Optimal protection against *Salmonella* infection requires noncirculating memory

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While CD4 Th1 cells are required for resistance to intramacrophage infections, adoptive transfer of Th1 cells is insufficient to protect against *Salmonella* infection. Using an epitope-tagged vaccine strain of *Salmonella*, we found that effective protection correlated with expanded *Salmonella*-specific memory CD4 T cells in circulation and nonlymphoid tissues. However, naive mice that previously shared a blood supply with vaccinated partners lacked T cell memory with characteristics of tissue residence and did not acquire robust protective immunity. Using a YFP–IFN-γ reporter system, we identified Th1 cells in the liver of immunized mice that displayed markers of tissue residence, including P2X7, ARTC2, LFA-1, and CD101. Adoptive transfer of liver memory cells after ARTC2 blockade increased protection against highly virulent bacteria. Taken together, these data demonstrate that noncirculating memory Th1 cells are a vital component of immunity to *Salmonella* infection and should be the focus of vaccine strategies.

Salmonella infection | CD4 T cell | protective immunity | tissue-resident memory | vaccines

Enteric fever is caused by infection with *Salmonella enterica serovar* Typhi (*S.* Typhi) and afflicts many individuals in low-income nations (1). *S.* Typhi uniquely infects humans and is transmitted via the oral-fecal route in geographical locations lacking access to clean water and/or sanitation (2, 3). Even after recovery from enteric fever, antibiotic-treated patients remain susceptible to reinfection, suggesting incomplete protective immunity after primary exposure (2, 4). Infection of inbred mice with *S. enterica serovar* Typhimurium (*S.* Typhimurium) causes a systemic infection with many similarities to human Salmonellosis and is used to study the mechanistic basis of effective *Salmonella*-specific immunity (5).

Two typhoid vaccines are licensed in the United States, one of which is a live vaccine strain (LVS) of *S.* Typhi that provides modest protection (6). Protective immunity can also be established in susceptible C57BL/6 mice using an LVS of *S.* Typhimurium (5). In this mouse model, LVS-mediated protection requires *Salmonella*-specific CD4$^+$ memory Th1 cells and B cells (7–10). Interestingly, both of these lymphocyte populations are actively targeted by bacterial immune evasion strategies (11–15). While LVS-immunized mice develop robust protection to *Salmonella*, acquired immunity is not easily transferred to naive mice (5). Transfer of immune spleen cells to naive mice does not provide complete protection, suggesting a requirement for antibody (16, 17). However, transfer of immune serum and immune spleen cells confers only partial protection (16, 18). Given these features of protective memory, it seems possible that noncirculating lymphocytes constitute a major component of protection mediated by LVS vaccination.

There is considerable heterogeneity within the T cell memory pool (19), and only a fraction of memory cells are accessible by sampling blood and lymphoid organs (20, 21). A pool of memory T cells resides in nonlymphoid tissues, and assessment of their contribution to protection is challenging (22). Here, we demonstrate that LVS immunization induces *Salmonella*-specific circulating and tissue-resident memory populations. When naive mice shared a circulation with LVS-immunized mice, they gained circulating memory CD4 T cells but remained unable to efficiently resolve *Salmonella* infection. Analysis of liver Th1 cells identified memory T cells displaying markers of tissue residence that could transfer protective immunity to naive recipients. Notably, this transfer required inhibition of P2X7 receptors, associating another feature of tissue-resident lymphocytes to these *Salmonella*-specific liver memory CD4 T cells. Our data therefore suggest that liver-associated memory CD4 T cells are a vital component of vaccine-mediated protection against *Salmonella* infection.

Results

Immunization with *Salmonella*-2W Provides Robust Protection. To visualize *Salmonella*-specific memory CD4 T cells, we generated recombinant AroA-deficient LVS *Salmonella* expressing 2W1S (BRD2W), a T cell epitope that allows identification of responding CD4 T cells by tetramer pull-down (23). The BRD2W strain colonized C57BL/6 mice for ∼5 wk (*SI Appendix*, Fig. S1). When BRD2W-immunized mice were subsequently challenged with virulent *Salmonella* (SL1344), bacterial burdens were two to three orders of magnitude lower than in naive mice (Fig. 1 *A* and *B*).

Significance

Effective vaccination against *Salmonella* infection requires the generation of memory T cells that can be reactivated upon exposure to bacteria in a natural setting. It is unclear whether immunity requires memory T cells that continuously patrol blood, lymphatics, and tissues, whether noncirculating T cells are important, or both. We demonstrate the generation of circulating and noncirculating memory pools after immunization. However, naive mice that previously shared a blood supply with vaccinated partners received recirculating memory T cells, but did not have T cell memory with characteristics of tissue residence, resulting in a failure to acquire robust protective immunity. Thus, noncirculating tissue-resident memory T cells are vital for effective protection against *Salmonella*.

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Furthermore, this protective immunity was long-lived and clearly evident at 45, 90, or 135 d postimmunization (Fig. 1 *C* and *D*).

LVS Immunization Generates Memory CD4 Cells in Lymphoid and Nonlymphoid Tissues. LVS immunization usually initiates expansion of CD4 T cells and subsequent generation of CD4-dependent protective immunity (24–27); however, individual subsets of *Salmonella*-specific memory CD4 T cells have not been carefully assessed. Using 2W1S MHC class-II tetramers, we detected an elevated frequency of *Salmonella*-specific CD4 T cells in blood and spleen 42 d after LVS immunization (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). The majority of antigen-specific CD4 T cells expressed high levels of CD44 and T-bet and produced IFN-γ upon restimulation with heat-killed bacteria (Fig. 2*B* and *SI Appendix*, Fig. S2). At this time point, mixed levels of CD27 were detected, consistent with the gradual development of *Salmonella*-specific memory CD4 T cells (26). Thus, LVS immunization generates an expanded population of memory Th1 cells with immediate effector function.

Tissue-resident memory (TRM) CD4 T cells have been described in multiple infection models (28–30), but have not yet been documented in *Salmonella* infection. It should be noted that *Salmonella* cause systemic infections and do not readily infect the intestinal epithelial and lamina propria in intact mice (4, 31). Indeed, the most appropriate nonlymphoid location to examine CD4 T cell-mediated protective immunity to *Salmonella* is the liver, where bacterial replication is effectively controlled in LVS-immunized mice (27, 32). Utilizing an intravascular stain (33), two populations of $CD69^+$ *Salmonella*-specific CD4 T cells were detected in the lung and liver of LVS-immunized mice, with variable access to the vasculature (Fig. 2 *C* and *D*). The fenestrated architecture of the liver endothelium can expose liver-resident T cells to blood, meaning that this

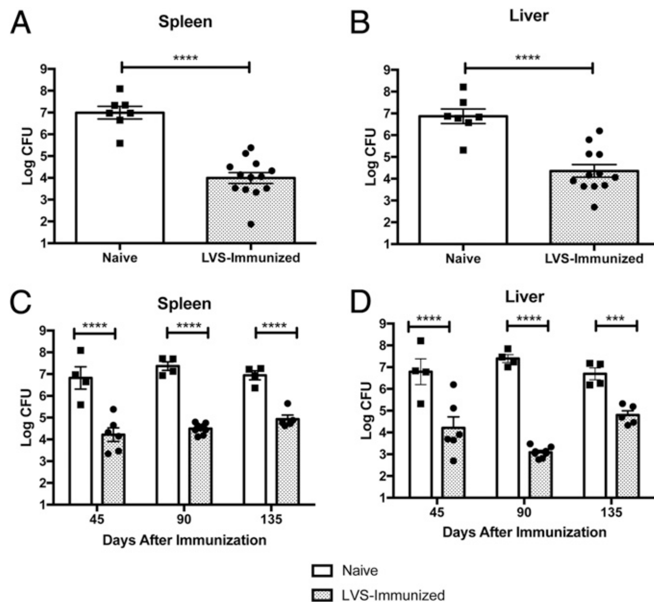

Fig. 1. Immunization with LVS *Salmonella* confers long-lasting protection against *Salmonella* infection. (*A* and *B*) Mice were immunized with 2.5×10^6 cfu/mouse intravenously (BRD509-2W1S). Mice were challenged orally 45 d later with 1×10^9 cfu/mL SL1344 via water bottle exposure. Mice were euthanized on day 5 post challenge, and bacterial burdens were analyzed in spleen and liver. Data show mean ± SEM of two replicative experiments with three to six mice per group. (*C* and *D*) Mice were immunized with 5×10^5 cfu/mouse intravenously (BRD509-2W1S), and on indicated days later mice were challenged with 1×10^9 cfu/mL SL1344 via water bottle exposure. Mice were euthanized on day 5 post challenge, and bacterial burdens were analyzed in the spleen and liver. Data show mean ± SEM with four to eight mice per group per time point. $****P < 0.0001$.

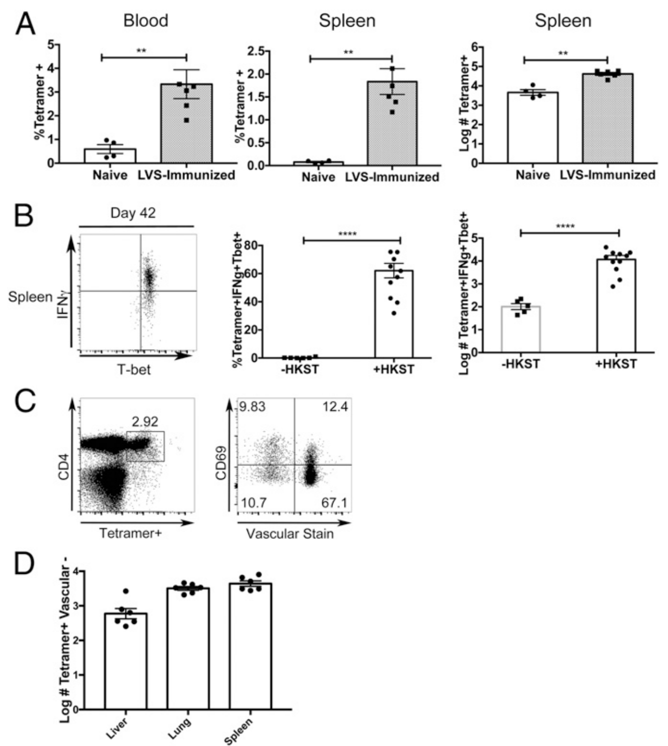

Fig. 2. Circulating and resident *Salmonella*-specific CD4 memory T cells are generated after LVS immunization. (*A*) Generation of circulating tetramer+ CD4 T cells by LVS immunization. Blood and spleen samples were collected from naive and LVS immunized mice (2.5×10^6 cfu/mouse intravenous BRD509-2W1S) 42 d after immunization. Lymphocytes were isolated, stained, and tetramer-positive cell events were quantified. Data show mean ± SEM of at least four mice per group. $**P < 0.01$. (*B*) Representative flow plot and quantitation of IFN-γ^+T-bet^{high} CD4 T cells from mice immunized as in *A*. Data show mean ± SEM of pooled data from two replicative experiments. $****P < 0.0001$. (*C* and *D*) Representative flow plots and pooled analysis from LVS-immunized mice (2.5×10^6 cfu/mouse i.v.). On day 45 after immunization, mice were administered anti-CD45.2 antibody (3 μg/mouse) 3 min before euthanasia. Organs were harvested and digested before lymphocytes were stained and quantified. Data show mean ± SEM with six mice per group.

intravascular staining approach underestimates tissue residency (34). Together, these data suggest that LVS immunization induces *Salmonella*-specific memory T cells with hallmarks of both circulating and tissue-resident memory CD4 T cells.

Immunity Induced by LVS Immunization Requires Circulating and Noncirculating Memory. Parabiosis of immunized and naive animals allows direct analysis of the contribution of circulating memory CD4 T cells transferred to naive individuals. Around 14 d after surgery, a mixed population of CD4 T cells was detected in the blood of parabiosed congenic mice, and an equivalent percentage of congenic CD4 T cells was detected at day 28 (*SI Appendix*, Fig. S3). After LVS immunization and separation surgery, circulating *Salmonella*-specific memory cells were detected in the spleens of both LVS-immunized and naive parabionts (Fig. 3*A*). However, LVS-immunized mice had a larger population of *Salmonella*-specific CD4 memory cells in the liver (Fig. 3*B*). Next, we assessed whether naive mice parabiosed to LVS-immunized mice were protected against *Salmonella* infection. LVS-immunized mice were parabiosed to naive mice for 1 month before separation surgery and then challenged with virulent *Salmonella*. The surgical control group and naive C57BL/6 mice had similarly high bacterial burdens in the spleen and liver, demonstrating that parabiosis surgery had no direct effect on immunity to *Salmonella* (Fig. 4). As expected, LVS-immunized mice that had been parabiosed

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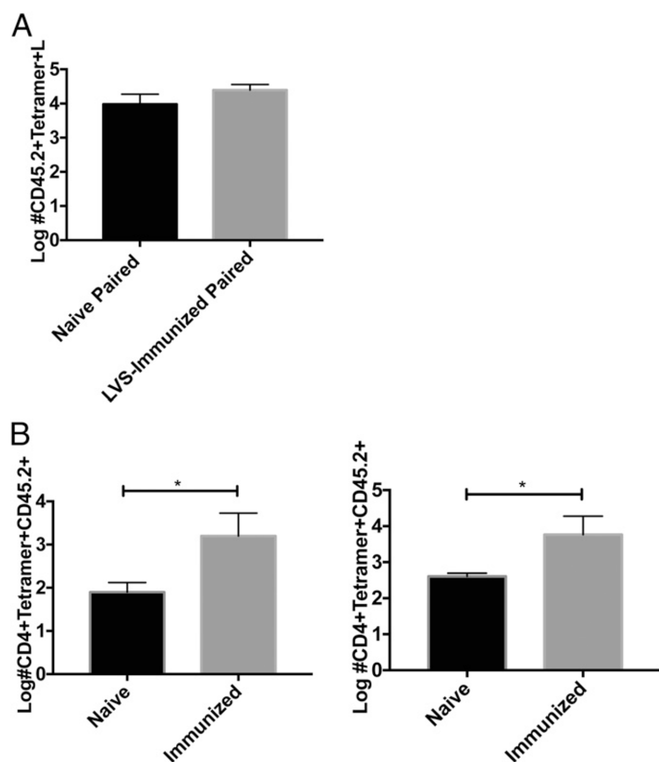

Fig. 3. LVS immunization induces noncirculating *Salmonella*-specific memory CD4 T cells. Congenically marked CD45.2 mice previously immunized intravenously with 2.5×10^6 cfu/mouse were surgically joined to CD45.1 mice for 28 d. Mice were separated, and tissues were harvested 14 d later. (*A*) Tetramer numbers in spleens from naive, naive parabiont, and LVS-immunized parabionts. Splenocytes were compared with naive and LVS-immunized parabionts for presence of the CD45.2 congenic marker. (*B*) Tetramer numbers in spleen (*Left*) and liver (*Right*) from naive and LVS-immunized parabionts. Tissue lymphocytes were compared between naive and LVS-immunized parabionts for presence of the CD45.2 congenic marker. Data show mean ± SEM from three mice per group. $^{*}P < 0.05$.

displayed low tissue bacterial burdens equivalent to unpaired LVS-immunized mice (Fig. 4). However, naive mice previously parabiosed to LVS-immunized mice displayed higher bacterial burdens than LVS-immunized mice, but lower than naive mice (Fig. 4). Taken together, these data demonstrate that a proportion of immunity is transferred via a shared circulation, but also that optimal protection against *Salmonella* requires noncirculating memory CD4 T cells.

Phenotypic Characterization and Protective Function of Liver-Resident Memory CD4 T Cells. To more carefully assess *Salmonella*-specific memory Th1 cells in the liver, we made use of an IFN-γ reporter line, in which transcriptional activity at the IFN-γ locus is visualized by eYFP expression (35). Reporter mice were immunized with an LVS of *Salmonella* (TAS2010) that provides robust protective immunity to *Salmonella* infection (36). A large population of memory CD4 T cells was detected in the liver that coexpressed IFN-γ and CD69 (Fig. 5 *A* and *B*). These IFN-γ/CD69$^+$ memory CD4 T cells also expressed high levels of P2X7, ARTC2, LFA-1, and CD101 (Fig. 5 *C*–*F*), but lacked CD103 (Fig. 5*G*), phenotypic markers recently associated with tissue-resident lymphocytes (37). Notably, IFN-γ/CD69$^+$ T cells lacked expression of KLRG1 (Fig. 5*H*), indicating that cells did not represent recently activated effector cells. To determine their protective capacity against *Salmonella* infection, we adoptively transferred liver memory T cells into naive *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice. In contrast to circulating T cells, tissue-resident T cells often fail to survive adoptive transfer due to P2X7-dependent potassium influx (34). However, survival after transfer can be enhanced by pretreating donors with a single-domain antibody (S+16a) to inhibit ARTC2. ARTC2 ADP ribosylates P2X7, and thus treatment with S+16a prevents the lethal opening of P2X7 ion channels on resident lymphocytes (34, 38, 39, 40). As expected, splenocytes from S+16a-treated immune mice or controls failed to confer protection against virulent *Salmonella* (Fig. 6*A*). However, adoptive transfer of liver cells from S+16a-treated immune mice, but not from untreated control mice, significantly enhanced survival (Fig. 6*A*). Consistent with a protective role for CD4 TRMs, CD4 depletion or neutralization of IFN-γ abrogated adoptively transferred protection (Fig. 6*B*). Furthermore, CD69^{hi} CD4$^+$ T cells preferentially relocated to the liver of recipient *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice (Fig. 6*C*). Taken together, these data indicate that liver-associated IFN-γ–producing CD4$^+$ T cells are protective against *Salmonella* infection.

Discussion

Intracellular pathogens like *Salmonella* cause high disease mortality and morbidity worldwide (3, 41). Next-generation Vi capsular polysaccharide-conjugate typhoid vaccines are likely to enhance protection against Vi-expressing typhoid serovars, but will not combat systemic salmonellosis caused by Vi-negative paratyphoid or nontyphoidal serovars (42, 43). Thus, the generation of effective vaccines for nontyphoidal systemic salmonellosis remains an important research goal, and this process would be assisted by a greater understanding of protective memory responses (1).

Salmonella-specific CD4 T cells are initially activated in the Peyer's patch and mesenteric lymph nodes (MLNs) before the development of an adaptive response in the spleen to systemic infection (26, 44, 45). This progression of activation correlates with the general pattern of bacterial dissemination although the

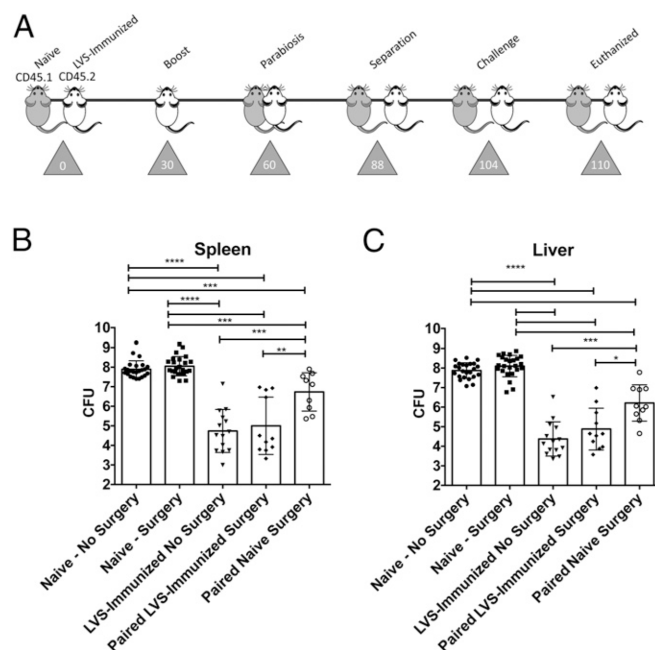

Fig. 4. Both tissue-resident and circulating memory are required for optimal protective immunity against *Salmonella* infection. (*A*) Congenically marked CD45.2 mice were immunized and boosted with 2.5×10^6 cfu/mouse, and 60 d later were surgically joined to CD45.1 mice for 30 d. Mice were separated and challenged intravenously 14 d later with 1×10^3 cfu/mouse SL1344. Mice were then euthanized 6 d post challenge. Spleens (*B*) and livers (*C*) were harvested, homogenized, diluted, and plated to determine bacterial burden in each tissue. Data show the mean of two replicative experiments ±SEM with five to seven mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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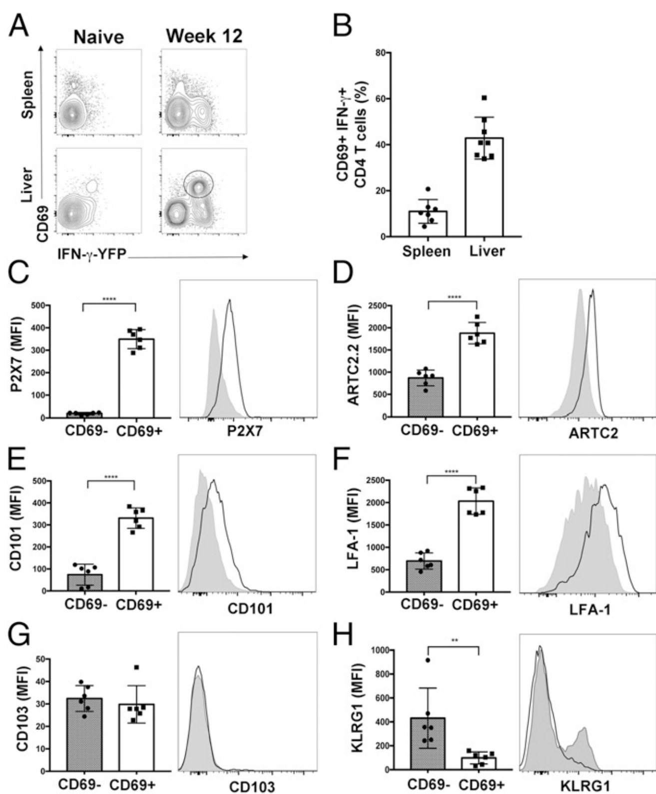

Fig. 5. CD69^{Hi} Th1 cells in the liver display markers of tissue residence. (*A*–*H*) IFN-γ-eYFP mice were immunized intravenously with 200 cfu of TAS2010, and 12 wk later spleens and livers were assessed by flow cytometry. (*A* and *B*) Representative plots and pooled percentages of CD69+ eYFP+ CD4+ T cells from immunized mice. (*C*–*H*) FACS plots comparing the expression of P2X7, ARTC2, CD101, LFA-1, CD103, and KLRG1 by eYFP+ CD69^{hi} (solid black line) and eYFP+ CD69^{lo} (gray shading) CD4+ T cells in the liver 12 wk after TAS2010 immunization. Data are representative of pools of (*A*–*H*) two independent experiments. (*B*) $n = 8$. (*C*–*H*) $n = 6$ per group. $^{**}P < 0.01$, $^{****}P < 0.0001$.

antigenic targets of intestinal and systemic responses may be distinct (26). The process of *Salmonella*-specific memory lymphocyte subset development is less clear and may be confounded by bacterial evasion strategies that can affect antigen-specific CD4 T cells (12, 15, 25, 46, 47). These inhibitory responses eventually wane, and memory CD4 Th1 cells emerge that can provide effective protection against secondary infection (48). It is not yet clear whether protective immunity depends upon circulating memory lymphocytes or memory subsets that provide local defense of previously infected tissues, such as the liver (49). Since *Salmonella* replicates within macrophages of lymphoid and nonlymphoid tissues, it is not immediately obvious which of these subsets would be critical.

Our data show that a robust *Salmonella*-specific CD4 memory T cell population is generated within the spleen and nonlymphoid tissues of LVS-immunized mice. A substantial pro-portion of memory T cells identified in the liver using an IFN-γ reporter expressed CD69, P2X7, ARTC2, LFA-1, and CD101, but lacked expression of KLRG1 and CD103 (34, 49, 50), consistent with a tissue-resident Th1 memory subset. In agreement with this characterization, a proportion of vascular stain-negative, CD69^{+} *Salmonella*-specific CD4 T cells failed to transfer after parabiosis between LVS-immunized and naive mice. Taken together, these data support the theory that LVS immunization effectively generates tissue resident CD4 Th1 cells in nonlymphoid tissues of immunized mice. This theory would be consistent with other infection models, where CD4^{+} TRM cells specific for *Chlamydia*, HSV, and TB have been detected in nonlymphoid tissues (29, 30, 50). This interpretation would also explain why adoptive transfers have so far been only partially successful in transferring *Salmonella*-specific protection (16, 17).

Importantly, our parabiosis experiments show that naive mice sharing circulation with LVS-immunized mice acquire only modest protection against *Salmonella* challenge. Since CD4 Th1 cells and antibody provide some immunity to *Salmonella* (8, 9, 16, 51), it is likely this, albeit incomplete, circulating protection is due to these factors. Indeed, a similar level of incomplete protection has been observed in models where immune serum and immune spleen cells have been transferred to naive mice (16). Importantly, protection in LVS-immunized mice was more robust than in parabiosed naive partners, demonstrating that noncirculating responses contribute to *Salmonella* immunity. Given the correlation between enhanced protection in LVS-immunized mice and the presence of antigen-specific tissue-resident Th1 cells, the simplest interpretation of these data is that tissue-resident memory CD4 Th1 cells form an essential component of protective immunity to *Salmonella* infection. However, alternative explanations are possible, such as antigen retention in tissues or the persistence of

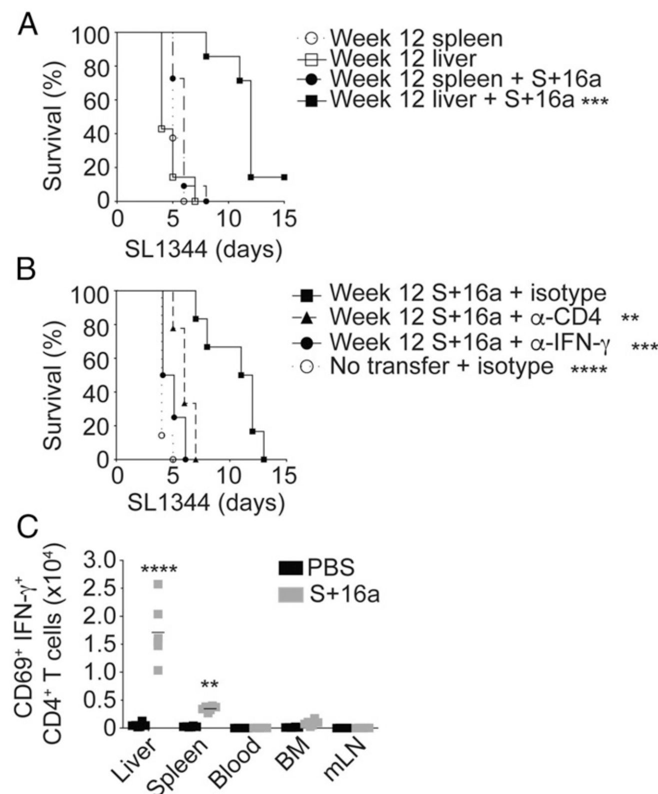

Fig. 6. Liver-associated IFN-γ^{+} CD4^{+} T cells protect against SL1344 infection. (*A*–*C*) Survival of *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice receiving 5×10^{7} splenocytes or 1×10^{7} lymphocyte-enriched liver cells from C57BL/6 mice that were previously infected for 12 wk and received 50 μg S+16a or PBS by intravenous injection 15 min before harvest. (*A*) Recipients were challenged intravenously with 200 cfu SL1344 24 h after adoptive transfer. (*B*) At the time of cell transfer, recipients of S+16a-treated liver cells received intraperitoneal injections of 250 μg of isotype control or anti-CD4 (GK1.5) or 200 μg anti–IFN-γ (HB-170-15), and 20 h later, recipients were challenged intravenously with 200 cfu SL1344. Antibody treatments were repeated intraperitoneally every 3–4 d. (*C*) Distribution of CD69^{+} eYFP^{+} CD4^{+} T cells in *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice that had been adoptively transferred with liver cells from C57BL/6 mice infected for 12 wk that received 50 μg S+16a or PBS. Data in *A* and *B* are representative of three independent experiments. Data in *C* are representative of two independent experiments. (*B*) $n = 7$–8. (*C*) $n = 7$–12 per group.

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low-level inflammation in previously immunized mice. The albeit modest protection observed in parabiosed naive mice is likely to be mediated by a combination of circulating memory cells and antibody (5). While transfer of immune spleen cells does not confer protection in this model, a modest degree of protection has been observed by combining spleen cells with immune serum transfer (16, 17, 52). The fact that adoptive transfer of liver cells can confer partial resistance to challenge with virulent *Salmonella* infection provides evidence that noncirculating liver CD4 T cells also make a contribution to protective immunity in this model. It should be noted that, although *Salmonella* is an intestinal infection, very few bacteria directly enter the lamina propria (4, 31), meaning that defense against infection is anatomically positioned within systemic lymphoid tissues and the liver. Thus, the liver-resident memory T cell population described in this study is likely to be a major contributing factor to the resolution of oral infection.

These data have significant implications for the design of *Salmonella* vaccines since they highlight an essential, but previously unrecognized, protective memory population. Although recent studies from our laboratories and others show the potential for subunit *Salmonella* vaccines to protect against *Salmonella* challenge, these approaches are usually less effective than LVS immunization (36, 53–55). Indeed, our recent studies suggest that subunit vaccine-mediated protection relies solely on circulating CD4 T cell memory (32). Thus, substantial improvements to the effectiveness of subunit vaccines or LVS *Salmonella* strains may require a new focus on the generation of *Salmonella*-specific TRMs. While *Salmonella* vaccine approaches have often focused upon the generation of intestinal immune responses, the generation of protective systemic TRM T cells is likely to require alternative strategies. Incorporation of this unexplored variable into vaccine design may allow for the generation of an effective subunit vaccine that could be administered safely to vulnerable individuals.

Materials and Methods

Experimental Model: Mice. Female C57BL/6J (stock #000664) and B6.SLJ-Ptprc^{a} Pepc^{b}/BobJ (B6 Cd45.1 Pep boy, stock #002014) 8-wk-old mice were purchased from The Jackson Laboratories. IFN-γ-eYFP$^{in/in}$ C57BL/6 mice were generously provided by R. Locksley, University of California, San Francisco (35). Mice were housed in specific-pathogen-free conditions and handled in accordance with institutional animal care and use committee practices at the University of California, Davis, University of Connecticut, and University of Melbourne.

***Salmonella* and Bacterial Culturing.** *S.* Typhimurium LVS strain BRD509 (Δ*aroA*) (56) was modified to express a short peptide sequence (EAWGALANWAVDSA) in frame with OmpC (BRD509-2W1S). It should be noted that, while BRD509 was initially reported as a mutant for *aroA* and *aroD*, recent genome sequencing has confirmed the presence of an intact *aroD* gene. Preliminary experiments confirmed correct gene targeting, peptide expression, and activation of 2W1S-specific CD4 T cells in vivo. *S.* Typhimurium Δ*edd* Δ*pfkA* Δ*pfkB* (TAS2010) is a LVS with enhanced protective capacity (36). Before use, bacteria were streaked out onto McConkey agar plates and grown statically at 37 °C in Luria–Bertani broth (supplemented with 50 μg/mL streptomycin for TAS2010). Before infection, OD_{600} of overnight cultures of BRD509 were diluted with PBS to 2.5×10^6 cfu/0.2 mL (BRD509-2W1S), 1×10^3 cfu/0.2 mL (SL1344), or 1 ± 10^9 cfu/mL (SL1344) (57). Dose estimates were confirmed by dilution of inoculum onto McConkey agar plates and by quantifying colonies 24 h later. For TAS2010 infections, 200 cfu were injected via the tail vein in a volume of 200 μL.

Bacterial Burdens. To evaluate bacterial burdens, mice were euthanized via cervical dislocation, and liver and spleen were harvested in 1× PBS. Liver and spleen were homogenized and brought to a final volume of 5 and 3 mL, respectively. Serial dilutions of organ homogenates were made, plated onto McConkey agar plates, and incubated overnight at 37 °C. The next day, colonies from serial dilutions were quantified and back-calculated to identify total cfu present in each organ.

Intravascular Staining. Intravascular staining was accomplished as previously established (33). Briefly, 3 μg anti-CD45.2 (BV650; Biolegend) or anti-CD90.2 (BV786; Biolegend) in 1× PBS was administered i.v. for 3 min to mice before euthanasia. Organs were then harvested, and lymphocytes were isolated from tissues and stained ex vivo.

Lymphocyte Isolation. Spleens were removed and placed in 2% FBS in 1× PBS. Single-cell suspensions were made by pressing spleens through a 70-μm mesh cell strainer filter unit (catalog no. 08–771-2; Fisher). Cellular suspensions were spun 720 × *g* for 5 min at 4 °C, and supernatant was decanted. To remove red blood cells, the pellet was resuspended in 1 mL of ammonium–chloride–potassium lysis buffer (catalog no. A1049201; Fisher) and incubated at room temperature for 5 min. Lysis was halted with 9 mL 2% FBS in 1× PBS. Cellular suspensions were spun and decanted as described previously. Pellets were resuspended in 2% FBS in 1× PBS, and cells were quantified (Nexcelom Cytometer Auto T4 Bright Field Counter). Livers were removed and placed in 2% FBS in 1× PBS. Single-cell suspensions were made as with spleens. Cellular suspensions were spun at 720 × *g* for 10 min at room temperature with no brake. Supernatant was decanted, and cells were suspended in 15 mL of 35% Percoll solution [P1644 (Sigma), 1× PBS, 6.5 mM Hepes, 1,500 units of heparin]. Cellular suspensions were spun as previously described. Hepatocytes were gently aspirated, and cellular pellets were washed in 40 mL 2% FBS in 1× PBS. Red blood cells were removed, and cells were resuspended in 2% FBS in 1× PBS and quantified.

Lungs were removed and placed in 1.3 mM EDTA solution. Tissue was cut into small pieces and incubated in 20 mL 1.3 mM EDTA for 30 min at 37 °C with stirring (700 × *g*). Tissue and EDTA were transferred to a 50-mL conical tube and spun at 720 × *g* at room temperature for 5 min. Supernatant was aspirated, and tissue was washed with 40 mL 2% FBS in 1× PBS. Tissue pieces were resuspended in 20 mL collagenase solution [4.7% FBS, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 U/mL collagenase (catalog no. 17018–029; Invitrogen)] and incubated for 1 h at 37 °C with stirring (700 × *g*). Single-cell suspensions were made by pressing tissue through a 70-μm mesh cell strainer filter unit. Cells were spun as previously described and washed in 40 mL 2% FBS in 1× PBS. Pellets were suspended in 8 mL of 44% Percoll solution (catalog no. 17–0891-02; GE Healthcare) and overlaid onto 5 mL of 67% Percoll solution. Cells were spun at 720 × *g* for 20 min at room temperature (minimal acceleration, no break). Lymphocytes were removed from Percoll and washed in 10 mL 2% FBS in 1× PBS. Cells were resuspended in 2% FBS in 1× PBS and then were quantified.

Staining and Flow Cytometry. Single-cell suspensions were made as stated above. Cells (2×10^6) were incubated with FC block solution (24G2 supernatant with 2% rat and 2% mouse serum). Cells were incubated with PE-conjugated 2W1S::I-A^{b} MHCII Tetramer for 1 h. Cells were washed with 2% FBS in 1× PBS and stained with the following antibodies: B220, F4/80, CD11b, CD11c (Tonbo Biosciences); CD4 (Alexa700; eBioSciences); CD8 (Pacific Orange; Thermo Fisher), CD44 (Pe-Cy7; Tonbo Biosciences), CD69 (eFluor 450; Thermo Fisher); CD62L (eBioSciences); CD27 (APC; Thermo Fisher), KLRG1 (BV605; BioLegend), CD103 (PE; BioLegend), CD101 (APC; BD Horizon), LFA-1 (PE; BioLegend), P2X7 (PE; BD Biosciences), and ARTC2 (APC; Novus Biologicals). Cells were fixed (2% paraformaldehyde in 1× PBS) for 20 min. Cells were then washed and spun as previously described. Samples were analyzed via flow cytometry using BD LSR Fortessa (BD Biosciences), and data were analyzed via FlowJo software (TreeStar). Comparison of datasets were performed using a Mann–Whitney *U* test and are displayed as SEM.

Parabiosis. Parabiosis was conducted as previously described (58). Briefly, 8-wk-old female C57BL/6J and B6CD45.1 Pep Boy mice were purchased from The Jackson Laboratories. C57BL/6J mice were immunized on day 0 with 2.5×10^6 cfu BRD509-2W1S intravenously. On day 30, mice were given a secondary immunization (boost) 2.5×10^6 cfu BRD509-2W1S intravenously. Immunized mice were cohoused with naive mice for 2 wk before surgical joining on day 60. Parabionts remained together for 28 d and were then surgically separated. Separated parabionts were allowed to recover for 2 wk before i.v. challenge with virulent bacteria (1×10^3 cfu SL1344 i.v.). Organs were harvested, and bacterial burdens were quantified as stated above.

Isolation, Enrichment, and Adoptive Transfer of Lymphocytes. Before organ harvesting, donor mice were perfused intracardially and via the portal vein with RPMI-1640 medium (no phenol red) supplemented with 5% (vol/vol) FCS. Spleen and liver, and in some experiments MLNs and bone marrow (left femur) were harvested and pushed through a 70-μm cell strainer. Red blood cells were lysed. Liver single-cell suspensions were taken up in 35% isotonic Percoll and centrifuged at 2,000 × *g* for 12 min at room temperature. Pellets from different donor mice were pooled and a second Percoll-based enrichment

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was performed. Single-cell suspensions from either spleen or enriched liver were incubated in RPMI-1640 medium supplemented with 5% (vol/vol) FCS and 100 μg/mL gentamycin for 20 min at 37 °C. Whole splenocytes (50×10^6) or liver lymphocytes (10×10^6) were diluted in 200 μL PBS and adoptively transferred to either Ly5.1 or $Rag2^{-/-} \times Il2rg^{-/-}$ recipients via the tail vein.

Nanobody-Mediated Inhibition of ARTC2. Mice were i.v. injected with 50 μg of S+16a nanobody (39) diluted in 200 μL PBS 15 min before organ harvest.

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1. Gilchrist JJ, MacLennan CA, Hill AV (2015) Genetic susceptibility to invasive Salmonella disease. *Nat Rev Immunol* 15:452–463.
2. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ (2002) Typhoid fever. *N Engl J Med* 347:1770–1782.
3. Keestra-Gounder AM, Tsolis RM, Bäumler AJ (2015) Now you see me, now you don't: The interaction of Salmonella with innate immune receptors. *Nat Rev Microbiol* 13: 206–216.
4. Griffin AJ, McSorley SJ (2011) Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* 4:371–382.
5. McSorley SJ (2014) Immunity to intestinal pathogens: Lessons learned from Salmonella. *Immunol Rev* 260:168–182.
6. Levine MM (2006) Enteric infections and the vaccines to counter them: Future directions. *Vaccine* 24:3865–3873.
7. Nauciel C (1990) Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection. *J Immunol* 145:1265–1269.
8. Hess J, Ladel C, Miko D, Kaufmann SH (1996) Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: Major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J Immunol* 156:3321–3326.
9. McSorley SJ, Jenkins MK (2000) Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. *Infect Immun* 68: 3344–3348.
10. Mastroeni P, Simmons C, Fowler R, Hormaeche CE, Dougan G (2000) Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. *Infect Immun* 68:46–53.
11. Cunningham AF, et al. (2007) Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection. *J Immunol* 178:6200–6207.
12. Kullas AL, et al. (2012) L-asparaginase II produced by Salmonella typhimurium inhibits T cell responses and mediates virulence. *Cell Host Microbe* 12:791–798.
13. Nanton MR, et al. (2015) Direct visualization of endogenous Salmonella-specific B cells reveals a marked delay in clonal expansion and germinal center development. *Eur J Immunol* 45:428–441.
14. Bayer-Santos E, et al. (2016) The Salmonella effector SteD mediates MARCH8-dependent ubiquitination of MHC II molecules and inhibits T cell activation. *Cell Host Microbe* 20:584–595.
15. Bedoui S, et al. (2010) Different bacterial pathogens, different strategies, yet the aim is the same: Evasion of intestinal dendritic cell recognition. *J Immunol* 184:2237–2242.
16. Mastroeni P, Villarreal-Ramos B, Hormaeche CE (1993) Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. *Infect Immun* 61:3981–3984.
17. Johanns TM, et al. (2011) Early eradication of persistent Salmonella infection primes antibody-mediated protective immunity to recurrent infection. *Microbes Infect* 13: 322–330.
18. Eisenstein TK, Killar LM, Sultzer BM (1984) Immunity to infection with Salmonella typhimurium: Mouse-strain differences in vaccine- and serum-mediated protection. *J Infect Dis* 150:425–435.
19. Jameson SC, Masopust D (2009) Diversity in T cell memory: An embarrassment of riches. *Immunity* 31:859–871.
20. Heath WR, Carbone FR (2013) The skin-resident and migratory immune system in steady state and memory: Innate lymphocytes, dendritic cells and T cells. *Nat Immunol* 14:978–985.
21. Schenkel JM, Masopust D (2014) Tissue-resident memory T cells. *Immunity* 41: 886–897.
22. Steinert EM, et al. (2015) Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell* 161:737–749.
23. Moon JJ, et al. (2007) Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27:203–213.
24. Mittrücker HW, Köhler A, Kaufmann SH (2002) Characterization of the murine T-lymphocyte response to Salmonella enterica serovar Typhimurium infection. *Infect Immun* 70:199–203.
25. Srinivasan A, Foley J, McSorley SJ (2004) Massive number of antigen-specific CD4 T cells during vaccination with live attenuated Salmonella causes interclonal competition. *J Immunol* 172:6884–6893.
26. Lee SJ, et al. (2012) Temporal expression of bacterial proteins instructs host CD4 T cell expansion and Th17 development. *PLoS Pathog* 8:e1002499.
27. O'Donnell H, et al. (2014) Toll-like receptor and inflammasome signals converge to amplify the innate bactericidal capacity of T helper 1 cells. *Immunity* 40:213–224.
28. Teijaro JR, et al. (2011) Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol* 187:5510–5514.
29. Stary G, et al. (2015) VACCINES. A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells. *Science* 348:aaa8205.
30. Iijima N, Iwasaki A (2014) T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* 346:93–98.
31. Carter PB, Collins FM (1974) The route of enteric infection in normal mice. *J Exp Med* 139:1189–1203.
32. Lee SJ, et al. (2017) Dual immunization with SseB/flagellin provides enhanced protection against *Salmonella* infection mediated by circulating memory cells. *J Immunol* 199:1353–1361.
33. Anderson KG, et al. (2014) Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc* 9:209–222.
34. Fernandez-Ruiz D, et al. (2016) Liver-resident memory $CD8^+$ T cells form a front-line defense against malaria liver-stage infection. *Immunity* 45:889–902.
35. Reinhardt RL, Liang HE, Locksley RM (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 10:385–393.
36. Kupz A, et al. (2013) Contribution of Thy1+ NK cells to protective IFN-γ production during Salmonella typhimurium infections. *Proc Natl Acad Sci USA* 110:2252–2257.
37. McNamara HA, Cockburn IA (2016) The three Rs: Recruitment, retention and residence of leukocytes in the liver. *Clin Transl Immunology* 5:e123.
38. Rissiek B, Haag F, Boyer O, Koch-Nolte F, Adriouch S (2015) ADP-ribosylation of P2X7: A matter of life and death for regulatory T cells and natural killer T cells. *Curr Top Microbiol Immunol* 384:107–126.
39. Rissiek B, Danquah W, Haag F, Koch-Nolte F (2014) Technical advance: A new cell preparation strategy that greatly improves the yield of vital and functional Tregs and NKT cells. *J Leukoc Biol* 95:543–549.
40. Rissiek B, et al. (2018) *In vivo* blockade of murine ARTC2.2 during cell preparation preserves the vitality and function of liver tissue-resident memory T cells. *Front Immunol* 9:1580.
41. Benoun JM, Labuda JC, McSorley SJ (2016) Collateral damage: Detrimental effect of antibiotics on the development of protective immune memory. *MBio* 7:e01520-16.
42. McGregor AC, Waddington CS, Pollard AJ (2013) Prospects for prevention of Salmonella infection in children through vaccination. *Curr Opin Infect Dis* 26:254–262.
43. Lin FY, et al. (2001) The efficacy of a Salmonella typhi Vi conjugate vaccine in two-to-five-year-old children. *N Engl J Med* 344:1263–1269.
44. Bumann D (2001) In vivo visualization of bacterial colonization, antigen expression, and specific T-cell induction following oral administration of live recombinant Salmonella enterica serovar Typhimurium. *Infect Immun* 69:4618–4626.
45. McSorley SJ, Asch S, Costalonga M, Reinhardt RL, Jenkins MK (2002) Tracking salmonella-specific CD4 T cells in vivo reveals a local mucosal response to a disseminated infection. *Immunity* 16:365–377.
46. Cheminay C, Möhlenbrink A, Hensel M (2005) Intracellular Salmonella inhibit antigen presentation by dendritic cells. *J Immunol* 174:2892–2899.
47. Srinivasan A, Nanton M, Griffin A, McSorley SJ (2009) Culling of activated CD4 T cells during typhoid is driven by Salmonella virulence genes. *J Immunol* 182:7838–7845.
48. Dougan G, John V, Palmer S, Mastroeni P (2011) Immunity to salmonellosis. *Immunol Rev* 240:196–210.
49. Gebhardt T, Palendira U, Tscharke DC, Bedoui S (2018) Tissue-resident memory T cells in tissue homeostasis, persistent infection, and cancer surveillance. *Immunol Rev* 283: 54–76.
50. Sakai S, et al. (2014) Cutting edge: Control of Mycobacterium tuberculosis infection by a subset of lung parenchyma-homing CD4 T cells. *J Immunol* 192:2965–2969.
51. Ravindran R, Foley J, Stoklasek T, Glimcher LH, McSorley SJ (2005) Expression of T-bet by CD4 T cells is essential for resistance to Salmonella infection. *J Immunol* 175: 4603–4610.
52. Nanton MR, Way SS, Shlomchik MJ, McSorley SJ (2012) Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. *J Immunol* 189:5503–5507.
53. Lee SJ, et al. (2012) Identification of a common immune signature in murine and human systemic salmonellosis. *Proc Natl Acad Sci USA* 109:4998–5003.
54. Barat S, et al. (2012) Immunity to intracellular Salmonella depends on surface-associated antigens. *PLoS Pathog* 8:e1002966.
55. Ferreira RB, et al. (2015) A highly effective component vaccine against nontyphoidal Salmonella enterica infections. *MBio* 6:e01421-15.
56. Strugnell R, et al. (1992) Characterization of a Salmonella typhimurium aro vaccine strain expressing the P.69 antigen of Bordetella pertussis. *Infect Immun* 60:3994–4002.
57. O'Donnell H, Pham OH, Benoun JM, Ravesloot-Chávez MM, McSorley SJ (2015) Contaminated water delivery as a simple and effective method of experimental Salmonella infection. *Future Microbiol* 10:1615–1627.
58. Donskoy E, Goldschneider I (1992) Thymocytopoiesis is maintained by blood-borne precursors throughout postnatal life. A study in parabiotic mice. *J Immunol* 148: 1604–1612.

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