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Optimal formation of Hepatic Tissue Resident Memory CD4 T cells Requires T-bet Regulation of CD18

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Abstract

Tissue resident memory (TRM) CD4 T cells allow robust protection of barrier surfaces against pathogens. We investigated the role of T-bet in the formation of liver CD4 TRM using mouse models. T-bet-deficient CD4 T cells did not efficiently form liver TRM when compared to wildtype. Additionally, ectopic expression of T-bet enhanced the formation of liver CD4 TRM, but only when in competition with wild-type CD4 T cells. Liver TRM also expressed higher levels of CD18 which was T-bet dependent. The wildtype competitive advantage was blocked by antibody neutralization of CD18. Taken together, our data show that activated CD4 T cells compete for entry to liver niches via T-bet-induced expression of CD18, allowing TRM precursors to access subsequent hepatic maturation signals. These findings uncover an essential role for T-bet in liver TRM CD4 formation and suggest targeted enhancement of this pathway could increase the efficacy of vaccines that require hepatic TRM.

Introduction

CD4 T cell memory is comprised of three main cellular subsets, central memory T cells (TCM), effector memory T cells (TEM) and tissue resident memory T cells (TRM). TCM and TEM circulate throughout the body while TRM do not circulate but remain in non-lymphoid tissues (NLTs) (1, 2). CD4 TRM provide a stable front line of defense against reinfection since they do not require recruitment and can rapidly secrete effector cytokines upon reactivation (3). Due to their role in protection against a wide range of pathogens, it is vital to understand how CD4 TRM could be induced during vaccination (4-9). While a mechanistic understanding of CD8 TRM genesis is rapidly being elucidated, the basic processes involved in CD4 TRM formation are poorly understood. Transcription factors are important for memory formation and TRM in different tissues can express different transcriptional profiles as they adapt to varying microenvironments (10). Specifically, the regulation of T-bet is essential during CD8 TRM formation in the skin where downregulation allows expression of receptors required for TRM maturation signaling (11, 12). However, the precise role that T-bet plays in liver CD4 TRM formation is currently unknown.

In this study we examined the generation of *Salmonella*-specific CD4 TRM using a simple infection model and tracking tools that allow precise identification of peptide-specific liver CD4 TRM. Hepatic CD4 TRM were formed after the 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 liver CD4 TRM formation and retroviral expression of T-bet provided a competitive advantage for cells entering the hepatic TRM pool. Increased surface expression of CD18 correlated with T-bet expression and was T-bet-dependent, suggesting a simple mechanism for competitive liver residence. Indeed, T-bet-dependent liver residence was significantly inhibited by blocking CD18 in vivo, providing a checkpoint for the formation of hepatic CD4 TRM.

Materials and Methods

Mouse strains

SM1 Rag-deficient mice expressing CD45.1 were created by back crossing SM1 mice to B6.SJL-PtprcaPep3b/BoyJ mouse strain (The Jackson Laboratory, Bar Harbor, ME) (13, 14). C.129S4(B6)-*Ifng*$^{tm3.1Lky}$/J (IFNγ-eYFP reporter mice) were a generously gift from Dr. R. Lockley, University of California, San Francisco (15). C57BL/6, B6.SJL-*Ptprc*^{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) via 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). Culturing of bacteria, infections and bacterial burdens were performed as described previously (4,16).

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 rested for at least 2 weeks after withdrawal of antibiotics before use in infection experiments.

Lymphocyte isolation

Liver and spleen single cell suspensions were generated by mechanical dissociation of tissue through 70μm mesh cell strainers (Corning). Liver lymphocytes were isolated by a 35% Percoll solution (GE). Single cell suspensions of lymph nodes were generated using frosted glass slides to gently disrupt the tissue. Red blood cells were lysed with ACK lysis buffer (Gibco).

Staining and Flow Cytometry

Single cell suspensions were counted and $1x10^6$ 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 and then incubated with PE conjugated 2W1S::I-A^b MHCII tetramer for 1 hour. Cells were then stained with the following antibodies: B220, F4/80, CD11b, CD11c, NK1.1, CD90.1, CD45.1, CD90.2, CD45.2, CD69 (Thermofisher), CD4, CD44, CD62L (BD Biosciences), P2RX7, CD18 (BioLegend). When staining for surface P2RX7 on liver CD4 T cells, mice were given 50μg s+16a nanobody (BioLegend) intravenously 15-30 minutes before euthanasia. For intracellular staining, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer set (eBioscience) following the manufacturer's protocol. Cells were stained with antibodies to T-bet (Thermofisher) in permeabilization buffer overnight. 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 SM1 T cells and adoptive transfer

SM1 T cells were cultured and transferred into C57BL/6 mice as previously described (16).

Retrovirus transduction

Retrovirus were produced by transfecting Plat-E cells (Cell Biolabs) with pMSCV-IRES vectors. Control-GFP and T-bet-GFP were generously gifted by Susan Kaech (Salk Institute, La Jolla) and Laura Mackay (University of Melbourne, Melbourne, Australia), respectively. Control-mAmetrine was purchased from Addgene (#52113). Briefly, Plat-E cells seeded 24 hours before transfection with 14μg of pMSCV-IRES vectors, 2μg gag plasmid, 2μg envelope plasmid and lipofectamine 2000 (Thermofisher). Viral supernatants were harvested and pooled at 48 and 72 hours after transfection. Day 3 *in vitro* activated SM1 T cells were resuspended in viral supernatant and "spinfected" at 2500rpm at 32°C for 90 minutes with 33 mM HEPES (Gibco) and 0.5μg/mL of polybrene (Sigma). At day five of culture, transduction efficiency averaged 50% as measured by fluorescent protein expression. For non-competition experiments, $5x10^6$ SM1 T cells were transferred intravenously. For co-transfer experiments, $5x10^6$ SM1 T cells transduced with either T-bet-GFP or Control-mAmetrine ($10x10^6$ cells total) were transferred intravenously.

CD18 antibody inhibition

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

Statistics and data figures

Statistical analysis was performed by using GraphPad Prism (GraphPad Software, Inc.). Significance is displayed as, p= *≥0.05, **≥0.01, ***≥0.001, ****≥0.0001. Experimental schematics were made using Biorender (https://biorender.com/).

Results

Protective LVS immunization forms CD4 TRM which upregulate T-bet

Robust protection against invasive non-typhoidal *Salmonellosis* (iNTS) in the mouse model requires CD4 TRM formation in the liver (4). C57BL/6 mice immunized with a live vaccine strain (LVS) of *Salmonella enterica* serovar Typhimurium were protected against wild type challenge by bacterial burden (Fig. 1A). At a memory time point (45 days post immunization), liver LVS specific CD4 T cells were identified using the 2W1S:I-A^b MHC class-II tetramer and the majority displayed a TRM surface phenotype ($CD69^{hI}$, $P2RX7^{hi}$ and $CD62L^{lo}$)(Fig. 1B). Vaccine-induced TRM were also identified using IFNγ-YFP reporter mice (4, 16). In the liver of LVS-immunized mice, there was significant expansion of $NK1.1^-$ $CD4^+$ T cells that expressed YFP, the majority of which also expressed CD69 (Fig. 1C). Additionally, liver CD4 TRM expressed higher levels of T-bet than corresponding $tetramer^+$ CD4 T cells in the spleen or non-CD4 TRM in the liver (Fig. 2A).

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

To determine if CD4 T cell intrinsic T-bet expression was essential 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 in irradiated wildtype ($CD90.2^+$/$CD45.1^+$) recipients (Fig. 2B). The contribution of wildtype and T-bet-deficient bone marrow to the chimerism of CD4 T cells was similar when blood was analyzed 6 weeks later (Fig. 2C). Chimeras were immunized with LVS-2W1S and 45 days later, livers and spleens were collected to assess TRM formation. Fold change of T-bet-deficient/wildtype CD4 T cells was calculated in the spleen, and for circulating memory CD4 T cells (Tcirc), and TRM in the liver, in order to determine the effect of T-bet deficiency on memory formation. There was a small negative fold change in the spleen but a much larger negative fold change among liver Tcirc and TRM, demonstrating a requirement for T-bet among all CD4 T cell memory cells that reside in the liver. However, the fold change of liver CD4 TRM was significantly greater than CD4 Tcirc. Thus hepatic CD4 TRM formation is highly dependent on T-bet expression (Fig 2D).

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

To examine if an increase T-bet expression would enhance the formation of liver CD4 TRM, we developed a model of *in vitro* activation and *in vivo* parking of *Salmonella*-specific SM1 TCR transgenic CD4 T cells. SM1 T cells were activated *in vitro* for five days and then adoptively transferred to naïve C57BL/6 mice. Two weeks after transfer, a population of SM1 T cells expressing CD69 and P2RX7, but lacking CD62L, was detected in the liver (Fig. 3A). Similar to vaccine induced CD4 TRM above, liver CD69+ SM1 T cells displayed greater T-bet expression than CD69− SM1 T cells, and both of these populations expressed higher T-bet than splenic SM1 T cells (Fig. 3B). To determine if ectopic T-bet expression drives TRM formation in the liver, activated SM1 T cells were transduced with T-bet-IRES-GFP or control-IRES-mAmetrine retroviral vectors (RV) and subsequently transferred into the same recipients (Supplemental Fig. 1). To determine if ectopic T-bet ectopic expression influenced overall CD4 T cell parking in the liver, the log fold change between liver and spleen was calculated for T-bet RV+ and Control RV+ SM1 T cells. Overexpression of T-bet

caused a significant preference for the liver over the spleen, while control RV+ SM1 T cells had no effect (Fig 3C). The overall number of T-bet RV+ SM1 T cells was reduced, consistent with a report showing that T-bet^{hi} CD4 T cells have lower survival in naïve mice, likely due to a requirement for additional pMHC interactions (17). However, despite this effect, T-bet transduced SM1 T cells formed TRM at significantly higher frequency compared to control transduced SM1 T cells (Fig. 3C). Thus, ectopic expression of T-bet increases the overall number of CD4 T cells in the liver and also preferentially enhances the formation of liver CD4 TRM. Interestingly, increased TRM formation by T-bet transduced SM1 T cells was not observed when transduced SM1 T cells were not placed in direct competition with wild-type T cells. (Supplemental Fig. 2).

To determine if this preference to reside in the liver by T-bet RV+ SM1 T cells occurred at an early stage, spleens and livers were analyzed at both day 3 and day 7 post transfer. Interestingly, there was no significant increase in the preference of T-bet RV+ SM1 T cells to reside in the liver at day 3. However, by day 7 the T-bet RV+ SM1 T cells displayed a strong preference to reside in the liver over the spleen, unlike control RV+ SM1 T cells (Fig. 3D). Thus, this contribution of T-bet does not affect early T cell migration to the liver.

T-bet enhances the expression of CD18

Our data suggest that T-bet is necessary and sufficient to promote liver CD4 TRM formation in addition to encouraging activated CD4 T cell parking in the liver, suggesting a potential mechanism involving integrins. A large family of integrins is the β2 integrins in which β2 (CD18) binds with CD11a, CD11b, CD11c and CD11d to form LFA-1, MAC-1, αXβ2, αDβ2; respectively (18). These integrins can bind ICAM-1, which is highly expressed in the liver, suggesting that β2 integrins might be involved in this T-bet-dependent effect (19). Expression of CD18 on LVS-specific liver CD4 TRM was assessed after immunization and it was found that this integrin was upregulated on CD69^{+} IFNγ^{+} CD4 T cells when compared to CD69^{-} IFNγ^{+} CD4 T cells in the liver. Both of these T cell populations displayed increased expression when compared to splenic IFNγ^{+} CD4 T cells (Fig. 4A). To determine if CD18 expression was regulated by T-bet expression, RV-transduced SM1 T cells were analyzed for CD18 expression. T-bet transduced SM1 T cells had increased expression of CD18 when compared to control transduced SM1 T cells (Fig. 4B).

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 residency and allows higher expression of CD18. To test whether CD18 was required for T-bet-mediated liver residency, we neutralized CD18 using 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. 5A). 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. 5B and C), as expected. In contrast, neutralization of CD18 ablated the advantage of cells ectopically expressing T-bet (Fig. 5B and C). Thus, increased CD18 expression by T-bet enables T-bet^{hi} CD4 T cells to outcompete other activated CD4 T cells during liver TRM formation.

Discussion

The transcription factor T-bet plays an important role in both CD4 and CD8 T cell memory development (17, 20, 21). T-bet^{hi} CD4 T cells preferentially become TEM in the spleen while T-bet^{int} CD4 T cells preferentially become TCM (17, 20). This effect on CD4 T cells is distinct from T-bet^{hi} CD8 T cells, which generally fail to survive clonal contraction, although persisting CD8 TEM express more T-bet than CD8 TCM (21, 22). T-bet also seems to play a tissue specific role for CD8 TRM, where in the skin the downregulation of T-bet is required for memory precursors to receive maturation signal; however, while T-bet is essential for liver CD8 TRM formation, the overexpression of T-bet does not appear to impact its formation (11, 23). The potential role of T-bet in liver CD4 TRM formation is unclear, despite a well-established role for this transcription factor in Th1-lineage formation (17).

Our data shows that T-bet is essential for optimal formation of liver CD4 TRM, since T cells lacking T-bet expression do not efficiently form hepatic TRM, consistent with liver CD8 TRM formation (23). However, liver CD4 TRM are unique in that increased T-bet activity was sufficient to enhance their formation and T-bet^{hi} CD4 T cells preferentially form liver TRM, compared to T-bet^{lo} CD4 T cells. Interestingly, this effect of T-bet was only evident in a competitive environment. We propose that this competitive effect of T-bet could be particularly important during an infection that drives heterogenous expression of T-bet, as occurs naturally during *Salmonella* infection (24). Differential expression of T-bet might reflect strong TCR and IL-12 signaling within in the lymph node or differential T-bet expression might simply reflect asymmetric cell division and be disconnected from TCR specificity (25).

It should be noted that T-bet RV+ SM1 T cells had poorer overall survival, compared to control RV+ SM1 T cells, even though input frequencies were similar. It has been reported that T-bet^{hi} CD4 T cells survived at a lower rate than T-bet^{int} CD4 T cells at a memory time point in naïve mice, suggesting that sustained peptide MHC contact may affect persistence (17). We were unable to assess this directly because SM1 TCR transgenic CD4 T cells model do not survive to a memory time point in *Salmonella*-infected mice (14). However, the use of relative frequencies with the spleen as a reference point allowed assessment of the effect of T-bet on the CD4 T cell liver residency.

Interestingly, the T-bet dependent effect on both liver residency and TRM formation was influenced by CD18 expression. T-bet^{hi} CD4 T cells expressed more surface CD18 than T-bet$^{int/lo}$ CD4 T cells. LFA-1 has previously been shown to be required for the formation of CD8 TRM and NKT cells in the liver (26, 27). However, the precise integrin partner that drives this T-bet dependent effect in CD4 T cells needs further assessment. The increased expression of β2 integrins by T-bet^{hi} CD4 T cells seems to allow CD4 T cells to outcompete T-bet$^{int/lo}$ CD4 T cells for a currently undefined liver niche. It is important to note that this T-bet-dependent preference for overall liver residency is observed whether or not CD4 T cells are placed in a competitive environment whereas the effect on increased TRM formation was only detected when in competition. Clearly, T-bet^{hi} CD4 T cells are allowed preferential access to the liver microenvironment niche and thus would be more likely access

TRM maturation signals which encourage TRM differentiation. Some maturation signals are IL-2 and IL-1α/β (16), while conversely IL-15 is not essential for liver CD4 TRM formation (data not shown), demonstrating unique development pathways for CD8 and CD4 cells in the liver. This model is distinct to the proposed role of T-bet in CD8 TRM formation in the skin where T-bet intrinsic signaling drives TRM differentiation (11, 12). Interestingly, the preference for liver residency by T-bet^{hi} CD4 T cells was not evident early after transfer suggesting that the T-bet dependent effect on liver residency (and resulting TRM formation) occurs relatively late during effector T cell maturation in infection.

The ability to influence the number and frequency of liver CD4 TRM could improve a wide variety of prototype vaccines, particularly for pathogens that target the liver. The failure of some vaccines to induce liver CD4 TRM might be due to their inability to induce an appropriate liver microenvironment to provide TRM maturation signals. However, a second possibility is simply that these vaccines induce suboptimal T-bet expression and therefore fail to generate liver TRMs. This latter model would be interesting since adjuvants that preferentially encourage T-bet^{hi} expression would be more likely to encourage liver, irrespective of whether the liver is inflamed or not. Greater understanding of the role of CD4 T cell priming and the local liver microenvironment in encouraging CD4 TRM memory formation could be key to the development of new vaccines for *Salmonella*, malaria, and other important pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

1. T-bet is required and sufficient to drive competitive liver CD4 TRM formation
2. T-bet drives CD18 expression on CD4 T cells
3. CD18 blockade ablated the T-bet dependent effect of TRM formation

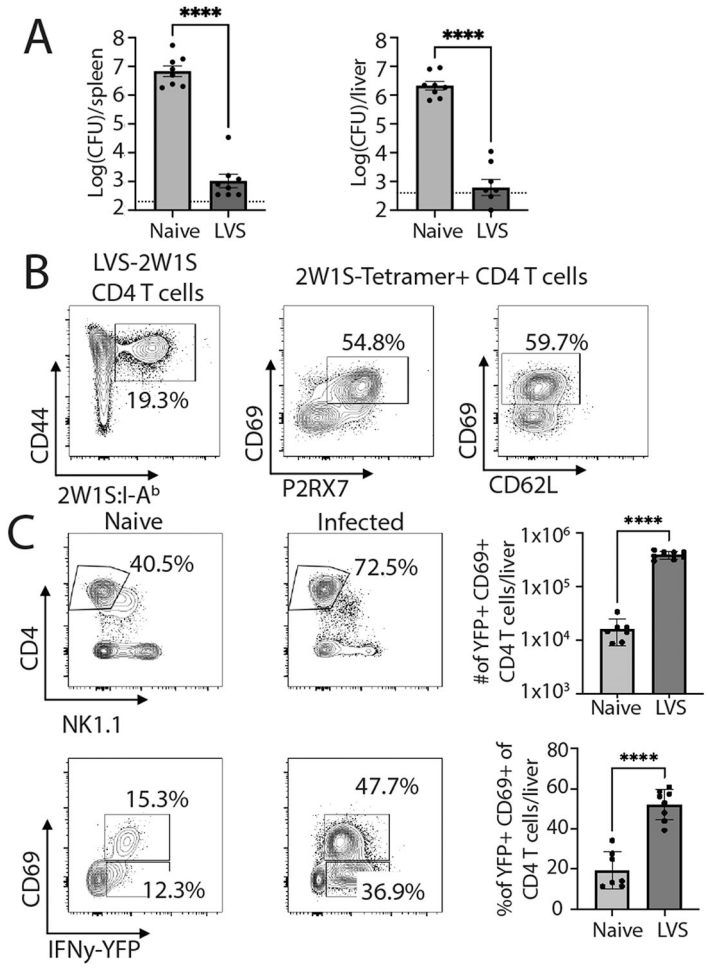

Figure 1. Protective LVS immunization form CD4 TRM in the liver

(A) 45 days post immunization, LVS immunized and naïve mice were challenged with wildtype *Salmonella* and bacterial burden of the spleen and liver was determined 4 days after challenge. (B, C) 45 days post immunization, livers and spleens were analyzed by flow cytometry. (B) Immunization specific TRM were identified as $2W1S^{+}$ $CD69^{+}$ $P2RX7^{+}$ $CD62L^{-}$ CD4 T cells. (C) In IFNγ-YFP reporter mice TRM were defined as $CD69^{+}$ YFP^{+} $NK1.1^{-}$ CD4 T cells. Data representative of two experiments with 4 mice in each group. Data is shown as mean ± SEM and analyzed by unpaired student t test (A-C).

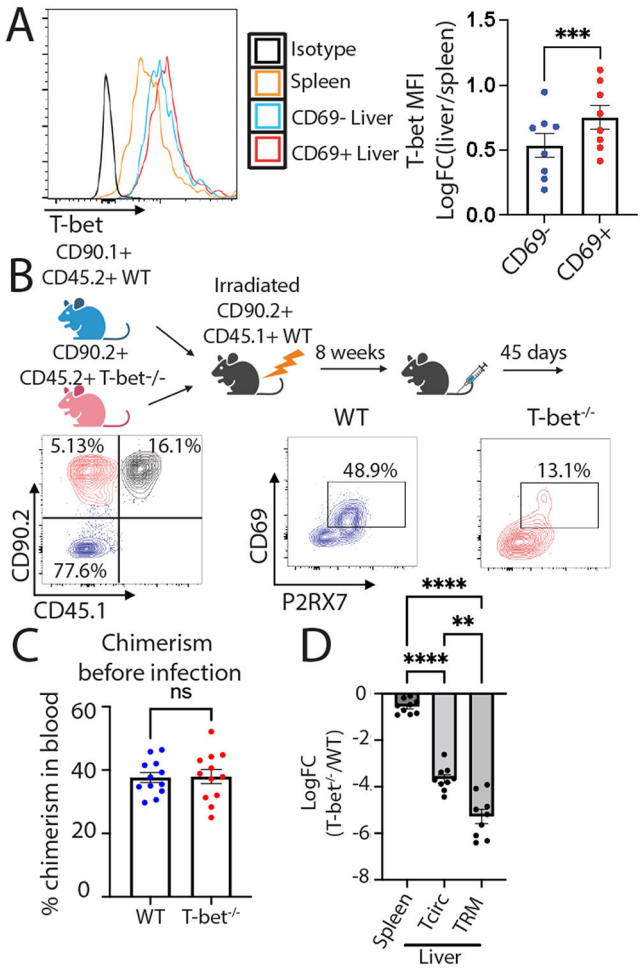

Figure 2. T-bet is upregulated in liver CD4 TRM and is required for their formation

(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^{+} and Tcirc was defined as 2W1S+ CD69-. Data representative of two experiments with 9 mice in each group. Data is shown as mean ± SEM and analyzed by paired student t test (A and C).

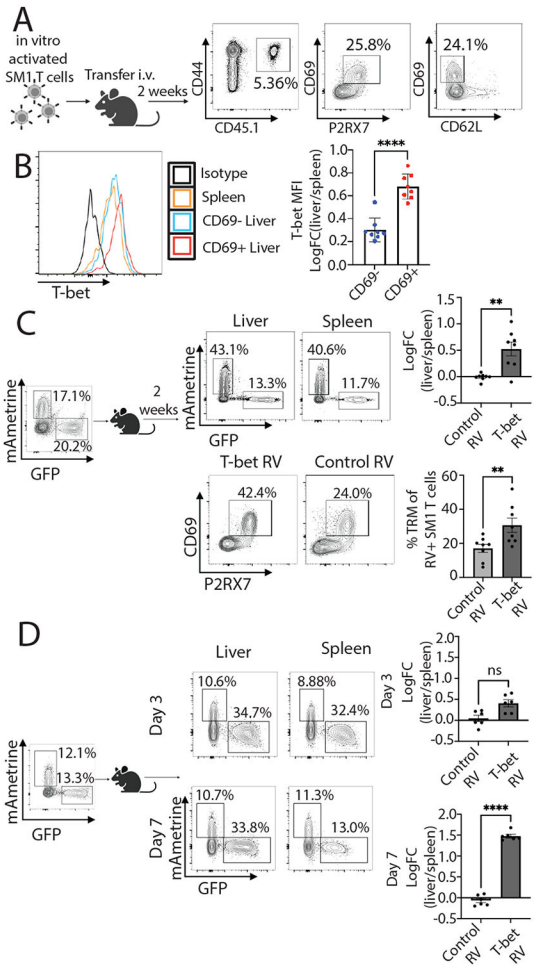

Figure 3. T-bet overexpression increases CD4 TRM formation in the liver

(A-D) 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 8 (B and C) or 6 (D) mice per group. Data is shown as mean ± SEM and analyzed by paired student t test (A-D).

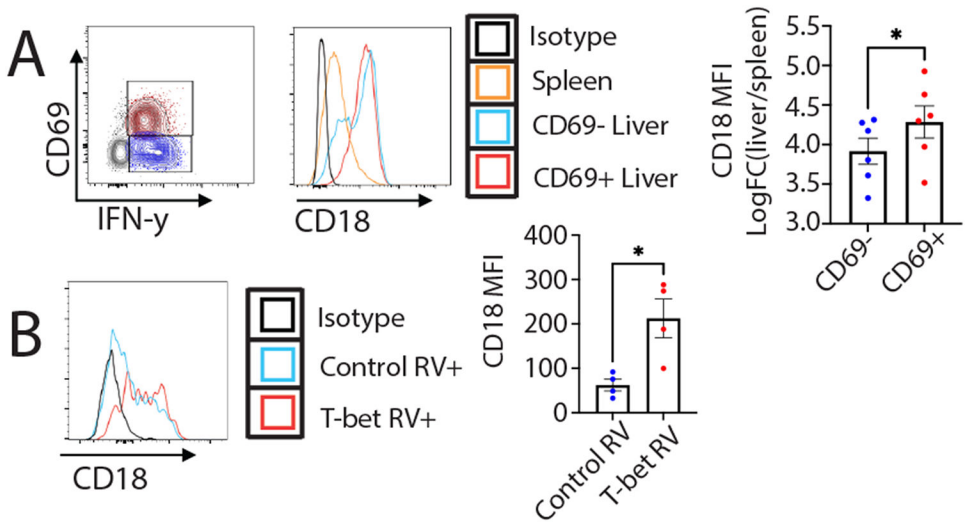

Figure 4. T-bet controls expression of CD18

(A) 45 days post LVS immunization, expression of CD18 of the livers and spleens of IFNγ-YFP reporters were analyzed by flow cytometry. (B) Two weeks post transfer, expression of CD18 of T-bet-GFP RV and control-mAmetrine RV transduced SM1 T Cells in the liver was analyzed by flow cytometry. Data representative of two experiments with 4 (A) or 6 (B) mice per group. Data is shown as mean ± SEM and analyzed by paired student t test (A and B).

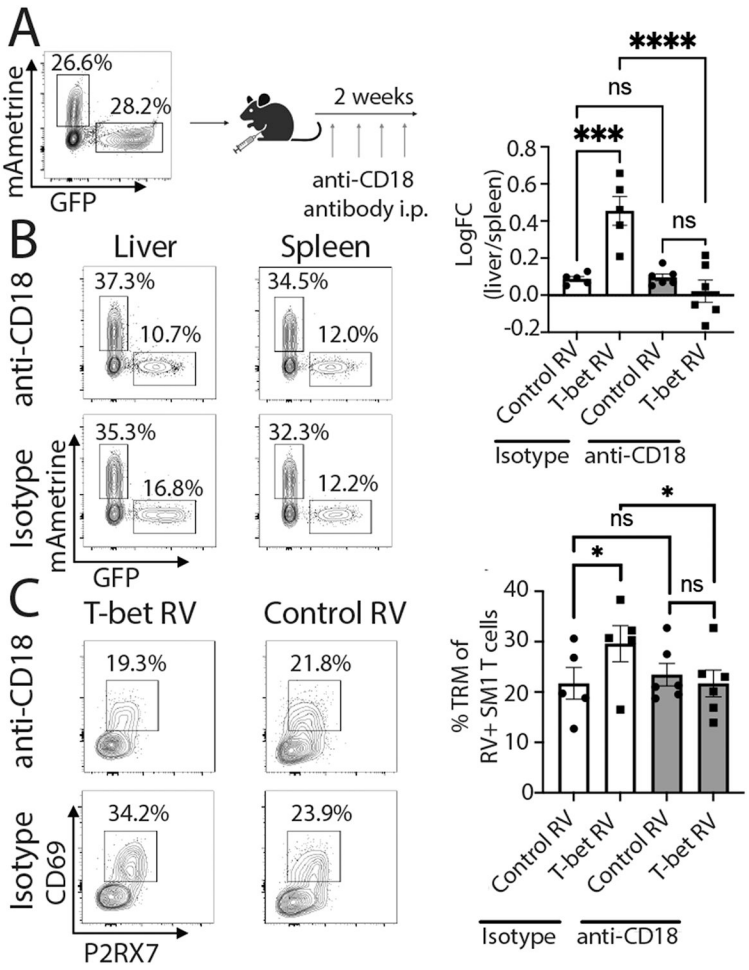

Figure 5. CD18 inhibition impedes T-bethi CD4 T cell competitional advantage in the liver

(A-C) T-bet-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. Data is shown as mean ± SEM and analyzed by one-way ANOVA (B and C).