The Journal of Immunology

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J Immunol 2016; 196:2153-2166; Prepublished online 29 January 2016;
doi: 10.4049/jimmunol.1401236
http://www.jimmunol.org/content/196/5/2153

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Context-Dependent Role for IL-21 in Modulating the Differentiation, Distribution, and Abundance of Effector and Memory CD8 T Cell Subsets

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The activation of naive CD8 T cells typically results in the formation of effector cells (T_E) as well as phenotypically distinct memory cells that are retained over time. Memory CD8 T cells can be further subdivided into central memory, effector memory (T_{EM}), and tissue-resident memory (T_{RM}) subsets, which cooperate to confer immunological protection. Using mixed bone marrow chimeras and adoptive transfer studies in which CD8 T cells either do or do not express IL-21R, we discovered that under homeostatic or lymphopenic conditions IL-21 acts directly on CD8 T cells to favor the accumulation of T_E/T_{EM} populations. The inability to perceive IL-21 signals under competitive conditions also resulted in lower levels of T_{RM} phenotype cells and reduced expression of granzyme B in the small intestine. IL-21 differentially promoted the expression of the chemokine receptor CX3CR1 and the integrin $\alpha_4\beta_7$ on CD8 T cells primed in vitro and on circulating CD8 T cells in the mixed bone marrow chimeras. The requirement for IL-21 to establish CD8 T_E/T_{EM} and T_{RM} subsets was overcome by acute lymphocytic choriomeningitis virus infection; nevertheless, memory virus-specific CD8 T cells remained dependent on IL-21 for optimal accumulation in lymphopenic environments. Overall, this study reveals a context-dependent role for IL-21 in sustaining effector phenotype CD8 T cells and influencing their migratory properties, accumulation, and functions. *The Journal of Immunology*, **2016, 196: 2153–2166.**

The local cytokine milieu contributes to the activation and differentiation of naive CD8 T cells as well as supports the maintenance of memory populations (1). One cytokine that regulates CD8 T cells is the common cytokine receptor γ-chain family member, IL-21 (1, 2). This cytokine is primarily produced by CD4 T cells, including T follicular helper and Th17 cells, as well as by NK T cells (3–6). IL-21 acts on multiple targets of the immune system, including T cells, B cells, dendritic cells (DCs), and NK cells (5, 6). Among its actions, IL-21 promotes the proliferation and function of CD8 T cells in conjunction with IL-15 (7), enhances the antitumor power of CD8 T cells (7, 8), facilitates the maturation of memory CD8 T cells through the activation of STAT3 (9), and controls chronic lymphocytic choriomeningitis virus (LCMV) infection in mice by sustaining polyfunctional effector CD8 T cells (10–12).

The development of peripheral CD8 T cell responses is triggered by the recognition of presented Ag in conjunction with costimulatory signals and cytokines. The ensuing CD8 T cell response is comprised of heterogeneous subsets that cooperate to protect the host, but differ in their phenotype, function, developmental fates, and anatomic location (13, 14). Massive populations of CD8 effector T (T_E) cells can develop following the activation of naive CD8 T cells, and these overwhelming responses operate to eliminate Ag-expressing target cells; however, the majority of these cells are prone to apoptosis and lack the self-renewal capacity necessary to constitute the memory pool (13). By contrast, CD8 memory T (T_M) cells are maintained over time following the peak of the response and contribute to long-lived immunity (13). They can be subdivided into central memory T (T_{CM}) and effector memory T (T_{EM}) subsets, as well as the more recently recognized tissue-resident memory (T_{RM}) population (14). T_{CM} cells preferentially home to lymphoid tissues and mount rapid proliferative recall responses that help to amplify and replenish the response during secondary antigenic exposures (15, 16). T_{EM} cells can traffic to nonlymphoid organs and are immediate producers of effector cytokines and cytotoxic proteins following reactivation, but are less proliferative (15–17). The ability of T_{EM} cells to rapidly elaborate effector activities may be vital for the control of certain chronic pathogens, such as SIV and malaria, before the infection is fully established (18–21). T_{RM} cells seed sites of pathogen entry and provide site-specific protection against viral infections, such as HSV and vaccinia virus prior to the recruitment of T_{EM} cells and the recall of T_{CM} cells (22–28).

Depending upon the priming conditions, IL-21 can promote, restrict, or have little, if any, impact on the development of CD8 T_E populations (8–12, 29–32). In certain cases, IL-21 has also been implicated in programming the proliferative recall potential of CD8 T_M cells (29–31). Moreover, it is plausible that IL-21 is especially important for sustaining specific CD8 T cell populations, such as exhausted T cells or T_{RM} cohorts, which cannot receive sufficient survival signals from IL-7 or IL-15 due to downregulation of their

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Received for publication May 14, 2014. Accepted for publication December 31, 2015.

This work was supported in part by National Institutes of Health Grants U01 AI082966, R01 AI049360, and U19 AI1009962 (to A.J.Z.), and T32 AI007051 (to M.A.C. and S.M.K.).

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Abbreviations used in this article: DC, dendritic cell; IEL, intraepithelial lymphocyte; LCMV, lymphocytic choriomeningitis virus; LPL, lamina propria lymphocyte; MLN, mesenteric lymph node; T_{CM}, central memory T; T_E, effector T; T_{EM}, effector memory T; T_M, memory T; T_{RM}, tissue-resident memory T.

respective receptor chains, CD127 and CD122 (25, 33–36). In this study, we set out to decipher whether IL-21 contributes to the formation and maintenance of distinct subsets of CD8 T cells. We report that the development of virus-specific CD8 T cell subsets is largely independent of IL-21 signaling following acute LCMV infection; however, IL-21 influences the differentiation of T_E/T_{EM} subsets as well as plays a role in regulating the abundance of CD8 T cells that reside in nonlymphoid tissues under homeostatic or lymphopenic conditions. Furthermore, IL-21 signaling is associated with the increased formation of CD8 T cell subsets that express CX3CR1 or $\alpha_4\beta_7$ both in vivo and in vitro. Thus, the requirement for IL-21 in supporting CD8 T cell development can vary and most likely depends upon the presence of other regulatory cytokines as well as the strength and repetitiveness of antigenic signals.

Materials and Methods

Mice and infections

C57BL/6J (B6), B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1), B6.129S7-*Rag1*^{tm1Mom}/J (RAG-1$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6;129S5-*Il21r*^{tm1Lex} (IL-21R$^{-/-}$) mice were obtained from the Mutant Mouse Regional Resource Center (Davis, CA) and backcrossed an additional 12–14 generations onto the B6 background (29). B6.PL Thy1^a/CyJ (Thy1.1) mice were purchased from The Jackson Laboratory. LCMV-specific P14 TCR transgenic (P14) mice crossed onto Thy1.1 and IL-21R$^{-/-}$ backgrounds were bred and maintained in fully accredited facilities at the University of Alabama at Birmingham. For acute LCMV infections, mice were infected by i.p. injection with 2×10^5 PFU LCMV-Armstrong (29).

Generation of mixed bone marrow chimeras

Bone marrow chimeras were generated essentially as previously described (29). Briefly, suspensions of bone marrow, obtained from the tibias and femurs of CD45.1 IL-21R$^{+/+}$, CD45.2 B6, and CD45.2 IL-21R$^{-/-}$ mice, were depleted of T cells using anti-CD5 (Ly-1) microbeads (Miltenyi Biotec, Auburn, CA). RAG-1$^{-/-}$ recipient mice were exposed to two doses of radiation (~500 rad each) from a ^{137}Cs source, given 3~4 h apart. Mice were then reconstituted by i.v. injection of 7×10^6 CD45.1 IL-21R$^{+/+}$ T cell–depleted bone marrow and equal numbers of CD45.2 IL-21R$^{+/+}$ or CD45.2 IL-21R$^{-/-}$ cells. Mice were provided chlorinated acidified water containing sulfamethoxazole, trimethoprim, and neomycin for 6 wk.

Cell preparations

Cells from the blood, spleen, liver, lungs, and kidneys were prepared essentially as previously described (29). For isolation of small intestine intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs), the small intestine was explanted and Peyer's patches removed. The intestine was opened longitudinally and cut into ~1-cm pieces, which were then sequentially incubated with 1 mM DTT, followed by 2 mM EDTA in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 2% FCS for 30 min at 37°C with gentle stirring to remove IELs. The remaining tissue was then digested with 1 mg/ml type IV collagenase (Sigma-Aldrich) and 20 μg/ml DNase (Sigma-Aldrich) in RPMI 1640 supplemented with 10% FCS for 30 min at 37°C with stirring to isolate LPLs. IELs and LPLs were then purified on a 40/75% Percoll (GE Healthcare) gradient by centrifugation at $830 \times g$ for 20 min at 20°C.

T cell enrichment

Naive P14 and polyclonal CD8 T cells were isolated from the spleens using a CD8 T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. In certain experiments, biotin-conjugated anti-CD44 (IM7; BioLegend) was used to ensure the removal of CD44^{high} cells from the donor cell pools, as previously described (37).

Lymphopenia-induced homeostatic proliferation

Mixtures containing equal numbers of naive IL-21R$^{+/+}$ and IL-21R$^{-/-}$ P14 or polyclonal CD8 T cells (2×10^6 or 10^6, respectively) were seeded by i.p. or i.v. injection into CD45.1 recipient mice that were sublethally irradiated with 650 rad from a ^{137}Cs source 1 d before transfer (38). In certain experiments, splenic P14 IL-21R$^{+/+}$ and P14 IL-21R$^{-/-}$ CD44^{high}CD62L^{high} T_{CM} phenotype CD8 T cells were isolated from irradiated recipients at 48–53 d posttransfer by cell sorting. Subsequently, these sorted populations were mixed at a 1:1 ratio and a total of 2×10^4 cells transferred by i.p. or i.v. injection to naive CD45.1 recipient mice that were then infected with LCMV-Armstrong 1 d later.

For the analysis of homeostatic proliferation by memory CD8 T cells, IL-21R$^{+/+}$ (Thy1.1) and IL-21R$^{-/-}$ (Thy1.2) mice were infected with LCMV-Armstrong. Between days 34 and 38 following infection, CD8 T cells were magnetically enriched from the spleens, as described above, and CD44^{high} memory CD8 T cells were isolated by cell sorting. Subsequently, purified IL-21R$^{+/+}$ and IL-21R$^{-/-}$ memory CD8 T cells were mixed at a 1:1 ratio, and the frequencies of LCMV gp33-specific cells from each donor population were measured by tetramer staining to confirm that the ratio of IL-21R$^{+/+}$ to IL-21R$^{-/-}$ gp33-specific memory CD8 T cells was close to 1. A total of 10^6 mixed cells was then transferred by i.p. injection into CD45.1 recipient mice that were sublethally irradiated (650 rad) 1 d before.

In vitro T cell differentiation

Naive P14 T cells were purified from the spleens, as described above, and activated with plate-bound anti-CD3 (2C11; 10 μg/ml) and soluble anti-CD28 (37.51; 1 μg/ml) for 2 d (39). Cells were then washed and recultured for 4 d at a concentration of 5×10^5 cells/ml with either IL-2 (100 U/ml) or IL-15 (10 ng/ml) to generate IL-2 T_E and IL-15 T_M cells, respectively (40, 41). During this differentiation period, certain cultures were supplemented with either a low (20 ng/ml) or high (100 ng/ml) dose of IL-21, and each day media was refreshed and resupplemented with the appropriate cytokines.

For activation of naive P14 T cells with DC subsets, DCs were enriched from the mesenteric lymph nodes (MLNs) using CD11c MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions, and subsequently FACS sorted to isolate CD103$^+$ and CD103$^-$ subsets, as previously described (42). Purified CD103$^+$ and CD103$^-$ DCs were pulsed with 1 μg/ml gp33–41 peptide for 1 h at 37°C. Following washing, 10^5 peptide-pulsed DC subsets were cultured with 2×10^5 purified naive responder P14 T cells in the absence or presence of 20 ng/ml (low) or 100 ng/ml (high) IL-21 for 4 d. Four days later, aliquots of cells cultured in the absence of IL-21 were washed and recultured with 10 ng/ml IL-7 in the absence or presence of 20 ng/ml or 100 ng/ml IL-21 for an additional 3 d.

Flow cytometry

Cell preparations from the blood, spleen, liver, lung, kidney, IEL, and LPL were stained using the following Abs: anti-$\alpha_4\beta_7$ allophycocyanin (DATK32; BioLegend), anti-CD8α PE or eFluor450 (53-6.7), anti-CD11c allophycocyanin (N418), anti-CD44 FITC or V500 (IM7; BD Biosciences), anti-CD45.1 allophycocyanin or allophycocyanin-eFluor780 (A20), anti-CD45.2 PerCP-Cy5.5 (104; BioLegend), anti-CD62L FITC or PE-Cy7 (MEL-14), anti-CD69 FITC or PE-Cy7 (H1.2F3), anti-CD103 FITC or allophycocyanin (2E7), anti-CD122 PE (TM-β1; BD Pharmingen), anti-CD127 PE-Cy7 (A7R34), anti-CX3CR1 PE (R&D Systems), anti-granzyme B PE (FGB12; Invitrogen), anti-KLRG1 allophycocyanin (2F1), and anti-Thy1.1 PE-Cy7 (HIS51) (all purchased from eBioscience, unless indicated otherwise). MHC tetramer staining was performed essentially as previously described (43). Intracellular staining for granzyme B was performed after fixation and permeabilization using the BD Biosciences Cytofix/Cytoperm kit. LIVE/DEAD fixable aqua stain kit (Life Technologies) was used to exclude dead cells from cell culture analysis. Samples were acquired using a LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Two-tailed paired Student *t* test was used to determine statistical significance between IL-21R$^{+/+}$ and IL-21R$^{-/-}$ T cell populations in the mixed bone marrow chimeras and irradiated recipients. Two-tailed unpaired Student *t* test was used to compare IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cell populations in intact mice. The *p* values were calculated using Prism software (GraphPad, La Jolla, CA).

Results

IL-21 signaling impacts the generation and maintenance of effector phenotype CD8 T cells in mixed bone marrow chimeras

To investigate how IL-21 influences the development of effector and memory phenotype CD8 T cells, we generated mixed bone marrow chimeras by reconstituting lethally irradiated RAG-1$^{-/-}$ mice with equal numbers of allelically marked IL-21R$^{+/+}$ and IL-21R$^{-/-}$ T cell–depleted bone marrow cells (Fig. 1A). In this way IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells are exposed to the same environmental

cues, and any differences observed between these two populations should be attributable to the effects of IL-21–mediated signaling on their differentiation. Control mixed chimeras were generated by reconstituting recipients with equal numbers of allelically marked CD45.1 IL-21R$^{+/+}$ and CD45.2 IL-21R$^{+/+}$ T cell–depleted bone marrow cells (Fig. 1A). We analyzed the blood of the chimeras for the presence of naive (CD44^{low}CD62L^{high}), T_{CM} (CD44^{high} CD62L^{high}), and T_E/T_{EM} (CD44^{high}CD62L^{low}) CD8 T cells (15, 16) and discovered that IL-21R$^{-/-}$ CD8 T_E/T_{EM} cells were underrepresented in the experimental chimeras at all time points checked (Fig. 1B, 1C). Enumeration of CD8 T cell subsets in the spleen showed that, although IL-21R$^{-/-}$ CD8 T cells displayed a minor reduction in T_{CM} cells, the defective accumulation of T_E/T_{EM} cells was more severe (Fig. 1D). Notably, the numbers of naive IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells were similar. Together, these data show that IL-21 signaling to CD8 T cells is necessary to generate and/or sustain T_E/T_{EM} subsets.

IL-21 signaling promotes the accumulation of effector phenotype CD8 T cells in mixed bone marrow chimeras

Because the loss of IL-21 signaling negatively affected the accumulation of T_E/T_{EM} phenotype CD8 T cells, we further dissected the differentiation state of the CD8 T cells that had become primed in the mixed bone marrow chimeras. Compared with the IL-21R$^{-/-}$ population, a greater fraction of IL-21R$^{+/+}$ CD44^{high} CD8 T cells expressed KLRG-1 (10 ± 4% versus 1 ± 1%) (Fig. 2A), and, similarly, higher absolute numbers of IL-21R$^{+/+}$ CD44^{high}KLRG1^{high} CD8 T cells were detected in the spleens of the experimental chimeras (Fig. 2B). The number of IL-21R$^{+/+}$ CD44^{high}KLRG1^{low} CD8 T cells was less significantly elevated in the experimental cohort (Fig. 2B). Thus, IL-21 signaling to CD8 T cells can facilitate the formation and/or maintenance of populations with a more activated, effector-like phenotype, which is consistent with the CD62L expression profiles reported in Fig. 1. To further investigate the roles of IL-21 in peripheral CD8 T cell differentiation, we evaluated the expression of CD122 (the IL-2R and IL-15R β-chain) and CD127 (IL-7Rα) (Fig. 2C, 2D). In the experimental chimeras, the most significant difference was the reduced fraction and number of IL-21R$^{-/-}$ CD44^{high}CD8 T cells that are CD122^{low}. Thus, IL-21 signaling may compensate for the downregulation of CD122 by subsets of activated CD8 T cells and operate to sustain these populations.

To better discriminate between CD8 T_E (CD127^{low}CD62L^{low}), T_{EM} (CD127^{high}CD62L^{low}), and T_{CM} (CD127^{high}CD62L^{high}) subsets (44, 45), we determined the patterns of CD127 and CD62L expression by IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD44^{high}CD8 T cells. In the experimental chimeras, by comparison with the IL-21R$^{+/+}$ populations, the fraction and number of CD127^{high}CD62L^{low} (T_{EM}) and CD127^{low}CD62L^{low} (T_E) IL-21R$^{-/-}$ subsets exhibited the most drastic reduction (Fig. 2E, 2F). Taken together, the results in Figs. 1 and 2 demonstrate that under competitive conditions IL-21 contributes to the accumulation of effector phenotype CD8 T cells in mixed bone marrow chimeras.

IL-21 influences the anatomic distribution of CD8 T cells in mixed bone marrow chimeras

Because effector and memory CD8 T cells can be present in both lymphoid and nonlymphoid organs, we compared the recoveries of

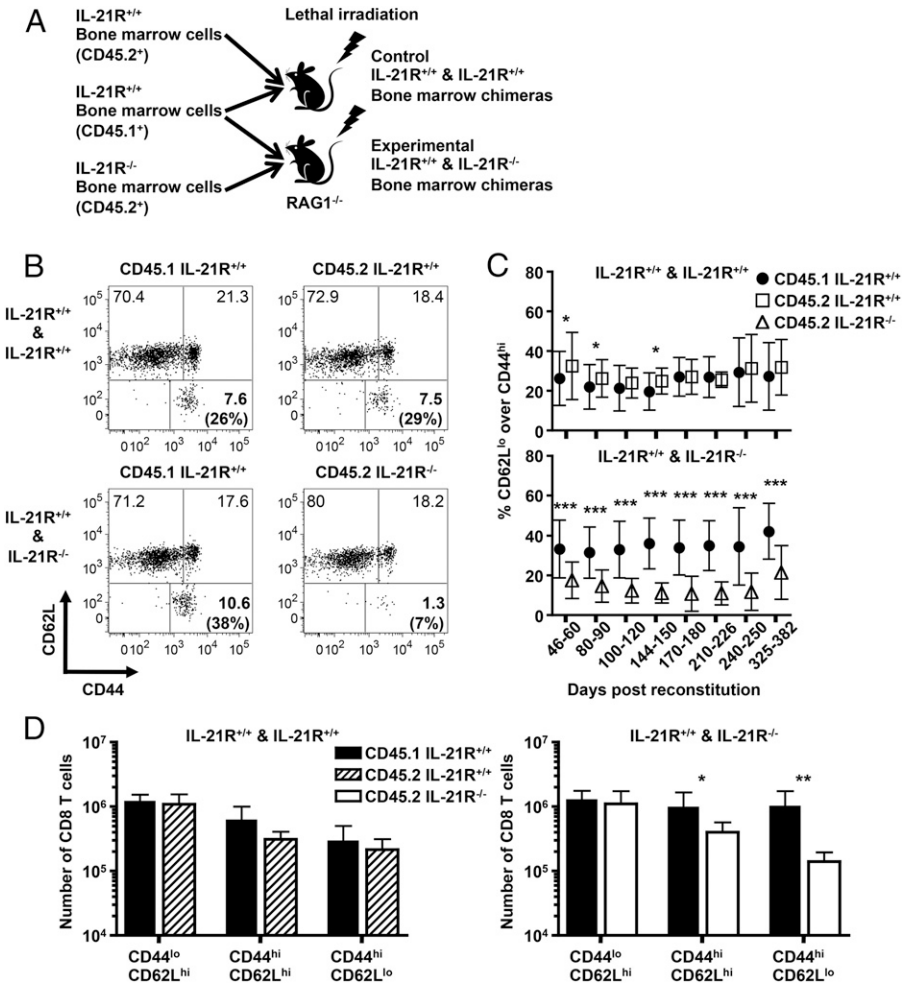

FIGURE 1. Defective accumulation of CD44^{high}CD62L^{low}CD8 T_E/T_{EM} cells in the absence of IL-21 signaling. PBMCs and splenocytes from cohorts of control IL-21R$^{+/+}$ (CD45.1) and IL-21R$^{+/+}$ (CD45.2) and experimental IL-21R$^{+/+}$ (CD45.1) and IL-21R$^{-/-}$ (CD45.2) mixed bone marrow chimeras were evaluated by flow cytometry. (**A**) Schematic depicting the generation of mixed bone marrow chimeras. (**B**) Donor-derived CD8 T cells were isolated from the blood 144 d following reconstitution and analyzed for the surface expression of CD44 and CD62L. Gated CD8 T cells are shown, and the percentages of CD8$^+$ CD44^{high} cells that are CD62L^{low} are indicated in parentheses. (**C**) The percentages of CD8$^+$CD44^{high} cells that are CD62L^{low} in the blood were evaluated over time following reconstitution. Representative or composite data are shown from five independent experiments with at least six mice per group. (**D**) Enumeration of splenic CD44^{low}CD62L^{high}, CD44^{high}CD62L^{high}, and CD44^{high}CD62L^{low} CD8 T cells 160–357 d following reconstitution. Composite results from three experiments are presented (n = 5 and 9 for IL-21R$^{+/+}$ and IL-21R$^{+/+}$ and IL-21R$^{+/+}$ and IL-21R$^{-/-}$ cohorts, respectively). Error bars show SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

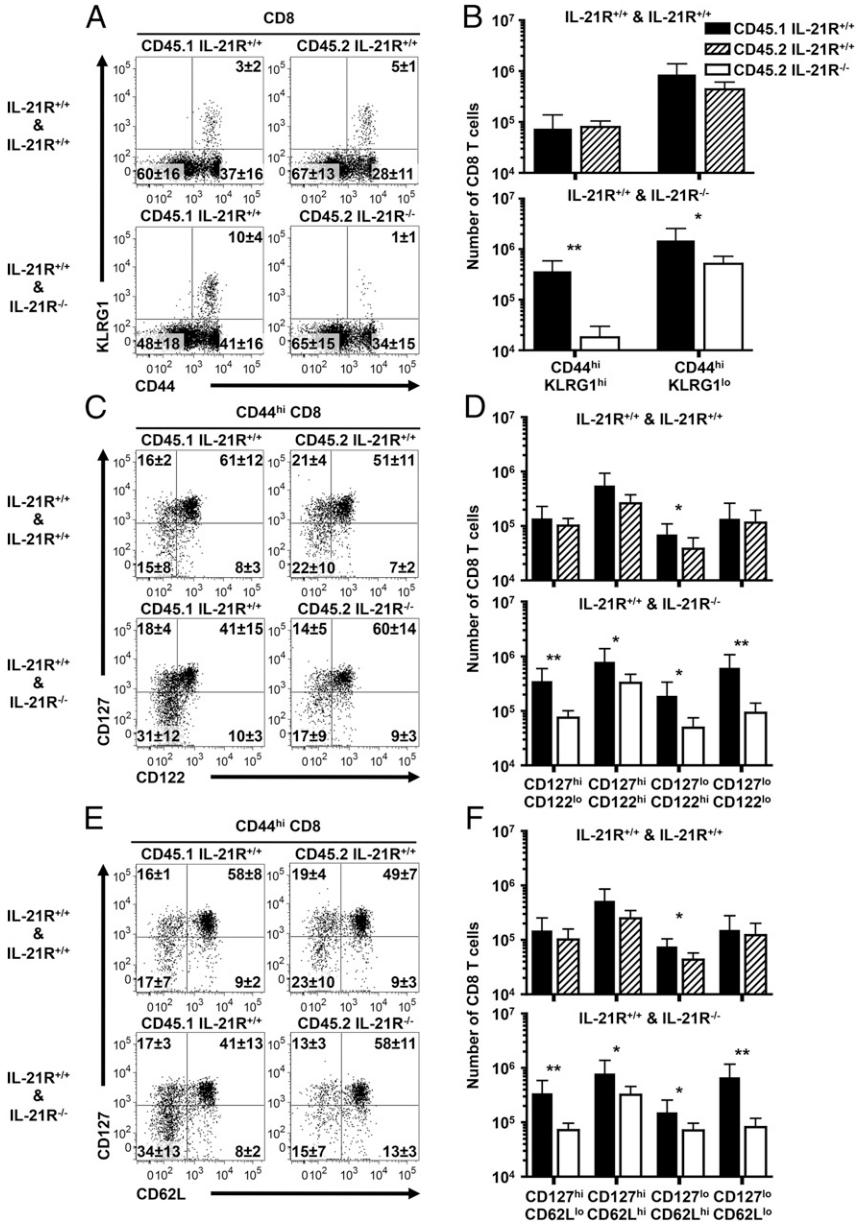

FIGURE 2. Altered CD8 T cell activation profiles in the absence of IL-21 signaling. Splenocytes from control (IL-21R$^{+/+}$ and IL-21R$^{+/+}$) and experimental (IL-21R$^{+/+}$ and IL-21R$^{-/-}$) mixed bone marrow chimeras were evaluated by flow cytometry at days 160–357 following reconstitution. (**A**) Dot plots show expression of CD44 and KLRG1 gated on CD8 T cells. The expression of (**C**) CD122 and CD127 and (**E**) CD62L and CD127 on gated CD44^{high}CD8 T cells is also shown. The mean ± SD percentages of cells in each quadrant are stated. (**B**, **D**, and **F**) Bar graphs indicate the numbers ± SD of the specified CD8 T cell subsets in the control (*upper panels*) or experimental (*lower panels*) cohorts. Representative or composite data are shown from three individual experiments ($n = 5$ and 9 for IL-21R$^{+/+}$ and IL-21R$^{+/+}$ and IL-21R$^{+/+}$ and IL-21R$^{-/-}$ cohorts, respectively). *$p < 0.05$, **$p < 0.01$.

IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells from various organs, including the spleen, liver, lung, and kidney, as well as from the small intestine IEL and LPL compartments in the mixed bone marrow chimeras (Fig. 3A). In the experimental IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mixed chimeras, lower percentages and numbers of IL-21R$^{-/-}$ CD8 T cells were recovered (Fig. 3B, 3C). Although there were 3.6-fold fewer IL-21R$^{-/-}$ CD8 T cells present in the spleen by comparison with the IL-21R$^{+/+}$ subset, the differences were more pronounced in certain nonlymphoid organs. The fold differences between the recoveries of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells in the liver, lung, kidney, IEL, and LPL were 5.8, 4.3, 5.6, 5.8, and 4.6, respectively. The overall level of CD4 T cell reconstitution was similar in the control and experimental bone marrow chimeras, in all compartments evaluated except in the LPL, which contained ~4-fold fewer CD4 T cells in the experimental chimeras (data not shown). Nevertheless, although the control and experimental chimeras contained similar overall numbers of LPL CD8 T cells, significantly fewer IL-21R$^{-/-}$ CD8 T cells were detected in the experimental cohort. Thus, in the mixed chimeras, IL-21 most dramatically influenced the accumulation of CD8 T cells in select nonlymphoid sites, including the liver, kidney, IEL, and LPL.

Canonical T_{RM} phenotype in the small intestine is partially impaired in the absence of IL-21 signaling in mixed bone marrow chimeras

Because lymphopenia-induced proliferation in the absence of infection can induce CD103^{+}CD69^{+}CD8 T_{RM} cells (33) and IL-21 influences the abundance of CD8 T cells in nonlymphoid organs (Fig. 3), we investigated whether the expression of IL-21R influenced the development of this population. By comparison with IL-21R$^{+/+}$ CD8 T cells, substantially lower numbers of small intestine CD8^{+} IL-21R$^{-/-}$ IELs were present in the mixed bone marrow chimeras (Fig. 3C). Although fewer IL-21R$^{-/-}$ IELs were present, both the IL-21R$^{+/+}$ and IL-21R$^{-/-}$ T cells displayed the prototypic CD103^{+}CD69^{+} IEL T_{RM} phenotype (84 ± 5% and 75 ± 13%, respectively) (Fig. 4A). Similarly, no defects in the expression of CD103 and CD69 by IL-21R$^{-/-}$ CD8 T cells were detected in the LPL (data not shown). In addition, both the IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T_{RM} cells expressed reduced levels of CD122,

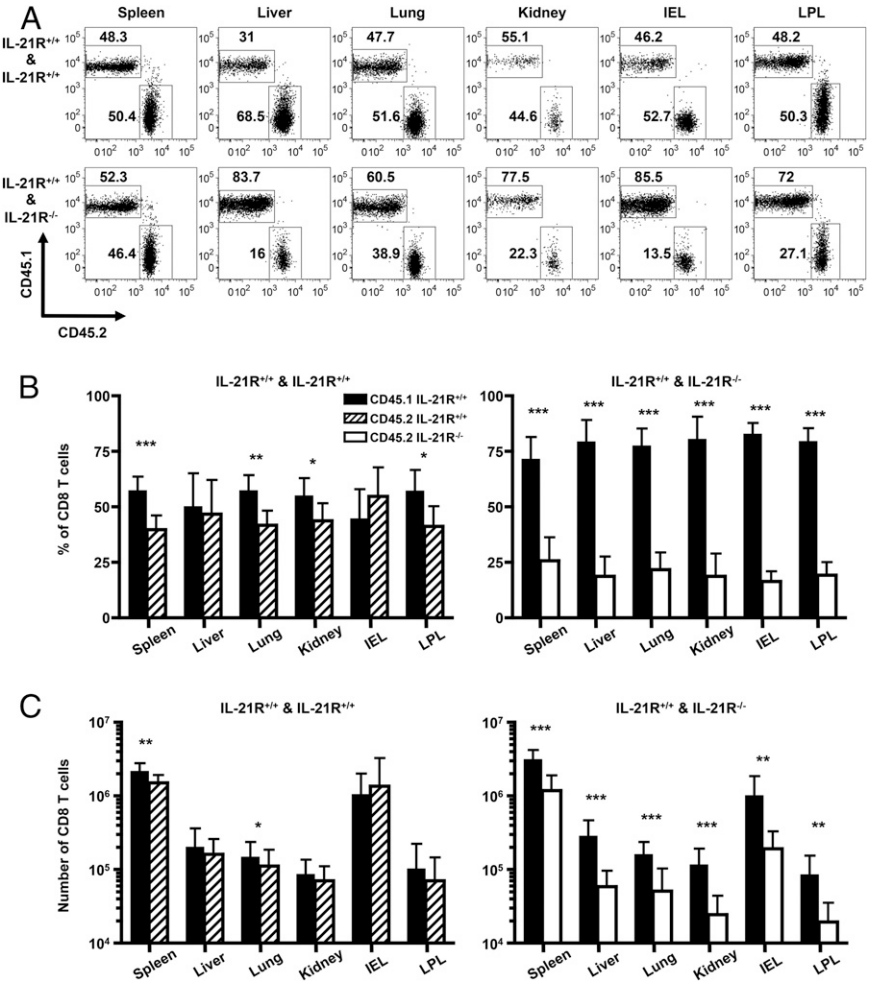

FIGURE 3. Defective accumulation of IL-21R$^{-/-}$ CD8 T cells in tissues. Control (IL-21R$^{+/+}$ and IL-21R$^{+/+}$) and experimental (IL-21R$^{+/+}$ and IL-21R$^{-/-}$) mixed bone marrow chimeras were sacrificed at days 160–388 following reconstitution, and CD8 T cells isolated from the indicated tissues were analyzed by flow cytometry. (**A**) Representative dot plots show the recoveries of CD45.1 and CD45.2 CD8 T cells. (**B** and **C**) Bar graphs show the (B) percentage or (C) number of donor-derived CD8 T cells with error bars indicating SD. Representative or composite data are shown from seven independent experiments analyzing at least nine mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CD127, and CD62L (Fig. 4B, 4C). We also compared the expression of granzyme B by the IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD103^{+}CD69^{+} T_{RM} phenotype CD8^{+} IELs. The levels of granzyme B were significantly lower in the IL-21R$^{-/-}$ IEL CD8 T_{RM} cells (Fig. 4D); however, both CD44^{high} splenic IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells expressed similarly low levels of granzyme B (Fig. 4D). Collectively, these findings demonstrate that under competitive conditions IL-21 signaling to CD8 T cells can influence the overall levels of T_{RM} phenotype CD8 T cells in the small intestine IEL compartment. The CD8 T_{RM} cells are also CD122^{low}, CD127^{low}, suggesting that this subset is sustained independently of IL-7 and IL-15, implicating a possible role for IL-21 in the maintenance of this subset. Nevertheless, the reduced numbers of IL-21R$^{-/-}$ CD8 T cells that populate the IEL attain a typical CD103^{+}CD69^{+} T_{RM} phenotype, which most likely aids their retention, but the lower levels of granzyme B expression may limit their protective efficacy in barrier tissues.

Recently, CD8 T_{RM} cells have been identified within secondary lymphoid organs, which are characterized by their expression of CD69, but, unlike their counterparts at epithelial sites, they do not express CD103 (46). Consistent with these results, we observed populations of CD69^{+}CD44^{high}CD8 T cells in the spleens of both control and experimental chimeras (data not shown). Notably, in the experimental chimeras, a greater fraction of IL-21R$^{+/+}$ CD44^{high}CD8 T cells expressed CD69 compared with the IL-21R$^{-/-}$ population (33 ± 10% versus 15 ± 5%, $p < 0.0001$), and the numbers of IL-21R$^{+/+}$ CD69^{+}CD8 T cells were 12-fold higher (data not shown). These data indicate that these putative secondary lymphoid organ T_{RM} cells are at least partially dependent on IL-21 for their generation and/or maintenance in the mixed bone marrow chimeras.

IL-21 signaling is required for the differentiation of CD8 T_E/T_{EM} cells and the distribution of CD8 T cells in nonlymphoid tissues after lymphopenia-induced homeostatic proliferation

Because we identified a role for IL-21 in regulating the phenotype, function, and abundance of CD8 T_E/T_{EM} and T_{RM} subsets that develop during the reconstitution of mixed bone marrow chimeras, we next investigated whether IL-21 also impacts the properties of CD8 T cells that differentiate in response to lymphopenia-induced homeostatic proliferation in sublethally irradiated recipients (38, 47–49). To address this, equal numbers of allelically marked naive IL-21R$^{+/+}$ and IL-21R$^{-/-}$ P14 TCR transgenic CD8 T cells were cotransferred into sublethally irradiated recipients (Fig. 5A). In response to these lymphopenic conditions, a smaller fraction of IL-21R$^{-/-}$ P14 T cells acquired the CD44^{high}CD62L^{low} T_E/T_{EM} phenotype in the blood, by comparison with their IL-21R$^{+/+}$ counterparts (Fig. 5B, 5C), which is consistent with the results from mixed bone marrow chimeras presented in Fig. 1B and 1C. Moreover, we also observed substantially fewer IL-21R$^{-/-}$ P14 T cells in the liver and small intestine IEL and LPL compartments, by comparison with their IL-21R$^{+/+}$ counterparts (Fig. 5D). The fold differences between the numbers of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ P14 T cells in the liver, IEL, and LPL were 2.3, 57.3, and 6.1, respectively, compared with a 1.3-fold difference in the spleen. Similar experiments in which naive polyclonal IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells were cotransferred into sublethally

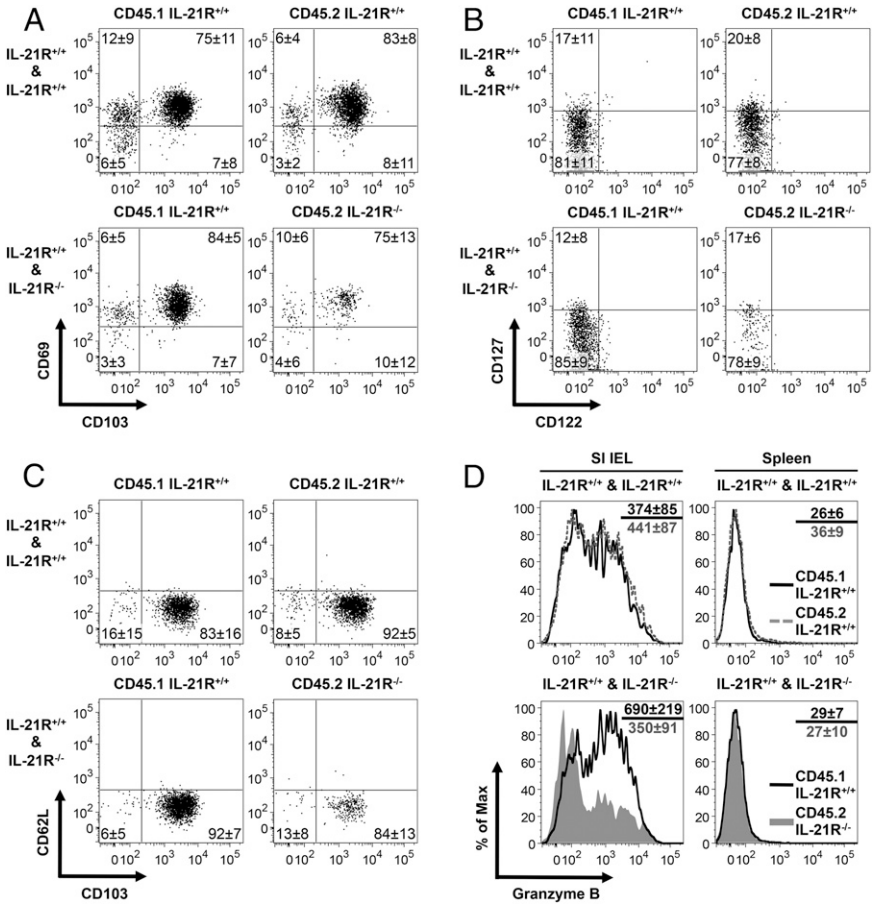

FIGURE 4. Phenotypic characterization of CD8 T cells accumulated in the small intestine (SI) epithelium. Control (IL-21R$^{+/+}$ and IL-21R$^{+/+}$) and experimental (IL-21R$^{+/+}$ and IL-21R$^{-/-}$) mixed bone marrow chimeras were sacrificed at days 160–388 following reconstitution and SI IEL CD8 T cells analyzed by flow cytometry. (**A**–**C**) Representative dot plots show expression of CD103, CD69, CD122, CD127, and CD62L by CD44^{high} CD8 T cells. Numbers show the percentages (mean ± SD) of cells in each quadrant. (**D**) Ex vivo granzyme B expression by CD103^{+}CD69^{+} CD8^{+} IELs (*left panels*) and CD44^{high} splenic CD8 T cells (*right panels*). Numbers show the mean fluorescence intensity (MFI) ± SD of CD45.1 (upper values) and CD45.2 (lower values) populations. Representative or composite data are shown from three to four independent experiments analyzing a total of 3–10 mice per group.

irradiated recipients also showed a marked reduction in the fraction and number of IL-21R$^{-/-}$ CD8 T cells in the liver, small intestine IEL, and LPL (Fig. 5E). Notably, IL-21R$^{-/-}$ P14 and polyclonal CD8 T cells showed substantially decreased expression of CD103 and CD69 in the small intestine LPL and reduced production of the effector molecule granzyme B (Fig. 5F and data not shown), suggesting that IL-21 is required for the formation of CD8 T_{RM} cells at mucosal sites following lymphopenia-induced homeostatic proliferation. Taken together, these data indicate that IL-21 modulates the abundance and differentiation of CD8 T cells in nonlymphoid organs during the reconstitution of lethally irradiated mixed bone marrow chimeras and also following lymphopenia-induced homeostatic proliferation.

Expression of CX3CR1 and integrin $\alpha_4\beta_7$ is reduced on IL-21R$^{-/-}$ T_E/T_{EM} CD8 T cells in the peripheral blood of mixed bone marrow chimeras

The defective accumulation of IL-21R$^{-/-}$ CD8 T cells in various tissues in the mixed bone marrow chimeras prompted us to investigate whether IL-21 signaling influences the ability of circulating CD8 T cells to seed these tissues. We assessed the expression of the chemokine receptor CX3CR1, which regulates the migration of CD8 T cells by interacting with its ligand CX3CL1 (50, 51) and is present on effector phenotype CD8 T cells, as well as serves as a marker for cytotoxic CD4 and CD8 T cells (51–55). We also evaluated the expression of integrin $\alpha_4\beta_7$, which has been shown to influence CD8 T cell homing to the intestines (56). CX3CR1 and $\alpha_4\beta_7$ were expressed at higher levels on IL-21R$^{+/+}$ CD44^{high}CD62L^{low} T_E/T_{EM} phenotype CD8 T cells than IL-21R$^{+/+}$ CD44^{high}CD62L^{high} T_{CM} CD8 T cells in mixed bone marrow chimeras (Fig. 6). However, substantially lower levels of CX3CR1 (mean fluorescence intensity: 61 ± 28 versus 131 ± 22 with $p < 0.0001$) (Fig. 6A) and $\alpha_4\beta_7$ (mean fluorescence intensity: 71 ± 24 versus 166 ± 29 with $p < 0.0001$) (Fig. 6B) were expressed on IL-21R$^{-/-}$ T_E/T_{EM} phenotype CD8 T cells when compared with the IL-21R$^{+/+}$ population in the experimental chimeras. Thus, IL-21 can play a role in configuring the homing properties of CD8 T cells, which most likely regulates their ability to populate nonlymphoid sites.

IL-21 increases the expression of CX3CR1 and $\alpha_4\beta_7$ on in vitro differentiated CD8 T cells

We investigated whether IL-21 directly promotes the expression of CX3CR1 and $\alpha_4\beta_7$ on effector and memory CD8 T cells differentiated in vitro using IL-2 and IL-15, respectively (39–41). Purified naive P14 CD8 T cells were stimulated with anti-CD3 and anti-CD28 Abs and then cultured with either IL-2 or IL-15, in the absence or presence of a low or high dose of IL-21 (Fig. 7A). Consistent with previous reports, IL-2–cultured cells (IL-2 T_E) displayed an effector phenotype as revealed by elevated expression of CD25, CD43, and CD44 and downregulation of CD62L and CD127, whereas cells cultured with IL-15 (IL-15 T_M) adopted a memory-like phenotype and expressed lower levels of CD25, CD43, and CD44, but higher levels of CD62L and CD127 (data not shown). Notably, IL-21 increased the percentage of $\alpha_4\beta_7^{high}$ cells among IL-2 T_E but not IL-15 T_M cells (Fig. 7B, 7C). IL-15 T_M cells expressed high basal levels of $\alpha_4\beta_7$, suggesting that IL-15 and IL-21 may have redundant roles in modulating the expression of $\alpha_4\beta_7$ on CD8 T cells. Interestingly, IL-21 enhanced the expression of CX3CR1 on IL-15 T_M cells in a dose-dependent manner while exerting minimal effects on IL-2 T_E cells (Fig. 7D, 7E). Similar results were obtained when splenocytes from P14

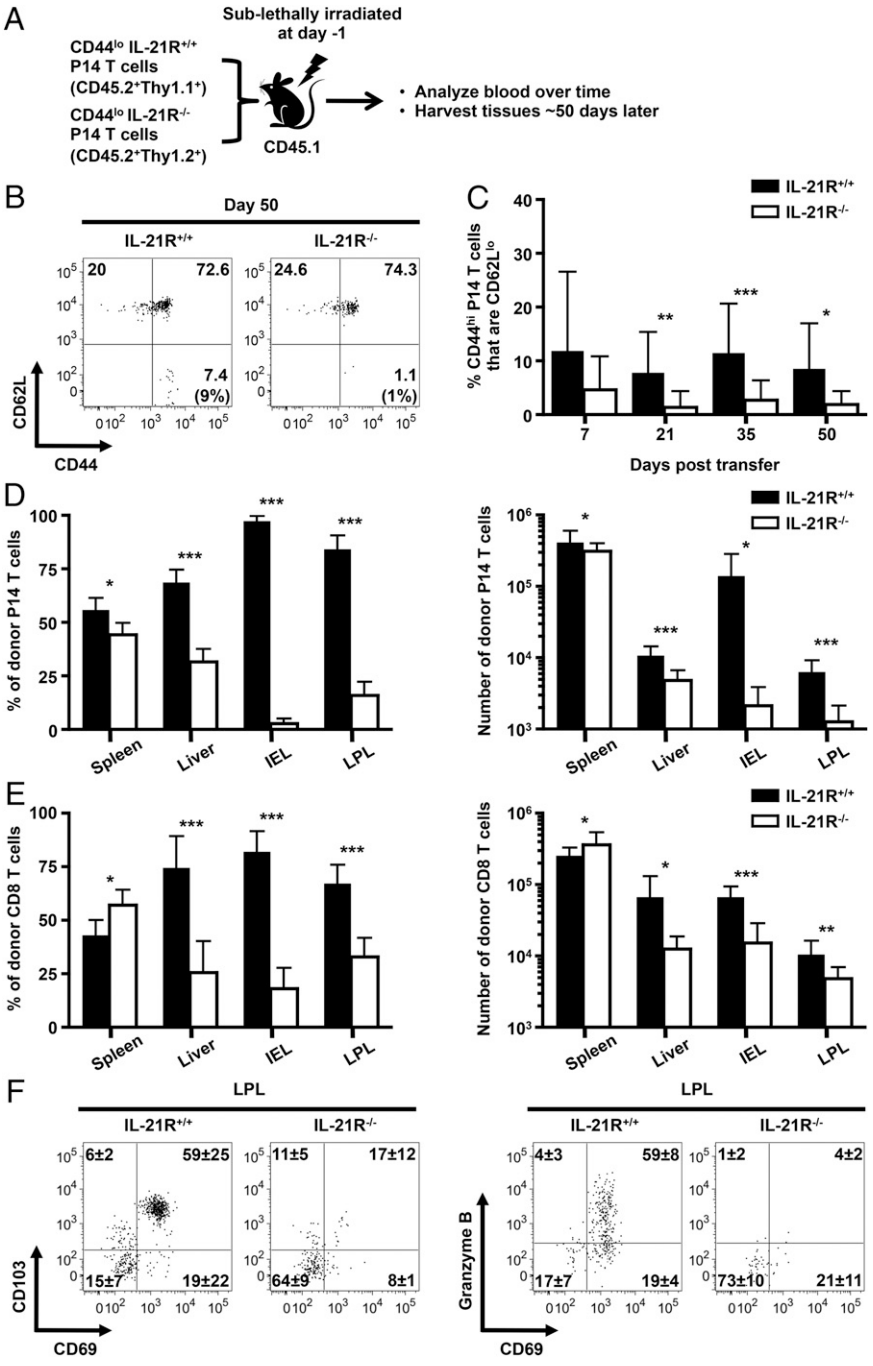

FIGURE 5. IL-21 signaling modulates CD8 T_E/T_{EM} responses and the accumulation of CD8 T cells in nonlymphoid tissues during lymphopenia-induced homeostatic proliferation. (**A**) Schematic depicting the adoptive transfer of equal numbers of P14 IL-21R$^{+/+}$ and P14 IL-21R$^{-/-}$ naive CD8 T cells into sublethally irradiated recipients. (**B** and **C**) Donor P14 CD8 T cells in the blood were analyzed for their expression of CD44 and CD62L over time following transfer. (B) Dot plots show expression of CD44 and CD62L at day 50 posttransfer. Gated P14 T cells are shown, and the percentages of CD44^{high} cells that are CD62L^{low} are indicated in parentheses. (C) Bar graph shows the percentages of CD44^{high} P14 T cells that are CD62L^{low} at the indicated time points. In (B) and (C), representative or composite data are shown from two independent experiments analyzing 16–20 mice. (**D**) Graphs show the percentage and number of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ donor P14 CD8 T cells recovered from various sites at days 48–53 posttransfer. Composite data are shown from two independent experiments analyzing eight mice. (**E**) Identical experimental design as in (A); however, polyclonal IL-21R$^{+/+}$ and IL-21R$^{-/-}$ naive donor CD8 T cells were used. Graphs show the percentage and number of donor CD8 T cells at days 53–57 following transfer. Composite data are shown from two independent experiments analyzing 10 mice. (**F**) Representative dot plots show the expression of CD69, CD103, and granzyme B gated on donor LPL IL-21R$^{+/+}$ and IL-21R$^{-/-}$ P14 T cells at 48–53 d posttransfer. Numbers indicate the percentages ± SD of cells in each quadrant. Representative or composite data are shown from two independent experiments analyzing eight mice. Error bars show SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

mice were stimulated with the cognate gp33–41 peptide and then cultured in the presence of the defined cytokines (data not shown). Taken together, our results indicate that the expression of CX3CR1 and $\alpha_4\beta_7$ is most likely independently regulated and that IL-21 can increase the expression of these molecules both in vivo and in vitro, depending upon the differentiation state of the responding cells.

It has been previously established that intestinal CD103$^+$ but not CD103$^-$ DCs can induce the expression of gut-homing molecules, including CCR9 and $\alpha_4\beta_7$ on CD8 T cells (42). Thus, we tested whether IL-21 modulates the expression of $\alpha_4\beta_7$ on CD8 T cells activated by these DC subsets isolated from the MLNs (Fig. 8A). Consistent with published results (42), CD103$^+$ DCs, but not CD103$^-$ DCs, were able to induce CCR9 expression on P14 T cells, and, as expected, CD103$^+$ DCs were also more efficient in inducing the expression of $\alpha_4\beta_7$ (Fig. 8B). Notably, at the 4-d time point, the presence of IL-21 modestly increased the expression of both CCR9 and $\alpha_4\beta_7$ on P14 T cells activated by CD103$^+$ but not CD103$^-$ DCs in a dose-dependent manner (Fig. 8B). In addition to the day 4 analysis, cell mixtures initially cultured without IL-21 were further expanded for 3 d with IL-7 in the presence or absence of IL-21. Although IL-21 did not enhance the expression of CCR9 during this phase, it did increase the levels of $\alpha_4\beta_7$ on P14 T cells activated by CD103$^+$ DCs (Fig. 8C). Strikingly, IL-21 also upregulated the expression of $\alpha_4\beta_7$ on P14 T cells activated by CD103$^-$ DCs to levels comparable to those detected on cells activated by CD103$^+$ DCs (Fig. 8C). We did not observe upregulation of CX3CR1 under these conditions (data not shown). Because MLN CD103$^+$ DCs induce the expression of $\alpha_4\beta_7$ on CD8 T cells via retinoic acid (57, 58), our data suggest that IL-21 may act as an alternative inducer of $\alpha_4\beta_7$ expression on CD8 T cells.

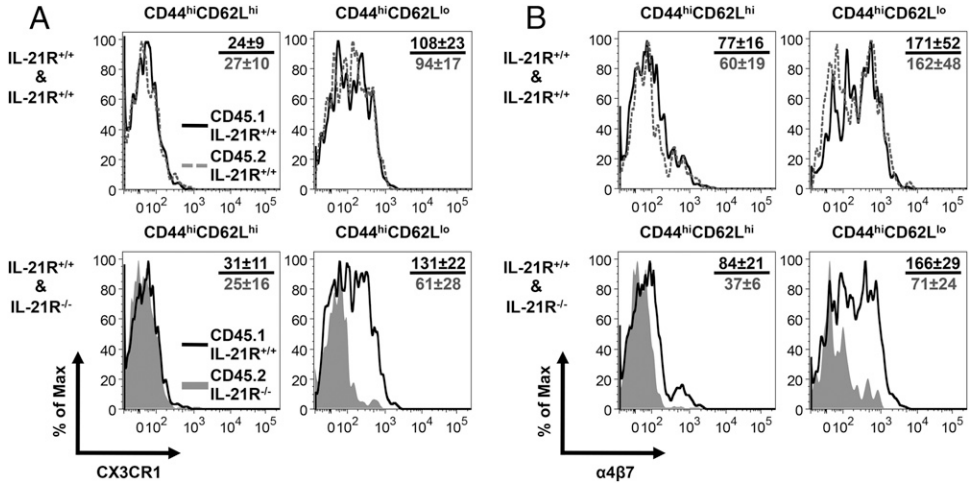

FIGURE 6. Impaired expression of CX3CR1 and $\alpha_4\beta_7$ by T_E/T_{EM} phenotype CD8 T cells in the absence of IL-21 signaling. PBMCs from control IL-21R$^{+/+}$ and IL-21R$^{+/+}$ and experimental IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mixed bone marrow chimeras were evaluated by flow cytometry at days 226–325 following reconstitution. Representative histograms show expression of (**A**) CX3CR1 and (**B**) $\alpha_4\beta_7$ gated on T_{CM} (CD44^{high}CD62L^{high}) and T_E/T_{EM} (CD44^{high}CD62L^{low}) CD8 T cells. Numbers show the mean fluorescence intensity of CX3CR1 and $\alpha_4\beta_7$ expression on the CD45.1$^+$ (upper values) and CD45.2$^+$ (lower values) populations. Representative or composite data are shown from two to three independent experiments with at least eight mice per group.

IL-21 signaling is dispensable for the generation of virus-specific CD8 T_E/T_{EM} cells and the accumulation of antiviral CD8 T cells in tissues after acute LCMV infection

To determine whether infection-associated cues can overcome the requirements for IL-21 in CD8 T cell differentiation, we infected intact naive IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mice with LCMV-Armstrong (an acute infection). Similar to previous reports (10, 11, 29–31), no marked differences in the patterns of CD44, CD62L, CD127, and KLRG1 by virus-specific IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD8 T cells were discernable at days 7 and 35 postinfection (data not shown). The expression of CX3CR1 (Fig. 9A) and $\alpha_4\beta_7$ (Fig. 9B) on LCMV-specific T_E cells was also similar between IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mice at day 7 postinfection. Although there were no marked differences between IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mice, analysis of the expression of CX3CR1 on CD62L^{high} and CD62L^{low} virus-specific CD8 T cells at days 35–38 postinfection revealed that CD62L^{low} T_E/T_{EM} phenotype cells expressed higher levels of CX3CR1 (Fig. 9C), which is consistent

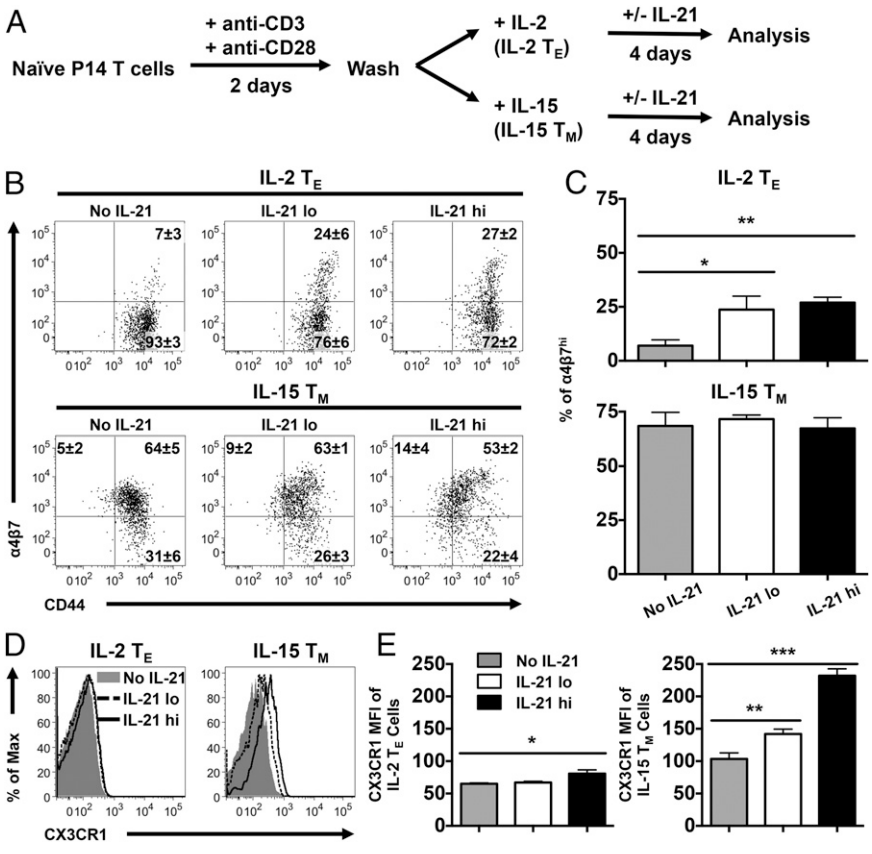

FIGURE 7. IL-21 increases the expression of CX3CR1 and $\alpha_4\beta_7$ on CD8 T cells in vitro. (**A**) Schematic depicting the experimental setup. Naive P14 T cells were activated with anti-CD3 and anti-CD28 Abs for 2 d, and then washed and cultured with IL-2 or IL-15 for 4 d to generate IL-2 T_E or IL-15 T_M cells, respectively. A low or high dose of IL-21 was added to IL-2 and IL-15 cultures, as indicated. (**B**) Representative dot plots show the expression of CD44 and $\alpha_4\beta_7$. Numbers indicate the percentages ± SD of cells in each quadrant. (**C**) Bar graph shows the percentages of live $\alpha_4\beta_7^{high}$ P14 T cells. (**D**) Representative histograms show the expression of CX3CR1. (**E**) Bar graphs show the mean fluorescence intensity of CX3CR1 on live P14 T cells. Representative or composite data are shown from three independent experiments analyzing three mice per group. Error bars show SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

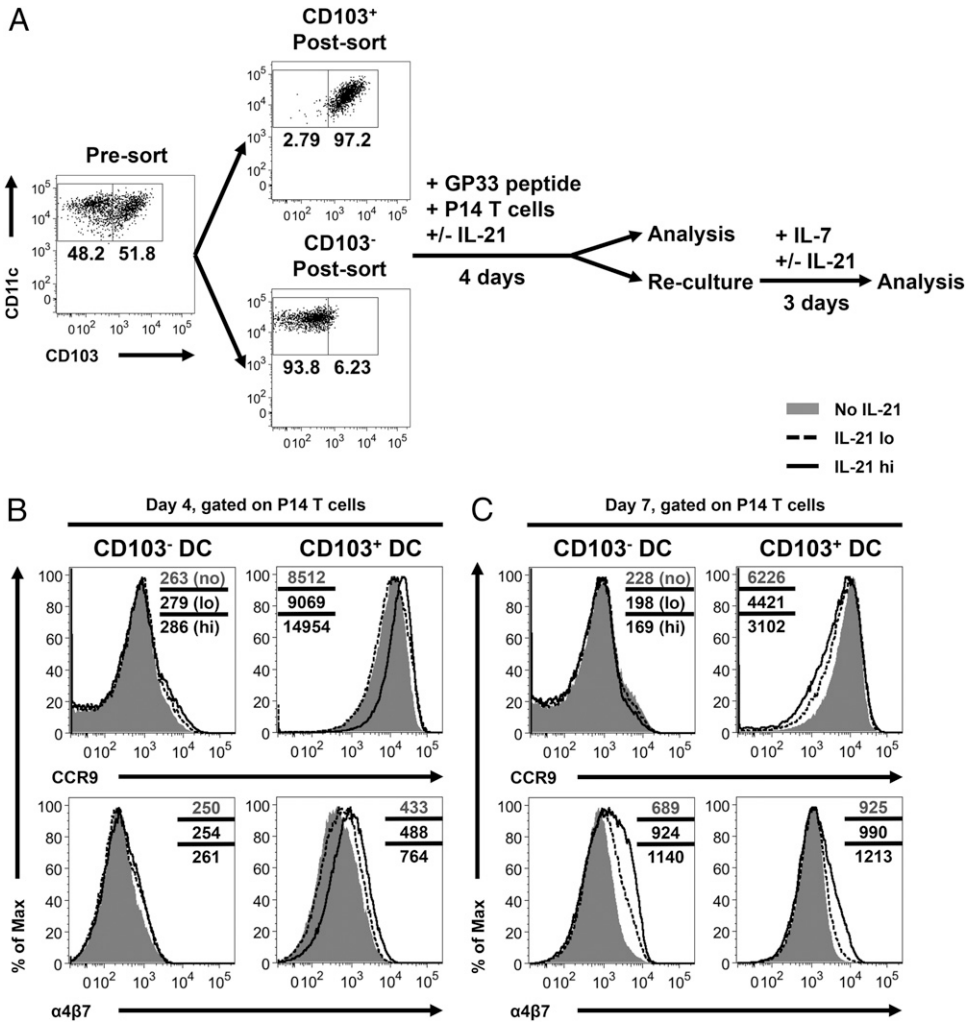

FIGURE 8. IL-21 enhances the capacity of DC subsets to induce $\alpha_4\beta_7$ expression on CD8 T cells in vitro. (**A**) Schematic depicting the experimental setup. $CD103^+$ and $CD103^-$ DCs from the MLNs were sorted and pulsed with gp33–41 peptide and then cultured with naive P14 T cells with or without the addition of IL-21. Four days later, cells were analyzed by flow cytometry. Cells activated without IL-21 were then recultured with IL-7 in the absence or presence of IL-21 for additional 3 d prior to analysis. (**B** and **C**) Histograms show expression of CCR9 and $\alpha_4\beta_7$ gated on live P14 T cells cultured with $CD103^-$ (*left panels*) and $CD103^+$ (*right panels*) DCs after (B) 4 d of initial culture and (C) 3 d of secondary culture. Numbers show the mean fluorescence intensity of P14 T cells cultured with no IL-21 (upper values), low dose of IL-21 (middle values), and high dose of IL-21 (lower values). Representative data are shown from two independent experiments.

with the results shown in Fig. 6A. In line with previous results (56), we did not detect $\alpha_4\beta_7$ expression on virus-specific CD8 T cells at memory time points (data not shown). Next, we evaluated the impact of IL-21 signaling on the distribution of memory CD8 T cells in tissues at days 35–38 postinfection. As shown in Fig. 9D, the numbers of virus-specific CD8 T cells in both lymphoid and nonlymphoid tissues were similar between the IL-$21R^{+/+}$ and IL-$21R^{-/-}$ mice, and virus-specific $CD69^+CD103^+$ T_{RM} phenotype CD8 T cells became established in the IEL compartment of both cohorts (Fig. 9E, 9F). Virus-specific CD8 T cells from both IL-$21R^{+/+}$ and IL-$21R^{-/-}$ mice also expressed similar levels of granzyme B (Fig. 9G).

To further evaluate whether IL-21 signaling influences the formation of effector phenotype CD8 T cells after acute LCMV infection, we cotransferred equal numbers of IL-$21R^{+/+}$ and IL-$21R^{-/-}$ T_{CM} phenotype ($CD44^{high}CD62L^{high}$) P14 T cells that had developed in the sublethally irradiated recipients into naive CD45.1 mice. These second sets of recipients were then infected with LCMV-Armstrong (Fig. 10A). Upon infection, both the P14 IL-$21R^{+/+}$ and IL-$21R^{-/-}$ donor CD8 T cell populations expanded in number and lost surface expression of CD62L, attaining a $CD44^{high}CD62L^{low}$ T_E/T_{EM} phenotype (Fig. 10B). Moreover, P14 IL-$21R^{-/-}$ CD8 T cells showed enhanced accumulation in all tissues tested at day 7 postinfection (Fig. 10C). Taken together, these data indicate that acute LCMV infection offsets the necessity for IL-21 to establish CD8 T_E/T_{EM} subsets and deposit CD8 T cells in nonlymphoid tissues, suggesting a context-dependent role for this cytokine in modulating the generation, accumulation, and function of effector phenotype CD8 T cell subsets.

LCMV-specific memory CD8 T cells require IL-21 for optimal accumulation in lymphopenic environments

The observation that acute LCMV infection drives the differentiation and tissue accumulation of CD8 T cells independently of IL-21 prompted us to test whether memory populations from LCMV-infected donors still required IL-21 under lymphopenic conditions. To address this, equal numbers of IL-$21R^{+/+}$ and IL-$21R^{-/-}$ $CD44^{high}$ memory phenotype CD8 T cells from LCMV-infected donors were mixed and cotransferred into sublethally irradiated recipients (Fig. 11A). Prior to transfer, the ratio of IL-$21R^{+/+}$ and IL-$21R^{-/-}$ gp33-specific CD8 T cells in the donor cell pool ranged from 0.9 to 1.1. At days 32–41 posttransfer, the frequencies and numbers of non-gp33–specific ($gp33^-$) donor CD8

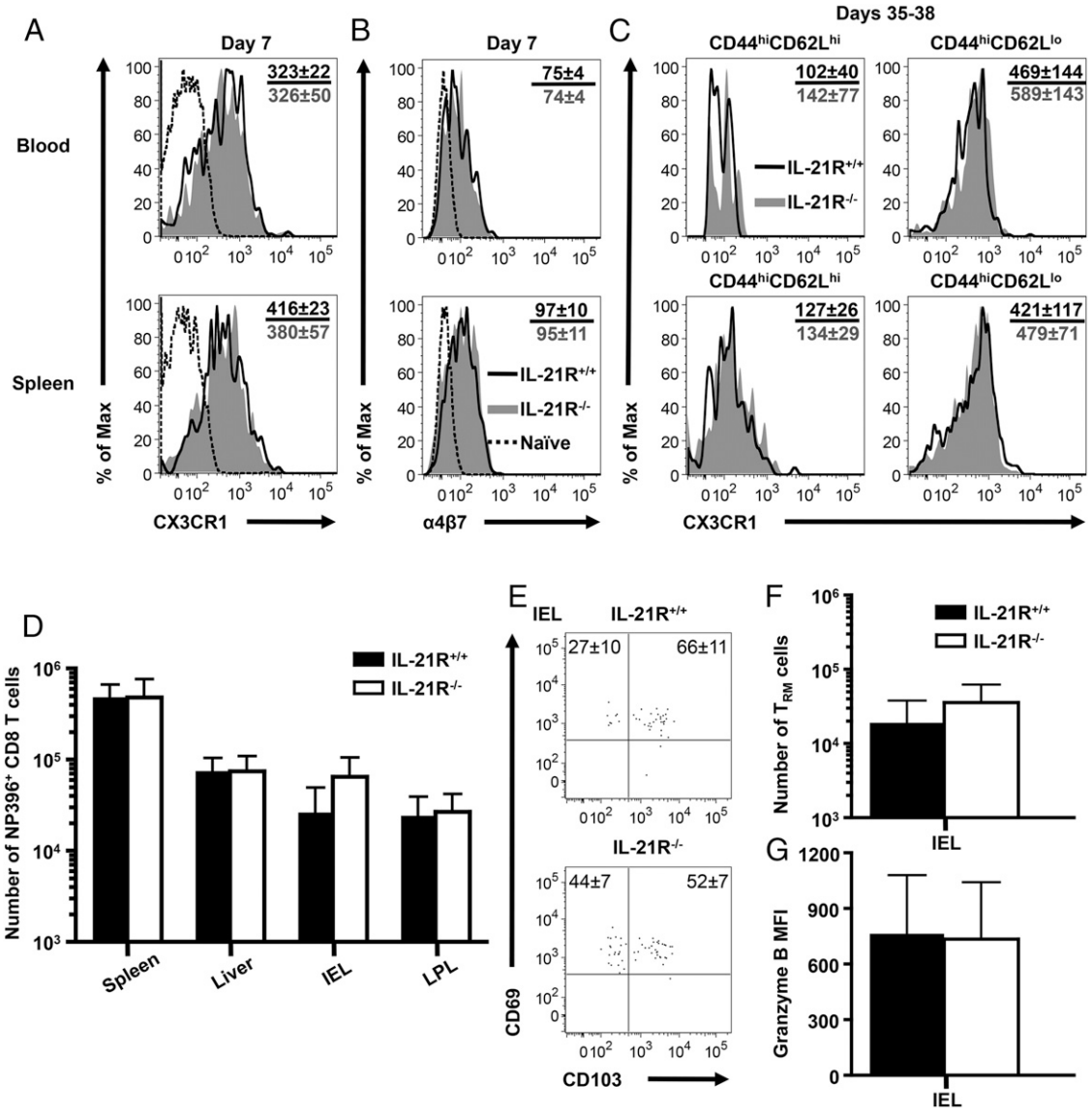

FIGURE 9. IL-21 signaling is dispensable for the differentiation of virus-specific CD8 T cell subsets during acute LCMV infection. IL-21R$^{+/+}$ and IL-21R$^{-/-}$ mice were infected with LCMV-Armstrong. Representative histograms show expression of (**A**) CX3CR1 and (**B**) $\alpha_4\beta_7$ on LCMV D^b(NP396)-specific CD8 T cells at day 7 postinfection. Numbers show the mean fluorescence intensity of expression on the IL-21R$^{+/+}$ (upper values) and IL-21R$^{-/-}$ (lower values) populations. Representative or composite data are shown from two independent experiments (n = 6 and 9 for IL-21R$^{+/+}$ and IL-21R$^{-/-}$ groups, respectively). (**C**) Representative histograms show expression of CX3CR1 gated on D^b(NP396)-specific T_{CM} (CD44^{high}CD62L^{high}) and T_{EM} (CD44^{high}CD62L^{low}) CD8 T cells at days 35–38 postinfection. Numbers show the mean fluorescence intensity of expression on the IL-21R$^{+/+}$ (upper values) and IL-21R$^{-/-}$ (lower values) populations. Representative or composite data are shown from two independent experiments (n = 7 and 9 for IL-21R$^{+/+}$ and IL-21R$^{-/-}$ groups, respectively). (**D**) Graphs show the numbers of D^b(NP396)-specific CD8 T cells in the indicated compartments at days 35–38 postinfection, with error bars indicating SD. Composite data are shown from two individual experiments (n = 7 and 9 for IL-21R$^{+/+}$ and IL-21R$^{-/-}$ groups, respectively). (**E**–**G**) D^b(NP396)-specific CD8 T cells isolated from the SI IEL compartment were analyzed at 35–38 d postinfection. (E) Representative dot plots show expression of CD103 and CD69. Numbers show the percentages (mean ± SD) of cells in each quadrant. (F) The numbers of CD103$^+$CD69$^+$ D^b(NP396)-specific CD8 T cells in the small intestine IEL, and (G) the ex vivo mean fluorescence intensity of granzyme B expression by these cells. Representative or composite data are shown from two independent experiments (n = 7 and 9 for IL-21R$^{+/+}$ and IL-21R$^{-/-}$ groups, respectively). Error bars show SD.

T cells recovered from the irradiated recipients were generally similar (Fig. 11B, 11D). Notably, by comparison with their IL-21R$^{+/+}$ counterparts, fewer IL-21R$^{-/-}$ gp33$^+$ donor CD8 T cells were detected in the spleen, and the differences between these two cell populations were more dramatic in the liver and LPL (Fig. 11C, 11E). The fold differences between the numbers of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ gp33$^+$ donor CD8 T cells in the spleen, liver, and LPL were 2.7, 4.4, and 6.8, respectively. It should be noted that few gp33$^+$ donor CD8 T cells were detected in the small intestine IEL compartment, and thus excluded from the analysis.

The expression of CX3CR1 was similar on IL-21R$^{+/+}$ and IL-21R$^{-/-}$ LCMV-specific memory CD8 T cells prior to transfer (Fig. 9C and data not shown); however, 32–41 d posttransfer, the frequencies of both gp33$^-$ and gp33$^+$ IL-21R$^{-/-}$ CD62L^{low} CX3CR1$^+$ donor CD8 T cells were 2-fold lower than their IL-21R$^{+/+}$ counterparts (13 ± 4% versus 6 ± 2% with p = 0.0037 and 23 ± 8% versus 10 ± 3% with p = 0.0017, respectively), whereas the frequencies of CD62L^{high}CX3CR1$^-$ cells were increased (Fig. 11F, 11G). Taken together, these data indicate that the priming and environment associated with acute LCMV infection

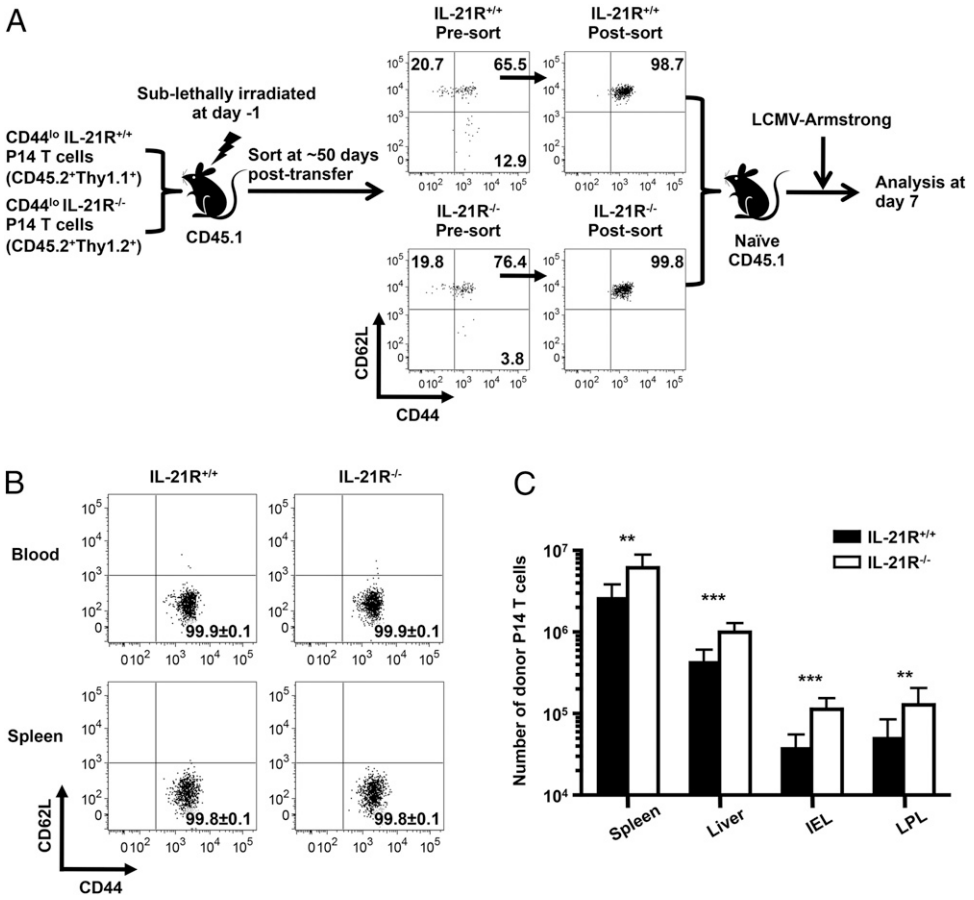

FIGURE 10. Homoeostatic proliferation-induced T_{CM} phenotype CD8 T cells mount IL-21–independent responses to acute LCMV challenge. (**A**) Schematic depicting the experimental setup. Equal numbers of lymphopenia-induced P14 IL-21R$^{+/+}$ and IL-21R$^{-/-}$ T_{CM} phenotype cells were adoptively transferred into second sets of recipients that were subsequently infected with LCMV-Armstrong, and responses were analyzed 7 d later. (**B**) Dot plots show expression of CD44 and CD62L on gated donor P14 CD8 T cells in the blood and spleens of the infected recipients. (**C**) Bar graphs show the numbers of donor P14 CD8 T cells recovered from various sites at day 7 postinfection. Representative or composite data are shown from two independent experiments analyzing a total of eight mice. Error bars show SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

circumvent the necessity for IL-21 for the differentiation and distribution of activated CD8 T cells. Moreover, virus-specific memory populations generated by this infection process are not programmed to become intrinsically IL-21 independent, as IL-21 signals are still required for their accumulation in tissues and the maintenance of a CX3CR1^{+} phenotype under irradiation-induced lymphopenic conditions.

Discussion

This study reveals context-dependent requirements for IL-21 in shaping the configuration and tissue distribution of effector and memory CD8 T cell pools. We demonstrate that IL-21 is more stringently required under competitive conditions as CD8 T cells reconstitute mixed bone marrow chimeras as well as following lymphopenia-induced homeostatic proliferation in sublethally irradiated recipients. Nevertheless, these requirements for IL-21 in CD8 T cell differentiation and maintenance are largely circumvented during acute LCMV infection. In mixed bone marrow chimeras and also in sublethally irradiated recipients, we show several roles for IL-21 in influencing CD8 T cell responses. First, IL-21 functions to increase the proportion of T_E/T_{EM} populations by acting directly on CD8 T cells. This is shown by both the greater proportion of IL-21R$^{+/+}$ CD44^{high}CD62L^{low}CD8 T cells and also by the decreased fraction of IL-21R$^{-/-}$ CD44^{high}CD8 T cells, which adopt a KLRG1^{high} and CD62L^{low}CD127^{low} effector-associated phenotype. Second, the expression of IL-21R aids the accumulation of activated CD8 T cells and their distribution into nonlymphoid tissues. Third, IL-21 signaling is associated with increased levels of the chemokine receptor CX3CR1 and the integrin $\alpha_4\beta_7$ on T_E/T_{EM} phenotype CD8 T cells. Fourth, IL-21R expression appears to be necessary for the expression of the cytotoxic effector molecule granzyme B on CD8 T_{RM} cells present in the small intestine of mixed bone marrow chimeras. Nevertheless, acute LCMV infection circumvents these requirements for IL-21, implying an intriguing context-dependent role of this cytokine in sustaining effector phenotype CD8 T cells and influencing their migratory properties, accumulation, and functions.

A property of CD8 T_{RM} cells is the downregulation of CD122 and CD127 (25, 33, 35). IL-7 and IL-15 are key regulators of T cell homeostasis, with IL-7 operating to promote survival and IL-15, mainly supporting basal homeostatic proliferation (38, 48, 59, 60). We show that the numbers of IL-21R$^{-/-}$ CD44^{high} CD122^{low}CD127^{low}CD8 T cells are dramatically decreased in the spleen of mixed bone marrow chimeras. Furthermore, the accumulation of IL-21R$^{-/-}$ CD8 T_{RM} cells in the small intestine is compromised in both mixed bone marrow chimeras and irradiated recipients. Notably, the exhaustion and deletion of antiviral CD8 T cells during chronic LCMV infections are markedly more severe in the absence of IL-21 (10–12), and exhausted T cells also downregulate the IL-7R and IL-15R (34, 36). Thus, we speculate that IL-21 contributes to the maintenance of CD8 T cells when

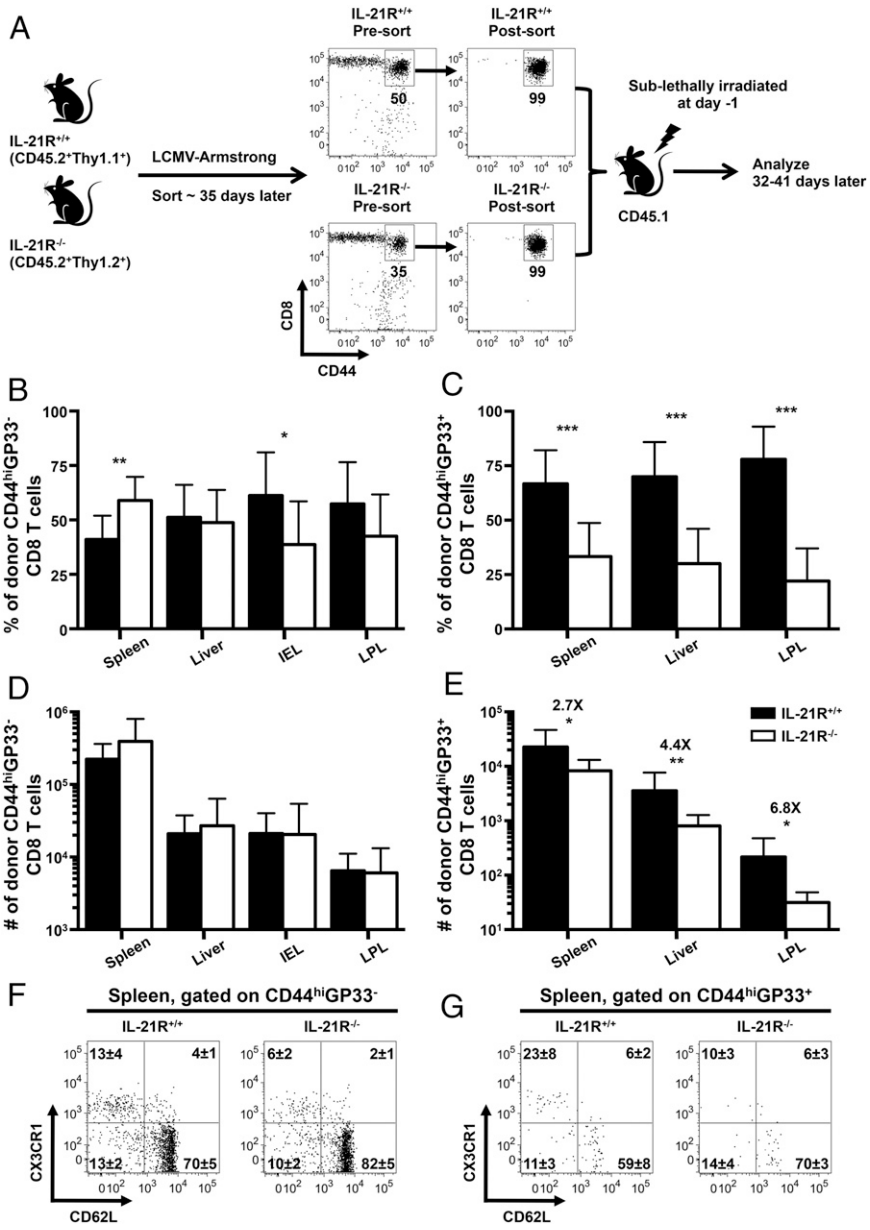

FIGURE 11. IL-21 signaling is required for the accumulation of virus-specific memory CD8 T cells in tissues and the maintenance of CX3CR1$^+$ subsets in lymphopenic environments. (**A**) Schematic depicting the adoptive transfer of equal numbers of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ CD44^{high} memory CD8 T cells after acute LCMV infection into sublethally irradiated recipients. Donor gp33$^+$ and gp33$^-$ CD8 T cells were analyzed 32–41 d later. (**B** and **C**) Bar graphs show the percentages of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ (B) CD44^{high}gp33$^-$ and (C) CD44^{high}gp33$^+$ donor CD8 T cells recovered from indicated tissues. (**D** and **E**) Bar graphs show the numbers of IL-21R$^{+/+}$ and IL-21R$^{-/-}$ (D) CD44^{high}gp33$^-$ and (E) CD44^{high}gp33$^+$ donor CD8 T cells recovered from indicated tissues. Composite data are shown from two independent experiments analyzing seventeen mice. (F and G) Representative dot plots show the expression of CX3CR1 and CD62L gated on splenic (**F**) CD44^{high}gp33$^-$ or (**G**) CD44^{high} gp33$^+$ CD8 T cells. Numbers show the percentages of cells in each quadrant. Representative or composite data are shown from two independent experiments analyzing seven mice. Error bars show SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

IL-7 and IL-15 signals are insufficient. This would most likely help prolong responses under conditions of persistent antigenic activation and also help the survival of sessile T_{RM} populations.

CD8 T_{RM} cells residing in the small intestine IEL compartment constitutively express granzyme B (33, 35), which is an important cytolytic granule constituent whose enzymatic activity causes target cell death. In the mixed bone marrow chimeras, fewer IL-21R$^{-/-}$ CD8 T cells are present in the small intestine IEL and other peripheral tissues. Nevertheless, the IL-21R$^{-/-}$ CD8 T_{RM} cells that are present in the IEL compartment share many of the canonical properties of their IL-21R$^{+/+}$ counterparts; they are CD69^{high}, CD103^{high}, CD122^{low}, and CD127^{low}. Strikingly, the majority of the small population of putative IL-21R$^{-/-}$ CD8 T_{RM} cells in the IEL of these hosts express markedly lower levels of granzyme B. This implies that IL-21 promotes the cytotoxic potential of CD8 T cells residing in certain tissues, which may occur via the upregulation of the transcription factor T-bet, which regulates the expression of cytolytic effector molecules (61).

The ability of CD8 T cells to migrate to nonlymphoid organs is governed by their expression of chemokine receptors, integrins, and selectins (14). We found that CX3CR1, the receptor for the chemokine CX3CL1 (fractalkine), is expressed by T_E/T_{EM} phenotype CD8 T cells, which is consistent with previously published results (52, 53). It has been reported that the interactions between CX3CR1 and fractalkine mediate the adhesion and migration of CD8 T cells and promote their migration in response to other chemokines such as CCL4 in vitro (50, 51). Integrin $\alpha_4\beta_7$, which binds to mucosal addressin cell adhesion molecule-1, mediates the migration of CD8 T cells to the small intestine (14, 56). The upregulation of CX3CR1 and $\alpha_4\beta_7$ on IL-21R$^{+/+}$ but not on IL-21R$^{-/-}$ T_E/T_{EM} phenotype CD8 T cells in the mixed bone marrow chimeras implicates a role for IL-21 in establishing the migratory properties of activated CD8 T cells and dictating their capacity to enter tissues. Furthermore, our in vitro studies demonstrate that IL-21 can differentially promote the development of CD8 T cell subsets that express CX3CR1 or $\alpha_4\beta_7$. Because IL-21 favors the expression of $\alpha_4\beta_7$ by IL-2 T_E but the expression of CX3CR1 by IL-15 T_M cells, this implies that these subsets mount distinct responses to IL-21 and that $\alpha_4\beta_7$ and CX3CR1 are independently regulated. We also discovered that IL-21 enhances the capacity of

DC subsets, especially CD103$^-$ DCs, to induce $\alpha_4\beta_7$ expression. It has been previously shown that MLN CD103$^+$ DCs promote $\alpha_4\beta_7$ expression on CD8 T cells via retinoic acid (57, 58), and the current findings suggest that IL-21 may amplify or substitute for these signals.

During several infections, including acute LCMV, vaccinia virus, and adenovirus, the patterns of CD62L expression by antiviral CD8 T cells are similar with and without IL-21 signals (10, 11, 29–31). Consistent with these reports, we confirmed that IL-21 signaling is not required for the generation of virus-specific CD8 T_E cells following acute LCMV infection. Moreover, under these conditions, the upregulation of CX3CR1 and $\alpha_4\beta_7$ by activated antiviral CD8 T cells as well as their accumulation in nonlymphoid tissues and the expression of granzyme B by CD8 T_{RM} cells did not require IL-21. By contrast, we consistently observe a lower frequency of IL-21R$^{-/-}$ CD44^{high}CD62L^{low} T_E/T_{EM} cells, as well as severely impaired accumulation of IL-21R$^{-/-}$ CD8 T cells in nonlymphoid organs both in mixed bone marrow chimeras and after lymphopenia-induced homeostatic proliferation. Thus, the inflammatory and antigenic signals during acute LCMV infection are sufficient to compensate for any role of IL-21 in forming and sustaining T_E/T_{EM} and T_{RM} CD8 T cell subsets (10, 11, 29). Context-dependent roles for IL-21 are further highlighted during the development of experimental autoimmune encephalomyelitis. IL-21 is required for the of development of spontaneous experimental autoimmune encephalomyelitis, but is not necessary for the induction of active disease, which involves immunization with CFA (62).

The priming conditions associated with acute LCMV infection do not render the responding CD8 T cells fully independent of IL-21, as gp33-specific memory cells still require IL-21 signals for their optimal accumulation and sustained expression of CX3CR1 following exposure to irradiation-induced lymphopenic conditions. Notably, the non-gp33–specific subset of memory CD8 T cells appears to less stringently require IL-21 in lymphopenic hosts. It has been estimated that LCMV-specific memory CD8 T cells constitute ~15% of the total CD8 T cell pool (63). Therefore, most of the bulk CD44^{high}CD8 T cells that were transferred are likely not LCMV specific, but instead reactive to self, environmental, or commensal Ags (47, 49, 64, 65). The disparities between the gp33-specific and nonspecific subsets may reflect cell-intrinsic differences between true virus-primed memory CD8 T cells and memory phenotype CD8 T cells generated by homeostatic and other mechanisms, supporting the concept that the state of differentiation and environmental circumstances shapes the demands for activation and maintenance signals, including IL-21.

In summary, our data demonstrate that IL-21 signaling can shape the CD8 T cell pool by preferentially promoting T_E/T_{EM} and T_{RM} populations. Additionally, IL-21 can influence the expression of CX3CR1 and $\alpha_4\beta_7$ by CD8 T cells that may impact their ability to accumulate in tissues. Notably, a substantially lower fraction of IL-21R$^{-/-}$ CD8 T cells is detected in nonlymphoid sites under homeostatic and lymphopenic conditions. Nevertheless, the necessity for IL-21 can be displaced by acute LCMV infection. Thus, although IL-21 can impact both systemic and local CD8 T cell responses, the precise requirements for this cytokine vary, illustrating the importance of the cytokine milieu and levels of antigenic activation in developing CD8 T_{EM} and T_{RM} responses, which may provide rapid and localized immunological protection.

Acknowledgments

We thank Marion Spell and the University of Alabama at Birmingham Center for AIDS Research Flow Cytometry Core Facility for services, as well as John Yi, Troy Randall, Chad Steele, and all of the members of the Zajac and Harrington laboratories for help and critical reading of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

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