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T-helper Type 1 Cells Drive Development of CD4 Tissue Resident Memory in the Small Intestine During Acute Viral Infection

A thesis submitted in partial satisfaction of the requirement for the degree of Master of Science

in

Biology

by

Shannon O'Shea

Committee in charge:
- Professor Ananda Goldrath, Chair
- Professor Matthew Daugherty
- Professor Li-Fan Lu

2022

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University of California San Diego

2022

DEDICATION

I would like to dedicate this to my family: Beth, Hank, Sean, Kate, Tim and Bandit.

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The dogs and their owners, who allow them to visit our lab, for the pure joy they brought me.

The ocean for every reason there is.

And above all, I would like to thank Ananda Goldrath for her patience and believing in me.

ABSTRACT OF THESIS

T-helper Type 1 Cells Drive Development of CD4 Tissue Resident Memory in the Small Intestine During Acute Viral Infection

By

Shannon O’Shea

Master of Science in Biology

University of California San Diego, 2022

Professor Ananda Goldrath, Chair

The adaptive immune system mounts a specialized response against invading pathogens and provides long lasting protection against secondary infection. During infections, antigen specific CD4 T cells differentiate into distinct effector T helper subsets, in which a portion persists after pathogen clearance and contributes to the generation of tissue-resident memory T cells (Trm) in non-lymphoid tissues (NLTs). In context of acute viral infections such as Lymphocytic choriomeningitis (LCMV-Armstrong), it is unknown which of the circulating helper T cell subsets, give rise to Trm populations during the effector phase. In LCMV infection, both T follicular helper (Tfh) or T helper type 1 (Th1) effector T cell subsets are generated and give rise to long-lived

memory T cell subsets in circulation, with Tfh memory T cells representing the majority of cells at memory time points. We studied these subsets in the secondary lymphoid organs (SLOs) and the intraepithelial layer (IEL) of the small intestine (SI) throughout LCMV infection to investigate whether Trm shared characteristics with effector populations and to understand the developmental relationship between circulating CD4 memory T cells and CD4 Trm. Circulating effector Th1 cells and Trm populations in the IEL displayed comparable surface protein and transcription factor expression. Trm populations solely expressed Th1 lineage marker, SLAM, and a substantial portion retained expression of the Th1-driving transcription factor, Blimp1. However, CD4 cells in the IEL expressed tissue retention marker CD69 significantly more than circulating Th1, confirming their Trm profile. These findings distinguish the Trm population in the IEL from circulating Th1 effector and memory populations and suggest Th1 effector cells are a Trm precursor. This study sheds light on CD4 Trm development and unveils subset programming that may serve as potential targets for improving vaccines that elicit CD4 Trm responses.

Introduction

The adaptive immune system is comprised of B and T lymphocytes. During infection, dendritic cells and other antigen presenting cells (APCs) present foreign peptides to naive cells in SLOs, beginning the early steps of the adaptive immune response. These antigen-specific T cells undergo rapid proliferation and differentiation to produce a wide range of T cell subsets that specialize in initial pathogen clearance and memory response. After pathogen clearance, most T cells generated during an immune response die during the contraction phase, however, a small population remains. This population, memory T cells, includes cells that serve as first responders that quickly mount an immune response to clear familiar pathogens before the host becomes ill. In the context of designing vaccines, generating memory T cells from initial immunization are key for quality, long lasting immunity. Thus, learning the developmental pathways that give rise to long-lived protective memory T cells can direct researchers towards specific targets that will help improve the production of T cell immunity from vaccination.

During the expansion phase of infection, antigen-specific CD4 T cells undergo clonal selection and differentiate into effector populations that help mediate the clearance of infection. CD4 T cells can be categorized into helper subsets that have distinct effector function, phenotype, and transcriptional profile. Which T helper subsets are generated in response to an infection is dependent on the type of invading pathogen; subsets include T follicular helper (Tfh), T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17). During LCMV-Armstrong infection in mice, Tfh and Th1 cells are generated in circulation (1). Tfh cells express chemokine receptor type 5

(CXCR5), which allows homing to the B cell follicle, where they facilitate antibody responses. Th1 cells specialize in intracellular pathogen clearance and express signaling lymphocytic activation molecule (SLAM), which drives proliferation and production of proinflammatory cytokine IFN-γ (2). IFN-γ drives pathogen clearance by not only directly inhibiting viral replication, but further activating macrophages and natural killer cells, thus increasing cytokine activity (3). Upregulation of MHC class I is induced by IFN-γ, promoting the recognition and lysis of infected cells by CD8 T cells (3).

Among the circulating effector T cells, phenotypic heterogeneity persists, and distinct surface markers are used to delineate populations into memory precursors (MP) or terminal effector (TE) populations. Identifying MP and TE populations in CD4 effector cells are based on P-selectin glycoprotein ligand-1 (PSGL-1) and lymphocyte antigen 6 complex (Ly6C) expression. MP populations, PSGL-1^{hi}Ly6C^{lo}, exhibit greater polyfunctionality and persist as memory cells after infection. TE populations, PSGL-1^{hi}Ly6C^{hi}, are terminally differentiated in the effector state and often die during contraction (4). Tfh demonstrate a bias towards memory phenotype, as majority of effector cells are MP, which can be attributed to their transcriptional profile that possesses intrinsic memory potential (5,6). Conversely, the Th1 effector population has both TE and MP cell subsets and the number of Th1 cells significantly contracts after infection in comparison to Tfh cells (4).

Memory T cells ensure immunity against reinfection by eliciting a robust recall response before the pathogenic load burdens the host. Memory T cells persist in the circulation and NLTs such as barrier sites of the IEL of the gastrointestinal tract, skin,

lungs, and female reproductive tract (1). Trm reside solely in the tissues and are powerful sentinels that elicit a robust secondary response to familiar antigens by expansive generation of effector cells and recruiting other immune cells to the cite of infection. Vesely and colleagues demonstrated that *Klebsiella pneumoniae* immunization generates effector Th17 cells that infiltrate the lungs and form a Trm population protective against carbapenem-resistant *Klebsiella pneumoniae* infection (7). Heterogeneity is identified within the CD4 Trm population. Not only is a Th17 related subpopulation identified, but a population that did not have a history of expressing IL-17 also persists, which, by RNA sequencing, is suspected to be from Th1/Th2 lineage. After primary *Leishmania major* infection, Glennie and colleagues observed a CD4 Trm population in the skin that offers protection against reinfection via IFN-γ production, which is the key function of the Th1 subset (8). Both studies identify a population of CD4 Trm that resemble effector cells but lack sufficient evidence that developmentally link Trm generation to a particular circulating Th subset. Utilizing circulating Th1 and Tfh subsets throughout LCMV infection as a point of comparison, I studied whether CD4 Trm in the IEL originate from effector Th1 or Tfh by examining protein expression of subset and Trm markers.

It is unclear whether CD4 memory generation can be linked to a particular helper subset or if memory precursors persist among multiple subsets (1). Examining the transcriptional profile of effector CD4 T cells gives insight to their memory potential. Unique transcriptional networks direct the development of Tfh and Th1 cells. T-bet, STAT1, and STAT4 are TFs that drive differentiation of Th1 lineage cells (9); Bcl6, Irf4, and c-Maf transcriptionally regulate Tfh development (10). Inhibitor of DNA-binding (ID)

proteins and E proteins direct Th subset differentiation (6). These proteins are ubiquitous heterodimers that belong to a class of transcriptional regulators called Helix-Loop-Helix (bHLH) proteins. ID proteins regulate differentiation of CD4 subsets by outcompeting other E proteins from homodimerization, neutralizing their transcriptional activity (11). For example, in *Toxoplasma gondii* infection, Id2 and Id3 possess a push and pull relationship that balances Th1 and Tfh effector polarization (6). Not only is Id3 associated with Tfh programming, but its expression in the circulating CD4 population indicates memory potential and multipotency in both Tfh and Th1 populations (12). Shaw and colleagues observed the frequency of an Id3-expressing memory Th1 population gradually emerge in the CD4 population during late memory time points of LCMV infection. Despite its increasing frequency, the absolute number of this population was established by peak infection (12). Further investigation revealed Id3-expressing memory CD4 T cells can give rise to both Th1 and Tfh in response to reinfection (12). However, the significance of Th linage markers in CD4 Trm establishment during viral infection has yet to be researched, and CD4 Trm programming remains elusive.

During viral infection, Blimp1 drives specific Th programming by actively repressing other lineage driving transcription factors (13). In Th1 cells, Blimp1 knockdown enhanced Tfh related genes, indicating Blimp1 acts as a mechanistic brake for Tfh programming in Th1 cells (14). Under polarizing conditions, such as LCMV infection, Blimp1 attenuates Th17 fate by repressing IL17 receptor (15). In CD4 T cell transfer colitis, Blimp1, along with its homolog, Hobit, were highly expressed in CD4 Trm. An induced KO of both Blimp1 and Hobit significantly decreased exacerbation of

disease, indicating the significance of Blimp1 in colitis related CD4 Trm cells (16). However, Blimp1, along with Id3, has yet to be explored in CD4 Trm formation in the infectious setting.

In CD8 T cells, both Blimp1 and Id3 expression show significance in the Trm population of the SI (17). With single-cell RNA sequencing, Blimp1 and Id3 were identified as markers that are highly expressed among CD8 Trm in the IEL (17). Milner and colleagues demonstrated that Blimp1 expression indicates a TE- like subpopulation while Id3 indicates a MP- like subpopulation. Because Blimp1 and Id3 have roles in CD4 T cell subset differentiation, CD4 memory, and delineating CD8 Trm subpopulations, it could be hypothesized that both Tfh and Th1 possess a transcriptional predisposition to Trm development. I will investigate the role of Id3 and Blimp1 in Trm generation of the IEL during LCMV infection and examine the developmental connection between effector Th1 and CD4 Trm. I hypothesize that effector Th1 and Trm cells will share comparable subset protein and transcription factor expression, which will serve as a foundation for linking development of CD4 Trm to effector Th1 cells. These findings will illuminate the differential pathway for CD4 Trm during viral infection and give researchers potential targets for vaccine development.

Results

We established the kinetics of Th1 and Tfh subsets in SLOs during LCMV infection, which served as a point of reference when investigating the role of circulating effector populations in the development of CD4 Trm in the IEL. Rather than studying LCMV-specific endogenous CD4 T cells, we transferred Smarta (SM) CD4 T cells into congenically distinct mice. SM transgenic T cells possess a TCR that recognizes LCMV epitope GP61-80 and ensures limited variability between experiments due to varying TCR strength. After transfer, we infected with LCMV-Arm, then at effector and memory timepoints of infection, isolated lymphocytes from the spleen (spl), mesenteric lymph nodes (mLN), and IEL. Isolated lymphocytes were then stained with florescent antibodies and cell surface receptor and transcription factor expression by transferred cells was evaluated via flow cytometry (Fig 2a, 3a).

To determine which subset markers Trm expressed, SLAM and CXCR5 expression were evaluated. SLAM expression indicates Th1, while CXCR5 indicates Tfh lineage. Both subset populations were present in circulation during the peak of infection, but only a Th1 population existed in the IEL at this time point (Fig 2b). By memory, Tfh cells were more prominent in the SLOs while Trm cells in the IEL retained express of molecules associated with Th1 subsets, suggesting a connection between effector Th1 cells and Trm generation in the IEL. To distinguish Trm from effector memory cells, resident markers CD69 and CD103 expression were evaluated. In CD8 SI Trm populations, co-expression of residency markers CD69 and CD103 is observed. Surprisingly, this characteristic was not seen in CD4 populations in the IEL, rather, only CD69 was expressed, which still confirmed their Trm phenotype (Fig 2c).

Id3 and Blimp1 drive Tfh and Th1, respectively, during the effector phase, however, it is unknown whether the function of these transcription factors hold the same role in the Trm population. To investigate this, Blimp1-YFP Id3-GFP double reporter (DR) SM CD4 T cells were transferred into congenically distinct mice and infected with LCMV-Arm (Fig 3a). When Blimp1 or Id3 is actively being expressed in a DR cell, YFP or GFP fluorescence, respectively, can be observed. The double reporter system was utilized so transcription factor expression could be evaluated without performing an intracellular stain as these transcription factors are difficult to detect by flow cytometry. At D7, both Blimp1-YFP and Id3-GFP populations were present in the spleen but only Blimp1 was expressed in the IEL. Blimp1 expression declined post contraction and the majority of circulating memory T cell were $Id3^{hi}$. Heterogeneity of Blimp1 expression was observed in the Trm IEL population but remained $Id3^{lo}$ (Fig 3b). Th lineage markers were assessed for the Id3-GFP and Blimp1- YFP populations to relate subset transcription factor expression to subset identification in effector and memory populations. On D7 and D21, nearly all cells in Blimp1-YFP populations of the circulation and IEL expressed Th1 marker SLAM. Conversely, Id3-GFP populations expressed Tfh marker CXCR5. Even in the IEL where Id3 expression is minimal, the majority of cells expressed CXCR5 (Fig 3c). Together, these data recapitulate the roles of Id3 and Blimp1 in the effector stage of infection and raise the question of whether Blimp1 expression may drive Trm formation of the IEL in a Th1-dependent manner.

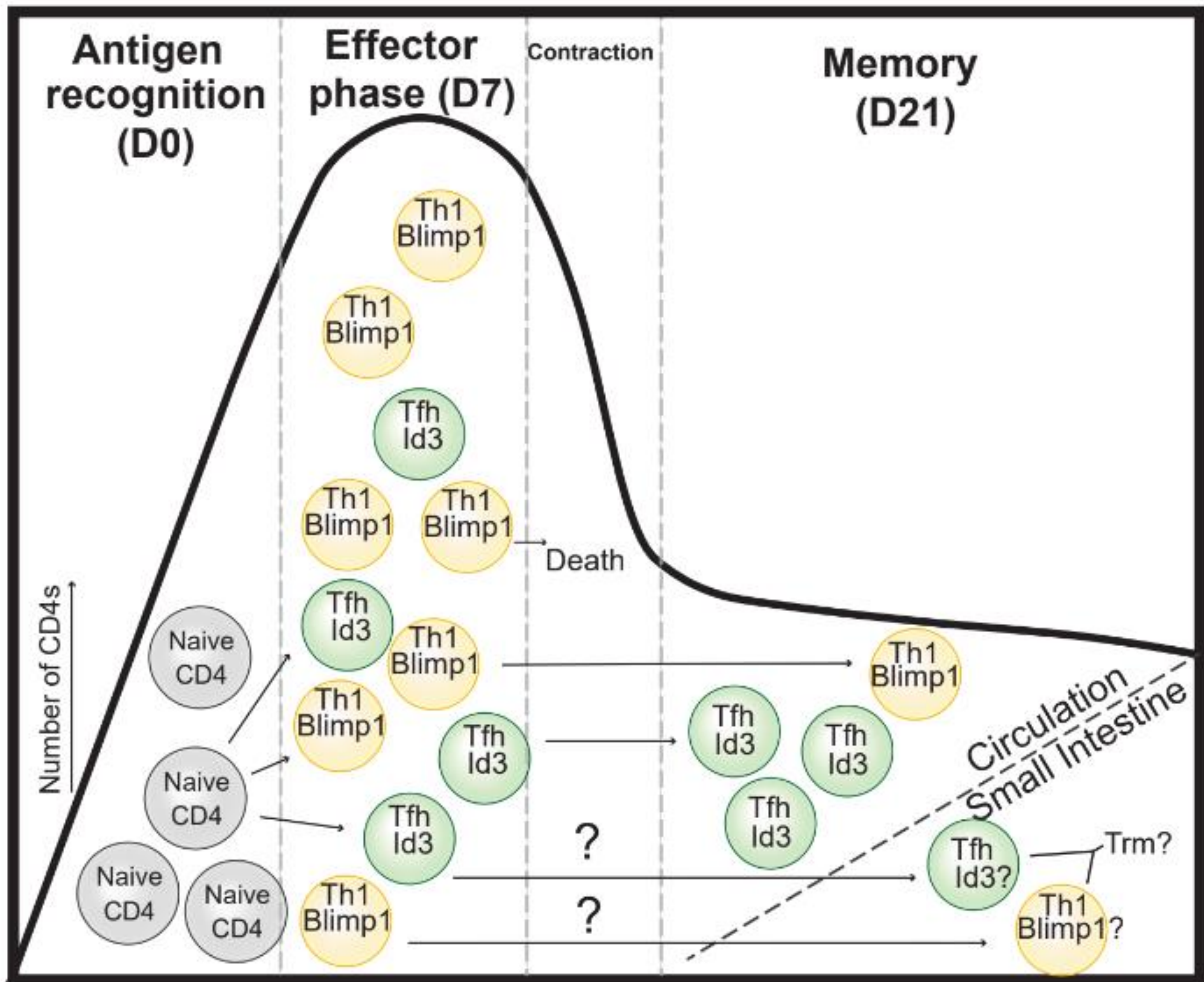
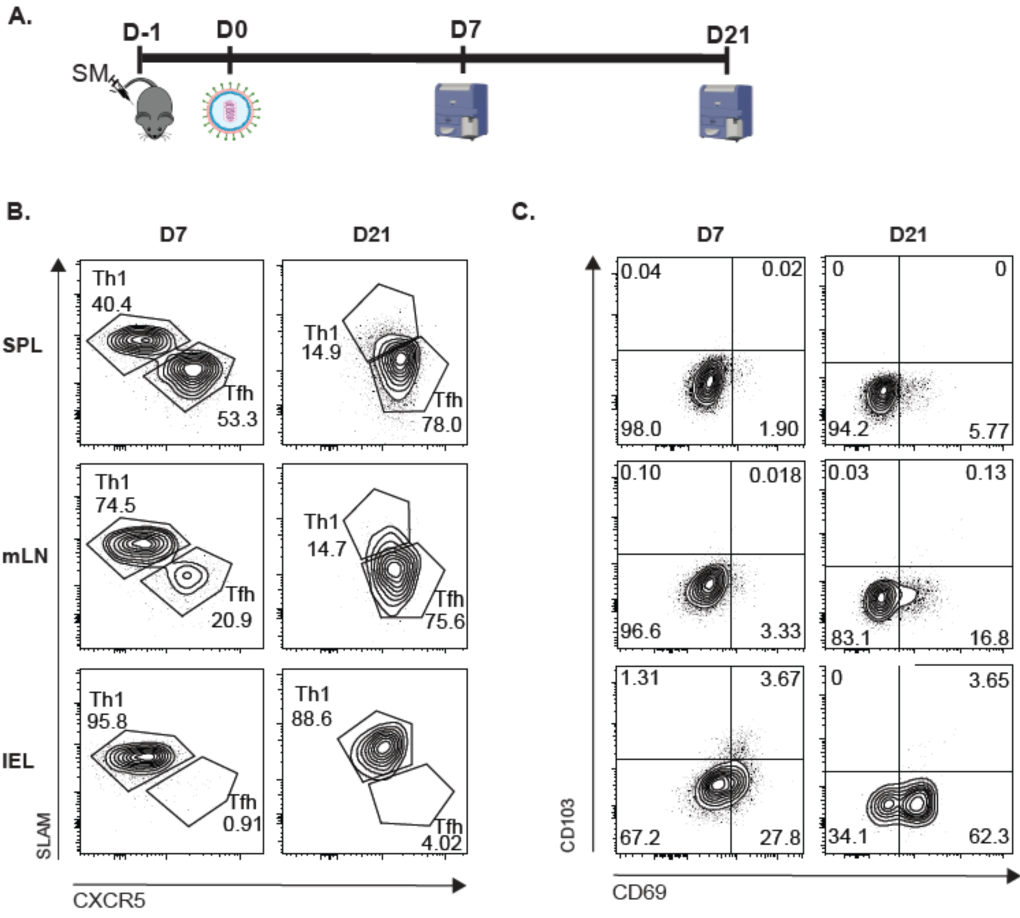
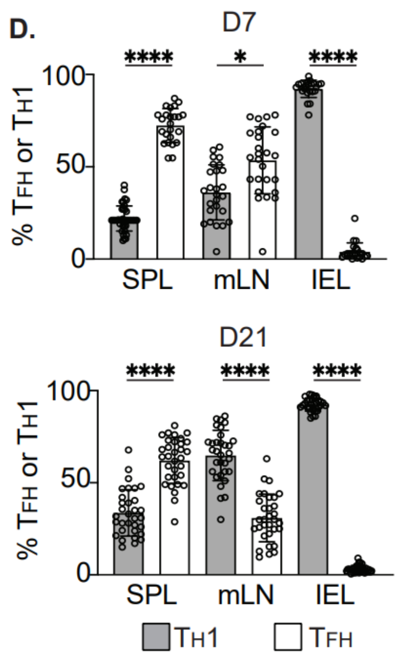


Figure 1. Effector Th1 cells LCMV infection

During LCMV infection, naive CD4 T cells proliferate and differentiate to produce the effector population comprised of Tfh and Th1 subsets. After contraction, it is unknown which of the effector subsets persist as Trm. Blimp1 and Id3 expression in CD4 cells during the effector phase of infection drives Th1 and Tfh lineage. The role of these TFs has yet to be explored in CD4 Trm of the small intestine.

Figure 2. CD4 Trm have comparable phenotype to circulating effector Th1 cells, yet are a distinguishable population.
A. Experimental setup. Naive SM CD4 T cells (SM) were transferred into congenically distinct mice. Mice were then infected with LCMV. D7 and D21 post infection lymphocytes were isolated from the spleen (SPL), mesenteric lymph nodes (mLN) and intra-epithelial layer of the small intestine (IEL), then stained with florescent antibodies and analyzed via flow cytometry. B-C Representative flow cytometry plots of indicated protein expression. Lymphocytes were gated based on FSC-A and SSC-A, and single cells were selected for using FSC-W/H and SSC-W/H. Of CD4 cells, transferred SM cells were selected based on CD45 congenics. B. Expression of Tfh marker chemokine receptor type 5 (CXCR5) and Th1 marker signaling lymphocyte activation molecule (SLAM) in SM populations. C. Expression of Trm markers CD69 and CD103 in SM populations. D. Quantification of Th1 (SLAM) and Tfh (CXCR5) SM T cells on D7 and D21 pi. E. Quantification of Trm markers CD69 and CD103 in SM populations D21 pi. Data are representative (b,c) or cumulative (d,e) of 3 experiments (b-e) with n=2-4 mice, or the average of 3 replicates. Graphs show mean ± SD; $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p< 0.0001$


A.
D-1
D0
D7
D21
SM
B.
D7
D21
SPL
mLN
IEL
Th1
40.4
Tfh
53.3
Th1
14.9
Tfh
78.0
Th1
74.5
Tfh
20.9
Th1
14.7
Tfh
75.6
Th1
95.8
Tfh
0.91
Th1
88.6
Tfh
4.02
SLAM
CXCR5
C.
D7
D21
0.04
0.02
98.0
1.90
0
0
94.2
5.77
0.10
0.018
96.6
3.33
0.03
0.13
83.1
16.8
1.31
3.67
67.2
27.8
0
3.65
34.1
62.3
CD103
CD69


D.
D7
% TFH or TH1
SPL
mLN
IEL
D21
% TFH or TH1
SPL
mLN
IEL
TH1
TFH

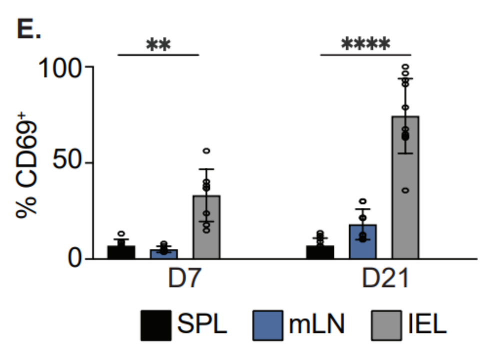
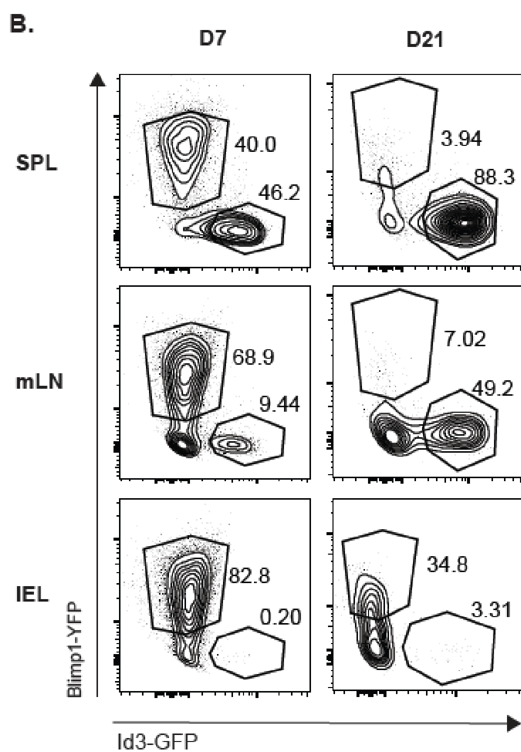
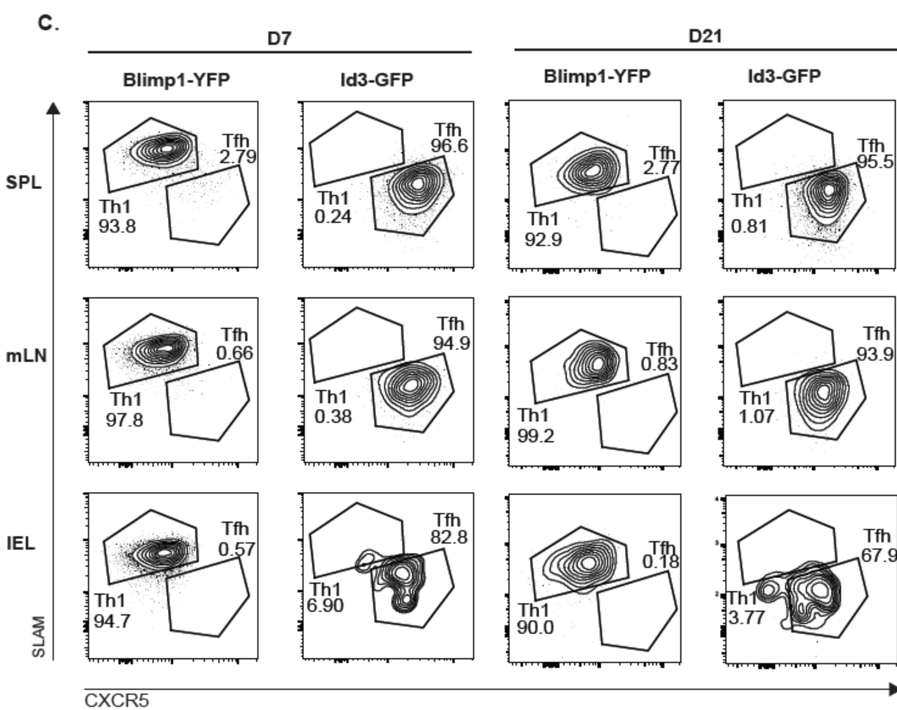
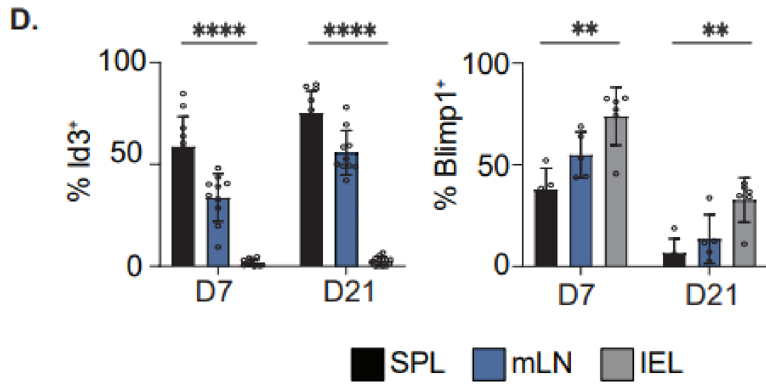

E.
% CD69+
D7
D21
SPL
mLN
IEL

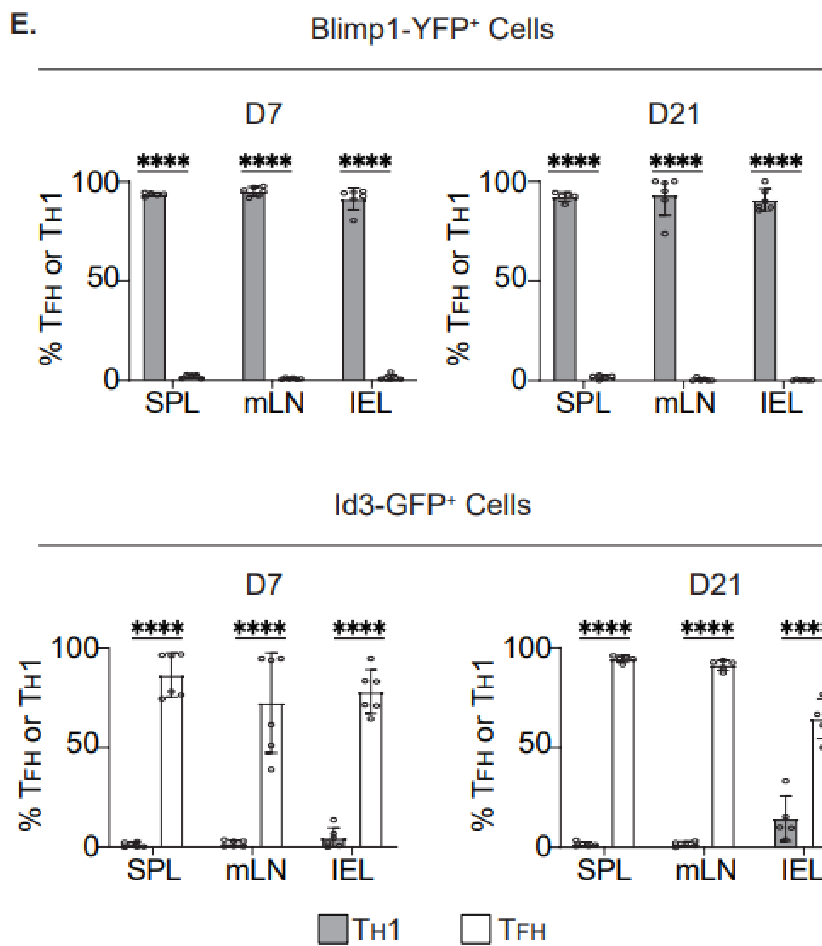
Figure 3. CD4 Trm express effector Th1 related TF, Blimp1.
A. Experimental setup. DR SM CD4 T cells (SM) were transferred into congenically distinct mice. Mice were then infected with LCMV. D7 and D21 post infection lymphocytes were isolated from the spleen (SPL), mesenteric lymph nodes (mLN) and intra-epithelial layer of the small intestine (IEL), then stained with florescent antibodies and analyzed via flow cytometry. B-C Representative flow cytometry plots of indicated protein expression. Lymphocytes were gated based on FSC-A and SSC-A, and single cells were selected for using FSC-W/H and SSC-W/H. Of CD4 cells, transferred SM cells were selected based on CD45 congenics. B. Expression of transcription factors Blimp1 and Id3 via YFP and GFP florescence, respectively, in DR SM populations. C. Expression of CXCR5 and SLAM in Blimp1-YFPhi and Id3-GFPhi populations gated in figure B. D. Quantification of Blimp1-YFP and Id3-GFP expression in SM T cells on D7 and D21 pi. E. Quantification of Th1 (SLAM) and Tfh (CXCR5) SM T cells on D7 and D21 pi. in either Blimp1-YFP or Id3-GFP SM populations D7 and D21 pi. Data are representative (b,c) or cumulative (d,e) of 2 experiments (b-e) with the average of 3 replicates SM populations D21 pi. Graphs show mean ± SD; $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p< 0.0001$

A.
D-1
D0
D7
D21
SM DR


B.
D7
D21
SPL
40.0
46.2
3.94
88.3
mLN
68.9
9.44
7.02
49.2
IEL
82.8
0.20
34.8
3.31
Blimp1-YFP
Id3-GFP


C.
D7
D21
Blimp1-YFP
Id3-GFP
Blimp1-YFP
Id3-GFP
SPL
Tfh 2.79
Th1 93.8
Tfh 96.6
Th1 0.24
Tfh 2.77
Th1 92.9
Tfh 95.5
Th1 0.81
mLN
Tfh 0.66
Th1 97.8
Tfh 94.9
Th1 0.38
Tfh 0.83
Th1 99.2
Tfh 93.9
Th1 1.07
IEL
Tfh 0.57
Th1 94.7
Tfh 82.8
Th1 6.90
Tfh 0.18
Th1 90.0
Tfh 67.9
Th1 3.77
SLAM
CXCR5


D.
****
****
100
50
0
% Id3+
D7
D21
**
**
% Blimp1+
SPL
mLN
IEL


E.
Blimp1-YFP+ Cells
D7
D21
****
% TFH or TH1
100
50
0
SPL
mLN
IEL
Id3-GFP+ Cells
TH1
TFH

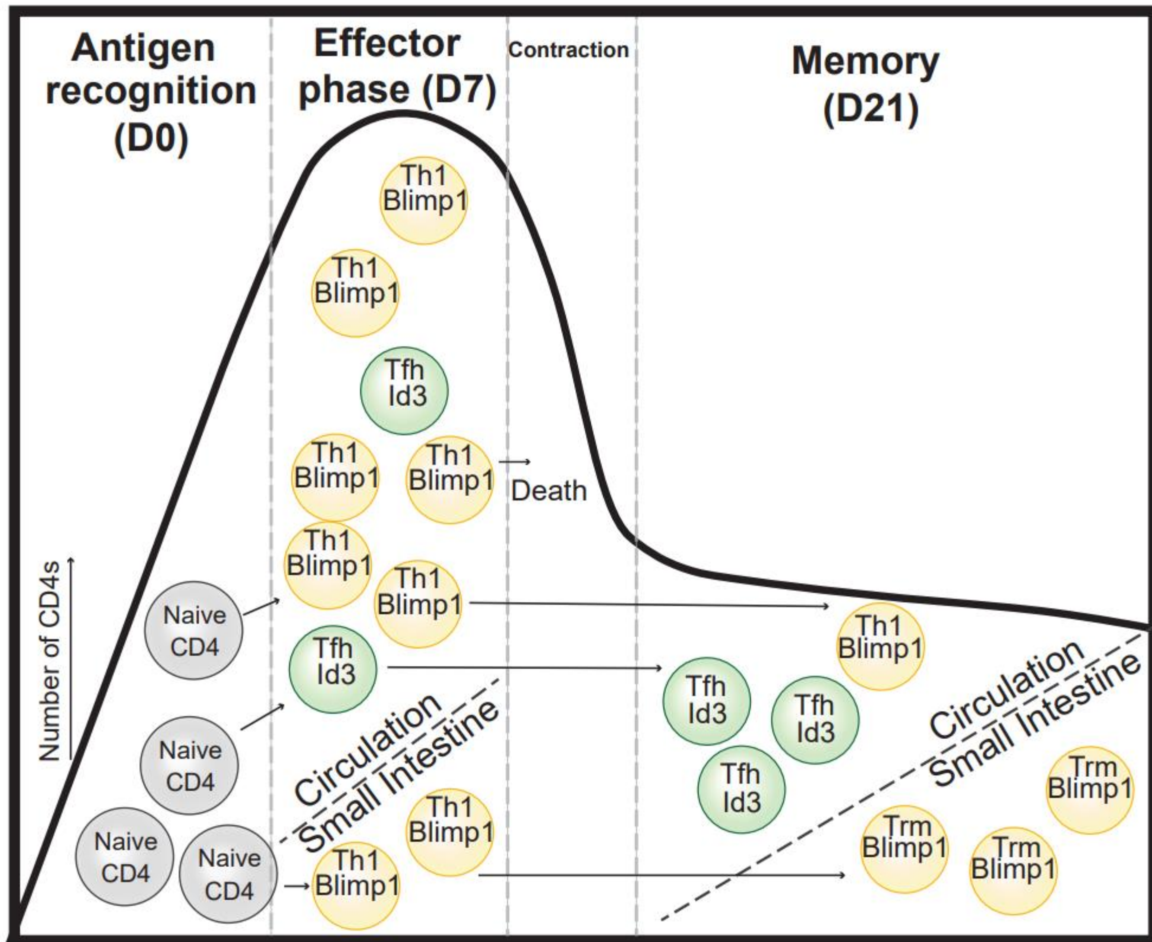


Figure 4. T-helper Type 1 Cells Drive Development of CD4 Tissue Resident Memory in the Small Intestine During Acute Viral Infection

During LCMV infection, naive CD4 T cells proliferate and differentiate to produce the effector population comprised of Tfh and Th1 subsets. Blimp1 expressing Th1 cells accumulate in the SI during peak infection. After contraction, Th1 phenotype and transcriptional programming is retained in the Trm SI population, thus developmentally linking Trm development to circulating Th1 effector cells.

Discussion

CD4 T cells proliferate into effector Th1 and Tfh subsets during acute viral infection, but it is unknown which effector populations give rise to Trm in the SI. My experiments were part of a larger effort to investigate this gap of knowledge in the field. My studies showed the CD4 Trm population in the gut is phenotypically and transcriptionally similar to Th1 effector cells, suggesting they may be a precursor for CD4 Trm in the gut. Further investigation solidified this conclusion through RNA sequencing and establishing clear roles of lineage associated TFs in Trm formation.

Bulk RNA sequencing demonstrated the gene signature of CD4 Trm in the gut had greater enrichment of genes associated with Th1 rather than Tfh. Interestingly, CD4 Trm cells expressed not only Th1 associated TF Blimp1 and Id2, but also Bcl-6 which drives Tfh lineage by antagonizing Th1 programing. RNA velocity analysis demonstrated transcriptional progression of effector splenic CD4 T cells towards effector SI populations, ultimately leading to a mature Trm transcriptional profile in the SI. Not only did the single-cell RNA sequencing show general acquisition of Trm signature in the CD4 population but indicated heterogeneity of effector and memory-associated genes, such as co-expression of Blimp1 and Bcl-6. Both subset lineage TFs were each found to be required for CD4 Trm formation and maintenance, respectively. KO of Blimp1, showed by peak infection, reduction of KO cells was specific to the SI and significantly impaired Trm accumulation, suggesting that Blimp1 promotes the migration of effector cells to SI and subsequently upregulation of the tissue-residency program. However, Id2 KO led to a comparable reduction of KO cells by D7 post infection across the SLOs and SI. By memory timepoints, the population of KO cells in the SI further declined. Together, these data points show the impairment of Trm accumulation in the Id2-KO population is likely

due to importance of Id2 in Th1 differentiation rather than a specific relevance to CD4 Trm formation. Interestingly, Bcl-6-KO initially increased accumulation of CD4 T cells in the SI but this was not sustained into memory and KO cells diminished, indicating that Bcl-6 may act as an inhibitor of the early Trm differentiation program but is necessary for its maintenance.

Recent studies demonstrate parallel results in human Trm populations. Human lymphocytes isolated from lung parenchyma tissues of patients with Mycobacterium tuberculosis show functional connections to CD4 Th1 cells (13). CD4 T cells from the lung that expressed CD103 and CD69 are considered protective Trm because, upon stimulation with TB, they produce anti-bacterial cytokines IFN-γ and TNF-α (13). As these two cytokines are key characteristics of effector Th1 cells, this study, alongside ours, suggests a strong link between effector Th1 and Trm generation. In our study, we showed that effector Th1 and Trm populations shared a phenotypic and transcriptional program. With SLAM expression, Trm resembled an effector Th1 phenotype. The majority of effector cells in the gut expressed Blimp1 and after contraction, some Trm in the gut retained Th1 transcription factor programing. These findings served as a strong foundation for concluding effector Th1 cells give rise to CD4 Trm in the gut. Together these studies propose that in response to intracellular pathogens, Th1 effector cells may be a precursor for Trm populations in not only the lungs and gut, but other tissues as well. However, further investigation of this trend may only be possible once better cell isolation protocols are established as CD4 T cells in other mouse tissues are very sparce, thus difficult to study.

Once it was established that effector Th1 cells may give rise to Trm in the SI, it was necessary to confirm their Trm profile. Th1 cells give rise to effector memory, which surveil both NLTs and circulation. It could be speculated that the population seen in the IEL at the time mice were sacrificed are Th1 effector memory that were surveilling the tissue. However, this is unlikely because as seen in our results, CD4 cells in the IEL expressed CD69 resulting in tissue retention, which is a key difference between effector and tissue resident memory. In order to truly prove a cell is bona fide Trm, a parabiosis study would need to be performed, where two congenically distinct mice are joined together by the skin producing a shared blood stream. Cells that are Trm will remain lodged in the NLTs and not enter the conjoined blood stream. While this type of experiment produces the utmost concrete data, it requires strict animal handling and care that is not available at our vivarium.

Little is known about the tissue environmental factors that drive CD4 Trm differentiation, as they are a more recent discovery when compared to CD8 Trm. However, both CD4 and CD8 Trm reside in the IEL and experience signals specific to that region of tissue. Thus, extrapolation of CD4 Trm development can be hypothesized based on previous findings in CD8 Trm development. CD8 effector cells located in the IEL during peak infection of LCMV have differential gene expression compared to that in the SLOs, suggesting that change in gene expression results from exposure to environmental factors (19). Trm differentiation could be contributed to the environmental signals such as cytokines, hypoxia and nutritional deprivation that effector cells see in the IEL (20). Cytokines that induce CD8 Trm retention are associated with phosphatidylinositol-3-OH kinase (PI(3)K)–Akt- activity, subsequent

downregulation of KLF2 and tissue retention (21). Our study illustrated effector cells in the IEL were Th1 lineage, while Tfh cells remained in the SLO. Perhaps lack of Blimp1 expression and the sheer abundance of LN homing molecules expressed on Tfh, which are needed for interacting with follicular B cells, decrease their time spent in the NLT and they therefore encounter less environmental signals that drive tissue retention in the IEL. In our studies, we saw Bcl-6, is upregulated in CD4 Trm and is crucial for its maintenance. Perhaps cytokines in the SI that trigger (PI(3)K)–Akt- activity lead to induction of Bcl-6 expression and subsequent repression of KLF2, as Bcl-6 has been previously demonstrated to target KLF2 in Tfh populations (22). Learning the unique transcriptional networking of CD4 Trm and how it promotes differentiation could unveil potential targets for developing immunotherapies.

Material and Methods

Mice

All mice were housed under specific pathogen-free conditions in an American Association of Laboratory Animal Care–approved vivarium at the University of California, San Diego (UCSD). C57BL/6 (B6) mice were either purchased from The Jackson Laboratory or bred in house. Smarta mice (JAX stock #030450) were bred in house and possess a TCR that is specific for H2-I-A^b-restricted LCMV epitope GP_{61-80}. Blimp1-YFP Id3-GFP double reporter (DR) (Milner et al. 2020) mice were crossed with WT SM lines to produce DR SM mice.

T Cell Transfer and LCMV Infection

Naive SM WT or DR CD4 T cells were isolated from the spleen and lymph nodes. SM T cells ($1x10^5$) were adoptively transferred via retro-orbital injection into recipient C57Bl/6 mice. Donor T cells and recipients were sex and age matched, yet congenically distinct for CD45 from recipient mice. Twenty-four hours later, recipient mice were infected with $2x10^5$ plaque-forming units (PFU) of lymphocytic choriomeningitis virus-Armstrong (LCMV-Arm) strain, via intraperitoneal injection.

Lymphocyte Isolation from Tissues

Protocol lymphocyte isolation from tissues from Milner et al. 2020 was utilized. Single-cell suspensions were prepared from spleen or lymph node by mechanical disruption. For small intestine (SI) preparations, Peyer's patches were excised, luminal contents were removed, and tissue was cut longitudinally then into 1 cm pieces. For

intraepithelial lymphocyte isolation, the SI pieces were incubated while shaking in 10% HBSS/HEPES bicarbonate solution containing 15.4mg/100μL of dithioerythritol (EMD Millipore) at 37°C for 30 minutes to extract SI-IEL. Cells were then filtered to remove any remaining tissue pieces. Lymphocytes from all tissue but spleen and lymph node were purified on a 44%/67% Percoll density gradient (8).

Antibodies, Flow Cytometry, and Cell Sorting

All antibodies were diluted and combined in Flow Cytometry Staining Buffer (PBS supplemented with 2% bovine growth serum and 0.1% sodium azide). The following antibodies were used for surface staining (all from eBioscience, unless otherwise specified): CD4 (GK1.5), CD45.1 (A20), CD45.2 (104), SLAM (TC15-12F12.2, BioLegend), CD8 (53-6.7, BioLegend), CD69 (H1.2F3, Biolegend), CD103 (2E7). Prior to surface staining, cell suspensions were incubated with biotin conjugated anti-CXCR5 (SPRCL5, 1:50; Invitrogen) for 30 minutes at 4°C. Cells were incubated with surface staining antibodies for 25 minutes at 4°C; secondary antibody PE-Cy7-labeled streptavidin (1:1000, eBioscience) was also included in the surface stain. Stained cells were analyzed using LSRFortessa X-20 cytometer (BD) and FlowJo software (TreeStar). When analyzing DR cells, the optic filter configuration needed to be changed, because YFP and GFP fluorescence will both emit wavelengths in the FITC channel. To distinguish YFP and GFP fluorescence, 515/20-nm Bandpass (BP) + 495-nm Longpass dichroic filter (LP) and 545/35-nm BP + 525-nm LP were utilized.

Work Cited

1. Nguyen, Q. P., Deng, T. Z., Witherden, D. A., & Goldrath, A. W. (2019). Origins of CD4+ circulating and tissue-resident memory T-cells. *Immunology, 157*(1), 3–12. https://doi.org/10.1111/imm.13059

2. Hamalainen, H., Meissner, S., & Lahesmaa, R. (2000). Signaling lymphocytic activation molecule (SLAM) is differentially expressed in human Th1 and Th2 cells. Journal of immunological methods, 242(1-2), 9–19. https://doi.org/10.1016/s0022-1759(00)00200-3

3. Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K., & Oliveira, M. J. (2018). Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Frontiers in immunology, 9, 847. https://doi.org/10.3389/fimmu.2018.00847

4. Marshall, H. D., Chandele, A., Jung, Y. W., Meng, H., Poholek, A. C., Parish, I. A., Rutishauser, R., Cui, W., Kleinstein, S. H., Craft, J., & Kaech, S. M. (2011). Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity,* 35(4), 633–646. https://doi.org/10.1016/j.immuni.2011.08.016

5. Choi, Y. S., Yang, J. A., Yusuf, I., Johnston, R. J., Greenbaum, J., Peters, B., & Crotty, S. (2013). Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. Journal of Immunology (Baltimore, Md. : 1950), 190(8), 4014-4026. https://doi.org/10.4049/jimmunol.1202963

6. Shaw, L. A., Bélanger, S., Omilusik, K. D., Cho, S., Scott-Browne, J. P., Nance, J. P., Goulding, J., Lasorella, A., Lu, L. F., Crotty, S., & Goldrath, A. W. (2016). Id2 reinforces TH1 differentiation and inhibits E2A to repress TFH differentiation. *Nature Immunology*, 17(7), 834–843. https://doi.org/10.1038/ni.3461

7. Amezcua Vesely, M. C., Pallis, P., Bielecki, P., Low, J. S., Zhao, J., Harman, C., Kroehling, L., Jackson, R., Bailis, W., Licona-Limón, P., Xu, H., Iijima, N., Pillai, P. S., Kaplan, D. H., Weaver, C. T., Kluger, Y., Kowalczyk, M. S., Iwasaki, A., Pereira, J. P., Esplugues, E., … Flavell, R. A. (2019). Effector TH17 Cells Give Rise to Long-Lived TRM Cells that Are Essential for an Immediate Response against Bacterial Infection. *Cell*, 178(5), 1176–1188.e15. https://doi.org/10.1016/j.cell.2019.07.032

8. Glennie, N. D., Yeramilli, V. A., Beiting, D. P., Volk, S. W., Weaver, C. T., & Scott, P. (2015). Skin-resident memory CD4+ T cells enhance protection against

Leishmania major infection. *The Journal of Experimental Medicine*, 212(9), 1405–1414. https://doi.org/10.1084/jem.20142101

9. Oestreich, K. J., & Weinmann, A. S. (2012). Transcriptional mechanisms that regulate T helper 1 cell differentiation. *Current Opinion in Immunology*, 24(2), 191–195. https://doi.org/10.1016/j.coi.2011.12.004

10. Liu, X., Nurieva, R. I., & Dong, C. (2013). Transcriptional regulation of follicular T-helper (Tfh) cells. *Immunological Reviews*, 252(1), 139–145. https://doi.org/10.1111/imr.12040

11. Wang, Lan-Hsin, and Nicholas E Baker. “E Proteins and ID Proteins: Helix-Loop-Helix Partners in Development and Disease.” *Developmental Cell* vol. 35,3 (2015): 269-80. doi:10.1016/j.devcel.2015.10.019

12. Shaw, L. A., Deng, T. Z., Omilusik, K. D., Takehara, K. K., Nguyen, Q. P., & Goldrath, A. W. (2022). Id3 expression identifies CD4+ memory Th1 cells. Proceedings of the National Academy of Sciences, 119(29). https://doi.org/10.1073/pnas.2204254119

13. Thomas Ciucci, Melanie S. Vacchio, Ting Chen, Jia Nie, Laura B. Chopp, Dorian B. McGavern, Michael C. Kelly, Rémy Bosselut; Dependence on Bcl6 and Blimp1 drive distinct differentiation of murine memory and follicular helper CD4+ T cells. *J Exp Med* 3 January 2022; 219 (1): e20202343. doi: https://doi.org/10.1084/jem.20202343

14. Oestreich, K. J., Mohn, S. E., & Weinmann, A. S. (2012). Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. Nature immunology, 13(4), 405–411. https://doi.org/10.1038/ni.2242

15. Cimmino, L., Martins, G. A., Liao, J., Magnusdottir, E., Grunig, G., Perez, R. K., & Calame, K. L. (2008). Blimp-1 attenuates Th1 differentiation by repression of ifng, tbx21, and bcl6 gene expression. Journal of immunology (Baltimore, Md. : 1950), 181(4), 2338–2347. https://doi.org/10.4049/jimmunol.181.4.2338

16. Zundler, S., Becker, E., Spocinska, M., Slawik, M., Parga-Vidal, L., Stark, R., Wiendl, M., Atreya, R., Rath, T., Leppkes, M., Hildner, K., López-Posadas, R.,

Lukassen, S., Ekici, A. B., Neufert, C., Atreya, I., van Gisbergen, K., & Neurath, M. F. (2019). Hobit- and Blimp-1-driven CD4+ tissue-resident memory T cells control chronic intestinal inflammation. Nature immunology, 20(3), 288–300. https://doi.org/10.1038/s41590-018-0298-5

17. Milner, J. J., Toma, C., He, Z., Kurd, N. S., Nguyen, Q. P., McDonald, B., Quezada, L., Widjaja, C. E., Witherden, D. A., Crowl, J. T., Shaw, L. A., Yeo, G. W., Chang, J. T., Omilusik, K. D., & Goldrath, A. W. (2020). Heterogenous Populations of Tissue-Resident CD8+ T Cells Are Generated in Response to Infection and Malignancy. *Immunity*, 52(5), 808–824.e7. https://doi.org/10.1016/j.immuni.2020.04.007

18. Yang, Q., Zhang, M., Chen, Q., Chen, W., Wei, C., Qiao, K., Ye, T., Deng, G., Li, J., Zhu, J., Cai, Y., Chen, X., & Ma, L. (2020). Cutting Edge: Characterization of Human Tissue-Resident Memory T Cells at Different Infection Sites in Patients with Tuberculosis. *Journal of Immunology* (Baltimore, Md. : 1950), 204(9), 2331-2336. https://doi.org/10.4049/jimmunol.1901326

19. Milner, J. J., & Goldrath, A. W. (2018). Transcriptional programming of tissue-resident memory CD8+ T cells. *Current Opinion in Immunology*, 51, 162–169. https://doi.org/10.1016/j.coi.2018.03.017

20. Chang, J. T., Wherry, E. J., & Goldrath, A. W. (2014). Molecular regulation of effector and memory T cell differentiation. *Nature Immunology*, 15(12), 1104–1115. https://doi.org/10.1038/ni.3031

21. Skon, C. N., Lee, J. Y., Anderson, K. G., Masopust, D., Hogquist, K. A., & Jameson, S. C. (2013). Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. Nature immunology, 14(12), 1285–1293. https://doi.org/10.1038/ni.2745

22. Choi, J., Diao, H., Faliti, C. E., Truong, J., Rossi, M., Bélanger, S., Yu, B., Goldrath, A. W., Pipkin, M. E., & Crotty, S. (2020). Bcl-6 is the nexus transcription factor of T follicular helper cells via repressor-of-repressor circuits. Nature immunology, 21(7), 777–789. https://doi.org/10.1038/s41590-020-0706-5