgCD69/sgCtrl closely mirrored the phenotype observed using germline knockouts (Fig. 1, Supplemental Fig. 4I, 4J). Interestingly, CD69-deficient cells were no longer present at an elevated frequency in the spleen. This is likely a result of in vitro activation, in vitro activated germline knockouts presented with the same phenotype upon cotransfer (data not shown). In the spleen and IEL, there was no difference in KLF2/GFP expression when comparing CD69-sufficient and -deficient cells. In the kidney, KLF2^{low} population was severely ablated, whereas the KLF2^{hi} cells were intact (Fig. 6B). This was apparent both by the ratio of cells (Fig. 6C) and by cell numbers (Supplemental Fig. 4K). These data indicated that the residency defect in the kidney was only among KLF2^{low} cells (potentially slated to develop into tissue-resident memory) and not in the KLF2^{hi} compartment. These data also reinforce the notion that reduced KLF2 expression is a defining feature of the tissue-resident population.

Discussion

The proposed role of CD69 expression as being critical for establishment of memory T cell tissue residency has been widely embraced, reaching the level of acceptance in immunology textbooks (41). Our data, however, argue against such a universal role for CD69. Rather, the functional significance of CD69 expression varies considerably with tissue site and immunization model. We also found that nonphysiological assays, such as direct injection of activated CD8^{+} T cells into NLT and adoptive transfer experiments involving high numbers of naive precursors may exaggerate the impact of CD69 in at least some tissues sites. Hence, we conclude that a key functional role of CD69 in dictating CD8^{+} T cell tissue residency is absent for several NLT.

We confirmed a role for CD69 in generating resident cells in the lung following influenza infection, consistent with other studies involving local infections (15, 18–20), and these might suggest that residual Ag and/or inflammation at the site of infection promotes or sustains a CD69-dependent recruitment or retention process. Even so, the impact of CD69 deficiency in the influenza-infected lung was relatively modest, as has been reported previously (20). It is possible that a functional role for CD69 may be stronger in situations of chronic infection, and it is notable that the most dramatic requirement for CD69 in driving CD8^{+} T cell residency comes from studies of the response to persistent HSV infection in the skin (19), but further studies will be required to compare the functional role for CD69 in situations of continued versus transient Ag exposure.

In contrast, a substantial impairment in generation of *Cd69*$^{-/-}$ T_{RM} was observed in the kidney regardless of the immunization strategy (including use of the TriVax subunit vaccine), suggesting that local infection is not the only circumstance for which a role for CD69 can be detected. Indeed, CD69 deficiency led to a failed development of a tissue-resident population in the kidney (as defined by parabiosis), whereas *CD69*$^{-/-}$ CD8^{+} T cell residency was unimpeded in the SI-IEL and SI-LP pools. This corresponded with defective recruitment of KLF2^{low}CD8^{+}

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T cells to the kidney following CD69 ablation. In previous studies, we showed that reduced expression of KLF2 and its transcriptional target S1PR1 was necessary for generation of CD8$^+$ T_{RM} in many nonlymphoid sites (17). Hence, there are two ways in which S1PR1 protein expression might be limited on T_{RM}: one involving loss of S1pr1 transcription (typically following loss of KLF2 expression), and the other involving upregulation of CD69, which can impede cell-surface expression of S1PR1 protein. Although one could imagine that these pathways could operate independently, it is noteworthy that, in the case of the kidney, where we find a clear-cut requirement for CD69 in establishing tissue residency, CD69 deficiency is not compensated by an increased frequency of KLF2^{low} cells. To the contrary, KLF2^{low} cells were virtually absent in the rare *Cd69*$^{-/-}$ cells from the kidney parenchyma. This might imply that, for the kidney, CD69 induction is a necessary prerequisite for downregulation of KLF2, as was previously proposed for skin T_{RM} (15). For other tissues, such as the SI-IEL, however, it appears that KLF2 downregulation is entirely independent of the ability of the T_{RM} precursors to express CD69.

It is unclear why formation of the kidney T_{RM} pool would so consistently and substantially depend on CD69 expression. Interestingly, S1PR1 and its ligand S1P are important for renal function, including sodium secretion (natriuresis) (42–44). Hence, although S1P is degraded in the parenchyma of most tissues (6, 7), there may be at least areas of the kidney that maintain elevated levels of S1P. In such a scenario, blocking cell-surface S1PR1 through CD69 upregulation might provide a key advantage for cells to avoid responding to S1P in the kidney. Although it is, of course, possible that the contribution of CD69 to residency in the kidney is independent of its interactions with S1PR1, the fact that the CD69-Δ31 mutant, which retains the entire extracellular domain of WT CD69, fails to enhance CD8$^+$ T cell residency in the kidney makes the role for alternative ligands less likely.

Our studies demonstrated that *Cd69*$^{-/-}$ CD8$^+$ T cells are present at elevated frequencies in the spleen (and, in some situations, the blood and lymph nodes). The basis for this finding is unclear, but may indicate that loss of basal CD69 expression promotes S1PR1-mediated recruitment or retention in the spleen. Regardless of the mechanism, this result may further exaggerate reports of impaired NLT residency by *Cd69*$^{-/-}$ CD8$^+$ T cells, because *Cd69*$^{-/-}$/WT ratios in the spleen have often been used as a reference tissue in these studies.

Consistent with other proposed models (15, 19), our data suggest that any discernable role for CD69 in regulating CD8$^+$ T cell residency in NLT residency manifest early in the immune response, at least in the models of acute infection/immunization studied in this study. We did not find that effects of CD69 deficiency became more or less marked when studying tissue-associated cells at the effector phase versus T_{RM} at long-term memory timepoints. Likewise, we were able to show that CD69-dependent recruitment to the kidney could be measured in short-term (16-h) homing assays. Together, these data suggest that a functional role of CD69, following acute immune responses, is likely to mediate initial recruitment and seeding of the NLT sites. Resident cells unable to make CD69 did not express higher levels of CD103 to compensate for CD69's absence. This reinforces the idea that CD69 and CD103 function at distinct timepoints. Seeming to contrast with this, sustained cell-surface CD69 expression is one of the most consistent features of long-term T_{RM}. Loss of S1PR1 expression [frequently associated with loss of KLF2 expression (17, 22)] may lead to cell-surface CD69 derived from basal expression in memory CD8$^+$ T cells. Still, one might expect that some CD8$^+$ T_{RM} may extinguish *Cd69* gene expression during their maintenance in NLT if it does not play a functional role. In fact, there is existing evidence for this idea, because analysis of tissue-resident CD8$^+$ T cells by quantitative immunofluorescence and parabiosis indicates that a significant fraction of T_{RM} in various sites (pancreas, salivary gland, but also kidney) lack expression of CD69 (13). In addition, a meaningful fraction of CD69 expressing cells in lymphoid tissues recirculate (28). These studies could not discern whether CD69 expression was critical for initial seeding to those NLT, but in conjunction, with our findings, it is likely that a significant fraction of T cells that do not express CD69 or relied on CD69 for their establishment are indeed resident in NLT. Likewise, our studies using forced CD69 expression, following retroviral transduction, suggested relatively mild impact on recruitment of cells to NLT. Together, such findings suggest considerable caution is warranted in interpreting the significance of CD69 expression as an indication of tissue residency by CD8$^+$ T cells in both lymphoid and NLT. Although there is a growing understanding of the unique gene expression characteristics of T_{RM} versus recirculating cells, most of these studies used CD69 expression as an essential marker for identifying "resident" cells and, hence, reinforce reliance on this molecule for their analysis. At least in mice, it would be better not to infer residency solely from CD69 surface expression.

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Disclosures

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