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The role and formation of hepatic tissue resident memory CD4 T cells during systemic
Salmonella enterica serovar Typhimurium infection

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DISSERTATION

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Abstract

Systemic *Salmonella enterica* infections are caused by serovars Typhi and Paratyphi, causing typhoid, and other serovars, which cause invasive non-typhoidal Salmonellosis (iNTS). These infections cause about 200,000 deaths per year and thus are a large public health burden. Currently, the vaccines that are approved for typhoid are not given to individuals who live in endemic areas due to low efficacy and there are no approved vaccines for iNTS. Thus, an effective vaccine is desperately needed to combat these infections. How the host naturally protects itself from pathogens is required to make an effective vaccine.

CD4 T cells are required for protection against systemic *Salmonella*. CD4 T cell memory is composed of three subsets: central, effector and tissue resident memory. Central memory T cells (TCM) circulate throughout the secondary lymphoid organs (SLOs) and blood while effector memory T cells (TEM) circulate throughout the SLOs, blood and non-lymphoid tissues (NLTs). Both TCM and TEM circulate the body looking for cognate antigen to be reactivated and therefore, together are termed circulating memory. The third subset tissue resident memory T cells (TRM) do not circulate and resides in previously infected tissues and become the front line of defense for subsequent infections. Previously, CD4 TRM in the liver has been shown to be required for complete protection against systemic *Salmonella*. Thus, to make an effective vaccine, this memory subset needs to be induced. However, how to induce the formation of CD4 TRM in the liver during vaccination is currently unknown.

Previously, transcription factors have been shown to be important in the formation of CD8 TRM in a variety tissues and allow CD8 TRM to adapt and survive in the tissue microenvironments. In this study we demonstrate a novel way transcription factors impact TRM formation. T-bet acts upstream of CD4 TRM differentiation in tissues and T-bet^{hi} CD4 T cells express higher levels of

LFA-1 which allow these TRM precursors to compete for the competitive liver niche where they can access signals in the liver microenvironment which will induce the CD4 T cells to become TRM. These TRM differentiation signals have previously been interrogated in CD8 TRM formation in a variety of tissues with inflammation and pro-inflammatory cytokines driving CD8 TRM formation. In this study we demonstrated that liver inflammation increases CD4 TRM formation in the liver with pro-inflammatory cytokines, IL-2 and IL-1 required for optimal CD4 TRM formation. Additionally, adding liver inflammation enhances the protection provided by *Salmonella* specific subunit vaccination which is partially protective.

Thus, this study has demonstrated there are two phases in CD4 TRM formation in the liver. In the secondary lymphoid organs, CD4 T cell priming impacts T-bet expression and T-bet^{hi} CD4 T cells have a higher chance of accessing the liver niche by increased LFA-1 expression. Once in the liver microenvironment, liver inflammation, specifically IL-2 and IL-1, stimulated by *Salmonella* infection induces CD4 T cells to become TRM. This understanding of CD4 TRM formation can further be applied to vaccine design for systemic *Salmonella* infection and aid in combating this large public health burden.

Chapter 1

Introduction

Salmonella enterica, a gram-negative intracellular bacterium, transmitted by the oral-fecal route causes a significant public health burden worldwide (1). The severity of this disease is determined by serovar, strain and host-related factors with serovars Typhi and Paratyphi causing systemic enteric fever while other serovars, such as Typhimurium, resulting in self-limiting enterocolitis or another systemic infection called invasive non-typhoidal *Salmonellosis* (iNTS) (2, 3).

Systemic enteric fever, or typhoid, causes about 110,000 deaths per year which mostly effects individuals living in south-central and south-east Asia (4-7). However, this number could be larger since there are problems with reporting typhoid since symptoms are very similar to other febrile infections and the limited resources of bacterial culturing in endemic areas, which can impede accurate diagnosis (2, 6). After initial infection, individuals usually do not show symptoms for 7-21 days but may continue to actively shed bacteria as shown by voluntary infection experiments (2, 8). The main symptoms of typhoid are fever and joint aches, but individuals can also have constipation, diarrhea or general abdominal pain and nausea (9). The serovars Typhi and Paratyphi are host restricted to humans making investigation of the host response to typhoid challenging to study in the laboratory (10). Instead, generalist serovars which cause iNTS are typically used to study systemic *Salmonella* infection in animal models (10).

iNTS causes about 77,500 deaths per year which mostly effects individuals in sub-Saharan Africa (5, 11, 12). Just like typhoid, the exact number of iNTS deaths could be higher due to the commonality of symptoms with other infections and limitations in diagnostic microbiology within endemic areas (6, 13). iNTS has non-specific symptoms, similar to typhoid, with fever and hepatosplenomegaly being the main symptom, while unlike enterocolitis, diarrhea is an uncommon

symptom of iNTS (14, 15). Unfortunately, iNTS has a high death rate of 36% when not treated with antibiotics, and even with treatment 12% of infected individuals die (16-18). Children under 5 years old are especially susceptible to this systemic infection with a concentration around ages 6-18 months (11, 19). There is second spike in infection rate from ages of 25-40 years, which is the age group with the highest rate of HIV, a major risk factor for iNTS (17, 19). Along with HIV, many other immune compromising factors increase risk of iNTS such as malaria, sickle cell anemia and malnutrition, all co-morbidities which are common in Africa (2, 20, 21). Along with increased comorbidities, areas endemic for iNTS tend to have strains a propensity for systemic infection via unknown mechanisms. For example, chickens infected with strain ST313, a strain endemic to Africa, displayed increased bacterial burdens in the spleen and less fecal shedding than chickens infected with strain ST19, a common strain found in Europe (22, 23). Due to all of these confounding factors, sub-Saharan Africa has the highest rates of iNTS in the world (11).

Current treatment for systemic *Salmonella* infection relies on antimicrobials, but multidrug resistant (MDR) strains are increasing in frequency (9, 24, 25). Fluoroquinolone resistance is so pervasive that extended-spectrum cephalosporins are now recommended for initial treatment (26). However, strains resistant to cephalosporins are also emerging with this being a larger problem in serovars that cause iNTS rather than typhoid (2). Azithromycin is prescribed to these extensively drug resistant strains (XDR) but the development of resistance to this antibiotic is highly likely (4). Therefore, development of highly efficacious vaccines are required to combat systemic *Salmonella* which required a greater understanding of how an infected host naturally clears the pathogen.

Mouse models for Systemic *Salmonella* infection

The biology of primary clearance and secondary protection to systemic *Salmonella* has been studied extensively using the iNTS mouse model. In this model, C57BL/6 mice naturally have a mutated NRAMP1 (SLC11A) gene which encodes a phagolysosome ion channel involved in depriving *Salmonella* of crucial nutrients such as iron and magnesium (27, 28). Due to this natural mutation, C57BL/6 mice are highly susceptible to wild type strains of *S. enterica* serovar Typhimurium and will typically die within 7-10 days of infection. However, these susceptible mice can be immunized with live vaccine strains (LVS) of *Salmonella* which they can naturally clear and subsequently become fully resistant to wildtype challenge (29). Thus, effective immune memory responses to systemic *Salmonella* are induced using this model. In contrast, 129/Sv mice have a functional NRAMP1 gene and do not die when challenged with a wild type *Salmonella* strain, but instead develop chronic *Salmonella* infection (30). Importantly, disruption of the gut microbiota is not a feature of either of these mouse models. This stands in contrast to the streptomycin-pretreatment model of *Salmonella* infection where mice are administered streptomycin to disrupt the natural microbiota prior to infection (31). In this model, the intestinal epithelium is hyper-infected and a useful model of colitis emerges (31), although the role of the adaptive immune system is limited. Since this current review is focused on vaccines against systemic *Salmonella*, we will primarily focus on lessons learned using the C57BL/6 iNTS mouse model. Additionally, since typhoid is highly host-restricted and there currently is no mouse model to understand this infection, discoveries in the iNTS mouse model will be extrapolated to typhoid whenever appropriate(10, 32).

Route of Infection of *Salmonella*

Salmonella is transmitted through the fecal-oral route and thus passes through the stomach where the bacterium has to survive a highly acidic environment and preferentially infects the distal ileum of the small intestine (33, 34). In the distal ileum, *Salmonella* infects specialized follicle-associated epithelial (FAE) cells called M cells which cover the Peyer's patches of the intestine creating the main way that *Salmonella* crosses the intestinal epithelium (35). Additionally, *Salmonella* can induce the formation of additional M cells from FAE cells and also destroy M cells and thus increase permissibility of intestinal epithelium through this specialized site (35, 36). Once *Salmonella* has crossed the intestinal epithelium and enters the Peyer's patches, the bacteria can reach the mesenteric lymph nodes, either as extracellular bacteria in lymph fluid or by infecting phagocytes which subsequently traffic to these lymph nodes (35). This infiltration of Peyer's patches and mesenteric lymph nodes is rapid and there are detectable *Salmonella* in the Peyer's patches and mesenteric lymph nodes at 3 hours and 6 hours post oral infection respectively (37). *Salmonella* can also infect intestinal epithelium directly or enter phagocytes cells that span the intestinal epithelial layer and thus bypass Peyer's patches to reach systemic site, however this is not the main way that *Salmonella* crosses the epithelium (35, 38, 39). Once *Salmonella* is present in the mesenteric lymph nodes, bacteria can infect the spleen, liver and bone marrow by unknown mechanisms. *Salmonella* prefers these tissue sites due the large number of available macrophages where *Salmonella* can grow within *Salmonella* containing vacuoles (SCVs), the preferred replicative niche in vivo (40-42). Indeed, in patients who are chronic typhoid carriers, the liver is the main anatomical location where *Salmonella* were detected by PCR (43). In addition to macrophages, *Salmonella* are known to reside in dendritic cells and inflammatory monocytes, but bacteria replicates at a lower rates inside these cells (42).

Immune response to *Salmonella*

Sensing of invading bacteria is the first step in the immune response and is largely accomplished by pattern recognition receptors (PRRs) expressed on innate cells. *Salmonella* is recognized by Toll-like receptors (TLRs) 2, 4, 5, and 9 (44). TLR4 which recognizes bacterial LPS, is crucial for the host immune response to *Salmonella* as evident by the fact that TLR4 deficient mice display increased susceptibility to infection (45). TLR5 induces a similar inflammatory response and is also required for optimal antigen processing and presentation of bacterial flagellin, an early target of *Salmonella*-specific CD4 T cells (46, 47). The other TLRs appear to be redundant and their absence is only noted when TLR4 is also deleted (48). Interestingly when TLR4, TLR9 (senses unmethylated CpG), and TLR2 (senses gram-positive bacteria cell wall) are all deleted together, these mice have increased resistance compared to double knockouts, demonstrating that TLR signaling is required for *Salmonella* pathogenesis (48).

Cytoplasmic *Salmonella* can be sensed by NOD-like receptors, NLRP3 and NLRC4, which induce the inflammasome response, leading to the release of IL-18 and IL-1 β through activation of caspase-1 (49). Increased susceptibility to *Salmonella* is only seen when mice are deficient in both NLRs, demonstrating a redundancy in signaling (50). Additionally, similar effects are seen when mice were deficient in caspase-1 or IL-18, although there was no effect on primary clearance when mice were deficient in IL-1 β (50, 51). The effect of caspase-1 deficiency can be reversed by supplementing mice with rIL-18 demonstrating that IL-18 is the required ultimate product of the inflammasome cascade (51). IL-18 is essential for non-cognate activation of T cells and thus loss of this cascade prevents *Salmonella* infected macrophages from inducing an environment where T cells can be activated by this mechanism (52).

Salmonella infects many cell types, but prefers to infect and reside within macrophages. Even though macrophages are phagocytic and kill bacteria, during *Salmonella* infection this population actually serve as a bacteria reservoir and depletion of macrophages reduces bacterial growth and decreases mortality (53). When *Salmonella* infects a macrophage, it forms the SCV via a process dependent on bacteria proteins encoded by the type III secretion system (T3SS), *Salmonella* pathogenicity Island 1 (SPI-1) (54). Once in the SCV, *Salmonella* uses another T3SS, *Salmonella* Pathogenicity Island 2 (SPI-2), to maintain the SCV and inhibit the host response (55-57). Specifically, *Salmonella* effectors inhibit the ability of the infected macrophage to produce iNOS and NADPH which stops the production of ROS and inhibits lysosome fusion with the SCV (58-60). Additionally, *Salmonella* inhibits the production of pro-inflammatory cytokines by the macrophage (54). This inhibition of macrophages can be impeded by the host through stimulating the macrophages with IFN γ . When cultured with recombinant IFN γ , macrophages infected with *Salmonella* had increased SCV fusing with the lysosome and increased NADPH and iNOS levels which coincided with increased macrophage killing (61-63).

IFN γ can be produced by many different immune cell types and initially the majority of the IFN γ is produced by innate cells such as NK cells (29). However, as the immune response progresses IFN γ is made mainly by CD4 T cells skewed to a Th1 polarization state (29, 64-67). Indeed, CD4 T cells are required for primary and secondary clearance of *Salmonella* and at the peak of infection the majority of activated CD4 T cells in the spleen are producing IFN γ (67, 68). Additionally, when T-bet deficient mice are infected with attenuated *Salmonella*, 50% of the mice die by day 25 which phenocopies the death rate of IFN γ R deficient mice (68, 69). Additionally, innate cell derived IFN γ can create iNOS⁺ granulomas in mice but in the absence of Th1 cells mice are unable

to reach sterile immunity and eventually succumb to the infection (70). Thus, Th1 skewed CD4 T cells are required for clearance of the primary infection and secondary protection.

In addition to inhibiting macrophage based killing mechanisms, *Salmonella* also downregulates MHC class-II on the surface of macrophages which inhibits the ability of CD4 T cells to exert their effector mechanisms via cognate interactions (71). Some CD4 T cells can circumvent this downregulation by being able to respond in a non-cognate manner in the absence of MHC class-II (52, 72). During a non-cognate response, CD4 T cells are activated by local inflammatory mediators instead of TCR stimulation, specifically through IL-18R and DR3 which allows CD4 T cells make large amount of IFN γ which can activate macrophages without the need of pMHCII:TCR interactions and enable efficient clearance of *Salmonella* (52).

As for the other adaptive immune cells, mice lacking CD8 T cells display only a slight increase in bacterial burden and mice deficient in B cells show no deficiency in clearance of the primary response (73, 74). Interestingly, B cells are required for secondary protection yet B cells lacking the ability to secrete antibody are fully protected against secondary challenge. Thus, the requirement for B cells in secondary protection is not antibody dependent and their exact role has yet to be determined (74, 75).

The importance of type 1 immune response to clearing *Salmonella* is also seen in humans. CD4 T cells are important in clearing *Salmonella* infection in humans since HIV+ individuals have an increased risk of iNTS as well as certain MHC alleles are a risk factor for systemic *Salmonella* infection as demonstrated by GWAS studies (76, 77). Specifically, Th1 cells are important for clearing *Salmonella* in humans since individuals who have inhibition of the Th1 pathway, such as a mutation in IL-12R β 1, have IFN γ autoantibodies, or are given anti-TNF α therapy, all have increased risk of iNTS (78-81). In this way, the lessons learned from the iNTS mouse model are

mirrored in the human clinic demonstrating that the mouse model robustly recapitulates salient aspects of clinical *Salmonella* infection (or something similar)

Current vaccines and vaccine development

Ty21 and Vi Capsular vaccines

The two main licensed vaccines against Typhoid are the live oral Ty21 vaccine and the Vi Capsular polysaccharide (ViCPS) vaccine (82). However, these vaccines have low rates of protection with Ty21 protecting only 1/3 of individuals for a year post vaccination and the ViCPS protecting 2/3 for 1 year, but this protection wanes after 2 years (82). Due to the short-lived efficacy of these vaccines, individuals need to be revaccinated around 3-6 years for the Ty21 vaccine and every three years for the ViCPS (83). As typhoid mainly impacts resource poor countries, it is not economical to vaccinate people living in endemic areas and these vaccines are usually given to travelers to these endemic regions (83). Additionally, the Ty21 vaccine cannot be given to children under the age of 6 while the ViCPS vaccine cannot be given to children under the age of 2 years, even though children are the largest demographic group affected by typhoid (2, 83). Thus, new and better vaccines against typhoid are urgently needed to protect individuals living in endemic areas.

In the past decade there have been the development of typhoid conjugate vaccine which shows promising results with the ViCPS being conjugated with different protein conjugates. The main conjugated proteins are the tetanus toxoid, diptheria toxin receptor or diptheria toxin (4, 84). By conjugating the ViCPS vaccine with a protein antigen, the vaccine can induce a T cell response which can help the B cell response (85). However, since the protein conjugate is not from *Salmonella*, a protective T cell response is not induced by this vaccine. In recent studies the ViCPS-typhoid toxin vaccine (Typbar TCV) provided about 80% protection against typhoid

infection in multiple sites for up to five years (4). With the high protection rate and long term stable protection, TCVs are currently licensed in India and Pakistan as of December 2020 (84). Therefore, TCVs are a promising therapeutic against typhoid. Unfortunately, these vaccines do not protect against iNTS and currently there are no approved vaccines against iNTS on the market.

Killed whole cell parental vaccines

Some of the first vaccines against typhoid were whole cell vaccines either inactivated by acetone or heat in 1896 by Pfeiffer, Kolle and Wright (86). They did show moderate protection but they were discontinued because there were substantial local and systemic side effects in 9-34% of recipients (86). They were later replaced with the current typhoid vaccines which were shown to induce a similar antibody response (83).

Live vaccines

While there is a live vaccine for typhoid, the Ty21 vaccine, there are no live vaccine for iNTS. Live vaccines are generated by attenuating the pathogen and this approach provides a good method of vaccination since these strains induce a robust humoral and cell mediated immune response to multiple pathogen antigens (87). However, live vaccines can be dangerous in immunocompromised individuals who cannot clear an attenuated pathogen, ultimately causing severe illness or death (88). Unfortunately, immunocompromised individuals, young children, and the elderly have the highest risk of iNTS and thus, a live vaccine might not be the best approach to vaccination for these groups. Additionally, a robust cold chain is usually required to keep live vaccines at correct temperatures which can be difficult for resource poor countries and adds a logistical challenge when distributing vaccines to countries where systemic *Salmonella* infection is endemic (89).

A live vaccine against *Salmonella enterica* Typhimurium, WT05, was created and tested in healthy human volunteers. While the protection against iNTS was not determined, this vaccine only induced detectable antibody responses at a very high dose. Additionally, at moderate to high doses, the vaccinated individuals shed bacteria in their feces for up to 23 days (90). This is an issue since long shedding times increases the chance that immunocompromised individuals will be exposed, especially since countries affected by iNTS do not have robust waste treatment facilities (2). A new live vaccine, CVD 1926, was created recently and tested in a gastroenteritis model in primates, but protection against systemic *Salmonella* infection was not examined. This new strain did show low shedding and moderate immune protection and thus, could be a future candidate for an iNTS vaccines (91).

Subunit vaccines

As immunocompromised individuals are at the highest at risk for systemic *Salmonella* infection, creating a subunit vaccine for iNTS is an attractive option and has been studied extensively (92-100). A subunit vaccine is created by selecting one or multiple protective antigens thus generating a more targeted immune response than whole cell vaccines (87). Subunit vaccines usually require pairing with an appropriate adjuvant since these antigens usually do not have intrinsic adjuvant properties (87). Therefore, subunit vaccines need to contain protective antigens and the correct adjuvant to be successful. The ViCPS vaccine for typhoid is a subunit vaccine but it is not protective against iNTS since iNTS causing serovars do not express the Vi capsule (101). As subunit vaccines are designed, it is important to note that *Salmonella* can express different antigens at different time points in vivo, partially controlled by the PhoP/PhoQ pathway (102). For example, in the extracellular phase, *Salmonella* express flagellin, but once

intracellular, the bacterium stops expressing this protein and instead secretes SPI effector proteins which allow the bacterium to establish a niche within the host cell (102, 103). When analyzing the CD4 T cell response to *Salmonella* infection, antigen specific CD4 T cell kinetics closely mirrors the tempo of bacteria protein expression (100). Thus, flagellin specific CD4 T cells are detected early in the response, primarily in the gut associated lymph node tissues and display a mixture of Th1 and Th17 cells (47, 104). However, T3SS specific CD4 T cells are detected later in the response and are primarily found in systemic tissues, when *Salmonella* is intracellular, and this population is mostly comprised of Th1 cells (104). Thus, whether analyzing human or mice for serodominant antigens, it is important to analyze the correct time point to identify the most robust protective antigens.

In recent year there have been many subunit vaccine studies in mice attempting to identify additional protective antigens (92-99). These studies have identified *Salmonella* LPS, *Salmonella* flagellin, or SPI-1 or SP1-2 proteins as possible targets with many combinations of these four different categories of antigens tested. However, none of these subunit vaccines have been tested in humans because most of them have shown only modest protection in mice.

Tissue Resident memory CD4 T cells in *Salmonella* infection and the future of vaccination

With the many failures of preclinical iNTS vaccines, our laboratory and others have attempted to gain a deeper understanding of the host immune response to *Salmonella* with the suspicion that something was missing in these formulations. In recent years a new memory T cell subset has been discovered termed tissue resident memory T cells (TRM) (105). Unlike the other memory T cell subsets, effector memory (TEM) and central memory (TCM), TRM do not circulate throughout the body and instead reside in tissues previously infected by a pathogen. In addition to being present in the tissue at the time of reinfection and therefore, not having to migrate to an

infected tissue like TEM and TCM, these TRM respond very quickly to antigenic peptide and become the front line of defense during secondary challenge (106). Thus, hosts require TRM for complete protection against many different pathogens suggesting that experimental vaccines could be failing due to this memory subset not being appropriately induced (107-110). Indeed, parabiosis experiments demonstrated that the partial protection provided by a subunit SseB vaccine (the protein that forms the needle complex of SP1-2 T3SS) and Flagellin, was provided by circulating memory alone (98). Further parabiosis experiments revealed that TRM are an essential component of host protection provided by LVS vaccination (111). Therefore, previous attempts to generate iNTS subunit vaccines likely failed due to the inability of these vaccines forming TRM.

Undeniably, this essential requirement for TRM in complete protection against systemic *Salmonella* resolves previous paradoxes noted in the iNTS mouse model. While CD4 T cells are required for complete protection against systemic *Salmonella*, as shown through depletion and gene-deficient models, susceptible were not protected by transfer of CD4 T cell memory splenocytes (112). How can CD4 T cells be required for protection but the transfer of splenic CD4 T cells not be protective? Interestingly, mice immunized with LVS showed a drastic decline of IFN- γ + CD4 T cells in the spleen and blood during the effector response at 3 to 5 weeks post infection while CD4 T cells increasingly homed to the liver during this time (113). Additionally, these hepatic IFN- γ + CD4 T cells were sensitive to P2RX7 mediated pyroptosis, a characteristic of TRM (113). Thus, effector CD4 T cells could be trafficking to peripheral tissues and become resident memory which would cause the transfer of splenic CD4 T cells ineffective but still have CD4 T cells required for protection.

Understanding systemic *Salmonella* infection is important to determine where protective CD4 TRM could be located. As stated above, systemic strains of *Salmonella* are fecal-oral pathogens but do not infect via the normal intestinal epithelium but instead becomes systemic by passing through the M cells and associated secondary lymphoid tissues (see “Route of Infection of *Salmonella*”). Thus, there is a very low chance that any intestinal CD4 TRM would be protective in this infection, however; the formation of intestinal CD4 TRM after Ty21 vaccination in humans has been detected (114). However, since there is no way to specifically identify Ty21 specific T cells, general TRM phenotype changes as well as stimulation assays were used. There was an increase in overall CD4 T cells as well as the CD69⁺ CD103⁻ CD4 TRM in the terminal ileum, but these changes were not seen in the lamina propria which is consistent with *Salmonella* preferentially infecting the terminal ileum. However, the contribution of these cells to protection against systemic *Salmonella* is unknown and should be fully interrogated in the iNTS mouse model to determine if the generation of intestinal TRM should be desirable for vaccine design. As stated above (see “Route of Infection of *Salmonella*”) one of the main reservoirs of *Salmonella* is found in liver macrophages and hepatomegaly is a common symptom of iNTS. Thus, the liver is another possible non-lymphoid tissue where protective CD4 TRM could be located. Biologically, having poised memory CD4 TRM to readily provide liver macrophages with IFN γ at the start of infection would be beneficial to the host. Indeed, analysis of LVS immunized mice show that immunization specific CD4 T cells are phenotypically TRM in the liver (111). Additionally, the transfer of liver CD4 TRM alone protected mice from *Salmonella* challenge (111). Thus, designing vaccines that induce CD4 TRM formation in the liver could help increase the efficacy of systemic *Salmonella* vaccines. However, how to induce this memory population during vaccination is unknown at this time. CD8 TRM formation in the liver

has been better studied that CD4 T cells, due to the requirement of these cells in protection against malaria (115). These studies have clearly shown that both inflammation and antigen in the liver increases CD8 TRM formation and a 'prime and trap' method of vaccination can enhance protection provided to the host (107, 116). However, whether CD4 TRM in the liver form following the same rules is still unknown and warrants further investigation given their potential importance to host protection.

Conclusion

Systemic *Salmonella* infection whether typhoid or iNTS causes a large public health burden but currently there are only ineffective vaccines in testing? or no licensed vaccines for these diseases. Understanding how the host naturally protects itself from these infections is crucial to make an effective vaccine. For decades it has been known that Th1 polarized CD4 T cells are required for protection but more recently a new subset of CD4 T cell memory has been discovered termed TRM which, through mouse studies, is required for complete protection against systemic *Salmonella*. Specifically, CD4 TRM in the liver are protective against *Salmonella* challenge. Thus, though many antigens have been tested as subunit vaccine candidates, a likely reason for their failure is their inability for generate CD4 TRM in the liver. A deeper understanding of how to form this memory subset is likely to lead to new effective vaccines for *Salmonella* infections.

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Chapter 2

Protective Tissue Resident Memory CD4 T cells against systemic *Salmonella* and the *in vivo* models to study their formation

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Introduction

Systemic *Salmonella enterica* infections such as typhoid, caused by serovars Typhi and Paratyphi, or invasive non-typhoidal *Salmonellosis* (iNTS), caused by other serovars such as Typhimurium, are a large public health burden resulting in a combined 200,000 deaths per year (1). Currently, the main treatment is antibiotics since vaccines for typhoid are ineffective and no vaccines for iNTS are licensed (2, 3). However, antibiotic resistance is a concern for systemic *Salmonella* infections and an effective vaccine is required (1, 4). How the host naturally protects itself needs to be understood to develop an effective vaccine. CD4 T cells are required for secondary protection against systemic *Salmonella* (5). Historically, CD4 T cell memory has been thought of as two cell subsets: central memory (TCM) and effector memory (TEM) (6, 7). TCM circulate throughout the secondary lymphoid organs (SLOs) and blood and when reactivated by antigen, these cells will produce IL-2 and proliferate. TEM circulate throughout the SLOs, blood and non-lymphoid tissues (NLTs) and when reactivated by antigen, these cells will produce effector cytokines. Since these cells both circulate throughout the body, combined they are termed circulating memory (6, 7). Relatively recently, a new subset of T cell memory has been discovered termed tissue resident memory T cells (TRM) which does not circulate and instead sits in tissues previously infected and becomes the front line of defense during secondary challenges (8, 9).

The liver is the main non-lymphoid tissues (NLT) infected by systemic *Salmonella* and thus, is the location with the highest potential for protective CD4 TRM (10). Indeed, previously, it has been

demonstrated that liver CD4 TRM are required for protection against systemic *Salmonella* through parabiosis and transfer studies (11). In these studies, circulating memory (TCM and TEM) was partially protective but to reach the complete protection provided by LVS vaccination, TRM was required to fully protect these mice. Therefore, inducing this memory subset during vaccination is required for robust protection. However, the mechanisms required for the formation of CD4 TRM in the liver is unknown. Thus, determining the mechanism of CD4 TRM formation in the liver and applying the findings to the vaccine design would allow for intelligent vaccine design for production of an efficacious vaccine against systemic *Salmonella* infection.

Thus, in this chapter we will confirm the protection studies performed previously as well as develop three different methods to form CD4 TRM in the liver: 2W1S+ tetramer+ CD4 TRM after immunization, IFN γ -eYFP reporter CD4 TRM after immunization and the transfer of *in vitro* activated *Salmonella*-specific TCR transgenic CD4 T cells (SM1 T cells) which form SM1 TRM in naïve mice. Using these models, how CD4 TRM form will be investigated in chapters 3 and 4.

Methods

Mouse Strains

C57BL/6 mice were purchased at 7 weeks from Jackson Laboratory. CD45.1 SM1 Rag-deficient mice were made by backcrossing the original strain to CD45.1 Rag-deficient mice from Jack Laboratory (12, 13). IFN γ -eYFP reporter mice were generously provided by Dr. R. Lockley from University of California, San Francisco (14).

Bacterial Culturing and Infection

LVS strain (BMM50) of *Salmonella enterica* serovar Typhimurium was created by introducing a null mutation into the *aroA* gene of SL1344 by the Baumler laboratory (15). BMM51 was created

by introducing a short peptide (EAWGALANWAVDSA) in frame with OmpC to create BMM51 (LVS-2W1S) (16, 17).

Before culturing, *Salmonella* strains were streaked out onto MacConkey agar plates and incubated at 37°C overnight. Single colonies were selected to inoculate Luria-Bertani broth and incubated at 37°C overnight to an OD₆₀₀ of 0.4-0.6. *Salmonella* cultures were then diluted with PBS to 5x10⁵ CFU/0.2mL (BMM50), 2.5x10⁶ CFU/0.2mL (BMM51), or 1x10³ CFU/0.2mL and then injected intravenously by tail vein at 0.2mL per mouse.

Bacterial Burden

Livers and spleens were collected and homogenized and serial dilutions of homogenates were performed. Serial dilutions were then plated on MacConkey agar plates.

Lymphocyte Processing

Spleens were crushed through a 70µm cell strainer and then incubated with ACK lysis buffer (Gibco) to remove red blood cells. Livers were crushed through a 70µm cell strainer and then separated by a 35% Percoll density gradient (GE) at 2000rpm with no brake. ACK lysis was then used to remove red blood cells.

Flow Cytometry

1x10⁶ cells were stained with zombie yellow (BioLegend) for 10 minutes at room temperature and then stained with PE conjugated 2W1S:I-A^b MHCII tetramer at 1:100 dilution for 1 hour. The cells were then stained for surface markers with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1, CD4, CD44, CD45.1, CD90.2, P2RX7, CD69, CD62L, CD103, KLRG1, CD11a, CXCR3, CD101. Then analyzed by BD flow cytometers and Flow Jo Software (TreeStar).

Lymphocyte Transfer

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

S+16a treatment

For flow cytometry analysis of hepatic T cell expression of P2RX7 or for transfer experiments, mice were given 50µg of s+16a nanobody (Biolegend) diluted in 200µL of PBS 30 minutes before organ harvest (18).

In vitro activated SM1 T cell 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 isolated with MACS CD4 T cell selection kit (Miltenyi) following the kit protocol. These cells were then cultured with 5µg/mL flagellin peptide (12), 5µg/mL LPS (Enzo), 10U/mL human IL-2 (Peprotech) and irradiated splenocytes from C57BL/6 mice in complete RPMI media. 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 x 10⁶ cells/0.2mL. Mice were injected with 0.2mL of cell suspension intravenously.

Statistics

Statistical analysis was performed by using paired or unpaired student 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/>)

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 days later (11) (Fig 1A). Mice clear *Salmonella* at day 30 and thus, at this early memory time point, more than 50% of tetramer⁺ CD4 T cells in the liver expressed CD69 (Fig. 1A), a surface marker expressed by many resident memory T cells (13). These *Salmonella*-specific CD69⁺ memory CD4 T cells expressed higher surface levels of P2RX7, CXCR3, CD11a and CD101, compared to CD69^{low} memory CD4 T cells (Fig. 1B). Additionally, CD69⁺ memory CD4 T cells expressed low levels of KLRG1 and similar levels of CD103 (Fig. 1B), consistent with a TRM phenotype.

Since these tetramer⁺ memory CD4 T cells respond to a single epitope expressed by *Salmonella*, it was of interest to examine the wider polyclonal endogenous *Salmonella*-specific memory CD4 T cell population within the same tissue. To visualize responding Th1 cells, we utilized a reporter mouse strain where IFN γ transcriptional activity causes expression of eYFP (14). In naïve mice, the vast majority of hepatic CD4 T cells expressed low levels of CD69 and lacked IFN- γ transcriptional activity (Fig. 1C). However, there was a small population of CD4 T cells which expressed YFP in mice and this is probably due to contamination of NKT cells in the gate and is an avoidable issue in the system (Fig 1C). In marked contrast, in mice immunized with LVS *Salmonella* 45 days previously, over 80% of hepatic CD4 T cells expressed the IFN- γ eYFP reporter (Fig. 1C), consistent with the strong Th1 polarization that occurs in response to *Salmonella* (13, 17, 19). The majority of IFN- γ ⁺ hepatic memory CD4 T cells also expressed increased levels of surface CD69 (Fig. 1C). Similar to tetramer⁺ CD4 T cells, eYFP⁺ CD69⁺

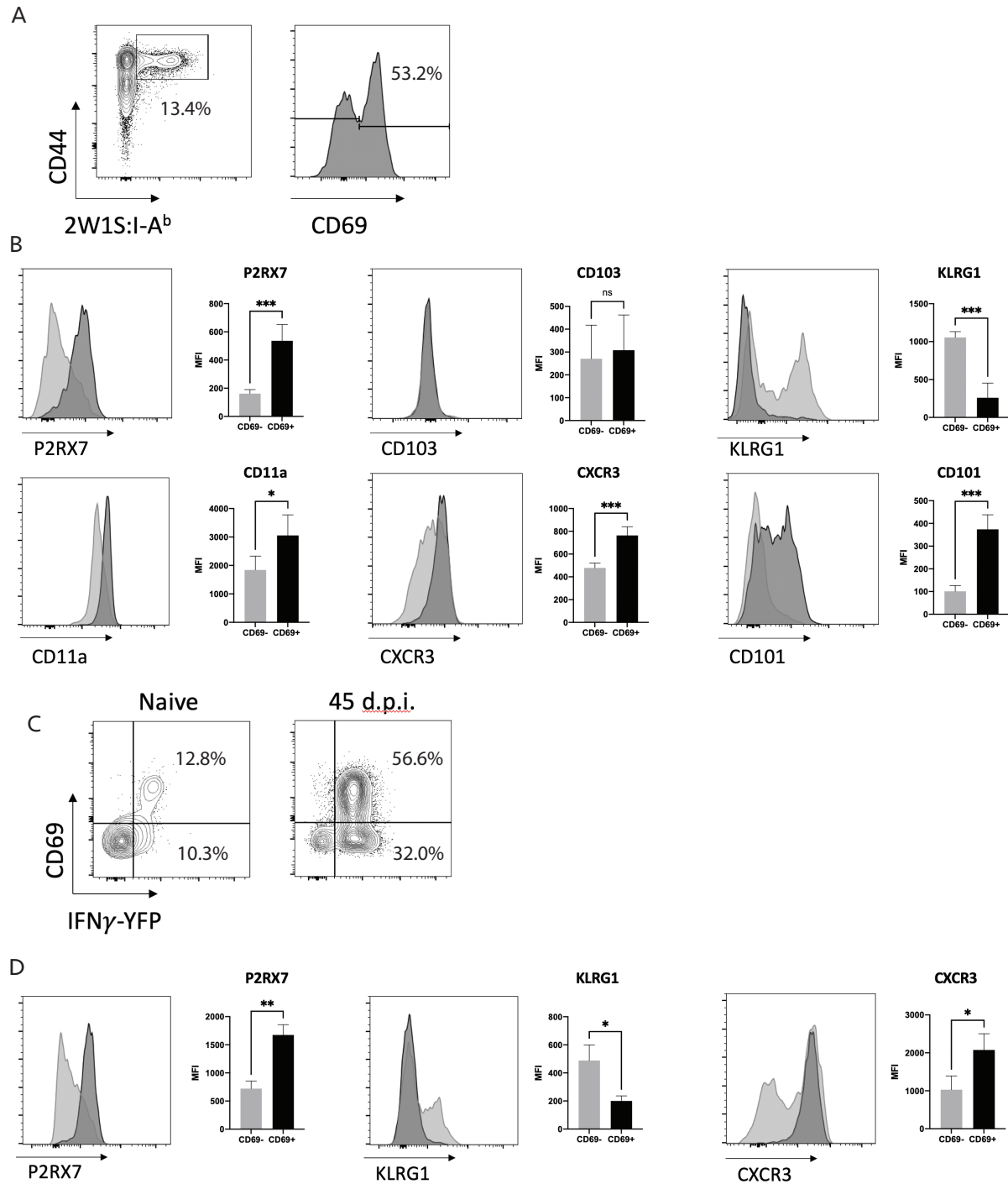


Figure 1. Systemic *Salmonella enterica* Typhimurium immunization forms CD4 TRM in the liver. (A-B) C57BL/6 mice were immunized with *Salmonella*-2W1S-LVS intravenously and 45 days later livers were collected and analyzed by flow cytometry. A) Immunization specific CD4 T cells were identified by 2W1S-tetramer staining and TRM were identified by CD69⁺ tetramer⁺ CD4 T cells. B) Expression of P2RX7, CD103, KLRG1, CD11a, CXCR3, and CD101 were determined for CD69⁺ and CD69⁻ tetramer⁺ cells by flow cytometry. (C-D) IFN γ -YFP reporter mice were immunized with *Salmonella*-LVS intravenously and 45 days later livers were collected and analyzed by flow cytometry. C) Immunization specific CD4 T cells were identified by YFP⁺ CD4 T cells and TRM were identified by CD69⁺ YFP⁺ CD4 T cells. D) Expression of P2RX7, KLRG1, and CXCR3 were determined for CD69⁺ YFP⁺ and CD69⁻ YFP⁺ CD4 T cells.

hepatic CD4 T cells expressed higher expression of P2RX7 and CXCR3, and low levels of KLRG1, compared to eYFP+ CD69- hepatic CD4 T cells (Fig. 1D). Thus, using two different experimental approaches to identify *Salmonella*-specific memory cells, LVS-*Salmonella* induced formation of CD4 TRM in the liver.

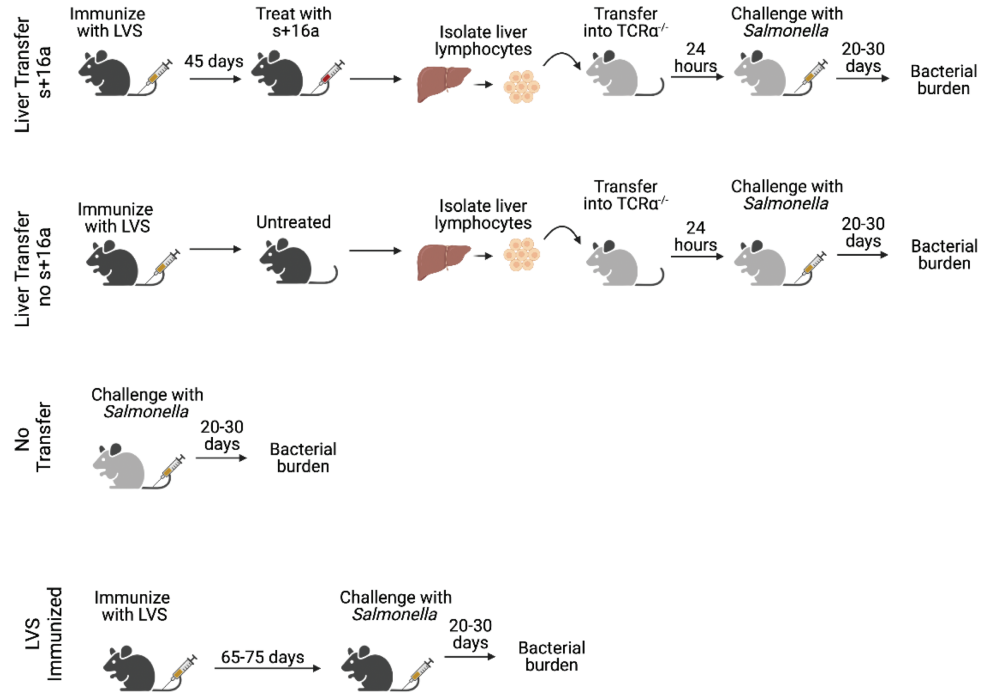
Transfer of Salmonella-specific TRM protects against Salmonella infection

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 days 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 TCR α -deficient recipients and challenged 24 hours later (Fig. 2A). This s+16a treatment prevents death of cells expressing high levels of P2RX7 during tissue processing (17, 18), 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. 2B, No transfer versus no s+16a). In contrast, mice that received a liver lymphocyte transfer from mice treated with s+16a had lower much bacterial burdens (Fig. 2B, s+16a versus no s+16a). Indeed, this protective effect was similar to LVS immunized controls (Fig. 2B, s+16a versus LVS immunized) confirming that s+16a sensitive lymphocytes are required for complete protection against *Salmonella* (17, 20).

In vitro activation of Salmonella specific TCR transgenic CD4 T cells form liver CD4 TRM

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 CD45.1 *Salmonella*-specific TCR transgenic CD4 T cells (SM1 T cells) were activated in vitro for five days before

A



B

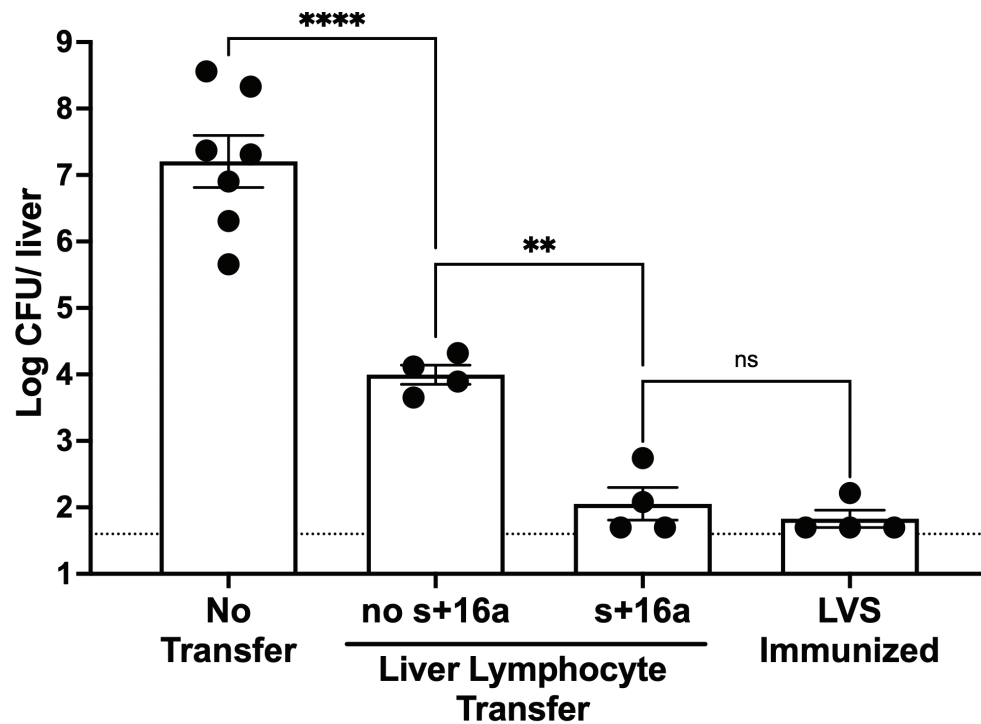


Figure 2. Hepatic CD4 TRM are required for complete protection against *Salmonella enterica* Typhimurium. (A, B) C57BL/6 mice were immunized with *Salmonella*-LVS intravenously. 45 days post infection livers were collected and liver lymphocytes were isolated by percoll gradient which were transferred intravenously into $TCR\alpha^{-/-}$ mice. A group of $TCR\alpha^{-/-}$ mice received lymphocytes from mice treated with nanobody s+16a 15 minutes before sacrifice. 24 hours after transfer, recipient mice were challenged intravenously with *Salmonella*. At 20-30 days post challenge livers were collected and bacterial burdens were calculated to determine protection.

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. 3A).

CD45.1⁺ SM1 T cells expressed low levels of CD62L and a subset expressed surface CD69 and P2RX7 (Fig. 3B). CD69⁺ SM1 T cells also expressed high levels of CXCR3, low KLRG1 and similarly low levels of CD103, compared to CD69⁻ SM1 T cells (Fig. 3C). This adoptive transfer system therefore provides a simple model to visualize establishment of *Salmonella*-specific CD4 TRM in the liver.

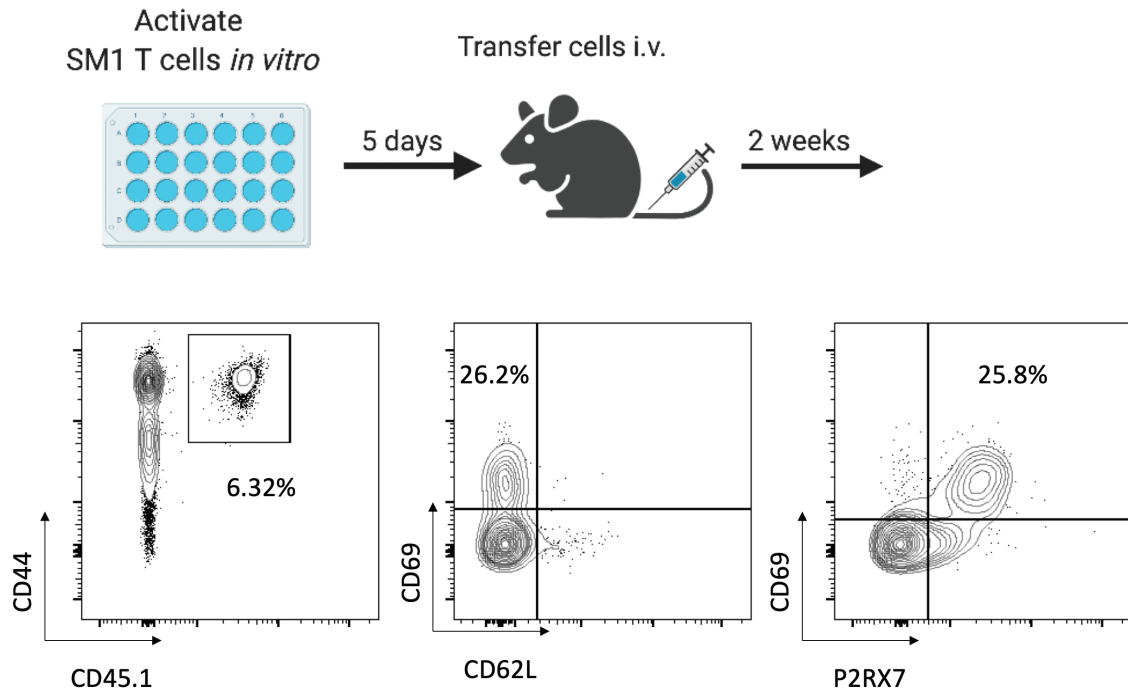
Discussion

As previously demonstrated, mice immunized with *Salmonella*-LVS form immunization specific CD4 TRM in the liver and these phenotypically resemble previously characterized TRM (11). These immunization specific CD4 TRM can be visualized by two different methods: tetramer, which is able to visualize immunization specific CD4 T cells to one epitope and IFN γ -eYFP, which is able to visualize immunization specific CD4 T cells that are reactive to multiple *Salmonella* epitopes. By using both methods, we are able to confirm what is observed is not an artifact of one system.

In addition, we confirmed that the transfer of s+16a sensitive liver TRM fully protects mice from *Salmonella* challenge when compared to LVS immunized mice. Thus, understanding how this memory population is formed is crucial to vaccine design.

A novel approach described in this chapter is the transfer of *in vitro* activated SM1 T cells as a reductionist approach to TRM formation. Though this method has been described with CD8 T cells, the ability of activated CD4 T cells to form TRM in the liver of naïve mice has not been reported (21). This method allows the study of liver CD4 TRM formation without the presence of liver inflammation or cognate antigen which would be present during infection. Thus, these factors can

A



B

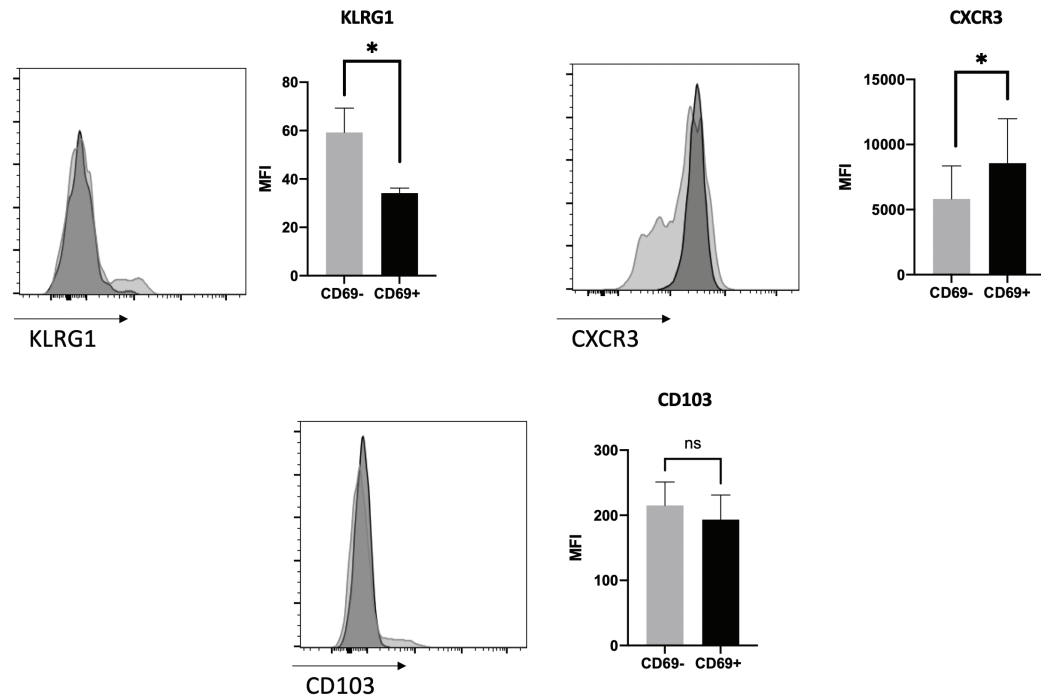


Figure 3. Adoptive transfer of *in vitro* activated SM1 T cells form resident memory in the liver of mice. (A-C) Naïve SM1 T cells were isolated and cultured *in vitro* with rhIL-2, LPS, rFlic, and irradiated splenocytes for 5 days and then transferred intravenously to naïve C57BL/6 mice. 2 weeks later livers were collected and analyzed by flow cytometry. B) SM1 T cells were identified by their congenic marker CD45.1 and a population of SM1 T cells were positive for CD69 and P2RX7 and negative for CD62L. C) Further analysis of CD69+ and CD69- SM1 T cells by staining for KLRG1, CXCR3 and CD103.

be added in individually or together or other factors can be added or removed and the impact of these variables on TRM formation can be determined.

Therefore, in this chapter we have shown that liver CD4 TRM mediate protection against systemic *Salmonella* and for complete protection against the pathogens circulating memory and resident memory are required. Additionally, we have provided three different methods to form and identify liver CD4 TRM which will allow us to investigate how to induce this subset during vaccination.

Limitations:

There are many limitations in this study which since we use these models in the further chapters, extend to these chapters as well. Firstly, using tetramer to identify immunization specific CD4 T cells can cause artifacts since T cells responding to a single epitope are analyzed and not a polyclonal population. We tried to mitigate this issue by using the IFN γ reporter mice whenever possible. However, even when gating on a NK1.1 negative population, there is an NKT contamination in our analysis seen by the small population of YFP+ CD69+ CD4 T cells in naïve mice which is inevitable and has been seen with other studies using these mice to study liver CD4 TRM (11). Since both systems have limitations, they are used together in our studies as confirmation and hopefully, mitigate these limitations seen.

Secondly, the transfer system to determine the requirement of liver TRM for protection against systemic *Salmonella* infection has many limitations. The best experiment to determine protection of CD4 TRM is to transfer this purified by sorting population into C57BL/6 mice. However, our group has tried over main years to get a large population of CD4 TRM by sorting and have been unsuccessful. Indeed, three mice had to be group together to get 300,000 CD4 TRM for RNA sequencing. Since immunized mice usually have around 500,000 CD4 TRM in the liver and when transferring there is usually a 10% ‘take’, the number of mice to perform this experiment would

not be worth animal suffering and knowledge gained. Thus, the transfer of total liver lymphocytes and relying on the inability of TRM to survive without s+16a was used. Additionally, using TCR $\alpha^{-/-}$ is also a limitation as transferring lymphocytes to lymphopenic mice cause proliferation of the transferred lymphocytes. This could have impacted the population transferred but we did not analyze this during our experiments. However, this variable was partially corrected for since the goal of the study is to study the protection provided by s+16a treated vs non-treated transfers and both of these transfers are being transferred into the same mice.

Lastly, the transfer of *in vitro* activated SM1 T cells in naïve mice is a highly artificial mouse model to study CD4 TRM but that is also the benefit of the model and anything discovered using this model should be confirmed in a more translational mouse model.

Therefore, even though there are many limitations in the mouse models used in these studies, we believe that they are the best available and that the biases are corrected for as much as possible.

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Chapter 3

Optimal formation of Hepatic Tissue Resident Memory CD4 T cells Requires T-bet

Regulation of LFA-1

Claire E. Depew, Marissa C. Franke, Alana T. Nguyen, Jessica Calderon, Roger Sciammas, and
Stephen J. McSorley

Introduction

Pathogen-specific naïve CD4 T cells are activated to proliferate by cognate antigen and subsequently gain relevant effector functions to limit microbial growth (1). This expanded pool eventually contracts to leave a small persisting population of antigen-experienced memory cells (2). At least three types of CD4 T cell memory populations have been defined - central, effector, and tissue resident subsets. Central memory T cells (TCM) circulate through secondary lymphoid organs (SLOs) and blood and when they re-encounter antigen will secrete IL-2 and engage in proliferation. Effector memory T cells (TEM) also circulate through the SLOs and blood, but additionally enter non-lymphoid tissues (NLTs) where they can immediately secrete effector cytokines upon secondary encounter with antigen. Since both of these populations circulate in blood, they comprise a rapidly deployable circulating pool of memory T cells (3, 4). In contrast, the third CD4 T cell memory population does not circulate in blood but resides in non-lymphoid tissues (NLTs) and is therefore termed tissue resident memory (TRM) T cells (3, 5). These TRM cells provide a stable front line of defense for subsequent infection since they do not have to be recruited to the infected tissue and can rapidly secrete effector cytokines (6). Since these CD4 TRM are crucial for protection against a wide range of pathogens, it is important to understand how to induce these cells during vaccination (7-12). While a mechanistic understanding of CD8 TRM genesis is rapidly developing, the processes involved in CD4 TRM formation are less well

known. Expression of a common transcriptional core appears to be crucial for the formation of CD8 TRM across species, but TRM that reside in different tissues also have varying transcriptional profiles that suggest they adapt to the microenvironment (13, 14). Along with transcription factors like Blimp1, Hobit, and Klf2, expression of T-bet, a member of the T-box transcription factors, is important during CD8 TRM formation in the skin (15, 16). Suppression of T-bet is essential to allow optimal TGF β signaling which allows CD103 expression and downregulation of S1P receptors that allow migration out of the skin (16, 17). Despite this requirement for T-bet downregulation, a low level of T-bet is still required for these cells to express IL-15R β which is essential to memory CD8 TRM survival (16). Conversely, there is a lesser role for T-bet in the development of liver CD8 TRM since T-bet overexpression does not impact hepatic CD8 TRM formation (18). Thus, the requirement for T-bet expression in CD8 TRM formation is tissue specific while the role of T-bet during hepatic CD4 TRM formation has not been fully investigated.

In this study, we examined the generation of *Salmonella*-specific CD4 TRMs using a simple infection model and tools that allow identification of peptide-specific TRM T cells in the liver. Hepatic CD4 TRM formed after resolution of primary *Salmonella* infection and expressed higher T-bet levels than circulating memory CD4 T cells. T cell-intrinsic T-bet expression was essential for optimal CD4 TRM formation in the liver and retroviral overexpression of T-bet provided a competitive advantage for cells entering the hepatic TRM pool. Increased surface expression of CD11a and CD18 correlated with T-bet expression and was T-bet-dependent, suggesting a mechanism for competitive liver residence. Indeed, T-bet-dependent liver residence was significantly inhibited by blocking LFA-1 in vivo, providing a simple checkpoint in the formation of protective *Salmonella*-specific TRMs.

Methods

Mouse strains

SM1 Rag-deficient mice expressing CD45.1 were created by back crossing SM1 C57BL/6 mice to B6.SJL-PtprcaPep3b/BoyJ mouse strain (The Jackson Laboratory, Bar Harbor, ME) (19, 20). C.129S4(B6)-*Ifng*^{tm3.1Lky}/J (IFN γ -eYFP reporter mice) were generously gifted by Dr. R. Lockley, University of California, San Francisco (21). C57BL/6, B6.SJL-*Ptprca*^a *Pepc*^b/BoyJ, B6.PL-*Thy1*^a/CyJ, and B6.129S6-*Tbx21*^{tm1Glm}/J were purchased from The Jackson Laboratory and used at 8-16 weeks of age. All mice were housed under specific pathogen-free conditions and cared for in accordance with IACUC policy.

Bacterial culture and infections

A live vaccine strain (LVS) of *S. enterica* serovar Typhimurium (BMM50) was previously generated by Dr. A. Baumler (University of California, Davis) by null mutation of the *aroA* gene of SL1344. This BMM50 strain was subsequently modified to express a short peptide sequence (2W1S) (EAWGALANWAVDSA) in frame with OmpC (BMM51). Before each use, bacteria were streaked onto MacConkey agar and individual colonies used to inoculate Luria-Bertani broth. After static overnight growth at 37°C (OD₆₀₀ of 0.4-0.6), cultures were washed and diluted in PBS to 5 x 10⁵ CFU/0.2mL (BMM50), 2.5 x 10⁶ CFU/0.2mL (BMM51) or 1 x 10³ CFU/0.2mL (SL1344) and 0.2mL injected via the tail vein of C57BL/6 mice. Dose estimates were confirmed by plating serial dilutions onto MacConkey agar plates and enumerating colonies 24 hours later. Bacterial burdens were evaluated by euthanizing mice by cervical dislocation and harvesting liver and spleen in 4mL and 2mL PBS, respectively. Liver and spleens were homogenized and the final volume was recorded for later calculations. Serial dilutions of each homogenate were plated on MacConkey agar plates and incubated overnight at 37 °C. Bacterial burden of the entire organ was calculated by back calculating from the serial dilution that provided clear countable colonies.

Mixed bone marrow chimeras

CD90.2⁺ CD45.1⁺ mice were irradiated with a single dose of 800 cGy. Sixteen hours later, mice were reconstituted by injecting 1:1 mix of bone marrow from CD90.1⁺ CD45.2⁺ WT and CD90.2⁺ CD45.2⁺ T-bet-deficient mice intravenously. After reconstitution, mice were maintained on Sulfatrim (Sulfamethoxazole and Trimethoprim Oral Suspension) for at least 4 weeks. Mice were bled by cheek bleed at 6 weeks to confirm successful chimerism by flow cytometry examining CD90 and CD45 congenic markers. Mice were rested for at least 2 weeks after withdrawal of antibiotics before use in infection experiments.

Lymphocyte isolation

Spleens single cell suspensions were generated by mechanical dissociation of tissue through 70µm mesh cell strainers (Corning) and then washed thoroughly. Red blood cells were lysed by incubation with 2mL ACK lysis buffer (Gibco) for 5 minutes which was halted by adding 5mL of 2% FBS in PBS. Remaining cells were spun down and supernatant removed before counting. Livers were turned into single cell suspensions by pressing the tissue through 70µm mesh cell strainer. Cells were spun down at 2000rpm for 15 minutes and supernatant removed. Cells were resuspended in 15mL of 35% Percoll solution (GE) and spun again at 2000rpm for 20 minutes without brake. Hepatocytes were removed and remaining cells washed with 2% FBS and PBS. Red blood cells were also lysed with ACK lysis buffer and cells counted. Single cell suspensions of lymph nodes were generated using frosted glass slides to gently disrupt the tissue. Large clumps were removed by straining through a 70µm mesh cell strainer.

Staining and Flow Cytometry

Single cell suspensions were counted and 1×10^6 cells/tube were incubated with Fc block (24G2 supernatant and 2% mouse serum and 2% rat serum). Cells were incubated with zombie yellow viability dye (Biolegend) for 15 minutes, washed with 2% FBS in PBS, and then incubated with PE conjugated 2W1S::I-A^b MHCII tetramer for 1 hour. Cells were then washed with 2% FBS and PBS and stained with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1 (Thermofisher), CD4 (BV786, BD Biosciences), CD44 (BD Biosciences), CD90.1 (Thermofisher), CD45.1 (Thermofisher), CD90.2 (Thermofisher), CD45.2 (Thermofisher), P2RX7 (Biolegend), CD69 (Thermofisher), CD62L (BD Biosciences), KLRG1 (BD Biosciences), CD11a (Thermofisher), CD18 (BioLegend), CD49a (BioLegend). When staining for surface P2RX7 on liver CD4 T cells, all mice were given 50µg s+16a nanobody (BioLegend) intravenously 15-30 minutes before sacrifice. After staining, cells were incubated with 2% paraformaldehyde (Thermofisher) in PBS for 15 minutes before being washed again and resuspended in 2% FBS in PBS. For intracellular staining, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer set (eBioscience) following the manufacturer's protocol. Briefly, cells were incubated for 30 minutes in fixation/permeabilization buffer and then washed twice with the permeabilization buffer. Cells were stained with T-bet (Thermofisher) in permeabilization buffer overnight. The next day the cells were washed with permeabilization buffer and resuspended in 2% FBS in PBS. All samples were analyzed by flow cytometry using BD LSR Fortessa or BD FACSymphony (BD Biosciences) and data was analyzed by FlowJo software (TreeStar).

In vitro activation of SMI T cells and adoptive transfer

SM1 mice were euthanized and inguinal, iliac, mesenteric, axillary and brachial lymph nodes and spleens harvested. Single cell suspensions were generated and CD4 T cells isolated using MACS CD4 T cell selection kit (Miltenyi) following the manufacturers protocol. Briefly, CD4 T cells were incubated with negative selection biotinylated cocktail for 10 minutes and then incubated with anti-biotin beads for 10 minutes. Cells were then passed through a pre-washed magnetic column and washed. The CD4 T cell enriched population was found in the flow through portion of the column and counted. A CD4 T cell enriched SM1 T cell population was cultured with 5µg/mL flagellin peptide, 5µg/mL LPS (Enzo), 10U/mL human IL-2 (Peprotech) and irradiated C57BL/6 splenocytes in complete RPMI media. Cells were fed with human IL-2 in complete RPMI media at day 3 of culture. SM1 cells were harvested at day 5 of culture and washed twice with PBS and adjusted to 5×10^6 cells/0.2mL. Mice were injected with 0.2mL of cell suspension intravenously.

Retrovirus transduction

Retrovirus were produced by transfecting Plat-E cells (Cell Biolabs) with pMSCV-IRES vectors. Control-GFP was generously gifted by Susan Kaech (Salk Institute, La Jolla), T-bet-GFP was generously gifted by Laura Mackay (University of Melbourne, Melbourne, Australia), Control-mAmetrine was generously provided by Dario Vignali through Addgene (#52113). Briefly, Plat-E cells were seeded at 5×10^6 cells in 100mm dishes 24 hours before transfection with 14µg of pMSCV-IRES vectors, 2µg gag plasmid, 2µg envelope plasmid and lipofectamine 2000 (Thermofisher). Viral supernatants were harvested 48 hours and 72 hours after transfection, pooled, and filtered with 0.45µm filters (Millipore). Day 3 *in vitro* activated SM1 T cells were resuspended in viral supernatant and “spininfected” at 2500rpm at 32°C for 90 minutes with 33 mM

HEPES (Gibco) and 0.5µg/mL of polybrene (Sigma). Washed SM1 T cells were further cultured for 48 hours. Transduction efficiency averaged 50% as measured by fluorescent protein expression on a flow cytometer. 5×10^6 SM1 T cells were transferred intravenously into individual cohorts of mice in non-competition experiments. 5×10^6 SM1 T cells transduced with either T-bet-GFP or Control-mAmetrine for a total of 10×10^6 SM1 T cells were co-transferred intravenously into the same cohorts of mice for competition experiments.

CD18 antibody inhibition

24 hours before SM1 T cell transfer, mice were injected intraperitoneally with 200µg anti-CD18 (BioXcell) resuspended in PBS. Mice were treated twice weekly until being euthanized for tissue collection. Control mice were also treated with 200µg Rat IgG2a isotype antibody at exactly the same time points.

Statistics and data figures

Statistical analysis was performed by using paired or unpaired student t-test, or one-way ANOVA as stated (Prism; GraphPad Software, Inc.). Significance is displayed as, $p = * \geq 0.05$, $** \geq 0.01$, $*** \geq 0.001$, $**** \geq 0.0001$. Graphical figures were made using Biorender (<https://biorender.com/>).

Results

T-bet is required for the formation of liver CD4 TRM

To determine if T-bet was differentially expressed between TRM and circulating memory, spleen and livers of mice that were immunized with *Salmonella*-LVS-2W1S were analyzed at an early memory time point. CD69⁺ and CD69⁻ tetramer⁺ CD4 T cells in the liver upregulated T-bet compared to splenic tetramer⁺ CD4 T cells; additionally, CD69⁺ tetramer⁺ CD4 T cells in the

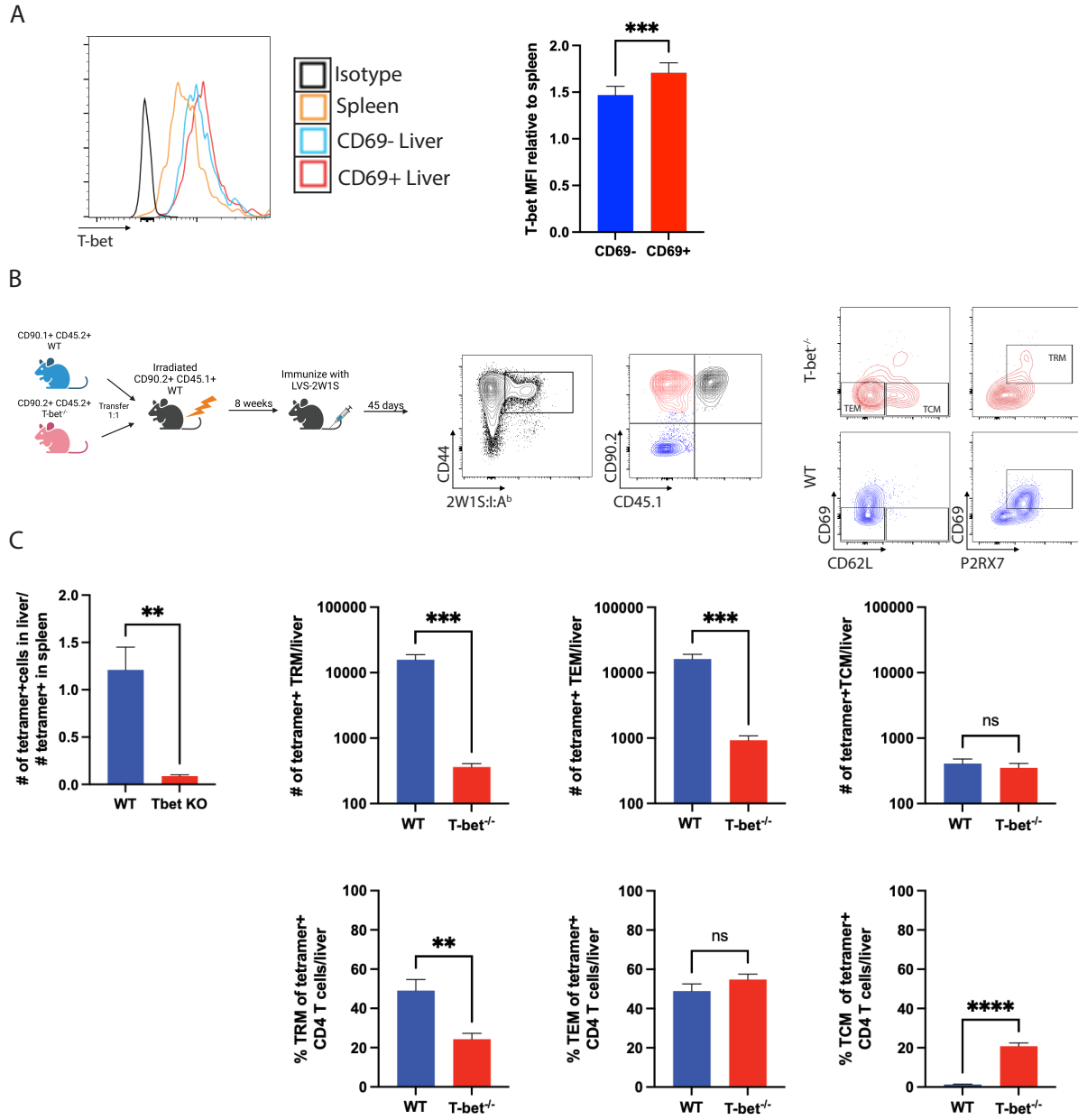
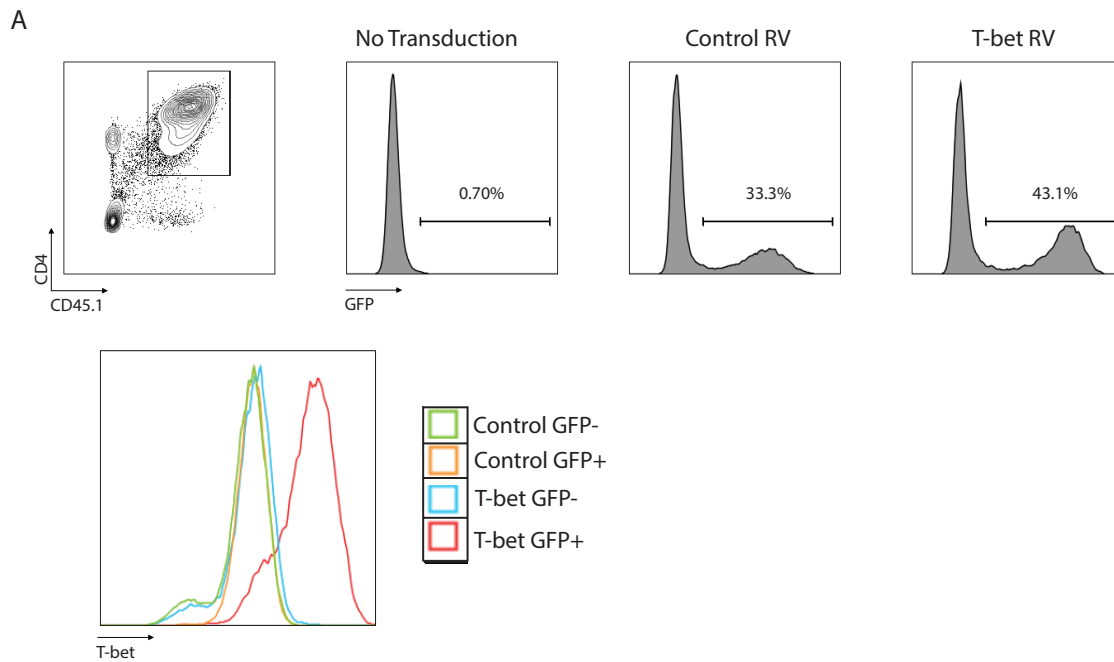


Figure 1. T-bet is required for the formation of CD4 TRM in the liver
 (A) 45 days post immunization, 2W1S⁺ tetramer CD4 T cells were stained for T-bet expression. (B, C). Mixed bone marrow chimeras were made by transferring a 1:1 ratio of wildtype and T-bet^{-/-} bone marrow into irradiated host. 8 weeks after transfer the mice were infected with LVS-2W1S. 45 days after immunization, immunization specific TRM were defined as 2W1S⁺ CD69⁺ P2RX7⁺, TEM were defined as 2W1S⁺ CD69⁻ CD62L⁻, and TCM were defined as 2W1S⁺ CD69⁻ CD62L⁺. Data representative of two experiments with 9 mice in each group.

liver expressed more T-bet than CD69⁻ tetramer⁺ CD4 T cells. Thus, CD4 TRM in the liver do express more T-bet than circulating memory CD4 T cells in either the spleen or liver (Figure 1A). To determine if T-bet expression was required for the formation of liver CD4 TRM, mixed bone marrow chimeras were generated using a 1:1 ratio of wildtype (CD90.1⁺/CD45.2⁺) and T-bet-deficient (CD90.2⁺/CD45.2⁺) bone marrow and irradiated wildtype (CD90.2⁺/CD45.1⁺) recipients. Chimeras were immunized with LVS-2W1S and 45 days later, livers and spleens collected to assess TRM formation by flow cytometry (Fig. 1B). To determine if the absence of T-bet impacts the CD4 T cell memory formation specifically in the liver, the ratio of the absolute number of liver tetramer⁺ CD4 T cells to that of splenic tetramer⁺ CD4 T cells was calculated. This calculation corrects for the possibility that T-bet impacts overall memory formation rather than liver TRM formation. In contrast to the even ratio of 1:1 for wildtype CD4 T cells, there was a significant reduction in the ratio for T-bet-deficient CD4 T cells. (Fig. 1C). This demonstrates that T-bet expression represents a critical checkpoint for CD4 T cells to either home to, or survive in the liver compared to the spleen. Furthermore, considering the mixed chimera experimental approach, the requirement for T-bet activity is cell intrinsic. As might be expected, the overall number of tetramer⁺ TRM was markedly reduced among T-bet-deficient CD4 T cells compared with wildtype CD4 T cells. To determine if other memory populations were impacted, T effector memory (TEM) and T central memory (TCM) formation by T-bet-deficient and wildtype CD4 T cells was also examined. CD4 TEM were defined by a CD44⁺ CD62L⁻ CD69⁻ phenotype, while CD4 TCM were defined by a CD44⁺ CD62L⁺ CD69⁻ phenotype. The number of tetramer⁺ T-bet-deficient TEM was reduced compared to wildtype TEM, while the number of tetramer⁺ T-bet-deficient TCM was not significantly different from wildtype (Fig. 1C). The frequency of each memory population formed among tetramer⁺ T-bet-deficient and wildtype CD4 T cells was also calculated. The

frequency of T-bet-deficient CD4 T cells that became TRM was significantly reduced compared to wildtype CD4 T cells (Fig. 1C) In summary, T-bet activity is required for efficient generation of liver TRM.



Supplemental Figure 1. T-bet-GFP retrovirus vector increases T-bet expression of SM1 T cells

(A) SM1 T cells were activated *in vitro* for five days and at day three of the culture, SM1 T cells were transduced with T-bet-GFP RV or control-GFP RV. At day five of the culture, SM1 T cells were sorted based on GFP expression and then stained for T-bet. T-bet expression was analyzed by flow cytometry.

Ectopic T-bet expression favors liver CD4 TRM formation during competition

Next, we wanted to determine whether increasing T-bet expression could enhance the formation of liver TRMs. To examine this possibility, we used a model of *in vitro* activation and subsequent *in vivo* parking of *Salmonella*-specific SM1 TCR transgenic CD4 T cells. SM1 T cells were activated *in vitro* for five days and then intravenously transferred to naïve C57BL/6 mice in the absence of cognate antigen or inflammation. Two weeks after transfer, a population of SM1 T cells expressing CD69 and P2RX7 and lacking CD62L was detected in the liver (Fig. 2A). Similar to vaccination data above, memory SM1 T cells in the liver expressed higher levels of T-bet than counterparts in the spleen and, more specifically, liver CD69⁺ SM1 T cells expressed more T-bet than liver CD69⁻ SM1 T cells (Fig. 2B). To determine if ectopic T-bet expression drives TRM

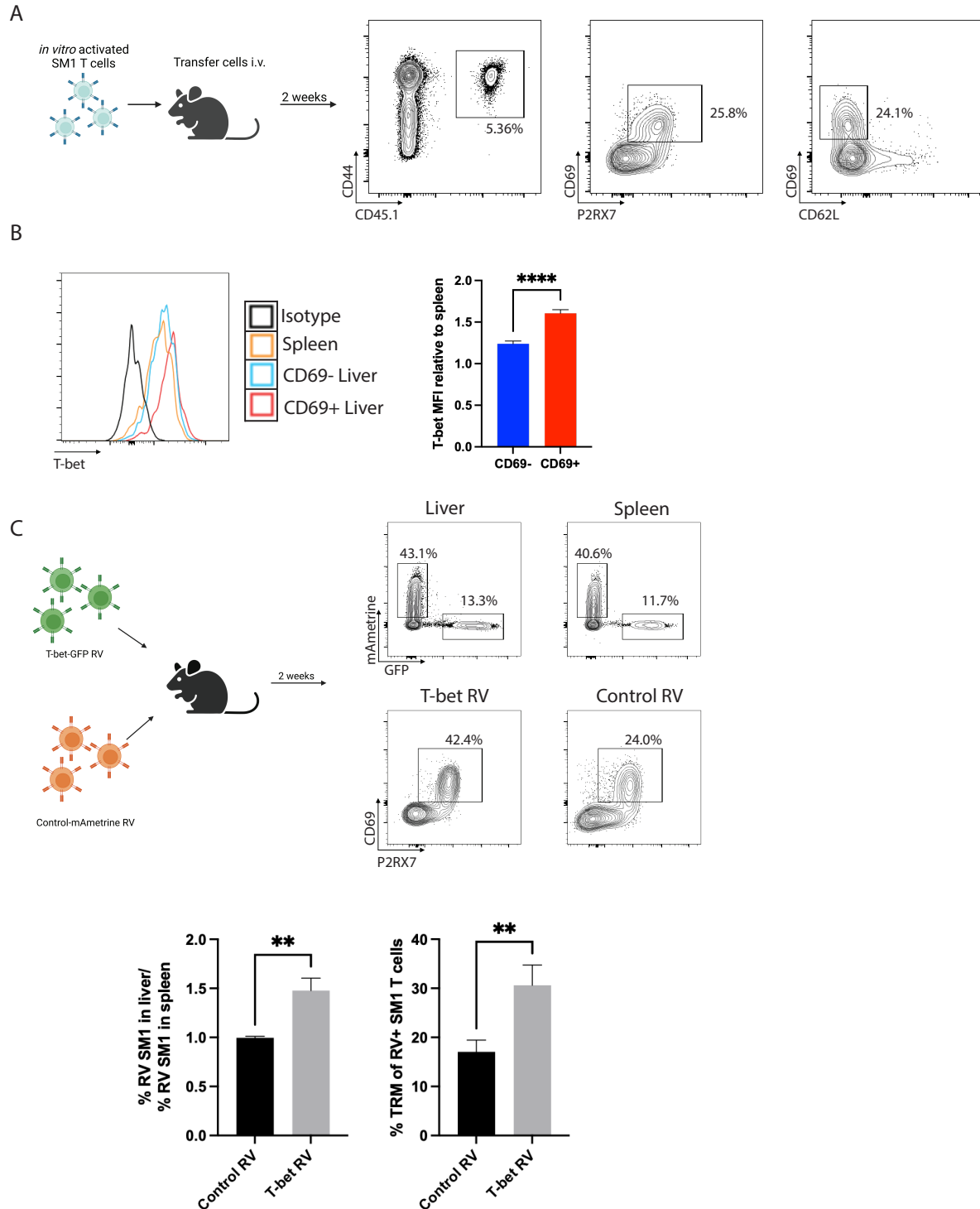
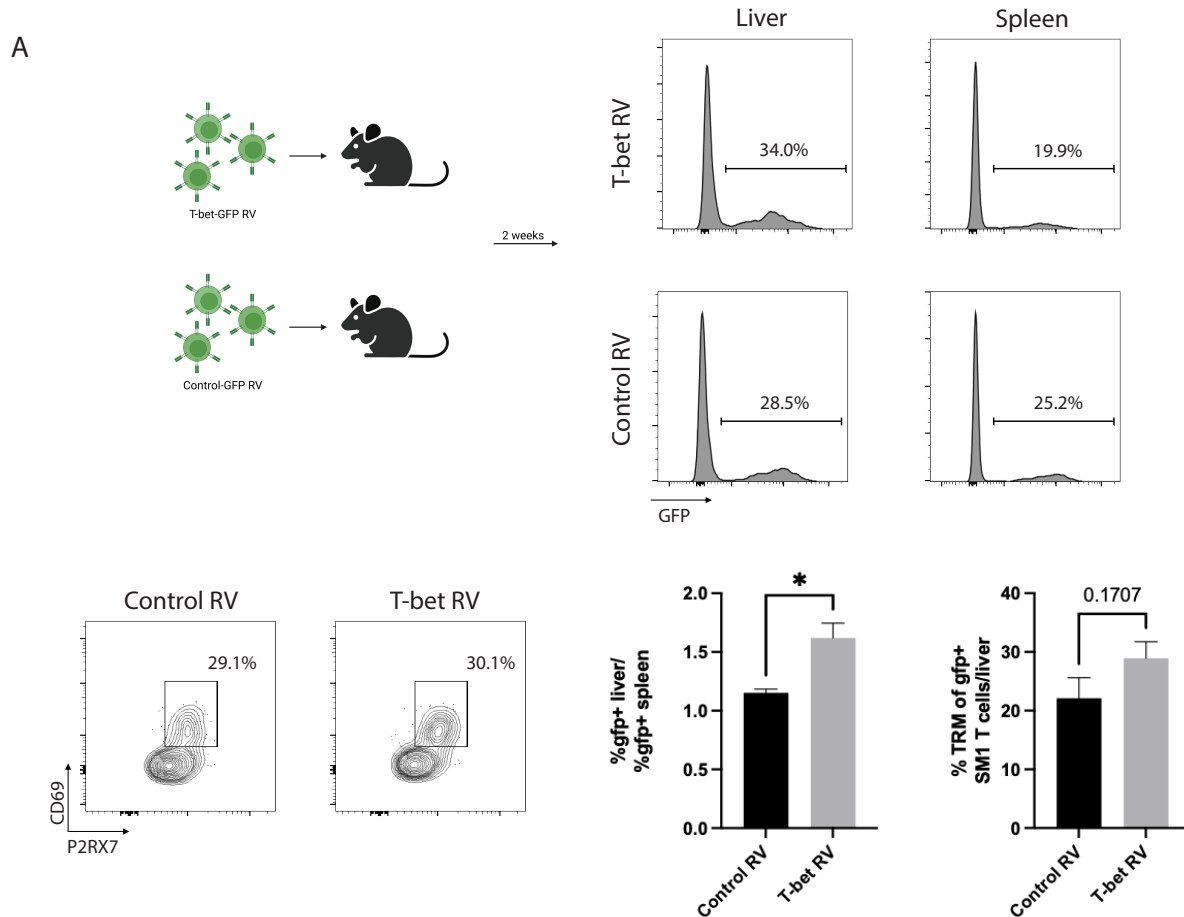


Figure 2. T-bet overexpression increases CD4 TRM formation in the liver
 (A-C) SM1 T cells were activated *in vitro* for five days and then transferred into naïve recipients and analyzed by flow cytometry at two weeks post transfer. (A) Liver SM1 T cells were identified by CD45.1⁺ CD4 T cells and SM1 TRM were defined as CD45.1⁺ CD69⁺ P2RX7⁺ CD62L⁻. (B) T-bet expression of SM1 T cells in livers and spleens was analyzed. (C) SM1 T cells were transduced with T-bet-GFP RV or control-mAmetrine RV during activation. Data representative of two experiments with 6 mice per group.

formation in the liver, activated SM1 T cells were transduced with T-bet-IRES-GFP or control-IRES-mAmetrine retroviral vectors (Fig. S1). T-bet or control transduced SM1 T cells were adoptively transferred into the same recipient and spleen and liver assessed two week later. T-bet transduction increased the overall frequency of SM1 T cells in the liver versus the spleen compared to the control vector (Fig. 2C). Furthermore, T-bet transduced SM1 T cells generated TRMs at a



Supplemental Figure 2. T-bet increases CD4 T cell preference of the liver but not TRM formation when not in competition

(A) SM1 T cells were activated *in vitro* for five days. SM1 T cells were transduced with T-bet-GFP RV or control-GFP RV during activation and transferred to different groups of naïve recipients. 2 weeks after transfer livers and spleens were collected and analyzed by flow cytometry where SM1 TRM were defined as CD45.1⁺ CD69⁺ P2RX7⁺. Data representative of repeat experiments.

significantly higher frequency than control transduced SM1 T cells (Fig. 2C). Thus, ectopic expression of T-bet increases the number of memory CD4 T cells in the liver and preferentially enhances formation of TRMs. Interestingly, cells with greater T-bet activity required a competitive

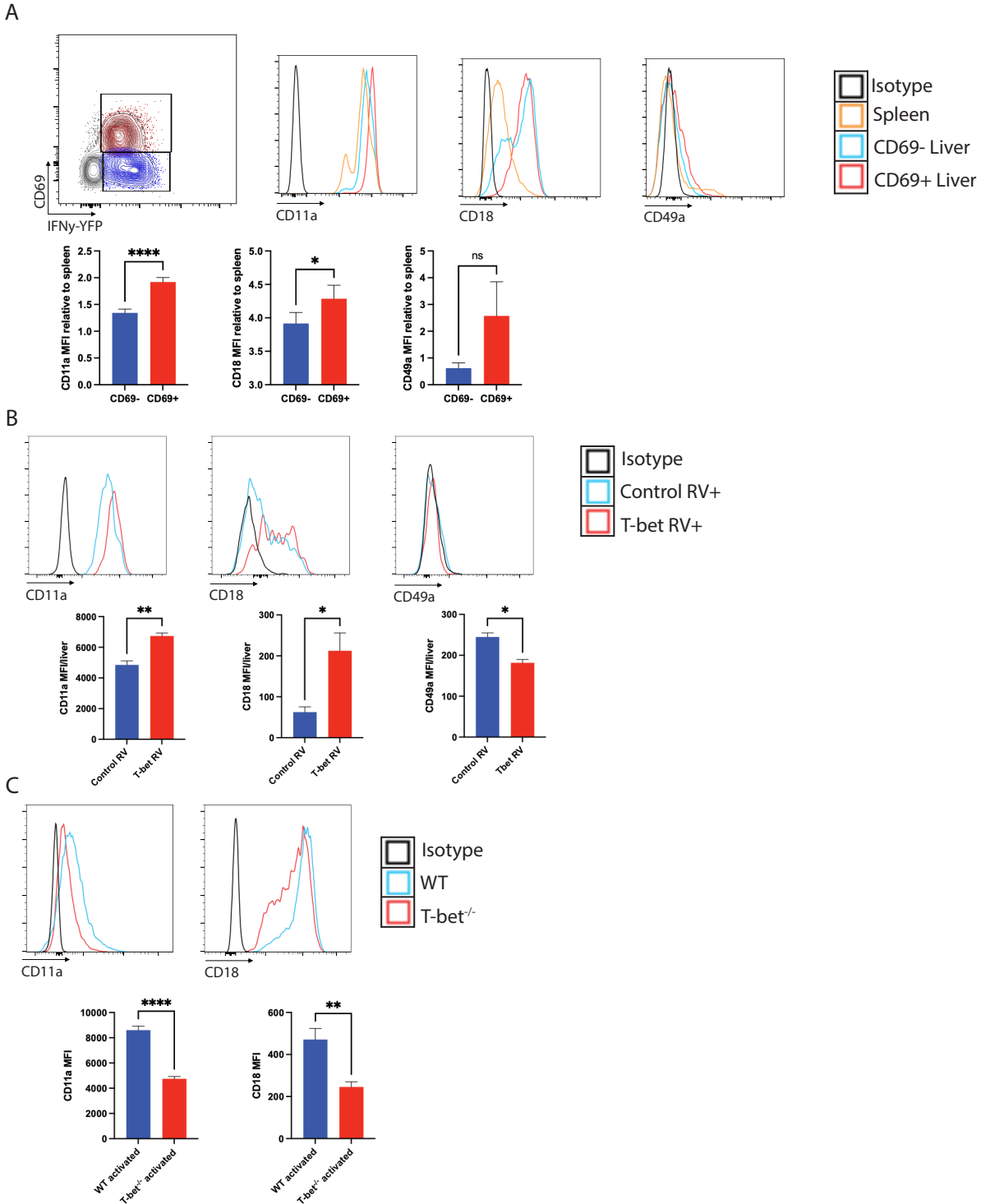
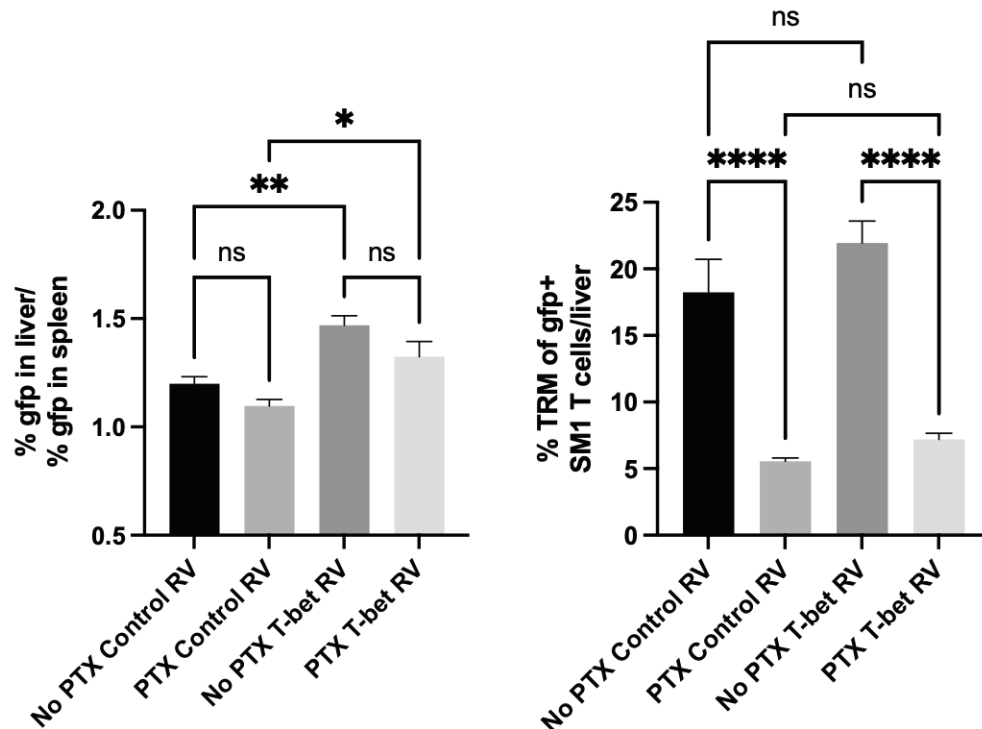


Figure 3. T-bet controls expression of LFA-1 subunits, CD18 and CD11a(A) 45 days post LVS immunization, expression of CD18 and CD11a of the livers and spleens of IFN γ -YFP reporters were analyzed by flow cytometry. (B) Two weeks post transfer, expression of CD18 and CD11a of T-bet-GFP RV and control-mAmetrine RV transduced SM1 T Cells in the liver was analyzed by flow cytometry. (C) Three days after *in vitro* activation with CD28 and CD3 antibodies, wildtype and T-bet-deficient CD4 T cells were analyzed for CD18 and CD11a expression by flow cytometry. Data representative of two experiments with 6 mice per group

environment since increased rates of TRM formation were not observed if transduced activated SM1 cells were individually transferred into separate cohorts of mice (Fig. S2). Importantly, total numbers were not calculated as T-bet RV transduced SM1 T cells were seen at a lower frequency than control RV transduced SM1 T cells in spleen and liver. This is probably due to the increased ectopic expression of T-bet caused the SM1 T cells to die at a larger rate than control RV SM1 T cells. Thus, ratios and frequencies were only analyzed to correct for the intrinsic bias caused by ectopic protein expression.

A



Supplemental Figure 3. Pertussis toxin treatment does not impact T-bet dependent effect

(A) SM1 T cells were activated *in vitro* for five days. SM1 T cells were transduced with T-bet-GFP RV or control-GFP RV during activation. SM1 T cells were either treated with pertussis toxin *in vitro* prior to transfer or left untreated. 2 weeks after transfer livers were analyzed by flow cytometry and SM1 TRM was defined as CD45.1 CD69+ P2RX7+.

T-bet enhances the expression of LFA-1 integrins, CD18 and CD11a

Our data suggest that T-bet is necessary and sufficient to promote CD4 TRM formation in the liver. Since T-bet overexpression also increases the overall number of CD4 T cells in the liver, this might be mediated via homing and retention molecules, such as chemokines and/or integrins. T-bet is known to regulate the expression of several chemokine receptors, especially CXCR3, and other chemokines participate in the formation of CD8 TRM (22-26). Additionally, the transfer of *Salmonella*-specific splenocytes into anti-CXCL16 treated mice reduced the ability of these cells to traffic to the liver (27). To examine the role of chemokines, transduced SM1 T cells were treated with pertussis toxin before adoptive transfer. Pertussis toxin treated SM1 T cells transduced with either control or T-bet retrovirus were unable to form TRM (Fig. S3), thus chemokine signaling is critical for CD4 TRM formation. However, the preference of T-bet transduced SM1 T cells to reside in the liver over the spleen was unaffected by pertussis toxin treatment (Fig. S3). Thus, the T-bet dependent effect of CD4 T cell residence in the liver is not chemokine receptor dependent.

Microenvironmental-specific integrin expression is important for TRM formation. For example, CD103 is important for TRM formation in E-cadherin rich tissues such as the intestine or skin, but is not expressed by CD8 or CD4 TRM in the liver (28). LFA-1 is crucial for CD8 TRM formation in the liver since these T cells need to interact with ICAM-1 expressed in sinusoids to patrol this tissue (29), and loss of T-bet in CD4 T cells can lead to lower expression of LFA-1 (30). We therefore examined the expression of the LFA-1 subunits, CD11a and CD18, on liver TRMs after vaccination with LVS. Indeed, both integrins were upregulated on liver IFN γ ⁺ CD4 T cells compared to splenic IFN γ ⁺ CD4 T cells (Fig. 3A). Similarly, liver CD4 TRM upregulated both integrins when compared to non-TRMs in the same tissue (Fig. 3A).

We also examined CD11a and CD18 expression after ectopic expression of T-bet. Control and T-bet transduced SM1 T cells were transferred into mice and two weeks later were stained for surface expression of CD11a and CD18. CD49a, which binds collagen, was used as an internal control since it is increased on TRM but T-bet regulation of CD49a has not been reported (28, 31). As expected, T-bet transduced SM1 T cells displayed increased expression of CD11a and CD18, while CD49a was modestly downregulated on these cells, compared to control cells (Fig. 3B). Finally, wildtype and T-bet-deficient CD4 T cells were activated *in vitro* to determine whether T-bet is required for the expression of LFA-1. Indeed, there was a notable reduction in surface expression of CD18 and CD11a in T-bet-deficient CD4 T cells compared to wild-type CD4 T cells (Fig. 3C).

CD18 inhibition removes T-bet^{hi} CD4 T cell competitive advantage for liver residence

Our data show that increased T-bet expression promotes CD4 T cell liver residence as well as expression of LFA-1. To test whether LFA-1 mediated liver residency, we neutralized CD18 with a monoclonal antibody in the SM1 adoptive transfer model. T-bet and control transduced SM1 T cells were co-transferred into recipient mice after treatment with a blocking anti-CD18 antibody or isotype control (Fig. 4A). In isotype-treated mice, T-bet transduced SM1 T cells exhibited preferential residence in the liver versus the spleen and developed an increased frequency of liver TRM compared to control transduced SM1 T cells (Fig. 4A) as shown above. In contrast, neutralization of CD18 ablated the advantage of cells ectopically expressing T-bet; a similar liver:spleen ratio and a comparable frequency of TRM in relation to control counterparts was observed (Fig. 4A). Thus, increased LFA-1 expression by T-bet enables T-bet^{hi} CD4 T cells to outcompete other CD4 T cells during TRM formation in the liver.

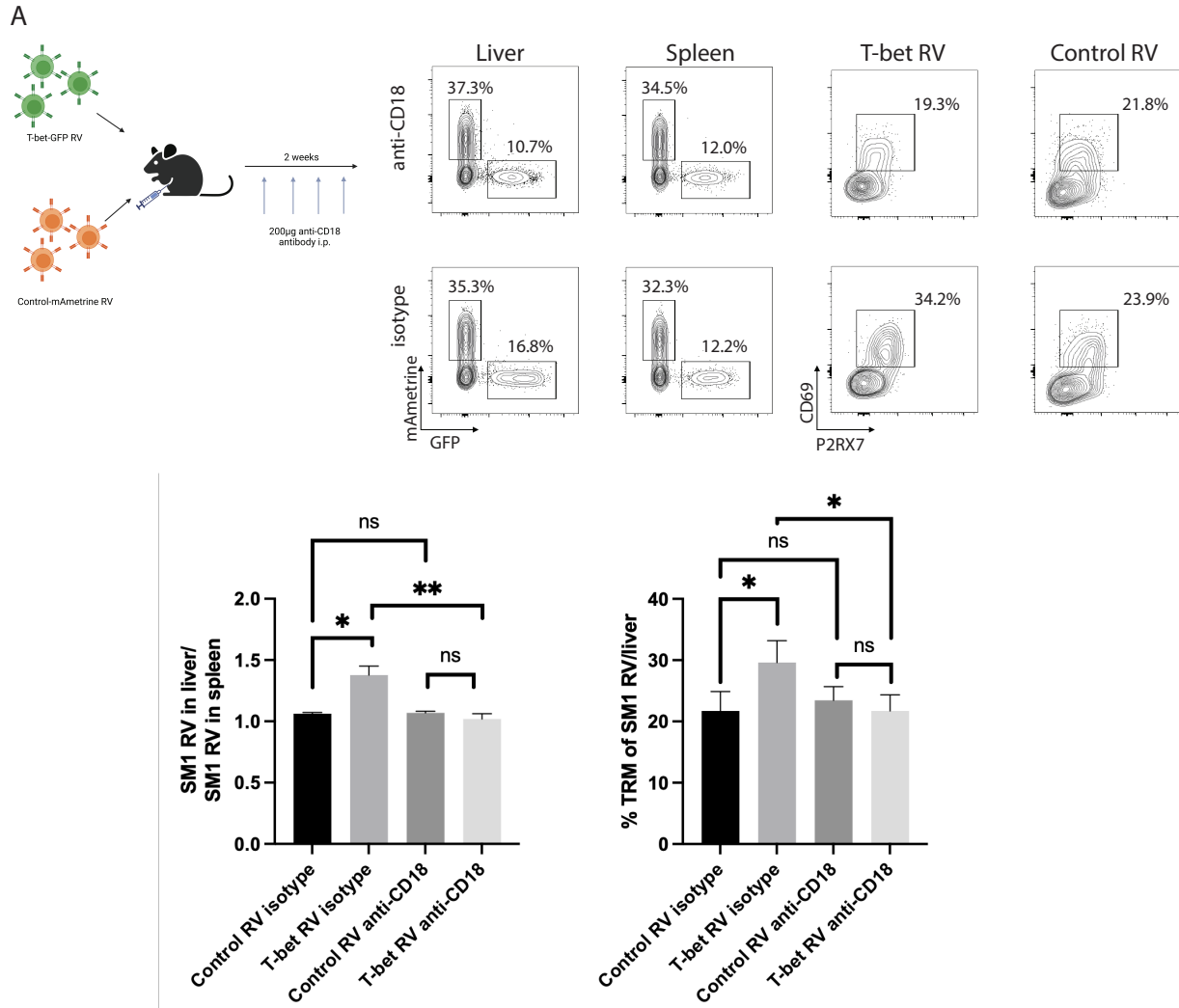


Figure 4. CD18 inhibition impedes T-bet^{hi} CD4 T cell competitive advantage in the liver

(A) Tbet-GFP RV and control-mAmetrine RV transduced SM1 T Cells were transferred into naïve recipients which received anti-CD18 inhibition antibody 24 hours before transfer. Recipient mice received anti-CD18 antibody twice weekly until take down, two weeks after transfer of SM1 T cells. TRM were defined as CD45.1⁺ P2RX7⁺ CD69⁺ CD4 T cells. Data representative of two experiments with 6 mice per group.

Discussion

CD4 TRM are required for protection against a wide variety of pathogens but a detailed understanding of how this memory cell population evolves during vaccination is lacking (7-12). Multiple transcription factors are important for programming activated T cells to adopt TRM or other memory subset characteristics (15, 32). Interestingly, although the transcriptional mechanisms that drive TRM within an individual tissue are shared between species, those mechanisms diverge in a tissue-specific manner by coordinating adaptation to local microenvironment and survival cues (13, 14). T-bet, a member of the T-box transcription factors, is notably important for CD8 TRM formation in the skin where it is downregulated to coordinate increased expression of the TGF β receptor and decreased expression of S1P receptors that allow migration out of the tissue (16, 17). However, a low level of T-bet is still essential for TRM formation due to its capacity to regulate IL-15R β expression (16). In marked contrast, liver CD8 TRM formation is not affected when T-bet is overexpressed within activated CD8 T cells (18). Thus, the importance of T-bet activity in TRM formation is variable depending on the specific tissue under investigation. Given these context-specific roles of T-bet, the role that it plays in CD4 TRM formation is not fully understood, especially in considering its role in the development of Th1 memory to combat intramacrophage pathogens (33).

Our data shows that T-bet is essential for optimal formation of CD4 TRM in the liver since T cells lacking T-bet expression do not efficiently form hepatic TRMs. In contrast, increased T-bet activity, by ectopic expression is sufficient to confer enhanced formation of liver CD4 TRM. Interestingly, this is only evident in a competitive environment against wild-type controls. Thus, this influence of T-bet on liver CD4 memory will most likely be accentuated when an infection drives a large clonally expanded lymphocyte population with variable expression of T-bet, as

occurs naturally during *Salmonella* infection. Given our observations, we propose that T-bet^{hi} CD4 T cells will preferentially form liver TRMs compared to T-bet^{lo} CD4 T cells; the latter may either contract or give rise to other memory subsets. Furthermore, in the case of *Salmonella* infection, this requirement of increased T-bet activity could retain liver CD4 TRM dependent on strong TCR and IL-12 signaling. A prediction of this model is a distinctive CD4 TCR repertoire of liver TRMs compared to other tissues which our laboratory is actively examining. Additionally, our lab has previously demonstrated that IL-18 induced T-bet⁺ CD4 T cells are able to respond in a non-cognate manner during primary *Salmonella* infection in which enables optimal clearance of bacteremia (34, 35). Whether increased T-bet expression exhibited by CD4 liver TRM synergizes with IL-18 signaling during non-cognate activation in memory responses that enhance clearance is also under investigation. The selection of distinctive clonotypes as well as activation mechanisms could be advantageous for preventing secondary infection of the liver or preventing reactivation of persisting bacteria at this location.

The selection of T-bet^{hi} CD4 T cells for liver residency appears to be directly due, in part, to increased LFA-1 expression. Responding T-bet^{hi} CD4 T cells express more surface LFA-1 than T-bet^{int/lo} cells allowing them to outcompete T-bet^{int/lo} cells for the ICAM-1⁺ liver niche. Due to this LFA-1:ICAM-1 dependent retention, T-bet^{hi} CD4 T cells are likely able to access additional signals in the liver microenvironment which allow them to reinforce a TRM transcriptional program. ICAM-1 is expressed by liver sinusoids, stellate cells, Kupffer cells and hepatocytes (36) and LFA-1 has previously been shown to be important for the formation of CD8 TRM in the liver. Thus, CD8 and CD4 T cells use a common means of liver retention even if the mechanism driving high LFA-1 expression is distinct (29).

The future practical ability to increase the number and frequency of liver TRM CD4 T cells is likely to improve a wide variety of prototype vaccines, particularly for pathogens that target the liver and require Th1 cells for clearance. Sub-unit *Salmonella* vaccines are often poor inducers of liver CD4 TRM, while LVS strains efficiently generate this population (37). This failure of sub-unit vaccines might relate directly to the lack of liver inflammation during T cell priming, preventing activated T cells from receiving access to cytokines or chemokines necessary to direct TRM formation. However, a second possibility is suggested by our experiments, specifically, suboptimal T-bet activity induced from basal subunit vaccines may preclude efficient formation of liver TRMs. We are currently examining whether novel adjuvant formulations in subunit vaccines preferentially induce the T-bet^{hi} state with a view to enhancing liver residency and ultimately protective immunity against liver infection. Overall, this study sheds light on the role of increased T-bet activity on liver CD4 TRM formation, the context-specific roles of T-bet in CD4 and CD8 TRM, and highlights a roadmap for enhancing memory CD4 T cell responses to intramacrophage pathogens.

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Chapter 4

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

Claire E. Depew, Jordan A. Rixon, and Stephen J. McSorley

Introduction

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 TRM 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 bacteria 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 *Salmonella enterica* serovar Typhi or distinct *Salmonella enterica* serovars which cause invasive Non-Typhoidal 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 non-typhoidal *Salmonella* infections. In highly susceptible inbred mouse models, a live vaccine strain of *Salmonella* (LVS) can fully protect against subsequent challenge with wildtype 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 partial protection provided by a SseB subunit vaccine was mediated primarily by circulating memory alone (21). Thus, the partial protection of this subunit vaccine could be increased by inducing protective liver CD4 TRM during vaccination since this cell subset has been shown to be required for complete protection against systemic *Salmonella* by parabiosis and transfer studies (25). Therefore, the genesis of hepatic TRMs is 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 towards a TRM phenotype, with sterile inflammatory agents and individual pro-inflammatory cytokines encouraging CD8 TRM formation in various tissues (26-32). In comparison, much less is known about requirements for tissue inflammation and cytokine signaling during CD4 TRM formation. In this 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.

Methods

Mouse strains

SM1 CD45.1 Rag-deficient mice expressing were produced by backcrossing the original C57BL/6 SM1 Rag deficient line to CD45.1 mouse strains (The Jackson Laboratory, Bar Harbor, ME) (33,

34). IFN γ -eYFP reporter mice (C.129S4(B6)-*Ifng*^{tm3.1Lky}/J) were generously provided by Dr. R. Lockley, University of California, San Francisco (35). B6.129S2-*Tcra*^{tm1Mom}/J and B6.129S7-*Il1r1*^{tm1Imx}/J were purchased from The Jackson Laboratory and used at 8-16 weeks 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 Baumler laboratory, University of California, Davis, by introducing a null mutation in the *aroA* gene of SL1344, as previously reported (36). BMM50 was subsequently modified to express a short peptide sequence (EAWGALANWAVDSA) in frame with OmpC (BMM51), using identical approach to our previous modification of BRD509 (4, 37). Preliminary experiments confirmed correct gene targeting, peptide expression, and activation of 2W1S-specific CD4 T cells in vivo. Before use, bacteria was 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 OD₆₀₀ of 0.4-0.6. *Salmonella* cultures were diluted in PBS to 5 x 10⁵ CFU/0.2mL (BMM50), 2.5 x 10⁶ CFU/0.2mL (BMM51) or 1 x 10³ CFU/0.2mL (SL1344) and 0.2mL was injected via the tail vein. *Listeria monocytogenes* Δ actA 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 OD₆₀₀ of 0.7-0.8. *L. monocytogenes* cultures were diluted in PBS to 1 x 10⁷ CFU/0.2mL and 0.2mL was injected via the tail vein. Liver 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 100µg of SseB with or without 1×10^6 CFU of *L. monocytogenes* intravenously and boosted 4 weeks later.

Bone marrow chimeras

CD90.2⁺ CD45.1⁺ mice were irradiated with a single dose of 800 cGy from a x-ray source. 16 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. Mice were maintained on 0.13mg/mL of sulfatrim ad libitum (Aurobindo) for at least 4 weeks. Mice were infected with BMM51 at 8-10 weeks and at least 2 weeks after withdrawal of antibiotics.

Lymphocyte Isolation

Spleens were turned into single cell suspensions by pressing the tissue through 70µm mesh cell strainer (Corning). Cells were incubated with 2 mL ACK lysis buffer (Gibco) to remove red blood cells and cells were counted. Livers were turned into single cell suspensions by pressing the tissue through 70µm mesh cell strainer. Cells were spun down at 2000rpm for 15 minutes without a brake. Cells were resuspended in 15mL of 35% Percoll solution (GE) and spun at 2000rpm for 20 minutes 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 μ L and injected into a TCR $\alpha^{-/-}$ mice 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 1x10⁶ cells were incubated with FC block (24G2 supernatant with 2% mouse serum and 2% rat serum). Cells were incubated with Zombie Yellow viability dye for 15 minutes. Cells were washed with 2% FBS in PBS and incubated with PE conjugated 2W1S::I-A^b MHCII tetramer for 1 hour. 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, CD101. Samples were analyzed by flow cytometry using BD LSR Fortessa or BD FACSymphony and data was 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 isolated with MACS CD4 T cell selection kit (Miltenyi) following the kit protocol. These cells were then cultured with 5 μ g/mL flagellin peptide (33), 5 μ g/mL LPS (Enzo), 10U/mL human IL-2

(Peprotech) and irradiated splenocytes from C57BL/6 mice in complete RPMI media. 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/0.2mL. Mice were injected with 0.2mL of cell suspension intravenously.

Acetaminophen treatment

Acetaminophen (Sigma) was dissolved in 0.9% saline at a concentration of 15mg/mL and sterile filtered. Mice were food restricted 16 hours before receiving acetaminophen overdose. Mice were injected intraperitoneally with 300mg/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 μ g of s+16a nanobody (Biolegend) diluted in 200 μ L of PBS 30 minutes before organ harvest (38).

RNA sequencing and analysis

Livers and spleens were collected from IFN γ -YFP reporter mice that were immunized with LVS-*Salmonella* and processed to single cell suspensions as described above. Each group was 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 EM were defined as CD4⁺ NK1.1⁻ CD69⁻ YFP⁺. Total RNA was collected from the cells using RNeasy kit (Qiagen). RNAseq and data processing was performed as in (39). Graphs were produced using RStudio. Raw data is

available in NCBI's Gene Expression Omnibus (40), and are accessible through GEO Series accession number GSE211181.

Statistics

Statistical analysis was performed by using paired or unpaired student t-test, or one-way ANOVA as stated (Prism; GraphPad Software, Inc.). Significance is displayed as, $p = * \geq 0.05$, $** \geq 0.01$, $*** \geq 0.001$, $**** \geq 0.0001$. Graphical figures were made using Biorender (<https://biorender.com/>)

Results

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 CD45.1 *Salmonella*-specific TCR transgenic CD4 T cells (SM1 T cells) were activated in vitro for five days 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. 1A).

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 two weeks later (Fig. 1A). 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. 1A and B). Thus, transferred activated SM1 T cell showed a better chance of becoming a TRM when encountering an inflamed liver. Next, inflammation was induced by *Listeria monocytogenes* Δ ActA, a liver tropic infection model which lacks the cognate antigen recognized by SM1 T cells (41) (Fig. 1C). Inflammation generated by *Listeria* infection also increased TRM formation in the liver (Fig 1D). It should be noted that there was variation in the percentage of

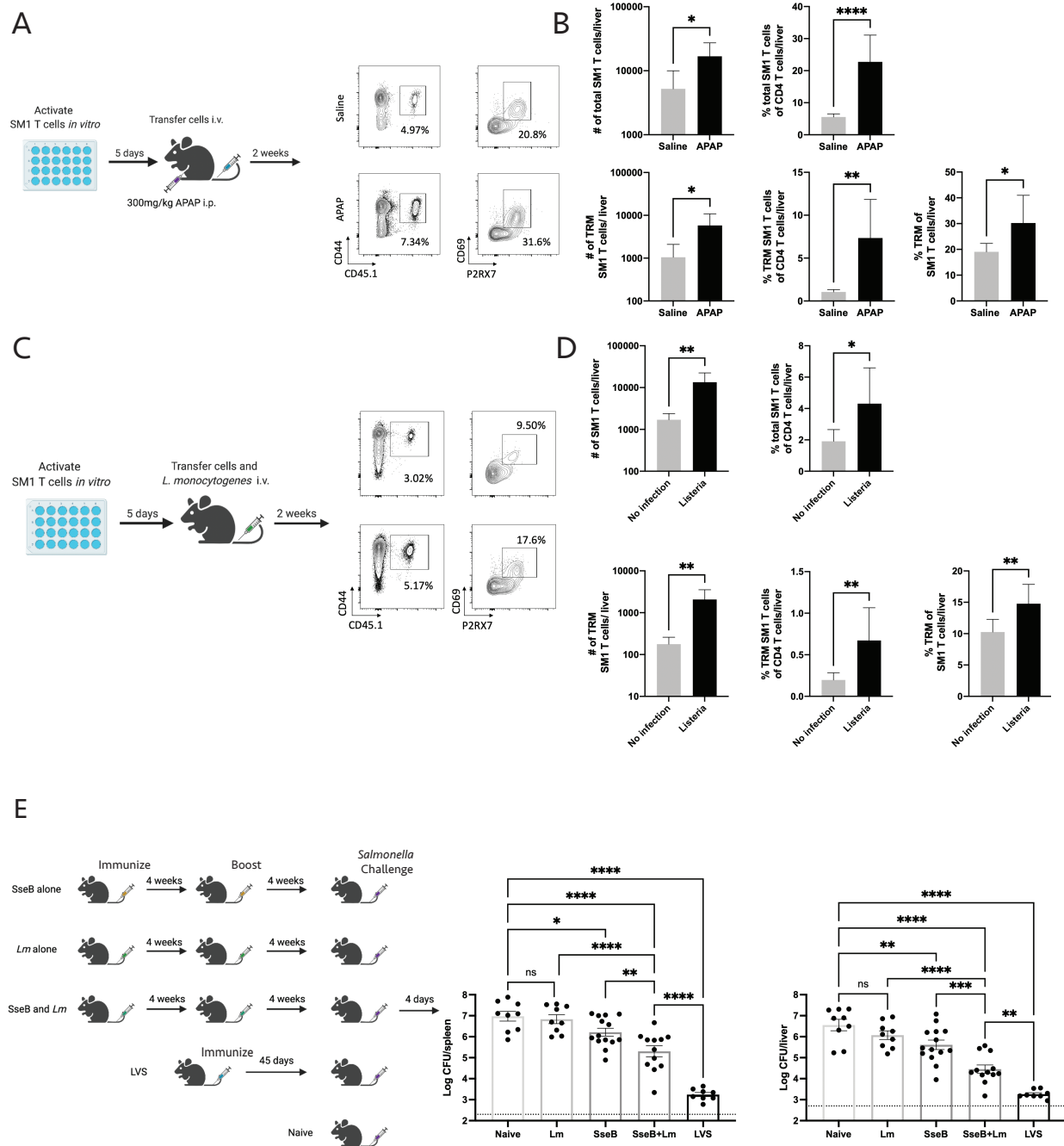


Figure 1. Liver inflammation increases TRM SM1 T cell formation in the liver and protection provided by subunit vaccine.

(A -D) SM1 T cells were activated *in vitro* for five days and transferred intravenously into naïve C57BL/6 mice. Inflammation was induced same day as transfer by 300mg/kg acetaminophen i.p. (A, B) or *L. monocytogenes* intravenously (C, D) and 2 weeks later livers were collected and analyzed by flow cytometry where SM1 T cells were identified by CD45.1 and TRM SM1 T cells were defined as CD45.1+ P2RX7+ CD69+. (E) Mice were immunized with either SseB alone, *L. monocytogenes* alone or SseB and *L. monocytogenes* intravenously. Four weeks later mice were boosted with the same vaccine and four weeks later challenged. A group of mice were immunized with *Salmonella*-LVS and 45 days later challenged. All groups were challenged with *Salmonella* intravenously and four days later protection was determined by bacterial burden of spleens and livers.

TRMs formed in individual experiments but that addition of inflammation enhanced TRM formation whatever 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 wildtype *Salmonella* and liver and spleen bacterial burdens determined. Naïve and *Listeria* infected mice had similar bacterial burden demonstrating that liver inflammation alone does not provide any protection (Fig. 1E, Naïve versus *Lm*). However, *Listeria* infection increased the protective efficacy of SseB immunization, although this effect was more noticeable in the liver (Fig. 1E, SseB versus SseB+*Lm*). Thus, liver inflammation can improve protection provided by a sub-optimal 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 TRMs.

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 IFN γ -YFP reporter mice. In MDS plots, liver CD4 TRM clustered distinctly from CD4 effector memory, and liver CD4 TRM displayed a similar transcriptional profile to CD8 TRM, including upregulation of *itga1*, *xcl1*, and *art2b* and downregulation of *klf2*, *slpr1* and *notch3* (Fig. 2B and C) (28, 42, 43). Chemokine receptors *cxcr3*, *cxcr6*, *ccr5*, and *ccr9*

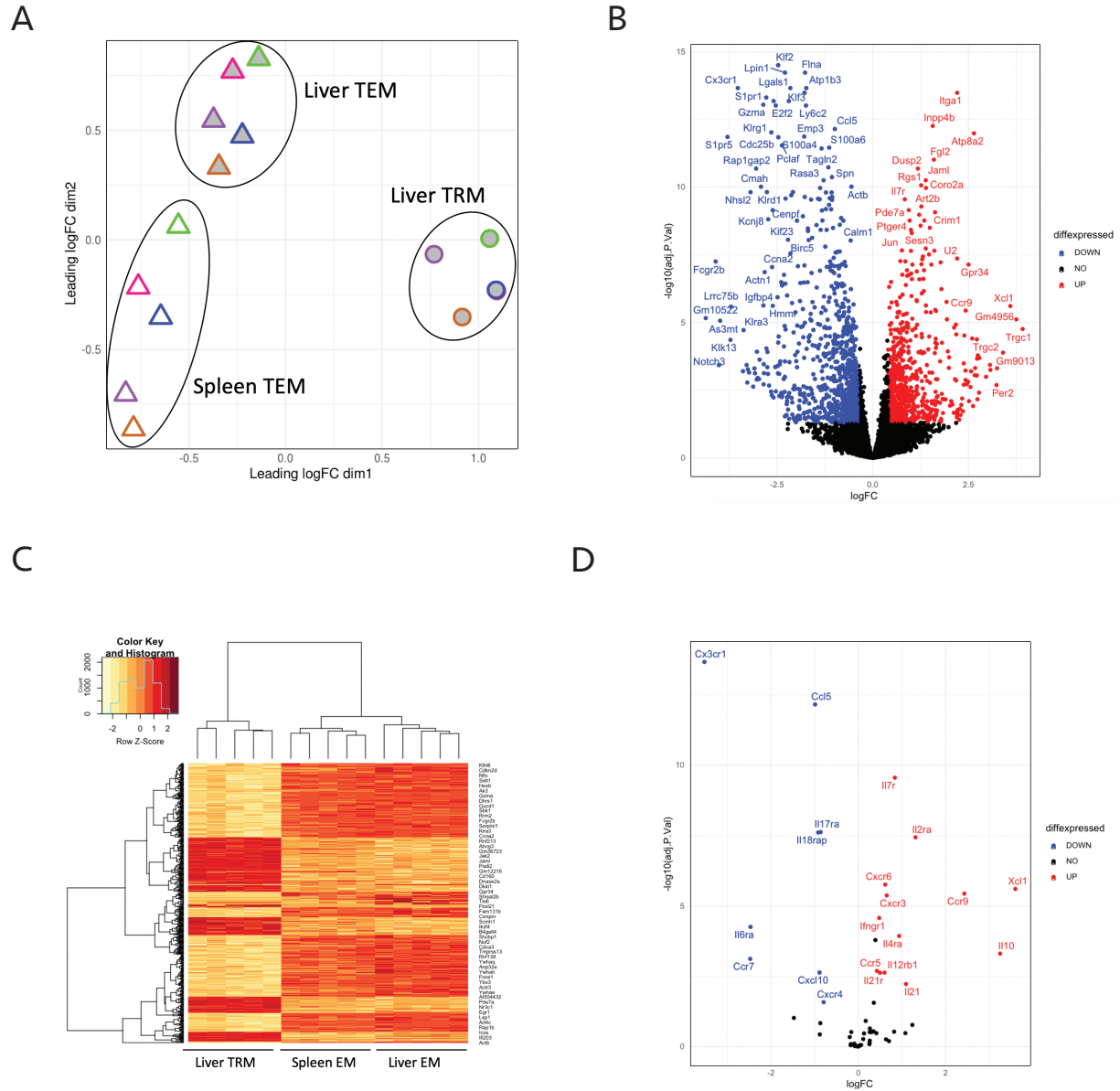


Figure 2. CD4 TRM are transcriptionally distinct from circulating memory

(A-E) IFN γ -YFP reporter mice were immunized with *Salmonella*-LVS and 45 days 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 was sequenced. (A) MDS plot of liver TRM, liver TEM and splenic TEM gene expression. (b) Volcano plot and (C) heatmap of all differentiated genes of liver TRM vs liver TEM. (D) Volcano plot of differentially expressed chemokines and receptors, and cytokines and receptors of liver TRM vs liver TEM.

were upregulated (Fig. 2D), similar to CD8 TRM transcriptional changes in the liver, and characterization of hepatic CD4 TRM by flow cytometry (42, 44). The cytokine receptors upregulated in CD4 TRM included *il7r*, *il2ra*, and to a lesser extent *il12rb1* and *il21r* (Fig. 2D).

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 (Fig. 2E and Fig. 3A). 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 two weeks (Fig. 3B). IL-2 depletion did not affect the number or frequency of SM1 T cells in the liver (Fig. 3C), 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. 3C). Since there was no effect on total SM1 T cells and thus, no impact on general proliferation, but a profound impact on TRM formation, IL-2 is required for optimal formation of CD4 TRM in the liver specifically. Interestingly, other receptors upregulated 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 (Fig. S2A 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 days later (Fig. 3D). Indeed, the number and frequency of SM1 T, the number and percentage of SM1 TRM, as well as the frequency of SM1 T cells becoming TRM were increased by hIL-2 administration (Fig. 3E). Interestingly, 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 hours (45, 46). Indeed, APAP

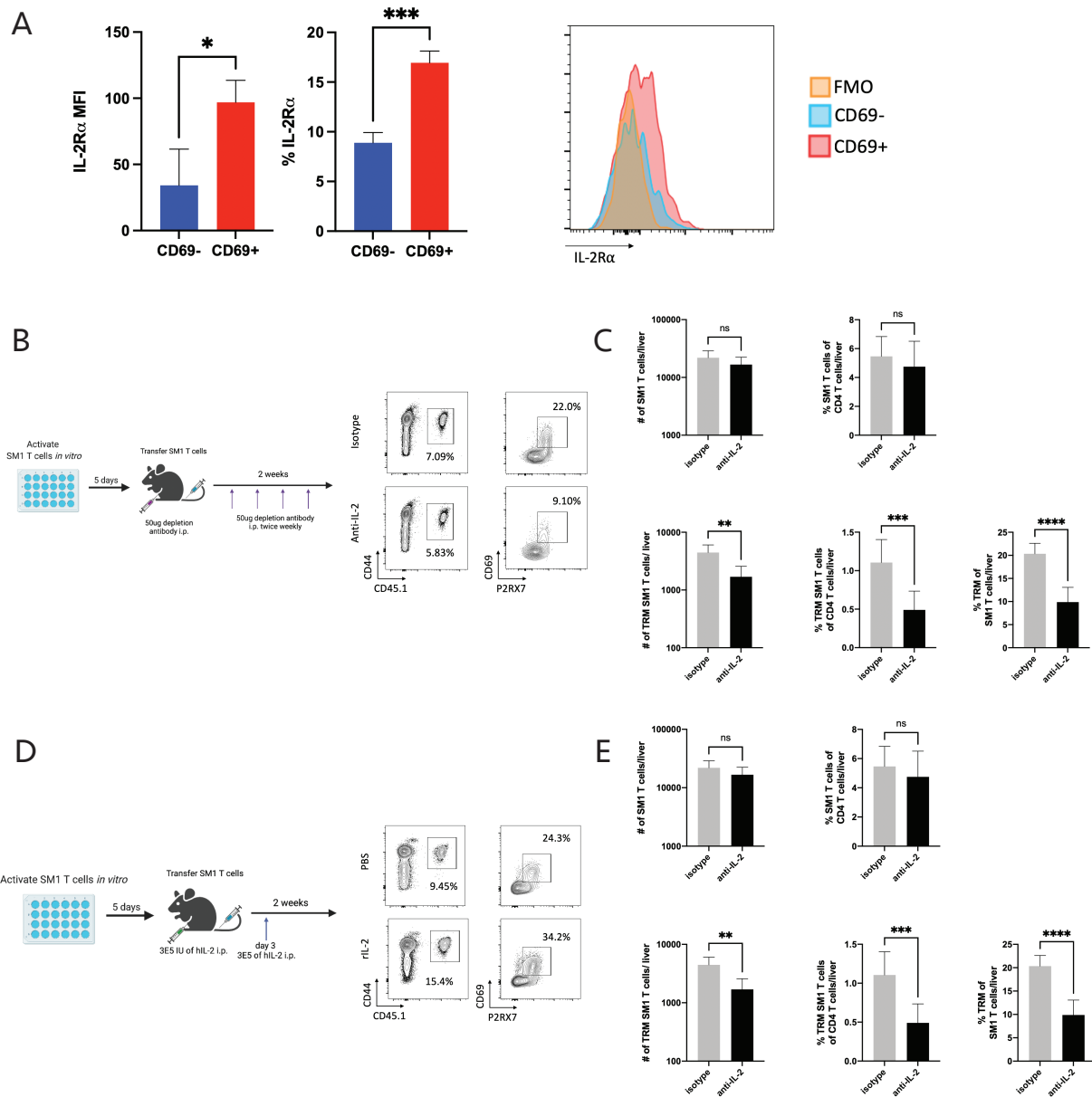
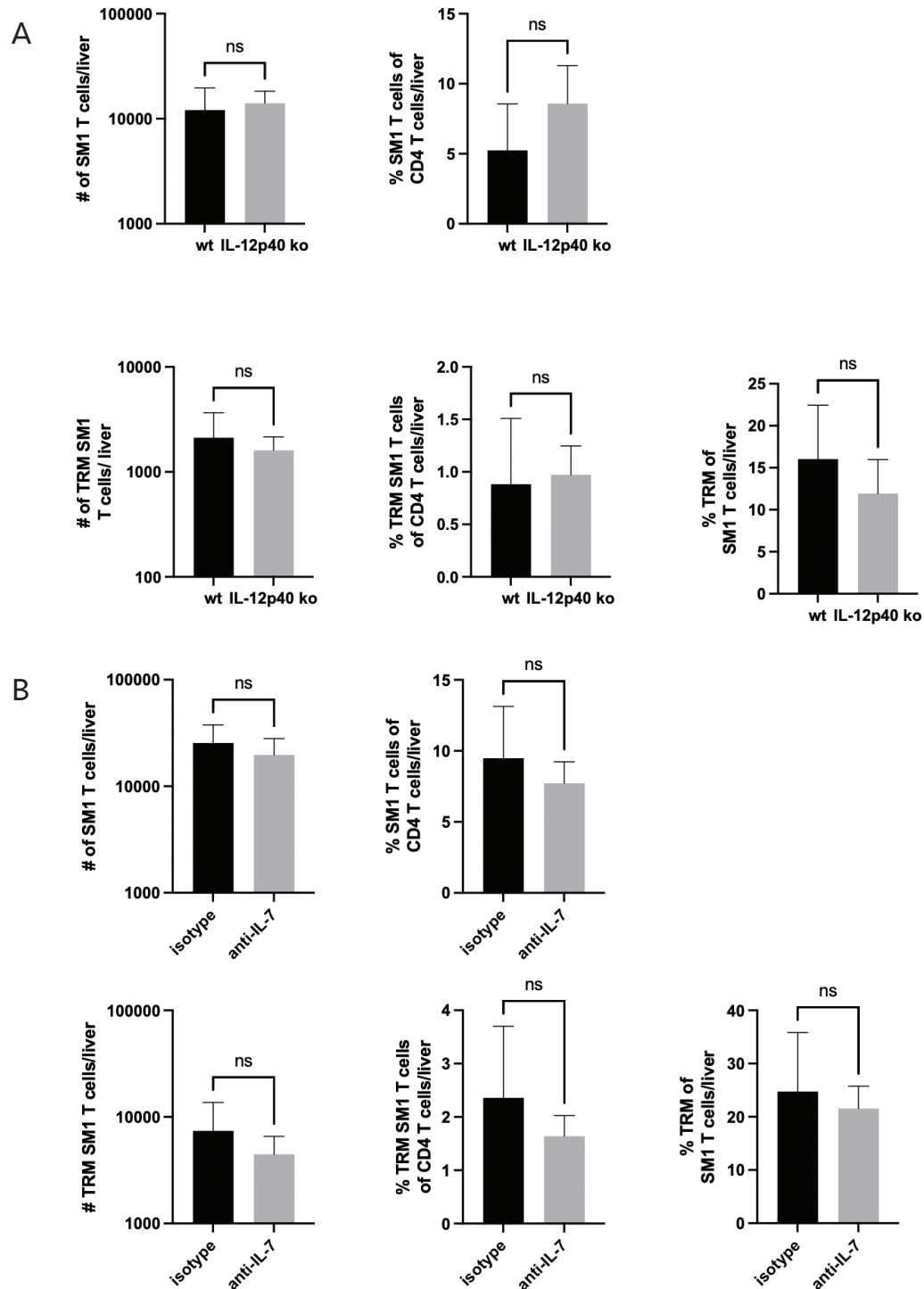


Figure 3. IL-2 is required and sufficient for optimal liver CD4 TRM formation

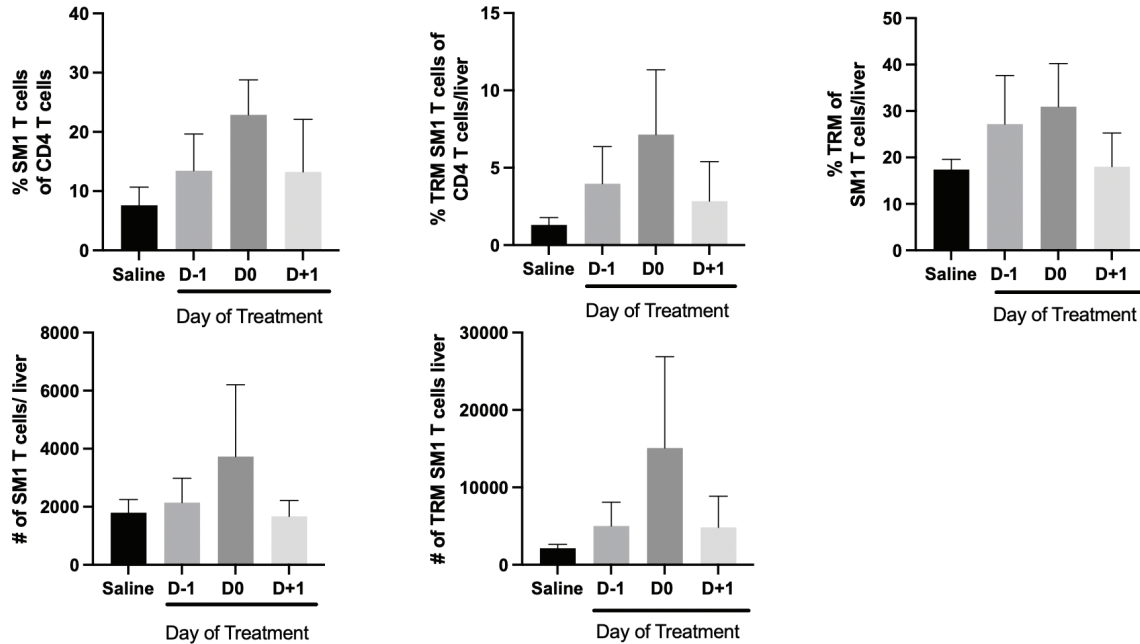
(A) C57BL/6 mice were immunized with *Salmonella*-LVS-2W1S and 45 days later liver lymphocytes were analyzed for the expression of IL-2R α of CD69 $^{+}$ and CD69 $^{-}$ 2W1S-tetramer $^{+}$ CD4 T cells. (B-E) SM1 T cells were activated *in vitro* for five days and transferred into naïve C57BL/6 mice. 2 weeks later livers were collected and analyzed by flow cytometry where SM1 T cells were identified by CD45.1 and TRM SM1 T cells were defined as P2RX7 $^{+}$ CD69 $^{+}$ CD45.1 $^{+}$ CD4 T cells. (B, 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, E) Same day as transfer of SM1 T cells, mice were treated with rIL-2 i.p. and a second dose three days after transfer.



Supplemental Figure 1. IL-7 and IL-12 signaling is not required for the formation of TRM SM1 T cells in the liver (A, B) SM1 T cells were activated *in vitro* for five days and transferred into naïve C57BL/6 mice. 2 weeks later livers were collected and SM1 T cells and TRM SM1 T cells were identified by flow cytometry. (A) *in vitro* activated SM1 T cells were transferred intravenously into IL-12p40^{-/-} mice or wildtype mice. (B) Same day as transfer of SM1 T cells, IL-7 was depleted with anti-IL-7 i.p. and twice weekly till take down. (A,B) n=6 for all experiments. Data is representative of two experiments. Significance calculated by student t-test and data are means ± SD

overdose 24 hours before or after SM1 transfer did not increase SM1 TRM formation (Fig. S3), suggesting a relatively short window in which liver inflammation contributes to TRM generation.

A



Supplemental Figure 2. Sterile inflammation same day as transfer of *in vitro* activated SM1 T cells has the largest effect on TRM SM1 T cell formation

(A) SM1 T cells were activated *in vitro* for five days and transferred intravenously into naïve C57BL/6 mice. Inflammation was induced 24 hours before (D-1), same day (D0) or 24 hours after (D+1) as transfer by 300mg/kg acetaminophen i.p. and 2 weeks later livers were collected and total SM1 T cells and CD69+ P2RX7+ TRM SM1 T cells were identified by flow cytometry. Significance calculated by student t-test and data are means $\pm$ SD.

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-1 α and IL-1 β expression was upregulated 6 hours after APAP administration (45) (Fig. 4A). RNAseq and flow cytometry analysis of CD4 TRM demonstrated that IL-1R1 is not significantly upregulated in our study (Fig. 4B). Nevertheless, we were interested to determine if IL-1R1 contributes to hepatic CD4 TRM formation. Mixed bone marrow chimeras were generated by reconstituting irradiated CD90.2⁺ CD45.1⁺ wildtype mice with a 1:1 ratio of CD90.1⁺ CD45.2⁺ wildtype bone marrow and CD90.2⁺ CD45.2⁺ *il1r1*-deficient bone marrow. After 8 weeks, chimeric mice were infected with *Salmonella*-LVS and memory T cell examined 45 days later using 2W1S tetramer.

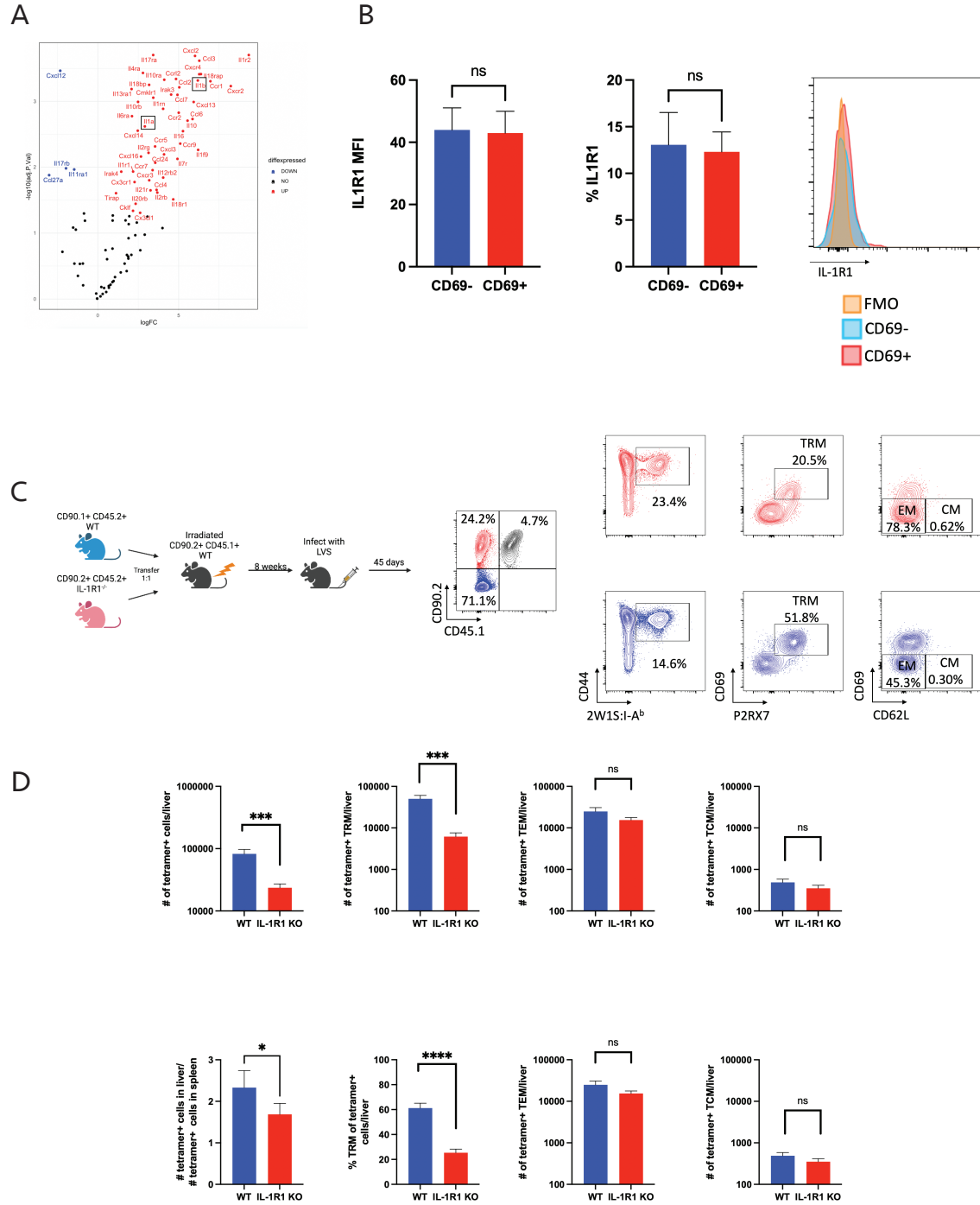


Figure 4. T cell intrinsic IL-1R1 signaling is required for optimal formation of liver CD4 TRM
 (A) Volcano plot of differentially expressed chemokine and receptors, and cytokine and receptors of 6 hours post acetaminophen overdose livers vs untreated livers. (B) C57BL/6 mice were immunized with *Salmonella*-LVS-2W1S and 45 days later liver lymphocytes were analyzed for the expression of IL-1R1 of CD69+ and CD69- 2W1S-tetramer+ CD4 T cells. (C, D) Mixed bone marrow chimeras were made by transferring 1:1 ratio of CD90.1+ CD45.2+ wildtype bone marrow and CD90.2+ CD45.2+ IL-1R1^{-/-} bone marrow into irradiated CD90.2+ CD45.1+ wildtype hosts. 8 weeks later the chimeras were infected with *Salmonella*-LVS-2W1S. 45 days post-infection livers and spleens were harvest and analyzed by flow cytometry. Immunization specific CD4 T cells were identified 2W1S-tetramer and TRM were defined as P2RX7+ CD69+, TEM were defined as CD69- CD62L⁻, TCM were defined as CD69- CD62L⁺.

TRM were defined as P2XR7⁺ CD69⁺ tetramer⁺ while TEM were defined as CD69⁻ CD62L⁻ tetramer⁺ CD4 T cells, and TCM defined as CD69⁻ CD62L⁺ tetramer⁺ CD4 T cells (Fig. 4C). 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 WT CD4 TRM, while the number of other CD4 T cell memory populations were not affected (Fig. 4D). 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 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 that showing that liver inflammation induced by a variety of mechanisms resulted in a higher proportion of CD8 TRM (29). Interestingly, both 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 to *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 formation of TRM populations at other tissue sites. In this study, hepatic CD4 TRM upregulated IL-2R α 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-1 α and IL-1 β which signal through this receptor (45). However, the IL-1R1 itself is not upregulated on CD4 TRM compared to CD4 TEM in the liver, as was noted for IL-2R α . 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-deficient 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 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,

delineating 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 (47). 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 under-studied. 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 have the potential to generate robust TRM population in the liver and markedly enhance protective efficacy of a future vaccine.

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Chapter 5

Conclusions

Historically, it has been difficult to create effective vaccines against systemic *Salmonella*, which causes typhoid and invasive non-typhoidal Salmonellosis (iNTS) (1). This could be due to the inability of vaccines to induce CD4 TRM in the liver. Tissue resident memory CD4 T cells (TRM) are a relatively newly identified/described subset of T cell memory which instead of circulating throughout the body looking for cognate antigen, reside in tissues and become the front line of defense in subsequent infections (2, 3). Previously, CD4 TRM specifically in the liver have been shown to be required for complete protection against systemic *Salmonella* infections (4). However, how to form TRM in tissues via vaccination has been a question since their discovery and to the dismay of researchers the process has proved complicated with a mixture of T cell intrinsic transcription factors and microenvironment signals that drive the formation of this memory subset (5-9). Additionally, the research focusing on TRM formation in tissues has been mostly focused on CD8 TRM and not CD4 TRM. Thus, how to form CD4 TRM in the liver has not been previously studied.

In this dissertation, we show the following: CD4 TRM in the liver form after LVS immunization and these cells are protective against systemic *Salmonella*, T-bet upregulation allows CD4 TRM precursors to access the liver niche more efficiently than other CD4 T cells through LFA-1 upregulation, and once in the liver niche the CD4 TRM precursors liver inflammation and specifically IL-2 and IL-1 increase TRM formation.

Mouse models to study CD4 TRM formation in the liver

We employ orthogonal approaches and three distinct models to study the formation of CD4 TRM in the liver. Vaccination models, using LAVs, where we can track *Salmonella*-specific CD4 TRM to a single epitope, with 2W1S tetramer, or to multiple epitopes, with IFN γ -eYFP reporters or

through a reductionist model where SM1 T cells are activated *in vitro* and transferred into naïve mice. With these models, we were able to study the impact of the transcription factor, T-bet, and liver inflammation, specifically the cytokines IL-2 and IL-1, in the formation of liver CD4 TRM.

Role of T-bet in the formation of liver CD4 TRM

Previously, transcription factors have been shown to be important in the formation and maintenance of TRM in a variety of tissues and allow the TRM to adapt to specific tissue microenvironments (5). T-bet is a transcription factor that is required for CD8 TRM formation in the skin and the downregulation of T-bet is required for proper TGF β signaling which induces integrin CD103 expression and the downregulation of migration receptors, S1PR (7, 10). However, a low level of T-bet is required for IL-15 signaling which is essential for CD8 T cell memory survival (7). Due to the requirement of the host response on T-bet⁺ CD4 T cells to clear *Salmonella*, the requirement of T-bet in the formation of CD4 TRM was of interest (11). Interestingly, we found that T-bet drives the expression of LFA-1 on CD4 T cells which allows for the CD4 T cells to compete for the liver niche in which the T cells can gain TRM signals in the liver microenvironment. Thus, unlike CD8 TRM in the skin, we propose that T-bet does not allow CD4 T cells in the liver to adapt to the microenvironment since ectopic expression of T-bet did not increase TRM formation unless in competition, instead we hypothesize that T-bet is upstream in the pathway and allows for CD4 T cells to compete for the limited space in the liver microenvironment, since increased liver seeding was seen when T-bet RV⁺ CD4 T cells were in competition or not, and once in this limited niche T-bet independent signaling can occur.

Role of liver inflammation and IL-2 and IL-2 in liver CD4 TRM formation

Since TRM formation once in the liver is not driven by T-bet, what are the signals in the liver microenvironment that drive TRM formation? Previously, inflammation and more specifically pro-inflammatory cytokines drive CD8 TRM formation in a variety of tissues (7, 9, 12-14). However, the role of liver inflammation has not been determined in CD4 TRM formation. We determined that sterile or pathogen induced inflammation increases CD4 TRM formation in the liver. Additionally, specifically, pro-inflammatory cytokines IL-2 and IL-1 α/β are required for the formation of CD4 TRM in the liver. Recombinant IL-2 was sufficient to increase liver CD4 TRM formation which therefore, suggests only one cytokine pathway possibly needs to be targeted when designing a vaccine. Interestingly, adding liver inflammation to the SseB vaccination, which is known to only protect mice by circulating memory, increased the protection of the vaccination (15). Thus, inducing liver inflammation could create a more effective vaccine through vaccine specific TRM formation.

CD8 TRM formation versus CD4 TRM formation: where are we now?

As stated multiple times in this dissertation, most of the work on resident memory T cell formation has been studied using CD8 T cell models. CD4 T cells and CD8 T cells are related cell types, their function and formation during the immune response differs. Thus, not all the rules discovered for CD8 TRM formation can be applied to CD4 TRM formation. Here we discuss what we have observed as the similarities in previously published studies in CD8 TRM formation and our research in CD4 TRM formation.

1. Mouse models to study formation

CD8 TRM have been able to be formed by multiple infection models in different tissues and when we immunized our mice with a live strain of *Salmonella* we also were able to form CD4 TRM in the liver (3, 13, 15-17). Additionally, CD8 TRM were able to be formed in the liver of naïve mice

after transferring *in vitro* activated TCR transgenic CD8 T cells and we are able to form CD4 TRM in the livers of naïve mice in the same way (14). Thus, the models we used to form CD4 TRM in the liver of mice is similar to models used in the CD8 TRM field.

2. Requirement of T-bet

Previously, the knockout of transcription factors such as T-bet, Hobit, and Blimp1 reduced the formation of CD8 TRM (6, 7, 10). Specifically, T-bet impacted CD8 TRM formation in the skin while liver CD8 TRM formation was impacted specifically in the deletion of Hobit while deletion in both Hobit and Blimp1 were required to reduce intestinal CD8 TRM (6, 7, 10). Thus, CD8 TRM in different tissues required different transcription factors.

When we look specifically at the work studying T-bet in CD8 TRM formation, most of the work has focused on CD8 TRM formation in the skin. T-bet needs to be downregulated in skin CD8 TRM so that the TGF β receptor can properly be expressed which allows for TGF β signaling inducing the expression of the integrin, CD103 which is required for the formation of CD8 TRM (7). Additionally, the downregulation of T-bet also downregulates the S1P receptors, S1PR1 and S1PR5 (10). The downregulation of these receptors is required to stop egress of the tissues to exit from the tissues. However, a low level of T-bet expression is required so that IL-15 receptor is expressed which is required for CD8 TRM survival in the skin (7).

In our study we found that unlike skin CD8 TRM, CD4 TRM in the liver actually express more T-bet than circulating memory. While T-bet-deficient T cells in both systems were unable to form TRM efficiently, when T-bet was ectopically expressed in CD4 T cells, more TRM were formed while this drove a reduction of CD8 TRM in the skin (7). Additionally, this effect of ectopically expressing T-bet in CD4 TRM formation was only seen when CD4 T cells were in competition which was not seen in the skin CD8 TRM study (7). Thus, we reached the hypothesis that T-bet

does not drive TRM formation like in CD8 TRM, instead T-bet acts upstream of the TRM differentiation process and increases the competition advantage of CD4 T cells to access the liver niche through increased LFA-1 expression. Therefore, the role of transcription factors, specifically T-bet, is different in CD8 TRM formation than CD4 TRM formation.

3. Inflammation and cytokine signaling

Previously, tissue inflammation and different pro-inflammatory cytokines have been shown to be required or increase CD8 TRM formation.

Sterile inflammatory agents such as DNFB (skin), N9 (female reproductive tract), and CPG and polyI:C (liver) increase CD8 TRM formation in the respective tissues (14, 18). In our study sterile inflammation induced by APAP overdose and pathogen induced inflammation by *L. monocytogenes* infection increased CD4 TRM formation in the liver and therefore, CD4 TRM in the liver behave like CD8 TRM.

As for specific cytokines that drive TRM formation, IL-15 and TGF β are required for skin CD8 TRM formation, though IL-15 is for survival in the tissue and probably not required for formation (7). IL-12 and type I IFN is required for CD8 TRM formation in the intestine (13). IFN γ and IL-15 are required for the formation of CD8 TRM in the liver, though again IL-15 is probably solely required for survival (14).

In our study we found that the cytokines IL-2 and IL-1 α/β are required for the formation of CD4 TRM in the liver and thus, we have found cytokines that have previously not been associated with CD8 TRM formation making CD4 TRM formation unique.

Future perspectives

Overall, we show that CD4 TRM formation in the liver is a two-step process where the first step is the optimal priming in the SLOs which would induce a T-bet^{hi} population and the second step

is the correct(?) signals in the liver microenvironment such pro-inflammatory cytokines IL-2 and/or IL-1 α/β . Thus, the current failure of vaccination against systemic *Salmonella* could be due to the inadequacy of a vaccine in either of both of these processes. As previously shown, SseB subunit vaccine does not induce protective TRM and this could be the failure of the vaccine to induce either initial priming or the correct liver microenvironment and this could be mitigated by adding the optimal priming environment or liver microenvironment by the use of optimal adjuvants. Additionally, strong TCR signaling can induce T-bet^{hi} expression of CD4 T cells and thus, finding antigen which induce T-bet^{hi} CD4 T cells could preferentially form TRM. We are currently investigating these areas in our lab.

This research is novel because not only the formation of CD4 TRM in the liver has not been studied but new mechanisms for the formation of TRM were discovered. Transcription factors allowing T cells to access a tissue niche and not inducing the differentiation into TRM has not been previously reported as a mechanism that transcription factors impact TRM formation. Additionally, cytokines IL-2 and IL-1 α/β have not been investigated fully as drivers for TRM formation. Thus, these studies further the field of not only liver CD4 TRM formation but also has applications for TRM formation in general by proposing new mechanism that have not been reported in CD8 TRM research. Additionally, not only will this research aid in expanding the basic research studying TRM formation but the mechanisms discovered in these studies can aid in the development of better vaccines against systemic *Salmonella* infections.

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