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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences Immunology

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The Maintenance of CD4 and CD8 T Cell Response to Persistent Antigens by Yu Xia

> A dissertation presented to The Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

> > August 2022 St. Louis, Missouri

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Acknowledgments

The completion of this work could not have been possible without the guidance and support from family, friends and colleagues.

First, I would like to thank my thesis mentor, Dr. Takeshi Egawa. Dr. Egawa values training both in terms of benchwork skills and in terms of critical thinking. He is always there when I need help, and pushes me to do better. The attitude with which he treats his job and people around him will always inspire me. I would like to thank our collaborator, Dr. Ansuman Satpathy at Stanford, for his expertise and for offering encouragement as well as constructive criticism. I would also like to thank the members of my thesis committee, Drs. Brian Edelson, Christina Stallings, Haina Shin, Chyi-Song Hsieh and Wayne Yokoyama, as well as Dr. Kenneth Murphy who served as a committee member in the early phase of my thesis study. They enabled me to view my projects from a different perspective, and they were always willing to give advice and feedback.

I would also like to thank past and present members of the Egawa lab. I would like to thank Saran Raju, for being my graduate student mentor. He challenged me to think like a scientist, and to pay attention to both the tree and the forest. I would like to thank Elena Tonc, for her guidance and help both within and outside of lab. I would also like to thank the current members, Sunnie Hsiung, Maegan Murphy, and Tenzin Yangdon for being my friends and for their feedback on my work. Acknowledgement of specific individuals outside of my lab who were involved in the work are at the end of each chapter.

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Finally, I would like to thank my support system outside of lab. I would also like to thank my friends within the MD/PhD and PhD programs, for going through the training with me together, as well as my friends in St. Louis and around the globe, who make the time to check in on me and do fun things with me. I would like to thank my parents for always believing in me and being my safe haven. I would also like to thank my fiancé, Yi'ou Wang, who takes great pride in me and offers support in every aspect, is willing to take adventures with me, big or small, and cheers me on when things do not look that bright (with occasional help from our dog Ada).

Yu Xia

Washington University in St. Louis August 2022

ABSTRACT OF THE DISSERTATION

Maintenance of CD4 and CD8 T Cell Response to Persistent Antigens by

Yu Xia

Doctor of Philosophy in Biology and Biomedical Sciences

Immunology

Washington University in St. Louis, 2022

Professor Takeshi Egawa, Chair

In response to transient antigen presence in the context of acute infections or vaccinations, antigen-specific naïve T cells clonally expand and differentiate. Most of the expanded cells undergo terminally differentiation and die following antigen clearance to reestablish immune homeostasis. In contrast, when the pathogen persists, such as in the context of chronic viral infections or anti-tumor immunity, while T cells undergo alternative differentiation known as exhaustion due to their reduced functionality, T cells still contribute to pathogen control and its response is sustained for longer duration due to unknown mechanisms. The goal of my thesis work is to understand how protective T cell response is sustained at the cellular levels and molecular levels for CD8 T cells and CD4 T cells in the presence of persistent antigens. The main model used is the mouse chronic lymphocytic choriomeningitis virus (LCMV-clone 13, c13) infection model, which causes viremia that lasts 3-4 months in immunocompetent mice and is subsequently resolved in a manner dependent on CD4 and CD8 T cells.

In the analyses of CD8 T cell responses, I identified previously undescribed populations of CD8 T cells that express the chemokine receptor CX3CR1 and further segregated into two populations based on expression of the inhibitory receptor TIM3. CX3CR1⁺ TIM3⁺ cells, despite the expression of inhibitory receptors, maintain proliferative states and express effector molecules, thus contributing to antiviral responses to the persisting virus. In contrast, the TIM3⁻ CX3CR1⁺ CD8 T cells are quiescent, require the transcription factor T-bet, express low but significant levels of TCF-1, and act as immediate precursors for the TIM3⁺ CX3CR1⁺ CD8 T cells. These findings reveal hierarchical differentiation of exhausted CD8 T cells which transit from the previously reported TCF-1^{hi} stem-like progenitor (TPEX) state, to T-bet^{hi} intermediate progenitor (TIM3⁺ CX3CR1⁺) state, to activated effector (TIM3⁺ CX3CR1⁺) state, which mediates sustained CD8 T cell immunity to persistent viral infection.

In studies of CD4 T cell responses, I demonstrated that in addition to the differentiated TH1 and follicular helper (Tfh) cells, activated CD4 T cells also differentiate into memory-like cells in a BCL6-dependent manner. While the initial TH1 cells and Tfh cells are generated through independent developmental pathways directly from naïve T cells, as infection persists, TH1 cells and Tfh cells are both generated from this newly identified memory-like cells, which is required to maintain both TH1 and Tfh responses for prolonged duration. In response to tumors, an analogous CD4 T cell population develops in draining lymph nodes. These findings reveal the heterogeneity and plasticity of CD4 T cells upon encountering persistent antigen and highlights their population dynamics through a stable and bipotent intermediate state.

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Chapter 1: Introduction

1.1 Overview of the immune system

Organisms big and small have evolved ways to defend against invading pathogens. Even prokaryotes have evolved molecular mechanisms that help them fend off invaders such as phages. An example is CRISPR-Cas, a microbial defensive strategy used by bacteria and archaea that uses RNA-guided nucleases to cleave foreign genetic elements¹. Organisms that are more complicated have evolved more sophisticated tools and strategies to protect themselves from a whole array of pathogens from virus to bacteria to fungi, and the system that enables the multifaceted protective response is termed the immune system, which consists of a variety of effector cells and molecules.

The first line of defense against pathogens are not made of immune cells, but rather physical and chemical barriers that keep most pathogenic microbes from entering into the body and becoming pathogenic. The skin, mucosa and epithelium provide a physical barrier that ward off many pathogens, and their barrier function is made obvious in patients with damaged skin or mucosa tissue, who are under high risk for infections. In addition, the skin and mucosa secret broad-spectrum antimicrobial proteins that act as natural antibiotics. This physical and chemical barrier is often enough to protect the host against pathogens. Sometimes, however, the pathogen can successfully bypass these barriers and gain entrance into the organism, which alerts and activates the immune system.

The immune system consists of the innate immune system and the adaptive immune system, with the former acting faster by recognizing general pathogen- or damage-related patterns and the latter mounted slower with more specificity and immune memory formation.

Both innate and adaptive immune responses depend on the white blood cells, or leukocytes, some of which circulate in the bloodstream or patrol in a specialized immune structure called the lymphatic system, and some of which are stationed within specific tissues and organs. Leukocytes are mostly derived from the hematopoietic system stem cells (HSCs) in the bone marrow, and can be segregated into two categories, namely, the myeloid lineage and the lymphoid lineage. Cells from the myeloid lineage comprise a majority of the innate immune system although some players are derived from the lymphoid lineage, and the rest of the cells from the lymphoid lineage make up the adaptive immune system.

The innate immunity functions as the second line of defense against pathogens. Upon barrier breach, complement gets quickly involved which, together with antibodies, can directly lyse the invading microbe². Macrophages and dendritic cells (DCs) act as the first cell responders, which is made possible by their location in the barrier tissues and their expression of specialized microbial-detecting sensors called pattern-recognition receptors (PRRs), which allow them to recognize molecular structures shared among a variety of pathogens called pathogenassociated molecular patterns (PAMPs)³. One example of PAMPs is lipopolysaccharide (LPS), which is commonly seen in Gram negative bacteria. Following PAMP recognition, macrophages secrete cytokines and chemokines that leads to the recruitment of more innate immune cells, such as granulocytes (include neutrophils, eosinophils and basophils), innate lymphoid cells (ILCs), and natural killer cells. Many of these cells are also equipped with sensors for danger signals, such as PAMPs and, in the case of NK cells, decrease of self-antigen presentation which is a common feature of many virally infected cells⁴. These recruited innate immune cells carry various effector functions, such as cell killing or production of cytokines with direct

antimicrobial activity. Together, the innate immune cells get quickly activated and protect the host from the majority of microbial invasion.

Sometimes, the innate immune system is insufficient to clear the pathogen, and the adaptive immune response is required. The immune cells that mediate the adaptive immunity are B lymphocytes and T lymphocytes, which carry unique B cell receptors or T cell receptors (BCR and TCR) that allow them to recognize unique molecules expressed by a particular pathogen with high specificity. The BCRs and TCRs are results of gene segment rearrangement called V(D)J recombination⁵, which yields an astoundingly high number of BCRs and TCRs. Upon BCR or TCR activation, the naïve B cells or T cells clonally expand and differentiate into cells with various effector functions.

Many B cells become antibody-secreting cells, and the antibody is a secreted form of the BCR that binds antigens in the extracellular department for marking of infected cells for destruction or neutralization of the pathogen to prevent its entry into tissues. The breadth of BCR goes beyond that produced by V(D)J recombination alone, as many activated B cells are selected to go through more genetic mutations carried out in a programmed process called somatic hypermutation in an effort to generate BCRs of higher affinity⁶.T cells, on the other hand, only carry membrane-bound TCRs that recognize peptides presented by major histocompatibility complex (MHC) or MHC-like molecules. Depending on the types of TCR chains, T cells can be categorized as alpha beta T cells or gamma delta T cells. Alpha beta T cells are found across the tissues and can be either CD4 or CD8 T cells, with CD4 and CD8 being the co-receptor for the TCR; gamma delta T cells are found primarily in epithelium and mucosa, and lack expression of the CD4 or CD8 co-receptors. CD4 T cells secrete cytokines and provide contact-dependent help to coordinate the overall immune response in addition to exert direct antimicrobial functions⁷.

CD8 T cells differentiate into cells with cytotoxic functions, thus also called cytotoxic T lymphocytes (CTLs), that lyse cells carrying intracellular pathogens⁸.

With the production of high-affinity antibodies and clonal expansion of antigen-specific T cells that carry various effector functions, the adaptive immunity is able to control the pathogen to achieve partial control or total eradication.

1.2 T cell activation and antigen recognition

After development in the thymus, naive T cells reach the bloodstream and traffic to the T cell zone of secondary lymphoid organs (SLOs) including the spleen and lymph nodes. Naive T cells continuously circulate between blood and those SLOs in search for its cognate antigen9. Upon antigen recognition via peptide: MHC (p: MHC) presentation by DCs under the appropriate context, which occurs in the SLOs, the naive T cells get activated (called priming) and undergo clonal expansion and differentiation to generate effector T cells.

The activation of T cells of DCs is a tightly regulated process, which requires the presence of 3 different signals: binding of the cognate p:MHC to the TCR (Signal 1); co-stimulation provided by DCs (Signal 2)¹⁰, which can only occur after DCs have matured due to inflammatory input and is a sign of uncontrolled pathogen invasion; and cytokines provided by DCs as well as other innate and adaptive cells types (Signal 3)¹¹ which carries instructive information on what kind of immune response is appropriate to mount. T cells that have received only signal 1 will remain anergic. In this way, the adaptive immune response is tailored to the needs of the host when and only when there is pathogen invasion.

CD4 T cells and CD8 T cells differ in one important regard, the type of MHC molecule that they can recognize at priming and during effector phase. CD4 T cells recognize peptides

presented on MHCII and CD8 T cells recognize peptides presented on MHCI. The two kinds of MHC molecules differ in the source of peptides loaded onto them and their expression pattern among different types of cells. The source of peptides will dictate what kind of antigens CD8 or CD4 T cells can see, and the expression patterns of MHC I or MHC II will dictate what kind of cells CD8 or CD4 T cells can recognize¹².

MHC I and MHC II have different loading mechanisms for peptides. Peptides loaded onto MHC I are products of proteasome-mediated degradation, which means that MHC I allows presentation of cytosolic proteins, such as mutated proteins from a tumor cells or viral proteins for an infected cell, to cytotoxic CD8 T cells that have been primed by DCs, and be killed subsequently. MHCII, on the other hand, is used to load epitopes derived from proteins in membrane-enclosed vesicles, which might contain intracellular pathogens such as mycobacteria and antigens taken from the extracellular space such as dead cells. The loading of intravesicular and extracellular antigens onto MHCII allows CD4 T cells to recognize a wide range of pathogens that are not accessible to the cytosol. This way, CD4 and CD8 T cells have a division of labor in terms of the source of antigens they recognize.

MHC I and MHC II also have different expression patterns among different cell types. Although only DCs can prime CD4 and CD8 T cells, more cell types can express MHC II and/or MHC I to present peptides and engage the effector function of CD4 T and CD8 T cells post priming. MHC II is expressed only on professional antigen presenting cells (APCs), including DCs, macrophages, and B cells, which means that only these cells can engage primed CD4 T cells to get help. This design allows a tight regulation of the delivery of CD4 T cell help, which is desirable considering the multifaceted role CD4 T cells play in coordinating the overall immune response. MHCI, on the other hand, is expressed on all nucleated cells, which allows

presentation of peptide to primed CD8 T cells in all tissues and almost all cell types in the body, a beneficial feature as it allows removal of any nucleated cell that has been infected or has mutated. Thus, CD4 and CD8 T cells act on different cell types to exert their effector or helper functions.

After priming, T cells need to traffic to the site of infection, which is achieved through chemokine and chemokine receptor pairing. Primed T cells upregulate receptors that allow them to enter into the bloodstream and subsequently the site of inflammation. Entry into the bloodstream is facilitated by sphingosine-1-phosphate (S1P)- S1P receptor (S1PR) interaction, where the blood maintains a high level of S1P which draws in primed T cells that have upregulated S1PR as a consequence of activation¹³. Tissue and innate immune cells at the site of infection produce chemokines that recruit T cells in a context-specific manner¹⁴. Once T cells enter the tissue, they recognize cognate p:MHC II either on the surface of professional APCs for CD4 T cells, or on the surface of infected nucleated cells, hematopoietic or non-hematopoietic, for CD8 T cells, to exert help or effector functions.

In summary, T cell response is highly regulated following pathogen invasion. T cell priming is tightly controlled to allow prompt activation when needed and avoidance of unnecessary activation to maintain homeostasis. Engagement of primed T cells with cognate p:MHC is controlled by design so that CD4 help is limited to professional APCs and infected/mutated cells across all tissues can be promptly removed by CD8 T cells. Regulation at the priming phase and later in the effector phage allow mounting of a T cell response that's appropriate in terms of magnitude and quality.

1.3 T cell immunity in acute infection and vaccination models

CD8 T cells following priming develop into cells with various degrees of cytotoxicity and memory potentials^{15,16}. This heterogeneity is at least partially achieved during priming, where the DCs present p:MHC to the CD8 T cells in a structure called the immune synapse¹⁷. It has been shown that the pole proximal to the immune synapse and the pole distal to it of the CD8 T cells have differential distribution of surface and intracellular molecules. Consequently, after the first cell division, the two daughter cells acquire an unequal amount of cytokine receptors and effector molecules. At the height of clonal expansion, some CD8 T cells express elevated levels of transcription factors RUNX3, T-bet and Eomesdermin (Eomes) that promote a cytotoxic phenotype, and express high level effector molecules IFN γ , granzymes and perform^{15,18,19}. In contrast, some CD8 T cells exhibit lower cytotoxicity and higher levels of molecules associated with memory cells such as the chemokine receptor IL7Ra and pro-survival transcription factor Bcl-2²⁰. Following antigen clearance, the highly cytotoxic CD8 T cells quickly contract and are thus termed as short-lived effector cells (SLECs). Whereas the less cytotoxic effector CD8 T cells that express memory markers contract less and are better at generating memory, and these effector cells are thus called memory precursor cells (MPECs). MPECs give rise to memory cells that can survive without antigen stimulation and quickly proliferate and differentiate into new SLECs and MPECs upon secondary antigen encounter. In this way, the CD8 T cell response achieves both prompt antigen control and memory formation upon pathogen invasion.

CD4 T cells play a central role in coordinating innate and adaptive immunity and achieve this goal through a high level of plasticity, which is the ability to differentiate down multiple pathways². In response to signals through TCR, co-stimulation, and cytokines provided by DCs, CD4 T cells differentiate into subtypes that provide contact-dependent help or context-specific

cytokines to coordinate response of the innate immune cells, CD8 T cells, as well as B cells. CD4 T cells that specialize in providing B cell help migrate from the T cell zone to the B cell zone and are called follicular helper T (Tfh) cells²¹, where they promote the expansion and affinity maturation of B cells which eventually leads to high-affinity neutralization antibody production. Differentiated non-Tfh CD4 T cells are collectively called non-Tfh effector cells, and depending on the types of infection and the cytokine milieu during priming, the effector CD4 T cells can take the form of a Th1, Th2 or Th17, which mounts appropriate response against intracellular pathogens, helminth/parasites, or extracellular pathogens, respectively. Like CD8 T cells, the antigen-specific CD4 T cell population also contracts and gives rise to memory CD4 T cells following antigen clearance^{22,23}. Memory CD4 T cells, like memory CD8 T cells, are capable of antigen-free survival as well as prompt proliferation and differentiation upon rechallenge.

In summary, the T cell response following acute infection or vaccination accomplish two goals, to generate enough cells with effector functions that allow prompt pathogen control, and to produce memory that protects against future encounters with the same pathogen.

1.4 T cell response in the presence of persistent antigens

T cell responses differ when the antigens persist for an extended period of time in scenarios such as chronic hepatitis C virus (HCV) infection for humans, chronic strains of lymphocytic choriomeningitis virus (LCMV) for mice, transplantations, and various kinds of tumors. In these cases, T cells are under persistent antigen exposure and undergo an alternative differentiation pathway^{24–27}.

The altered CD8 T cell response in the presence of persistent antigens has been heavily studied and is termed exhaustion, which is thus named based on their prolonged expression of

inhibitory receptors such as PD-1 and TIM3, decreased cytokine production such as IFN- γ and TNF, and blunted proliferation compared to their acute counterparts during the chronic phase of the infection^{28–31}. Like cytotoxicity for CD8 T cells during the acute infections, exhaustion also exists as a spectrum with CD8 T cells occupying various positions on that spectrum²². While some CD8 T cells are terminally exhausted with multiple inhibitory receptor expression and little turnover, some CD8 T cells are less exhausted and carry more effector functions. Despite the connotation of the term exhaustion, a protective role of the exhausted CD8 T cells have been demonstrated, as depletion of CD8 T cells has been shown to exacerbate infection control and tumor control in various models^{31,33}. In summary, exhausted CD8 T cells are a heterogeneous group and despite their name those exhausted CD8 T cells are required for active pathogen control.

In recent years, the mechanism of the durable CD8 T cell response is in part worked out through the discovery of a memory-like CD8 T cell population (progenitor exhausted, or TPEX)^{34,35}. TPEX are less exhausted, more quiescent, and require the transcription factor TCF-1 which is required for CD8 memory formation. This CD8 subset mostly resides in the T cell zone as opposed to the red pulp in the spleen and is thought to be more shielded from antigen exposure³⁵. They demonstrate superior proliferation and differentiation capacity in a transfer setting, and, importantly, PD-1 blockade acts on the TPEX population by facilitating their mobilization and differentiation into cells with more effector functions. In contrast to TPEX, CD8 T cells that have lost TCF-1 expression acquire a higher cytotoxic or exhaustion phenotype. Because they are terminally differentiated and contract quickly³⁵, those TCF-1⁻ cells are thought as short-lived like SLECs and need constant replenishment. Based on those observations, the TPEX cells are thought to replenish the more exhausted CD8 T cell pool, thus maintaining a

durable CD8 T cell response in the presence of persistent antigens. This progenitor-progeny relationship is initially worked out in LCMV-c13, and subsequently has been observed in tumors and autoimmunity models³⁶.

However, much remains to be learned about the CD8 T cell response in the presence of persistent antigens. If TCF-1⁺ TPEX is quiescent in vivo, it is unlikely that there is frequent mobilization from the TPEX to the more exhausted cells, which leaves the question how exactly CD8 T cell dynamics is maintained. It is also unclear what the CD8 subset are that carry more effector functions. My first part of the thesis project attempts to tackle this question of CD8 population dynamics and heterogeneity.

Similar to CD8 T cells, activated CD4 T cells are also required for pathogen control and demonstrate altered phenotypes in the presence of chronic antigens. Our current knowledge of the altered CD4 T cell response is largely derived from studies using LCMV-c13. Compared to their acute counterparts, the inhibitory receptor expression of CD4 T cells is higher, leading some groups to also describe them as being exhausted^{37,38}. Although producing less IL-2 compared to their acute counterparts and thus delivering less help to CD8 T cells and effector CD4 T cells, these CD4 T cells produce a higher level of IL-21, which help maintain the CD8 T cell response that is required for the control of the virus³⁹⁻⁴¹. CD4 T cells in chronic infection also produce more IL-10, which is thought to be driven by the transcription factor BLIMP-1 that promotes terminal effector differentiation and antagonizes the transcription factor BCL-6⁴². IL-10 is thought to be immunoregulatory and mice lacking IL-10 are capable of clearing LCMV-c13 within the first 9 days post infection⁴³. However, it is unclear if IL-10 might play some beneficial role once the chronic infection sets in by mechanisms such as protecting the hosts from overt immunopathology or preserve the CD8 T cells from getting more exhausted, a phenomenon that

has been described in anti-tumor immunity and another chronic strain of IL-10^{44–46}. There is also a bias toward cells that express Bcl6 and CXCR5⁴⁷, in line with a need for continued Tfh activity that promotes production of high-affinity neutralizing Abs, which is required for the eventual clearance of the virus. Aside from those aforementioned info, little is known about how the CD4 T cell response to persistent antigens is coordinated or how such a response is maintained, despite the crucial role CD4 T cells play in chronic viral infections, which is what motivates the second part of my thesis project.

References

- Barrangou, R. The roles of CRISPR-Cas systems in adaptive immunity and beyond. *Curr. Opin. Immunol.* 32, 36–41 (2015).
- Dunkelberger, J. R. & Song, W.-C. Complement and its role in innate and adaptive immune responses. *Cell Res.* 20, 34–50 (2010).
- Janeway, C. A., Jr & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216 (2002).
- Raulet, D. H. Missing self recognition and self tolerance of natural killer (NK) cells. *Semin. Immunol.* 18, 145–150 (2006).
- 5. Roth, D. B. V(D)J Recombination: Mechanism, Errors, and Fidelity. *Microbiol Spectr* 2, (2014).
- Nina Papavasiliou, F. & Schatz, D. G. Somatic Hypermutation of Immunoglobulin Genes: Merging Mechanisms for Genetic Diversity. *Cell* 109, S35–S44 (2002).
- Zhu, J., Yamane, H. & Paul, W. E. Differentiation of effector CD4 T cell populations (*). *Annu. Rev. Immunol.* 28, 445–489 (2010).
- Zhang, N. & Bevan, M. J. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35, 161–168 (2011).
- 9. Girard, J.-P., Moussion, C. & Förster, R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat. Rev. Immunol.* **12**, 762–773 (2012).
- Dong, C. *et al.* ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409, 97–101 (2001).
- Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* 22, 333–340 (2010).

- Neefjes, J., Jongsma, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* 11, 823–836 (2011).
- Matloubian, M. *et al.* Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427, 355–360 (2004).
- Griffith, J. W., Sokol, C. L. & Luster, A. D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32, 659–702 (2014).
- Joshi, N. S. *et al.* Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27, 281–295 (2007).
- Pipkin, M. E. *et al.* Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* **32**, 79–90 (2010).
- 17. Chang, J. T. *et al.* Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* **315**, 1687–1691 (2007).
- Banerjee, A. *et al.* Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J. Immunol.* 185, 4988–4992 (2010).
- Intlekofer, A. M. *et al.* Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6, 1236–1244 (2005).
- Kaech, S. M. *et al.* Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4, 1191–1198 (2003).
- Crotty, S. T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. *Immunity* 50, 1132–1148 (2019).
- Pepper, M., Pagán, A. J., Igyártó, B. Z., Taylor, J. J. & Jenkins, M. K. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 35, 583–595 (2011).

- Ichii, H. *et al.* Bcl6 is essential for the generation of long-term memory CD4+ T cells. *Int. Immunol.* 19, 427–433 (2007).
- Wieland, D., Hofmann, M. & Thimme, R. Overcoming CD8+ T-Cell Exhaustion in Viral Hepatitis: Lessons from the Mouse Model and Clinical Perspectives. *Dig. Dis.* 35, 334–338 (2017).
- Coss, S. L. *et al.* CD4+ T cell restoration and control of hepatitis C virus replication after childbirth. *J. Clin. Invest.* 130, 748–753 (2020).
- McLane, L. M., Abdel-Hakeem, M. S. & Wherry, E. J. CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annu. Rev. Immunol.* (2019) doi:10.1146/annurev-immunol-041015-055318.
- Angeletti, A., Cantarelli, C., Riella, L. V., Fribourg, M. & Cravedi, P. T-cell Exhaustion in Organ Transplantation. *Transplantation* 106, 489–499 (2022).
- Shin, H. *et al.* A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31, 309–320 (2009).
- Wherry, E. J., Barber, D. L., Kaech, S. M., Blattman, J. N. & Ahmed, R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16004–16009 (2004).
- Wherry, E. J. *et al.* Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27, 670–684 (2007).
- 31. Matloubian, M., Concepcion, R. J. & Ahmed, R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056–8063 (1994).
- Barber, D. L. *et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439, 682–687 (2006).

- Ferris, S. T. *et al.* cDC1 prime and are licensed by CD4+ T cells to induce anti-tumour immunity. *Nature* 584, 624–629 (2020).
- 34. He, R. *et al.* Follicular CXCR5- expressing CD8(+) T cells curtail chronic viral infection. *Nature* 537, 412–428 (2016).
- Im, S. J. *et al.* Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 537, 417–421 (2016).
- Kwong, C.-T. J. *et al.* Harnessing CD8+ T-cell exhaustion to treat type 1 diabetes. *Immunol. Cell Biol.* 99, 486–495 (2021).
- Crawford, A. *et al.* Molecular and transcriptional basis of CD4⁺ T cell dysfunction during chronic infection. *Immunity* 40, 289–302 (2014).
- Hwang, S., Cobb, D. A., Bhadra, R., Youngblood, B. & Khan, I. A. Blimp-1-mediated CD4 T cell exhaustion causes CD8 T cell dysfunction during chronic toxoplasmosis. *J. Exp. Med.* 213, 1799–1818 (2016).
- Elsaesser, H., Sauer, K. & Brooks, D. G. IL-21 is required to control chronic viral infection. Science 324, 1569–1572 (2009).
- Fröhlich, A. *et al.* IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection. *Science* 324, 1576–1580 (2009).
- Yi, J. S., Du, M. & Zajac, A. J. A Vital Role for Interleukin-21 in the Control of a Chronic Viral Infection. *Science* 324, 1572–1576 (2009).
- Parish, I. A. *et al.* Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. *J. Clin. Invest.* **124**, 3455–3468 (2014).
- 43. Brooks, D. G. *et al.* Interleukin-10 determines viral clearance or persistence in vivo. *Nat. Med.*12, 1301–1309 (2006).

- 44. Guo, Y. *et al.* Metabolic reprogramming of terminally exhausted CD8+ T cells by IL-10 enhances anti-tumor immunity. *Nat. Immunol.* **22**, 746–756 (2021).
- 45. Hanna, B. S. *et al.* Interleukin-10 receptor signaling promotes the maintenance of a PD-1int TCF-1+ CD8+ T cell population that sustains anti-tumor immunity. *Immunity* 54, 2825–2841.e10 (2021).
- Richter, K., Perriard, G. & Oxenius, A. Reversal of chronic to resolved infection by IL-10 blockade is LCMV strain dependent. *Eur. J. Immunol.* 43, 649–654 (2013).
- Fahey, L. M. *et al.* Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J. Exp. Med.* 208, 987–999 (2011).

<u>Chapter 2: Identification of a CX3CR1 expressing exhausted</u> <u>CD8 T Cell Subpopulation</u>

The contents of this chapter have been previously published in Journal of Immunology.

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2.1 Abstract

Persistent Ag induces a dysfunctional CD8 T cell state known as "exhaustion" characterized by PD-1 expression. Nevertheless, exhausted CD8 T cells retain functionality through continued differentiation of progenitor into effector cells. However, it remains illdefined how CD8 T cell effector responses are sustained in situ. In this study, we show using the mouse chronic lymphocytic choriomeningitis virus infection model that CX3CR1⁺ CD8 T cells contain a T-bet–dependent TIM3⁻PD-1^{lo} subpopulation that is distinct from the TIM3⁺CX3CR1⁺PD-1⁺ proliferative effector subset. The TIM3⁻CX3CR1⁺ cells are quiescent and express a low but significant level of the transcription factor TCF-1, demonstrating similarity to TCF-1^{hi} progenitor CD8 T cells. Furthermore, following the resolution of lymphocytic choriomeningitis virus viremia, a substantial proportion of TCF-1⁺ memory-like CD8 T cells show evidence of CX3CR1 expression during the chronic phase of the infection. Our results suggest a subset of the CX3CR1⁺ exhausted population demonstrates progenitor-like features that support the generation of the CX3CR1⁺ effector pool from the TCF-1^{hi} progenitors and contribute to the memory-like pool following the resolution of viremia.

2.2 Introduction

Upon encountering Ag, naive CD8 T cells undergo robust clonal expansion and acquire effector functions for host protection against pathogens and tumors. However, in the context of persistent Ag exposure during chronic viral infections, they are subject to alternative differentiation known as T cell exhaustion, which is characterized by the continued expression of inhibitory receptors, decreased cytokine secretion, and blunted proliferation^{1, 2}. Nevertheless, exhausted CD8 T cells are not inert and can actively control Ag burden³. This function is in part facilitated by a progenitor–progeny relationship within exhausted CD8 T cells that facilitates sustainable generation of functional effector cells. However, our understanding of the progenitor cell dynamics that supports this continuous differentiation process is limited.

The TCF-1^{hi} (CXCR5⁺Ly-108/SLAMF6⁺) progenitor exhausted CD8 T cells (TPEX) give rise to TCF1⁻ effector-like cells, of which a substantial fraction expresses the inhibitory receptor/exhaustion marker TIM3⁴⁻⁸. CX3CR1 is also expressed in a subset of TCF-1⁻ effector-like cells, which exhibit enhanced proliferative activity and cytolytic function compared with CX3CR1⁻ cells ^{9, 10}. Because TPEX cells are quiescent in situ and mainly present in the white pulp of the spleen and are thus physically separated from red pulp–predominant TCF-1^{lo/-} cells ⁴, it is possible that proliferative CX3CR1⁺ CD8 T cells are supported by a non-TPEX intermediate progenitor population. Prior work identified T-bet–expressing exhausted CD8 T cells as having progenitor-like features with a low intrinsic turnover ¹¹; however, their relationship to TCF-1^{hi} TPEX cells remains unclear as a vast majority of TPEX express T-bet at a low level. The CX3CR1⁺ population is enriched for T-bet–expressing cells compared with TCF-1^{hi} TPEX cells

^{4,5}, suggesting that they could be composed of a progenitor-like subpopulation, even though they lack cells with high TCF-1 expression.

In this work, we found using single-cell RNA sequencing (RNA-seq) (scRNA-seq) that CX3CR1⁺ cells are composed of two clusters. Cells present in one of the CX3CR1⁺ cell clusters are quiescent, highly enriched for expression of T-bet, and distinguished from the proliferating cluster of CX3CR1⁺ cells by TIM3 expression. The quiescent cluster also resembles TPEX cells by basal expression of *Tcf7* and *Bach2*, the transcription factor genes highly expressed in TPEX. Slow replenishment of the total CX3CR1⁺ cells by CX3CR1⁻ cells, dependency on the transcription factor T-bet, and trajectory based on scRNA-seq diffusion pseudotime analysis suggest that TIM3⁻CX3CR1⁺ cells serve as upstream precursors for the TIM3⁺CX3CR1⁺ effector-like cells. By contrast, the resistance of a vast majority of TIM3⁺ CX3CR1⁻ cells to T-bet deletion and CD4 T cell depletion suggests that they are independent of CX3CR1⁺ cells or develop through an alternative pathway. Furthermore, a substantial fraction of TCF-1^{hi} "memory-like" cells that persist after the resolution of viremia are marked by a history of Cx3cr1 expression during high viral burden and display continued expression of CX3CR1 and the exhaustion-associated gene TOX. These results collectively suggest multiple trajectories of CD8 T cell differentiation dynamics under exhausting conditions and the development of memory-like cells that persist after the resolution of viremia ¹²⁻¹⁴.

2.3 Results

CX3CR1 defines two populations of exhausted CD8 T cells with distinct properties

We first sought to identify additional heterogeneity in the CX3CR1⁺ subset, given that they are enriched for T-bet–expressing cells¹¹.To this end, we performed scRNA-seq analysis of

LCMV gp33–specific CD8 T cells during the chronic phase of LCMV-c13 infection in C57BL/6 mice. UMAP of the data demonstrated four major clusters of exhausted CD8 T cells, including the previously defined TCF-1^{hi} (encoded by *Tcf7*) and TIM3 (*Havcr2*)⁺ subsets, and also two distinct clusters of *Cx3cr*1⁺ cells segregated by the expression of *Havcr2* (**Figs. 1A, 2A**). Consistent with the scRNA-seq data and prior reports ^{10, 23}, ~40% of LCMV-gp33–specific CD8 T cells in mice infected with LCMV-c13 expressed CX3CR1 in the chronic phase, and its expression was seen exclusively in TCF-1^{10/-} cells (**Figs 1B, 2B**) The frequency of CX3CR1⁺ cells was notable in both TIM3⁻ and TIM3⁺ cells, confirming the presence of two unique CX3CR1⁺ populations (**Fig. 1C**). We also observed TIM3⁻CX3CR1⁻TCF1^{10/-} population; however, this subset was not a focus for the rest of this study (**Fig. 1C**).

The amount of surface PD-1 expression is known to correlate with the extent of exhaustion, with higher levels corresponding to more severe exhaustion and unresponsiveness to the checkpoint blockade ^{11, 24, 25}. Notably, the surface PD-1 level in TIM3⁻CX3CR1⁺ cells was even lower than that in TCF-1^{hi} TPEX cells (**Fig. 1D**). PD-1 was elevated in the TIM3⁺ fraction of CX3CR1⁺ cells, suggesting that TIM3⁺CX3CR1⁺ cells are more differentiated or in a more activated state than TCF-1^{hi} and TIM3⁻CX3CR1⁺ cells. Accordingly, the expression of GZMB was significantly higher in TIM3⁺CX3CR1⁺ cells compared with TIM3⁻CX3CR1⁺ cells, whereas GZMB expression was barely detectable in TCF-1^{hi} cells (**Fig. 1E**)

We next examined cell cycle states between the two CX3CR1⁺ populations by measuring BrdU incorporation, which directly reflects DNA synthesis during the S phase of the cell cycle, by pulse labeling in the chronic phase (22 dpi) of LCMV-c13 infection (**Fig. 1F**) We confirmed the quiescence of TCF-1^{hi} cells as <5% of cells incorporated BrdU over 12 h following one injection of BrdU. TIM3⁺CX3CR1⁺ cells exhibited the highest incorporation of BrdU (~30%),

whereas BrdU incorporation by the TIM3⁻CX3CR1⁺ cells was significantly lower than the TIM3⁺ counterpart, which was also reflected in Ki-67 expression (**Fig. 2C**). However, at an earlier time point on 8 dpi, cells in all subpopulations, including TPEX and TIM3⁻CX3CR1⁺ cells, were active in the cell cycle and upregulated Nur77 (**Fig. 2C, 2D**). Notably, expression of Nur77 in TIM3⁻CX3CR1⁺ cells was slightly lower (**Fig. 2D**), suggesting that they equally or slightly weakly received a TCR stimulus following infection. These results highlight distinct characteristics between the two subpopulations of CX3CR1⁺PD-1⁺ CD8 T cells. Whereas TIM3⁺ CX3CR1⁺ cells are actively proliferating effector cells similar to the cells described in previous studies ^{9,10}, TIM3⁻CX3CR1⁺ cells appear progenitor-like, with phenotypic similarities to TPEX with regards to low to moderate cell cycle activity and low expression of PD-1 and GZMB, and are thus distinct from TIM3⁺CX3CR1⁺ cells.

Effector cells are typically found in both blood and tissue at higher frequencies compared with memory-like cells²⁶. To examine the migratory behavior of CX3CR1⁺ cells, we performed in vivo labeling of T cells by an i.v. injection of anti-CD45.2, which allowed us to discriminate between tissue resident (CD45.2⁻) and circulating (CD45.2⁺) Ag-specific CD8 T cells present in the lung. In contrast to TIM3⁺CX3CR1⁻ cells that were predominantly localized in the lung parenchyma, distribution of both CX3CR1⁺ subpopulations was significantly enriched in the circulating compartment (**Fig. 2E**) Within the CX3CR1⁺ populations, more TIM3⁺ cells were found in the tissues compared with TIM3⁻ cells, suggesting that TIM3⁺CX3CR1⁺ cells preferentially migrate or are retained in tissue parenchyma and are likely key mediators of effector function.

TIM3⁻CX3CR1⁺ cells can give rise to TIM3⁺CX3CR1⁺ effectors

To examine the development and dynamics of each subset, we next performed a timecourse analysis. In the early phase of the acute phase response on 5 dpi, the TIM3⁺CX3CR1⁻ subset was dominant; however, CX3CR1⁺ cells were present with uniformly intermediate TIM3 expression without a clear segregation between TIM3⁻ and TIM3⁺ populations (**Fig. 3A, B**). TIM3⁻CX3CR1⁺ cells emerged around 8 dpi, and they continued to increase in numbers until 15 dpi, possibly because a subset of Ag-specific CD8 T cells receive weaker TCR stimulus and slow down their proliferation during this period (**Figs. 2C, 2D, 3A, 3B**).

To gain additional insight into the two CX3CR1⁺ populations of CD8 T cells, we purified the two subsets of CX3CR1⁺PD-1⁺ CD8 T cells based on TIM3 expression as well as TPEX and terminally exhausted (TIM3⁺CX3CR1⁻) CD8 T cells for population-based RNA-seq on 16 dpi. Confirming our scRNA-seq data, PCA and K-means clustering showed that the two CX3CR1⁺ populations have distinct gene expression patterns (**Figs. 4A, 5A**). A gene set enrichment analysis for differentially expressed genes between the two CX3CR1⁺ populations showed significant representation of pathways associated with cell division, MYC target genes, IL2–STAT5 signaling, and GTPase effectors, consistent with effector functions, high proliferative activity, and tissue migration (**Fig. 5B**). This result was supported by a gene-level analysis showing *Gzmb, Prdm1, Ifng, Prf1, Lag3, Entpd1* (CD39), *and Tigit* being higher in TIM3⁺CX3CR1⁺ compared with the TIM3⁻CX3CR1⁺ populations (**Fig. 5C**). In addition, Kmeans clustering showed that many genes that are differentially expressed between the CX3CR1⁺ populations were similarly expressed between TPEX and TIM3⁻CX3CR1⁺ cells (**Fig. 5A**, clusters 2 and 5), suggesting that there is transcriptomic similarity between TPEX and TIM3⁻CX3CR1⁺ cells. Among genes encoding transcription factors, the two

CX3CR1⁺ populations expressed high *Tbx21* compared with the TPEX subset that is marked by high expression of *Tcf7*, *Id3*, *Bcl6*, and *Bach2*. It is notable that these TCF-1^{hi} population– specific factors were also expressed at lower but significant levels in the

TIM3⁻CX3CR1⁺ population, whereas the TIM3⁻CX3CR1⁺ population expressed higher amounts of *Zeb2*, which cooperates with T-bet ^{27, 28}, than TIM3⁺CX3CR1⁺ cells and other PD-1⁺ CD8 T cells (**Fig. 4B**). Consistently, TCF-1 protein was also expressed in a fraction of gp33-specific TIM3⁻CX3CR1⁺ CD8 T cells, and the frequency of TCF-1⁺ cells was substantially reduced in TIM3⁻CX3CR1⁺ cells (**Figs. 2B, 4C**).

To assess the differentiation potentials of CX3CR1⁺ CD8 T cells, we transferred CFSElabeled TIM3⁻ and TIM3⁺CX3CR1⁺ and SLAMF6⁺TIM3⁻ (TPEX) CD8 T cells isolated from LCMV-c13–infected C57BL/6 mice (CD45.2) into infection-matched congenic CD45.1 mice and analyzed donor-derived cells 14 d after transfer (**Fig. 4D**). Approximately 40% of donorderived TIM3⁻CX3CR1⁺ cells upregulated TIM3 (**Fig. 4E, F**), and TIM3 upregulation preferentially occurred in CFSE¹⁰ cells (**Fig. 4H**). In addition, the majority of CFSE^{mid} and a fraction of CFSE¹⁰ cells remained as TIM3⁻ (**Fig. 4E, H**). TIM3⁺CX3CR1⁺ cells barely generated TIM3⁻ cells (**Fig. 4E-G**). However, expansion of both TIM3⁻ and TIM3⁺ CX3CR1⁺ cells was 2– 3-fold lower compared with TPEX cells, which gave rise to more differentiated cell types. Inefficient differentiation to CX3CR1⁻ cells from the transferred TPEX could be affected by timing or a complication in trafficking or cell death in a setting of infection-matched adoptive transfer. It is also notable that expansion of TIM3⁺CX3CR1⁺ cells is substantially compromised upon adaptive transfer, although they were highly proliferating in situ. To gain additional insights, we treated LCMV-infected mice with anti–PD-L1 Abs, which releases TPEX from PD- 1–mediated suppression and specifically drives their expansion (**Fig. 3D, E**)⁴. After three doses of anti–PD-L1, we detected proportional ratios between TIM3⁻ and TIM3⁺ CX3CR1⁺ cells, suggesting that proliferating TPEX cells give rise to both populations in situ (**Fig. 3D, E**). These results collectively indicate that TIM3⁻CX3CR1⁺ cells can be derived from TPEX and differentiate into TIM3⁺CX3CR1⁺ cells. This result was also supported by diffusion pseudotime analysis of scRNA-seq data, showing a trajectory of TPEX differentiation toward TIM3⁺CX3CR1⁺ cells via a TIM3⁻CX3CR1⁺ intermediate (**Fig. 4H**). These results suggest that TIM3⁻ cells are positioned at an intermediate between TPEX and upstream of TIM3⁺ cells in terms of developmental hierarchy within the CX3CR1⁺PD-1⁺ CD8 T cells.

The loss of CD4 help via IL-21 reduces the absolute numbers of CX3CR1⁺ CD8 T cells during the chronic phase of the response to LCMV-c13 infection ¹⁰. In CD4-depleted LCMVc13–infected mice, we did not observe a reduction in the number of CX3CR1⁺ CD8 T cells on 8 dpi at the peak of the Ag-specific CD8 T cell response (**Fig. 4A, C**). However, in the chronic phase on 22 dpi, the number of CX3CR1⁺ CD8 T cells in CD4-depleted mice was substantially reduced in both TIM3⁻ and TIM3⁺ subpopulations, which is consistent with prior results ¹⁰ (**Fig. 4A, C**). Thus, CD4 T cell help appears to be required for the maintenance of the CX3CR1⁺ cells rather than their generation. Interestingly, the loss of TIM3⁻CX3CR1⁺ CD8 T cells preceded that of TIM3⁺ cells, with an ~15-fold reduction of the former population compared with an ~4-fold reduction of the latter on 15 dpi. The severe loss of TIM3⁺CX3CR1⁻ cells followed by TIM3⁺CX3CR1⁺ cells could reflect their developmental relationship or highlight differential sensitivity to IL-21 for their maintenance.

T-bet is critical for the maintenance of CX3CR1⁺ exhausted CD8 T cell populations

To more rigorously test the possibility that the TIM3⁻CX3CR1⁺ population overlaps the previously identified T-bet^{hi} progenitor population ¹¹, we first examined the proportion of each subset with T-bet^{hi} cells by flow cytometry. Indeed, TIM3⁻CX3CR1⁺ cells comprised ~40% of total T-bet^{hi} cells followed by the TCF-1^{hi} population (30%) (**Fig. 6A**). Co-staining for T-bet and EOMES showed a marked difference in T-bet and EOMES expression between the two CX3CR1⁺ populations (**Fig. 6B**). Notably, the TIM3⁻CX3CR1⁺ subset was predominantly composed of T-bet^{hi} cells (~70%) compared with the rest of the exhausted CD8 T cell subsets, in which a majority of cells were EOMES^{hi} but T-bet^{lo} (**Fig. 6B**). Together with low PD-1 expression, basal expression of TPEX-associated transcription factors, low proliferative activity, and capacity for differentiation to TIM3⁺ effector/exhausted cells, our data suggest that TIM3⁻CX3CR1⁺ CD8 T cells overlap with the previously reported T-bet^{hi} progenitor cells ¹¹.

The loss of T-bet in T cells during chronic infection has been associated with more severe exhaustion ^{11, 29}. To determine whether T-bet is required for TPEX, T-bet^{hi}TIM3⁻CX3CR1⁺, cells or both, we analyzed CD8 T cell responses to LCMV-c13 infection in mice with CD8 T cell– specific *Tbx21* deletion. During the chronic phase of LCMV infection (29 dpi), frequencies of total LCMV gp33-specific CD8 T cells in the spleen were reduced by 2.5-fold in CD8- cre *Tbx21^{F/F}* mice compared with control cre(–) *Tbx21^{F/F}* mice (**Fig. 6C**, **6D**). The absolute number of TPEX cells did not significantly change by CD8 T cell–specific deletion of *Tbx21*. In contrast, the reduction of total Ag-specific CD8 T cells was attributed to a marked reduction of CX3CR1⁺ CD8 T cells, most notably to that of TIM3⁻CX3CR1⁺ cells that expressed the highest amounts of T-bet among all fractions (**Fig. 6B**, **6D**). We also observed a decreased frequency and absolute number of TIM-3⁺CX3CR1⁺ effector-like cells, which could reflect the loss of input
from TIM3⁻CX3CR1⁺ cells or defective differentiation of TPEX cells. Consistent with previous studies ^{11, 30, 31}, ex vivo stimulation of splenocytes from *Tbx21*-deficient mice showed preserved GZMB expression, with decreased IFN- γ expression in CD8 T cells, which likely contributes to defective viral control (**Fig. 7A–D**). To determine the impact of the loss of CX3CR1⁺ CD8 T cells on viral control, we tracked LCMV-c13–infected CD8-cre⁺ *Tbx21*^{F/F} and control *Tbx21*^{F/F} mice for 100 d, by which time the majority of immune-competent mice resolve viremia ³². Although control mice were able to resolve viremia, *Tbx21*-deficient mice still demonstrated significant viremia (**Fig. 6E**). These results indicate that T-bet is required for the maintenance of CX3CR1⁺ cells despite a minimal impact on TCF-1^{hi} cells.

Eomes and Bach2 antagonistically control differentiation of TPEX into effector-like exhausted CD8 T cells

We next defined the relationship between the TCF-1^{hi} and CX3CR1⁺ populations by examining the function of transcription factors that are highly expressed in and potentially important for differentiation of the TPEX population. Based on our gene expression data (**Fig. 4B**), we first examined the role of *Eomes* in the differentiation of CD8 T cells during the chronic phase of LCMV infection. *Eomes* expression has been associated with severe exhaustion, and its loss results in reduced Ag-specific CD8 T cells numbers ^{11, 33}. We thus anticipated that *Eomes* deletion would result in a reduction in TCF-1^{hi} cells. Contrary to our prediction, in *CD8-cre⁺Eomes*^{F/F} mice, there was a 3-fold increase in both frequency and absolute numbers of total LCMV-gp33–specific CD8 T cells (**Fig. 8A**, 8**B**). We suspect our result is discrepant with the prior results of *Eomes* deficiency, possibly because of an additional deletion of Eomes in CD4 T cells using CD4-cre in prior studies, whereas the increased proportion of TPEX is consistent with the reported reduced *Prdm1* expression in Ag-specific CD8 T cells ^{11, 34}. This increase was most attributable to a >7-fold increase in TPEX cells (**Fig. 8B**). In addition, TIM3⁻CX3CR1⁺ CD8 T cells were increased by 3-fold (**Fig. 8A**, 8C). However, we did not observe a change in the total TCF-1^{10/-}PD-1⁺ CD8 T cells or TIM3⁺CX3CR1⁺ CD8 T cells, and within TCF-1^{10/-} cells, TIM3 cells were increased in number (**Fig. 8A**, 8C). Increased numbers of the two quiescent subpopulations of exhausted CD8 T cells in *Eomes* deficiency suggest that EOMES promotes the development of TPEX cells after T cell activation and/or represses the differentiation of progenitor-like CD8 T cells, including TPEX and TIM3⁻CX3CR1⁺ cells, to downstream effector cells.

As a consequence of altered differentiation, the turnover of downstream effector cells derived from TPEX cells or TIM3⁻CX3CR1⁺ cells may be impaired, and fitness of these effector cells could be compromised in *Eomes*-deficient mice. To test this possibility, we first examined IFN- γ and TNF- α production by CD8 T cells following the stimulation of splenocytes with LCMV-specific peptides ex vivo, which showed no difference between *Eomes*-deficient and control CD8 T cells (**Fig. 7E, 7F**). However, we observed a significant reduction of GZMB expression in *Eomes*-deficient Ag-specific CD8 T cells (**Fig. 7G, 7H**). Consistent with the defects in differentiation of PD-1⁺ CD8 T cells, the majority of CD8-cre *Eomes*^{F/F} mice failed to resolve viremia on \geq 100 dpi (**Fig. 7I**). These results together indicate unique roles of the two Tbox proteins, T-bet and EOMES, in CD8 T cell responses to persisting Ag and a specific role for T-bet in the maintenance of CX3CR1-expressing cells.

Bach2 is another transcription factor highly expressed specifically in TPEX cells. CD8 T cell–specific deletion of *Bach2* resulted in a similar increase in the number of gp33-specific CD8 T cells to *Eomes* deletion (**Fig. 8D–G**). However, the overall increase was attributable to TCF-

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1^{lo/-} cells with a marked reduction in the frequency of TPEX as well as absolute numbers. In TCF-1^{lo/-} cells, contrary to *Eomes*-deficient mice, the most notable increase was seen in the TIM3⁺CX3CR1⁺ proliferating population. These results indicate that, although both EOMES and BACH2 are highly expressed in the TPEX population, their roles may be antagonistic with BACH2-protecting TPEX cells from differentiation into the downstream effector cells.

CX3CR1⁺ CD8 T cells are largely self-sustainable with limited input from TPEX cells

We next sought to measure the turnover of the CX3CR1⁺ cells by TCF-1^{hi} TPEX-derived cells by performing genetic labeling of CX3CR1⁺ cells by using the Cx3cr1-creER^{T2} allele crossed to the ROSA26-loxP-stop-loxP-tdTomato (R26tdT) reporter (Fig. 9A). Then, 16 h after one dose of TAM on 8 dpi, it showed labeling of $\sim 10-25\%$ of TCF-1^{lo/-} CD8 T cells in contrast to the little labeling of TPEX cells in the spleen, demonstrating specific, low-background labeling of TPEX cells by the TAM-inducible cre activity (Fig. 9B). Then, 48 h after TAM administration, ~80% of CX3CR1⁺ CD8 T cells in the peripheral blood became tdT positive (Fig. 9C). Given that TPEX cells can differentiate into downstream effector and exhausted CD8 T cells, we expected to observe robust displacement of tdT⁺ cells by TPEX-derived tdT⁻ cells within the CX3CR1⁺ populations over the course of the antiviral response. However, over a 2-wk chase period, when TAM is no longer bioavailable in vivo ^{35, 36}, the frequencies of tdT⁺ cells in the CX3CR1⁺ CD8 T cells were preserved with only a modest decay to 60% on 22 dpi (Fig. 9C). In the spleen, both TIM3⁻ and TIM3⁺CX3CR1⁺ cells retained high percentages of tdT⁺ cells, whereas $\sim 30\%$ of TIM3⁺CX3CR1⁻ cells were labeled with tdT (Fig. 9D), suggesting some transition from CX3CR1⁺ into CX3CR1⁻ cells. These results highlight a slow turnover of CX3CR1⁺ cells, which could reflect a long population half-life or be consistent with a relatively

minor contribution of TPEX cells to the pool of TIM3⁻ and TIM3⁺CX3CR1⁺ exhausted CD8 T cell pool.

CX3CR1⁺ cells exhibit longevity following viral clearance with the capability to respond to Ag rechallenge

In immunocompetent mice, LCMV-c13 viremia is eventually resolved by both T and B cell responses around 3 mo after infection ^{16, 32, 37}. We next sought to determine the long-term fate of CX3CR1⁺ cells as the TIM3⁻CX3CR1⁺ subset expressed low but detectable levels of the memory-related factor, *Tcf7*. To this end, we administered TAM to Cx3cr1-creER^{T2} R26tdT mice during the chronic phase of infection on 28 dpi and chased the tdT-labeled cells for 120 d. Similar to acute LCMV infection, we were able to detect the persistence of LCMV-gp33– specific CD8 T cells expressing intermediate levels of PD-1 in the PBMCs and in the spleen of LCMV-c13-immune mice (Fig. 10A). These cells likely correspond to "chronic memory" cells or memory-like cells that were previously reported ^{14, 38}. In contrast to LCMV-Arm-immune mice, in which TCF-1^{hi} memory CD8 T cells were mostly CX3CR1⁻, a substantial fraction of gp33-specific chronic memory CD8 T cells in LCMV-c13-infected mice showed an effector memory phenotype with the expression of CX3CR1 (Fig. 10A). TAM administration to LCMVc13–infected Cx3cr1-creER^{T2} fate-mapping mice on 28 dpi resulted in the marking of ~40% of LCMV-gp33-specific CD8 chronic memory T cells on 120 dpi (Fig. 10A, 10B). Almost all tdT⁺ cells continued to express surface CX3CR1, and \sim 40% of the tdT⁺ cells expressed the memory transcription factor TCF-1 at the levels slightly lower than the CX3CR1⁻ chronic memory cells (Fig. 10A, 10B). The majority (~80%) of CX3CR1⁺ chronic memory CD8 T cells were also tdT⁺ and thus were derived from CX3CR1⁺ cells during the labeling period (Fig. 10B). In contrast to PD-1⁺ CD8 T cells during high Ag burden, the CX3CR1⁺ and CX3CR1⁻ chronic memory CD8 T cells were able to produce considerable IFN- γ following stimulation with LCMV-gp33 ex vivo (Fig. 10C). Notably, ~50% of IFN- γ^+ cells also expressed TNF- α , indicating partial restoration of polyfunctionality in these chronic memory CD8 T cell populations (Fig. 10C). The transcription factor TOX has been closely linked to CD8 T cell exhaustion in the context of antiviral and anti-tumor immunity and is implicated in programming the unique genetic and epigenetic signatures associated with chronic Ag stimulation ³⁹⁻⁴³. Interestingly, the vast majority of chronic memory CD8 T cells continued to express TOX (Fig. **10D**). Their TOX expression was lower than that of TCF-1⁺ progenitors during active infection, which mirrors continued expression of low PD-1 by chronic memory CD8 T cells³⁸. Furthermore, both CX3CR1⁺ and CX3CR1⁻ chronic memory CD8 T cells from LCMV-c13– infected mice were capable of secondary expansion following adoptive transfer to congenic wildtype mice followed by LCMV-Arm infection (Fig. 10E). Because CX3CR1⁺TCF-1⁺ cells barely expressed TIM3 after the resolution of viremia (data not shown), it is likely that a substantial fraction of CX3CR1⁺ chronic memory CD8 T cells is derived from the TCF-1^{lo/-}TIM3⁻CX3CR1⁺ cells or a minor population of TPEX, which had turned on expression of Cx3cr1 as they initiated differentiation toward TCF-1^{lo/-} cells. These results collectively indicate that a substantial fraction of Ag-experienced TCF-1⁺ cells are derived from exhausted T cells that initiated differentiation away from TPEX, suggesting the plasticity for long-term persistence, polyfunctionality, and secondary expansion after chronic exposure to Ag and *Tcf*7 downregulation.

2.4 Discussion

The mechanisms underlying the maintenance of functional CD8 T cell responses in the context of persistent Ag exposure remains unresolved. A substantial body of work has identified various subsets of exhausted CD8 T cells with distinct phenotypic and gene expression profiles. However, how these populations interact and ultimately promote control of viral or tumor burden remains an active area of investigation. In this study, we identify a TIM-3 negative subset of PD-1^{lo} CD8 T cells within the CX3CR1⁺ population, which is highly enriched for T-bet^{hi} cells. This population is quiescent and exhibits low TCF-1 expression but also demonstrates features of effector-like cells. These results highlight underappreciated heterogeneity within exhausted CD8 T cells and provide a more complete framework for understanding the dynamics of T cell responses to chronic Ag.

Prior work has established that TCF-1^{hi} TPEX cells act as progenitors for BLIMP-1⁺ effector-like and terminally exhausted CD8 T cells. In comparison, an analogous relationship was described between T-bet^{hi} cells (progenitor) and Eomes^{hi} cells (progeny), which become fully exhausted as Eomes expression elevates ^{11, 29}. However, the nature of the T-bet^{hi} progenitor cells has remained unclear because TCF-1^{hi} TPEX cells are predominantly T-bet^{lo}EOMES^{hi 4}. Two recent papers described a subset of PD-1⁺ CD8 T cells enriched for CX3CR1 expression and T-bet ^{9, 10}. These cells are highly proliferative and express effector molecules, which contribute to the control of chronic viral infection and tumors. They also overlap with another subpopulation of exhausted CD8 T cells, Ly108(SLAMF6)⁻CD69⁻T_{EX}-int cells, which are proposed to be a transitory population between TPEX and terminally exhausted cells ⁴⁴. Our work has identified a TIM3-negative subpopulation within the CX3CR1⁺ population, which is almost exclusively T-bet^{hi} cells and distinct from the transitory populations in their cell cycle status and gene expression. The TIM3⁻ cells are phenotypically similar to TPEX with their quiescence, basal expression of TCF-1, and low levels of effector gene expression. Because each study used different markers to define populations and phenotypic changes, further work will be necessary to reconcile the discrepancies between the studies. In addition, although not a focus of this study, we identified a TIM3⁻CX3CR1⁻TCF1^{10/-} population whose function and development remain yet to be determined. This population was resistant to the loss of CD4 T cell help and exhibited low proliferation. We speculate this could be an intermediate to TIM3⁺CX3CR1⁻ cells, which may develop from TPEX or naive CD8 T cells independent of CX3CR1⁺ cells.

A key question that remains unsettled is the developmental trajectory and nature of the progenitor-progeny relationships between the exhausted CD8 T cell subsets. The external cues that drive this process remain incompletely defined; however, a role for type I IFNs and IL-12 in promoting differentiation has been proposed ^{6, 45, 46}. This issue has been largely studied in the setting of adoptive transfer of various subsets to congenic recipients. Indeed, adoptively transferred TPEX cells exhibit higher proliferative potential than TCF-1⁻ or CXCR5⁻PD-1⁺ CD8 T cells and can give rise to TIM3⁺ differentiated progeny ⁴⁻⁶. However, it should be noted that the behavior of transferred cells may not faithfully reflect the kinetics of this transition in situ because of inefficient engraftment of transferred cells that are removed from their native microenvironment. This issue is also seen in long-term hematopoietic stem cells, which show a limited contribution to homeostatic hematopoiesis, although they are capable of robust expansion and reconstitution of cells in multiple lineages ⁴⁷⁻⁴⁹. This biology may also be the case in exhausted CD8 T cells as exemplified by the loss of quiescence of TPEX cells upon adoptive transfer and limited proliferative activity of TIM3⁺ CX3CR1⁺ cells in our study and T_{EX}-int cells in another study ⁴⁴. In this study, we used an in vivo lineage-tracing approach to overcome this

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limitation and found that displacement of pulse-labeled CX3CR1⁺PD-1⁺ CD8 T cells by newly generated cells derived from TPEX or naive CD8 T cells occurs slowly with a half-life longer than a few weeks. This result suggests that, although transferred TPEX cells robustly give rise to CX3CR1⁺ and CX3CR1⁻ effector/exhausted CD8 T cells, they are mostly quiescent in situ and do not rapidly replace downstream differentiated cells. This is consistent with low BrdU incorporation and KI67 expression as well as a pseudotime modeling of our scRNA-seq data. We recognize two caveats that need to be considered. First, we cannot rule out the possibility that a small fraction of TCF-1^{hi} cells that were labeled with tdT, instead of TCF-1^{lo/-}CX3CR1⁺ cells, replenish the CX3CR1⁺ CD8 T cell pool. However, this result also suggests that the vast majority of TPEX, except for those with poised Cx3cr1 expression, are dormant with limited contribution to TCF-1^{lo/-} cells in situ, which is largely consistent with our interpretation. Second, future work is necessary to evaluate TIM3⁻CX3CR1⁺ cells recruitment to TIM3⁺CX3CR1⁺ cells, which is technically limited because of the lack of a specific cre driver. Nevertheless, given the similarity to TPEX, TIM3⁻CX3CR1⁺ cells may function as intermediate progenitors for TIM3⁺ CD8 T cells with limited self-renewing capabilities, like short-term hematopoietic stem and progenitor cells, which are still dependent on largely dormant long-term hematopoietic stem cells but mainly support homeostasis of hematopoiesis ^{47, 48}.

The Ag-specific cells present in the host after the resolution of viremia no longer demonstrate a high expression of PD-1 and have been termed chronic memory ¹⁴. Using our CX3CR1 lineage-tracing approach, we were able to track cells labeled during the chronic phase of the infection and found that a substantial portion of TCF-1⁺ chronic memory cells are labeled by previous expression of CX3CR1 cells. The labeled cells are exclusively found in CX3CR1⁺ chronic memory cells, suggesting earlier activation of *Cx3cr1* imprints in its

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continued expression, which is consistent with trajectories of CX3CR1⁺ cells, and TIM3⁻CX3CR1⁻TCF-1^{10/-} cells are mostly segregated during the chronic phase. Although we cannot formally rule out that these labeled cells were derived from a small proportion of labeled TPEX cells, it is unlikely this would be the case based on a much larger fraction of TCF-1⁺ chronic memory cells being labeled by tdT. A previous study demonstrated that memory cells that have experienced chronic Ag stimulation retain persistent PD-1 expression ³⁸. Likewise, we found that TCF-1⁺ cells generated through LCMV-c13 infection retain persistent TOX expression, similar to effector memory cells or anti-CMV memory CD8 T cells in humans^{12, 13,} ^{50, 51}. These findings suggest that cells other than TPEX or central memory-like cells possess the capacity to retain or re-express TCF-1 under certain conditions and highlight additional heterogeneity of TCF-1⁺ CD8 memory T cells that may be imprinted by the kinetics of Ag stimulation or inflammatory milieu. An equivalent type of memory T cell following cancer elimination is likely ⁵², which may be important to prevent relapse. It remains to be determined how this memory cell type differs from CX3CR1⁻ memory cells through the acquisition of imprinted identity during the effector phase. This knowledge will potentially transform into methods that enhance long-term immunity against chronic viral infection or cancers.

2.5 Materials and Methods

Mice and infection

Male C57BL/6N and B6-CD45.1 mice were purchased from Charles River Laboratories. *Prdm1-EYFP*, CD8 (*E8I*)-*Cre*, *Tbx21^F*, *Eomes^F*, *Cx3cr1^{CreER}*, and *Rosa26^{LSL-}* ^{*dT*} mice were originally obtained from The Jackson Laboratory. *Bach2*^F mice are described previously ¹⁵. All mice were housed in a specific pathogen-free facility at Washington University in St. Louis and were used for infection at 8–12 wk of age, unless stated otherwise. All experiments were performed according to a protocol approved by Washington University's Institutional Animal Care and Use Committee. Stocks of lymphocytic choriomeningitis virus (LCMV) were made by propagating virus by infection of BHK21 cells, followed by determination of titer in culture supernatants by plaque assay on Vero cells. For LCMV infection, mice were infected with 2×10^5 PFUs of LCMV-Armstrong (LCMV-Arm) via the i.p. route or 2×10^6 PFU of LCMV-clone 13 (LCMV-c13) by i.v. injection. For the quantification of plasma viral load, RNA was extracted from 10 µl of plasma using TRIzol (Thermo Fisher_Scientific). Before RNA extraction, a spike-in of exogenous control External RNA Controls Consortium RNA (Thermo Fisher_Scientific) was carried out and used to normalize viral loads following quantitative PCR as described previously ^{16, 17}.

Bulk RNA-seq

Polyclonal PD-1⁺ CD8 T cells, which are enriched for LCMV-Ag–specific CD8 T cells, were purified and sorted based on the indicated cell surface markers on 16 d after infection (dpi) of LCMV-c13 from *Prdm1*-YFP mice as described previously ^{6, 18}. Briefly, CD8⁺ T cells were first enriched from splenocytes using the Dynabeads FlowComp Mouse CD8 T Cell Kit (Thermo Fisher Scientific) followed by surface staining. We then sorted *Prdm1*-*EYFP*⁻TIM3⁻CX3CR1⁻ cells, which corresponded to TCF-1^{hi} CD8 TPEX cells, and divided *Prdm1*-EYFP⁺ cells into TIM3⁻CX3CR1⁺, TIM3⁺CX3CR1⁺, and

TIM3⁺CX3CR1⁻ populations. Total RNA was extracted from 20,000–50,000 sorted cells using Direct-Zol Micro Kit (Zymo Research), according to the manufacturer's instructions. cDNA

synthesis and amplification were performed with Next Ultra RNA Library Preparation Kit (New England BioLabs). Libraries were sequenced on a HiSeq 3000 (Illumina) in single-read mode, with a read length of 50 nucleotides producing ~25 million reads per sample. Sequencing reads were mapped to the National Center for Biotechnology Information mm9 mouse genome assembly using STAR with default parameters, and mapping rates were higher than 90%. Transcripts with 4 TPM in at least one sample were initially filtered, followed by principal component analysis (PCA), unsupervised clustering, and profiling of subpopulation-specific gene expression using Phantasus and limma packages

(V.15.1, <u>https://artyomovlab.wustl.edu/phantasus/</u>). The raw data have been deposited to National Center for Biotechnology Information Gene Expression Omnibus.

scRNA-seq

scRNA-seq libraries were prepared using the 10× Single-Cell Immune Profiling Solution Kit (v1 Chemistry), according to the manufacturer's instructions. Briefly, FACS-sorted cells were washed once with PBS + 0.04% BSA. Following reverse transcription and cell barcoding in droplets, emulsions were broken and cDNA purified using Dynabeads MyOne SILANE, followed by PCR amplification (98°C for 45 s; 14 cycles of 98°C for 20 s, 67°C for 30 s, and 72°C for 1 min; and 72°C for 1 min). For gene expression library construction, 50 ng of amplified cDNA was fragmented and end-repaired, double-sided size selected with SPRIselect beads, PCR amplified with sample indexing primers (98°C for 45 s; 14 cycles of 98°C for 20 s, 54°C for 30 s, and 72°C for 20 s; and 72°C for 1 min), and double-sided size selected with SPRIselect beads. The prepared single-cell RNA libraries were sequenced on an Illumina HiSeq 4000 to a minimum sequencing depth of 25,000 reads/cell using the read lengths 28 bp read1, 8 bp i7 index, and 91 bp read2. Reads were aligned to the mm10 reference genome and quantified

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using cellranger count (10× Genomics, version 3.1.0). Filtered gene-barcodes matrices containing only barcodes with unique molecular identifier counts passing the threshold for cell detection were used for further analysis.

Additional analysis was performed using Seurat (version 3.1.2) ¹⁹. Cells with less than 200 genes detected or greater than 5% mitochondrial RNA content were excluded from analysis. For clustering, raw unique molecular identifier counts were log normalized and variable genes identified based on a variance stabilizing transformation. We assigned scores for S and G2/M cell cycle phase based on previously defined gene sets ²⁰ using the CellCycleScoring function. Scaled z-scores for each gene were calculated using the ScaleData function and regressed against the S-phase score and G2/M-phase score to reduce clustering based on cell cycle. Scaled z-scores for variable genes were used as input into PCA. Clusters were identified using shared nearest neighbor–based clustering based on the first 10 principal components with k = 15 and resolution = 0.23. The same principal components were used to generate the Uniform Manifold Approximation and Projection (UMAP) projections (Refs. 21 and L. McInnes, J. Healy, and J. Melville, manuscript posted on arXiv, DOI: arXiv:1802.03426), which were generated with a minimum distance of 0.1 and 30 neighbors. Expression of selected genes was plotted using log-normalized gene expression values.

Pseudotime and diffusion map analysis of scRNA-seq data were performed using Destiny (version 3.2.0) ²². The normalized, log-transformed transcript count matrix was used to create a diffusion map using the DiffusionMap function with default parameters. Cells were plotted using the first three components of the diffusion map. A randomly selected cell from the TCF-1⁺ cluster was used as the root cell for diffusion psuedotime computation using the DPT function with default parameters.

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Treatments

For depletion of CD4 T cells, 200 μ g of anti-CD4 (GK1.5, Leinco Technologies) was injected on -1 and +1 dpi. For administration of tamoxifen (TAM, Sigma), 10 mg/ml solutions were prepared in Corn oil (Sigma) and 1 mg gavaged orally.

Cell preparation, cell staining, and flow cytometry

Single-cell suspensions of splenocytes were prepared by manual disruption with frosted glass slides. Lungs were minced with scissors and digested with Collagenase D (Sigma) and DNase I (Sigma) with agitation for 1 h at 37°C followed by enrichment of lymphocytes by a 40/70 Percoll gradient. Absolute live cell counts were determined by trypan blue exclusion using Vi-CELL (Beckman Coulter). Tetramer staining was performed using iTag-PE and allophycocyanin LCMV gp33-41 and gp276-286 (MBL International). The following monoclonal Abs were purchased from BioLegend, unless otherwise indicated: FITC-conjugated anti-CD45.2 (104), PerCP-Cy5.5-conjugated anti-CD86 (YTS156.7), anti-CD4 (GK1.5), anti-Ki-67 (16A8), PerCP-eFluor 710-conjugated anti-Eomes (eBioscience, Dan11mag), allophycocyanin-conjugated CD366 (TIM-3, RMT3-23), anti–TNF-α (MP6-XT22), anti– granzyme B (GZMB) (QA16A02), anti-T-bet (4B10), anti-CX3CR1 (SA011F11), Alexa Fluor 700-conjugated anti-CD44 (IM7), anti-CD45.2 (104), Brilliant Violet (BV) 421-conjugated anti-TIM3 (RMT3-23), BV605-conjugated CD4 (GK1.5), anti-CD45.1 (A20), anti-CX3CR1 (SA011F11), BV711-conjugated anti-CD4 (RM4-5), PE-conjugated anti-IFN-γ (XMG1.2), anti-PE-Cy7-conjugated anti-PD-1 (29F.1A12), PE-Dazzle 594-conjugated anti-B220 (RA3-6B2), BUV395-conjugated anti-CD8 (53-6.7, Becton Dickinson), and anti-TCF-1 Ab (Cell Signaling Technology, C63D9) and detected by Alexa Fluor 488-conjugated donkey anti-rabbit polyclonal

IgG (Thermo Fisher_Scientific, catalog no. R37118). Staining for transcription factors was performed using the Foxp3 staining kit (eBioscience), according to the manufacturer's instructions.

For intracellular cytokine staining, splenocytes were cultured in RPMI-1640 supplemented with 10% FBS in the presence of 1 ug/ml of LCMV-gp33-41 peptide (GenScript Biotech) and 5 ug/ml of brefeldin A (BioLegend) for 4 h. Cells were stained for surface makers and then subject to LIVE/DEAD Aqua staining (Thermo Fisher_Scientific) for 30 min at 4°C before being fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were then washed twice with 0.03% saponin in 2% FBS/PBS before being stained with the indicated Abs in 0.3% saponin in 2% FBS/PBS for 20 min at 4°C.

BrdU incorporation assay was performed using the allophycocyanin BrdU Flow Kit (BD Biosciences), according to the manufacturer's instruction with one-time i.p. injection of 1 mg of BrdU followed by tissue collection after 12 h.

Stained samples were analyzed with BD FACS LSR Fortessa, X20, or Symphony A3 or sorted on Aria II or III. Data were processed with FlowJo Software (FlowJo).

Infection-matched adoptive transfer

SLAMF6⁺, SLAMF6⁻TIM3⁻CX3CR1⁺, or SLAMF6⁻TIM3⁺CX3CR1⁺PD-1⁺ CD8 T cells were sorted from C57BL/6 mice on 21 dpi with LCMV-c13, and 3 × 10⁵ CFSE-labeled cells were i.v. transferred into infection-matched CD45.1 congenic recipients. Splenocytes from the recipient mice were harvested 14 d after transfer, and donor-derived CD8 T cells were analyzed by flow cytometry following magnetic bead–enrichment with a MojoSort Mouse CD8 T Cell Isolation Kit (<u>BioLegend</u>). For transfer of memory CD8 T cells, CD44⁺CX3CR1⁺ or CD44⁺CX3CR1⁻ CD8 T cells were sorted from the spleen of mice that had been infected with LCMV-c13 >120 d prior. The sort-purified memory CD8 T cells that contained 2,000–20,000 gp33-tetramer⁺ CD8 T cells were transferred to uninfected CD45.1 congenic recipients and infected the next day. We quantitated donor-derived CD8 T cells in the recipients without LCMV-Arm infection 24 h after transfer, and donor cell expansion was calculated by dividing the donor cell number in infected recipients on 6 dpi by that in uninfected control gp33-tetramer⁺ CD8 T cells.

Statistical analysis

The *p* values were calculated with an unpaired two-tailed Student *t* test or Mann– Whitney *U* test for two-group comparisons and by one-way ANOVA for multigroup comparisons with the Tukey post hoc test using Prism 8 software: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

2.6 Acknowledgements

We thank M. Colonna and M. Cella for LCMV stocks, K. Kim and G. Randolph for *Cx3cr1*-creER^{T2} mice, C. Fuji and T. Yangdon for the maintenance of the mouse colony, and C-S. Hsieh for discussion and critical reading of the manuscript.

2.7 References

1. Gallimore, A., J. Hombach, T. Dumrese, H. G. Rammensee, R. M. Zinkernagel, and H. Hengartner. 1998. A protective cytotoxic T cell response to a subdominant epitope is influenced by the stability of the MHC class I/peptide complex and the overall spectrum of viral peptides generated within infected cells. *Eur. J. Immunol.* 28: 3301-3311.

2. Zajac, A. J., K. Murali-Krishna, J. N. Blattman, and R. Ahmed. 1998. Therapeutic vaccination

against chronic viral infection: the importance of cooperation between CD41 and CD81 T cells. *Curr. Opin. Immunol.* 10: 444-449.

Cartwright, E. K., L. Spicer, S. A. Smith, D. Lee, R. Fast, S. Paganini, B. O. Lawson, M. Nega,
 K. Easley, J. E. Schmitz, et al. 2016. CD8(1) lymphocytes are required for maintaining viral suppression in SIV-infected macaques treated with short-term anti- retroviral therapy. *Immunity*. 45: 656-668.

4. Im, S. J., M. Hashimoto, M. Y. Gerner, J. Lee, H. T. Kissick, M. C. Burger, Q. Shan, J. S. Hale, J. Lee, T. H. Nasti, et al. 2016. Defining CD81 T cells that pro- vide the proliferative burst after PD-1 therapy. *Nature*. 537: 417-421.

5. Utzschneider, D. T., M. Charmoy, V. Chennupati, L. Pousse, D. P. Ferreira, S. Calderon-Copete, M. Danilo, F. Alfei, M. Hofmann, D. Wieland, et al. 2016. T cell factor 1-expressing memory-like CD8(1) T cells sustain the immune response to chronic viral infections. *Immunity*. 45: 415-427.

6. Wu, T., Y. Ji, E. A. Moseman, H. C. Xu, M. Manglani, M. Kirby, S. M. Anderson,

R. Handon, E. Kenyon, A. Elkahloun, et al. 2016. The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness. *Sci. Immunol.* 1:eaai8593.

7. Leong, Y. A., Y. Chen, H. S. Ong, D. Wu, K. Man, C. Deleage, M. Minnich, B. J. Meckiff, Y. Wei, Z. Hou, et al. 2016. CXCR5(1) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat. Immunol.* 17: 1187-1196.

8. He, R., S. Hou, C. Liu, A. Zhang, Q. Bai, M. Han, Y. Yang, G. Wei, T. Shen, X. Yang, et al. 2016. Follicular CXCR5- expressing CD8(1) T cells curtail chronic viral infection. [Published erratum appears in 2016 *Nature*. 540: 470.] *Nature*. 537: 412-428.

9. Hudson, W. H., J. Gensheimer, M. Hashimoto, A. Wieland, R. M. Valanparambil, P. Li, J.-X.

Lin, B. T. Konieczny, S. J. Im, G. J. Freeman, et al. 2019. Proliferating tran-sitory T cells with an effector-like transcriptional signature emerge from PD-1¹ stem-like CD8¹ T cells during chronic infection. *Immunity*. 51: 1043-1058.e4.

10. Zander, R., D. Schauder, G. Xin, C. Nguyen, X. Wu, A. Zajac, and W. Cui. 2019. CD4¹ T cell help is required for the formation of a cytolytic CD8¹ T cell subset that protects against chronic infection and cancer. *Immunity*. 51: 1028-1042.e4.

11. Paley, M. A., D. C. Kroy, P. M. Odorizzi, J. B. Johnnidis, D. V. Dolfi, B. E. Barnett, E. K. Bikoff, E. J. Robertson, G. M. Lauer, S. L. Reiner, and E. J. Wherry. 2012. Progenitor and terminal subsets of CD81 T cells cooperate to contain chronic viral infection. *Science*. 338: 1220-1225.

12. Galletti, G., G. De Simone, E. M. C. Mazza, S. Puccio, C. Mezzanotte, T. M. Bi, A.N. Davydov, M. Metsger, E. Scamardella, G. Alvisi, et al. 2020. Two subsets of stem-like CD8⁺ memory T cell progenitors with distinct fate commitments in humans. *Nat. Immunol.* 21: 1552-1562.

13. Sekine, T., A. Perez-Potti, S. Nguyen, J.-B. Gorin, V. H. Wu, E. Gostick, S. Llewellyn-Lacey, Q. Hammer, S. Falck-Jones, S. Vangeti, et al. 2020. TOX is expressed by exhausted and polyfunctional human effector memory CD8¹ T cells. *Sci. Immunol.* 5: eaba7918.

14. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigenindependent memory CD8 T cells do not develop during chronic viral infection. *Proc. Natl. Acad. Sci. USA.* 101: 16004-16009.

15. Kometani, K., R. Nakagawa, R. Shinnakasu, T. Kaji, A. Rybouchkin, S. Moriyama, K. Furukawa, H. Koseki, T. Takemori, and T. Kurosaki. 2013. Repression of the transcription factor Bach2 contributes to predisposition of IgG1 memory B cells toward plasma cell differentiation.

Immunity. 39: 136-147.

16. Chou, C., D. J. Verbaro, E. Tonc, M. Holmgren, M. Cella, M. Colonna, D. Bhattacharya, and T. Egawa. 2016. The transcription factor AP4 mediates resolution of chronic viral infection through amplification of germinal center B cell responses. *Immunity*. 45: 570-582.

17. McCausland, M. M., I. Yusuf, H. Tran, N. Ono, Y. Yanagi, and S. Crotty. 2007. SAP regulation of follicular helper CD4 T cell development and humoral immunity is independent of SLAM and Fyn kinase. *J. Immunol.* 178: 817-828.

18. Fooksman, D. R., M. C. Nussenzweig, and M. L. Dustin. 2014. Myeloid cells limit production of antibody-secreting cells after immunization in the lymph node. *J. Immunol.* 192: 1004-1012.

19. Stuart, T., A. Butler, P. Hoffman, C. Hafemeister, E. Papalexi, W. M. Mauck III,

Y. Hao, M. Stoeckius, P. Smibert, and R. Satija. 2019. Comprehensive integration of single-cell data. *Cell*. 177: 1888-1902.e21.

Tirosh, I., B. Izar, S. M. Prakadan, M. H. Wadsworth II, D. Treacy, J. J. Trombetta, A. Rotem,
 C. Rodman, C. Lian, G. Murphy, et al. 2016. Dissecting the multicellular ecosystem of metastatic
 melanoma by single-cell RNA-seq. *Science*. 352: 189-196.

21. Becht, E., L. McInnes, J. Healy, C.-A. Dutertre, I. W. H. Kwok, L. G. Ng, F. Gin-houx, and E. W. Newell. 2018. Dimensionality reduction for visualizing single- cell data using UMAP. *Nat. Biotechnol.* 37: 38-44.

22. Angerer, P., L. Haghverdi, M. B€uttner, F. J. Theis, C. Marr, and F. Buettner. 2016. Destiny: diffusion maps for large-scale single-cell data in R. *Bioinformatics*. 32: 1241-1243.

23. Miller, B. C., D. R. Sen, R. Al Abosy, K. Bi, Y. V. Virkud, M. W. LaFleur, K. B. Yates, A. Lako, K. Felt, G. S. Naik, et al. 2019. Subsets of exhausted CD8⁺ T cells differentially mediate

tumor control and respond to checkpoint blockade. [Published erratum appears in 2019 Nat. Immunol. 20: 1556.] Nat. Immunol. 20: 326-336.

24. Ahmadzadeh, M., L. A. Johnson, B. Heemskerk, J. R. Wunderlich, M. E. Dudley, D. E. White, and S. A. Rosenberg. 2009. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood.* 114: 1537-1544.

25. Blackburn, S. D., A. Crawford, H. Shin, A. Polley, G. J. Freeman, and E. J. Wherry. 2010. Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: implications for CD8 T-cell exhaustion. *J. Virol.* 84: 2078-2089.

26. Jung, Y. W., R. L. Rutishauser, N. S. Joshi, A. M. Haberman, and S. M. Kaech. 2010. Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection. *J. Immunol.* 185: 5315-5325.

27. Guan, T., C. X. Dominguez, R. A. Amezquita, B. J. Laidlaw, J. Cheng, J. Henao-Mejia, A. Williams, R. A. Flavell, J. Lu, and S. M. Kaech. 2018. ZEB1, ZEB2, and the miR-200 family form a counterregulatory network to regulate CD8¹ T cell fates. *J. Exp. Med.* 215: 1153-1168.

28. Omilusik, K. D., J. A. Best, B. Yu, S. Goossens, A. Weidemann, J. V. Nguyen, E. Seuntjens, A. Stryjewska, C. Zweier, R. Roychoudhuri, et al. 2015. Transcrip- tional repressor ZEB2 promotes terminal differentiation of CD81 effector and memory T cell populations during infection. *J. Exp. Med.* 212: 2027-2039.

29. Kao, C., K. J. Oestreich, M. A. Paley, A. Crawford, J. M. Angelosanto, M.-A. A. Ali, A. M. Intlekofer, J. M. Boss, S. L. Reiner, A. S. Weinmann, and E. J. Wherry. 2011. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD81 T cell responses during chronic infection. *Nat. Immunol.* 12: 663-671.

30. Intlekofer, A. M., A. Banerjee, N. Takemoto, S. M. Gordon, C. S. Dejong, H. Shin, C. A.

Hunter, E. J. Wherry, T. Lindsten, and S. L. Reiner. 2008. Anomalous type 17 response to viral infection by CD81 T cells lacking T-bet and eomesodermin. *Science*. 321: 408-411.

31. Cruz-Guilloty, F., M. E. Pipkin, I. M. Djuretic, D. Levanon, J. Lotem, M. G. Lichtenheld, Y. Groner, and A. Rao. 2009. Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J. Exp. Med.* 206: 51-59.

32. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J. Virol.* 77: 4911-4927.

33. Li, J., Y. He, J. Hao, L. Ni, and C. Dong. 2018. High levels of eomes promote exhaustion of anti-tumor CD8⁺ T cells. *Front. Immunol.* 9: 2981.

34. Chen, Z., Z. Ji, S. F. Ngiow, S. Manne, Z. Cai, A. C. Huang, J. Johnson, R. P. Staupe, B. Bengsch, C. Xu, et al. 2019. TCF-1-centered transcriptional network drives an effector versus exhausted CD8 T cell-fate decision. *Immunity*. 51: 840-855.e5.

35. Wilson, C. H., I. Gamper, A. Perfetto, J. Auw, T. D. Littlewood, and G. I. Evan. 2014. The kinetics of ER fusion protein activation in vivo. *Oncogene*. 33: 4877-4880.

36. Robinson, S. P., S. M. Langan-Fahey, D. A. Johnson, and V. C. Jordan. 1991. Metabolites, pharmacodynamics, and pharmacokinetics of tamoxifen in rats and mice compared to the breast cancer patient. *Drug Metab. Dispos.* 19: 36-43.

37. Harker, J. A., G. M. Lewis, L. Mack, and E. I. Zuniga. 2011. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science*. 334: 825-829.

38. Utzschneider, D. T., A. Legat, S. A. Fuertes Marraco, L. Carrie, I. Luescher,

D. E. Speiser, and D. Zehn. 2013. T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. *Nat. Immunol.* 14: 603-610.

39. Alfei, F., K. Kanev, M. Hofmann, M. Wu, H. E. Ghoneim, P. Roelli, D. T. Utzschneider, M. von Hoesslin, J. G. Cullen, Y. Fan, et al. 2019. TOX reinforces the phenotype and longevity of exhausted T cells in chronic viral infection. *Nature*. 571: 265-269.

40. Khan, O., J. R. Giles, S. McDonald, S. Manne, S. F. Ngiow, K. P. Patel, M. T. Werner, A. C. Huang, K. A. Alexander, J. E. Wu, et al. 2019. TOX transcription- ally and epigenetically programs CD8¹ T cell exhaustion. *Nature*. 571: 211-218.

41. Scott, A. C., F. Dundar, P. Zumbo, S. S. Chandran, C. A. Klebanoff, M. Shakiba, P. Trivedi,L. Menocal, H. Appleby, S. Camara, et al. 2019. TOX is a critical regulator of tumour-specific Tcell differentiation. *Nature*. 571: 270-274.

42. Seo, H., J. Chen, E. Gonz alez-Avalos, D. Samaniego-Castruita, A. Das, Y. H. Wang, I. F. Lopez-Moyado, R. O. Georges, W. Zhang, A. Onodera, et al. 2019. TOX and TOX2 transcription factors cooperate with NR4A transcription factors to impose CD8¹ T cell exhaustion. [Published erratum appears in 2019 *Proc. Natl. Acad. Sci. USA.* 116: 19761.] *Proc. Natl. Acad. Sci. USA.* 116: 12410-12415.

43. Yao, C., H.-W. Sun, N. E. Lacey, Y. Ji, E. A. Moseman, H.-Y. Shih, E. F. Heuston, M. Kirby,
S. Anderson, J. Cheng, et al. 2019. Single-cell RNA-seq reveals TOX as a key regulator of CD8⁺ T
cell persistence in chronic infection. *Nat. Immunol.* 20: 890-901.

44. Beltra, J.-C., S. Manne, M. S. Abdel-Hakeem, M. Kurachi, J. R. Giles, Z. Chen, V. Casella, S. F. Ngiow, O. Khan, Y. J. Huang, et al. 2020. Developmental relationships of four exhausted CD81 T cell subsets reveals underlying transcriptional and epigenetic landscape control mechanisms. *Immunity*. 52: 825-841.e8.

45. Danilo, M., V. Chennupati, J. G. Silva, S. Siegert, and W. Held. 2018. Suppression of Tcf1 by inflammatory cytokines facilitates effector CD8 T cell differentiation. *Cell Rep.* 22: 2107-

2117.

46. Snell, L. M., B. L. MacLeod, J. C. Law, I. Osokine, H. J. Elsaesser, K. Hezaveh,

R. J. Dickson, M. A. Gavin, C. J. Guidos, T. L. McGaha, and D. G. Brooks. 2018. CD8⁺ T cell priming in established chronic viral infection preferentially directs differentiation of memory-like cells for sustained immunity. *Immunity*. 49: 678-694.e5.

47. Sun, J., A. Ramos, B. Chapman, J. B. Johnnidis, L. Le, Y.-J. Ho, A. Klein, O. Hofmann, and F. D. Camargo. 2014. Clonal dynamics of native haematopoiesis. *Nature*. 514: 322-327.

48. Busch, K., K. Klapproth, M. Barile, M. Flossdorf, T. Holland-Letz, S. M. Schlenner, M. Reth,

T. Hofer, and H.-R. Rodewald. 2015. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature*. 518: 542-546.

49. Busch, K., and H.-R. Rodewald. 2016. Unperturbed vs. post-transplantation hematopoiesis: both in vivo but different. *Curr. Opin. Hematol.* 23: 295-303.

50. Hensel, N., Z. Gu, D. Sagar, D. Wieland, K. Jechow, J. Kemming, S. Llewellyn- Lacey, E. Gostick, O. Sogukpinar, F. Emmerich, et al. 2021. Memory-like HCV- specific CD8⁺ T cells retain a molecular scar after cure of chronic HCV infection. *Nat. Immunol.* 22: 229-239.

51. Heim, K., B. Binder, D. Sagar, N. Wieland, S. Hensel, E. Llewellyn-Lacey, E. Gostick, D. A. Price, F. Emmerich, H. Vingerhoet, et al. 2020. TOX defines the degree of CD81 T cell dysfunction in distinct phases of chronic HBV infection. *Gut.* DOI: 10.1136/gutjnl-2020-322404.

52. Yan, Y., S. Cao, X. Liu, S. M. Harrington, W. E. Bindeman, A. A. Adjei, J. S. Jang, J. Jen, Y. Li, P. Chanana, et al. 2018. CX3CR1 identifies PD-1 therapy- responsive CD81 T cells that withstand chemotherapy during cancer chemoimmunotherapy. *JCI Insight*. 3: e97828.



Figure 1. CX3CR1 marks two distinct subsets of exhausted T cells.

(A) UMAP analysis of scRNA-seq data of 2141 gp-33 specific CD8 T cells on 21 dpi with LCMV-c13. Right panels show the expression of the indicated genes. (**B and C**) Flow cytometry showing expression of TIM3, TCF-1, CX3CR1, and CD44 in LCMV gp33-specific CD8 T cells 4 wk after infection of C57BL/6 mice with LCMV-c13. Numbers indicate percentages of gated cells in each parental population shown in the figure. Data are representative of >5 independent experiments with >20 mice in total. (**D**) Expression of PD-1 measured by flow cytometry in the indicated population of LCMV-gp33-specific CD8 T cells on 22 dpi, quantified in the right panel. Data are representative of three independent experiments with *n* 5 3 mice each. (**E**) GZMB expression ex vivo as measured by flow cytometry of LCMV gp33-specific CD8 T cells in the indicated subpopulations on 22 dpi with LCMV-c13. Data are representative of two independent experiments with *n* = 4-6 mice each. (**F**) BrdU incorporation as measured by flow cytometry of LCMV gp33-specific CD8 T cells in the indicated subpopulations on 22 dpi with LCMV-c13 to the indicated subpopulation of 1 mg BrdU. Data are representative of three independent experiments with *n* 5 3 mice each. (**F**) BrdU incorporation as measured by flow cytometry of LCMV gp33-specific CD8 T cells in the indicated subpopulations on 22 dpi with LCMV-c13 to the indicated subpopulations of 1 mg BrdU. Data are representative of three independent experiments with *n* 5 3 mice each. (**F**) (p < 0.05), (p < 0.01)



Figure 2. Characterization of CX3CR1 expressing CD8 T cells in infection.

(A) UMAP plots of scRNA-seq data of LCMV-gp33-specific CD8 T cells in C57BL/6 mice on 21 dpi withLCMV-c13 showing expression of indicated genes. (B) Flow cytometry showing gating strategy for analysis of exhausted CD8 T cell subsets showing expression of CD44, TIM3, CX3CR1, and TCF-1 byLCMV gp33-specific cells on Day 22 after LCMV-c13 infection. Data are representative of greater than10 independent experiments. (C) Expression of Ki-67 in subpopulations of gp33-specific CD8 T cells defined by TIM3 and CX3CR1 in LCMV-c13infected C57BL/6 mice at different time points after infection. Data are representative of two experiments with n = 5 mice for each. (D) Flow cytometry showing expression of Nur77 in indicated subset of gp33 specific CD8 T cells 8dpi with LCMV-c13. Data in right panel are representative of two independent experiments with n = 3 mice each. (E) Flow cytometry analyzing expression of TIM3, CX3CR1, and TCF-1 by LCMV gp33-specific CD8 T cells in lungs of C57BL/6 mice that were intravenously injected with 3 ug of anti-CD45.2 three minutes before euthanasia. Data are representative of 2 independent experiments with n=3 mice each. Each dot in the graphs indicates an individual mouse that was examined, and data are shown by mean± SD. Statistical differences were tested using one-way ANOVA with a Tukey post-hoc test (B) and student's t-test (C).



Figure 3. Development and dynamics of CX3CR1 positive subsets of exhausted CD8 T cells. (A-C) Flow Cytometry showing expression of CX3CR1 and TIM3 (A) in TCF-1^{lo/-} gp33-specific CD8 T cells in control and CD4 T cell-depleted mice on the indicated dpi with LCMV-c13. Pooled data from two independent experiments with n=4-5 mice in total per group. Statistical analyses are shown in (B) and (C) as mean \pm SD. (D) Flow cytometry plots showing H-2D^b(gp33-41) binding CD8 T cells and expression of TCF-1, CX3CR1, and TIM3 on 30 dpi in mice treated with PBS or anti-PD-L1 initiated on22 dpi. (E) Frequencies and absolute numbers of indicated populations from (D) shown as mean \pm SD. Data are pooled from two independent experiments with n=5-6 mice per group in total.



Figure 4. TIM3⁻CX3CR1⁺ cells are progenitor-like cells upstream of TIM3⁺CX3CR1⁺ proliferating effector CD8 T cells.

(A) PCA plot derived from population-level RNA-seq analysis of the indicated subpopulations of PD-1⁺ CD8 T cells on 16 dpi. The populations were defined using *Prdm1*-YFP mice as *Prdm1*-YFP⁻ (TCF-1^{hi}) and subpopulations of *Prdm1*-YFP⁺ (TCF-1^{lo/-}) with a combination of CX3CR1 and TIM3. (B) Heat map showing expression of the indicated genes by the indicated subsets of PD-1⁺ CD8 T cells. Expression values were averaged from three independent samples for each population and color-coded based on row mean and fold changes. (C) Flow cytometry plots

showing expression of TCF-1 in the indicated subsets of H-2Db (gp33–41)⁺ CD8 T cells 22 dpi LCMV-c13 infection. Data in the right panel are representative of two independent experiments with n = 3-4 mice each. (**D**) Experimental schematic of infection-matched adoptive transfer. (**E–G**) Flow cytometry showing expression of SLAMF6, CX3CR1, and TIM3 in sorted donor CD45.2⁺PD-1⁺ CD8 T cells prior to transfer (top) and of the donor-derived (CD45.2) PD-1⁺ CD8 T cells 14 d after transfer. Frequencies of cells in each population and total cell yield from each donor population were quantified in (E) and (F). (**H**) CFSE dilution and expression of TIM3 of donor cells derived from TIM3⁻CX3CR1⁺ cells. Expression of TIM3 in CFSE^{lo}, CFSE^{med}, and CFSE^{hi} subpopulations is shown by a histogram overlay and geographical mean fluorescence intensity (gMFI). (**I**) Pseudotime and diffusion map analysis of scRNA-seq data from (Fig. 1A performed using destiny package.

Data shown in (D–G) are pooled from two independent experiments with n = 4-6 mice recipient mice analyzed for each donor transfer and shown as shown as mean \pm SD. Statistical analysis by one-way ANOVA was performed on data in (G). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.



Figure 5. Profiling of differentially expressed genes between distinct subpopulations of PD-1⁺ CD8 T cells following LCMV-c13 infection by bulk RNA-seq.

(A) Heatmap showing K-mean clustering of genes differentially expressed between distinct subpopulations on 16 dpi. Gene expression levels are shown as z-score normalized to each row mean.

(B) Gene Set Enrichment Analysis (GSEA) showing pathways associated with genes that were differentially expressed between TIM3⁻ CX3CR1⁺ and TIM3⁺ CX3CR1⁺ populations.

(C) Heat maps showing expression of indicated genes by distinct subsets of $PD-1^+$ CD8 T cells. Expression values were averaged from three independent samples for each population and color-codedbased on row mean and fold-changes.



Figure 6. T-bet is critical for the maintenance of CX3CR1+ exhausted CD8 T cell populations.

(A) Flow cytometry plots showing the expression of the indicated molecules within total and Tbethi gp33-specific CD8 T cells 22 dpi LCMV-c13 infection. Data are representative of three independent experiments with n = 4-6 mice each. (B) Flow cytometry plots showing expression of T-bet and Eomes in the indicated subsets of gp33-specific CD8 T cells in the spleen on 22 dpi with LCMV-c13 infection. Data are representative of three independent experiments with n 5 5 mice each. (C-E) Flow cytometry showing expression of TCF-1, CX3CR1, and TIM3 by gp33-specific CD8 T cells in control Tbx21^{F/F} and CD8-cre Tbx21^{F/F} mice on 29 dpi with LCMV-c13. Representative flow cytometry plots are shown in (C) with the frequencies of gated cells in each parental population, and statistical analyses are shown in (D) and (E). Data are shown as mean \pm SD and are pooled from three independent experiments with *n* > 8 mice per genotype. Dots in the graphs in (A), (C), and (D) indicate individual mice. Data in (A), (C), and (D) are shown as mean \pm SD. Statistical differences in (A) were tested using oneway ANOVA with a Tukey post hoc test. For (C), Student t test was used. For (D), Mann-Whitney U test was used. (F) LCMV-glycoprotein mRNA abundance in plasma as assessed by quantitative RT-PCR in *Tbx21*^{F/F} and CD8-cre *Tbx21*^{F/F} mice on 100 dpi with LCMV-c13. Data are pooled from two independent experiments with n = 4 (*Tbx21*^{F/F}) and n=5 (*CD8-cre*⁺ $Tbx21^{F/F}$) mice per genotype in total. Horizontal bars indicate median for samples in each genotype, and the statistical differences were assessed by Mann-Whitney U test. The dotted line indicates the limit of detection. p < 0.05, p < 0.01, p < 0.001.



Figure 7. Characterization of CD8 T cell-specific Tbx21- and Eomes- deficient mice during chronic infection.

(A-B, E-F) Flow cytometry plots showing expression of IFN- γ and TNF- α in CD8 T cells following *ex vivo* peptide re-stimulation of splenocytes from $Tbx21^{F/F}$ and CD8-cre $Tbx21^{F/F}$ (A-B), and *Eomes*^{F/F} and CD8-cre *Eomes*^{F/F} (E-F) on 30 dpi of LCMV-c13 infection. Statistical analysis is shown in (B) and (F). (C-D, G-H) Flow cytometry showing expression of Granzyme B by intracellular staining in the indicated subsets of gp33-specific CD8 T cells from $Tbx21^{F/F}$ and

CD8-cre *Tbx21*^{F/F} (C-D) and *Eomes*^{F/F} and CD8-cre *Eomes*^{F/F} (G-H). Data in (B, D, F, and H) are shown as mean \pm SD and are representative data from 2-3 independent experiments with n=4-5 mice analyzed per genotype; unpaired student's t-test was used for statistical analysis. (I) Viral abundance as assessed by qRT-PCR in plasma from *Eomes*^{F/F} and CD8-cre *Eomes*^{F/F} mice on 100 dpi with LCMV-c13. Data are pooled from 3 independent experiments with n=7 (*Eomes*^{F/F}) and n=9 (CD8-cre *Eomes*^{F/F}) in total. The horizontal line indicates median and statistical analysis was performed using Mann-Whitney U-test. The dotted line indicates the limit of detection.



Figure 8. Opposing roles of EOMES and BACH2 in the differentiation of progenitor-like subsets of exhausted CD8 T cells.

(A-C) Flow cytometry showing expression of CD44, TCF-1, CX3CR1, and TIM3 of gp33tetramer⁺ CD8 T cells in the spleen of control *Eomes*^{F/F} and CD8-cre *Eomes*^{F/F} mice on 28 dpi with LCMV-c13. Representative flow cytometry plots are shown in (A) with the frequencies of gated cells in each parental population, and statistical analyses are shown in (B) and (C). Data are representative of three independent experiments with n = 4-6 mice per genotype in each experiment. (**D-G**) Flow cytometry showing the expression of TCF-1, CX3CR1, and TIM3 of gp33-tetramer⁺ CD8 T cells in *Bach2*^{F/F} and *CD8-Cre⁺Bach2*^{F/F} mice 30 d after LCMV-c13 infection. Representative flow cytometry plots are shown in (D) with frequencies of gated cells in each parental population, and statistical analyses are shown in (E)-(G). Data are pooled from two independent experiments with n = 4 (*Bach2*^{F/F}) and n = 5 (*CD8-Cre⁺Bach2*^{F/F}) in total. All statistical analysis plots are shown as mean \pm SD with statistics performed using an unpaired *t* test. *p < 0.05, **p < 0.01, ***p < 0.001.



Figure 9. Lineage tracing reveals the stability and persistence of CX3CR1⁺PD-1⁺ CD8 T cells.

(A) Experimental schematic of TAM administration to $Cx3cr1^{creERT2/+}$ R26tdT mice infected with LCMV-c13 and analysis. (B) Flow cytometry plots showing the expression of tdT and TCF-1 in gp33-specific CD8 T cells 16 h following TAM administration on 8 dpi. Data from replicates are shown in the right panel. Data are representative of two independent experiments with n = 3-4 mice each. A paired *t* test for the statistical analysis. (C) Representative flow cytometry plots showing expression of tdT and CX3CR1 in PD-1⁺ CD8 T cells in PBMCs and frequencies of tdT⁺ cells within CX3CR1⁺PD-1⁺ CD8 T cells in the PBMC in $Cx3cr1^{CreER/+}$ R26tdT mice at the indicated time points after infection and TAM administration. Data are representative of two independent experiments with n=3 mice each. (D) Flow cytometry plots showing expression of the tdT reporter in each of gp33-specific CD8 T cell subpopulations in the spleen of $Cx3cr1^{creERT2/+}$ R26tdT mice on 22 dpi. Data are representative of two independent experiments with n=3 mice each. **p < 0.01, ***p < 0.001.



Figure 10. CX3CR1⁺ CD8 T cells during the chronic phase give rise to TCF-1⁺ chronic memory cells following the resolution of viremia.

(A and B) Flow cytometry plots showing expression of tdTomato (tdT), TCF-1, CX3CR1, and TIM3 in gp33-specific CD8 T cells in the splenocytes of LCMV- c13-infected $Cx3cr1^{creERT2/+}$ R26tdT mice treated with TAM on 28 dpi and analyzed on >120 dpi. Representative data from two independent experiments with n = 3 mice are shown. Flow plots are shown in (A), and replicates from one experiment are shown as mean \pm SD. (B). (C) Expression of IFN- γ and TNF- α in the indicated subsets of CD8 T cells in the spleen of 29 dpi LCMV-c13-infected mice, >120 dpi LCMV-Arm-infected mice, or >120 dpi LCMV-c13-infected mice following stimulation with LCMV-gp33 peptide *ex vivo* for 4 h. Data are representative of two independent experiments with n = 3 mice each. (D) Expression of TCF-1

and CX3CR1 in LCMV-gp33-specific splenic CD8 T cells (top) and expression of PD-1 and TOX in TCF-1⁺LCMV-gp33-specific CD8 T cells at the indicated time point after LCMV-Arm or LCMV-c13 infection. Frequencies of TOX⁺ cells and levels of TOX expression are shown with mean \pm SD. (E) Schematic of adoptive transfer experiment to assess the capability of secondary expansion of CX3CR1⁺ and CX3CR1⁻ CD8 T cells that were sorted from LCMV-Arm- and LCMV-c13-infected mice. The right panel shows data from replicates. Data are representative of two independent experiments with n = 2 donor mice and n= 5 recipient mice per experiment. Fold expansion was calculated by fold change of gp33-specific CD8 T cells followed by normalization to no infection control.

<u>Chapter 3: BCL6-dependent TCF-1+ progenitor cells</u> <u>maintain effector and helper CD4 T cell responses to</u> <u>persistent antigen</u>

The contents of this chapter are from a paper that is accepted at Immunity

Xia, Y.*, Sandor, K. S.*, Pai, J. A.*, Daniel, B., Raju, S., Wu, R., Hsiung, S., Qi, Y., Yangdon, T., Okamoto, M., Chou, C., Hiam-Galvez, K. J., Schreiber, R. D., Murphy, K. M., Satpathy, A. T., & Egawa, T. (2022). BCL6-dependent TCF-1⁺ progenitor cells maintain effector and helper CD4 T cell responses to persistent antigen. [Accepted].

Submitted as a preprint. doi.org/10.1101/2021.08.06.455141. (*: equal contribution).

3.1 Abstract

Shortly after priming, the fate of activated CD4 T cells is segregated into BCL6⁺ follicular helper T (Tfh) and BCL6⁻ effector (Teff) cells. However, it remains unknown how these subsets are sustained in the presence of chronic antigen stimulation. Using a combination of single cell- and population-based approaches, we show that in chronic viral infection, activated CD4 T cells differentiate in a BCL6-dependent manner into TCF-1⁺ progenitor cells with superior capacity to expand and give rise to both Teff and Tfh . They share properties with progenitor-exhausted CD8 T cells and are required for the continued generation of antigen-specific CD4 T cells as antigen persists. In response to tumors, an analogous CD4 T cell population develops in draining lymph nodes. Our study reveals the heterogeneity and plasticity
of CD4 T cells upon encountering persistent antigen and highlights their population dynamics through a stable, bipotent, intermediate state.

3.2 Introduction

CD4 T cells play a central role in coordinating innate and adaptive immunity. In response to signals through T cell receptors (TCR) and cytokines provided by dendritic cells, they functionally differentiate into T effector (Teff) cells and produce context-specific cytokines to coordinate activation of innate immune cells ^{1–3}. Activated CD4 T cells also differentiate into follicular helper T (Tfh) cells that provide contact-dependent help to B cells for their expansion and affinity maturation. Shortly after priming, CD4 T cells diverge into BCL6[–] and BCL6⁺ subsets depending on the strength of TCR and IL-2R signaling in a B cell-independent manner ^{4–} ⁷. Stronger TCR signaling and subsequent upregulation of IL-2 is associated with the expression of BCL6 and differentiation into Tfh cells, whereas activation of IL-2R signaling and IL-12R signaling induces upregulation of the transcription factor Blimp1 (PRDM1), which promotes Teff differentiation and suppresses Tfh fate via downregulation of BCL6 and TCF-1^{4,8–10}. In studies using acute pathogen infection or non-replicating antigen, the early binary fate decision of CD4 T cells is stable as early BCL6⁺ cells exhibit skewed developmental potential towards Tfh at the express of Teff cells ^{4,11}.

Following antigen clearance, the antigen-specific CD4 T cell population contracts, giving rise to memory CD4 T cells that confer long-lasting protection against re-infection. These cells are classified into central (Tcm) and effector (Tem) memory subpopulations ¹². Tem cells, similar to Teff cells, express tissue homing receptors, promptly produce effector cytokines such as interferon-gamma (IFN-g) upon rechallenge, and decay faster than Tcm. In contrast, Tcm cells

express homing receptors, such as CCR7 and CD62L, that facilitate their homing to lymphoid organs. Additionally, Tcm cells are capable of robust IL-2 production immediately after stimulation, resembling BCL6⁺ T helper cells that emerge early after priming. In acute viral or intracellular bacterial infections, Tcm cells are derived from central memory precursor CD4 T cells (Tcmp) that are formed at the peak of the T cell response 3,13,14 . Tcmp cells already express Tcm-related genes such as *Tcf7*, *Ccr7*, *Id3* and *Bcl2*, but phenotypically overlap with pre-Tfh cells with an intermediate level of BCL6 and CXCR5 expression 15,16 . Thus, the relationship between Tfh and memory populations and the nature of memory precursor cells has remained incompletely understood.

CD4 T cells also play essential roles in controlling chronic viral infection and tumors, in which their responses must be sustained for substantially longer periods, compared to acute infection or vaccine-elicited immune responses. Similar to CD8 T cells, activated CD4 T cells acquire distinct phenotypes in the presence of chronic antigen compared to acute viral infection ^{17–19}. Whereas increased expression of inhibitory receptors is the hallmark feature of exhausted CD8 T cells, CD4 T cells exposed to persistent antigen exhibit a unique pattern of gene expression changes, including elevated expression of *Bcl6* and additional similarities to Tfh cells ²⁰, while expression of multiple inhibitory receptors is less pronounced compared to CD8 T cells ¹⁷. In CD8 T cells, a series of recent studies identified a subset of stem-like or progenitor-like exhausted CD8 T cells, referred to as T progenitor exhausted (TPEX) cells, that express the transcription factors (TF) TCF-1 and BCL6, and the canonical Tfh marker CXCR5 ^{21–25}. TPEX cells are necessary to sustain CD8 T cell effector responses and critical for enhanced antiviral and anti-tumor immunity in response to immune checkpoint blockade (ICB). Given the terminally differentiated nature of CD4 Teff and Tfh cells, the continued CD4 T cell response by

Teff and Tfh cells may similarly be supported by their continued differentiation from less differentiated stem-like, or progenitor cells, which could be similar to previously proposed TCF-1⁺ cells generated by asymmetric divisions ²⁶. However, it remains unknown whether sustained CD4 T cell response depends on such a progenitor population, or how they are distinct, if any, from pre-Tfh or memory CD4 T cells.

To address this question, we performed an unbiased characterization of the heterogeneity of antigen-specific CD4 T cells in response to infection with a chronic strain of Lymphocytic choriomeningitis virus (LCMV, clone 13 (c13)), which achieves prolonged antigen persistence, infection and identified a population of PD-1⁺ progenitor CD4 T (Tprog) cells that were TCF-1⁺ BCL6^{lo/-} and distinct from TCF-1⁻ BCL6⁻ Teff and TCF-1⁺ BCL6^{hi} Tfh cells. This cell population is detectable at the peak of T cell response and persists into the chronic phase of infection in a B cell-independent manner. Trajectory analysis, TCR clonal tracing, and adoptive transfer experiments demonstrate that they serve as common progenitors for both Teff and Tfh cells. Epigenomic analysis with single-cell ATAC-seq (scATAC-seq) suggests that TCF-1⁺ BCL6^{lo/-} progenitor cells require NFAT and AP-1 TF activity for early differentiation, followed by bifurcation to the two distinct terminal fates. Their development also requires BCL6 and CD4 T cell-specific conditional deletion of Bcl6 not only results in the expected loss of BCL6^{hi} TCF-1⁺ Tfh but also the total ablation of TCF-1⁻ BCL6⁻ Teff cells two weeks after infection, despite an intact initial expansion. Finally, in tumor-bearing mice, phenotypes of tumor-reactive CD4 T cells were distinct between the tumor microenvironment and tumor-draining lymph nodes (tdLNs). An analogous heterogeneity of CD4 T cells was observed in tdLNs, while antigenspecific CD4 T cells in the tumor were predominantly TCF-1⁻ Teff cells, suggesting tumorspecific CD4 T cells are maintained in secondary lymphoid organs rather than in the tumor

microenvironment. These results collectively indicate that following an initial wave of naive T cell-derived differentiation of Teff and Tfh cells, CD4 Teff and Tfh cell responses to persistent antigen are maintained by a pool of BCL6-dependent TCF-1⁺ common Tprog cells, akin to how the CD8 response is maintained by CD8 TPEX.

3.3 Results

scRNA-seq and flow cytometry reveals heterogeneity within antigen-specific CD4 T cells responding to chronic viral infection.

To obtain an unbiased overview of the CD4 T cell response to persisting antigen, we performed paired single-cell RNA-seq (scRNA-seq) and TCR sequencing of I-A^b-LCMV-gp66specific (Tet⁺) and the remaining total CD4 T cells (Tet⁻) in C57BL/6 (B6) mice infected either with LCMV-c13 (collected 8 or 21 days post infection - dpi) or with an acute strain of LCMV (Armstrong strain (Arm)) (collected 8 or 21 dpi) (Fig. 11A). In total, we obtained 80,785 cells after quality control filtering based on the number of genes captured and percent mitochondrial reads per cell (# of genes within 2 standard deviations of mean; % mitochondrial reads < 95thpercentile; Methods), and we obtained paired TCR $\alpha\beta$ sequences in 89.0% of these cells (Fig. 11B). Dimensionality reduction and clustering of the cells based on their gene expression profiles identified 15 cell type clusters, most of which were grouped into 5 large clusters: $Sell^+$ Ccr7⁺ Tcf7⁺ Slamf6⁻ naive (Cluster 1 from RNA-seq as abbreviated as C1r), Tcf7⁺ Slamf6⁺ Bcl6⁻ Cxcr5^{lo/-} resting memory-like (C2r), Tcf7⁺ Slamf6⁺ activated memory-like (C3r and 4r), Tcf7⁺ Slamf6⁺ Bcl6⁺ Cxcr5⁺ Tfh (C5r), and Prdm1⁺ Slamf1⁺ Cxcr6⁺ Teff cells (C6r and C7r) (Fig. 12A, **B**; Fig. 11C). C3r and C4r were characterized by the expression of *Tcf7*, *Slamf6*, *Pdcd1*, *Zbtb32*, and Bcl2 at similar levels to C2r, and additionally expressed Cd69 and Mif, which are upregulated by activated T cells ^{27,28}. They also expressed additional genes induced by TCR signaling, including *Egr1*, *Egr2*, *Nr4a1*, *Tnfrsf4*, *Nfkbia* and *Batf* (**Fig. 12A-C; Fig. 13A, B**), suggesting that they represent activated memory-like cells. We also identified 3 clusters of regulatory T cells (Tregs), including thymic *Il2ra^{hi}* and peripheral *Il2ra^{lo}* Tregs (C8r and C9r, respectively), as well as a population of proliferating Tregs (C10r). C11r comprised CD4 T cells expressing high levels of genes related to the IFN response, including *Irf7* and IFN-stimulated genes *Isg15*, *Ifit1*, and *Ifit3*. Lastly, we found several additional small clusters, including an undefined population of non-Treg CD4 T cells with elevated expression of the chemokine receptors, *Ccr4* and *Ccr6* (C12r), *Myb* (C13r), *Gzmk* (C14r), and proliferating markers including *Hmgb2* and *Stmn1* (C15r).

Arm-8 dpi Tet⁻ cells, which contained a mixture of bystander naive cells, resting memory CD4 T cells, and LCMV-reactive CD4 T cells recognizing non-dominant epitopes, were present in all 5 major clusters, with a substantial proportion (~30%) in the C1r Naive cluster (**Fig. 12A**). In contrast, Tet⁺ cells more frequently exhibited Teff and Tfh phenotypes, constituting approximately 40% of the whole population. A large proportion of the remaining Tet⁺ cells displayed the resting memory phenotype (**Fig. 12A**), likely corresponding to Tcmp ^{13,14}. In c13 infection at 8 and 21 dpi, the relative frequency of Teff cells in Tet⁻ CD4 T cells was reduced compared to CD4 T cells responding to Arm infection, while that of Tfh cells was similar, indicating Tfh-skewing of expanding CD4 T cells in c13 infection. However, compared to Tet⁺ cells in Arm infection, the percentage of Teff cells within Tet⁺ cells in c13 infection was notably reduced on 8 dpi (**Fig. 12A**). Frequencies of Tet⁺ cells with the resting memory phenotype (C2r) were decreased in c13 infection compared to Arm infection on 8 and 21 dpi. However, c13-Tet⁺

cells in this cluster differed qualitatively from their Arm counterparts with a relative upregulation of activation-induced genes (e.g., Pdcd1, *Lag3*, *Tnfrsf4*) and IFN-I-induced genes (e.g., *Ifi27l2a*), which are associated with Tfh cells¹⁴, and downregulation of the Th1/Teff-associated genes, *Ccl5*, *Nkg7* and *Ly6c2*^{3,29,30}(**Fig. 12C, right and Fig. 13C, D**). These changes in gene expression suggest that cells in the memory-like cell populations may be biased towards Tfh over Teff. In addition, activated memory-like cells in C3r and C4r were significantly increased in c13-Tet⁺ samples compared to Arm infection samples on 21 dpi (Fig. 12A and Fig. 13E). Altogether, these results suggest the presence of a distinct *Tcf7*⁺ resting memory population (C2r) in c13 infection and increased mobilization to the activated memory-like cell pool (C3/4r) and into more differentiated cells.

To validate the scRNA-seq dataset with analysis of the subset-specific proteins, we used flow cytometry to analyze splenocytes of c13-infected B6 mice for BCL6 and TCF-1 expression (**Fig. 12D-F**). On 5 dpi, TCF-1⁺ cells uniformly upregulated BCL6, which is gradually and uniformly downregulated on 8 and 15 dpi (**Fig. 12D**). On 22 dpi, TCF-1⁺ cells constituted a large proportion of Tet⁺ CD4 T cells (**Fig. 12E, F**). and a subfraction of these cells expressed higher BCL6 as they further underwent differentiation into Tfh cells and supported expansion of germinal center (GC) B cells. A similar change was observed in expression of CXCR5 (**Fig. 12E**). Consistent with the scRNA-seq data, approximately 15% of Tet⁺ CD4 T cells expressed CD69, and about half of them were TCF1⁺ BCL6^{10/-}. TCF-1⁻ cells minimally expressed CD69 (**Fig. 12G**). Both TCF-1⁺ populations expressed Ly-108 (encoded by *Slam/6*) but lacked expression of the Teff marker *Prdm1* based on a *Prdm1*-YFP reporter (**Fig. 12E, H**). TCF-1⁺ BCL6^{10/-} cells expressed lower PD-1 and higher Ly-108 compared to TCF-1⁺ BCL6^{hi} cells, and a minority (~5%) of them expressed CCR7, in contrast to resting Tcm. TCF-1⁻ cells upregulated T-bet, CXCR6 and CD150 (encoded by *Slamf1*) as well as the *Prdm1* reporter, indicating that they were highly enriched for differentiated Teff cells. TCF-1⁻ cells also contained a small percentage (~5%) of Foxp3⁺ Treg cells (**Fig. 13F**). Consistent with previous reports, CXCR5 was a reliable surrogate marker of BCL6, while Ly-108 was a reliable surrogate for TCF-1 expression (**Figs. 12E and 13G**). In terms of tissue distribution, Tfh cells were barely detected in the bone marrow or liver, whereas Teff cells were detected at higher percentages (40-50%) in those organs compared to the spleen, and TCF-1⁺ BCL6^{10/-} cells were also present in those organs (**Fig. 13H, I**). While CD4 T cells from all three populations produced cytokines following stimulation with gp61-77 peptide *ex vivo*, production of TNF and IL-2 was significantly reduced in TCF-1⁻ cells (**Fig. 13J**), suggesting that they are relatively exhausted compared to two other populations of PD-1⁺ CD4 T cells.

To investigate the similarities between CD4 and CD8 T cells in c13 infection, we compared the transcriptional profiles of each CD4 population to a single-cell CD8 T cell dataset from LCMV-Arm and c13 infection ³¹. We calculated gene signature scores of CD8 T cell populations, including CD8 memory (Tmem), TPEX (Texprog), KLR-expressing effector cells (TexKLR), intermediate/transitory cells (Texint), and terminally exhausted cells (Texterm) (Methods). First, we observed that the CD4 *Tcf*7⁺ C2r population exhibited the highest similarity to CD8 Tmem in acute infection (mean CD8 Tmem signature score: 0.231; **Fig. 13**), supporting their similarity to resting memory CD8 T cells as observed by our scRNA-seq analysis. Second, CD4 Teff cells aligned closely to the exhausted CD8 clusters TexKLR, Texint, and Texterm in c13 infection, with the C7r Teff-Ifng cluster displaying the highest similarity to Texterm (mean CD8 Texterm signature score: 0.295), in line with the elevated expression of exhaustion-related markers (e.g. Havcr2, Lag3) in this cluster (**Fig. 13B**). Lastly, using the Texprog gene signature,

which comprised 108 genes including *Tcf7*, *Slamf6*, and *Id3*, we observed that CD8 TPEX cells were most transcriptionally similar to *Tcf7*⁺ *Cd69*⁺ *Pdcd1*⁺ CD4 T cells (mean CD8 TPEX signature score: 0.354), and also showed similarity to Tfh cells (mean CD8 TPEX signature score: 0.328; **Fig 13K**). In line with this similarity, the frequency of *Tcf7*⁺ *Slamf6*⁺ *Bcl2*⁺ *Pdcd1*⁺ memory-like cells was increased in c13 infection, compared to Arm infection (**Fig 13E**), indicative of progenitor potential, which actively supports the maintenance of Teff and Tfh cells in response to persistent antigen.

Collectively, our results indicate that with chronic persistence of antigen, a substantial fraction of antigen-specific CD4 T cells acquire a TCF-1⁺ PD-1⁺ memory-like phenotype. These memory-like CD4 T cells resemble CD8 TPEX and contain a subpopulation of cells with a gene expression signature associated with TCR-mediated activation. They may thus act as progenitor cells to support the maintenance of Teff and Tfh cells in response to persistent antigen.

Memory-like cells are enriched for common progenitors for both Tfh and Teff

To investigate the potential developmental relationships between CD4 T cell clusters, including memory-like, Teff, and Tfh cells, we performed trajectory inference analysis (Monocle 3; Methods) using the scRNA-seq data. To specifically analyze the transition of cells in response to chronic antigen stimulation, we reclustered Tet⁺ cells from c13 infection on 8 and 21 dpi and labeled the cells according to the previously defined phenotypic clusters (**Fig. 14A, 15A**). Tfh (C5r) and Teff (C6/7r) were mapped separately and most distantly in pseudotime from the naive cluster (C1r), supporting their terminally differentiated states. Naive cells were closely projected to the resting memory-like populations (C2r), but did not directly transition into either terminally

differentiated population. Instead, cells in C2r transitioned into mature Tfh (C5r) or Teff (C6/7r) cells after passing through the activated memory-like state (C3r) and a subsequent bifurcation, suggesting that the memory-like state serves as a common progenitor for both Tfh and Teff fates. Along the trajectory from naive to mature Tfh cells (**Fig. 14B, top**), *Tcf7* expression remained roughly constant across all differentiation time points (**Fig. 14C**). As cells differentiated from C3/4r, *Bcl6* and *Cxcr5* expression was increased following commitment to the mature Tfh phenotype (**Fig. 14C**). In contrast, *Tcf7* expression decreased along the Teff trajectory (**Fig. 14B, top**), while *Runx3*, *Prdm1*, and *Ifng* expression increased (**Fig. 14D**).

To further support our hypothesis that cells in the memory-like clusters constitute a common progenitor pool for Tfh and Teff cells, we paired single-cell phenotyping with TCR sequence information to define clonal developmental trajectories of memory-like (C2r and C3/4r), Tfh (C5r), and Teff (C6/7r) cells. We defined TCR clones based on identical TCR $\alpha\beta$ CDR3 sequences and identified 10,330 total clones from c13 infection 21 dpi cells (Fig. 15B). Most of the expanded clones (>1 cell per clone) in all infection conditions and timepoints were observed in the Tet⁺ population, while the majority of Tet⁻ clones were detected as unexpanded singletons (Fig. 15C-D). Among expanded Tet⁺ clones on 21 dpi in LCMV-c13 (880 total expanded clones; 9,803 total cells), 483 clones were found in two or more of the three broad phenotypic clusters, and 442 of the 483 (819%) expanded clones were detected in the memorylike population, in addition to Tfh and/or Teff populations (Fig. 14E). Indeed, the most highly expanded clones from c13-21 dpi all comprised cells with the memory-like phenotypes (Fig. 14F). In contrast, only 41 expanded clones (7.8%) were detected in both Tfh and Teff populations, but not in the memory-like population (Fig. 14E). Taken together, these results suggest that during LCMV-c13 infection, the vast majority of the long-term clonal CD4 T cell

response is maintained by the memory-like cells that replace the initially expanded naive poolderived clones and function as putative common Tprog cells.

Epigenetic differentiation trajectories of progenitor CD4 T cells

To identify epigenetic pathways of TCF-1⁺ progenitor CD4 T cell differentiation, we performed single-cell assay for transposase accessible chromatin with sequencing (scATAC-seq) on PD-1⁺ CD4 T cells from the spleens of c13-infected mice on 21 dpi (Fig. 16A). In total, we obtained high quality scATAC-seq profiles from 11,611 cells (unique fragment count >1,000, transcription start site enrichment >11; Fig. 16B, C) and performed dimensionality reduction using iterative latent semantic indexing followed by UMAP³², which identified 8 distinct chromatin state clusters after removing thymic Tregs (Fig. 14A). Next, we integrated scATACseq and scRNA-seq datasets to define the identity of each cell cluster, and link pseudo-scRNAseq expression values to each cell in the scATAC-seq data (Fig. 17B; Fig. 16D; scRNA-seq for integration analyses generated from the same experiments, Methods). To perform integrative analysis, we first visualized the accessibility of cell cluster marker genes identified in scRNA-seq data shown in Fig. 12 by calculating gene scores, which is a measurement of the overall accessibility of the gene body and surrounding open chromatin regions (OCR) ³³; (see Methods). Second, we performed unconstrained integration of single-cell transcriptomic and chromatin accessibility profiles to calculate gene integration scores, yielding gene expression profiles at the single-cell level in the scATAC-seq defined UMAP space (see Methods). Importantly, these analyses identified cells with Tfh (C4a and C5a), progenitor (C6a and C7a) and Teff (C2a and C3a) phenotypes; C6a and 7a were marked by intermediate accessibility of Tcf7 and Slamf6 and

low to intermediate accessibility of *Cxcr5* and *Bcl6*, corresponding to the putative Tprog cell clusters identified in the transcriptomic analysis (**Fig. 17C**). Despite the overall epigenetic similarity between C6a and C7a, hierarchical clustering of differentially activated loci revealed increased accessibility in C6a at several loci related to T cell activation, including *Cd69*, *Batf*, *Nr4a2*, *Irf4*, and *Tox* (**Fig. 17C**; **Fig. 16E**, **F**). The result suggests that C7a and C6a correspond to the resting (C2r) and activated (C3r) Tprog cell clusters identified by scRNA-seq, respectively. To further support these findings, we defined the differential OCRs for each cluster and performed motif enrichment analysis between C6a and C7a, identifying several motifs associated with NFAT and AP-1 TF families in C6a compared to C7a (**Fig. 17D**). In contrast, Tfh cells showed high TF motif enrichment of TCF/LEF TF family members, while Teff cells showed high enrichment of TBX and RUNX motifs (**Fig. 17D**).

To further investigate the epigenetic dynamics between the Tprog, Teff, and Tfh populations, we constructed cellular trajectories for the transition to either terminally differentiated CD4 subset, and analyzed differential TF motif accessibility along the two paths (**Fig. 17E, top**). Tprog cells are clustered in between the Tfh and Teff cells, and TCR-based analysis uncovered extensive clonal sharing of Teff and Tfh cells with this population (**Figs. 12, 14**). Therefore, we used Tprog cells as a starting point, and reconstructed two differentiation trajectories using a nearest-neighbor approach (sequential selection of similar chromatin states based on Euclidean distance, see Methods). As cells transitioned from C7a to C6a (representing the common path of the two trajectories), they gained accessibility at NFAT, BACH, and BATF/AP-1 motifs, representing the early chromatin remodeling events of Tprog commitment (**Fig. 17E**). After this common differentiation step, cells bifurcated into terminally differentiated Teff and Tfh phenotypes. At these later stages of the trajectory, cells lost accessibility at

common progenitor TF motifs and gained accessibility at Tfh- (e.g., TCF7, ASCL, CEBP, and MAF) ^{9,34–36}, and Teff-related TF motifs (e.g., RUNX, TBX/EOMES) ^{37–40}(**Fig. 17E**).

To quantify chromatin remodeling activities across the different phenotypic populations, we performed differential chromatin accessibility analysis between Tprog, Tfh and Th1 effector cells (Fig. 17F). Comparison of C7a and C6a Tprog cells identified 1,988 and 5,797 differentially accessible OCRs, while a comparison of C4a (Tfh) and C6a (Tprog) cells yielded 4,525 and 4,290 differential OCRs, respectively. Comparing C2a (Teff) to C6a (Tprog) cells identified 5,482 and 2,616 differentially accessible OCRs, respectively. We performed motif enrichment analyses at these specific OCRs and found that the Tprog populations exhibited several motifs associated with NFAT and AP-1 binding in C6a, compared to C7a (Fig. 17G), recapitulating the trajectory analysis. Enrichment of motifs for NFAT and AP-1 was lost during the early transition from Tprog to Teff in addition to loss of TCF/LEF-related motifs (Fig. 17G), which was consistent with loss of Tcf7 accessibility and expression (Fig. 17H; Fig. 16G). In contrast, the most significantly enriched motifs were associated with RUNX activity, which have been implicated in the commitment to the Th1 lineage ^{37,38}. During the transition from Tprog to Tfh, we detected a similar reduction of accessibility to NFAT and AP-1 motifs, while several motifs associated with the binding of basic helix-loop-helix (bHLH) proteins were enriched, which have been shown to promote Tfh differentiation at the expense of Th1/Teff differentiation 35,41

Altogether, these results suggest that activated CD4 T cells adopt two distinct states of TCF-1⁺ progenitor phenotypes: resting Tcmp-like (C2r/C7a) and activated memory (C3r/C6a) states. Resting memory-like cells are mobilized to terminal differentiation by transient TCR signaling, initially without polarization towards either lineage, and the specification of each

terminal cell type is likely initiated after cells complete the transition to C6a through the activation of lineage-specific TF binding motifs. These two-step processes may be distinct from the initial differentiation towards either lineage as an immediate consequence of T cell activation during the initial priming of naive T cells.

TCF-1⁺ *BCL6*^{*lo/-*} *cells exhibit superior proliferative capacity and give rise to both TCF-1*⁻ *Teff and TCF-1*⁺ *BCL6*^{*hi*} *Tfh following adoptive transfer*

To demonstrate the progenitor function of the TCF-1⁺ BCL6^{lo/-} cells *in vivo*, we compared repopulation and differentiation capacities of the three major subsets of PD-1⁺ CD4 T cells isolated from LCMV-c13 infected mice. Each population was adoptively transferred into congenic B6-CD45.1 mice, which were either subsequently infected with LCMV-c13, or infection-matched at the time of transfer (**Figs. 18 and 19**). We used Ly-108 and CXCR5 as surrogate surface markers for the expression of TCF-1 and BCL6 (**Fig. 13G**). Ly-108⁺ CXCR5⁻ (enriched for TCF-1⁺ BCL6^{lo/-} cells), Ly-108⁺ CXCR5⁺ (enriched for TCF-1⁺ BCL6^{hi} cells), and Ly-108⁻ CXCR5⁻ (enriched for TCF-1⁻ BCL6⁻ cells) PD-1⁺ CD4 T cells were harvested as donor cells from B6 mice that had been infected with LCMV-c13 for 21-24 days (**Fig. 19A**).

Following transfer to naive host mice and a subsequent challenge with LCMV-c13 infection (**Fig. 19B**), the donor-derived Ly-108⁺ CXCR5⁻ cells expanded at a significantly higher magnitude compared to the two other populations of donor cells (**Fig. 19C, D**). They repopulated all three major populations of the PD-1⁺ CD4 T cell pool at similar ratios compared to those of endogenous CD4 T cells (**Fig. 19C, middle, and D**), indicating their progenitor function. In contrast, Ly-108⁻ CXCR5⁻ Teff cells remained predominantly as TCF-1⁻ BCL6⁻ cells and

generated few TCF-1⁺ CD4 T cells with low overall proliferation (**Fig. 19C, left, and D**), an indication of their terminal differentiation. Ly-108⁺ CXCR5^{hi} Tfh cells expanded the least among the three donor populations and gave rise to a higher frequency of TCF-1⁺ BCL6⁺ cells compared to the other populations (**Fig. 19C, right, and D**). We detected TCF-1⁺ BCL6⁻ and TCF-1⁻ CD4 T cells in the mice that had received donor Tfh cells; however, the absolute numbers of these cells were much lower than those derived from donor Teff or memory-like cells (**Fig. 19D**).

To more stringently test the ability of cells in each CD4 subset to proliferate and differentiate, we performed transfers to infection-matched recipient mice (Fig. 18A-C). Spleens from recipient mice were analyzed 15 days after transfer (36 dpi). Similar to the transfer to naive host mice followed by rechallenge, total and Tet⁺ Ly-108⁺ CXCR5⁻ PD-1⁺ cells demonstrated the highest proliferation capacity with more than 10-fold expansion compared to Teff and Tfh donor cells (Fig. 18B-E). They gave rise to all three populations at a ratio similar to endogenous CD4 T cells, further demonstrating their lineage plasticity and self-maintenance. Since the numbers of donor-derived cells 24 hour after transfer were comparable between Ly-108⁺ CXCR5⁻ cells and Ly-108⁻ Teff cells (Fig. 19E-G), we concluded that Teff cells are short-lived with limited expansion capacity. In contrast, the engraftment of Tfh cells was substantially lower compared to the other two populations (Fig. 19E-G), suggesting their impaired migration possibly due to CXCR5 blockade as a result of cell sorting. While we detected small numbers of TCF-1⁺ BCL6^{lo/-} cells in recipients of Teff or Tfh cells, it is likely that the observed phenotypic changes were caused by infrequent, contaminating memory-like cells during purification (Fig. 18E, F).

Together, the results from these adoptive transfer experiments demonstrated that the Ly-108⁺ CXCR5⁻ activated CD4 T cells retain high proliferative capacity and plasticity to differentiate into both TCF-1⁻ Teff and TCF-1⁺ Tfh cells. These results suggest that they act as progenitor cells to maintain the pool of differentiated Teff and Tfh cells.

TCF-1⁺ BCL6^{lo/-} PD-1⁺ CD4 cells are progenitors that maintain TCF-1⁻ effector cells *in vivo*

Our results thus far have shown that in the presence of chronic antigen, CD4 T cells preferentially adopt the TCF-1⁺ BCL6^{lo/-} phenotype after a wave of binary differentiation from naive CD4 T cells into Teff and Tfh cells. TCF-1⁺ BCL6^{lo/-} cells can give rise to TCF-1⁻ Teff cells following adoptive transfer and thus can function as progenitors for antigen-specific CD4 T cells. To determine whether they are required for sustained CD4 Teff response, we conditionally deleted Bcl6 in a CD4 T cell-specific manner. To achieve this, we generated a novel cre transgenic driver that facilitates deletion of a loxP-flanked gene in CD4 T cells - with minimal impact on CD8 T cells - by knocking-in cre into the Cd40lg locus on the X chromosome (Fig. 20A). This cre driver deleted a loxP-flanked transcriptional stop cassette in the Rosa26-stop-YFP allele in virtually all CD4⁺ T cells, whereas its activity in CD8 T cells was substantially lower in naive, Arm- and c13-infected mice (Fig. 20B-G). YFP⁺ cells were barely detectable in B cells, myeloid cells, and NK cells (Fig. 20C, E). GC B cells were formed comparably in Cd40lg-cre male mice compared to control mice (Fig. 20H). Comparable contribution of YFP⁺ and YFP⁻ CD4 T cells to Tfh cells in female Cd40lg-cre Rosa26-stop-YFP mice further indicates that cre knock-in to the 3' UTR preserved the function of the *Cd40lg* gene (Fig. 20I).

Using this animal model, we investigated the role of *Bcl6* in the maintenance of longterm antigen-specific CD4 T cell response. On 8 dpi with LCMV-c13 infection, the number of Tet⁺ cells was comparable in *Cd40lg*-cre *Bcl6*^{F/F} compared to control *Cd40lg*-cre *Bcl6*^{+/+} mice (Fig. 21A, B). In Tet⁺ CD4 T cells, TCF-1⁺ cells were practically absent in *Cd40lg*-cre *Bcl6*^{F/F} mice with the number of TCF-1⁻ CXCR6⁺ Teff cells remaining intact (Fig. 21C, D). This result indicates that the deletion of *Bcl6* was complete and that the development of both TCF-1⁺ BCL6^{hi} Tfh and TCF-1⁺ BCL6^{lo/-} cells requires *Bcl6*. In LCMV-Arm infected mice, *Bcl6* deletion caused not only a loss of Tfh cells as defined by CXCR5⁺ PD1⁺ CD4 T cells or Tet⁺ TCF-1⁺ BCL6^{hi} cells, but also a loss of TCF-1⁺ BCL6^{lo/-} cells, which contains CCR7⁺ Tcmp cells (Fig. 22A, B) that express low levels of BCL6⁻¹³. The CD4 Teff cells in c13-infected *Cd40lg*-cre *Bcl6*^{F/F} and control mice expressed comparable levels of the inhibitory receptor TIM3, indicating they are qualitatively similar (Fig. 22C). Although the number of Teff cells was comparable between *Cd40lg*-cre *Bcl6*^{F/F} mice (Fig. 21C, D), indicating that the initially generated BCL6-independent Teff cells derived from naive CD4 T cells are short-lived and that *Bcl6* and BCL6-teff cells.

The TF Blimp1 plays antagonistic roles to BCL6 in the differentiation of Tfh versus Teff CD4 T cells, memory versus effector CD8 T cells, and memory B versus plasma cells $^{9,34,42-47}$. In the absence of Blimp1 in *Cd40lg*-cre *Prdm1*^{F/F} mice, expansion of Tet⁺ CD4 T cells was increased by 3-fold compared to control mice on 8 dpi with c13 (**Fig. 22D, E**). In addition to the greater expansion, the vast majority of Tet⁺ CD4 T cells in *Cd40lg*-cre *Prdm1*^{F/F} mice were TCF-1⁺. Thus, Blimp1 not only regulates the expansion of antigen-specific CD4 T cells, but also promotes the differentiation of TCF-1⁺ into TCF-1⁻ CD4 T cells.

To determine whether TCF-1⁺ BCL6^{hi} or Ly-108⁺ CXCR5⁺ Tfh cells are necessary for the development of TCF-1⁺ BCL6^{lo/-} cells or the maintenance of TCF-1⁻ Teff cells, we analyzed

MuMT mice lacking TCF-1⁺ BCL6^{hi} Tfh cells. Although total Tet⁺ cells were mildly diminished on 21 dpi, the frequency of cells in the TCF-1⁺ BCL6^{lo/-} compartment was comparable to control WT mice despite the total loss of TCF-1⁺ BCL6^{hi} cells (**Fig. 21E, F**). These results indicate that BCL6^{hi} CXCR5^{hi} cells are dispensable for the maintenance of antigen-specific CD4 T cells.

Furthermore, to determine whether Ly- 108^+ CXCR5⁻ Tprog cells are sufficient to support CD4-dependent immune response, we adoptively transferred distinct subsets of PD-1⁺ CD4 T cells to infection-matched *Cd40lg*-cre *Bcl6*^{F/F} mice on 21 dpi, in which host-derived LCMV-specific CD4 T cells were severely reduced (**Fig. 21G-I**). Four weeks after the transfer, the numbers of GL7⁺ Fas⁺ B cells, enriched for GC B cells, were increased by 10-fold in recipients of Ly- 108^+ CXCR5⁻ donor CD4 T cells compared to those of either of the two other subsets, indicating that transferred Tprog cells are sufficient to provide help to B cells. Taken together, our results establish *Bcl6* as a critical regulator of the differentiation of TCF-1⁺ BCL6^{lo/-} Tprog cells, which are indispensable for long-term, antigen specific CD4 T cell responses during chronic viral infection.

Finally, to gain insights into the contribution of Tprog or Tfh CD4 T cells to the maintenance of IL-21-dependent CX3CR1⁺ exhausted CD8 T cells ^{48,49}, we examined LCMV-gp33-specific CD8 T cells in LCMV-c13-infected MuMT mice, lacking BCL6⁺ Tfh, and *Cd40lg*-cre *Bcl6*^{F/F}, in which antigen-specific CD4 T cells are severely reduced in the chronic phase of LCMV-c13 infection. Although CD4 T cell depletion prior to infection almost completely depletes the CX3CR1⁺ population of exhausted CD8 T cells, frequencies of CX3CR1⁺ TCF-1⁻ cells in gp33-specific CD8 T cells were only mildly reduced in both MuMT and *Cd40lg*-cre *Bcl6*^{F/F} mice (**Fig. 23**). Although the observed reduction confirms that antigen-specific CD4 T cells, or more specifically BCL6⁺ Tfh cells, play roles in the maintenance of

CX3CR1+ exhausted CD8 T cells, these results suggest the presence of alternative sources of IL-21 that are dependent on CD4 T cells specifically in early phases of infection.

Tumor antigen-specific CD4 T cells differentiate predominantly into TCF-1⁺ BCL6^{10/-} cells in tumor-draining lymph nodes

Our results using viral infection models raise the question of whether a similar progenitor population might support sustained antigen-specific CD4 responses during anti-tumor immune responses. In order to examine CD4 T cell differentiation in anti-tumor immunity and determine if it shows similarities to chronic LCMV infection, we examined the differentiation and proliferation of OT-II CD4 T cells expressing an I-A^b-restricted OVA-specific TCR in response to the subcutaneously transplanted ovalbumin (OVA) expressing 1956 sarcoma cell line ⁵⁰. We adoptively transferred CFSE-labeled, CD45.1/2 OT-II CD4 T cells one day prior to tumor inoculation and tracked their proliferation by CFSE dilution and differentiation by staining for TCF-1 and BCL6 in the tumor and tdLNs (**Fig. 24A**).

In tdLNs, OT-II CD4 T cells differentiated into all three populations defined by the expression of TCF-1 and BCL6 (**Fig. 24B, C**). As we observed in LCMV-c13 infected mice, the frequency of the TCF-1⁺ BCL6^{lo/-} cells was the highest among expanded OT-II cells. Although TCF-1⁻ Teff, BCL6⁺ Tfh and the majority of TCF-1⁺ BCL6^{lo/-} cells fully diluted CFSE over several days, approximately 10% of TCF-1⁺ BCL6^{lo/-} cells underwent limited rounds of division and expressed higher levels of BCL6 compared to fully divided TCF-1⁺ cells (**Fig. 24D-F**). Thus, the tumor-reacting TCF-1⁺ BCL6^{lo/-} cells were heterogeneous, ranging from BCL6^{int/hi} PD-1^{lo} dormant cells to extensively divided BCL6^{lo/-} PD-1⁺ cells, which resembled Tprog found in

LCMV-infected mice. In contrast to cells in tdLNs, OT-II cells isolated from the tumors were predominantly TCF-1⁻ CXCR6⁺ Teff cells (**Fig. 24B, C**). Since a small fraction of extensively divided OT-II CD4 T cells in tdLN upregulated CXCR6 before downregulating TCF-1 expression (**Fig. 24G**), tumor-reactive CD4 T cells likely initiate their Teff differentiation during the TCF-1⁺ state prior to migration to the tumor microenvironment. These results suggest that in the setting of chronic antigen stimulation, rather than binary differentiate into TCF-1⁻ BCL6⁻ or TCF-1⁺ BCL6⁺ populations, CD4 T cells preferentially differentiate into TCF-1⁺ BCL6^{lo/-} cells, which may serve as progenitors and continuously generate fully differentiated Teff and Tfh cells as they continue to recognize antigen. These results also suggest that such continued differentiation mainly takes place in tdLNs rather than in tumors, possibly because only the tdLNs contain the appropriate microenvironment for TCF-1⁺ BCL6^{lo/-} cells to retain a partially differentiated phenotype.

3.4 Discussion

Using a combination of single-cell genomics and population-based experiments in the chronic LCMV-c13 model, we demonstrate that antigen-specific CD4 T cells differentiate into TCF-1⁺ BCL6^{lo/-} PD-1⁺ CD4 Tprog cells that serve as progenitor cells and give rise to Teff and Tfh cells in response to persistent antigen. During an acute infection or in response to vaccination, significant proportions of Teff and Tfh cells are derived directly from naive CD4 T cells ^{4,6}. In contrast, CD4 T cell response to chronic antigen is more complicated and requires progenitor cells that can support long-term Teff responses as the initial Teff cells decay. This process is illustrated in our *Cd40lg*-cre *Bcl6*^{F/F} model, where the initial Teff cell expansion from

naive cells was intact, but Teff cells fail to persist due to a lack of Tprog cells that generate new Teff cells. Thus, the developmental pathway of CD4 Teff cells is shifted from BCL6-independent to -dependent as antigen persists.

The CD4 Tprog cells identified in this study exhibit similarities to CD4 Tcm, Tcmp, and pre-Tfh cells that also express Tcf7, Slamf6, and Bcl2, which allows for their long-term survival in contrast to differentiated Teff and Tfh cells ^{1,6,14}. Shortly after activation, ranging from several hours to a few days, CD4 T cells diverge into IL2Ra⁺ Blimp1⁺ Teff cells and IL2Ra⁻ BCL6⁺ cells in a B cell-independent manner ^{6,7}. These IL2Ra⁻ BCL6⁺ cells contain Tcmp and pre-Tfh ¹¹. This binary fate choice between Teff and Tfh is stable, since differentiation of adoptively transferred IL2Ra⁻ BCL6⁺cells is substantially biased towards Tfh, and the depletion of these early IL2Ra⁻ BCL6⁺ cells leads to a significant reduction of Tfh cells ^{4,11}. In the contexts of acute infection or immunization with non-replicating antigen, both antigen levels and inflammation wane by the time pre-Tfh or Tcmp cells develop. In contrast, following LCMV-c13 infection, antigen levels remain high for the first several days, rendering TCF-1⁺ PD-1⁺ CD4 T cells that might otherwise become pre-Tfh or Tcmp exposed to continued signals through TCR and other receptors. Such continued stimulation may result not only in changes in surface marker expression, such as increased PD-1 and reduced CCR7 and CXCR5 compared to Tcmp¹⁴, but also epigenetic changes that preserve Teff differentiation, although the newly defined Tprog population is heterogeneous and may contain or overlap with committed pre-Tfh with a similar surface phenotype.

Our single-cell analysis revealed two states of cells in the progenitor pool with distinct activation signatures. The transition between the two states is associated with NFAT- and AP-1target gene activation; increased chromatin accessibility of these TF binding motifs was the dominant change between the two states. Trajectories between the resting progenitor state to Tfh or Teff using both transcriptomic and epigenetic analyses suggest that the specification to either lineage occurs independently of the initial transition from the resting to activated states. Activation of E-protein targets, which may be mediated by E2A and ASCL2 35,41 , in progenitor-Tfh trajectory and activation of RUNX targets for the Teff trajectory 37,38 were detected as CD4 Tprog cells acquire transient NFAT and AP-1 activation. Although a stringent validation may require fate tracing at the single cell level, this conclusion is also supported by the substantially overlapping TCR clonality among resting and activated Tprog, Tfh and Teff populations, while Teff and Tfh are derived from distinct clones in the context of acute infection 51 . The activated Tprog cells may overlap with previously described, BCL6-dependent PD-1⁺ CD4 T cells that develop during *M. tuberculosis* infection 52 .

Finally, our study highlights the similarities between CD4 Tprog and CD8 TPEX cells. Both cell types develop in the presence of persistent antigen, express a shared transcriptional program, including *Tcf7*, *Slamf6*, and *Pdcd1*, and continue generating differentiated effectors while they self-renew. Although these cell types resemble CD4 Tcmp and CD8 MPEC, respectively, they have unique gene expression and epigenetic signatures, such as those associated with activation. In addition, we observed the emergence of PD-1⁺ CD4 T cells in tumor-bearing mice, which phenocopy progenitor CD4 T cells in response to LCMV infection. Many studies have demonstrated the importance of CD8 TPEX in enabling durable anti-tumor immunity and the immune response to ICB therapies. CD8 TPEX cells are found in tumors and extratumoral tissues, such as lymph nodes. In mouse tumor transplantation models, it has been demonstrated that intratumoral CD8 TPEX cells are sufficient to promote anti-tumor immunity in response to vaccine or ICB ⁵³. In our tumor transplantation experiments, we found tumorreactive TCF-1⁺ PD-1⁺ CD4 T cells exclusively in the tdLNs while almost all antigen-specific CD4 T cells in the tumor were differentiated TCF-1⁻ Teff cells. In the LNs, a small fraction of tumor-reactive TCF-1⁺ CD4 T cells initiate Teff differentiation as indicated by CXCR6 upregulation. CD4 T cell-dependent anti-tumor immunity is dependent on expression of BCL6 in tumor-specific CD4 T cells, and enhanced by the presence of cognate B cells ⁵⁴. The abundance of non-Teff CD4 T cells suggests that such interaction between CD4 T and B cells may occur in tdLNs, and in turn promote anti-tumor immunity in multiple mechanisms, including promoting CD4 Teff differentiation and establishing an IL-21-rich environment.

During the revision of our manuscript, an independent study demonstrated the presence of a CXCR5⁻ CXCR6⁻ memory-like CD4 T cell subset during chronic LCMV infection ⁵⁵. This study shows that Tfh cells derived from the memory-like cells are an essential source of IL-21 to sustain CX3CR1⁺ exhausted CD8 T cells. It is likely that the described memory-like CD4 T cell population overlaps with Tprog cells defined in the current work. However, it also remains to be determined whether CXCR5⁺ Tfh cells are the predominant source of IL-21, given that both MuMT and *Cd40lg*-cre *Bcl6*^{F/F} mice largely maintain CX3CR1⁺ exhausted CD8 T cells despite a specific lack of Tfh and a substantial loss of total antigen-specific CD4 T cells, respectively.

In summary, our analysis decoded the heterogeneity of CD4 T cells that respond to persistent antigen in the context of antiviral and anti-tumor immunity and highlighted a population of TCF-1⁺ BCL6^{lo/-} PD-1⁺ CD4 T cells as progenitor cells that support the continued generation of differentiated effectors and helper CD4 T cells. Progenitor CD4 T cells go through a transitory state in which they retain an unbiased epigenetic signature towards either terminal fate, and subsequently resolve the bipotential states, which is distinct from the binary differentiation of naive CD4 T cells into Teff and Tfh early in an immune response. Our results

reveal the population dynamics and differentiation hierarchy of CD4 T cells for their sustained responses to persistent antigen.

3.5 Materials and Methods

Mice and infection

Male C57BL/6N (B6) and B6-CD45.1 mice were purchased from Charles River Laboratories and JAX. *Prdm1*-EYFP, *Bcl6*-flox mice were obtained from the Jackson Laboratory. *Cd40lg*-cre mice were generated by knocking in a mammalian codon optimized cre coding sequence following an internal ribosomal entry sequence (IRES) into 3' UTR of the Cd40*lg* locus by homologous recombination in JM8.N4 embryonic stem cells. After germline transmission, the FRT-flanked selection cassette was removed by crossing to *Actb*-Flpe transgenic mice (JAX). Because the *Cd40lg* locus is on X-chromosome, all female mice used in the *Bcl6* deletion experiments were homozygous for cre knock-in. All mice were housed in a specific pathogenfree facility at Washington University in St. Louis and were used for infection at 8–12 wk of age, unless stated otherwise. LCMV infection was performed essentially as described ⁵⁶. All experiments were performed according to a protocol approved by Washington University's Institutional Animal Care and Use Committee.

scRNA-seq and TCR-seq sample and library generation

Single-cell RNA-seq libraries were prepared using the 10X Chromium Next Gem Single Cell V(D)J Reagent Kit (v1.1 Chemistry), according to the manufacturer's instructions. Briefly,

FACS sorted cells were washed once with PBS + 0.04% BSA and resuspended in PBS containing 0.04% BSA. Following reverse transcription and cell barcoding in droplets, emulsions were broken, and cDNA purified using Dynabeads MyOne SILANE followed by PCR amplification (98°C for 45 sec; 14 cycles of 98°C for 20 sec, 67°C for 30 sec, 72°C for 1 min; 72°C for 1 min). Amplified cDNA was then used for both 5' gene expression library construction and TCR enrichment. For gene expression library construction, 50 ng of amplified cDNA was used for fragmentation, following by and end-repair, double-sided size selection with SPRIselect beads, PCR amplification with sample indexing primers (98°C for 45 sec; 14 cycles of 98°C for 20 sec, 54°C for 30 sec, 72°C for 20 sec; 72°C for 1 min), and double-sided size selection with SPRIselect beads. For TCR library construction, TCR transcripts were enriched from 2 µl of amplified cDNA by PCR (primer sets 1 and 2: 98 °C for 45 s; 10 cycles of 98 °C for 20 s, 67 °C for 30 s, 72 °C for 1 min; 72 °C for 1 min). Following TCR enrichment, 5 - 50 ng of enriched PCR product was fragmented and end-repaired, size-selected with SPRIselect beads, PCRamplified with sample-indexing primers (98 °C for 45 s; 9 cycles of 98 °C for 20 s, 54 °C for 30 s, 72 °C for 20 s; 72 °C for 1 min), and size-selected with SPRIselect beads._scRNA/TCR-seq libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and a HighSensitivity DNA chip run on a Bioanalyzer 2100 system (Agilent). Sequencing was performed on NovaSeq S4 (Illumina) with paired-end reads (2 x 150 cycles).

scRNA-seq and TCR-seq library processing

Reads from 10x scRNA expression libraries were aligned to mouse genome assembly GRCm38 (mm10) and quantified using cellranger *count* (10x Genomics, version 3.1.0). The filtered

feature-barcode matrices containing only cellular barcodes were used for further analysis. Single cell gene expression matrices were imported into R (version 3.6.1) and analyzed using Seurat (version 3.1.1)⁵⁷. Cells for which the number of genes captured fell within two standard deviations of the mean of all cells in the library were kept. Additionally, cells with mitochondrial RNA content percentages above the 95th-percentile were excluded from subsequent analyses.

Single cell TCR reads were aligned to mouse genome assembly GRCm38 (mm10) and assembled into reconstructed TCR consensus sequences using cellranger *vdj* (10x Genomics, version 3.1.0). Only productive TCR α and TCR β sequences were considered for further analysis. Overall, TCR sequences were annotated for 80,785 cells that passed RNA quality filtering, with paired TCR $\alpha\beta$ sequences detected for 71,818 cells (89.0%). Only cells with conventional paired TCR chain combinations $\alpha\beta$ or $\alpha\alpha\beta$ were kept for downstream clonotype analyses. Cells sharing the same CDR3 $\alpha\beta$ amino acid sequences were defined as belonging to the same TCR clone.

scRNA-seq data integration and clustering

scRNA-seq libraries of I-A^b-LCMV-gp66 tetramer-sorted CD4 T cell populations from LCMV-Arm and LCMV-c13 at 8 dpi and 21 dpi were normalized individually while regressing out cell cycle score. Cells passing quality filtering (80,785 cells) from all 19 samples (Fig. 11B-C) were then integrated by identifying anchors between datasets using 30 reciprocal PCA dimensions. TCR genes were excluded from the selection of integration anchors to prevent TCR chain driven biases. Dimensionality reduction of the integrated matrix was performed using Uniform Manifold Approximation and Projection (UMAP) with the first 15 principal components. Phenotypic clusters were defined by constructing a k-nearest neighbors graph and identifying groups of cells using the Louvain algorithm with resolution of 0.5.

CD8 gene signature scoring

To compare the CD4 T cell populations in LCMV infection identified by scRNA-seq to previously described CD8 T cell populations, we computed gene signature scores for each cell with Seurat's AddModuleScore() function ⁵⁷ using gene signatures of CD8 T cell subsets in LCMV-c13 infection from Daniel et al., 2021 ³¹. Mitochondrial, TCR, and BCR genes were removed from all gene sets in order to prevent biases due to library quality or TCR clonal composition. Scores were then averaged per CD4 cluster to generate a mean composite signature score for each CD4 population.

Trajectory inference

To perform trajectory analysis of the CD4 T cell response in LCMV-c13 infection, dimensionality reduction of I-A^b-LCMV-gp66 Tet⁺ cells on 8 dpi (2 samples) and 21 dpi (4 samples) was performed using UMAP as described above after excluding cells belonging to the Treg clusters C8-10r and other infrequent populations (C12-15r). Pseudotime analysis was then performed with Monocle 3 ⁵⁸ by learning a principal graph for the data and ordering cells along the graph using the cells in the naive phenotype cluster to select a root node.

scATAC-seq sample and library generation

Single cell ATAC-seq dataset is obtained from two biological replicates. Experiments were performed on the 10x Chromium platform as described previously³². Briefly, following sorting, cells were subjected to nuclei isolation according to the manufacturer's recommendation. After tagmentation, nuclei were processed for generating scATAC-seq libraries and loaded to the 10x Chromium controller. For GEM incubation the standard thermocycler conditions were used and library construction was done as described by 10x Genomics for scATAC-seq. Libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and a HighSensitivity DNA chip run on a Bioanalyzer 2100 system (Agilent). Sequencing was performed on NovaSeq S4 (Illumina) with paired-end reads (2 x 150 cycles), and demultiplexed using CellRanger-ATAC v1.2.

scATAC-seq analysis

scATAC-seq datasets were processed as described previously ⁵⁹. Briefly, reads were filtered, trimmed, and aligned to the mm10 reference genome using the 10x cellranger atac-count pipeline. Fragment files were loaded into ArchR for additional processing and analysis. Doublets were identified and removed using ArchR's default doublet simulation and calling procedures. Barcodes were removed that had an enrichment of Tn5 insertions in transcription start sites (TSS enrichment) less than 4 or less than 1000 fragments. Tiles and GeneScores matrices were computed by summing Tn5 insertions in predefined genomic windows. After clustering the cells, peaks were called by macs2 ⁶⁰ on pseudoreplicates sampled from each cluster to obtain a reproducible peak set retaining cell type specific peaks. scATAC-seq and scRNA-seq datasets were integrated using the ArchR addGeneIntegrationMatrix() function, which directly aligns cells from scATAC-seq with cells from scRNA-seq by comparing the scATAC-seq gene score

matrix with the scRNA-seq gene expression matrix. For each cell in the scATAC-seq data, this integration process finds the cell in the scRNA-seq data that looks most similar and assigns the gene expression data from that scRNA-seq cell to the scATAC-seq cell. Dynamics of scRNA-seq expression data can then be computed using this paired cell matrix. TF motif deviations were computed using chromVar ⁶¹. Imputation was performed using Magic ⁶².

Cell preparation, cell staining, and flow cytometry

Single-cell suspensions of splenocytes were prepared by manual disruption with frosted glass slides. Bone marrow cells were dissociated from the femur with mortar and pestle. Liver cells were prepared by manual disruption with frosted glass slides followed by gradient centrifugation with 40% Percoll. Absolute live cell counts were determined by trypan blue exclusion using Vi-CELL (Beckman Coulter). PE- or APC-labeled I-A^b-LCMV-gp66 tetramer reagents were obtained from the NIH Tetramer Core at Emory and PE- or APC-labeled H2-D^b-LCMV-gp33 tetramer reagents were obtained from MBL (TB-M512-1). Total splenocytes were stained with MHC-tetramers at room temperature for 60 min. For analysis of T cells in the liver and lung, circulating T cells were excluded by staining for biotinylated anti-CD3e that was intravenously injected (3 ug) 3 minutes before euthanasia. For intracellular cytokine staining, total splenocytes were cultured in RPMI-1640 supplemented with 10% FBS in the presence of 1 ug/ml of LCMV-gp61-80 peptide (GenScript Biotech) and 5 ug/ml of Brefeldin A (BioLegend) for 4 h before staining. Cells for intracellular staining were staining for surface proteins and then labeled with LIVE/DEAD Aqua (Thermo Fisher Scientific) before fixation and intracellular staining using the Foxp3 staining kit (eBioscience) according to the manufacturer's instructions.

The following antibodies were purchased from BioLegend, unless otherwise indicated:

Alexa Fluor (AF)-conjugated donkey polyclonal anti-rabbit IgG (Thermo Fisher Scientific, catalog no. R37118); AF647-conjugated goat polyclonal anti-rabbit IgG (Cell Signaling, catalog no. 4414S); AF700-conjugated anti-CD44 (IM7); APC-conjugated anti-PD-1 (29F.1A12); APC-Cv7-conjugated anti-CD8a (53-6.7, BD Biosciences); BV421- or BV711- conjugated anti-CXCR5 (L138D7); Biotinylated anti-CD3e (145-2C11); PE-conjugated anti-GL7 (GL7); PerCP-Cy5.5-conjugated anti-CD4 (GK1.5); BUV395-conjugated anti-CD4 (GK1.5, BD Biosciences); PerCP-Cv5.5-conjugated anti-CD45.1 (A20), A700-conjugated anti-CD45.2 (A20); anti-Ki67 (B56); PE-Cy7-conjugated anti-PD-1 (29F.1A12), anti-CD95 (Fas)(Jo2); PE-Dazzle 594conjugated anti-B220 (RA3-6B2), anti-CXCR6 (SA051D1); BV605-conjugated anti-Ly-108 (13G3, BD Biosciences); BV650-conjugated anti-CD150 (SLAM)(TC15-12F12.2); FITCconjugated anti-CD69 (H1.2F3); BV605-conjugated anti-CX3CR1 (SA011F11); PE-Cy7conjugated anti-LAG3 (C9B7W); BV421- or APC- conjugated anti-Tim-3 (RMT3-23); eFluor660-conjugated anti-TOX (TXRX10, Thermofisher); unconjugated anti-TCF-1 (Cell Signaling Technology, C63D9), BV421-conjugated anti-BCL6 (K112-91); PE-conjugated anti-IFN-g (XMG1.2); APC-conjugated anti-IL-2 (JES6-5H4, BD Pharmingen); and APC-conjugated TNF (MP6-XT22). Stained samples were analyzed with BD FACS LSR Fortessa, X20, or Symphony A3 or sorted on Aria II or III. Data were analyzed using FlowJo Software (FlowJo).

Adoptive Transfer

For transfer into naive mice, CD4 T cells from the spleen and peripheral lymph nodes of B6 mice on 22-24 dpi with LCMV-c13 were harvested and enriched for CD4 T cells using a MojoSort Mouse CD4 T Cell Isolation Kit (BioLegend) prior to surface staining. 180,000 - 250, 000 Ly-108⁻ CXCR5⁻, Ly-108⁺ CXCR5⁻, or Ly-108⁺ CXCR5⁺ PD-1⁺ CD4 T cells were sorted and intravenously transferred into naive CD45.1 congenic recipients, which were infected with LCMV-c13 the next day. For infection-matched transfer, donor CD4 T cells from B6 mice on 21 dpi with LCMV-c13 were bead-enriched, surface stained and sorted as described above. 1-2 million Ly-108⁻ CXCR5⁻, Ly-108⁺ CXCR5⁻, or Ly-108⁺ CXCR5⁺ PD-1⁺ CD4 T cells were transferred into infection-matched CD45.1 congenic recipients. Splenocytes from the recipient mice were analyzed at time points described in Figure legends.

Tumor transplantation experiment

500,000 CFSE-labeled CD45.1/2 OT-II cells were intravenously transferred into B6 mice, which were subcutaneously inoculated with 1 million 1956-mOVA cells⁵⁰ the next day. Tumor infiltrating lymphocytes (TILs) and cells in the tdLN were harvested 8 to 9 days after inoculation. LN cells were prepared by manual dissociation with frosted glass slides and TILs were prepared by digestion with Collagenase B, D1, and DNaseI, followed by staining with fluorescently labeled antibodies as described above.

Statistical analysis

The *P* values were calculated with an unpaired two-tailed Student's *t*-test and by one-way ANOVA for multigroup comparisons with the Tukey post hoc test using Prism 9 software (GraphPad): *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Data availability

All single-cell sequencing data generated in this paper have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE181474.

3.6 Acknowledgements

We thank the NIH tetramer core at Emory for providing the I-A^b-LCMV-gp66 tetramers. This study was supported by NIH grants R01AI130152 (to T.E.), R21AI161040 (to T.E.), 5T32AI007290 (to J.A.P.), K08CA230188 (to A.T.S.), U01CA260852 (to A.T.S.), UMHG012076 (to A.T.S.), R01CA190700 (to R.D.S), P30AR073752 (Rheumatic Diseases Research Resource-Based Center at Washington University), the Leukemia and Lymphoma Society Scholar Award (to T.E.), Stanford Propel Scholarship (to KJHG), the Parker Institute for Cancer Immunotherapy (to A.T.S. and R.D.S), the Burroughs Wellcome Fund Career Award for Medical Scientists (to A.T.S.), a Technology Impact Award from the Cancer Research Institute (to A.T.S.), a Pew-Stewart Scholars for Cancer Research Award (to A.T.S.), and a Baxter Foundation Scholar Award (to A.T.S.).

3.7 References

- Pepper, M. & Jenkins, M. K. Origins of CD4(+) effector and central memory T cells. *Nat. Immunol.* 12, 467–471 (2011).
- Crotty, S., Johnston, R. J. & Schoenberger, S. P. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat. Immunol.* 11, 114–120 (2010).

- 3. Marshall, H. D. *et al.* Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity* **35**, 633–646 (2011).
- 4. DiToro, D. *et al.* Differential IL-2 expression defines developmental fates of follicular versus nonfollicular helper T cells. *Science* **361**, (2018).
- Hondowicz, B. D., Kim, K. S., Ruterbusch, M. J., Keitany, G. J. & Pepper, M. IL-2 is required for the generation of viral-specific CD4+ Th1 tissue-resident memory cells and B cells are essential for maintenance in the lung. *Eur. J. Immunol.* 48, 80–86 (2018).
- Choi, Y. S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34, 932–946 (2011).
- Baumjohann, D., Okada, T. & Ansel, K. M. Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. *J. Immunol.* 187, 2089–2092 (2011).
- Johnston, R. J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325, 1006–1010 (2009).
- Wu, T. *et al.* TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. *CellReports* 12, 2099–2110 (2015).
- Sheikh, A. A. & Groom, J. R. Transcription tipping points for T follicular helper cell and Thelper 1 cell fate commitment. *Cell. Mol. Immunol.* 18, 528–538 (2021).
- 11. Choi, Y. S. *et al.* Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory. *J. Immunol.* **190**, 4014–4026 (2013).

- Ruterbusch, M., Pruner, K. B., Shehata, L. & Pepper, M. In Vivo CD4 T Cell Differentiation and Function: Revisiting the Th1/Th2 Paradigm. *Annual Review of Immunology* vol. 38 705–725 (2020).
- Pepper, M., Pagán, A. J., Igyártó, B. Z., Taylor, J. J. & Jenkins, M. K. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 35, 583–595 (2011).
- Ciucci, T. *et al.* The Emergence and Functional Fitness of Memory CD4+ T Cells Require the Transcription Factor Thpok. *Immunity* 50, 91–105.e4 (2019).
- Crotty, S. T Follicular Helper Cell Biology: A Decade of Discovery and Diseases. *Immunity* 50, 1132–1148 (2019).
- 16. Crotty, S. Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. 29, 621-663 (2011).
- Crawford, A. *et al.* Molecular and Transcriptional Basis of CD4+ T Cell Dysfunction during Chronic Infection. *Immunity* 40, 289–302 (2014).
- Wherry, E. J. *et al.* Molecular Signature of CD8 T Cell Exhaustion during Chronic Viral Infection. *Immunity* vol. 27 824 (2007).
- Hashimoto, M. *et al.* CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu. Rev. Med.* 69, 301–318 (2018).
- Fahey, L. M. *et al.* Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. *J. Exp. Med.* 208, 987–999 (2011).
- 21. Im, S. J. et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy.

Nature **537**, 417–421 (2016).

- 22. Utzschneider, D. T. *et al.* T Cell Factor 1-Expressing Memory-like CD8+ T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity* **45**, 415–427 (2016).
- Leong, Y. A. *et al.* CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. *Nat. Immunol.* 17, 1187–1196 (2016).
- 24. Wu, T. *et al.* The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness. *Sci Immunol* **1**, (2016).
- He, R. *et al.* Follicular CXCR5-expressing CD8+ T cells curtail chronic viral infection.
 Nature 537, 412–428 (2016).
- Nish, S. A. *et al.* CD4+ T cell effector commitment coupled to self-renewal by asymmetric cell divisions. *J. Exp. Med.* 214, 39–47 (2017).
- 27. Choi, S. *et al.* Role of macrophage migration inhibitory factor in the regulatory T cell response of tumor-bearing mice. *J. Immunol.* **189**, 3905–3913 (2012).
- Ziegler, S. F., Ramsdell, F. & Alderson, M. R. The activation antigen CD69. *Stem Cells* 12, 456–465 (1994).
- Jenner, R. G. *et al.* The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17876–17881 (2009).
- Lund, R. *et al.* Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur. J. Immunol.* 35, 3307–3319 (2005).

- Daniel, B. *et al.* Divergent clonal differentiation trajectories of T cell exhaustion. *bioRxiv* 2021.12.16.472900 (2021) doi:10.1101/2021.12.16.472900.
- 32. Satpathy, A. T. *et al.* Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nat. Biotechnol.* **37**, 925–936 (2019).
- Pliner, H. A. *et al.* Cicero Predicts cis-Regulatory DNA Interactions from Single-Cell Chromatin Accessibility Data. *Mol. Cell* 71, 858–871.e8 (2018).
- 34. Shao, P. *et al.* Cutting edge: Tcf1 instructs T follicular helper cell differentiation by repressing Blimp1 in response to acute viral infection. *J. Immunol.* **203**, 801–806 (2019).
- Liu, X. *et al.* Transcription factor achaete-scute homologue 2 initiates follicular T-helpercell development. *Nature* 507, 513–518 (2014).
- Tanaka, S. *et al.* CCAAT/enhancer-binding protein α negatively regulates IFN-γ expression in T cells. *J. Immunol.* **193**, 6152–6160 (2014).
- Naoe, Y. *et al.* Repression of interleukin-4 in T helper type 1 cells by Runx/Cbfβ binding to the II4 silencer. *Journal of Experimental Medicine* vol. 204 1749–1755 (2007).
- Djuretic, I. M. *et al.* Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence II4 in T helper type 1 cells. *Nature Immunology* vol. 8 145–153 (2007).
- Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655–669 (2000).
- 40. Mullen, A. C. *et al.* Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* **292**, 1907–1910 (2001).

- 41. Shaw, L. A. *et al.* Id2 reinforces TH1 differentiation and inhibits E2A to repress TFH differentiation. *Nat. Immunol.* **17**, 834–843 (2016).
- Shaffer, A. L. *et al.* Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17, 51–62 (2002).
- 43. Shapiro-Shelef, M. *et al.* Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* **19**, 607–620 (2003).
- Nutt, S. L., Fairfax, K. A. & Kallies, A. BLIMP1 guides the fate of effector B and T cells. *Nat. Rev. Immunol.* 7, 923–927 (2007).
- 45. Welsh, R. M. Blimp hovers over T cell immunity. *Immunity* vol. 31 178–180 (2009).
- 46. Rutishauser, R. L. *et al.* Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31, 296–308 (2009).
- Kallies, A., Xin, A., Belz, G. T. & Nutt, S. L. Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8+ T Cells and Memory Responses. *Immunity* 31, 283– 295 (2009).
- Zander, R. *et al.* CD4+ T Cell Help Is Required for the Formation of a Cytolytic CD8+ T Cell Subset that Protects against Chronic Infection and Cancer. *Immunity* vol. 51 1028– 1042.e4 (2019).
- 49. Raju, S. *et al.* Identification of a T-bethi Quiescent Exhausted CD8 T Cell Subpopulation That Can Differentiate into TIM3+ CX3CR1+ Effectors and Memory-like Cells. *The Journal of Immunology* 206, 2924–2936 (2021).
- 50. Ferris, S. T. *et al.* cDC1 prime and are licensed by CD4 T cells to induce anti-tumour immunity. *Nature* vol. 584 624–629 (2020).
- 51. Khatun, A. *et al.* Single-cell lineage mapping of a diverse virus-specific naive CD4 T cell repertoire. *J. Exp. Med.* **218**, (2021).
- Moguche, A. O. *et al.* ICOS and Bcl6-dependent pathways maintain a CD4 T cell population with memory-like properties during tuberculosis. *J. Exp. Med.* 212, 715–728 (2015).
- Siddiqui, I. *et al.* Intratumoral Tcf1+PD-1+CD8+ T cells with stem-like properties promote tumor control in response to vaccination and checkpoint blockade immunotherapy. *Immunity* 50, 195–211.e10 (2019).
- 54. Cui, C. *et al.* Neoantigen-driven B cell and CD4 T follicular helper cell collaboration promotes anti-tumor CD8 T cell responses. *Cell* **184**, 6101–6118.e13 (2021).
- 55. Zander, R. *et al.* Tfh-cell-derived interleukin 21 sustains effector CD8 T cell responses during chronic viral infection. *Immunity* (2022) doi:10.1016/j.immuni.2022.01.018.
- Chou, C. *et al.* The Transcription Factor AP4 Mediates Resolution of Chronic Viral Infection through Amplification of Germinal Center B Cell Responses. *Immunity* 45, 570– 582 (2016).
- Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* 177, 1888–1902.e21 (2019).
- Cao, J. *et al.* The single-cell transcriptional landscape of mammalian organogenesis. *Nature* 566, 496–502 (2019).

- 59. Granja, J. M. *et al.* ArchR is a scalable software package for integrative single-cell chromatin accessibility analysis. *Nat. Genet.* **53**, 403–411 (2021).
- 60. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).
- Schep, A. N., Wu, B., Buenrostro, J. D. & Greenleaf, W. J. chromVAR: inferring transcription-factor-associated accessibility from single-cell epigenomic data. *Nat. Methods* 14, 975–978 (2017).
- van Dijk, D. *et al.* Recovering Gene Interactions from Single-Cell Data Using Data Diffusion. *Cell* 174, 716–729.e27 (2018).



Figure 11. Sorting and library quality metrics of scRNA/TCR-seq samples from splenic CD4 T cells in LCMV-Arm and LCMV-c13.

(A) Sorting strategy of $gp66-Tet^+$ and $-Tet^-$ scRNA/TCR-seq samples from LCMV-Arm and LCMV-c13.

(B) Sequencing metrics of paired scRNA/TCR-seq libraries, including number of reads per cell (top left), number of genes per cell (top right), percent mitochondrial read per cell (bottom left), and TCR $\alpha\beta$ chain capture efficiency (bottom right).

(C) UMAP of cells from each scRNA-seq replicate colored by phenotypic cluster.



Figure 12. scRNA-seq and flow analysis reveals heterogeneity within antigen-specific CD4 T cells in chronic LCMV infections.

(A) UMAP of LCMV-Arm 8 and 21 dpi, LCMV-c13 8 and 21 dpi CD4 T splenocytes sorted for gp66 Tet⁺ and Tet⁻ populations. Cells are clustered by scRNA-seq gene expression profiles and colored by phenotype (top). Density UMAP of CD4 T cells from each condition and tetramer-

sorted population (bottom left). Proportion of cells from each condition in each phenotypic cluster (bottom right).

(B) Expression of cluster-defining markers.

(C) Differential gene expression of cells in the resting memory cluster C2r compared to the activated memory-like cluster C3r (left). Differential gene expression of gp66-Tet+ resting memory C2r cells from LCMV-Arm 21 dpi compared to LCMV-c13 21 dpi (right).

(D-G) Expression of TCF-1, BCL6, CXCR5, Ly-108, and CD69 by gp66-specific splenic CD4 T cells and expression of Fas and GL7 in B cells in the spleen from in LCMV-c13-infected B6 mice at indicated time points. Representative flow cytometry plots are shown with pooled data for the frequencies of each PD-1⁺ CD4 T cell population from 2 experiments (n = 2-3 / experiment) as mean±SD in (F). Unpaired *t*-test.

(H) Expression of indicated proteins or a *Prdm1*-YFP reporter in gp66 specific splenic CD4 T cell subpopulations from LCMV-c13 infected B6 mice. Data are representative of 2 experiments with n = 2-3 / experiment.



Figure 13. Characterization of antigen-specific CD4 T cells in chronic LCMV infections.

(A) Heatmap of the top 10 differentially expressed markers for each scRNA-seq cluster. Colors represent normalized (z-scored) expression per gene among each cluster.

(B) Expression levels of select cluster markers and T cell subset defining genes within each cluster.

(C) Differential gene expression of gp66-Tet+ resting memory C2r cells from LCMV-Arm d8 dpi compared to LCMV-c13 8 dpi.

(D) Expression levels of progenitor, memory, and activation-induced markers among gp66-tetramer-sorted cells in the resting memory C2r cluster on 21 dpi in LCMV-Arm and LCMV-c13 infection.

(E) Frequencies of memory and memory-like clusters among gp66-tetramer-sorted samples on 21 dpi in LCMV-Arm and LCMV-c13 infection. Each dot indicates one replicate.

(F) Representative flow cytometry plots showing expression of PD-1 and Foxp3 in gp66-specific splenic CD4 T cells in B6 mice infected with LCMV-c13 22 days before the analysis. Pooled data from 2 experiments with 2-3 mice / experiment are shown with mean±SD. Statistical analysis by one-way ANOVA.

(G) Representative flow plots showing expression of CXCR5, BCL6, Ly-108, and TCF-1 in gp66 -specific splenic CD4 T cells in B6 mice infected with LCMV-c13 8 day or 22 days before the analysis.

(H, I) Representative flow plots showing expression of BCL6 and TCF-1 in PD-1⁺ CD4 T cells in B6 mice infected with LCMV-c13 15 day before the analysis (G). Pooled data are shown in (H) with mean±SD. Statistics analysis with one-way ANOVA.

(J) IFN-g, IL-2 and TNF production of CD4 T cells in splenocytes harvested from B6 mice at 21 dpi and stimulated with gp61-77 peptide *ex vivo*. Cytokine production by the three CD4 T cell populations is shown on the left, and quantification is shown on the right with data pooled from 2 experiments with 2-3 mice / experiment. Data is shown as mean±SD. Statistical analysis done by one-way ANOVA.

(K) Heatmap of CD8 gene signature scores ³¹ for each CD4 cluster. Colors represent normalized (z-scored) means per CD8 gene signature.



Figure 14. Memory-like cells are enriched for common progenitors for both Tfh and Teff.

(A) UMAP trajectory graph of gp66-Tet⁺ CD4 T cells from LCMV-c13 infected B6 mice on 8 and 21 dpi colored by phenotypic clusters as in Fig. 12A. The Treg clusters and other infrequent populations were excluded from the trajectory analysis for clarity.

(B) UMAP trajectory of gp66-Tet⁺ CD4 T cells from LCMV-c13 infected B6 mice on 8 and 21 dpi along the Tfh branch (top) or Teff branch (bottom). Cells are colored by pseudotime.

(C) Gene expression dynamics of activation and Tfh lineage markers along the Tfh pseudotime trajectory.

(D) Gene expression dynamics of activation and Teff markers along the Teff pseudotime trajectory.

(E) Phenotypic overlap of expanded (>1 cell) T cell clones among the resting memory, activated memory-like, Tfh, and Teff clusters. Bars are colored by the category of clonal phenotypic overlap.

(F) Phenotypic composition of the top 10 most highly expanded gp66-Tet⁺ clones from LCMVc13-infected B6 mice on 21 dpi.



Figure 15. Clonal expansion of gp66 Tet⁺ T cells from LCMV-c13-21 dpi.

(A) UMAP of gp66-Tet⁺ CD4 T cells from LCMV-c13-infected B6 mice on 8 (top) and 21 (bottom) dpi colored by pseudotime.

(B) UMAP of LCMV-Arm and LCMV-c13 CD4 T splenocytes on 8 dpi and 21 dpi sorted for gp66-Tet⁺ and Tet⁻ populations, colored by clone size.

(C) Clonal expansion levels of TCR clones from gp66-Tet⁺ and Tet⁻ splenic CD4 T cells on 8 and 21 dpi in LCMV-Arm and LCMV-c13 infection.

(**D**) Frequencies (top) and Gini indices (bottom) of TCR clones from gp66-Tet⁺ and Tet⁻ splenic CD4 T cells on 8 and 21 dpi in LCMV-Arm and LCMV-c13 infection.



Figure 16. scATAC-seq identifies chromatin features of Tprog, Tfh and Teff populations.

(A) Sorting strategy of scATAC-seq and scRNA-seq samples used for the integrative analysis of $CD4^+$ PD-1⁺ T cells.

(B) UMAP of scATAC-seq biological replicates of PD-1⁺ CD4 T cells sorted on 21 dpi of LCMV-cl13 infection.

(C) Quality control of scATAC-seq data. Fragment distribution of scATAC-seq libraries (left), Normalized insertion profile at the transcription start site (TSS) of genes (middle). Density plot of TSS read enrichment and unique fragments in single cells (right).

(D) Violin plot representation of gene scores for *Cxcr5*, *Tcf7*, *Slamf6* and *Tbx21*.

(E) Heatmap of marker gene accessibility scores of the progenitor population.

(F) UMAP of gene score (accessibility) values for the indicated genes.

(G) Pseudotime analysis of the gene scores (accessibility) and gene integration scores (integrated expression values) of the indicated genes.





(A) UMAP of scATAC-seq data of PD1⁺ CD4 T cells on 21 dpi of LCMV-cl13 infection.
(B) UMAP of gene score (accessibility) and gene integration matrix (expression) for *Cxcr5*,

Slamf6 and *Tbx21*.

(C) Heatmap visualization of marker gene accessibility scores across clusters represented by gene score values.

(D) Heatmap of marker peak scores of 5,227 *cis*-regulatory elements in scATAC-seq clusters (left). Heatmap of enriched motifs in marker peaks of the specific clusters (right).

(E) UMAP projection of Tfh and Teff differentiation trajectory, respectively. Cells that are not part of the trajectory are colored grey (top). Pseudotime heatmaps of motif deviation scores on the two differentiation trajectories (bottom).

(F) Volcano plot visualization of the differential peak analysis between the indicated clusters (FDR ≤ 0.1 and a Log₂FC ≥ 0.5)

(G) Hockey plot representation of the enriched motifs under the cluster specific peak sets.

(H) Pseudotime analysis of the gene scores (accessibility) and gene integration scores (integrated expression values) of the indicated genes.



Figure 18. TCF-1⁺ BCL6^{10/-} PD-1⁺ CD4 T cells are capable of superior expansion and differentiation into both Teff and Tfh following adoptive transfer.

(A) Experimental design of adoptive transfer of distinct populations of PD-1⁺ CD4 T cells from LCMV-c13 infected mice to infection-matched recipient mice for the experiments shown in (B, C) and (D, E). For the experiments shown in (B, C), 1×10^6 PD-1⁺ CD4 T cells were transferred. For the experiments shown in (D, E), 1 to 2 x 10⁶ PD-1⁺ CD4 T cells containing an estimated amount of 4.5 x 10⁴ Tet⁺ cells were transferred.

(B-E) Representative flow cytometry data showing frequencies of total donor-derived cells (B) and Tet⁺ donor-derived cells (D) and their expression of TCF-1 and BCL6 15 days after transfer are shown. Pooled data from 2 experiments with n = 2.4 / transfer group / experiment are shown in (C, E) with mean±SD and statistical analysis by One-way ANOVA. Frequencies and total numbers of donor-derived cells in (B, C) were normalized to 1 x 10⁶ transferred donor cells, and frequencies and total numbers of Tet⁺ donor-derived cells in (D, E) were normalized to 4.5 x 10⁴ Tet⁺ transferred donor cells.



Figure 19. Sorting strategy and "take" analysis of adoptive transfer experiments

(A) Sorting strategy of donor cells using PD-1, Ly-108 and CXCR5 for all experiments performed in Fig. 18 and Fig. 19.

(B-D) Adoptive transfer of distinct populations of PD-1⁺ CD4 T cells from LCMV-c13 infected mice to naive mice subjected to subsequent infection with LCMV-c13. (B) shows the experimental design.1.8 - 2.5 x 105 PD-1+ cells were transferred. Representative flow cytometry data showing frequencies of donor-derived cells and their expression of TCF-1 and BCL6 15 days after infecting the recipient mice with LCMV-c13 were shown in (C). Pooled data from 2 experiments with n = 3 / transfer group / experiment are shown in (D) with mean±SD with statistical analysis with One-way ANOVA. Percentages and numbers of donor-derived cells shown in (D) are normalized to a transferred amount of 1 x 105 cells.

(E-G) Measurement of donor cell engraftment following infection-matched adoptive transfer of distinct populations of PD-1⁺ CD4 T cells. 1 x 106 PD-1⁺ cells were transferred. Experimental design is shown in (E) where spleens of recipients were harvested one day after the transfer. Representative flow cytometry data showing frequencies of donor-derived cells and their

expression of Ly-108 and CXCR5 were shown in (F). Pooled data from 2 experiments with n = 2-3 / transfer group / experiment are shown in (G) with mean±SD with statistical analysis with One-way ANOVA.



Figure 20. The generation of Cd40lg-cre knock-in mice and validation of cre activity.

(A) Targeted insertion of a codon-optimized cre (hCre) coding sequence followed by an internal ribosomal entry sequence into the 3' UTR of Cd40lg by homologous recombination in B6-derived JM8 ES cells. After obtaining germline transmission of the targeted allele, the FRT-flanked selection cassette was removed *in vivo* by crossing to Flpe transgenic mice.

(B-E) Expression of YFP in thymocyte (B), splenocyte (C), peripheral blood mononuclear cell (D) and liver mononuclear cell (E) subpopulations from uninfected Cd40lg-cre Rosa26(loxP-stop-loxP-YFP) mice. Data are shown with representative flow cytometry plots and mean±SD from 3-8 mice.

(F, G) Expression of YFP in splenic CD4 and CD8 T cells (F) and expression of GL7 and Fas by splenic B cells (G) in LCMV-Arm-infected *Cd40lg*-cre *Rosa26*(stop-YFP) mice. Data are shown

with representative flow cytometry plots and mean±SD from 3 mice. *P*-values by *t*-test: % of B220⁺ CD19⁺/spleen: P = 0.815, % of GL7⁺ Fas⁺/B220⁺ CD19⁺: P = 0.122.

(H) Expression of YFP in CD62L⁺ CD44⁻ CD4 splenocytes and CXCR5⁺ PD1⁺ CD4 splenocytes of female $Cd40lg^{cre/+}$ Rosa26-stop-YFP mice 15 days post LCMV-Arm infection. Representative flow plots and data pooled from 2 experiments with 3-4 mice / experiments are shown.

(I) Expression of YFP in PD-1⁺ splenic CD4 and CD8 T cells from LCMV-c13-infected Cd40lg- cre *Rosa26*-stop-YFP mice on 21 dpi. Data is representative of 2 experiments with 3-4 mice / experiments.



Figure 21. The maintenance of TCF-1⁻ Teff cells requires BCL6-dependent, B cellindependent, TCF-1⁺ BCL6^{lo/-} PD-1⁺ memory-like CD4 T cells

(A-B) Representative flow cytometry plots showing expression of CD44 and gp66-specific TCR in splenic CD4 T cells in Cd40lg-cre $Bcl6^{F/F}$ and control Cd40lg-cre mice infected with LCMVc13, analyzed on 8 dpi, 15 dpi or 28 dpi. Pooled data from 2 experiments with 2-3 mice/group/time point are shown with mean±SD. Unpaired *t*-test. (C-D) Expression of TCF-1, BCL6, Ly-108, CXCR5 and CXCR6 in gp66-specific splenic CD4 T cells from LCMV-c13-infected Cd40lg-cre $Bcl6^{F/F}$ and control Cd40lg-cre mice on 8 dpi. Data pooled from two experiments with n = 2-4 per genotype are shown with mean±SD.

(E-F) Expression of TCF-1, BCL6 and CXCR6 in gp66-specific splenic CD4 T cells from LCMV-c13-infected MuMT and age-matched B6 (WT) mice on 21 dpi. Data from two experiments with n = 2-5 per genotype per experiment are shown with mean±SD.

(G-I) Adoptive transfer of distinct populations of PD-1⁺ CD4 T cells from LCMV-c13 infected B6-CD45.1 mice to infection-matched *Cd40lg*-cre $Bcl6^{F/F}$ recipients (CD45.2).

Experimental design (G): adoptive transfer of 0.7 to 1 x 10^6 cells of distinct PD-1⁺ CD4 T cell population from LCMV-c13 infected donor mice to infection-matched *Cd40lg*-cre *Bcl6*^{F/F} recipients. 27 days after transfer, frequencies of GL7⁺ Fas⁺ B cells in the spleen from recipient mice were analyzed. Representative flow cytometry plots (H) and pooled data from 2 experiments (n = 2-4 / transfer group / experiment) are shown in (I) with mean±SD with statistical analysis by One-way ANOVA.



Figure 22. BCL6 and Blimp1 are essential for the differentiation of TCF-1⁺ and TCF-1⁻ antigen-specific CD4 T cells, respectively, during LCMV infection.

(A, B) Expression of PD-1, CXCR5, TCF-1, BCL6 and CCR7 in splenocytes from LCMV-Arminfected *Cd40lg*-cre or *Cd40lg*-cre *Bcl6*^{F/F} mice on 8 dpi. Representative flow plots (A) and pooled data from 2 experiments with n = 2-3 / genotype /experiment are shown with mean±SD in (B).

(C, D) Expression of TCF-1 and TIM3 in PD-1⁺ CD8 T cells (C, top) and gp66-Tet⁺ CD4 T cells (C, bottom) in the spleen of LCMV-c13-infected *Cd40lg*-cre or *Cd40lg*-cre *Bcl6*^{F/F} mice on 8dpi. Histogram overlays of TCF-1⁻-gated cells from each of the parental populations are shown (C, right). Mean fluorescence intensity (MFI) of TIM3 from 2 experiments with n = 2-4/genotype/experiment is shown with mean±SD in (D).

(E, F) Expression of TCF-1 and BCL6 by gp66-Tet⁺ CD4 T cells in the spleen from LCMV-c13infected *Cd40lg*-cre or *Cd40lg*-cre *Prdm1*^{F/F} mice on 8 dpi. Representative flow plots (E) and pooled data from 2 experiments with n = 2-4 / genotype /experiment are shown in (F) with mean±SD. Statistical differences were assessed by unpaired *t*-test.



Figure 23. IL-21-dependent CX3CR1+ exhausted CD8 T cells in LCMV-c13-infected mice are maintained in the absence of Bcl6 in CD4 T cells.

(A, B) Expression of PD-1, TCF-1 and CX3CR1 in LCMV-gp33-specific CD8 T cells in the spleen from LCMV-c13-infected MuMT mice on 21 dpi. Representative flow cytometry plots (A) and pooled data from 2 experiments with n = 2-3 / genotype /experiment are shown with mean±SD. Unpaired t-test.

(C, D) Expression of PD-1, TCF-1 and CX3CR1 in LCMV-gp33-specific CD8 T cells in the spleen from LCMV-c13-infected Cd40lg-cre Bcl6^{F/F} mice on 21 dpi. Representative flow cytometry plots (C) and pooled data from 2 experiments with n = 2-3 / genotype /experiment are shown with mean±SD. Unpaired t-test

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Figure 24. Antigen-specific CD4 T cells differentiate into TCF-1⁺ PD-1⁺ cells following cell division in tumor-draining lymph nodes, but not in the tumor microenvironments.

(A) Experiment design to examine CD4 T cell response to tumor antigen.

(B-C) Representative flow plots showing expression of TCF-1 and BCL6 by donor-derived OT-II CD4 T cells harvested from tumor (TIL) and the tumor draining lymph node (tdLN). Data are pooled from two experiments (n = 6 - 8 per experiment) and shown with mean±SD. **(D-F)** CFSE dilution and expression of BCL6, TCF-1, CXCR6, PD-1 and CD44 of donor-derived OT-II cells. The CFSE level of naive OT-II cells was determined by control recipient mice without tumor transplantation sacrificed at the same time points. Left panels in (E) show only tdLN-derived OT-II cells without overlay of TIL or naive cells. Pooled data are shown with mean±SD in (F) with assessment of statistical differences by one-way ANOVA.

(G) Representative flow cytometry plots showing expression of CXCR6, TCF-1 and BCL6 by donor-derived OT-II CD4 T cells harvested from tdLN and the tumor. Numbers shown are mean±SD.

Chapter 4: Discussion

In this work, we have sought to understand the mechanisms that maintain CD8 and CD4 T cell responses in the presence of persistent antigens, using the chronic strain of LCMV as the main model. In Chapter 2, we focused on understanding the population dynamics of CD8 T cells, where we identified and characterized a previously unknown exhausted CD8 subset marked by the expression of CX3CR1. CX3CR1⁺ cells can be further divided into a TIM3⁻ portion that expresses low levels of TCF-1 and a TIM3⁺ portion that carry more effector functions. CX3CR1⁺ TIM3⁻ cells give rise to CX3CR1⁺ TIM3⁺ cells, and together comprise a relatively stable exhausted/effector CD8 subset that requires little input from the previously identified TCF1^{hi} progenitor exhausted (TPEX) subset. In Chapter 3, we turned our attention toward CD4 T cells and discovered that a memory-like CD4 T cell population exists which serves as a bipotential progenitor for both TFH and non-Tfh effector cells (Teff), thus enabling a durable CD4 T cell response in the presence of persistent antigens. This progenitor CD4 population relies on the transcription factor BCL-6 for formation. Our studies shed light on the heterogeneity of CD8 and CD4 T cells during chronic viral infections and on the mechanism of how T cells maintain those heterogeneity as the infection goes on, a challenge that T cells in acute infection or vaccination models do not need to tackle.

The identification of CX3CR1⁺ CD8 population is significant as it challenges the idea that exhausted cells are dysfunctional and the idea that the TPEX population constantly feeds into the TCF1^{10/-} terminally differentiated CD8 T cell compartment. For the first point, although CD8 T cell response in chronic LCMV infection, on the population level, exhibit less effector functions compared to that in acute infections¹⁻³, caution need to be taken regarding what the

data means and to whether the CD8 T cell response in acute infection models should be deemed as a "better" or more "normal" response. Previous data looked at the exhausted TCF1^{lo/-} population as a whole^{5, 6}, and here we report that within the exhausted TCF1^{lo/-} population there are cells with higher effector functions, namely the CX3CR1⁺ TIM3⁺ cells. For the second point, our data suggests that the previously identified TPEX cells likely feed into the more exhausted CD8 T cells at a rate lower than previously thought. Although the TPEX cells are what responds to the anti-PD-1 or anti-PDL1 blockade and give rise to more effector/exhausted cells, there is no reliable data aside from our study to this day regarding the rate at which this mobilization happens in vivo. Because TPEX cells express a high level of and are dependent on TCF-1, Tcf7 (which encodes for TCF-1) knockout (KO) models have been used as a model that specifically lacks the TPEX population^{5, 7, 8}, and phenotypes discovered using those models have been used to infer the function of TPEX. However, TCF-1 is required for thymic development² and is also expressed by the CX3CR1⁺ TIM3⁻ cells, albeit at a lower level, which draws into question to what extent the *Tcf*7-KO phenotypes are solely due to a lack of TPEX. With our $Cx3cr1^{creERT2/+}$ *R26*tdT fate-mapping mice, we showed evidence that the CX3CR1⁺ compartment is stable with little loss of fate-mapped cells, which suggest minimal input from the TPEX cells within the window of observation in our study. Therefore, our study contributes to our understanding of CD8 T cell response in the presence of persistent antigens by providing a non-biased characterization of the CD8 T cell heterogeneity and a careful study of their population dynamics in vivo.

CD4 T cell plasticity is a well-known phenomenon and this study furthers our understanding in this regard by the identification of a bipotential progenitor population in the presence of persistent antigens that could differentiate into either Tfh or non-Tfh effector cells. Plasticity between Th17 and Th1 has been documented in various infection and autoimmunity settings, where Th17 cells can give rise to cells with Th1 characteristics^{10, 11}. It is unclear if the Th17 and Th1 conversion can occur by direct trans-differentiation or de-differentiation of Th17 cells first followed by differentiation into Th1 cells. Results of our study are more in line with the second possibility, although it is likely that CD4 T cells in different contexts utilize different differentiation approaches to achieve plasticity depending on the epigenetic landscape of the cells and the microenvironment that they are in.

Since its discovery as a transcription factor required for Tfh differentiation^{12, 13}, BCL6 has been called the Tfh lineage-defining transcription factor. However, our study shows that BCL6 functions beyond Tfh differentiation by promoting formation of memory-like progenitor cells. Likely, BCL6 antagonizes BLIMP-1 early during CD4 T cell differentiation¹³⁻¹⁵, thus preserving the stemness of the BCL6⁺ cells and their progenies. This is in line with previous reports that BCL6 is required for CD4 central memory formation following acute bacterial or viral infections^{16, 17}, and might partially explain the perplexing Tfh memory phenotype that Tfh memory cells are multipotent and upon rechallenge give rise to both Tfh and effector T (Th1) cells, as BCL6 might also function in Tfh cells to preserve their stemness^{9, 18, 19}. In this sense, our work points to new functions of BCL6 and provides a unifying explanation for the multipotency shown by Tfh memory cells, T central memory cells, and progenitor cells.

Although not a main focus, our work also provides some information on the mechanism of CD4 help for CD8 T cell response in the presence of persistent antigens. CD4 depletion in the beginning of the chronic LCMV infection has no impact on the formation of CX3CR1⁺ cells but impairs their maintenance. By 21 days post infection, there are few CX3CR1⁺ CD8 T cells left. *Cd40lg*-cre *Bcl6*^{F/F} mice, on the other hand, have CD4 T cells for the first week of infection and maintain a largely intact CX3CR1⁺ CD8 T cell response by 21 days post infection. These two lines of evidence suggest that it is the CD4 T cell response during the first week of infection that is crucial for a maintenance of CX3CR1⁺ CD8 T cell response. In other words, a large part of the CD4 T cell help to CD8 T cells likely occurs during the priming phase of the infection.

References

- 1. Matloubian, M., Concepcion, R. J. & Ahmed, R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* **68**, 8056–8063 (1994).
- Wherry, E. J. *et al.* Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27, 670–684 (2007).
- Zajac, A. J. *et al.* Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med.* 188, 2205–2213 (1998).
- 4. Blank, C. U. et al. Defining 'T cell exhaustion'. Nat. Rev. Immunol. 19, 665–674 (2019).
- Im, S. J. *et al.* Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 537, 417–421 (2016).
- He, R. *et al.* Follicular CXCR5- expressing CD8(+) T cells curtail chronic viral infection. *Nature* 537, 412–428 (2016).
- Wu, T. *et al.* The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness. *Sci Immunol* 1, (2016).

- Utzschneider, D. T. *et al.* T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity* 45, 415–427 (2016).
- Weber, J. P., Fuhrmann, F. & Hutloff, A. T-follicular helper cells survive as long-term memory cells. *Eur. J. Immunol.* 42, 1981–1988 (2012).
- Hirahara, K. *et al.* Mechanisms underlying helper T-cell plasticity: implications for immunemediated disease. *J. Allergy Clin. Immunol.* 131, 1276–1287 (2013).
- Liu, H.-P. *et al.* TGF-β converts Th1 cells into Th17 cells through stimulation of Runx1 expression. *Eur. J. Immunol.* 45, 1010–1018 (2015).
- Nurieva, R. I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science* 325, 1001–1005 (2009).
- Johnston, R. J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325, 1006–1010 (2009).
- Baumjohann, D., Okada, T. & Ansel, K. M. Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. *J. Immunol.* 187, 2089–2092 (2011).
- 15. Ciucci, T. *et al.* Dependence on Bcl6 and Blimp1 drive distinct differentiation of murine memory and follicular helper CD4+ T cells. *J. Exp. Med.* **219**, (2022).
- Ichii, H. *et al.* Bcl6 is essential for the generation of long-term memory CD4+ T cells. *Int. Immunol.* 19, 427–433 (2007).

- Pepper, M., Pagán, A. J., Igyártó, B. Z., Taylor, J. J. & Jenkins, M. K. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* 35, 583–595 (2011).
- Hale, J. S. *et al.* Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity* 38, 805–817 (2013).
- 19. Künzli, M. *et al.* Long-lived T follicular helper cells retain plasticity and help sustain humoral immunity. *Science Immunology* **5**, (2020).