PNAS RESEARCH ARTICLE | IMMUNOLOGY AND INFLAMMATION OPEN ACCESS

Optimal generation of hepatic tissue-resident memory CD4 T cells requires IL-1 and IL-2

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Edited by Roy Curtiss III, University of Florida, Gainesville, FL; received August 30, 2022; accepted February 23, 2023

Hepatic CD4 tissue-resident memory T cells (TRM) are required for robust protection against *Salmonella* infection; however, the generation of this T cell population is poorly understood. To interrogate the contribution of inflammation, we developed a simple *Salmonella*-specific T cell transfer system that allowed direct visualization of hepatic TRM formation. *Salmonella*-specific (SM1) T cell receptor (TCR) transgenic CD4 T cells were activated in vitro and adoptively transferred into C57BL/6 mice while hepatic inflammation was induced by acetaminophen overdose or *L. monocytogenes* infection. In both model systems, hepatic CD4 TRM formation was accentuated by local tissue responses. Liver inflammation also enhanced the suboptimal protection provided by a subunit *Salmonella* vaccine which typically induces circulating memory CD4 T cells. To further elucidate the mechanism of CD4 TRM formation in response to liver inflammation, various cytokines were examined by RNAseq, bone marrow chimeras, and in vivo neutralization. Surprisingly, IL-2 and IL-1 were found to enhance CD4 TRM formation. Thus, local inflammatory mediators enhance CD4 TRM populations and can boost the protective immunity provided by a suboptimal vaccine. This knowledge will be foundational for the development of a more effective vaccine against invasive nontyphoidal salmonellosis (iNTS).

tissue-resident memory | CD4 T cells | *Salmonella* | vaccination

During an immune response to infection, T cells can greatly expand to perform various effector functions, but will eventually contract to form a much smaller memory population (1). T cell memory is largely composed of circulating cells [effector memory (TEM) and central memory (TCM)] and tissue-resident cells (TRM), which are maintained in tissues as a frontline defense against secondary infection (2, 3). Due to their importance in T cell-mediated protection, it is vital to understand how these TRMs are generated at local tissue sites. The vast majority of previous studies have focused on the generation of CD8 TRM even though CD4 TRM play a critical role in combating a wide range of intracellular bacteria (4–9). Understanding CD4 TRM formation will aid vaccine design, thus addressing the serious public health concerns associated with many of these diseases.

One intracellular bacterium that requires CD4 TRM for secondary protection is *Salmonella enterica* which causes over 200,000 deaths per year predominantly in sub-Saharan Africa and Southeast Asia, due to typhoid fever caused by *S. enterica serovar* Typhi or distinct *S. enterica serovars* which cause invasive nontyphoidal salmonellosis (iNTS) (10–12). The two licensed vaccines for typhoid are not widely used by people living in endemic areas and there are no licensed vaccines for iNTS (13–15). Therefore, there is an urgent need to develop effective vaccines against typhoidal and nontyphoidal *Salmonella* infections. In highly susceptible inbred mouse models, a live vaccine strain of *Salmonella* (LVS) can fully protect against subsequent challenge with wild-type (WT) strains via the activity of Th1 CD4 T cells (16–18). However, live vaccines are not ideal for those most at risk for *Salmonella* infections (immunocompromised individuals and infants) and a subunit vaccine would be preferable (19). While there have been multiple attempts to develop a subunit *Salmonella* vaccine, none are fully protective (20–24). We previously reported that the protection provided by an SseB subunit vaccine was mediated primarily by circulating memory alone (21). This incomplete protection was most likely due to the absence of CD4 TRM in the liver, meaning that the genesis of hepatic TRM is therefore important for *Salmonella* vaccine development.

Inflammatory cues within infected tissues are thought to play a major role in the terminal differentiation of activated T cells toward a TRM phenotype, with sterile inflammatory agents and individual proinflammatory cytokines encouraging CD8 TRM formation in various tissues (25–31). In comparison, much less is known about requirements for tissue inflammation and cytokine signaling during CD4 TRM formation. In this

Significance

Systemic *Salmonella* causes about 371,500 deaths a year, predominantly impacting young or immunocompromised individuals, and an effective subunit vaccine is needed. CD4 (TRM) tissue-resident memory T cells are required for complete protection against *Salmonella,* but subunit vaccines typically induce circulating memory cells rather than tissue-resident populations. This study demonstrates that liver inflammation increased the formation of hepatic CD4 TRM and enhanced protection provided by SseB subunit vaccination. Cytokines IL-1 and IL-2 were required for optimal formation of hepatic CD4 TRM. This provides mechanistic insight into the formation of protective memory T cells against an important human pathogen.

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Author contributions: C.E.D. and S.J.M. designed research; C.E.D. and J.A.R. performed research; C.E.D. and S.J.M. analyzed data; and C.E.D. and S.J.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2214699120/-/DCSupplemental.

Published April 11, 2023.

study, we developed a simple model system that allowed direct visualization of *Salmonella*-specific CD4 TRM generation in the liver, allowing an examination of local inflammation on CD4 TRM formation. These data show that liver inflammation influenced TRM formation in the liver and enhanced protective immunity to *Salmonella* infection. Inflammatory cytokines, IL-1 and IL-2, were identified as necessary mediators for optimal formation of hepatic *Salmonella*-specific CD4 TRM.

Results

Immunization with LVS *Salmonella* Generates Hepatic CD4 TRM. In order to visualize *Salmonella*-specific memory CD4 T cells, C57BL/6 mice were immunized with LVS *Salmonella*-expressing 2W1S (BMM51), and 2W1S MHC class-II tetramer was used to identify responding CD44$^+$ CD4 T cells 45 d later (4) (Fig. 1*A*). At this memory time point, more than 50% of tetramer$^+$ CD4 T cells in the liver expressed CD69 (Fig. 1*A*), a surface marker commonly expressed by resident memory T cells. *Salmonella*-specific CD69$^+$ memory CD4 T cells expressed higher surface levels of P2RX7, CD11a, CXCR3, and CD101, compared to CD69− memory CD4 T cells, as well as low levels of KLRG1 and similar levels of CD103 (Fig. 1*B*), consistent with a TRM phenotype. IFN-γ-YFP reporter mice were used to confirm that this TRM phenotype was also evident when analyzing endogenous polyclonal *Salmonella*-specific CD4 T cells (32) (*SI Appendix*, Fig. S1 *A* and *B*).

Transfer of *Salmonella*-Specific TRM Protects against *Salmonella* Infection. We next wanted to determine the protective capacity of these hepatic CD4 TRM that arise during vaccination. C57BL/6 mice were immunized with LVS-*Salmonella* and 45 d later, mice were administered nanobody s+16a or left untreated prior to isolation of liver lymphocytes. Isolated hepatic lymphocytes from immunized mice were adoptively transferred to TCRα-deficient recipients and challenged 24 h later (Fig. 2*A*). This s+16a treatment prevents death of cells expressing high levels of P2RX7 during tissue processing, like TRM (4, 33), and thus allows TRM to survive adoptive transfer. Mice that did not receive any lymphocyte transfer displayed high bacterial burdens in the liver, while mice that received untreated lymphocytes had lower burden, demonstrating the modest protective effect of hepatic lymphocytes without s+16a treatment (Fig. 2*B*, no transfer versus no s+16a). In contrast, mice that received a liver lymphocyte transfer from mice treated with s+16a had much lower bacterial burdens (Fig. 2*B*, s+16a versus no s+16a). Indeed, this protective effect was similar to LVS-immunized controls (Fig. 2*B*, s+16a versus LVS immunized), confirming that s+16a-sensitive lymphocytes are required for complete protection against *Salmonella* (4, 21).

Liver Inflammation Enhances CD4 TRM Formation and Vaccine-Mediated Protection. Given the importance of hepatic TRM to *Salmonella* immunity, understanding how these cells are generated will aid vaccine development. We created a mouse model where

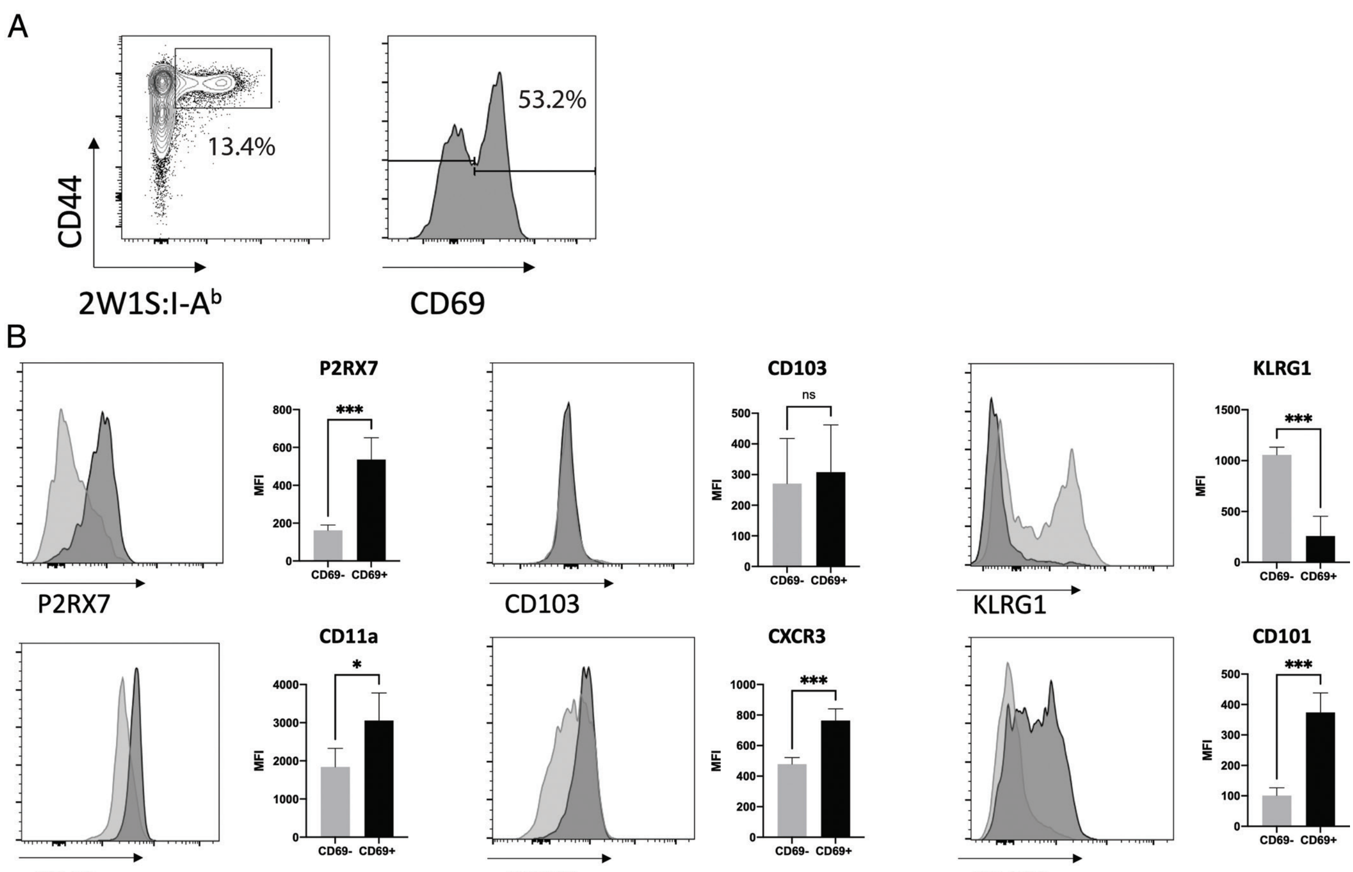

Fig. 1. Systemic *S. enterica* Typhimurium immunization forms CD4 TRM in the liver. (*A* and *B*) C57BL/6 mice were immunized with *Salmonella*-2W1S-LVS intravenously and 45 d later, livers were collected and analyzed by flow cytometry. (*A*) Immunization-specific CD4 T cells were identified by 2W1S-tetramer staining and TRM were identified as CD69+ tetramer+ CD4 T cells. (*B*) Expression of tetramer+ CD44+ CD4 T cells was determined for CD69+ (black) and CD69− (gray) tetramer+ cells by flow cytometry. (*B*) n = 4 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SD.

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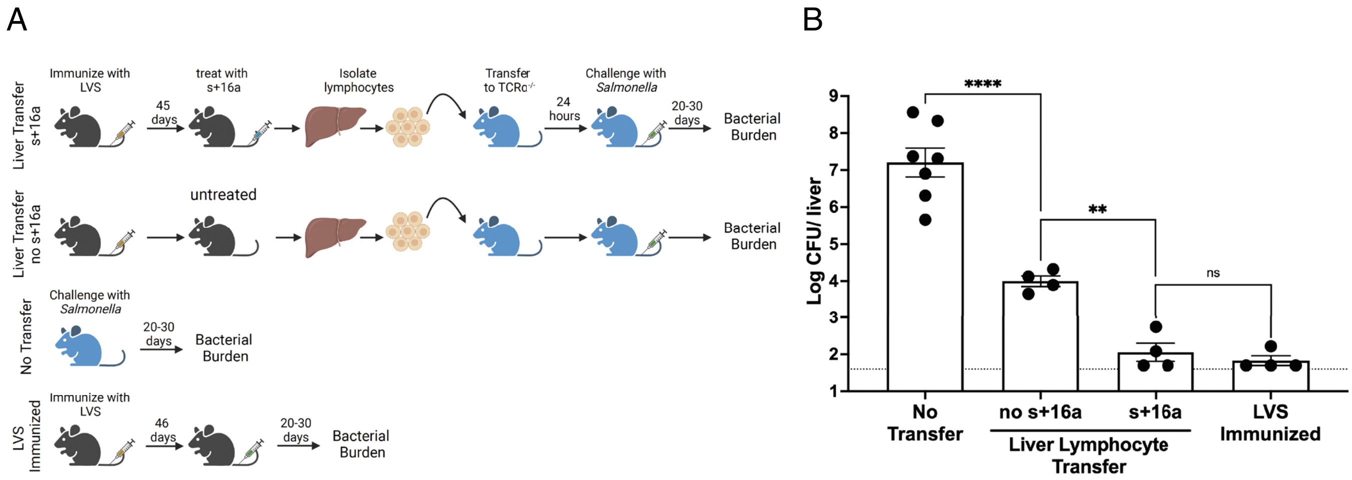

Fig. 2. Hepatic CD4 TRM are required for complete protection against *S. enterica* Typhimurium. (*A* and *B*) C57BL/6 mice were immunized with *Salmonella*-LVS intravenously. Forty-five days post infection, liver lymphocytes were isolated by Percoll gradient, which were transferred intravenously into TCRα$^{-/-}$ mice. One group of TCRα$^{-/-}$ mice received lymphocytes from mice treated with nanobody s+16a 15 min before killing while the second did not. Twenty-four hours after transfer, recipient mice were challenged intravenously with *Salmonella* BMM50. At 20 to 30 d post challenge, livers were collected and bacterial burdens were calculated to determine protection. (*B*) n = 7 for no transfer group; n = 4 for with s+16a transfer group, no s+16a transfer group, and LVS group. Data are representative of two experiments. Significance was calculated by one-way ANOVA and data are means ± SEM.

CD45.1 *Salmonella*-specific TCR transgenic CD4 T cells (SM1 T cells) were activated in vitro for 5 d before being transferred to naïve CD45.2 C57BL/6 mice. Two weeks later, a population of SM1 T cells was detected in the liver that phenotypically resembled LVS-specific CD4 TRM (Fig. 1*B*, Fig. 3*A*). CD45.1$^+$ SM1 T cells expressed low levels of CD62L and a subset expressed surface CD69 and P2RX7 (Fig. 3*B*). CD69+ SM1 T cells also expressed high levels of CXCR3, low levels of KLRG1, and similarly low levels of CD103, compared to CD69− SM1 T cells (Fig. 3*C*). This adoptive transfer system therefore provides a simple model to visualize the establishment of *Salmonella*-specific CD4 TRM in the liver.

To determine the effect of inflammation on TRM formation, mice were given an overdose of acetaminophen (APAP) or saline after transfer of activated SM1 T cells and then assessed 2 wk later (Fig. 4*A*). Sterile liver inflammation increased the number and frequency of SM1 T cells and encouraged the rate by which SM1 T cells became TRM compared to saline controls (Fig. 4 *A* and *B*). Thus, transferred activated SM1 T cell has a better chance of becoming a TRM when encountering an inflamed liver. Next, inflammation was induced by *Listeria monocytogenes* ΔActA, a liver tropic infection model, which lacks the cognate antigen recognized by SM1 T cells (34) (Fig. 4*C*). Inflammation generated by *Listeria*

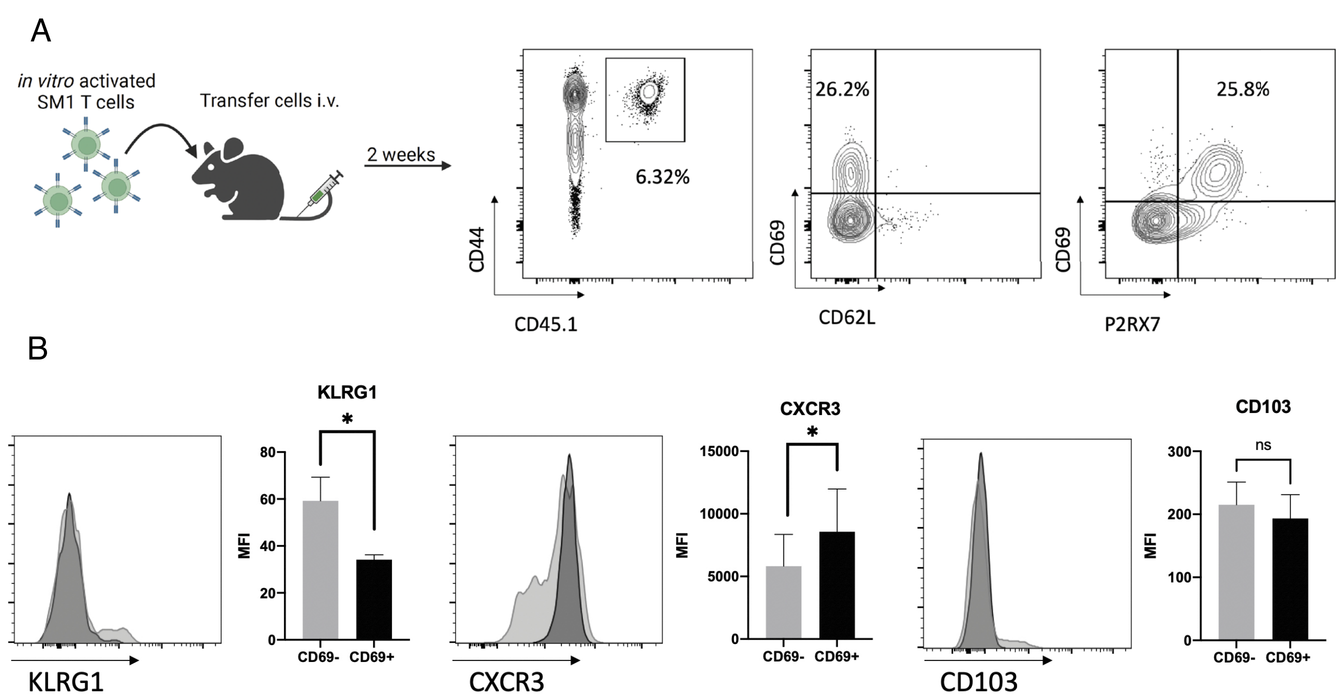

Fig. 3. Adoptive transfer of in vitro activated SM1 T cells form CD69+ P2RX7+ CD62L− SM1 TRM in the liver of mice. (*A* and *B*) Naïve SM1 T cells were isolated and cultured in vitro with rhIL-2, LPS, rfliC, and irradiated splenocytes for 5 d and then transferred intravenously to naïve C57BL/6 mice. Two weeks later, livers were collected and analyzed by flow cytometry. (*A*) Total SM1 T cells and SM1 TRM were identified by flow cytometry (*B*) Expression was determined by flow cytometry for CD69+ (black) and CD69− (gray) SM1 T cells. (*B*) n = 4 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SD.

infection also increased TRM formation in the liver (Fig. 4*D*). It should be noted that there was variation in the percentage of TRM formed in individual experiments but that addition of inflammation enhanced TRM formation irrespective of the baseline.

Protein immunization using the *Salmonella* T3SS protein, SseB, provides partial protection despite the fact that it does not generate resident memory (21). We therefore examined whether liver inflammation could enhance vaccine-specific hepatic TRM and increase protective immunity. Liver inflammation was induced by infecting mice with *Listeria* on the same day as SseB immunization. As controls, mice were immunized with SseB alone, *Listeria* alone, or *Salmonella*-LVS. To determine the protection provided, mice were subsequently challenged with WT *Salmonella,* and liver and spleen bacterial burdens were determined. Naïve and *Listeria*-infected mice had similar bacterial burden, demonstrating that liver inflammation alone does not provide any protection (Fig. 4*E*, naïve versus *Lm).* However, *Listeria* infection increased the protective efficacy of SseB immunization, although this effect

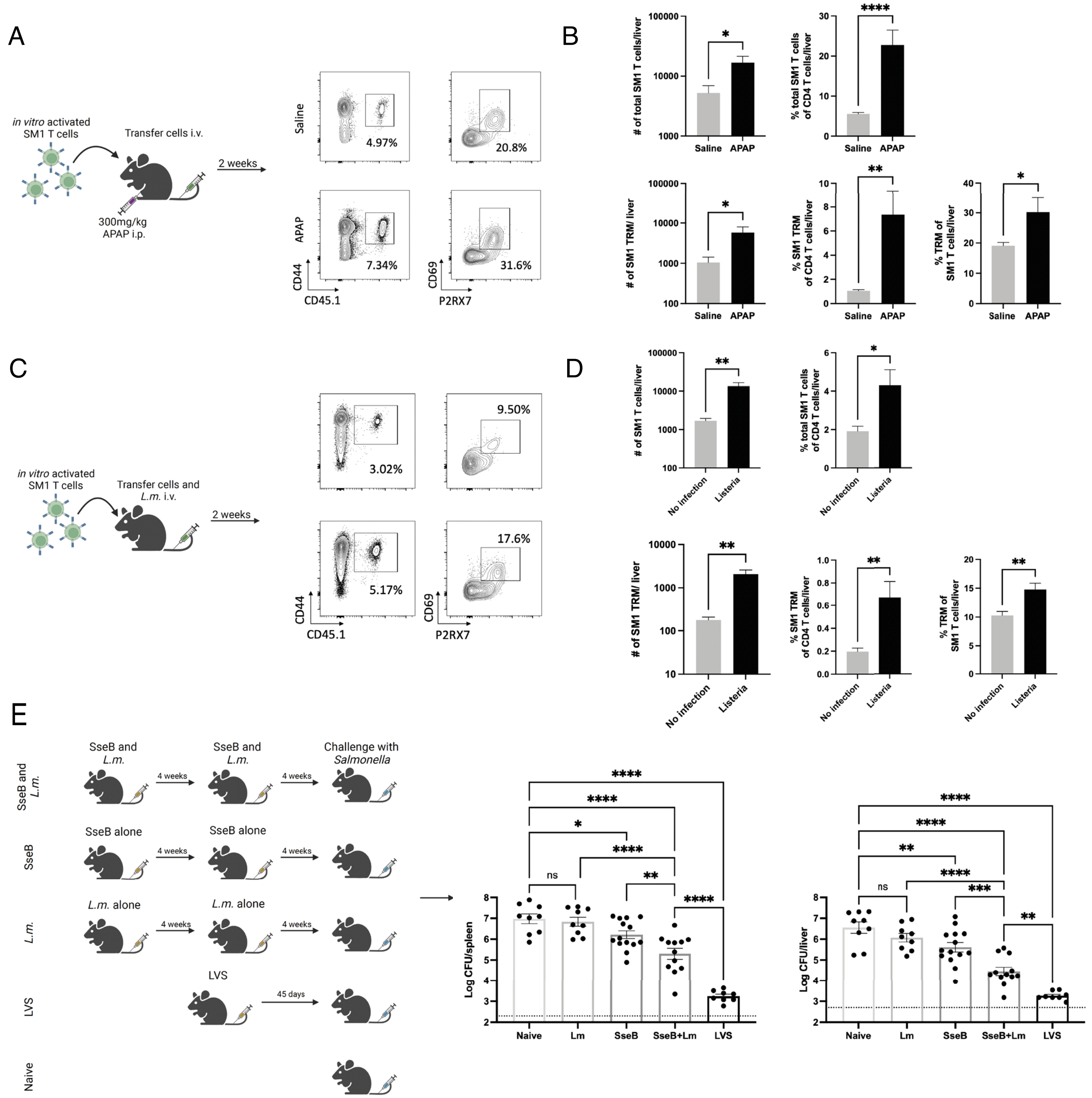

Fig. 4. Liver inflammation increases SM1 TRM formation in the liver and protection provided by subunit vaccine. (*A*–*D*) SM1 T cells were activated in vitro for 5 d and transferred intravenously into naïve C57BL/6 mice. Inflammation was induced same day as transfer by 300 mg/kg acetaminophen i.p. (*A* and *B*) or 1E7 CFU *L. monocytogenes* intravenously (*C* and *D*) and 2 wk later, livers were collected and total SM1 T cells and CD69+ P2RX7+ SM1 TRM were identified by flow cytometry (*E*) Mice were immunized with either SseB alone, *L. monocytogenes* alone, or SseB and *L. monocytogenes* intravenously. Four weeks later, the mice were boosted with the same vaccine and 4 wk later challenged. A control group of mice were immunized with *Salmonella*-LVS and challenged 45 d later. All groups were challenged with *Salmonella* SL1344 intravenously and 4 d later, protection was determined by bacterial burden of spleens and livers. (*B* and *D*) n = 6 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SEM. (*E*) Data are compiled from two experiments. Significance was calculated by one-way ANOVA and data are means ± SEM.

was more noticeable in the liver (Fig. 4*E*. SseB versus SseB+*Lm*). Thus, liver inflammation can increase the protection provided by a suboptimal subunit vaccine. It is formally possible that this enhancement could be due to effects on other cell populations, but the most logical interpretation is that this is due to an effect of inflammation on liver TRM.

Hepatic CD4 TRM Are Transcriptionally Distinct from Circulating Memory. Given the enhancing effect of liver inflammation, it was important to identify specific mediators induced by this response. Bulk RNAseq was performed on sorted memory populations from *Salmonella*-LVS immunized IFNγ-YFP reporter mice (35). In Multidimensional Scaling (MDS) plots, liver CD4 TRM clustered distinctly from CD4 TEM, and liver CD4 TRM displayed a similar transcriptional profile to CD8 TRM, including upregulation of *Itga1*, *Xcl1*, and *Art2b* and downregulation of *Klf2*, *S1pr1*, and *Notch3* (Fig. 5 *B* and *C*) (27, 36, 37). Chemokine receptors *Cxcr3*, *Cxcr6*, *Ccr5*, and *Ccr9* were up-regulated (Fig. 5*D*), similar to CD8 TRM transcriptional changes in the liver and characterization of hepatic CD4 TRM by flow cytometry (36, 38). The cytokine receptors up-regulated in CD4 TRM included *Il7r*, *Il2ra*, and, to a lesser extent, *Il12rb1* and *Il21r* (Fig. 5*D*).

IL-2 Is Sufficient and Necessary for Optimal Formation of CD4 TRM in the Liver. We decided to interrogate the role of IL-2 in the formation of hepatic CD4 TRM since the *Il2ra* was the cytokine receptor displaying the highest fold change by RNAseq and also confirmed by flow cytometry (Figs. 5*E* and 6*A*). To examine the role of IL-2, we used the activated SM1 T cell transfer model with same-day administration of an IL-2 depletion antibody, followed by twice-weekly administration of anti-IL-2 for 2 wk (Fig. 6*B*). IL-2 depletion did not affect the number or frequency of SM1 T cells in the liver (Fig. 6*C*); thus, IL-2 is not required for homing or survival of CD4 T cells in the liver. However, there was a significant reduction in the number and frequency of hepatic SM1 TRM, and the rate of SM1 T cells becoming TRM in IL-2-depleted mice (Fig. 6*C*), indicating that IL-2 is required for optimal formation of CD4 TRM in the liver. Interestingly, other receptors up-regulated on CD4 TRM were not required for TRM formation since transfer into IL-12p40-deficient recipients or depletion of IL-7 failed to affect SM1 TRM formation (*SI Appendix*, Fig. S2 *A* and *B*). To determine whether IL-2 was sufficient to encourage the formation of CD4 TRM, SM1 T cells were transferred into recipient mice treated with recombinant human IL-2 (hIL-2) on the day of transfer and 3 d later (Fig. 6*D*). Indeed, the number and frequency of SM1 T cells, the number and percentage of SM1 TRM, and the frequency of SM1 T cells becoming TRM were increased by hIL-2 administration (Fig. 6*E*). Notably, hIL-2 was only administered at the beginning of the SM1 T cell transfer, which is similar to transient APAP inflammation which lasts less than 24 h (39, 40). Indeed, APAP overdose 24 h before or after SM1 transfer did not increase SM1 TRM formation (*SI Appendix*, Fig. S3), suggesting a relatively short window in which liver inflammation contributes to TRM generation.

T Cell Intrinsic IL-1R1 Signaling Is Required for Optimal Formation of Hepatic CD4 TRM. Using a published APAP overdose RNAseq dataset (GEO: GSE136679), we noted that IL-1α and IL-1β expression was up-regulated 6 h after APAP administration (39) (Fig. 7*A*). RNAseq and flow cytometry analysis of CD4 TRM demonstrated that IL-1R1 is not significantly up-regulated in our study (Fig. 7*B*). Nevertheless, we were interested to determine whether IL-1R1 contributes to hepatic CD4 TRM formation. Mixed bone marrow chimeras were generated by reconstituting irradiated CD90.2$^+$ CD45.1$^+$ WT mice with a 1:1 ratio of CD90.1$^+$ CD45.2$^+$ WT bone marrow and CD90.2$^+$ CD45.2$^+$ *Il1r1*-deficient bone marrow. After 8 wk, chimeric mice were infected with *Salmonella*-LVS and memory T cells were subsequently examined 45 d later using flow cytometry and 2W1S tetramer. TRM were defined as P2XR7$^+$ CD69$^+$ tetramer$^+$, while effector memory T cells (TEM) were defined as CD69– CD62L– tetramer$^+$ CD4 T cells and central effector memory T cells (TCM) were defined as CD69– CD62L$^+$ tetramer$^+$ CD4 T cells (Fig. 7*C*). The number of *Il1r1*-deficient CD4 TRM in the liver was reduced and the frequency of tetramer+ CD4 T cells that became TRM was also reduced compared to that of WT CD4 TRM, while the number of other CD4 T cell memory populations was not affected (Fig. 7*D*). Therefore, IL-1R1 signaling is also required for optimal resident memory development.

Discussion

Systemic *Salmonella* infections are a major public health concern, especially since there is a lack of efficacious vaccines for people

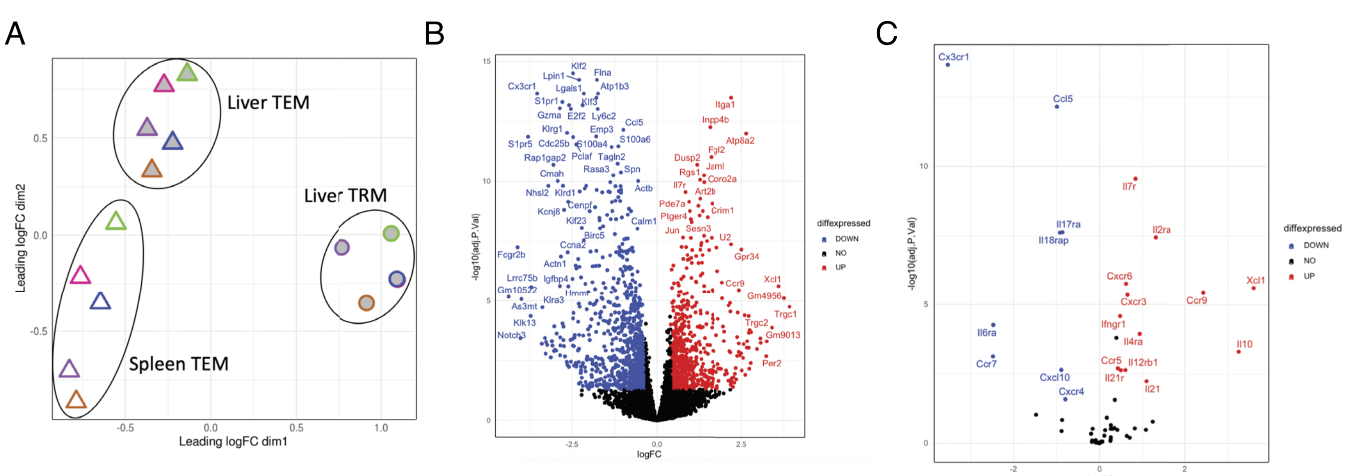

Fig. 5. CD4 TRM are transcriptionally distinct from circulating memory cells. (*A*–*C*) IFNγ-YFP reporter mice were immunized with *Salmonella*-LVS and 45 d later, livers and spleens were collected and CD4 T cell memory populations were sorted. TRM was defined as CD4+ YFP+ CD69+, and TEM was defined as CD4+ YFP+ CD69−. RNA was isolated from sorted CD4 T cell memory populations and sequenced. (*A*) MDS plot of liver TRM, liver TEM, and splenic TEM gene expression. Same colors denote samples are from same mice, TEM (triangles), TRM (circles), liver (filled in symbol), spleen (open symbols). (*B*) Volcano plot of all differentially expressed genes of liver TRM vs. liver TEM. (*C*) Volcano plot of a selection of differentially expressed chemokines and receptors and cytokines and receptors of liver TRM vs. liver TEM. n = 6; differential gene expression calculated by limma in R.

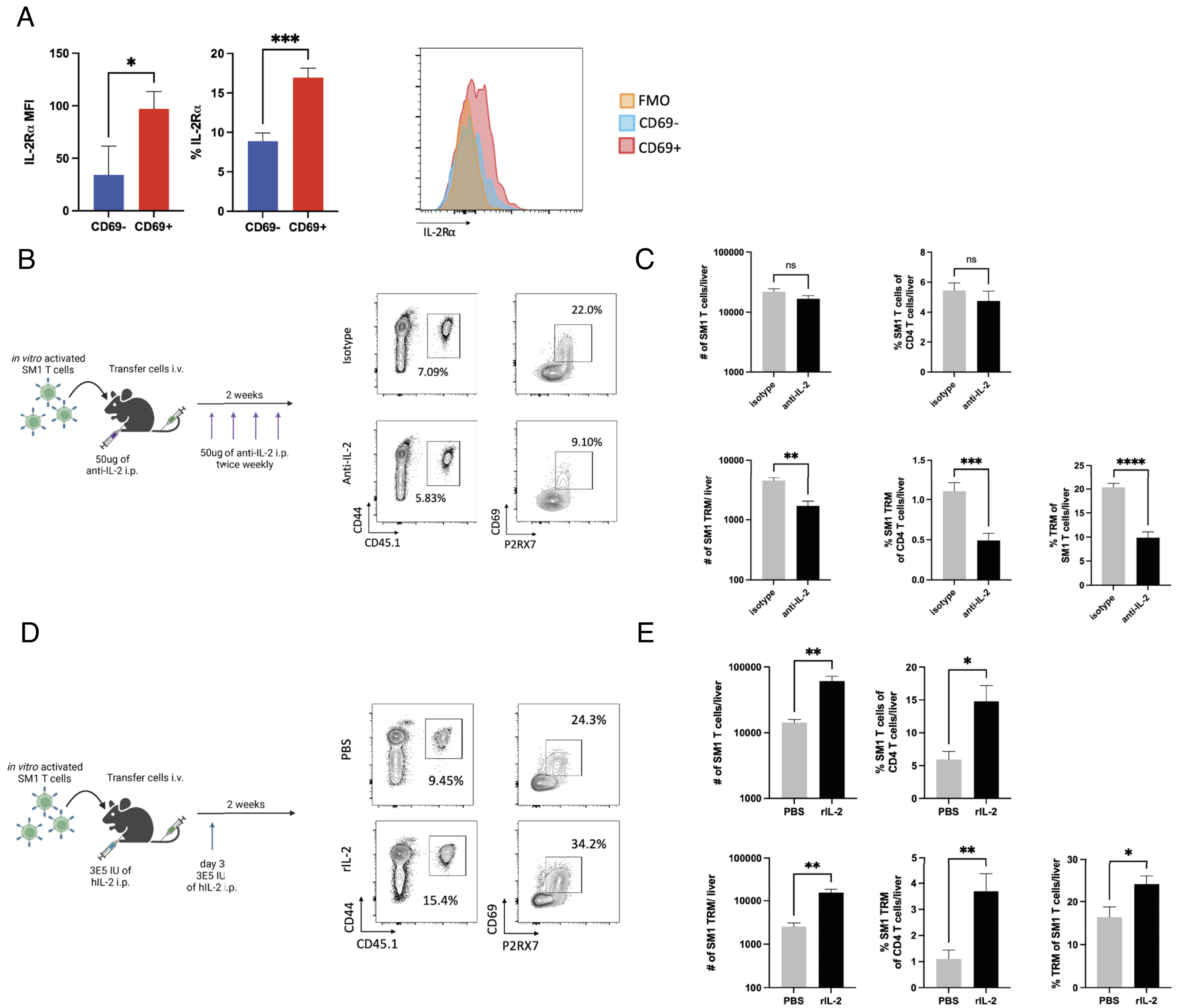

Fig. 6. IL-2 is required and sufficient for optimal liver CD4 TRM formation. (*A*) C57BL/6 mice were immunized with *Salmonella*-LVS-2W1S and 45 d later, liver lymphocytes were analyzed for the expression of IL-2Rα of CD69+ and CD69− 2W1S-tetramer+ CD4 T cells. (*B*–*E*) SM1 T cells were activated in vitro for 5 d and transferred into naïve C57BL/6 mice. Two weeks later, livers were collected and SM1 T cells and SM1 TRM were identified by flow cytometry. (*B* and *C*) Same day as transfer of SM1 T cells, IL-2 was depleted with anti-IL-2 i.p. and twice weekly till take down. (*D* and *E*) Same day as transfer of SM1 T cells, mice were treated with hIL-2 i.p. and a second dose 3 d after transfer. (*A*) "Fluorescence Minus One" (FMO) control staining. n = 4 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SD. (*C* and *E*) n = 6 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SD.

living in endemic areas (13). While a subunit vaccine would be ideal for those most at risk of *Salmonella* infection, experimental data show that they provide partial protection and lack the ability to induce TRM (19, 21). Our data show that induction of hepatic inflammation by sterile or infectious means can enhance *Salmonella*-specific CD4 TRM formation and the protective efficacy of a subunit vaccine. This outcome suggests that gains in protective efficacy could be achieved by focusing on encouraging liver T cell residency during immunization. This mirrors a study of *Plasmodium*-specific CD8 T cells which showed that liver inflammation induced by a variety of mechanisms resulted in a higher proportion of CD8 TRM (28). Interestingly, both of these studies support the idea that activated T cells form TRM in the liver of naïve recipients, but the presence of local inflammation enhances this process.

Given the importance of TRM development in *Salmonella* vaccination, it was crucial to define specific inflammatory signals that might affect *Salmonella*-specific CD4 TRM formation. We observed numerous changes in cytokine receptor expression by CD4 TRM, but subsequent experiments identified IL-1 and IL-2 signaling as critical to hepatic TRM formation. Previously, there has not been definitive evidence that IL-2 encourages the formation of TRM populations at other tissue sites. In this study, hepatic CD4 TRM up-regulated IL-2Rα expression when compared to CD4 TEM by flow cytometry and RNA-seq. Using our activated SM1 T cell model, CD4 T cells were activated before being adoptively transferred into an IL-2-limited environment, which substantially reduced SM1 TRM formation. Thus, IL-2 appears to be an essential component of hepatic TRM formation, but further work is needed to carefully examine the role of IL-2 in hepatic CD4 TRM formation during infection. As with IL-2, few studies have examined a role for IL-1 in the formation of TRM. We initially examined IL-1R1 signaling due to the observation that APAP overdose induces expression of IL-1α and IL-1β which

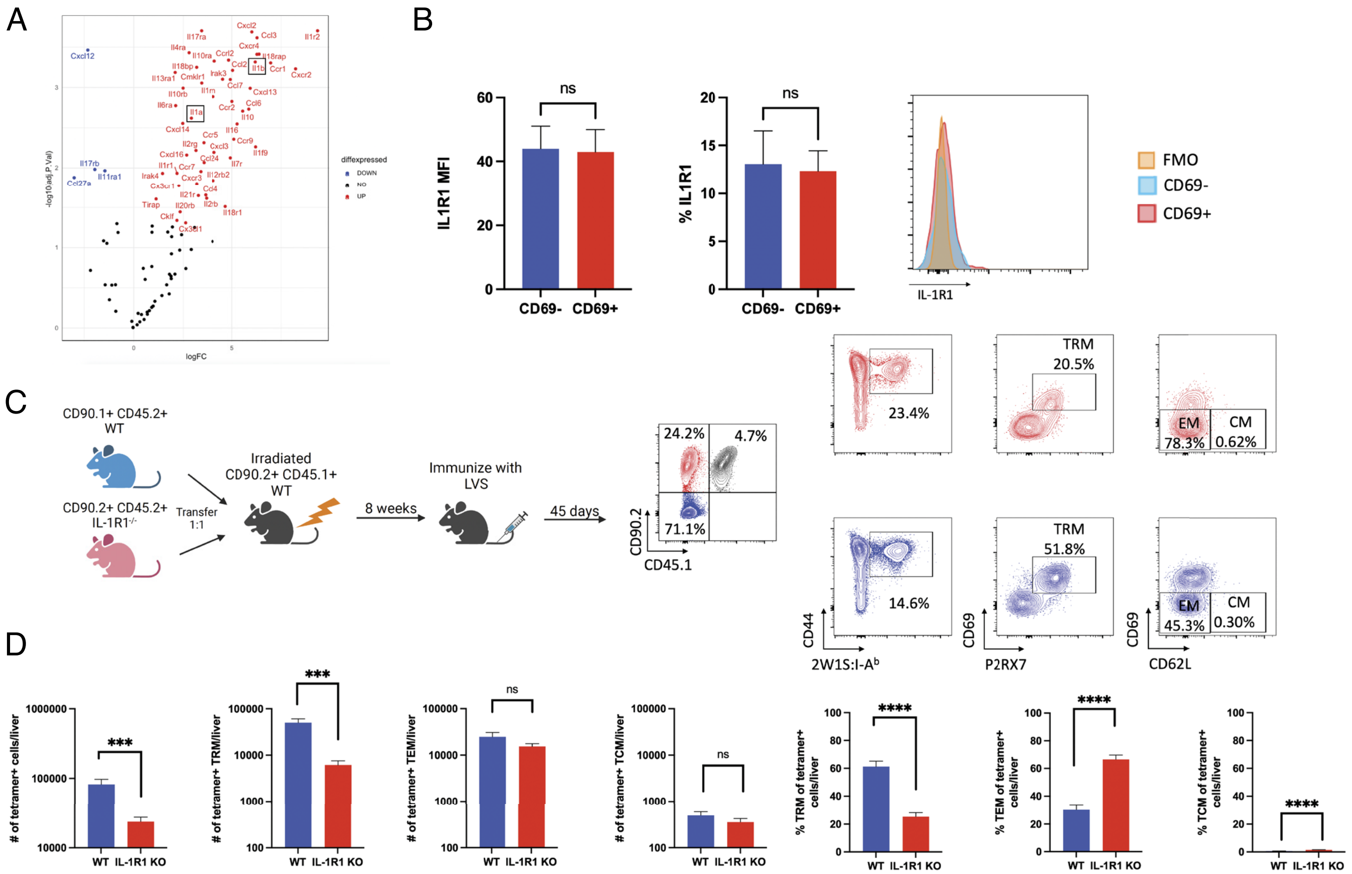

Fig. 7. T cell intrinsic IL-1R1 signaling is required for optimal formation of liver CD4 TRM. (*A*) Volcano plot of differentially expressed chemokines and receptors and cytokines and receptors of 6 h post acetaminophen overdose, livers vs. untreated livers from GEO: GSE136679. (*B*) C57BL/6 mice were immunized with *Salmonella*-LVS-2W1S and 45 d later, liver lymphocytes were analyzed for the expression of IL-1R1 of CD69+ and CD69− 2W1S-tetramer+ CD4 T cells. (*C* and *D*) Mixed bone marrow chimeras were made by transferring a 1:1 ratio of CD90.1+ CD45.2+ WT bone marrow and CD90.2+ CD45.2+ *Il1r1*$^{-/-}$ bone marrow into irradiated CD90.2+ CD45.1+ WT hosts. Eight weeks later, the chimeras were infected with *Salmonella*-LVS-2W1S. Forty-five days postinfection, livers and spleens were harvested and analyzed by flow cytometry. Immunization-specific CD4 T cells were identified as 2W1S-tetramer, and TRM were defined as P2RX7+ CD69+, TEMs were defined as CD69− CD62L−, TCMs were defined as CD69− CD62L+. FMO control staining. n = 9 for all experiments. Data are representative of two experiments. Significance was calculated by Student's *t* test and data are means ± SEM.

signal through this receptor (39). However, the IL-1R1 itself is not up-regulated on CD4 TRM compared to CD4 TEM in the liver, as was noted for IL-2Rα. Thus, modification in either cytokine receptors, such as IL-2 signaling, or the availability of local inflammatory mediators, such as IL-1 signaling, can each affect CD4 TRM formation. When we analyzed IL-1R1-deficent mixed bone marrow chimeras, we detected a slight reduction in CD4 T cells homing to the liver but this reduction in CD4 T cell memory derived specifically from a reduction in the CD4 TRM compartment, while the other memory populations were unaffected. Thus, there is a specific requirement for IL-1 signaling in memory CD4 T cell formation in the liver. Overall, the lack of either IL-1 and IL-2 signaling reduced CD4 TRM formation and thus both are required for optimal formation of hepatic TRM. Thus, future subunit vaccine design for *Salmonella* may be able to make substantial gains in protection simply by targeting one pathway that enhances TRM formation. However, further interrogation of these cytokine pathways is needed to understand the downstream effects and to determine which pathway to target for vaccine design.

A limitation of our study is that modifications of cytokine signaling were not specifically localized to the liver microenvironment and it is possible that systemic responses prior to liver trafficking were the cause of inhibition or accentuation of hepatic TRM formation. Indeed, understanding the source of IL-1 and IL-2 will be important to fully understanding the process of CD4 TRM formation in the liver. Previously, lymphoid aggregates have been detected in the liver, and CD8 T cell proliferation in these structures termed iMATE (intrahepatic myeloid-cell aggregates for T cell population expansion) can be increased by TLR stimulation (41). Therefore, cells within iMATE structures could be a source of IL-1 and/or IL-2 and promote the formation of CD4 TRM. Further research that includes imaging CD4 TRM by histology will be required to answer this question.

Overall, our study expands our understanding of CD4 TRM formation in the liver, a memory cell type that has historically been understudied. The frequency of CD4 T cells that become TRM was markedly increased by inflammation which also increased efficacy of the SseB subunit vaccine. The identification of the origin of IL-1 and IL-2 signaling for optimal formation of hepatic CD4 TRM will be of interest for vaccine design, since targeting either of these cytokine signaling pathways has the potential to generate robust TRM population in the liver and markedly enhance the protective efficacy of a future vaccine.

Materials and Methods

Mouse Strains. RAG2-deficient SM1 CD45.1 mice were produced by backcrossing the original C57BL/6 SM1 RAG2 deficient line to CD45.1 mouse strains (The Jackson Laboratory, Bar Harbor, ME) (42, 43). IFNγ-eYFP reporter mice (C.129S4(B6)-*Ifng*$^{tm3.1Lky}$/J) were generously provided by R. Lockley, University of California, San Francisco (32). B6.129S2-*Tcra*^{tm1Mom}/J (strain# :002115) and

B6.129S7-*Il1r1*^{tm1Imx}/J (strain# 003245) were purchased from The Jackson Laboratory and used at 8 to 16 wk of age. All mice were housed in specific pathogen-free conditions and cared for in accordance with the University of California, Davis Institutional Animal Care and Use Committee, and Institutes of Health guidelines.

Bacterial Growth and Burden. The LVS strain of *S. enterica* serovar Typhimurium (BMM50) was generated by the Andreas Baumler Laboratory, University of California, Davis, by introducing a null mutation in the aroA gene of SL1344, as previously reported (44). BMM50 was subsequently modified to express a short peptide sequence (EAWGALANWAVDSA) in frame with OmpC (BMM51), using an identical approach to our previous modification of BRD509 (4, 45). Preliminary experiments confirmed correct gene targeting, peptide expression, and activation of 2W1S-specific CD4 T cells in vivo. Before use, bacteria were streaked out onto a MacConkey agar plate and individual colonies were used to inoculate Luria-Bertani broth and grown statically overnight at 37 °C to an OD_{600} of 0.4 to 0.6. *Salmonella* cultures were diluted in Phosphate Buffered Saline (PBS) to 5×10^5 CFU/0.2 mL (BMM50), 2.5×10^6 CFU/0.2 mL (BMM51), or 1×10^3 CFU/0.2 mL (SL1344), and 0.2 mL was injected via the tail vein. *L. monocytogenes* ΔactA was streaked out onto brain–heart infusion (BHI) agar plate and individual colonies were used to inoculate BHI broth and grown statically overnight at 37 °C to an OD_{600} of 0.7 to 0.8. *L. monocytogenes* cultures were diluted in PBS to 1×10^7 CFU/0.2 mL, and 0.2 mL was injected via the tail vein. Livers and spleens of mice were homogenized, and serial dilutions of the homogenates were plated on MacConkey agar plates and incubated overnight at 37 °C. Bacterial burden of the entire organ was calculated by back calculating the counted serial dilution.

Generation of SseB and Immunization. Purified recombinant sseB protein was produced as previously described (21). Mice were immunized with 100 μg SseB with or without 1×10^6 CFU of *L. monocytogenes* intravenously and boosted 4 wk later.

Bone Marrow Chimeras. CD90.2+ CD45.1+ mice were irradiated with a single dose of 800 cGy from a X-ray source. Sixteen hours later, the mice received a 1:1 mix of bone marrow from CD90.1+ CD45.2+ WT and CD90.2+ CD45.2+ IL-1R1$^{-/-}$ mice intravenously. The mice were maintained on 0.13 mg/mL sulfatrim ad libitum (Aurobindo) for at least 4 wk. The mice were infected with BMM51 at 8 to 10 wk and at least 2 wk after withdrawal of antibiotics.

Lymphocyte Isolation. Spleens were turned into single-cell suspensions by pressing the tissue through a 70 μm mesh cell strainer (Corning). Cells were incubated with 2 mL Ammonium-Chloride-Potassium (ACK) lysis buffer (Gibco) to remove red blood cells, and the cells were counted. Livers were turned into single-cell suspensions by pressing the tissue through a 70 μm mesh cell strainer. The cells were spun down at 2,000 rpm for 15 min without a brake. The cells were resuspended in 15 mL 35% Percoll solution (Cytiva) and spun at 2,000 rpm for 20 min without a brake. The red blood cells were lysed with ACK lysis buffer, and the cells were counted. Single-cell suspensions were made of the lymph nodes by using frosted glass slides to gently disrupt the lymph node. The red blood cells were lysed with ACK lysis buffer, and the cells were counted.

Adoptive Transfer of Lymphocytes. Liver lymphocytes of LVS-immunized mice were resuspended in 300 μL and injected into a TCRα$^{-/-}$ mouse so that one recipient mouse received all of the liver lymphocytes from one immunized donor mouse.

Antibody Staining and Flow Cytometry. Single-cell suspensions were made as described above and 1×10^6 cells were incubated with FC block (24G2 supernatant with 2% mouse serum and 2% rat serum). The cells were incubated with Zombie Yellow (BioLegend) viability dye for 15 min. The cells were washed with 2% fetal bovine serum (FBS) in PBS and incubated with PE-conjugated 2W1S::I-A^b MHCII tetramer for 1 h. The cells were washed with 2% FBS and PBS and stained with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1, CD4, CD44, CD45.1, CD90.2, P2RX7, CD69, CD62L, CD103, KLRG1, CD11a, CXCR3, and CD101. Samples were analyzed by flow cytometry using Becton Dickinson (BD) LSR Fortessa or BD FACSymphony, and data were analyzed by FlowJo software (TreeStar).

In Vitro Activation of SM1 T Cells and Adoptive Transfer. Inguinal, iliac, mesenteric, axillary, and brachial lymph nodes and spleen of SM1 mice were harvested. Single-cell suspensions were made as described above, and CD4 T cells were isolated with MACS CD4 T cell selection kit (Miltenyi) following the kit protocol. These cells were then cultured with 5 μg/mL flagellin peptide (42), 5 μg/mL LPS (Enzo), 10 U/mL human IL-2 (PeproTech), and irradiated splenocytes from C57BL/s6 mice in complete Roswell Park Memorial Institute Medium (RPMI) media. The cells were fed with human IL-2 in complete RPMI media at day 3 of culture before being harvested at day 5 of culture and washed twice with PBS and adjusted to 5×10^6 cells/0.2 mL. The mice were injected with 0.2 mL of cell suspension intravenously.

Acetaminophen Treatment. Acetaminophen (Sigma) was dissolved in 0.9% saline at a concentration of 15 mg/mL and sterile filtered. Mice were food restricted 16 h before receiving acetaminophen overdose. The mice were injected intraperitoneally with 300 mg/kg of acetaminophen.

Nanobody Blockade of ARTC2.2. For flow cytometry analysis of hepatic T cell expression of P2RX7 or for transfer experiments, mice were given 50 μg of s+16a nanobody (BioLegend) diluted in 200 μL PBS 30 min before organ harvest (33).

RNA Sequencing and Analysis. Livers and spleens were collected from IFNγ-YFP reporter mice that were immunized with LVS-*Salmonella* and processed to single-cell suspensions as described above. Each group comprised pooled cells from three mice for a final count of six groups. Cells were sorted with BD FACSAria Cell Sorter. TRM were defined as CD4+ NK1.1− CD69+ YFP+ and TEM were defined as CD4+ NK1.1− CD69− YFP+. Total RNA was collected from the cells using RNeasy kit (Qiagen). RNAseq and data processing were performed as in ref. 46. Graphs were produced using RStudio. Raw data are available in NCBI's Gene Expression Omnibus (47) and are accessible through GEO Series accession number GSE211181.

Statistics. Statistical analysis was performed by using paired or unpaired Student's *t* test, or one-way ANOVA as stated (Prism; GraphPad Software, Inc.). Significance is displayed as, P = *≥0.05, **≥0.01, ***≥0.001, ****≥0.0001. Graphical figures were made using BioRender (https://biorender.com/).

Data, Materials, and Software Availability. Bulk RNAseq data have been deposited in Gene Expression Omnibus (GSE211181) (35).

ACKNOWLEDGMENTS. Sequencing was carried out at the UC Davis Genome Center DNA Technologies and Expression Analysis Core, supported by NIH Shared Instrumentation Grant 1S10OD010786-01. This study was supported by funding from NIH (R01AI139047, R01AI139402, and T32 AI060555).

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