Expansive residence decentralizes immune homeostasis

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUTE SCHOOL
UNIVERSITY OF MINNESOTA
BY

Sathi Philip Wijeyesinghe

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: David Masopust, Ph.D.

October 2020

ACKNOWLEDGEMENTS

First, thanks to you, the reader, who have taken your time to read this. This thesis dissertation represents several years of hard work full of many failures and a few successes in pursuit of a modestly better understanding of the immune system. I could not have accomplished this work without help from innumerable individuals – some who made overt contributions and others whose part was so subtle that I will never know their names. A few of these individuals are acknowledged below, but I have a tremendous sense of gratitude to all those who stopped to provide help to me along the way.

To all past, present, and future members of the Masopust and Vezys laboratories, I am in awe of your tenacity, curiosity, and humility in your pursuit of science. During my time in the lab, I was fortunate to be part of an inclusive and collaborative environment that embraced me as a scientist, a peer, a colleague, and a friend. It was a true pleasure to get to know every member of the lab in my time there. Even when experiments yielded negative results (or worse, no results), I had a strong support network of friends and colleagues who encouraged me to persevere. A few lab colleagues warrant further mention. Lizzie, your patience and guidance in my early days in the lab were essential in my introduction to the lab and your work itself was a touchstone for my own thesis. Lalit, some of my fondest memories stem from the many experiments we did together and the sushi we ate afterwards.

The diligent, yet light-hearted, tone in the lab, was set by its leadership. Vaiva and Dave, thank you for providing excellent scientific role models over the years. Vaiva, I appreciate all of your counsel, scientific and otherwise. Dave, it is difficult for me to fully recognize the full breadth of knowledge and experience you've imparted to me. I think back to my first days in the lab and know that I have matured profoundly as a scientist and a person. I hope that I can continue to carry with me your passion for science and your uniquely creative approach to problem solving. For all of your wisdom, support, and guidance over the years, thank you.

To every member of the Center for Immunology, from the leadership to the support staff, thank you for developing one of the best places in the world to conduct my doctoral research and pursue science. I had conversations with many of you, some science-related, some not, but all of your support, advice and help has been much appreciated. In particular, thank you Ryan Langlois, Bryce Binstadt, Brian Fife, and Kris Hogquist for providing invaluable feedback and serving on my thesis committee. I greatly appreciate the efforts of Empowering Women in Science for promoting a culture of inclusivity at the CFI.

Without the programmatic support at the University of Minnesota, I would not have been able to succeed. Specifically, thanks to the MICaB program, particularly Louise Shand, Meg Ruf, Wade Bresnahan, and Steve Jameson, as well as the MSTP, particularly Susan Shurson and Nick Berg. Yoji Shimizu, thank you for recruiting me to Minnesota and fostering my growth as a medical scientist. Your passion for mentorship is inspiring and provides a model to emulate for the rest of my career. I also would like to thank all my previous mentors who helped me to refine my scientific pursuits. Thanks to Dr. June Kwak and Dr. Fabien Jammes, who gave me an introduction to basic scientific research at the University of Maryland. I am also indebted to Dr. Daniel Douek and Samuel Darko at the Vaccine Research Center who introduced me to the field of immunology and encouraged me to pursue this career.

I owe a great deal to my friends and family who have supported me. Audrey, you are a true partner in this work. Your infinite patience, encouragement, and enduring love have been a rare constant in my life the past several years and I look forward to continuing my medical career and scientific research in partnership with you. Lastly, I want to acknowledge my family's role in this work. Sita, you've been a sounding board for many of my thoughts and ideas for most of my life. Despite being my junior, I strive to learn from your fierce independence and maturity beyond your years. To my parents, I will never be able to truly express my deepest sense of gratitude for all of your support. Dad, you gave me the freedom to be an independent thinker. Mom, you instilled in me your own curiosity for the natural world and passion for scientific endeavor. Any small successes I have are truly a reflection of your hard work and sacrifices.

DEDICATION

For my parents, Jeyarany and Rohan Wijeyesinghe,
and Audrey, the most important resident in my life.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 The immune system in a diverse world

1.1.1 Biodiversity

It is difficult to fully appreciate the magnitude of biodiversity in the natural world. Large-scale collaborative projects have begun experimentally cataloguing distinct archaeal and bacterial species, but will likely only scratch the surface (1). Theoretical ecologists estimate at current rates, it could take a full millennium to identify the nearly 1 trillion unique microbial species on earth (2). It is even more difficult to quantify viral diversity, given controversies surrounding the delimitation of viral populations (3). Still, it is evident that viruses are also extraordinarily diverse (4) and the sheer number of viruses approaches (or exceeds) astronomical territory – the number of viruses on earth outnumbers the stars in the universe by a factor of ten million (5–7). Eukaryota is far simpler with just over one million eukaryotic species identified (though computational models suggest this number is also an underrepresentation by ~one order of magnitude) (8). Humans are one of these species and have evolved myriad mechanisms to contend with the extraordinary biodiversity of this planet.

Most often, humans coexist harmoniously with microbial organisms. There are thousands of commensal species that innocuously colonize human barrier surfaces to form the human microbiome (9, 10). Only ~1,400 microbes (one in a billion) are classified as human pathogens (11). Still, the sudden emergence of SARS-CoV2 serves as a stark reminder that this number is ever-changing, and a single novel microbial species can create a profound disturbance in the delicate equilibrium between species. So how does the human body parse through a constant exposure to microbes and identify potentially harmful ones? And then, how do we prevent pathogen entry, mitigate harmful infectious events, and control or eliminate infectious agents? These are primary functions of a complex and coordinated network of diverse cell types situated throughout the body, collectively known as the immune system.

1.1.2 Origins of immunology

While it is now understood that the immune system performs vital functions unrelated to pathogen defense, the discipline of immunology owes its genesis to seminal advances in microbiology and the study of infectious diseases in the late 19th century (12, 13). The word "immunity" from the Latin *immunis* refers to an exemption from taxation or military service offered to certain individuals (14). As the germ theory was embraced following advances from scientific rivals Louis Pasteur and Robert Koch, early immunologists borrowed the concept of *immunis* to describe protective mechanisms employed by hosts to avoid or mitigate consequences of microbial infection (12, 13, 15). The 1908 Nobel prize in Medicine was awarded to both Elie Metchnikoff and Paul Ehrlich 'in recognition of their work on immunity'(16, 17). This prize confirmed the importance of the nascent field, but its shared receipt would foretell two key dichotomies that remain evident today. While Ehrlich focused on humoral antitoxins (among other things), Metchnikoff identified an evolutionarily conserved population of cells (phagocytes) that could uptake extracellular material, including microbes. The relative significance of protective immunity conferred by either humoral or cellular mechanisms continues to prompt debate on immunization strategies today (18, 19). The 1908 Nobel prize also led to a foundational division of the immune system into two broad protective strategies: non-specific innate and highly specific adaptive immunities.

1.1.3 Innate immunity

Innate mechanisms are defined by their rapid, but non-specific antimicrobial action. Given that most microbes enter the body through mucosal membranes and skin, the physical tissue barriers (formed by epithelial tissue and underlying layers) constitute a critical component of innate immunity. Beyond physical and chemical antimicrobial mechanisms which parenchymal cells may initiate, there are several innate hematopoietic cell types. Macrophages are now well-appreciated for their phagocytotic function and may non-specifically engulf microbial intruders throughout the body. Granulocytic populations may also engage in phagocytosis but are named for their prepackaged granules of toxic and

degradative antimicrobial enzymes that may be released to promote an inflammatory response. More recently appreciated, innate lymphoid cells can mediate non-specific inflammation in response to pathogen entry, while related natural killer cells express many innate receptors that may recognize and kill infected or cancerous cells (20).

Most lymphocytes are considered part of the adaptive immune system, but a few lymphocytic populations with restricted receptor binding capacity straddle the intersection between innate and adaptive immunity. These include invariant natural killer T (iNKT) cells specific for glycolipids (21), mucosa-associated invariant T cells (MAIT) specific for bacterial metabolites (22), γδ T cells restricted to lipid antigens and B-1a cells which recognize a limited range of bacterial and host antigens. Dendritic cells (DCs) are innate cells that provide the crucial link between the innate and adaptive responses. DCs phagocytose antigen but are specialized antigen-presenting cells that can relocate from nonlymphoid organs to lymphocyte-rich areas and alert lymphocytes of potential pathogens.

1.1.4 Adaptive immunity

At the University of Minnesota in the 1960's, Max Cooper manipulated chickens to distinguish between bursa- and thymus-derived lymphocytes, culminating in the current classification of B and T lymphocytes, respectively (23–25). In mammals, B cell maturation occurs in the bone marrow and gives rise to the antibody-producing cells responsible for Ehrlich's antitoxins. Antibodies have precise specificity against their targets and can rapidly mediate sterilizing neutralization of a pathogen. Indeed, most approved vaccinations rely on a productive and long-lasting antibody response (26).

T cells possess a panoply of functions, including cytotoxicity and antipathogen cytokine production, but also immune suppression, germinal center maturation (where antibodies further evolve), and tumor clearance. Nearly contemporaneous studies of the thymus from Jacques Miller and Delphine Parrot revealed the organs' essential role in T cell development (27, 28). T cells can be further bifurcated into $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells recognize antigen presented on MHC-I, which is expressed on most nucleated cells

of the body. $CD4^+$ T cells recognize antigen presented on MHC-II molecules, which is expressed on a much narrower fraction of cells in the body. 'Professional' APCs express an abundance of MHCII and typically uptake extracellular antigen to be processed and presented on the surface. In this way, $CD8^+$ T cells tend to survey for viral or cancerous antigens expressed within cells of the body, while $CD4^+$ T cells typically detect antigens derived from extracellular pathogens such as fungi and bacteria. Temporal kinetics of innate and adaptive responses differs markedly. While innate responses are generally deployed within hours to days after an initial infection, a specialized T or B cell response can take days to weeks to form during primary infections.

1.1.5 Unconventional immune cells

Conventional immune mediators of myeloid and lymphoid lineages are borne from hematopoietic niches (29), but other cell types have been reported to participate in the immune response. A recent study shows that stromal cells (thought to primarily support organ structure and integrity) are poised to initiate and coordinate immune functions in response to acute viral infection (30). There are several reports describing antigen presentation and T cell priming by parenchymal cells including hepatocytes (31, 32). Several elegant studies of antipathogen activity and immune stimulation by neuronal cells extend immune function to the nervous system (33–37). Thus, seemingly every cell type functions in concert to generate an immune response, highlighting the outsized evolutionary pressure that microbial species have exerted on mammalian development.

1.2 1.2 Generation of immunological memory

With respect to antipathogen functions, *immunity* encompasses broad protections against new and repeated pathogen exposures. *Immunological memory* is a component of *immunity* and a defining feature of the vertebrate immune system. While there has been recent evolution of the term, *immunological memory* will be defined here by four distinct elements: 1) immunological memory is generated by an initial pathogen exposure (or vaccination); 2) immunological memory allows for enhanced protection against future exposures; 3) this protection is specific for the initial or perhaps related pathogens; 4)

immunological memory is maintained in the absence of the initial stimuli. This working definition may not accommodate all descriptions of immunological memory, particularly as it relates to innate immunological memory and contexts in which antigen is perpetually present (e.g. chronic infections, tumors) (38–40).

The generation of adaptive immunological memory can be exemplified by examining the response of pathogen-specific T cells over the course of an acute infection. When frontline barriers are breached, and innate immunity is insufficient to eliminate a viral infection, viral proteins or whole virions will eventually reach the draining lymph node of an infected tissue. It is primarily in lymph nodes or other secondary lymphoid organs (SLOs) such as spleen, where naïve (antigen-inexperienced) lymphocytes can initiate the adaptive immune response.

1.2.1 Naïve T cell surveillance

The number of lymphocytes that emerge from the thoracic duct daily could replace the bloodborne pool several times over (41). Given that the bloodborne population of lymphocytes is relatively stable, speculation arose regarding the fate of the many ‘disappearing lymphocytes’ (42). James Gowans used an elegant apparatus to radioactively label lymphocytes sourced from thoracic duct and intravenously reinfuse these labelled cells to trace their fate *in vivo* (43, 44). While the predominant theories at the time suggested lymphocytes in blood were fated to rapidly differentiate or die, experiments by Julie King and Gowans demonstrated small lymphocytes continuously trafficked from blood to lymph nodes to lymph, only to rejoin the circulation *ab initio*. Naïve T cells follow this circuitous route in their surveillance of the body and are largely excluded from nonlymphoid organs.

1.2.2 Lymph node entry

High endothelial venules (HEVs) are post-capillary vascular sites found in lymph nodes and other organized lymphoid structures such as Peyer’s patches (45). They are distinguished by their cuboidal cellular appearance, as compared to more flattened

conventional endothelium. This specialized vascular tissue allows for the entry of lymphocytes into most SLOs (the spleen being a notable exception). While hemodynamic forces are lower in post-capillary venules, relative to arteries, the shear force remains a considerable obstacle (up to 30 dynes/cm^2) for leukocyte tethering to endothelium (46, 47). As erythrocytes overtake other hematogenous cells in blood, leukocytes can be displaced to the margins of the blood vessel (48, 49).

Canonically, leukocytes transmigrate through endothelium in a three-step process comprised of rolling, chemoattraction, and integrin-mediated arrest (47, 50, 51). Following margination, an interaction between fucosylated carbohydrates and selectin molecules curbs the speed of the leukocyte, causing it to 'roll' on the endothelium. L-selectin (expressed on naïve T cells) binds to sialyl Lewis (x) carbohydrate moieties that are abundantly expressed on HEVs and this interaction is responsible for the initial naïve T cell tethering (51, 52). This deceleration provides an opportunity for chemoattractant receptors expressed by leukocytes to search for chemokines that decorate the HEV extracellular membrane. CCL21 decorates the luminal surface of the HEV and can interact with CCR7, expressed on naïve T cells (53). Like all chemokine receptors, CCR7 is a G-protein coupled receptors and its activation initiates a signal transduction cascade that induces conformational changes and clustering of integrin proteins already present on the cell surface (51, 54). This is known as 'inside-out' integrin activation and for naïve T cells, increased LFA-1 avidity for its integrin ligands (ICAM1/2) mediates complete cell arrest, followed finally by diapedesis into the lymph node (55, 56).

1.2.3 Lymph node surveillance and egress

Naïve T cells traffic to the paracortical region of the lymph node, following chemokine gradients established by fibroblastic reticular cells. Over ~6-24 hours, T cells may probe hundreds of antigen-presenting cells (57, 58). In the absence of cognate antigen, continuous exposure to chemokine ligands induces T cell internalization of CCR7 (59, 60). The desensitization of T cells to CCR7 ligands is balanced by re-sensitization to another chemokine gradient. Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that is

abundantly found in blood and lymph, but largely absent from lymph nodes (61). T cells can express several sphingosine-1-phosphate receptors (S1PR). Naïve T cells in lymph nodes re-express S1PR1 and become highly sensitive to S1P, prompting lymph node egress via efferent lymphatics (61–63). Thus, a balance between opposing chemokine gradients controls T cell egress from lymph nodes.

1.2.4 Clonal selection and the GOD problem

In 1947, Macfarlane Burnet (contemporaneously with David Talmage (64)) provided the conceptual framework to understand the adaptive response to antigen (65). Building on ideas of Niels Jerne (66), he proposed that each lymphocyte had one and only one specificity and upon antigenic recognition, a few 'clones' would undergo proliferation. In this manner, the requisite immune response overcomes an immensely diverse repertoire through preferential amplification. From an endless sea of possible lymphocyte receptors, the optimal few are 'naturally selected' and expand. With remarkable prescience, Burnet also anticipated that each receptor would arise from somatic mutations, but it would take decades for the precise mechanisms of somatic recombination to be unraveled (65).

Known as the GOD ('generation of diversity') problem, pioneering research (culminating in a Nobel Prize to Dr. Susumu Tonegawa) identified elegant somatic gene recombination events which lymphocytes employ to amplify the collective number of unique lymphocyte receptors (67–69). It is now thought that the amount of genetic information stored in regions encoding T cell receptor (TCR) and B cell receptor (BCR) proteins is ~10^4 greater than the amount of information stored in the rest of the human genome (70). Functionally, this allows a human being to possess ~10^7 unique TCRs, largely expressed on naïve T cells (71, 72). In the face of a biodiverse world, the immune system responds in kind with remarkable diversity of recognition.

1.2.5 T cell activation and expansion

The adaptive immune system must balance considerable clonal diversity in the naïve lymphocyte repertoire with surveillance efficiency. Lymphocytes, particularly T cells, are

tactile and probe for antigen on the surface of cells. To increase the expediency by which very rare naïve T cell clones detect cognate antigen, they largely limit their surveillance to secondary lymphoid organs rather than explore the entire organism. Upon recognition of a viral antigen (i.e. activation), a rare population of activated T cells rapidly proliferates resulting in a massive clonal expansion of antigen-specific T cells. A given antigen-specific naïve T cell population consists of ~10-1000 cells in a mouse (73). Following an acute lymphocytic choriomeningitis virus (LCMV) infection, 10^7 antigen-specific T splenocytes can be isolated eight days later, indicating a cellular expansion of three orders of magnitude within a week (74). To achieve this expansion, activated T cells undergo a dramatic shift from catabolic quiescence to anabolic biosynthesis of macromolecules and organelle biogenesis (75). This metabolic switch allows for the impressive proliferation rates observed on activated T cells. *In vivo* studies suggest the division rate of activated T cells can approach 2 hours, approaching the rate at which yeast divide (76, 77).

1.2.6 T cell 'effector' program

T cell activation also unleashes a wide range of functions as part of the cell-mediated 'effector' program. $CD4^+$ T cells have several potential fates following TCR stimulation. These fates may correspond to three general types of immunity: type 1, type 2, or type 3. Type 1 immunity is directed against intracellular pathogens such as viruses, while Th1 T cells may produce antipathogen cytokines (IFN-γ) or induce macrophage activation. Type 2 immune responses and Th2 T cells are essential for host resistance against helminth infections, but are also thought to mediate aberrant allergic responses. Type 3 immune responses and Th17 T cells mediate antimicrobial responses at epithelial barrier surfaces. 'Master' transcription factors that induce distinct Jak-Stat signaling cascades have been identified for Th1, Th2, and Th17 cells. Beyond these cell subsets, activated $CD4^+$ T cells may also differentiate into Tfh cells that may provide survival signals to B cells and promote natural selection of high-affinity B cells within the lymph node follicles. While $CD8^+$ T cells are appreciated for their targeted killing of infected cells, $CD4^+$ T cells can also express cytotoxic molecules and mediate cell death (78).

In addition to cell killing, $CD8^+$ T cell functions include expression of antipathogen cytokines (e.g., IFNγ, TNFα). More recently, it has been recognized that $CD8^+$ T cells (as well as ILCs and iNKT cells) can also mediate all three arms of immunity, each reliant on the same respective transcription factor that controls CD4 differentiation (20, 21, 79). It is becoming increasingly evident that there is substantial redundancy between various immune cell players and the full breadth of T cell function remains an active area of research.

1.2.7 T cell activation: the immunological synapse

Given the dramatic consequences of T cell activation, this process is tightly regulated to prevent aberrant cell-mediated responses that may induce allergic or autoimmune pathologies. While dendritic cells are the predominant APCs during an acute infection, B cells, macrophages, basophils, and stromal cells can also serve in this role (80–82). APCs may express a combination of various costimulatory factors (e.g. B7, CD40, OX-40, ICOS, PD-L1/2, CTLA-4) that can augment or abate T cell stimulation. Of note, PD-1 and CTLA-4 signaling can inhibit T cell activation via dephosphorylation of cytoplasmic domain of CD28 (83, 84) or directly competing for CD28 binding (85), an activating costimulation molecule expressed by T cells (86). Since the inhibitory nature of these two molecules was identified from basic research (87, 88), several successful drugs have targeted this pathway in humans to provoke an antitumor immune response (89, 90). Additionally, cytokine production from APCs or other neighboring cells can direct the subsequent response (91).

1.2.8 Redistribution of activated T cells

Following activation and proliferation, T cells egress from SLOs and rejoin the peripheral blood. Ultimately, many T cells traffic to nonlymphoid organs, where pathogen entry and replication often begins. Canonically, leukocyte diapedesis follows the same 3-step sequence as described for naïve T cell entry into lymph nodes. The precise combination of selectin, chemokine receptor, and integrin is highly contextual and will vary depending on several factors (e.g., priming, infection model, route of infection). It has been suggested that T cell priming in SLOs induces expression of homing molecules specific for the

upstream tissue draining the SLO. For example, there is evidence that T cell activation in Peyer's patches or the mesenteric LN induces trafficking to the intestinal mucosa (92, 93). Similar evidence exists for skin homing (94, 95), but whether similar SLO imprinting directs T cells to the many other nonlymphoid organs in the body has not yet been fully explored. Once at the sites of infection, activated T cells may produce antipathogen cytokines or mediate direct killing of infected cells. Thus, activated T cells radiate out to solid organs and barrier sites where they survey the tissue, recognize infected cells, and execute effector functions that contribute to the restoration of homeostasis. This could be considered an *inside* (originating in draining LNs) *out* (then migrating to peripheral tissues) immune response (Fig. 1.1a).

1.2.9 T cell contraction

Following resolution of infection, the host is left with a stable pool of memory T cells that patrol the body for reinfection. Such memory T cells are orders of magnitude more numerous than the naive cells they differentiated from, but it should be noted that the vast majority of activated T cells die shortly after activation, resulting in a T cell 'contraction' by 90-95% in blood (74, 96, 97). This crash is highly reproducible and does not seem to be controlled by the infective dose or cytokine scarcity, though there is evidence that limiting inflammation mitigates the extent of the contraction (98, 99). Mechanistically, Fas/Fas-L interactions, tumor necrosis factor and reactive oxygen species have been implicated in T cell apoptosis during this phase (100–102). While much attention has been given to the ontogeny of memory T cells, identifying additional regulators of contraction (e.g., competition for limited metabolites) may provide new avenues for manipulating T cell responses.

1.3 Memory T cell surveillance

Fundamentally, T cell immunity provides a more rapid and efficient response to pathogen re-exposure than naïve T cells were able to achieve during the initial infection. Understanding how memory T cells accomplish this will provide an opportunity to exploit cell-mediated immunity via vaccination to prevent intractable infections. Numerically, an

antigen-specific memory T cell pool is present at a higher frequency than its ancestral naïve population, which provides enhanced surveillance of the entire body. However, memory T cells are also advantaged in their distribution throughout the body. While naïve T cells limit their surveillance to SLOs, memory T cells can be found in nonlymphoid tissues (Fig. 1.1b).

1.3.1 Resident T cell memory

'Local immunities' in nonlymphoid organs have long been appreciated (103). It is now understood that many memory T cells are resident within nonlymphoid organs. Resident memory T cells (T_{RM}) are remarkably abundant and broadly distributed throughout most of the body including lymphoid organs, visceral organs, skin, and the mucosal barriers that form the most common sites of pathogen entry (104–111). Generally, T_{RM} are defined as a population of memory T cells in tissues that do not exchange with memory T cells in peripheral blood. In mice, the residency of antiviral memory CD8 T cell populations has been demonstrated using a number of complementary experimental techniques including tissue grafting (105, 112), parabiosis (106, 113), and *in vivo* depletion of circulating cells (114).

1.3.1.1 T_{RM} formation

T_{RM} arise from recently activated T cells that migrate to NLTs (112), and are thought to result from an inductive differentiation program that depends on tissue-derived developmental cues (115, 116). Following a herpes infection in mice, skin T_{RM} formation depended on local signaling by IL-15 and TGF-β, resulting in upregulation of CD69 and CD103, respectively (115). Several additional studies have further validated the importance of local IL-15 and TGF-β signaling for T_{RM} formation in other contexts (116–123). Still, not all T_{RM} necessarily follow this developmental pathway. T_{RM} can form in the absence of IL-15 signaling and without expression of CD69 (124, 125). Other putative extracellular regulators of T_{RM} formation include type 1 interferon, TNF-α, IL-12, IL-33 and ATP (116, 126–128). Several reports show that antigen within the tissue also augments formation of T_{RM} (129, 130), though antigen is not strictly required (116, 131). Recent

studies also indicate that T_{RM} commitment may be regulated even prior to tissue entry. Priming in SLOs by specific APCs including DNGR1$^+$ DCs, DC3-like cells, monocytes, and stromal cells all appear to promote T_{RM} formation (82, 132–134). A particularly intriguing study recently reported that some naïve T cells were preconditioned to form T_{RM} even before priming via epigenetic modifications induced by DC-mediated TGF-β activation (135).

1.3.1.2 Markers of T_{RM}

CD69 and CD103 are well-described markers of residence and their expression unequivocally correlates with residence of memory CD8 T cells (106, 115). CD69 is a C-type lectin that is also a well-known marker of activated T cells. When expressed at high levels, CD69 can interact with the S1PR1 and induce its internalization and degradation. In nonlymphoid tissues, the expression of CD69 provides a post-translational mechanism for S1PR1 downregulation and tissue residency. CD103 represents the αE integrin protein that can form the heterodimer αEβ7, which mediates adhesion between E-cadherin on epithelial cells (136). CD49a is another integrin subunit that forms the α1β1 (VLA-1) integrin heterodimer, which can also bind to ECM components, including collagen. CD49a is expressed on a fraction of resident memory T cells, notably in lung and skin, and is associated with cytotoxic capacity (137, 138). These integrins may also modulate T_{RM} motility *in situ* (139).

T_{RM} are not monolithic. The property of residence (as evaluated by *in vivo* migration asays) and markers of residence do not overlap completely. Resident memory T cells may acquire neither CD69 nor CD103 (106), while CD69 can be upregulated transiently on non-resident T cells stimulated by antigen or inflammatory cytokines (140, 141). While a common set of genes is shared by T_{RM} isolated from a few tissues (115, 142), it is unlikely that any single marker of combination of markers will distinguish all resident memory T cells. With advancing technologies, it has become apparent that there is considerable heterogeneity among resident memory T cells (143, 144). Such diversity will need to be embraced, as

current conceptualizations of T cell surveillance have become encumbered by delineating memory T cells into a handful of discrete subsets.

1.3.1.3 Molecular regulation of T_{RM}

At a molecular level, several transcription factors have been reported to modulate T_{RM} formation. KLF2, a central regulator of naïve T cell trafficking, can induce CD62L and S1PR1 expression (145–147). T_{RM} are characterized by low KLF2 expression, relative to bloodborne T cell populations, and the absence of S1PR1 expression may prevent T_{RM} egress from NLT (148, 149). Hobit, Blimp-1, T-bet and Runx3 have all been shown to be positive regulators of T_{RM} formation, though no single transcription factor appears to be universally instructive (142, 150, 151).

1.3.1.4 T_{RM} reactivation

T_{RM} that encounter cognate antigen rapidly alert neighboring cells of a reinfection event, which has been termed a "sensing and alarm" function (114, 152, 153). Within hours of antigen sensing, T_{RM} alarm the immune system through the release of proinflammatory cytokines, establishing an antiviral state locally within the tissue, activating NK cells and T cells, promoting dendritic cell maturation and recruiting circulating innate and adaptive immune cells into the tissue (Fig. 1.1d). These rapid T_{RM}-mediated responses can accelerate protection against reinfection and in many cases are sufficient for protection without the contribution of circulating memory T cells (113, 153–155).

1.3.1.5 T_{RM} relocation

T_{RM} have been reported to express markers, transcripts, and functions that are shared with effector and exhausted T cells, supporting one view that T_{RM} are terminally differentiated cells; unable to mount recall responses that give rise to an amplified resident memory population, or differentiate into other subsets (156–158). In contrast to this view, recent and ongoing work reveals that T_{RM} are able to drive autonomous expansion of their population in response to antigen (157, 159), can relocate to SLOs (160), and even stably re-enter circulation where they may transmogrify into memory T cells with different

properties (161–163). This can be likened to an 'outside-in' topology; recall responses can be initiated at barrier sites (on the *outside* of the body) where T_{RM} will proliferate, differentiate, migrate, and, after resolution of infection, may stably populate other tissue compartments including blood and lymphoid tissue (*inside* the body) (Fig. 1.1c). In the event of systemic reactivation, those 'ex-T_{RM}' that have joined the circulating pool may remain poised for preferential homing back to previous sites of residence and reacquisition of a stable T_{RM} program (162, 164). Of note, while T_{RM} contributions to circulating immunity occur following local reactivation, it is unclear whether this phenomenon can occur at the steady state.

1.3.2 Bloodborne T cell memory

1.3.2.1 A 'division of labour'

In addition to memory T cells resident in tissues, memory T cells can be found in circulation. A seminal study proposed 'a division of labour' between two populations in blood: central and effector memory T cells (165). Akin to naïve T cells, central memory T cells (T_{CM}) express lymph node entry molecules such as CD62L and/or CCR7 and circulate through SLOs (165–167). Cells lacking these markers were named effector memory T cells (T_{EM}), given evidence of heightened cytotoxic capacity. T_{EM} were also shown to express molecules associated with tissue-homing and it was speculated that T_{EM} recirculated through nonlymphoid organs. Thus, Sallusto *et al.* married two properties of T cell differentiation in migration and 'effector' function. Going further, this paper proposed a linear differentiation schema whereby T_{CM} beget T_{EM}, thereby superimposing developmental potential as a third axis by which to delineate T cells. Given limitations of human studies of the time, the authors did not probe antigen specificity or evaluate T cells in tissues. Still, this study has become foundational for memory T cell studies. While others had already reported heterogeneity among human memory T cell populations and even evaluated expression of tissue-homing molecules and LN entry molecules (168), Sallusto *et al*. provided an appealing conceptual framework for T cell surveillance that neatly bifurcated organism-wide T cell immunity by location, function, and developmental potential.

1.3.2.2 Memory T cell heterogeneity

Perhaps the true division of labor has been between those evaluating easily accessible SLOs and those studying NLT immunities. Until recently, most studies of T cell memory have evaluated bloodborne and circulating memory. This intensive focus has resulted in the discovery of significant heterogeneity among memory T cells (169). As the initial T_{CM}/T_{EM} framework did not capture such diversity, countless amendments have been proposed over the past two decades. Still, new memory T cell subsets have not been embraced by all, resulting in an unwieldy and inconsistent lexicon.

For example, the emergence of T_{RM} as dominant tissue surveyors has raised questions regarding the true function of CD62L- memory T cells in blood (originally T_{EM}). Several studies have identified bimodal expression of KLRG1 within this population (170, 171). The KLRG1$^+$ T_{EM} are largely absent from tissues, possess enhanced relative cytotoxicity upon intravenous pathogen re-exposures, but exhibit reduced homeostatic proliferation (171–173), suggesting that KLRG1$^+$ T_{EM} may constitute a 'blood-resident' surveyor for hematogenous reinfection. Amongst the KLRG1- T_{EM}, a recent study combined parabiosis, thoracic duct lymphocyte isolation, and HEV-entry blocking antibody to infer that memory T cells expressing an intermediate level of CX3CR1 were enriched in a small population of bloodborne cells that could traffic through nonlymphoid organs (173). These rare cells partially fit the original conception of T_{EM} but were termed T_{PM} for peripheral memory. It is too early to know if this term will be adopted into the evolving T cell nomenclature.

1.3.2.3 Memory T cell ontogeny

Bimodal expression of KLRG1 has also been observed on activated T cells during the effector phase. This KLRG1 expression inversely correlates with expression of CD127 (a subunit of the IL-7 receptor). Adoptive transfers of activated T cells sorted on the basis of CD127 and KLRG1 revealed these two markers can enrich for precursor cells to the memory T cell pool. Specifically, CD127$^+$KLRG1$^-$ cells were demonstrated to be memory precursor effector cells (MPECs), while KLRG1$^+$CD127$^-$ cells were comprised short-lived effector cells (SLECs) (174, 175). One intriguing explanation for this bifurcation of the activated T cell pool is asymmetric division. Descendants of activated T cells have been

demonstrated to have unequal expression of CD3, CD8, and LFA-1, as well as transcription factors TCF-1 and T-bet (176, 177). A recent report indicated that the transcription factor c-Myc was apportioned unequally during early divisions after activation, regulated subsequent asymmetric divisions, and metabolically programmed descendant T cells (178). Transcription factors Id2 and Id3 have also been identified as central regulators of SLECs and MPECs, respectively (179). Evaluation of these opposing transcription factors allows for the bisection of memory T cell populations, including resident memory T cells as recently described (143, 180).

Two models of T cell differentiation summarize an ongoing debate on memory T cell ontogeny. The progressive differentiation model proposes that naïve T cells differentiate directly into a stem-like memory population that is self-renewing. This pluripotent progenitor can then give rise to more terminally differentiated subsets and ultimately 'effector' T cells (156, 181, 182). In support of this model, memory T cells in humans possess longer telomeres indicative of fewer ancestral divisions, as compared to activated T cells (183). Elegant studies have examined daughter cells arising from allogeneic hematopoietic stem cell transplantation (HSCT) and detected a stem cell memory T cell population borne directly from naïve T cells (184, 185).

The linear differentiation model suggests that upon activation, naïve T cells uniformly proceed through an 'effector' T cell phase. Several factors shape the subsequent transition of activated T cells to memory T cells, including relative antigen stimulation, co-stimulatory signals, and cytokines). To test this model, reporter mice have been developed which allow for indelible labeling of cells that express putative markers of 'effector' T cells such as granzyme B and IFN-γ (186–188). From studies of these mice, memory T cells do appear to be borne from activated T cells that express cytotoxic proteins or antiviral cytokines. However, it has been argued that such mice do not identify bona fide 'effector' T cells as they may be oversensitive reporters (189) or do not account for asymmetric divisions that partition the 'effector' molecule unequally in daughter cells (190).

Two recent studies directly addressed such questions of T cell differentiation with complementary approaches (191, 192). Combining *in vivo* deuterium cell labeling in humans after yellow fever vaccination, Akondy *et al*. found that memory $CD8^+$ T cells originate from presumably activated cells that divide extensively initially after immunization (192). Youngblood *et al*. identified an epigenetic signature in murine memory $CD8^+$ T cells and propose that T cell activation allows for demethylation of 'effector' genes in memory precursor populations (191). Bolstering earlier evidence, these recent studies provide compelling experimental support in favor of the progressive differentiation model, whereby memory T cells largely form from potent effector T cells.

1.4 Homeostasis of T cell memory

1.4.1 Longevity

The durability of immunological memory underlies its protective value. The earliest observers of immunity were struck by the length of the intervening period between infections. The Greeks recognized that 'the same man was *never* attacked twice' during the plague of Athens in 430 B.C (193). In his study of measles' epidemics on the remote Faroe Islands, Peter Panum wrote 'it was noteworthy in that a high age by no means lessened the susceptibility to measles' (194). Edward Jenner boldly rejected the possibility of waning immunity after observing that cowpox immunization protected an individual for 'fifty-three years before the effect of the smallpox was tried upon him' [with no effect] (195).

We can now appreciate that Panum and Jenner were observing natural experiments of humoral immunity. For measles and smallpox, antibodies are primarily responsible for immunological protection and elegant longitudinal analyses have revealed remarkably durable serum antibody titers over time (196–198). The antibody half-life for vaccinia-specific antibodies is ~92 years (197). And if still alive today, it is possible Thucydides himself would have detectable antibodies against measles ($t_{1/2}$ = 3014 y). Were these experiments of nature representative of most immune responses? Not necessarily. Antibody responses to tetanus and diphtheria wane much more quickly ($t_{1/2}$ = 11 y; $t_{1/2}$ = 19 y), necessitating occasional booster immunizations (197). Notably, antibodies to the

2003 SARS coronavirus virus have been reported to wane completely within ~6 months after infection (199, 200). While it is understood that long-lived plasma cells are responsible for continuously producing antibodies (201, 202), the cause of such variation in longevity for distinct humoral responses remains a major outstanding question.

The longevity of antigen-specific T cell memory in humans has been even less comprehensively evaluated. There are several reports detecting human memory T cells years to decades after infection (192, 203–205), but fewer quantitative assessments of longitudinal decay. One cross-sectional analysis of vaccinia-specific responses demonstrated $CD4^+$ T cells and $CD8^+$ T cells exhibit half-lives of 8-13 years or 8-15 years, respectively (203). A second study reported a similar value of $t_{1/2}$ = 14 years for vaccinia-specific $CD4^+$ T cells (206). An additional study of measles-specific T cells did not quantify half-lives but detected both $CD4^+$ and $CD8^+$ T cells in human blood 34 years after vaccination (207). The SARS-CoV-2 pandemic has fueled new studies into T cell longevity with reports of T cell immunity to the initial SARS virus lasting up to 17 years, though these T cell populations may be intermittently reactivated given the endemic nature of related coronaviruses (207). Nevertheless, further longitudinal evaluation of antigen-specific human memory T cell populations is warranted to assess whether existing characterizations are generalizable.

Studies of T cell memory are more feasible in mice, where one can control infectious exposures and more easily execute repeated longitudinal sampling. Following an acute infection, mouse memory $CD8^+$ T cells are stably maintained indefinitely (>900 days) (167, 208). Over the same time period, $CD4^+$ T cells markedly decline in multiple models of infection (208–210). It is difficult to know how to integrate these findings with evidence for human memory T cell attrition over decades. There are likely limitations on extrapolating from mouse to human, given differences in T cell homeostasis mechanisms between the two species (211).

Tissue immunities are not equivalent to those in circulation and the longevity of resident memories warrants a rigorous evaluation. Certainly, memory T cells can be detected in

tissues at protracted time points after initial infection (105, 212), but the precise population kinetics are largely unknown. Conventional lymphocyte isolation strategies are inefficient and tend to underestimate the true number of cells, adding an additional obstacle to accurately addressing the question (106, 213). $T_{RM,}$ in particular, are susceptible to anoikis or DAMP-induced apoptosis (214, 215) making it difficult to interpret existing longitudinal studies (120, 216). Still, mouse studies suggest that T_{RM} can be found up to ~1 year after an initial infection in most tissues (105, 212). One notable exception is the lung, where it has long been appreciated that cell-mediated immunity wanes over time, corresponding with a loss of heterosubtypic (cross-reactive) immunity (217, 218).

1.4.2 Antigen and TCR dependence

As defined here, *immunological memory* does not require the persistent presence of antigen or continuous antigen stimulation. Whether immunological memory truly exists in the absence of antigen was a much-contested question for decades among both B and T cell immunologists (219–221). Studies of antigen-specific B and T lymphocytes suggested that even after presumably acute infections, antigen depots might linger throughout the body and provide occasional reminders (222–224), though even these data were experimentally debated (225). Follicular dendritic cells (fDCs), stromal cells of lymphoid tissue that can acquire antigen and present it for years (226), provided a compelling mechanism for long-term retention of antigen that might maintain humoral immune responses, in particular (220, 227).

Convincing resolutions arrived nearly contemporaneously for B and T cells at the end of the century. Using an elegant mouse model, in which BCR specificity could be altered after the establishment of memory B cells, Klaus Rajewsky's group demonstrated that lingering antigen was unlikely to mediate long-term maintenance of humoral immunity (228). In parallel, memory $CD8^+$ T cells were transferred into mice deficient in molecules necessary for antigen presentation ($D^{b-/-}$ x $K^{b-/-}$ x $\beta2M^{-/-}$) and there was no decay over the course of 240 days (229). Similar results were observed for memory $CD4^+$ T cells (230).

With a few exceptions (231), antigen-independent maintenance of memory T and B cells is now largely accepted. Interestingly, such studies revealed a related homeostatic mechanism for naïve lymphocyte maintenance. With another elegantly conceived mouse model, Rajewsky *et al*. demonstrated naïve B cells did depend on constitutive BCR signaling. And while memory T cell durability was independent of MHC interactions, naïve $CD4^+$ and $CD8^+$ T cells decayed in the absence of tonic self-peptide–MHC complexes (232, 233). Studies of antigen and TCR dependence have largely focused on memory populations in SLOs. While there is evidence that T_{RM} can form in the absence of antigen (116, 131, 234), it is also clear that the presence of local antigen in tissues augments their formation. Additionally, T_{RM} resemble activated T cells raising an unaddressed possibility of whether they depend on antigen or constitutive TCR signaling for their homeostasis.

1.4.3 Cytokines

Various cytokines have also been identified as important regulators of T cell longevity. Naïve T cells primarily depend on IL-7 signaling for their maintenance, though others have been implicated (235, 236). Circulating memory T cells rely on both IL-7 and IL-15 for long-term survival and proliferation (235–237). For memory $CD8^+$ T cells, IL-15 is of particular importance for homeostatic proliferation (237). For skin-resident memory $CD8^+$ T cells, IL-15 has been found to be critical for generation and maintenance. Interestingly, T_{RM} dependence on IL-15 varies by tissue. For example, T_{RM} exhibit no defect in formation or maintenance in the small intestine or female reproductive tract of $IL15^{-/-}$ mice (124). Thus, while most circulating memory T cells depend on IL-7 and IL-15, T_{RM} are not axiomatically constrained by cytokine availability, suggesting that the size of the T_{RM} population may be less regulated to allow for expansion of this compartment. Whether T_{RM} depend on IL-7 signaling remains a major unresolved question.

1.4.4 Boosting immunity

Intermittent antigen exposures do serve to amplify the abundance of memory lymphocyte populations. Upon reactivation, primary memory T cells follow a similar cascade of

proliferation, ‘effector’ molecule upregulation, and relocation to sites of infection or inflammation. Following resolution, a secondary memory T cell population remains that is numerically more abundant than the primary memory population. Memory T cell populations will continue to expand following each subsequent activation (238). In mice, following two successive boosts, antigen-specific memory T cell splenocytes can expand by greater than one order of magnitude (239). In settings of latent and/or persistent infections, T cell reactivation can occur *ad infinitum* resulting in unrestrained expansion of T cell populations. In individuals seropositive for HCMV, ~10% of bloodborne memory-phenotype T cells are HCMV-specific (240). This expansive property of T cell immunity has been leveraged via CMV-based vectors that have been demonstrated to offer protection from tuberculosis or simian immunodeficiency virus challenge in non-human primate models. (241, 242).

Does the generation of new memory T cells via boosting or unencountered pathogens erode preexisting memories? Early evidence suggested that the bloodborne T cell compartment was finite and new infections induced attrition of established memory T cell populations (243–247). As a counterpoint, a heterologous prime-boost immunization enormously expanded the CD62L- compartment, but not the $CD62L^{+}$ compartment, leading some to conclude that T_{CM} are fixed in size, while T_{EM} are not (236, 248). Studies of this question in tissues have been limited and it is unclear whether nonlymphoid organs restrain the expansibility of T cell memory or other immune populations. In skin and liver (120, 157), a carrying capacity of T_{RM} has not yet been observed, though the generation of memory T cells can displace innate dendritic epidermal T cell (DETC) populations (249).

1.4.5 Metabolism

The heterogenous nature of memory T cells extends to their energy demands and metabolism. While activated T cells are largely supported by aerobic glycolysis, central memory T cells are relatively more dependent on oxidative phosphorylation for ATP generation (250). To synthesize acetyl-CoA, T_{CM} intake extracellular glucose and glycerol to generate fatty acids that are then broken down to generate ATP via beta-oxidation and

oxidative phosphorylation (251, 252). However, forced glycolytic metabolism is not incompatible with memory, but instead induces the formation of T_{EM}-phenotype memory T cells, suggesting that T_{EM} may utilize aerobic glycolysis (akin to activated T cells) (253). T_{RM} take yet another approach. T_{RM} utilize fatty acid oxidation and oxidative phosphorylation, but acquire free fatty acids from the extracellular environment (instead of producing it from sugars) (254). T_{RM} appear to adapt further to their local environment as the specific fatty acid uptake protein is tissue-dependent (255). T_{RM} may differ further in unexamined locations. For example, resident memory T cells in lymph nodes may uptake extracellular free fatty acids (as NLT T_{RM} do) or they may adopt a metabolic program resembling the many neighboring circulating T cells trafficking through the lymph node.

1.5 Implications of resident immune surveillance

1.5.1 T_{RM} beyond viruses

T cell responses, particularly $CD8^+$ T cell responses, are often critical for antiviral immunity. The study of antiviral $CD8^+$ T cells was essential for the discovery of tissue residence (105, 112, 212, 256). However, early indications of residence were also observed in presumably sterile human pathologies, such as fixed drug eruption, a drug-induced dermatosis (257, 258). Since then, T_{RM} have been implicated in a number of allergic and autoimmune responses including asthma (259, 260), atopic dermatitis (261), vitiligo (122, 137, 262), psoriasis (137, 263), experimental autoimmune encephalomyelitis (EAE) (264), rheumatoid arthritis (265), and multiple sclerosis (266). Additionally, $CD4^+$ and $CD8^+$ T_{RM} can mediate protection against bacterial, fungal and parasitic infections (109, 267–269). In human tumors, the frequency of T_{RM} can correlate with prognosis (270) and a recent study found that T_{RM} can durably control microscopic tumors in mouse skin (271).

1.5.2 Residence in humans

Studies in mice enable reductionist *in vivo* migration analyses that rigorously evaluate tissue residence, such as parabiosis, tissue engraftment and cell depletion. While studies of humans are more difficult to fully control, there are several indications that tissue residence extends to humans. Prior to the full appreciation of T_{RM}, studies of human skin and lung

revealed the magnitude of memory T cells at these sites, as well as their expression of CD69 and CD103 (104, 258, 272). In collaboration with the New York Organ Donor Network, Donna Farber's group has reported several in-depth characterizations of lymphocyte populations in nonlymphoid tissues from recently deceased individuals, finding that a majority of human memory T cells in nonlymphoid tissues express known markers of residence (107, 273).

Recently, several reports have assayed tissues after organ transplant and discriminated between host and donor on the basis of HLA-type (274–277). From these studies, it appears that the degree of tissue residence varies markedly by tissue. One year after duodenal transplant, ~60% of $CD4^+$ or $CD8^+$ memory T cells expressing $CD103^+$ remained donor-derived (indicative of residence) (275, 278). Slightly more host replenishment was observed in lung tissue (274). These results are compelling evidence in support of human tissue-residence, but it is difficult to infer longevity of T_{RM} from these data. Host-derived cells may be de novo T_{RM}, graft-reactive T cells, or circulating surveyors of these tissues. The characterization of antigen-specificity in human transplant experiments would be highly informative in these types of experiments. Interestingly, in studies of kidney and liver, there was near complete replacement of donor T cells by host T cells following transplantation, though this may be attributed to graft rejection or perfusion of donor tissue in these respective studies (276, 277). An unexplored area of clinical interest will likely be the impact of resident T cells on organ transplant success (269, 279). Quantifying the degree of tissue residency in humans remains an important, but challenging goal. Animal models that are more reflective of human immune experience may prove valuable and allow more reductionist experiments than analogous human studies.

1.6 Footnotes

Portions of this text have been previously published and adapted here:

P. C. Rosato*, S. Wijeyesinghe*, J. M. Stolley, D. Masopust, Integrating resident memory into T cell differentiation models. *Curr. Opin. Immunol.* **63**, 35–42 (2020). *Equal contribution.

Author contributions: S.W., P.C.R, and D.M wrote the manuscript. P.C.R. and Nancy Fares-Frederickson are credited with the illustration in Figure 1.1.

1.7 Figures

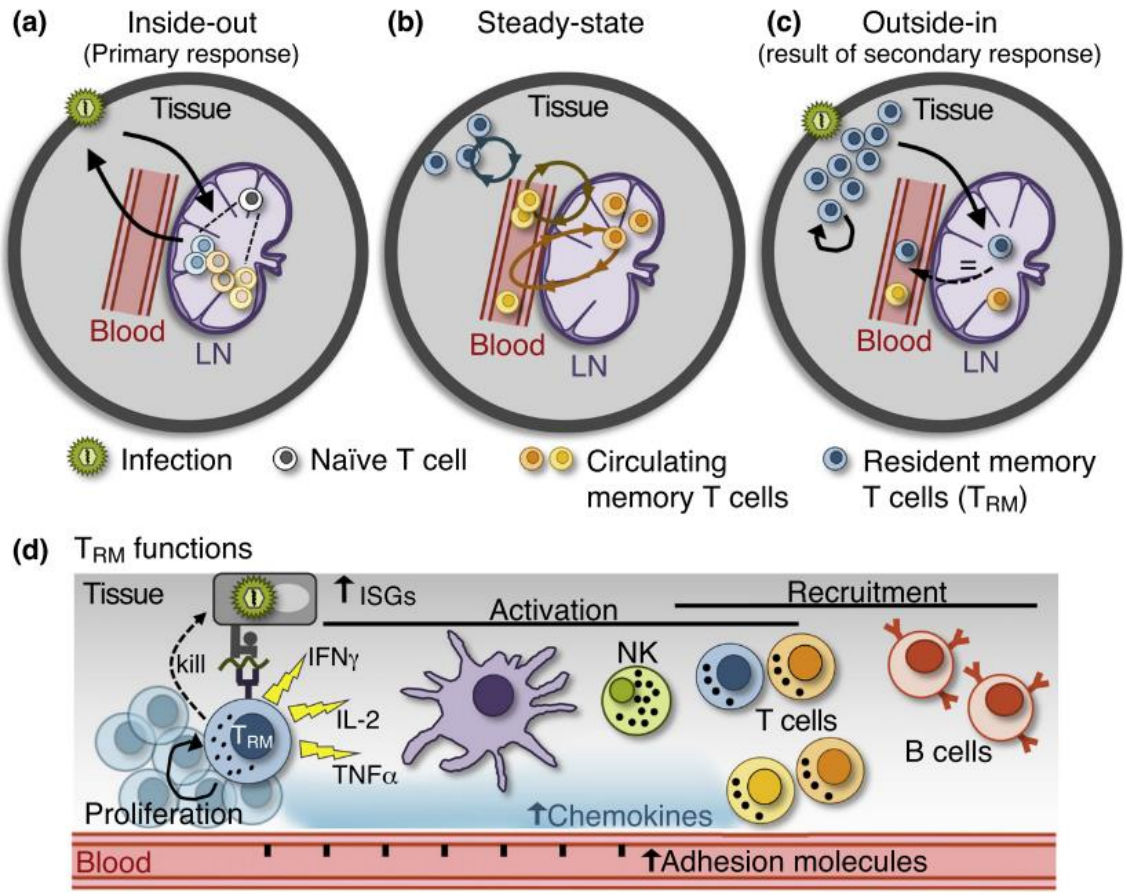


Fig. 1.1 The anatomic topology of primary and recall T cell responses. a) During a primary infection, antigen drains from peripheral tissues to draining lymph nodes (LN) which activates naïve T cells specific for that pathogen. Cells expand in lymphoid tissue and migrate out to peripheral tissues to control the infection (inside-out immune response). **b**) At steady state, memory T cells patrol for reinfection and are heterogenous, consisting tissue-resident cells (T_{RM}) and memory T cells in peripheral blood circulation. **c)** Following secondary exposure to antigen, T_{RM} can proliferate, give rise to an expanded local population of long-lived progeny, redistribute to draining LNs where they may remain resident, or possibly rejoin the circulation (outside-in immune response). **d**) Upon sensing cognate antigen, T_{RM} reactive and alert the local immune system via cytokine production. This elicits interferon-stimulated gene (ISG) upregulation, DC maturation, T cell and NK cell activation, and recruitment of $CD8^+$ T cells and B cells. T_{RM} also proliferate and upregulate cytotoxic molecules, likely contributing to direct pathogen control.

Chapter 2: Methods and Materials

2.1 Mice

Female SPF C57BL/6J (CD45.2$^+$ B6) and B6.SJL-Ptprc^{a}Pepc^b/BoyJ (CD45.1$^+$ B6) mice 6-8 weeks of age were purchased from The Jackson Laboratory. For co-housing experiments, female SPF CD45.2$^+$ and CD45.1$^+$ B6 mice 6-8 weeks of age were purchased from Charles River Laboratories. Female pet shop mice (age not provided by vendor) were purchased from various pet shops in the greater Minneapolis-St. Paul metropolitan area. Co-housing of SPF mice with sex-matched pet shop mice was performed as described (280) within the University of Minnesota BSL-3 facility. TCR$\alpha^{fl/fl}$ mice (281, 282) were fully backcrossed to UBC-CreER^{T2} mice (283) (JAX stock no. 007001) to generate UBC-CreER^{T2} TCR$\alpha^{fl/fl}$ mice (provided by K. Hogquist). Male and female UBC-CreER^{T2} TCR$\alpha^{fl/fl}$ mice 6-10 weeks of age were used in tandem with age- and sex-matched Thy1.1$^+$ B6 mice. CD45.1$^+$ sex-matched mice 6-12 weeks of age were used as recipient mice. P14 CD8$^+$ T cell transgenic, Thy1.1$^+$ B6, and CD45.1$^+$ B6 mouse strains were maintained in house. In experiments with multiple groups, littermates of the same sex were randomly assigned to experimental groups. All mice were used in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Minnesota.

2.2 Adoptive transfers and infections

P14 immune chimeras were generated by intravenous (i.v.) adoptive transfer of 5 x 10^4 P14 CD8$^+$ splenocytes into naive female B6 mice and infection with 2 x 10^5 plaque-forming units (PFU) of the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) via intraperitoneal (i.p.) injection on the subsequent day. For experiments using TCR$\alpha^{fl/fl}$ mice, TCR$\alpha^{fl/fl}$ mice and WT Thy1.1$^+$ B6 mice were intraperitoneally infected with 2 x 10^5 PFU of LCMV. After 30 days, 10^7 lymphocytes, isolated from secondary lymphoid organs, were i.v. transferred into naive CD45.1$^+$ B6 mice. On the subsequent day, CD45.1$^+$ recipients were i.p. infected with 10^6 PFU of LCMV. For heterologous prime boost immunization, three viruses were administered by i.v. injection at 30-day intervals in the following order,

as described (248): 1°, 10^6 PFU of vesicular stomatitis virus (VSV), New Jersey serotype, 2°, 2 x 10^6 PFU of recombinant vaccinia expressing the VSV nucleoprotein, 3°, 10^7 PFU of VSV, Indiana serotype.

2.3 Quantitative immunofluorescence microscopy

Harvested murine tissues were fixed in 2% paraformaldehyde for 2 hours, followed by overnight cryoprotection in 30% sucrose solution at 4°C. Sucrose-treated tissue was embedded in optimal cutting temperature (OCT) compound and frozen in a chilled isopentane bath. Alternatively, harvested murine tissues were directly embedded in OCT compound and snap-frozen in a chilled isopentane bath. Studies of the salivary gland focused exclusively on the submandibular gland. Frozen tissue blocks were sectioned at 7-μm in a Leica cryostat. Sections were stained with primary and secondary antibodies, counterstained with either 4′,6-diamidino-2-phenylindole (DAPI) or SYTOX Green to detect nuclei, and immunofluorescence microscopy was performed using a Leica DM6000 B microscope. Monoclonal anti-mouse antibodies used were: CD8α (53-6.7), CD8β (YTS156.7.7), CD45 (30-F11), CD45.1 (A20), Thy1.1 (OX-7), B220 (RA3-6B2), EpCAM (G8.8), PNAd (MECA-79) from BioLegend. Polyclonal goat anti-mouse collagen type IV antibody from MilliporeSigma and secondary bovine anti-goat IgG (H+L) antibody from Jackson ImmunoResearch were used. Images were processed using FIJI software (284) and cell enumeration was performed manually as previously described (106) or using ImageJ scripts developed in house.

2.4 Leukocyte isolation and phenotyping of cells

An intravascular staining method was used to discriminate between cells within the vasculature and those within the parenchyma of tissues. Three minutes prior to sacrifice, mice were intravenously injected with either 3 μg of biotinylated- or fluorophore-conjugated CD8α (53-6.7) antibody or 2 μg of fluorophore-conjugated CD45 (30-F11) antibody (285). Tissues were harvested and leukocytes isolated as previously described (106). Studies of the salivary gland focused exclusively on the submandibular gland. Isolated leukocytes were surface-stained with monoclonal anti-mouse monoclonal

antibodies: CD4 (GK1.5), CD5 (53-7.3), CD8α (53-6.7), CD8β (53-5.8), CD11c (N418), CD43 (1B11), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD64 (X54-5/7.1), CD69 (H1.2F3), CD103 (2E7), Thy1.1 (OX-7), B220 (RA3-6B2), F4/80 (BM8), Ly6C (HK1.4), Ly6G (1A8), NKp46 (29A1.4), PD.1 (29F.1A12) from BioLegend; CD11b (M1/70), CD19 (1D3), NK1.1 (PK136), Siglec-F (E50-2440) from BD; CD3ε (145-2C11), CD127 (A7R34), KLRG1 (2F1), TCRβ (H57-597) from Tonbo Biosciences. Cell viability was determined using Ghost Dye Violet 510 or Ghost Dye Red 780 (Tonbo Biosciences). To identify VSV nucleoprotein-specific $CD8^+$ T cells, leukocytes were stained with H-$2K^b$/N (MHC class I tetramer), conjugated to PE. Staining for intracellular transcription factors and proteins was performed using a transcription factor staining buffer kit (Tonbo Biosciences) with monoclonal anti-mouse antibodies: T-bet (4B10) from BioLegend; Eomes (Dan11mag), Foxp3 (FJK-16s), Gata-3 (TWAJ), Ki67 (SolA15), Rorγt (B2D) from ThermoFisher Scientific. The stained samples were acquired using LSRII or LSR Fortessa flow cytometers (BD) and analyzed with FlowJo software (BD). Neutrophils were distinguished by expression of CD11b and Ly6G. Eosinophils were identified by Siglec-F expression. Innate lymphoid, natural killer, B cell, and monocyte/macrophage populations were distinguished after excluding lineage-positive cells using combinations of CD3, CD5, CD19, B220, and Ly6G and then using recommended lineage defining-markers as described (33, 286). Monocytes were further subdivided into classical and patrolling populations on the basis of Ly6C expression. Lung macrophages were subdivided into alveolar and interstitial populations on the basis of Siglec-F and CD11b expression.

2.5 Parabiosis and separation surgeries

Parabiosis and separation surgeries were performed with age-matched female mice as described (113) with some modifications. For surgeries, anesthesia to full muscle relaxation was achieved using avertin (250 mg/kg) by i.p. injection and surgical site preparation included betadine application to surgical site in a gradually enlarging circular pattern. For parabiosis, corresponding lateral aspects of mice were thoroughly shaved with

electric clippers from ~1 cm superior to the shoulder and ~1 cm inferior to the hip. Excess hair was wiped off with alcohol prep pads. Following surgical site preparation, matching skin incisions were made ~0.5 cm superior to the shoulder and ~0.5 cm inferior to the hip. Subcutaneous fascia was bluntly dissected to create ~0.5 cm of free skin. Dorsal and ventral skins of adjacent mice were approximated by interrupted horizontal mattress stitches with 3-0 Prolene suture and overlying surgical wound clips. To separate parabiotic mice, preexisting suture and wound clips were removed. Excess hair was shaved and wiped off with alcohol prep pads. After surgical site preparation, a longitudinal incision was made with sharp scissors lateral to the initially conjoined skin. Newly formed fascia was gently detached with a pair of curved forceps. The superior and inferior aspects of the skin of each mouse were then approximated by running stitch with a single 4-0 Vicryl suture. For analgesic treatment, mice received preoperative subcutaneous buprenorphine (2mg/kg) and postoperative subcutaneous bupivacaine (2mg/kg) and carprofen (5mg/kg). Mice were kept on heating pads during and after surgery and their recovery was monitored continuously.

2.6 Tamoxifen administration

Tamoxifen was dissolved in corn oil at 37°C with shaking overnight to a working concentration of 20 mg/ml. Working stocks were freshly prepared for each experiment. For $CreER^{T2}$ induction, tamoxifen was administered to mice i.p at a dose of 75 mg/kg every 24 hours over five consecutive days.

2.7 In vitro stimulation assays

Isolated lymphocytes were incubated at 37°C for 4 hours in stimulation media with or without gp_{33-41} peptide (0.2 μg/mL) or phorbol myristate acetate (PMA) and ionomycin (Cell stimulation cocktail, ThermoFisher Scientific). Stimulation media consisted of RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-mercaptoethanol, and brefeldin A (GolgiPlug, BD). Intracellular staining for cytokines was performed using the Cytofix/Cytoperm kit (per manufacturer's directions, BD) with anti-mouse antibodies: IFN-γ (XMG1.2) from BioLegend and TNF-α (MP6-XT22) from ThermoFisher Scientific.

2.8 Quantification and Statistical Analysis

Specific statistical tests, sample size (*n*), and *p*-values can be found in figure legends. Individual data points represent biological replicates. All statistical tests were two-tailed and generally, non-parametric tests were used to test for significance (Mann-Whitney U test for unpaired samples, Wilcoxon matched-pairs signed rank test for paired samples). All statistical analysis was done using Prism (GraphPad). For all experiments, a *p*-value < 0.05 was considered significant. Mean and standard error of the mean are used to represent the center and dispersion, unless otherwise stated. Nonlinear regression analysis using Prism (GraphPad) was used to model memory T cell population kinetics data, using data points between 30 and 450 days post infection. Model constraints imposed that decay plateau = 0 and a positive rate constant. An exponential decay model was fit to tissue populations if $R^2 > 0$. Either one or two-phase exponential decay models were selected after comparison using extra sum-of-squares F test and the Akaike information criterion (AIC).

2.9 Data and code availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. ImageJ scripts developed in house for cell enumeration are available upon request.

Chapter 3: Expansive residence decentralizes immune homeostasis

3.1 Introduction

Metazoans relegate specific tasks to dedicated organs that are established early in development, occupy discrete anatomic locations, and typically remain fixed in size. The adult immune system arises from a centralized hematopoietic niche that maintains self-renewing potential (29, 287), and upon maturation, becomes distributed throughout the body to monitor environmental perturbations, regulate tissue homeostasis, and mediate organism-wide defense. This study examines how immunity is integrated within adult mouse tissues while addressing issues of durability, expansibility, and contribution to organ cellularity. Focusing on antiviral T cell immunity, we observe durable maintenance of resident memory T cells (T_{RM}) up to 450 days after infection. Once established, resident T cells did not require the T cell receptor for survival or retention of a poised effector-like state. While resident memory indefinitely dominated most mucosal organs, surgical separation of parabiotic mice unexpectedly revealed a tissue-resident provenance for bloodborne memory T cells, and circulating memory slowly made substantial contributions to tissue immunity in some organs. Following additional microbial experiences via serial immunizations or pet shop mice co-housing, we find tissue pliancy allows for the accretion of tissue-resident memory, without axiomatic erosion of preexisting antiviral T cell immunity. Extending these findings, we demonstrate tissue residence and organ pliancy are generalizable aspects underlying the homeostasis of innate and adaptive immunity. The immune system-at-large grows commensurate to microbial experience reaching up to 25% of visceral organ cellularity. Regardless of location, many white blood cell populations adopted a tissue residency program within nonlymphoid organs. Thus, residence, rather than renewal or recirculation, typifies nonlymphoid immune surveillance, and organs serve as a pliant storage reservoir that can accommodate the continuous expansion of the cellular immune system throughout life. While hematopoiesis (‘to make blood’) restores certain elements of the immune system, in parallel, nonlymphoid organs sustain an accrual of durable tissue-autonomous cellular immunity, resulting in the progressive decentralization of organismal immune homeostasis.

3.2 Results

Most immune cells function locally (288, 289). For example, T cells require contact with neighboring cells to sense and eliminate infections or tumors (269, 290, 291). To systematically evaluate the longevity of memory T cells in different locations, we transferred CD45.1$^+$ or Thy1.1$^+$ P14 CD8$^+$ T cells into naive female C57Bl/6J mice followed by LCMV Armstrong infection (referred to as P14 immune chimeras). Nonlymphoid tissues are surveyed by T_{RM}, which are vulnerable to apoptosis upon tissue digestion making enumeration by this method inadequate (106, 214). To mitigate this issue, we visually enumerated P14 CD8$^+$ T cells by quantitative immunofluorescent microscopy (QIM) between five and 450 days after LCMV infection in tissues of 80-96 mice (Fig. 3.1a-e). The response to LCMV peaked within 5-9 days before contracting. A stable population of memory was established in the lymph node by 16 days after infection. We found considerable variation in the durability of nonlymphoid T_{RM}, which were stable in salivary gland, decayed modestly in the small intestine (best modeled by biphasic decay), and underwent continued attrition in the uterus. T_{RM} attrition was particularly noted within uterine endometrium, which underwent vacuolation and glandular atrophy in aged mice (Supplementary Data Fig. 3.1a-c), coinciding with infertility. Aging was also associated with the development of prominent peripheral node addressin-expressing tertiary lymphoid organs in salivary glands (excluded from T_{RM} enumeration in Fig. 3.1c; Supplementary Data Fig. 3.1d, e). These data indicate that memory CD8$^+$ T cell longevity varies by location, but in some compartments can persist indefinitely.

Naïve and central memory (T_{CM}) T cells recirculate between blood and lymph nodes. Naive T cells depend on constitutive T cell receptor (TCR) signaling for survival, while T_{CM} do not (229). Unlike T_{CM}, T_{RM} share many properties with T cells undergoing recent TCR stimulation (144, 161). To test whether T_{RM} persistence depends on the TCR, we crossed mice that possess a floxed region of the TCRα gene to UBC-CreER^{T2} mice. The hybrid strain (referred to as TCR$\alpha^{fl/fl}$) allowed us to genetically ablate the TCR by tamoxifen treatment. LCMV-specific memory CD8$^+$ T cells were established in Thy1.1$^+$/WT and

CD45.2$^+$/TCR$\alpha^{fl/fl}$ mice (without tamoxifen treatment). Memory CD8$^+$ T cells were co-transferred to naïve mice, and then recalled by LCMV infection to expand a secondary memory CD8$^+$ T cell population (Supplementary Data Fig. 3.2a-c). 40 days later, TCR was removed from CD45.2$^+$/TCR$\alpha^{fl/fl}$ cells via tamoxifen treatment, and surface TCRβ expression was significantly diminished within 12 days (Fig. 3.1f, g). CD45.2 was used as a surrogate for identifying LCMV-specific CD8$^+$ T cells, as cells could no longer be assessed for specificity by TCR dependent assays (Supplementary Data Fig. 3.2d, e). Once secondary memory populations were established, no significant difference was observed between the longevity of WT and TCR-ablated T_{RM} in all tissues examined, nor was expression of CD69 and CD103 affected (Fig. 3.1h-j and Supplementary Data Fig. 3.2f, g). Thus, CD8$^+$ T_{RM} are not maintained by persistent viral antigen, self-peptide–MHC I complexes, or cross-reactivity with microbial TCR ligands.

The defining characteristic of T_{RM} is the absence of migration. However, migration experiments are typically short-term, raising the question of whether T_{RM} populations are gradually replenished by T_{CM} (166) or slowly recirculating (292–294). To address this issue, we generated mice containing either CD45.1$^+$ or Thy1.1$^+$ memory P14 CD8$^+$ T cells by LCMV infection. 30 days later, we conjoined mice by parabiosis, which resulted in equilibration of circulating memory P14 CD8$^+$ T cells in the blood of paired mice. Parabionts were separated 30 days after conjoining, which allowed for prolonged monitoring of residence (Fig. 3.1k). 260 days after LCMV infection (200 days after separation), memory T cells were of host origin in most nonlymphoid tissues, indicating that T_{RM} autonomously dominate immunosurveillance for the lifespan of the mouse (Fig. 3.1l). Organized lymphoid structures, including tertiary lymphoid organs in salivary gland and Peyer's patches in small intestine, were surveyed by both recirculating and resident memory T cells. However, extravascular memory T cells in the lung parenchyma demonstrated near-complete equilibration regardless of CD69 expression (Fig. 3.1l and Supplementary Data Fig. 3.3a, b). Although T_{RM} seed the lung shortly after infection, these data indicate that long-term pulmonary surveillance may depend on circulating memory T cells that enter the tissue, which has ramifications for the durability of T cell-dependent

protection against respiratory infections (218). Circulating memory T cells also eventually made substantial contributions to the maintenance of memory in liver and kidney (Fig. 3.1l). While these observations offer support for a centralized source for nonlymphoid memory in certain locations (166), for most tissues, residence is responsible for long-term surveillance after clearance of primary infections in mice housed under specific pathogen free (SPF) laboratory conditions.

Primary infections induce CD62L^{-}/KLRG1^{-} long-lived effector memory T cells (T_{EM}) that patrol blood without entering lymph nodes. After surgical separation, we unexpectedly observed a gradual disequilibrium in blood, in favor of host-derived P14 CD8^{+} T cells (Fig. 3.1l-n). These data indicate that T cells that failed to equilibrate during parabiosis (i.e., T_{RM}) slowly join the blood circulation. These ex-T_{RM} were significantly enriched within the KLRG1^{-} T_{EM} subset (Fig. 3.1m, n). Expression of the CD43 glycoform, recognized by the 1B11 antibody, further distinguished ex-T_{RM}, consistent with expression of this glycoform on tissue resident memory cells (Fig. 3.1o and Supplementary Data Fig. 3.4a, b). Thus, T_{RM} give rise to bloodborne T_{EM} over time.

Cell populations can be numerically fixed by specific niches that regulate abundance. This principle extends to naïve and T_{CM} populations (295). Unlike humans, naïve SPF mice have a paucity of T_{RM} prior to intentional LCMV infection (280). Thus, our analyses of T_{RM} longevity may represent idealized circumstances exempt from the potential competitive influence of new T_{RM} populations arising from subsequent microbial exposures. If T_{RM} occupy an inflexible numerical niche, then the addition of new T_{RM} populations may be balanced by erosion of LCMV-specific T_{RM}. We previously employed a reductionist approach to test whether the circulating memory CD8^{+} T cell population in blood, spleen, and lymph nodes was expansible or rigidly defined (248), and apply that approach here for the analysis of nonlymphoid tissues dominated by T_{RM} (Fig. 3.1). P14 CD8^{+} T cell memory was established by LCMV infection (as in Fig. 3.1a-e).

Sixty days after LCMV infection, we initiated a potent heterologous prime-boost (HPB) vaccination regimen that established a large, broadly distributed memory CD8^{+} T cell

population specific for the immunodominant nucleoprotein ('N') epitope of vesicular stomatitis virus (VSV) (Fig. 3.2a, b). Concomitant with the expansion of N-specific memory T cells, there was a reduction in the relative frequency of preexisting P14 $CD8^+$ T cells as a fraction of total $CD8^+$ T cells (Fig. 3.2c). However, enumeration of P14 $CD8^+$ T cells via QIM demonstrated that T_{RM} were numerically preserved (Fig. 3.2d). In contrast to a previous study (296), new immunizations did not compromise the capacity of preexisting memory $CD8^+$ T cells to produce antiviral cytokines (Fig. 3.2e and Supplementary Data Fig. 3.5a). These data indicate that the immunological 'space' or carrying capacity for T_{RM} is malleable, allowing for the coexistence of established immunity and de novo immune responses.

To assess whether preexisting T_{RM} were also durably maintained following diverse physiologic microbial exposure, we took advantage of a recently developed co-housing model that effects pronounced changes in innate and adaptive immunity. Co-housing SPF mice with pet shop mice results in transmission of bacteria, viruses, fungi, and helminths, and induces extensive immune activation and adoption of a more human-like immune system (280). Sixty days after LCMV, P14 chimeras were co-housed with pet shop mice for two months (Fig. 3.2f, g). In most tissues, we observed little attrition of P14 $CD8^+$ T cells, indicating that circulating and resident memory $CD8^+$ T cells can persist in the face of substantial microbial exposure (Fig. 3.2h, i). Of note, the resident property of memory P14 $CD8^+$ T cells in tissues was also preserved in mice subject to co-housing with pet shop mice (Fig. 3.2j) suggesting that residence itself is not an artifact of artificially clean mouse husbandry.

Homeostasis balances cellular self-renewal with cell death and maintains organ size in adult organisms. This process preserves organ function while preventing cancer. The expansible nature of the T cell compartment raised questions about whether the immune system *in toto* adheres to a fixed size or exhibits pliancy in proportion to microbial experience. Indeed, co-housing induced a durable increase in nearly all leukocyte cell types in blood and persistent enlargement of lymph nodes and spleen (Fig. 3.3a-c). Even so-called 'innate' populations of the immune system demonstrated an 'adaptive' ability to

durably expand in magnitude. Extensive QIM analysis revealed that 5-20% of most nonlymphoid tissues of SPF mice were composed of white blood cells. However, the frequency of immune cells was significantly and durably expanded after co-housing, indicating that the immune system occupies a considerable fraction of visceral and mucosal organs, is flexible in size, and is capable of long-lived adaptation in relation to microbial exposure (Fig. 3.3d, e).

Memory T cells achieve durable immune surveillance of the entire organism through prolonged residence in most nonlymphoid tissues (Fig. 3.1). We asked whether residence is a common mechanism employed by the immune system-at-large to achieve broad immunological surveillance throughout the body. To recapitulate physiologic microbial exposure, we co-housed B6 mice with pet shop mice for two months and then performed parabiotic surgery (Supplementary Data Fig. 3.6a). Leukocytes (distinguished by CD45) equilibrated completely within peripheral blood of parabiotic mice (Supplementary Data Fig. 3.6b), but tissue-resident immune populations occupied and often dominated most mouse organs (Fig. 3.4a). Over one month, many $CD4^+$ and $CD8^+$ T cells of the adaptive immune system, as well as macrophages, innate lymphoid cells, and NK cells of the innate immune system stably occupied nonlymphoid tissues (Fig. 3.4b and Supplementary Data Fig. 3.6c-f). Consistent with their rapid turnover, granulocytes in tissues relied on continuous replenishment from blood (Fig. 3.4b and Supplementary Data Fig. 3.6g). B cells, which differentiate into the antibody secreting cells of the immune system, largely equilibrated between parabionts (Fig. 3.4b and Supplementary Data Fig. 3.6h). Overall, residence was a shared feature exhibited by many adaptive and innate immune cell types in mice exposed to diverse microbes.

3.3 Discussion

Here, we demonstrate that tissue-resident T cell immunity can autonomously endure, without displacement by subsequent inflammation, infection, or competition for a fixed immunological niche – relevant findings for immunization strategies that seek to employ resident T cells for pathogen protection or tumor clearance (297, 298). Extending these

observations, we show that the properties of tissue residence and expansibility are generalizable features of many immune cell types. Historically, studies of blood and lymphoid organs have emphasized the renewable aspects of the immune system via centralized niches of stem or stem-like populations (165, 182, 299–301). Our study finds that tissue immunity can function independently and as such, tissue residence serves to decentralize the homeostasis of the immune system. In a reversal of expectations, a tissue-resident provenance for certain circulating memory T cells indicates that bloodborne immunity is an amalgam that partly reflects the broadly distributed resident T cell diaspora throughout the organism. Indeed, most long-lived T_{EM} may be ex-T_{RM} (Fig. 3.1) and reactivated T_{RM} likely contribute even further to circulating memory (159). The regionalization of immunity results in specialized responses suited for each location of the body and the durability of tissue-autonomous immunity may mitigate the consequences of hematopoietic senescence (302). Unlike neurological memory which relies on plastic connections between existing neurons (303), preservation of immunological memory depends on tissue pliancy that accommodates the addition of stably resident cells, each specific for new hazards encountered throughout life. Future studies are needed to address whether accumulation of such expansive immunity bears any cost, perhaps by crowding out parenchymal structures or elevating the total metabolic burden of the organism. Our study supports the view of the immune system as a uniquely dynamic sensory organ although its resident integration within tissues draws parallels to somatosensory modes of nociception or tactile sensation (304, 305). Locally autonomous immunities are durably interwoven throughout mammalian organs to register and localize encountered stimuli and anticipate future perturbations of tissue homeostasis.

3.4 Footnotes

This chapter has been modified from a submitted manuscript:

Wijeyesinghe S, Beura LK, Pierson MJ, Adam OA, Ruscher R, Stolley JM, Rosato PC, Masopust D. Expansible residence decentralizes immune homeostasis.

Author contributions: S.W., L.K.B., M.J.P., O.A.A., R.R., J.M.S., and P.C.R. performed the experiments; S.W. and D.M. designed the experiments and wrote the manuscript.

3.5 Figures

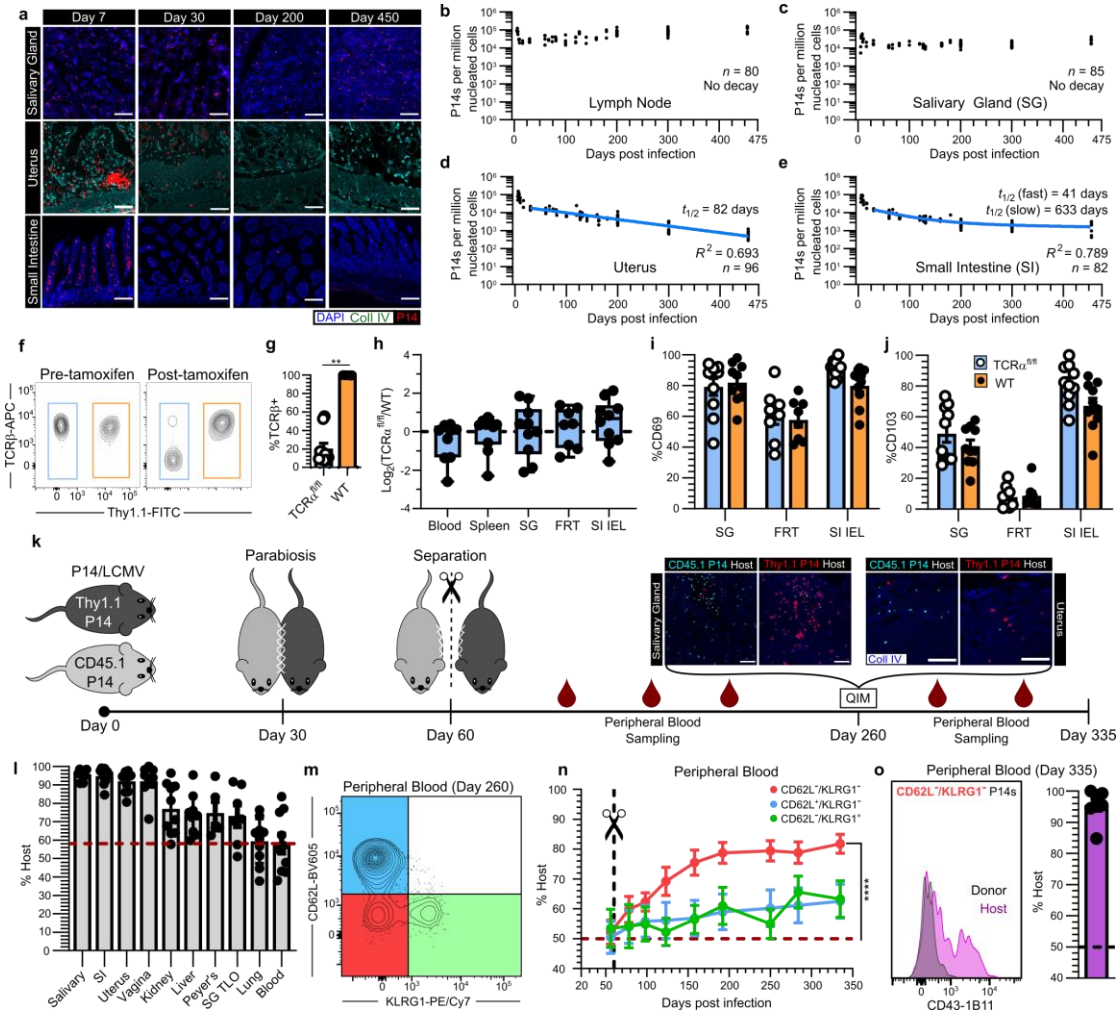


Fig. 3.1. Residence sustains organism-wide autonomous T cell immune surveillance. a-e, Using quantitative immunofluorescent microscopy (QIM) (**a**), P14 memory CD8$^+$ T cells were enumerated five to 450 days after LCMV infection in mice (n = 80-96) (**b-e**). **f-j,** Secondary LCMV-specific TCR$\alpha^{fl/fl}$ and WT memory T cells were established (see methods and Supplementary Data Fig. 2a) and mice were treated with tamoxifen to selectively ablate TCR, as evaluated 12 days post-treatment (**f**, **g**). To assess the impact of TCR ablation on T cell survival, the ratio of TCR$\alpha^{fl/fl}$/WT CD8$^+$ T cells was quantified 60 days after tamoxifen and normalized to pre-tamoxifen values from blood (**h**). Expression of canonical residence markers was compared between WT and TCRβ$^-$ TCR$\alpha^{fl/fl}$ T_{RM} (**i**, **j**). Data pooled from four independent experiments for a total of n = 8-10 mice (**f-j**). **k-o,** Congenically distinct parabiotic P14 chimeras (n = 10) were surgically separated **(k)** and after 260 days after LCMV, tissues of n = 5-10 mice were examined by QIM to evaluate durability of residence among extravascular P14 memory CD8$^+$ T cells (**l**). After separation, peripheral blood of parabiotic P14 chimeras (n = 7) was serially monitored for reemergence of disequilibrium amongst P14 memory CD8$^+$ T cells, which were subdivided based on CD62L/KLRG1 expression (**m**, **n**). 335 days after LCMV, CD62L$^-$/KLRG1$^-$ donor and host P14 memory CD8$^+$ T cells were evaluated for CD43-1B11 staining (**o**). Coll IV, collagen type IV. FRT, female reproductive tract. IEL, intestinal epithelial lymphocytes. TLO, tertiary lymphoid organ. 100 μm scale bars (**a**, **k**). Statistical significance was determined by Wilcoxon

matched-pairs signed rank test (**g**) and one sample *t* test using 0 (**h**) or 50% (**n**) as a hypothetical mean, where $^{**}p = 0.0020$, $^{****}p < 0.0001$.

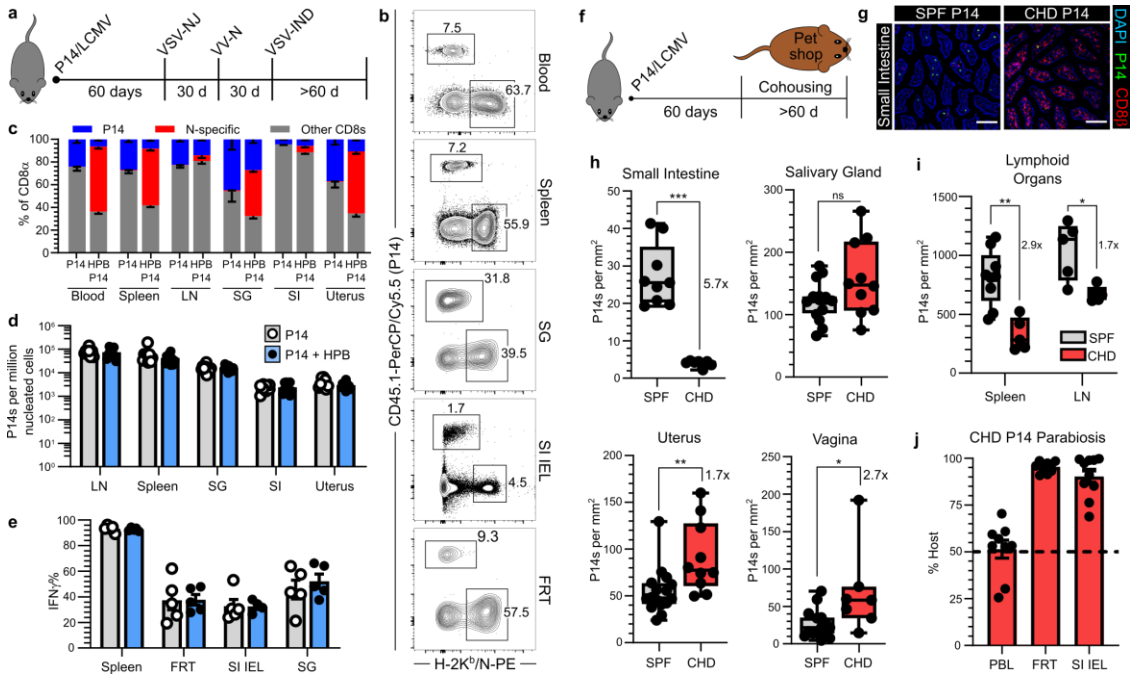
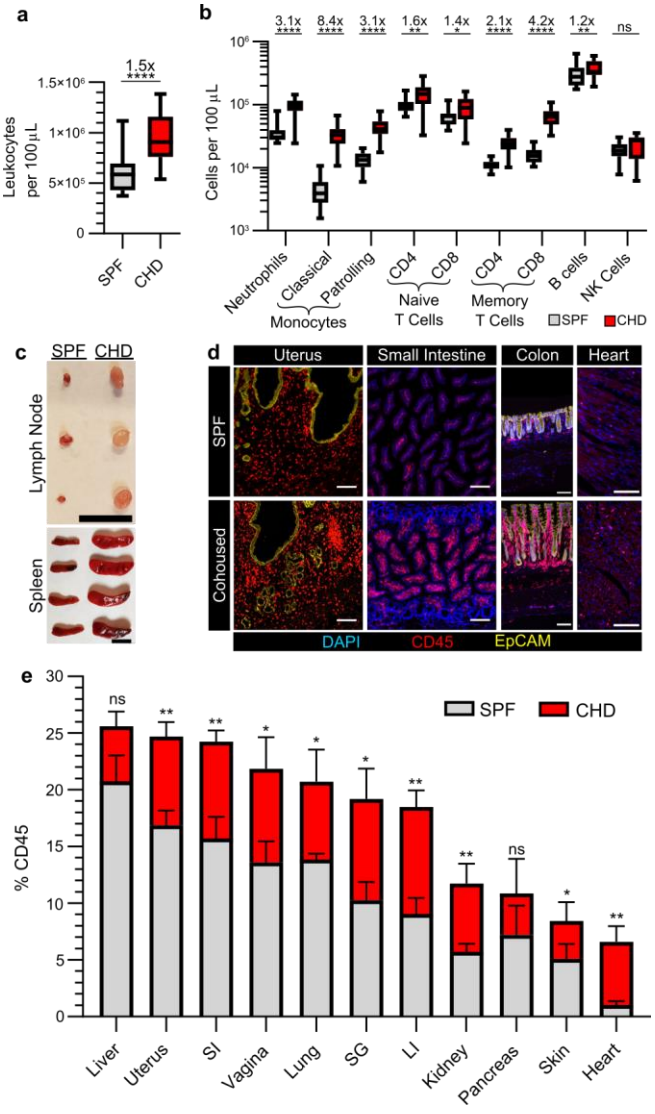


Fig. 3.2. The CD8+ T cell compartment expands to accommodate new and preexisting resident memory. a-e, 60 days after LCMV, P14 immune chimeras were subjected to heterologous prime-boost (HPB) regimen to generate a robust CD8$^+$ T cell response against the nucleoprotein (N) epitope of VSV (**a**, **b**). Numerical abundancy, evaluated by flow cytometry (**c**) and QIM (**d**), and ex vivo functionality (**e**) of memory P14 CD8$^+$ T cells in various tissues was compared and not statistically significant ($p > 0.05$) (**d**, **e**) between mice receiving HPB and age-matched control mice. Number (n = 4-5) of mice per group per experiment and one shown of two independent experiments with similar results (**c**, **e**). Data pooled from two independent experiments for a total of n = 8-10 mice per group (**d**). **f-i,** 60 days after LCMV, P14 immune chimeras were co-housed with pet shop mice to facilitate microbial exposure. Memory P14 CD8$^+$ T cells in nonlymphoid (**h**) and lymphoid (**i**) tissues were enumerated by QIM in co-housed (CHD) P14 immune chimera mice and compared to P14 immune chimeras housed in SPF conditions. Data pooled from two independent experiments for a total of n = 5-16 mice per group (**h**, **i**). **j,** P14 chimeras (n = 9-10) were co-housed with pet shop mice for >60 days, conjoined via parabiosis for 30 days, at which point tissue disequilibrium of memory P14 CD8$^+$ T cells was assessed. LN, lymph node. 100 μm scale bars (**g**). Statistical significance was determined by Mann-Whitney U test where $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig. 3.3. Tissue pliancy allows for immune expansion following microbial conditioning. a, b, The abundance of total leukocytes (**a**) and immune subpopulations (**b**) in peripheral blood was compared between B6 mice co-housed (CHD) with pet shop mice for 90 days (n = 21) and B6 mice housed in conventional SPF conditions (n = 21). **c,** Representative lymphoid organs from SPF and CHD mice. **d, e,** The frequency of immune cells contributing to total organ cellularity (quantified by enumeration of total nucleated cells) in SPF and CHD mice was evaluated by QIM. CHD mice were co-housed for 90-120 days (**c-e**). Data pooled from four independent experiments for a total of n = 3-16 mice per group, dependent on the tissue (**d**, **e**). LI, large intestine. 1 cm scale bar (**c**), 100 μm scale bars (**d**). EpCAM staining shown for uterus and colon only; DAPI staining excluded for uterus (**d**). Statistical significance was determined by Mann-Whitney U test where $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.



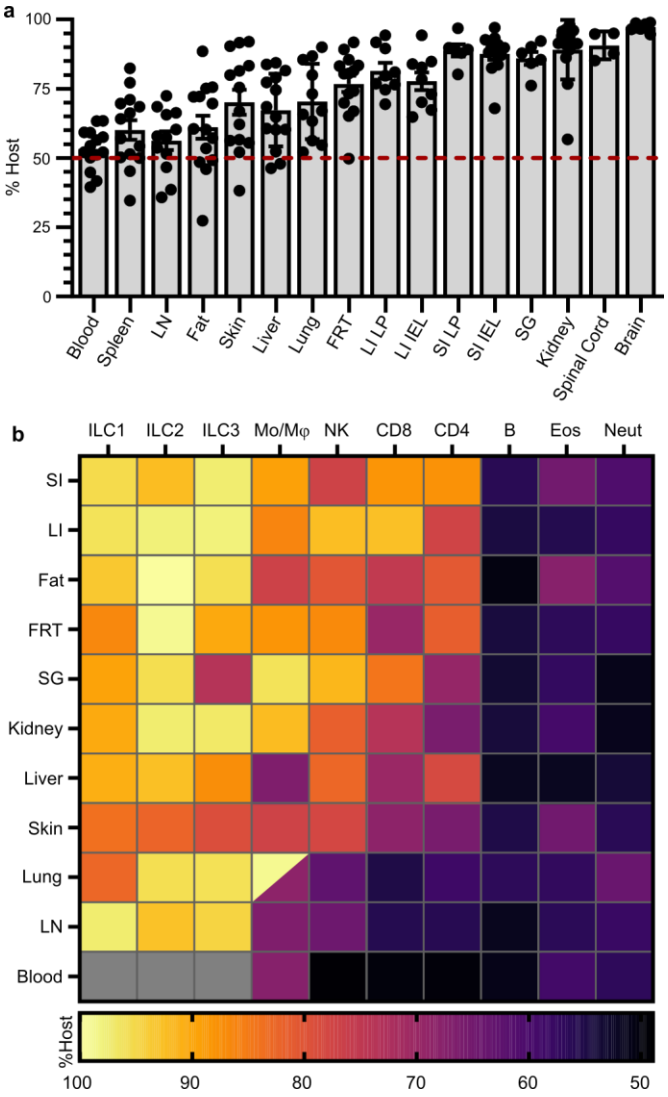
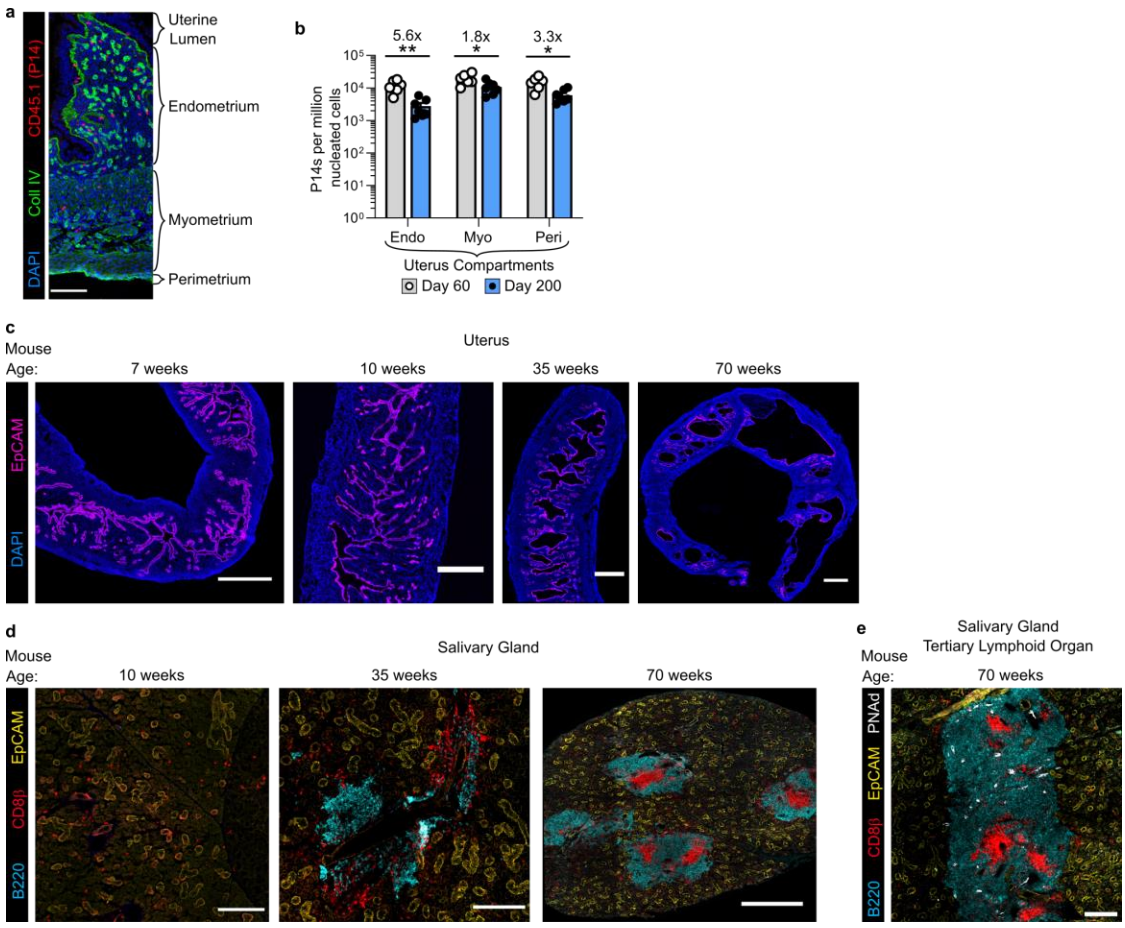
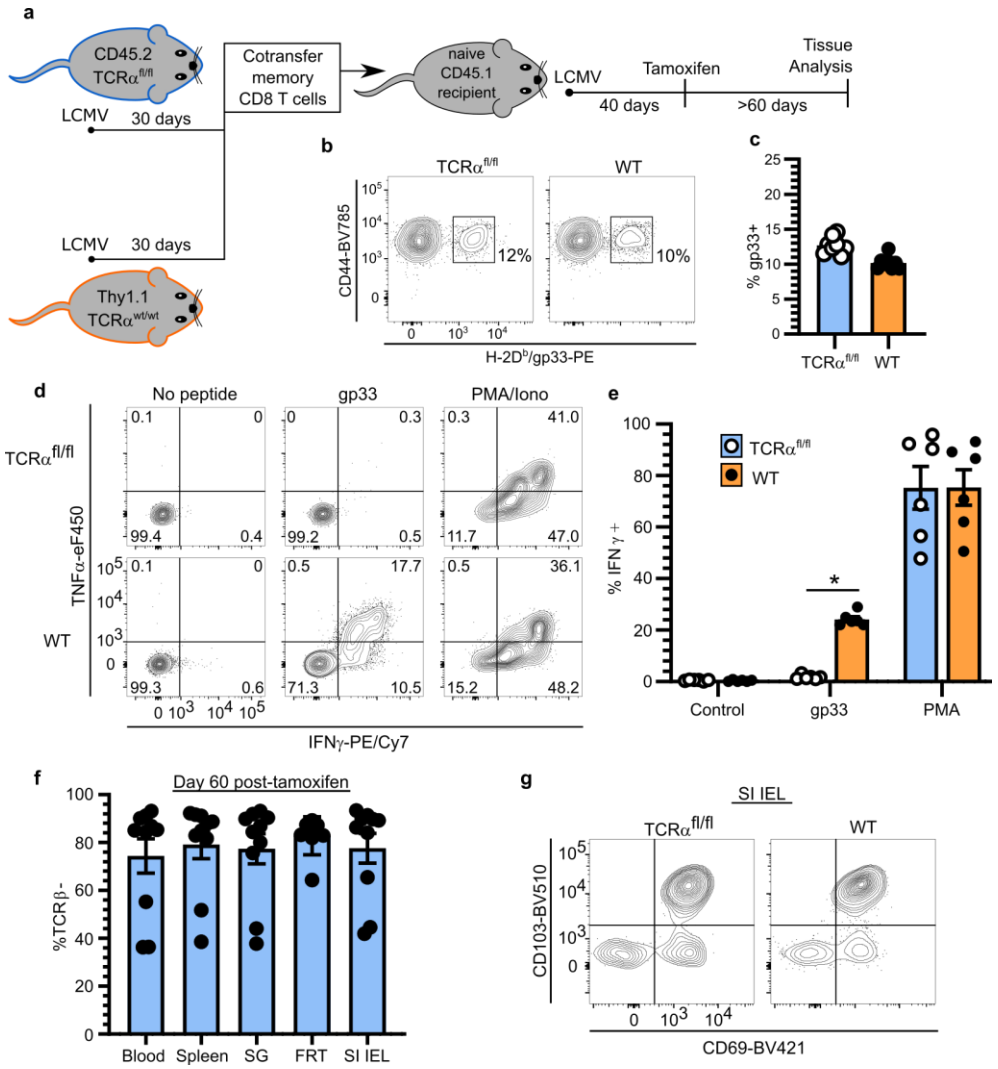


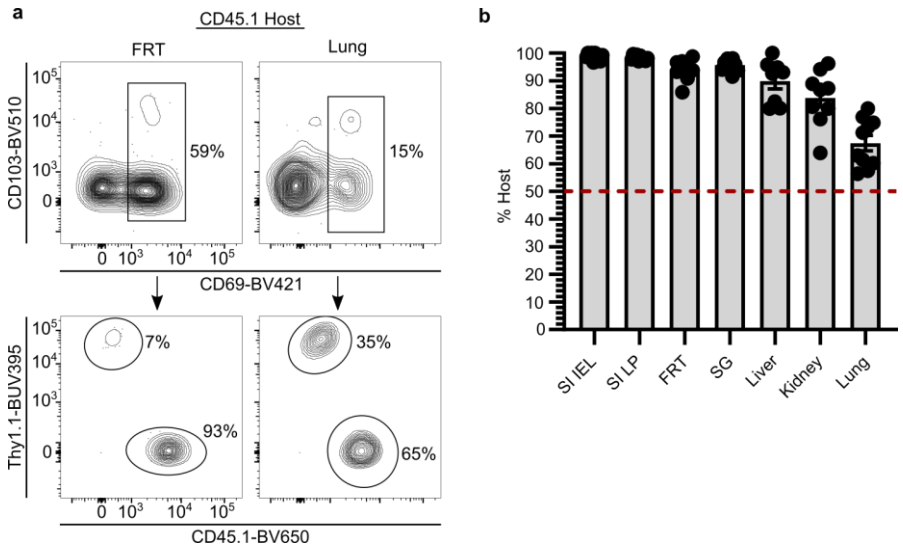
Fig. 3.4. Tissue residence typifies organism-wide immune surveillance. a, b, Congenically distinct B6 mice were co-housed with pet shop mice. After >60 days, mice were conjoined by parabiosis for 28-32 days. Equilibration of total leukocytes (**a**) and immune populations (**b**) was evaluated in peripheral blood and tissues. Lung and kidney populations exclude intravascular leukocytes. Data pooled from four independent experiments for a total of n = 4-14 mice, dependent on tissue (**a**). In heatmap (**b**), lung macrophages are divided into alveolar (top left) and interstitial (bottom right) populations, intestinal populations were isolated from lamina propria (LP), and gray denotes populations that were not detectable. Heatmap shows mean percentage value of host-derived cells with data pooled from four independent experiments for a total of n = 2-14 mice. (**b**). ILC, innate lymphoid cells. Mo/Mφ, monocytes/macrophages. NK, natural killer cells. CD4/CD8 refers to memory T cells ($CD44^+/PD1^-$). B, B cells. Eos, eosinophils. Neut, neutrophils.



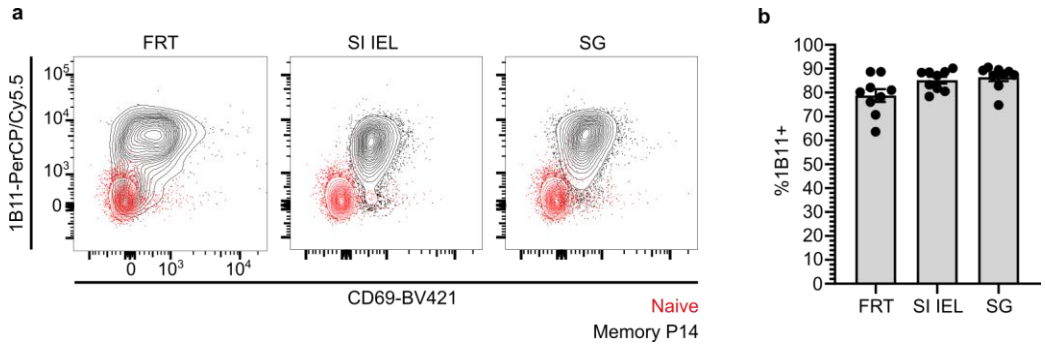
Supplementary Data Fig. 3.1. Compartmentalized decay of uterine T cells concomitant with morphological changes in tissue architecture over time. a, Representative immunofluorescence of uterine tissue. **b**, The frequency of P14 memory $CD8^+$ T cells in uterine compartments was assessed by quantitative immunofluorescent microscopy at day 60 (n = 6 mice) and day 200 (n = 7 mice) after LCMV infection. **c**, Representative immunofluorescence images of mouse uterine tissue at various ages demonstrating endometrial vacuolations in older mice. **d**, **e**, Representative immunofluorescence images of mouse salivary gland at various timepoints demonstrating emergence of salivary gland tertiary lymphoid organs in older mice (**d**) and expression of peripheral node addressin (PNAd) (**e**). Coll IV, collagen type IV. 100 μm scale bar (**a**), 500 μm scale bars (**c**), 200 μm scale bars (10/35 weeks) and 500 μm scale bar (70 weeks) (**d**), 200 μm scale bar (**e**). Statistical significance was determined by Mann-Whitney U test (**b**) where *$p < 0.05$, **$p = 0.0023$.



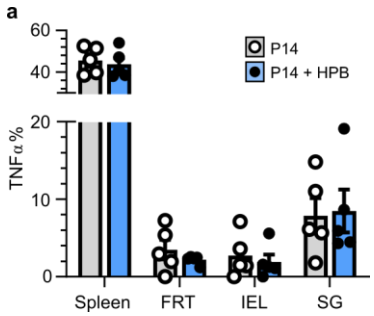
Supplementary Data Fig. 3.2. Selective TCR ablation using TCRα$^{fl/fl}$ mice reveals TCR-independent homeostasis of T_{RM}. a, Experimental model. CD45.2$^+$ UBC-CreER^{T2} TCRα$^{fl/fl}$ mice (TCRα$^{fl/fl}$ mice, for brevity) and WT Thy1.1$^+$ B6 mice were infected with LCMV. After 30 days, 10^7 lymphocytes, isolated from secondary lymphoid organs, were transferred into naive CD45.1$^+$ B6 mice. Recipients were infected with LCMV and treated with tamoxifen, beginning at 40 days post infection. **b**, **c**, Representative flow cytometry (**b**) and quantification (**c**) of secondary LCMV-specific memory T cells in peripheral blood 40 days after LCMV infection (before tamoxifen treatment, staining with H-2D^b/gp33 MHC I tetramer that comprises an immunodominant epitope). Data pooled from three independent experiments for a total of $n = 8$ mice (**c**). **d**, **e**, Selective TCR ablation of TCRα$^{fl/fl}$ memory CD8$^+$ T cells as measured by ex vivo peptide stimulation assay. 60 days after tamoxifen treatment of CD45.1$^+$ B6 recipients, splenocytes were isolated and stimulated in vitro with gp_{33-41} peptide. Representative flow cytometry (**d**) and quantification (**e**) shows cytokine production by TCR$^-$ TCRα$^{fl/fl}$ memory CD8$^+$ T cells and TCR$^+$ WT memory CD8$^+$ T cells from spleen. Data shown reflects $n = 6$ mice (**e**). **f**, The frequency of cells that lack TCRβ expression on TCRα$^{fl/fl}$ memory CD8$^+$ T cells is shown. Data pooled from four independent experiments for a total of n = 8-10 mice (**f**). **g**, Representative flow cytometry depicts expression of tissue-resident markers on small intestinal epithelial (SI IEL) memory CD8$^+$ T cells 60 days after tamoxifen treatment. Statistical significance was determined by Wilcoxon matched-pairs signed rank test (**e**), where $*p = 0.0313$.



Supplementary Data Fig. 3.3. CD69 does not unequivocally distinguish long-lived resident memory T cells in the lung. a-b, Representative flow cytometry (**a**) and graph (**b**) demonstrating degree of disequilibrium among $CD69^+$ extravascular memory P14 $CD8^+$ T cells in tissues of separated parabiotic mice (n = 10), 260 days after LCMV infection. Upper panels are gated on extravascular memory $CD8^+$ P14 T cells (**a**). FRT, female reproductive tract. SI, small intestine. IEL, intestinal epithelial lymphocytes. LP, lamina propria. SG, salivary gland.

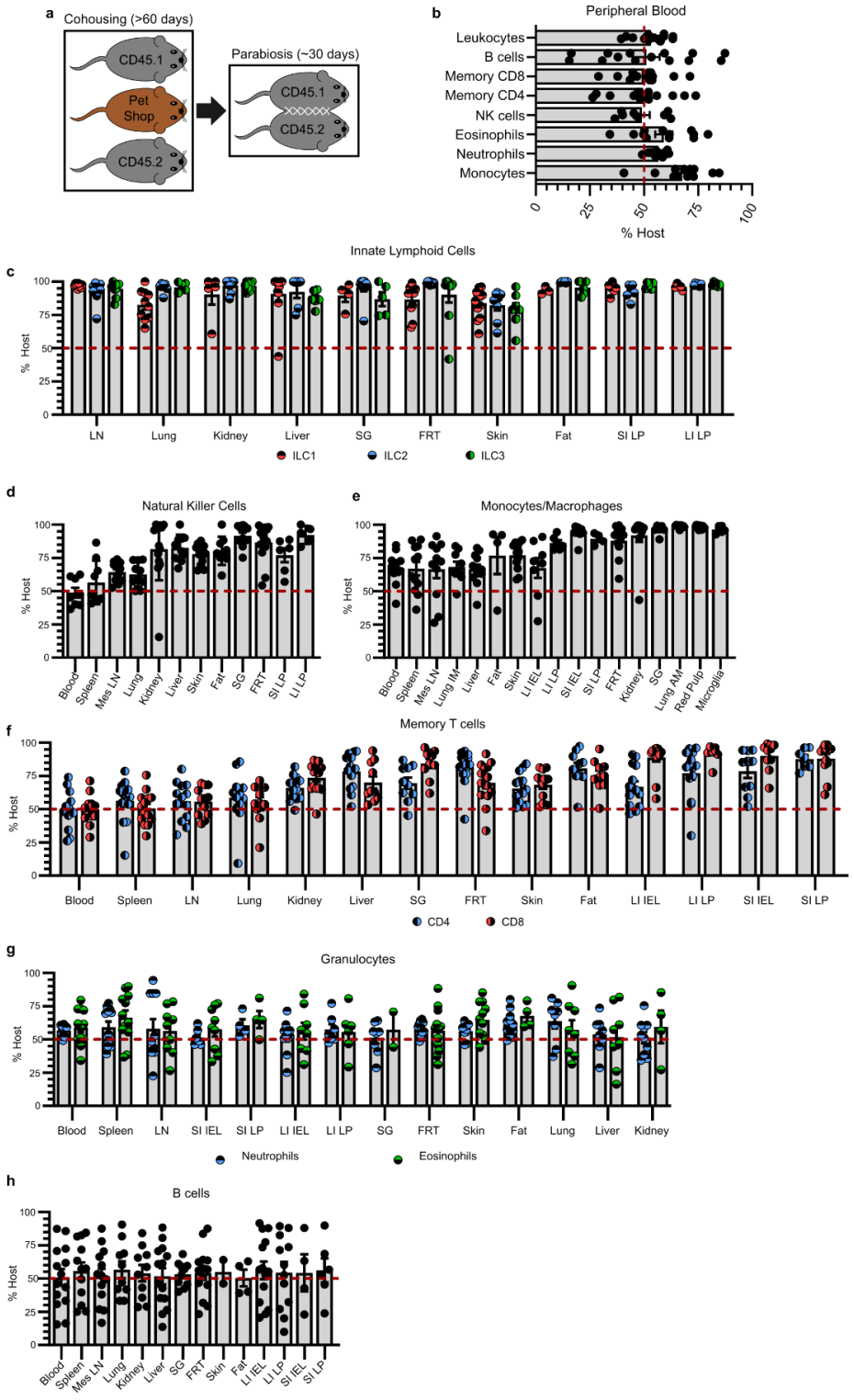


Supplementary Data Fig. 3.4. The 1B11-recognized glycoform of CD43 is expressed on resident memory CD8$^+$ T cells. a, **b**, Representative flow cytometry (**a**) and quantification (**b**) of CD43-1B11 antibody staining on memory P14 CD8$^+$ T cells in nonlymphoid tissues of mice (n = 9) 200 days after LCMV. Naive CD8$^+$ T cells isolated from peripheral blood (in red) serve as basis for comparison (**a**). FRT, female reproductive tract. SI IEL, small intestinal epithelial lymphocytes. SG, salivary gland.



Supplementary Data Fig. 3.5. Preexisting memory T cells retain functional potency following heterologous prime-boost immunization. a, 60 days after LCMV, P14 immune chimeras were subjected to heterologous prime-boost (HPB) regimen. Ex vivo functionality of memory P14 $CD8^+$ T cells in various tissues was compared and not statistically significant ($p > 0.05$) between mice receiving HPB and age-matched control mice. Number ($n = 5$) of mice per group per experiment and one shown of two independent experiments with similar results. Statistical significance was determined by Mann-Whitney U test.

Supplementary Data Fig. 3.6. See next page for caption.



Supplementary Data Fig. 3.6. Tissue residence typifies immune surveillance for many cell types. a, Model depicting co-housing CD45.1^{+} and CD45.2^{+} C57Bl/6 SPF lab mice with pet shop mice for >60 days followed by parabiosis of lab mice for 28-32 days (*n* = 14). **b**, 28-32 days after parabiosis, equilibration of leukocyte populations in peripheral blood was evaluated. **c-h**, 28-32 days after parabiosis, tissue disequilibrium of innate lymphoid cells (**c**), natural killer cells (**d**), monocytes/macrophages (**e**), CD44^{+}/PD1^{-} memory T cells (**f**), granulocytes (**g**) and B cells (**h**) was evaluated. Data pooled from four independent experiments for a total of *n* = 2-14 mice. *n* varies dependent on tissue and population of interest, as not all cell populations were abundantly detected in each experiment. IM, interstitial macrophages. AM, alveolar macrophages. Mes LN, mesenteric lymph node.

Chapter 4: Discussion

riverrun, past Eve and Adam's, from swerve of shore to bend of bay, brings us by a commodious vicus of recirculation back to Howth Castle and Environs.

James Joyce, *Finnegans Wake*

Starting mid-sentence, *Finnegans Wake* begins with this particularly cryptic opening salvo. The rest of the novel is equally enigmatic, and the introductory lines quoted here complete the last phrase of the book, creating a never-ending loop of sorts. The ambiguous language of *Finnegans Wake* is in part why literary scholars have pored over Joyce's final and most incomprehensible work. The language we use matters. Without a precise and agreed-upon vocabulary, the meaning of our work is left open to interpretation by the reader. While Joyce may have intended to be ambiguous, presumably immunologists are striving for a higher degree of clarity.

Conceptualizations of immunological memory, particularly memory T cells have been encumbered by several controversial debates over the past few decades. Does immunological memory depend on antigen (219)? Do effector T cells form memory T cells (189)? Is immunological space fixed or expansible (247)? Do memory T cells pass through tissues or reside in them (166)? Immunologists have conceived of elegant and exhaustive studies in response to these questions (113, 192, 228). However, our vocabulary in these discussions is not universally accepted so regardless of the experimental outcomes, all parties continue to argue their case.

If immunological memory is defined as stably maintained memory T cells, then it is undoubtedly antigen-independent and even TCR-independent (Fig. 3.1). Three decades after the initial debate began, Rolf Zinkernagel and David Gray would still take issue with that definition though and argue that true immunological memory is defined by its protective efficacy (220, 231). They may have a point, as two of the most promising immunization strategies in development for HIV and tuberculosis infections likely depend on antigen persistence to bolster the T cell response (306, 307).

What does effector T cell mean? If effector T cells are simply activated T cells, then of course, memory T cells are derived from effector T cells – all T cells require TCR stimulation to progress to memory. However, if effector T cells are terminally differentiated cells restricted to imminent death (as most activated T cells are), then these cells cannot possibly constitute memory precursors. This unresolved semantic issue has obscured the true spirit of the question. That is, how do activated T cells form protective memory populations? To some degree, this question has been answered. Asymmetric division allows for partitioning of the activated T cell pool, so that memory precursor and short-lived populations divergently form (174, 176). Despite these findings from more than a decade ago, studies have shifted towards a more esoteric argument on the fate of 'effector' T cells. Without established definitions, we risk engaging in a 'commodious vicus' (vicious cycle) of recirculating ideas, without progressing forward.

4.1 Residency: an exception that may prove the rule

Faced with the question of 'disappearing lymphocytes', James Gowans identified their recirculatory nature via the lymphatic system. Gowans and others would go on to attribute the 'carriage of immunological memory' to recirculating lymphocytes, as irradiated rats given thoracic duct cells from immunized donors elicited a secondary immune response (308). At the time, Gowans was puzzled by the finding that a secondary immune response could still be produced by animals whose lymphocytes were depleted from lymphoid tissue (42). This unexpected result went largely unnoticed, and has largely been forgotten, but may have provided one of the earliest indications of an occult tissue-resident immune response (309). Following Gowans' work, subsequent characterizations of memory focused on circulating populations because it was assumed that these cells were reflective of the T cell diaspora throughout the body. This perception was further bolstered by the finding that T cells isolated from afferent lymph of a sheep's hind leg bore markers of memory, suggestive of memory T cells leaving nonlymphoid tissues. Ultimately, over four decades of attention to recirculating T cells culminated in the T_{CM}/T_{EM} framework.

While many T cell immunologists before and including Sallusto *et al*. may have overlooked tissue residence, the perception that immune homeostasis is incompatible with autonomous

tissue immunity was and still is widely believed. In the early 1960's, the identification of hematopoietic stem cells in centralized hematopoietic niches revealed that seemingly all leukocytes could be borne from a single cell in the bone marrow (300). Shortly after Gowans' series of seminal papers describing recirculating lymphocytes, Ralph van Furth and Zanvil Cohn formulated the framework for the monocyte phagocyte system (299, 310), suggesting that bloodborne monocytes continuously replenished terminally differentiated macrophages in tissues. The prevailing weltanschauung among 20th century immunologists was that most leukocytes in tissues were short-lived but could be replaced by precursors populations in blood or lymphoid tissue.

A small blow to this concept came in 2002, when Merad *et al*. used a combination of bone marrow chimeras and parabiosis to unexpectedly demonstrate dendritic cells in the skin, known as Langerhans cells, were largely self-maintained independently of blood (311). It would be ten years later that the same parabiotic mice approach was used to definitively demonstrate tissue residence of memory CD8^{+} T cells in the skin (113). Several groups including Miriam Merad's have now demonstrated that many macrophage populations in tissues, as well as microglia, are generated early during life and are self-renewing (312, 313).

Do these isolated discoveries of self-autonomous populations reflect a more general observation? If so, it remains unclear to what extent tissue residency extends to human immune surveillance. Studies of human organ transplants are ambiguous with considerable residency in some organs (274, 275) and vanishingly few tissue-resident immunities in others (276, 277). It is unclear whether ILCs are resident in mice and human studies have suggested tissue ILCs are replenished from bloodborne ILC precursors (314–316).

My thesis work makes a concerted effort to resolve these questions in 'dirty mice' (where results are not limited to a single infection model and reflect the full breadth of immunological experience that humans face). In this context, most white blood cells populations (including T_{RM}, macrophages, and ILCs) are resident in nonlymphoid organ and collectively, expansive tissue-resident immunities are largely responsible for the

surveillance of microbially experienced mouse tissues. Thus, tissue residency is essential for organismal immune homeostasis *writ large*.

4.2 A case for recirculation

There remain several historical and recent pieces of evidence that would still support memory T cells recirculation. T cells have also been demonstrated to egress from lung and skin, following activation or inflammation (317, 318). More recently, T_{RM} have been shown to be capable of exiting nonlymphoid tissues and establishing residence in the upstream lymph node (160, 319). My thesis work unexpectedly revealed a T_{RM} provenance for bloodborne memory T cells, particularly $1B11^+$ T_{EM}. Thus, egress of T cells may reflect activated T cells, be induced by inflammation, or is explained by the relocation of T_{RM} to the draining lymph node or blood. Such egress appears to be relatively rare, consistent with the bias for residency exhibited by most leukocytes.

Collectively, these observations may explain historical observations of memory T cells in afferent ovine lymph (320, 321) and validate the longstanding search for recirculating T cells. Some of these egressing cells may even conform to the T_{EM} model predicting memory T cells that enter and exit tissues at steady state. My thesis work provides evidence suggestive of tissue-egressing memory T cells and enrichment of these cells amongst $1B11^+$ T_{EM}. Future studies evaluating whether these same cells enter tissues and complete the circuit will be critical. To that end, conventional parabiosis of LCMV-immune P14 chimeras revealed that there is significant nonlymphoid equilibration amongst $1B11^+$ T_{EM} that lack a tissue-resident phenotype (Fig 4.1a-c). These data suggest that $1B11^+$ T_{EM} are capable of tissue diapedesis at the steady state. Many alternative fates for $1B11^+$ T_{EM} may exist including conversion to T_{CM} and/or reestablishment of tissue residency in other lymphoid or nonlymphoid tissues.

4.3 Memory T cell subsetting

"We must be clear that when it comes to atoms, language can be used only as in poetry. The poet, too, is not nearly so concerned with describing facts as with creating images and establishing mental connections"

Niels Bohr

4.3.1 Developmental relationships

Our language concerning memory T cells often applies terms (and associated concepts) from developmental biology. Cellular differentiation is a progression of specification and determination events that result in discrete cell types that have reduced developmental potential. A cell lineage traces this differentiation history of a cell or tissue and 'lineage commitment' is the developmental point where a cell is irreversibly committed to becoming a certain cell type (322). The T cell lineage, as with other cell types, ultimately begins with a single zygote, followed by iterative cell divisions and differentiation. Eventually, hematopoiesis gives rise to many lineage-committed cells of the immune system, including T cells. Thus, naïve T cells are the result of many cell divisions and differentiation steps. T cells themselves branch developmentally into clear lineages that do not typically transdifferentiate. Branches may include commitment to αβTCR vs γδTCR expression and/or single positive CD4 vs. CD8 expression. When mature naïve T cells recognize antigen, that likely also initiates an irreversible differentiation process coupled with division, that results in a large population of activated cells; many of which will die and some of which will further develop into memory cells. Important questions arise when we consider the possible branch points, or lack thereof, in this differentiation path following activation of naïve T cells. The way we conceptualize this process is intertwined with how we define subsets of cells and infer their developmental relationship.

Naïve T cells differentiate into 'effector' cells. Effector T cells have a somewhat ambiguous definition; sometimes being defined based on a functional property such as cytolysis or antiviral cytokine production, sometimes being defined as a cell that will soon die, and sometimes being defined as a cell that was 'recently' activated. The definition of memory is not particularly precise either, but usually invokes the idea that a significant period of time has elapsed since the naïve T cell was primed (although even this ambiguous definition could be considered controversial). Superimposed on these vagaries, memory T cells are dissected into multiple discrete subsets, on the basis of phenotype, function, migration, perceived cell fate, or combinations of properties. There is obvious importance in being able to categorize cells into groups with shared properties, and subsetting imputes

a division of labor among T cell varieties best suited to different desirable biological outcomes. Accordingly, vaccines that favor ‘central’ vs. ‘effector’ memory may have different efficacies that are contextual, rejuvenation of the ‘exhausted’ T cell subset may aid in clearing chronic infections or cancer (301, 323), and chimeric antigen receptor (CAR) T cells might perform optimally if they are comprised of stem-like populations . This has inspired a need to not only classify memory, but also to understand the developmental processes that contribute to the ontogeny of one subset or another.

But, how should we think about memory T cell subsets? To what extent does subsetting comprise an extension of irreversible developmental decisions or lineage commitments? Do some subsets give rise to others, but not the other way around? Or, could some subsets represent alternative differentiation states that are interconvertible? Are the qualitative axes by which subsets have been delineated robustly capturing differences in developmental relationships? Or rather, are they just highlighting functional differences of interest, without being founded on a more traditional developmental strategy for classification? Given recent advances in the field, has an opportunity been presented to restructure memory T cell classification, or to at least be more explicit about the definitions of each class and acknowledge when development principles are being eschewed in favor of (sometimes loosely defined) properties?

4.3.2 Criteria for subsetting and ontogeny models

There are a number of properties by which memory T cells have been Balkanized into subsets, and this process has carried implications, both semantic and conceptual. Firstly, subsets imply largely discrete homogenous entities (Fig. 4.2a). Moreover, subset ontogeny has been diffracted through the prism of developmental biology, which imputes unidirectional lineage relationships and progressive loss of plasticity, and there has been a temptation to insert newly defined subsets into an existing lineage hierarchy. Confounding understanding, many subsets have been proposed, though not always clearly defined, with some gaining more traction than others. Often, a designation is based on a conveniently measurable cell-surface marker or transcription factor. These markers may imperfectly correlate with diverse and difficult-to-measure properties that relate more closely to

function (e.g. what cytokines are made, trafficking patterns, proliferative capacity, developmental plasticity, etc.). Soon, the marker, and an associated subset name, becomes a synonym or substitute for the biology. In addition, the semantics (e.g. central vs. effector memory) can belie the actual properties (central memory T cells make effector cytokines while 'effete' might be a better label for some effector memory T cells) (167). These problems are not unfamiliar to anyone working in the field of T cell memory, and can be especially vexing to those in other fields (324, 325).

It bears consideration whether immunology should borrow a page from physics. Niels Bohr elegantly visualized electrons as occupying discrete orbits, which advanced the field in its time and enabled further discoveries. However, the Bohr model had limitations, as many models do, and it was superseded by Erwin Schrodinger's proposal that electrons fall within a probabilistic distribution that only approximate Bohr's orbits, which was conceptualized as the 'electron cloud' (Fig. 4.2b). T cell subsets may be fuzzy at the margins, or even represent a continuum of differentiation states upon which borders (and labels) have been roughly assigned.

4.3.3 Terrace model

If Linnaeus was the father of taxonomy, memory T cell 'subsetology' would undoubtedly be an ugly step-child. With no single agreed upon property to distinguish T cells, putative T cell subsets often reflect several imperfectly correlated properties shared amongst a population of heterogenous cells. To structure the natural world, Linnaeus created taxa, grouping organisms with similar traits. As taxonomy has evolved, guided by Darwinian principles, phylogenetic relationships reflecting ontogeny have taken precedence over shared morphologies.

We propose a framework in which the central axis of memory T cell taxonomy is developmental potential. Cellular differentiation, including T cell differentiation is axiomatically unidirectional (i.e., a differentiated cell has inherently lost some potential to develop along other differentiation paths). This model restricts T cell movement to one direction along an unambiguous axis of ontogeny. Differentiation along this axis does not

occur in a step-wise fashion, which would presuppose the existence of discrete cell lineages with successive developmental potential. Instead, T cells fall on a continuum of developmental potential, likened to the many gradations of terraced farming. Discrete subsets may reflect shared features between some memory T cells, but an ontogenetic connection should not be inferred, without an evaluation of developmental potential at the single cell level.

This ‘terrace model’ uses migration status to further stratify memory T cells along an axis independent of developmental potential. Resident and recirculating memory T cells differentiate in parallel and may possess equivalent developmental potential. This model allows for some fluidity between resident and recirculating populations, as migration status and differentiation are not inherently linked. Indeed, recirculating memory T cells can become resident and resident cells may join the recirculating pool, with restimulation and inflammation promoting interconversion. This model offers a different perspective on the immunological topology of the body. As the first responders, Trm can autonomously control reinfection events, self-proliferate in situ, and emigrate from tissues to potentially contribute to the recirculating pool. In certain contexts, Trm may shoulder the burden of amplifying anamnestic responses throughout the body. Our language (e.g., ‘central memory’) strongly implies that nonlymphoid tissues are the terminus for immune responses, but this model offers a vantage point in which tissue-resident and recirculating immune responses cooperate equally. The property of residence or recirculation is not necessarily a fixed property, and TCR stimulus or inflammation may be a catalyst for change. Thus, developmental potential (irreversible), and migration status (amenable to change) form two continuous axes along which memory T cells may be placed.

4.4 Concluding remarks

My thesis work demonstrated 1) the size of the immune system adapts to microbial experience, 2) the immune system has the potential to comprise a remarkable fraction of nonlymphoid organs, and 3) most immune cells stably reside in, rather than recirculate through, nonlymphoid organs. The concept of ‘expansible tissue residence’ stands as a counterpoint to the widely accepted paradigm of continuous immune replenishment from

centralized sources. This work offers a fundamental reconceptualization of how to think about the immune system. Tissues autonomously sustain local adaptive and innate resident immunities that are durably flexible in cellularity, adapting to the experience of the host.

Initially, my studies focused on longstanding, but specific questions of T_{RM} biology including their longevity and homeostatic carrying capacity. T_{RM} resemble short-lived effector T cells and their discovery was incongruent with the T_{CM}/T_{EM} paradigm which espoused a centralized source of immune homeostasis. Here we show that T_{RM} are autonomously durable, but longevity varies considerably by anatomic location. Durability is a cardinal feature of immunological memory, as evidenced by recent scrutiny on longitudinal kinetics of humoral and cellular immune responses after SARS-CoV-2 infection (207, 326). Generalizing these results to other infection models and human experience will further contribute to our understanding of cell-mediated immunity.

T_{RM} decay in some locations is contemporized by the accumulation of ex-T_{RM} in blood. The ontogeny and function of bloodborne T_{EM} has been debated, particularly since the discovery of resident surveillance of tissues. Instead of T_{EM} surveillance of nonlymphoid organs, parabiosis and separation revealed that T_{RM} mediate long-term surveillance of tissues and directly contribute to bloodborne T_{EM}. This tissue-resident provenance reveals the unappreciated developmental potential of T_{RM} and raises new questions regarding the ultimate fate and function of T_{EM}, particularly $1B11^+$ T_{EM}. It is not unreasonable to speculate that $1B11^+$ T_{EM} may be imprinted by their time in nonlymphoid organs and return to their tissues of origin at steady state or upon recall (162).

Naïve T cells require constitutive TCR signaling and cytokine availability for their maintenance. Using a mouse model initially conceived by Klaus Rajewsky's group (281), we demonstrated that T_{RM} did not depend on constitutive TCR signaling or persistent antigen for their maintenance or poised cytotoxic state. T_{CM} require IL-7 and IL-15 signaling for durable maintenance (236). It is thought that these signaling mechanisms serve as a homeostatic rheostat to maintain a numerically fixed T cell pool (244). Unlike T_{CM}, T_{RM} do not depend axiomatically depend on IL-15 for their maintenance (124) and

we find that T_{RM} are unrestrained in their expansive capacity, even following *de novo* T_{RM} generation and significant immunological/inflammatory experience. At this juncture, it is unclear what, if any, signal does control the size of the T_{RM} population. Identifying additional cytokines that may control the size of the T_{RM} pool may inform translational efforts to harness these cells for pathogen prevention or tumor control.

These findings significantly advance our understanding of memory T cell surveillance. However, they also exemplify broader concepts related to the immune system at-large. Tissue residence, rather than recirculation, is the norm and recirculating immunity has been the anomalous observation. And although nonlymphoid organs are fairly fixed in size, the immune system within these organs is not. This work shows that tissue immunity is considerably compartmentalized and subsequently operates under distinct homeostatic pressures that may defy expectations drawn from studies of blood. Collectively, this thesis offers a reoriented conceptualization of the immune system and prompts greater recognition of its varied recognition, ontogeny, and numerical abundance throughout the body.

4.5 Footnotes

Portions of this text have been previously published:

P. C. Rosato*, S. Wijeyesinghe*, J. M. Stolley, D. Masopust, Integrating resident memory into T cell differentiation models. *Curr. Opin. Immunol.* **63**, 35–42 (2020). *Equal contribution.

Author contributions: S.W., P.C.R, and D.M wrote the manuscript. J.M.S. is credited for illustration of the terrace model as depicted in Figure 4.2c.

4.6 Figures

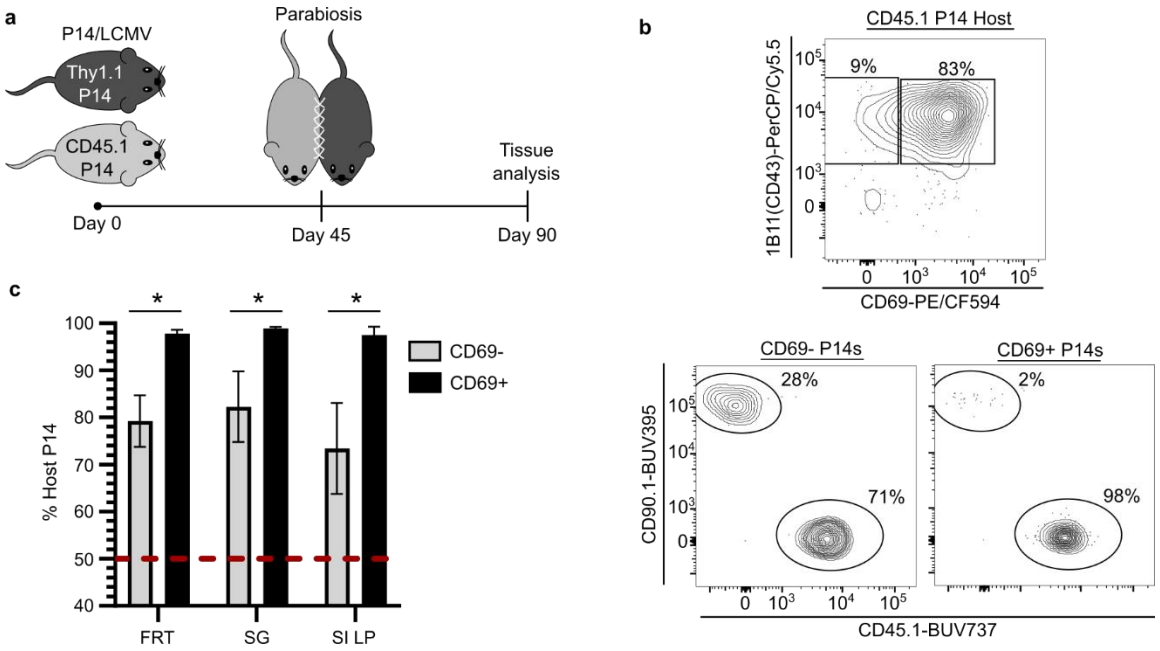


Fig. 4.1. 1B11^{+} T$_{EM}$ preferentially enter tissues at steady state. **a**, Model depicting generation and parabiosis of congenically mismatched P14 immune chimeras. **b-c,** 45 days following parabiosis surgery, nonlymphoid tissues were examined for equilibration of memory P14 T cells. Representative flow cytometry of the female reproductive tract from a CD45.1^{+} P14 host is depicted showing gating strategy for 1B11^{+} memory T cells bisected based on CD69 expression (**b**, upper panel). Representative flow cytometry shows equilibration between parabiotic mice among 1B11^{+}CD69^{-} and 1B11^{+}CD69^{+} memory T cells (**b**, lower panel) and quantification of equilibration for multiple tissues (**c**). *n = 6* mice.

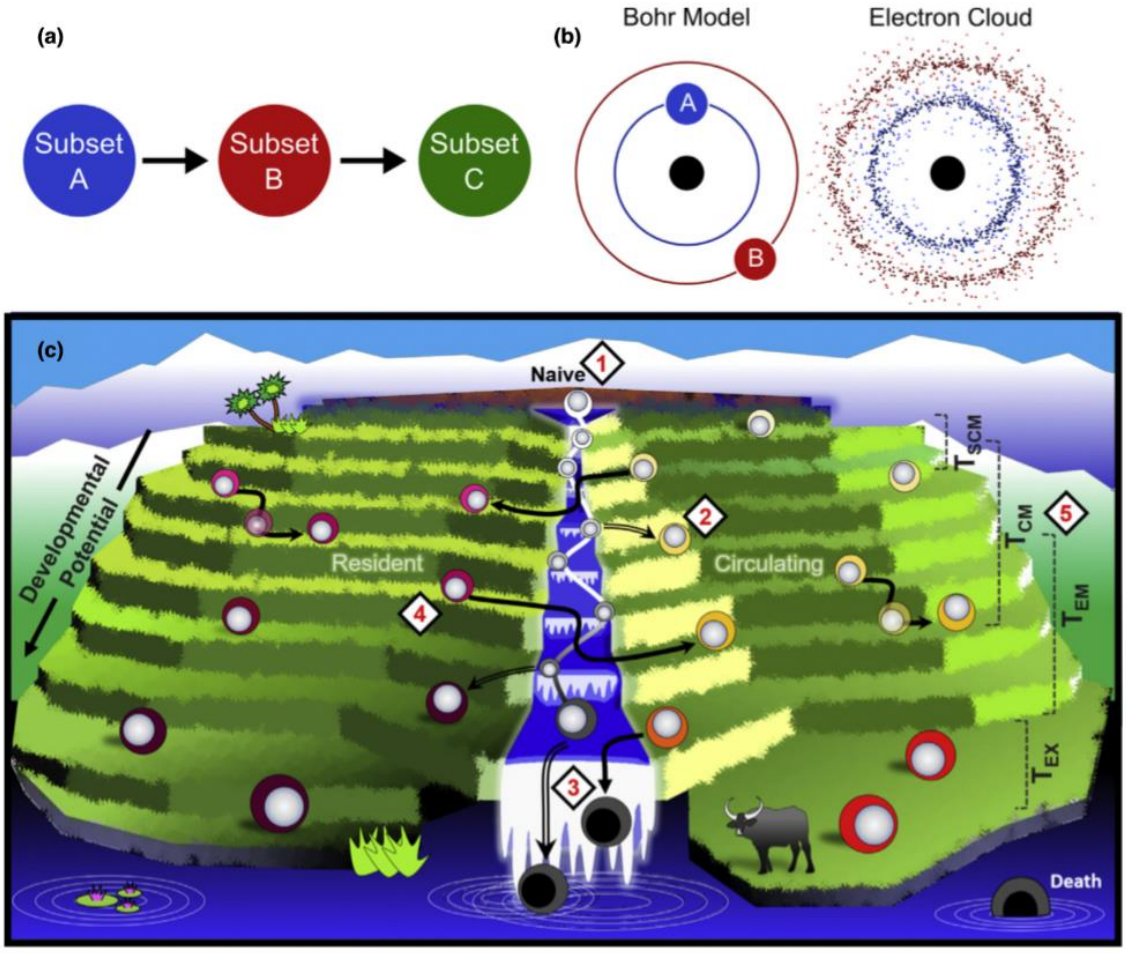


Fig. 4.2. Models of T cell differentiation. a) Defined subsets of memory T cells imply unidirectional differentiation between discrete cell types. **b)** Initial modeling of electron dynamics implied discrete orbits of movement (left), whereas the modern view highlights a probabilistic distribution, represented as an electron cloud (right). **c)** The 'terrace' model depicts naive T cell activation (1) and differentiation into memory T cells (2) along a continuum of developmental potential (depicted as terraces), though most activated T cells (depicted in the river) die (3). Migration status is used to further bifurcate memory T cells into a resident and circulating pool. This migration status is largely fixed, though may not be permanent for all cells (4). Subset nomenclature of circulating memory T cells is approximated, but does not reflect discrete cell types with stepwise loss of differentiation potential (5). The 'terrace' model is based on developmental potential and migration properties. Stem cell memory T cells (TSCM); Central memory T cells (TCM); Effector memory T cells (TEM); Exhausted T cell (TEX).

References

1. L. R. Thompson, *et al.*, A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* **551**, 457–463 (2017).

2. K. J. Locey, J. T. Lennon, Scaling laws predict global microbial diversity. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 5970–5975 (2016).

3. L. M. Bobay, H. Ochman, Biological species in the viral world. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 6040–6045 (2018).

4. A. C. Gregory, *et al.*, Marine DNA Viral Macro- and Microdiversity from Pole to Pole. *Cell* **177**, 1109-1123.e14 (2019).

5. R. W. Hendrix, M. C. M. Smith, R. N. Burns, M. E. Ford, G. F. Hatfull, Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. *Proc. Natl. Acad. Sci.* **96**, 2192–2197 (1999).

6. A. R. Mushegian, Are There 10 31 Virus Particles on Earth, or More, or Fewer? *J. Bacteriol.* **202**, 18–22 (2020).

7. , Microbiology by numbers. *Nat. Rev. Microbiol.* **9**, 628 (2011).

8. C. Mora, D. P. Tittensor, S. Adl, A. G. B. Simpson, B. Worm, How Many Species Are There on Earth and in the Ocean? *PLoS Biol.* **9**, e1001127 (2011).

9. J. A. Gilbert, *et al.*, Current understanding of the human microbiome. *Nat. Med.* **24**, 392–400 (2018).

10. Y. Belkaid, T. W. Hand, Role of the Microbiota in Immunity and Inflammation. *Cell* **157**, 121–141 (2014).

11. L. H. Taylor, S. M. Latham, M. E. J. Woolhouse, Risk factors for human disease emergence. *Philos. Trans. R. Soc. London. Ser. B Biol. Sci.* **356**, 983–989 (2001).

12. S. H. E. Kaufmann, Immunology's coming of age. *Front. Immunol.* **10**, 1–13 (2019).

13. A. M. Silverstein, *A history of immunology* (2009).

14. P. C. Doherty, S. J. Turner, The Challenge of Viral Immunity. *Immunity* **27**, 363–365 (2007).

15. S. M. Blevins, M. S. Bronze, Robert Koch and the 'golden age' of bacteriology. *Int. J. Infect. Dis.* **14**, e744–e751 (2010).

16. S. H. E. Kaufmann, Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat. Immunol.* **9**, 705–712 (2008).

17. , THE NOBEL PRIZE FOR 1908: METCHNIKOFF—EHRLICH. *JAMA* **301**, 335 (2009).

18. L. K. Beura, S. C. Jameson, D. Masopust, Is a Human CD8 T-Cell Vaccine Possible, and if So, What Would It Take? *Cold Spring Harb. Perspect. Biol.* **10**, a028910 (2018).

19. H. Li, B. Javid, Antibodies and tuberculosis: finally coming of age? *Nat. Rev. Immunol.* **18**, 591–596 (2018).

20. C. S. N. Klose, D. Artis, Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat. Immunol.* **17**, 765–774 (2016).

21. H. Wang, K. A. Hogquist, How Lipid-Specific T Cells Become Effectors: The Differentiation of iNKT Subsets. *Front. Immunol.* **9** (2018).

22. J. D. Berkson, M. Prlic, The MAIT conundrum – how human MAIT cells distinguish bacterial colonization from infection in mucosal barrier tissues. *Immunol. Lett.* **192**, 7–11 (2017).

23. B. Glick, T. S. Chang, R. G. Jaap, The Bursa of Fabricius and Antibody Production. *Poult. Sci.* **35**, 224–225 (1956).

24. M. D. Cooper, R. D. A. Peterson, R. A. Good, Delineation of the thymic and bursal lymphoid systems in the chicken. *Nature* **205**, 143–146 (1965).

25. M. D. COOPER, R. D. PETERSON, R. A. GOOD, DELINEATION OF THE THYMIC AND BURSAL LYMPHOID SYSTEMS IN THE CHICKEN. *Nature* **205**, 143–6 (1965).

26. M. K. Slifka, I. Amanna, How advances in immunology provide insight into improving vaccine efficacy. *Vaccine* **32**, 2948–2957 (2014).

27. J. F. A. P. Miller, IMMUNOLOGICAL FUNCTION OF THE THYMUS. *Lancet* **278**, 748–749 (1961).

28. D. M. V. Parrott, J. East, Role of the thymus in neonatal life. *Nature* **195**, 347–348 (1962).

29. C. M. Sawai, *et al.*, Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals. *Immunity* **45**, 597–609 (2016).

30. T. Krausgruber, *et al.*, Structural cells are key regulators of organ-specific immune responses. *Nature* **583**, 1–7 (2020).

31. A. P. Bénéchet, *et al.*, Dynamics and genomic landscape of CD8+ T cells undergoing hepatic priming. *Nature* **574**, 200–205 (2019).

32. R. M. Sutherland, *et al.*, Cognate antigen engagement on parenchymal cells stimulates CD8+ T cell proliferation in situ. *Nat. Commun.* **8**, 14809 (2017).

33. C. S. N. Klose, *et al.*, The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature* **549**, 282–286 (2017).

34. V. Cardoso, *et al.*, Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature* **549**, 277–281 (2017).

35. J. A. Cohen, *et al.*, Cutaneous TRPV1+ Neurons Trigger Protective Innate Type 17 Anticipatory Immunity. *Cell* **178**, 919-932.e14 (2019).

36. J. R. Huh, H. Veiga-Fernandes, Neuroimmune circuits in inter-organ communication. *Nat. Rev. Immunol.* **20**, 217–228 (2020).

37. C. Chu, D. Artis, I. M. Chiu, Neuro-immune Interactions in the Tissues. *Immunity* **52**, 464–474 (2020).

38. J. Ordovas-Montanes, S. Beyaz, S. Rakoff-Nahoum, A. K. Shalek, Distribution and storage of inflammatory memory in barrier tissues. *Nat. Rev. Immunol.* **20**, 308–320 (2020).

39. S. Naik, *et al.*, Inflammatory memory sensitizes skin epithelial stem cells to tissue damage. *Nature* **550**, 475–480 (2017).

40. S. L. Park, T. Gebhardt, L. K. Mackay, Tissue-Resident Memory T Cells in Cancer Immunosurveillance. *Trends Immunol.* **40**, 735–747 (2019).

41. J. L. GOWANS, The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. *Br. J. Exp. Pathol.* **38**, 67–78 (1957).

42. J. L. Gowans, The lymphocyte — a disgraceful gap in medical knowledge. *Immunol. Today* **17**, 288–291 (1996).

43. J. L. GOWANS, The recirculation of lymphocytes from blood to lymph in the rat. *J. Physiol.* **146**, 54–69 (1959).

44. J. L. GOWANS, E. J. KNIGHT, THE ROUTE OF RE-CIRCULATION OF LYMPHOCYTES IN THE RAT. *Proc. R. Soc. London. Ser. B, Biol. Sci.* **159**, 257–82 (1964).

45. J.-P. Girard, C. Moussion, R. Förster, HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat. Rev. Immunol.* **12**, 762–73 (2012).

46. K. Ley, P. Gaehtgens, Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric vanules. *Circ. Res.* **69**, 1034–1041 (1991).

47. E. C. Butcher, L. J. Picker, Lymphocyte homing and homeostasis. *Science* **272**, 60–6 (1996).

48. G. W. Schmid-Schönbein, S. Usami, R. Skalak, S. Chien, The interaction of leukocytes and erythrocytes in capillary and postcapillary vessels. *Microvasc. Res.* **19**, 45–70 (1980).

49. C. Sun, C. Migliorini, L. L. Munn, Red blood cells initiate leukocyte rolling in postcapillary expansions: a lattice Boltzmann analysis. *Biophys. J.* **85**, 208–22 (2003).

50. D. N. Granger, P. Kubes, The microcirculation and inflammation: Modulation of leukocyte-endothelial cell adhesion. *J. Leukoc. Biol.* **55**, 662–675 (1994).

51. T. A. Springer, Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* **57**, 827–72 (1995).

52. C. Foxall, *et al.*, The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. *J. Cell Biol.* **117**, 895–902 (1992).

53. M. D. Gunn, *et al.*, A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 258–263 (1998).

54. Y. Shimizu, W. Newman, Y. Tanaka, S. Shaw, Lymphocyte interactions with endothelial cells. *Immunol. Today* **13**, 106–112 (1992).

55. M. L. Dustin, T. A. Springer, Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J. Cell Biol.* **107**, 321–331 (1988).

56. C. L. A. and C. A. Lowell, The Ins and Outs of Leukocyte Integrin Signaling. *Annu. Rev. Immunol.* **185**, 974–981 (2013).

57. M. Tomura, *et al.*, Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 10871–10876 (2008).

58. J. N. Mandl, *et al.*, Quantification of lymph node transit times reveals differences in antigen surveillance strategies of naive CD4+ and CD8+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 18036–41 (2012).

59. M. R. Britschgi, A. Link, T. K. A. Lissandrin, S. A. Luther, Dynamic Modulation of CCR7 Expression and Function on Naive T Lymphocytes In Vivo. *J. Immunol.* **181**, 7681–7688 (2008).

60. U. H. Von Andrian, T. R. Mempel, Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* **3**, 867–878 (2003).

61. J. G. Cyster, S. R. Schwab, Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu. Rev. Immunol.* **30**, 69–94 (2012).

62. M. Matloubian, C. G. Lo, G. Cinamon, Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. **1231**, 355–360 (2002).

63. A. Baeyens, V. Fang, C. Chen, S. R. Schwab, Exit Strategies: S1P Signaling and T Cell Migration. *Trends Immunol.* **36**, 778–787 (2015).

64. D. W. Talmage, Diversity of antibodies. *J. Cell. Comp. Physiol.* **50**, 229–246 (1957).

65. F. M. Burnet, A Modification of Jerne's Theory of Antibody Production using the Concept of Clonal Selection. *CA. Cancer J. Clin.* **26**, 119–121 (1976).

66. N. K. Jerne, THE NATURAL-SELECTION THEORY OF ANTIBODY FORMATION. *Proc. Natl. Acad. Sci.* **41**, 849–857 (1955).

67. G. M. Edelman, J. A. Gally, SOMATIC RECOMBINATION OF DUPLICATED GENES: AN HYPOTHESIS ON THE ORIGIN OF ANTIBODY DIVERSITY. *Proc. Natl. Acad. Sci.* **57**, 353–358 (1967).

68. S. Tonegawa, C. Steinberg, S. Dube, A. Bernardini, Evidence for Somatic Generation of Antibody Diversity. *Proc. Natl. Acad. Sci.* **71**, 4027–4031 (1974).

69. S. Tonegawa, Somatic generation of antibody diversity. *Nature* **302**, 575–581 (1983).

70. B. Briney, A. Inderbitzin, C. Joyce, D. R. Burton, Commonality despite exceptional diversity in the baseline human antibody repertoire. *Nature* **566**, 393–397 (2019).

71. T. P. Arstila, *et al.*, A direct estimate of the human αβ T cell receptor diversity. *Science (80-.).* **286**, 958–961 (1999).

72. D. J. Laydon, C. R. M. Bangham, B. Asquith, Estimating T-cell repertoire diversity: Limitations of classical estimators and a new approach. *Philos. Trans. R. Soc. B Biol. Sci.* **370** (2015).

73. M. K. Jenkins, J. J. Moon, The role of naive T cell precursor frequency and recruitment in dictating immune response magnitude. *J. Immunol.* **188**, 4135–40 (2012).

74. K. Murali-Krishna, *et al.*, Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–87 (1998).

75. N. M. Chapman, M. R. Boothby, H. Chi, Metabolic coordination of T cell quiescence and activation. *Nat. Rev. Immunol.* **20** (2020).

76. H. Yoon, T. S. Kim, T. J. Braciale, The cell cycle time of CD8+ T cells responding in Vivo is controlled by the type of antigenic stimulus. *PLoS One* **5** (2010).

77. M. L. Slater, S. Sharrowt, J. J. Gartt, Cell- cycle of Saccharomyces cerevisiae in populations growing at different rates. **74**, 3850–3854 (1977).

78. D. I. Kotov, J. A. Kotov, M. F. Goldberg, M. K. Jenkins, Many Th Cell Subsets Have Fas Ligand–Dependent Cytotoxic Potential. *J. Immunol.* **200**, 2004–2012 (2018).

79. O. J. Harrison, *et al.*, Commensal-specific T cell plasticity promotes rapid tissue adaptation to injury. **6280**, 1–18 (2018).

80. S. J. Turley, A. L. Fletcher, K. G. Elpek, The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat. Rev. Immunol.* **10**, 813–825 (2010).

81. C. V. Jakubzick, G. J. Randolph, P. M. Henson, Monocyte differentiation and antigen-presenting functions. *Nat. Rev. Immunol.* **17**, 349–362 (2017).

82. F. D. Brown, *et al.*, Fibroblastic reticular cells enhance T cell metabolism and survival via epigenetic remodeling. *Nat. Immunol.* **20**, 1668–1680 (2019).

83. A. O. Kamphorst, *et al.*, Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science* **0683**, 1–10 (2017).

84. E. Hui, *et al.*, T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* **355**, 1428–1433 (2017).

85. A. M.L., F. K.A., T. C.B., T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol.* **1**, 220–228 (2001).

86. M. K. Jenkins, P. S. Taylor, S. D. Norton, K. B. Urdahl, CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* **147**, 2461–6 (1991).

87. D. L. Barber, *et al.*, Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682–7 (2006).

88. D. R. Leach, M. F. Krummel, J. P. Allison, Enhancement of antitumor immunity by CTLA-4 blockade. *Science (80-.).* **271**, 1734–1736 (1996).

89. J. Larkin, *et al.*, Five-year survival with combined nivolumab and ipilimumab in advanced melanoma. *N. Engl. J. Med.* **381**, 1535–1546 (2019).

90. S. L. Topalian, *et al.*, Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *N. Engl. J. Med.* **366**, 2443–2454 (2012).

91. J. M. Curtsinger, D. C. Lins, M. F. Mescher, Signal 3 determines tolerance versus full activation of naive CD8 T cells: Dissociating proliferation and development of effector function. *J. Exp. Med.* **197**, 1141–1151 (2003).

92. B. Johansson-Lindbom, *et al.*, Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): Requirement for GALT dendritic cells and adjuvant. *J. Exp. Med.* **198**, 963–969 (2003).

93. J. R. Mora, *et al.*, Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88–93 (2003).

94. J. J. Campbell, *et al.*, The chemokine receptor CCR4 in vascular recognition by

cutaneous but not intestinal memory T cells. *Nature* **400**, 776–780 (1999).

95. J. R. Mora, *et al.*, Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* **201**, 303–316 (2005).

96. E. A. Butz, M. J. Bevan, Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* **8**, 167–75 (1998).

97. V. P. Badovinac, B. B. Porter, J. T. Harty, Programmed contraction of CD8+ T cells after infection. *Nat. Immunol.* **3**, 619–626 (2002).

98. V. P. Badovinac, B. B. Porter, J. T. Harty, CD8+ T cell contraction is controlled by early inflammation. *Nat. Immunol.* **5**, 809–817 (2004).

99. M. Prlic, M. J. Bevan, Exploring regulatory mechanisms of CD8+ T cell contraction. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 16689–16694 (2008).

100. M. R. Alderson, *et al.*, Fas ligand mediates activation-induced cell death in human T lymphocytes. *J. Exp. Med.* **181**, 71–77 (1995).

101. L. Zheng, *et al.*, Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* **377**, 348–351 (1995).

102. D. A. Hildeman, T. Mitchell, J. Kappler, P. Marrack, T cell apoptosis and reactive oxygen species. *J. Clin. Invest.* **111**, 575–581 (2003).

103. C. J. Whitby, Influenza and Local Immunity. *Hosp. (Lond. 1886).* **41**, 207–208 (1906).

104. R. A. Clark, *et al.*, The Vast Majority of CLA + T Cells Are Resident in Normal Skin. *J. Immunol.* **176**, 4431–4439 (2006).

105. T. Gebhardt, *et al.*, Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10**, 524–30 (2009).

106. E. M. Steinert, *et al.*, Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* **161**, 737–49 (2015).

107. T. Sathaliyawala, *et al.*, Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* **38**, 187–97 (2013).

108. L. M. Wakim, A. Woodward-Davis, M. J. Bevan, Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 17872–9 (2010).

109. D. Fernandez-Ruiz, *et al.*, Liver-Resident Memory CD8+ T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity* **45**, 889–902 (2016).

110. N. Li, *et al.*, Memory CD4+ T cells are generated in the human fetal intestine. *Nat.*

Immunol. **20**, 301–312 (2019).

111. J. M. Schenkel, K. A. Fraser, D. Masopust, Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. *J. Immunol.* **192**, 2961–4 (2014).

112. D. Masopust, *et al.*, Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* **207**, 553–64 (2010).

113. X. Jiang, *et al.*, Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483**, 227–31 (2012).

114. J. M. Schenkel, K. A. Fraser, V. Vezys, D. Masopust, Sensing and alarm function of resident memory $CD8^+$ T cells. *Nat. Immunol.* **14**, 509–13 (2013).

115. L. K. Mackay, *et al.*, The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat. Immunol.* **14**, 1294–301 (2013).

116. K. A. Casey, *et al.*, Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J. Immunol.* **188**, 4866–75 (2012).

117. N. Zhang, M. J. Bevan, Transforming Growth Factor-β Signaling Controls the Formation and Maintenance of Gut-Resident Memory T Cells by Regulating Migration and Retention. *Immunity* **39**, 687–696 (2013).

118. B. S. Sheridan, *et al.*, Oral infection drives a distinct population of intestinal resident memory CD8(+) T cells with enhanced protective function. *Immunity* **40**, 747–57 (2014).

119. R. T. Sowell, *et al.*, IL-15 Complexes Induce Migration of Resting Memory CD8 T Cells into Mucosal Tissues. *J. Immunol.* **199**, 2536–2546 (2017).

120. L. E. Holz, *et al.*, CD8+ T Cell Activation Leads to Constitutive Formation of Liver Tissue-Resident Memory T Cells that Seed a Large and Flexible Niche in the Liver. *Cell Rep.* **25**, 68-79.e4 (2018).

121. C. Ma, S. Mishra, E. L. Demel, Y. Liu, N. Zhang, TGF-β Controls the Formation of Kidney-Resident T Cells via Promoting Effector T Cell Extravasation. *J. Immunol.* **198**, 749–756 (2017).

122. J. M. Richmond, *et al.*, Antibody blockade of IL-15 signaling has the potential to durably reverse vitiligo. *Sci. Transl. Med.* **10**, 1–10 (2018).

123. T. Adachi, *et al.*, Hair follicle-derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. *Nat. Med.* **21**, 1272–9 (2015).

124. J. M. Schenkel, *et al.*, IL-15-Independent Maintenance of Tissue-Resident and Boosted Effector Memory CD8 T Cells. *J. Immunol.* **196**, 3920–6 (2016).

125. D. A. Walsh, *et al.*, The Functional Requirement for CD69 in Establishment of

Resident Memory CD8+ T Cells Varies with Tissue Location. *J. Immunol.* **203**, 946–955 (2019).

126. T. Bergsbaken, M. J. Bevan, P. J. Fink, Local Inflammatory Cues Regulate Differentiation and Persistence of CD8+ Tissue-Resident Memory T Cells. *Cell Rep.* **19**, 114–124 (2017).

127. B. Slütter, *et al.*, Dynamics of influenza-induced lung-resident memory T cells underlie waning heterosubtypic immunity. *Sci. Immunol.* **2** (2017).

128. H. Borges da Silva, *et al.*, Sensing of ATP via the Purinergic Receptor P2RX7 Promotes CD8+ Trm Cell Generation by Enhancing Their Sensitivity to the Cytokine TGF-β. *Immunity* **53**, 158-171.e6 (2020).

129. T. N. Khan, J. L. Mooster, A. M. Kilgore, J. F. Osborn, J. C. Nolz, Local antigen in nonlymphoid tissue promotes resident memory CD8+ T cell formation during viral infection. *J. Exp. Med.* **213**, 951–66 (2016).

130. B. Davies, *et al.*, Cutting Edge: Tissue-Resident Memory T Cells Generated by Multiple Immunizations or Localized Deposition Provide Enhanced Immunity. *J. Immunol.* **198**, 2233–2237 (2017).

131. L. K. Mackay, *et al.*, Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7037–42 (2012).

132. S. Iborra, *et al.*, Optimal Generation of Tissue-Resident but Not Circulating Memory T Cells during Viral Infection Requires Crosspriming by DNGR-1+ Dendritic Cells. *Immunity* **45**, 847–860 (2016).

133. E. A. Thompson, *et al.*, Monocytes Acquire the Ability to Prime Tissue-Resident T Cells via IL-10-Mediated TGF-β Release. *Cell Rep.* **28**, 1127-1135.e4 (2019).

134. P. Bourdely, *et al.*, Transcriptional and Functional Analysis of CD1c+ Human Dendritic Cells Identifies a CD163+ Subset Priming CD8+CD103+ T Cells. *Immunity* **0**, 1–18 (2020).

135. V. Mani, *et al.*, Migratory DCs activate TGF-β to precondition naïve CD8+ T cells for tissue-resident memory fate. *Science* **366** (2019).

136. K. L. Cepek, *et al.*, Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. *Nature* **372**, 190–193 (1994).

137. S. Cheuk, *et al.*, CD49a Expression Defines Tissue-Resident CD8+ T Cells Poised for Cytotoxic Function in Human Skin. *Immunity* **46**, 287–300 (2017).

138. M. Richter, *et al.*, Collagen Distribution and Expression of Collagen-Binding α 1 β 1 (VLA-1) and α 2 β 1 (VLA-2) Integrins on CD4 and CD8 T Cells during Influenza Infection . *J. Immunol.* **178**, 4506–4516 (2007).

139. E. C. Reilly, *et al.*, TRM integrins CD103 and CD49a differentially support adherence and motility after resolution of influenza virus infection. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 12306–12314 (2020).

140. J. G. Cyster, M. Matloubian, CD69 acts downstream of interferon- a / b to inhibit S1P 1 and lymphocyte egress from lymphoid organs. **440**, 4–8 (2006).

141. D. Masopust, K. Murali-Krishna, R. Ahmed, Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. *J. Virol.* **81**, 2002–11 (2007).

142. L. K. Mackay, *et al.*, Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459–63 (2016).

143. J. J. Milner, *et al.*, Heterogenous Populations of Tissue-Resident CD8+ T Cells Are Generated in Response to Infection and Malignancy. *Immunity* **52**, 808-824.e7 (2020).

144. N. S. Kurd, *et al.*, Early precursors and molecular determinants of tissue-resident memory CD8+ T lymphocytes revealed by single-cell RNA sequencing. *Sci. Immunol.* **5**, eaaz6894 (2020).

145. C. M. Carlson, *et al.*, Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature* **442**, 299–302 (2006).

146. E. Sebzda, Z. Zou, J. S. Lee, T. Wang, M. L. Kahn, Transcription factor KLF2 regulates the migration of naive T cells by restricting chemokine receptor expression patterns. *Nat. Immunol.* **9**, 292–300 (2008).

147. M. A. Weinreich, *et al.*, KLF2 Transcription-Factor Deficiency in T Cells Results in Unrestrained Cytokine Production and Upregulation of Bystander Chemokine Receptors. *Immunity* **31**, 122–130 (2009).

148. L. G. Ledgerwood, *et al.*, The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat. Immunol.* **9**, 42–53 (2008).

149. C. N. Skon, *et al.*, Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat. Immunol.* **14**, 1285–93 (2013).

150. L. K. Mackay, *et al.*, T-box Transcription Factors Combine with the Cytokines TGF-β and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101–11 (2015).

151. J. J. Milner, *et al.*, Runx3 programs CD8+ T cell residency in non-lymphoid tissues and tumours. *Nature* **552**, 253–257 (2017).

152. S. Ariotti, *et al.*, T cell memory. Skin-resident memory $CD8^+$ T cells trigger a state of tissue-wide pathogen alert. *Science* **346**, 101–5 (2014).

153. J. M. Schenkel, *et al.*, T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* **346**, 98–101 (2014).

154. N. Iijima, A. Iwasaki, T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **346**, 93–8 (2014).

155. P. C. Rosato, *et al.*, Virus-specific memory T cells populate tumors and can be repurposed for tumor immunotherapy. *Nat. Commun.* **10**, 567 (2019).

156. D. L. Farber, N. A. Yudanin, N. P. Restifo, Human memory T cells: generation, compartmentalization and homeostasis. *Nat. Rev. Immunol.* **14**, 24–35 (2014).

157. S. L. Park, *et al.*, Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. *Nat. Immunol.* **19**, 183–191 (2018).

158. B. V. Kumar, *et al.*, Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Rep.* **20**, 2921–2934 (2017).

159. L. K. Beura, *et al.*, Intravital mucosal imaging of CD8+ resident memory T cells shows tissue-autonomous recall responses that amplify secondary memory. *Nat. Immunol.* **19**, 173–182 (2018).

160. L. K. Beura, *et al.*, T Cells in Nonlymphoid Tissues Give Rise to Lymph-Node-Resident Memory T Cells. *Immunity* **48**, 327-338.e5 (2018).

161. D. Masopust, V. Vezys, E. J. Wherry, D. L. Barber, R. Ahmed, Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J. Immunol.* **176**, 2079–83 (2006).

162. R. Fonseca, *et al.*, Developmental plasticity allows outside-in immune responses by resident memory T cells. *Nat. Immunol.* **21**, 412–421 (2020).

163. F. M. Behr, *et al.*, Tissue-resident memory CD8+ T cells shape local and systemic secondary T cell responses. *Nat. Immunol.* https:/doi.org/10.1038/s41590-020-0723-4.

164. M. Hofmann, H. Pircher, E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 16741–6 (2011).

165. F. Sallusto, D. Lenig, R. Förster, M. Lipp, A. Lanzavecchia, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708–12 (1999).

166. F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* **22**, 745–63 (2004).

167. E. J. Wherry, *et al.*, Lineage relationship and protective immunity of memory CD8

T cell subsets. *Nat. Immunol.* **4**, 225–34 (2003).

168. D. Hamann, *et al.*, Phenotypic and functional separation of memory and effector human CD8+ T cells. *J. Exp. Med.* **186**, 1407–1418 (1997).

169. S. C. Jameson, D. Masopust, Diversity in T cell memory: an embarrassment of riches. *Immunity* **31**, 859–71 (2009).

170. S. Sarkar, *et al.*, Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* **205**, 625–40 (2008).

171. J. A. Olson, C. McDonald-Hyman, S. C. Jameson, S. E. Hamilton, Effector-like $CD8^+$ T cells in the memory population mediate potent protective immunity. *Immunity* **38**, 1250–60 (2013).

172. M. A. Huggins, *et al.*, KLRG1+ Memory CD8 T cells Combine Properties of Short-lived Effectors and Long-lived Memory. *J. Immunol.* **204** (2020).

173. C. Gerlach, *et al.*, The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis. *Immunity* **45**, 1270–1284 (2016).

174. N. S. Joshi, *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**, 281–95 (2007).

175. S. M. Kaech, *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**, 1191–8 (2003).

176. J. T. Chang, *et al.*, Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* **315**, 1687–91 (2007).

177. W.-H. W. Lin, *et al.*, CD8 + T Lymphocyte Self-Renewal during Effector Cell Determination. *Cell Rep.* **17**, 1773–1782 (2016).

178. K. C. Verbist, *et al.*, Metabolic maintenance of cell asymmetry following division in activated T lymphocytes. *Nature* **532**, 389–93 (2016).

179. B. N. Weber, *et al.*, A critical role for TCF-1 in T-lineage specification and differentiation. *Nature* **476**, 63–8 (2011).

180. C. Y. Yang, *et al.*, The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat. Immunol.* **12**, 1221–1229 (2011).

181. D. T. Fearon, P. Manders, S. D. Wagner, Arrested differentiation, the self-renewing memory lymphocyte, and vaccination. *Science* **293**, 248–50 (2001).

182. L. Gattinoni, D. E. Speiser, M. Lichterfeld, C. Bonini, T memory stem cells in health and disease. *Nat. Med.* **23**, 18–27 (2017).

183. P. Romero, *et al.*, Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. *J. Immunol.* **178**, 4112–9 (2007).

184. N. Cieri, *et al.*, Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood* **125**, 2865–74 (2015).

185. A. Roberto, *et al.*, Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood* **125**, 2855–64 (2015).

186. J. Jacob, D. Baltimore, Modelling T-cell memory by genetic marking of memory T cells in vivo. *Nature* **399**, 593–7 (1999).

187. L. E. Harrington, K. M. Janowski, J. R. Oliver, A. J. Zajac, C. T. Weaver, Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* **452**, 356–60 (2008).

188. O. Bannard, M. Kraman, D. T. Fearon, Secondary replicative function of CD8+ T cells that had developed an effector phenotype. *Science* **323**, 505–9 (2009).

189. N. P. Restifo, L. Gattinoni, Lineage relationship of effector and memory T cells. *Curr. Opin. Immunol.* **25**, 556–63 (2013).

190. J. Oliaro, *et al.*, Asymmetric cell division of T cells upon antigen presentation uses multiple conserved mechanisms. *J. Immunol.* **185**, 367–75 (2010).

191. B. Youngblood, *et al.*, Effector CD8 T cells dedifferentiate into long-lived memory cells. *Nature* **552**, 404–409 (2017).

192. R. S. Akondy, *et al.*, Origin and differentiation of human memory CD8 T cells after vaccination. *Nature* **552**, 362–367 (2017).

193. Thucydides, *History of the Peloponnesian War, Volume 1. Translated by C.F. Smith.* (1920).

194. P. L. Panum, Observations made during the epidemic of measles on the Faroe Islands in the year 1846 (originally published in the Bibliothek for Laeger, Copenhagen, 3R. 1847;1:270-344).

195. E. Jenner, *A continuation of facts and observations relative to the variolae vaccinae, or cow pox* (1800).

196. S. A. Plotkin, Correlates of protection induced by vaccination. *Clin. Vaccine Immunol.* **17**, 1055–65 (2010).

197. I. J. Amanna, N. E. Carlson, M. K. Slifka, Duration of humoral immunity to common viral and vaccine antigens. *N. Engl. J. Med.* **357**, 1903–15 (2007).

198. S. Crotty, *et al.*, Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J. Immunol.* **171**, 4969–73 (2003).

199. S. C. Chang, *et al.*, Longitudinal analysis of severe acute respiratory syndrome (SARS) coronavirus-specific antibody in SARS patients. *Clin. Diagn. Lab.*

Immunol. **12**, 1455–1457 (2005).

200. W.-C. Cao, W. Liu, P.-H. Zhang, F. Zhang, J. H. Richardus, Disappearance of Antibodies to SARS-Associated Coronavirus after Recovery. *N. Engl. J. Med.* **357**, 1162–1163 (2007).

201. R. A. Manz, A. Thiel, A. Radbruch, Lifetime of plasma cells in the bone marrow [3]. *Nature* **388**, 133–134 (1997).

202. M. K. Slifka, R. Antia, J. K. Whitmire, R. Ahmed, Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363–72 (1998).

203. E. Hammarlund, *et al.*, Duration of antiviral immunity after smallpox vaccination. *Nat. Med.* **9**, 1131–7 (2003).

204. J. D. Miller, *et al.*, Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* **28**, 710–22 (2008).

205. S. a. Fuertes Marraco, *et al.*, Long-lasting stem cell-like memory CD8+ T cells with a naïve-like profile upon yellow fever vaccination. *Sci. Transl. Med.* **7**, 282ra48 (2015).

206. S. Crotty, *et al.*, Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination. *J. Immunol.* **171**, 4969–4973 (2003).

207. N. Le Bert, *et al.*, Different pattern of pre-existing SARS-COV-2 specific T cell immunity in SARS-recovered and uninfected individuals. *bioRxiv*, 2020.05.26.115832 (2020).

208. D. Homann, L. Teyton, M. B. Oldstone, Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* **7**, 913–9 (2001).

209. C. C. DeNucci, Y. Shimizu, β1 integrin is critical for the maintenance of antigen-specific CD4 T cells in the bone marrow but not long-term immunological memory. *J. Immunol.* **186**, 4019–26 (2011).

210. M. Pepper, *et al.*, Different routes of bacterial infection induce long-lived T H 1 memory cells and short-lived T H 17 cells. *Nat. Immunol.* **11**, 83–89 (2010).

211. I. den Braber, *et al.*, Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity* **36**, 288–97 (2012).

212. D. Masopust, V. Vezys, A. L. Marzo, L. Lefrançois, Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413–7 (2001).

213. G. C. Preza, O. O. Yang, J. Elliott, P. A. Anton, M. T. Ochoa, T lymphocyte density and distribution in human colorectal mucosa, and inefficiency of current cell isolation protocols. *PLoS One* **10**, 1–13 (2015).

214. R. Stark, *et al.*, TRM maintenance is regulated by tissue damage via P2RX7. *Sci. Immunol.* **3**, eaau1022 (2018).

215. H. Borges da Silva, H. Wang, L. J. Qian, K. A. Hogquist, S. C. Jameson, ARTC2.2/P2RX7 Signaling during Cell Isolation Distorts Function and Quantification of Tissue-Resident CD8+ T Cell and Invariant NKT Subsets. *J. Immunol.* **202**, 2153–2163 (2019).

216. V. Davé, *et al.*, Cervicovaginal tissue residence imprints a distinct differentiation program upon memory CD8 T cells. *bioRxiv* (2020).

217. S. Liang, K. Mozdzanowska, G. Palladino, W. Gerhard, Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J. Immunol.* **152**, 1653–61 (1994).

218. T. Wu, *et al.*, Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J. Leukoc. Biol.* **95**, 215–24 (2014).

219. R. Ahmed, D. Gray, Immunological memory and protective immunity: understanding their relation. *Science* **272**, 54–60 (1996).

220. D. Gray, A role for antigen in the maintenance of immunological memory. *Nat. Rev. Immunol.* **2**, 60–65 (2002).

221. R. M. Zinkernagel, On differences between immunity and immunological memory. *Curr. Opin. Immunol.* **14**, 523–536 (2002).

222. D. Gray, H. Skarvall, B-cell memory is short-lived in the absence of antigen. *Nature* **336**, 70–73 (1988).

223. D. Moskophidis, F. Lechner, H. Pircher, R. M. Zinkernagel, Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* **362**, 758–761 (1993).

224. D. Gray, P. Matzinger, T cell memory is short-lived in the absence of antigen. *J. Exp. Med.* **174**, 969–974 (1991).

225. L. L. Lau, B. D. Jamieson, T. Somasundaram, R. Ahmed, Cytotoxic T-cell memory without antigen. *Nature* **369**, 648–52 (1994).

226. B. A. Heesters, R. C. Myers, M. C. Carroll, Follicular dendritic cells: Dynamic antigen libraries. *Nat. Rev. Immunol.* **14**, 495–504 (2014).

227. M. F. Bachmann, B. Odermatt, H. Hengartner, R. M. Zinkernagel, Induction of Long-lived Germinal Centers Associated with Persisting Antigen after Viral Infection By Martin F. Bachmann, Bernhard Odermatt, Hans Hengartner, and Rolf M. Zinkernagel. *J. Exp. Med.* **183**, 2259–69 (1996).

228. M. Maruyama, K.-P. Lam, K. Rajewsky, Memory B-cell persistence is independent

of persisting immunizing antigen. *Nature* **407**, 636–642 (2000).

229. K. Murali-Krishna, *et al.*, Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377–81 (1999).

230. S. L. Swain, H. Hu, G. Huston, Class II-independent generation of CD4 memory T cells from effectors. *Science* **286**, 1381–3 (1999).

231. R. M. Zinkernagel, What if protective immunity is antigen—driven and not due to so-called "memory" B and T cells? *Immunol. Rev.* **283**, 238–246 (2018).

232. S. Takeda, H. R. Rodewald, H. Arakawa, H. Bluethmann, T. Shimizu, MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity* **5**, 217–228 (1996).

233. C. Tanchot, F. A. Lemonnier, B. Pérarnau, A. A. Freitas, B. Rocha, Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science (80-.).* **276**, 2057–2062 (1997).

234. E. J. Lauron, *et al.*, Viral MHCI inhibition evades tissue-resident memory T cell formation and responses. *J. Exp. Med.* **216**, 117–132 (2019).

235. S. C. Jameson, Maintaining the norm: T-cell homeostasis. *Nat. Rev. Immunol.* **2**, 547–56 (2002).

236. C. D. Surh, J. Sprent, Homeostasis of naive and memory T cells. *Immunity* **29**, 848–62 (2008).

237. T. C. Becker, *et al.*, Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* **195**, 1541–8 (2002).

238. D. L. Woodland, Jump-starting the immune system: Prime-boosting comes of age. *Trends Immunol.* **25**, 98–104 (2004).

239. D. Masopust, S.-J. Ha, V. Vezys, R. Ahmed, Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J. Immunol.* **177**, 831–9 (2006).

240. A. W. Sylwester, *et al.*, Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* **202**, 673–685 (2005).

241. S. G. Hansen, *et al.*, A live-attenuated RhCMV/SIV vaccine shows long-term efficacy against heterologous SIV challenge. *Sci. Transl. Med.* **11** (2019).

242. S. G. Hansen, *et al.*, Prevention of tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine. *Nat. Med.* **24**, 130–143 (2018).

243. J. Mestas, C. C. W. Hughes, Of mice and not men: differences between mouse and human immunology. *J. Immunol.* **172**, 2731–8 (2004).

244. P. Marrack, *et al.*, Homeostasis of αβ TCR+ T cells. *Nat. Immunol.* **1**, 107–111 (2000).

245. L. K. Selin, K. Vergilis, R. M. Welsh, S. R. Nahill, Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. *J. Exp. Med.* **183**, 2489–99 (1996).

246. L. K. Selin, *et al.*, Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity* **11**, 733–42 (1999).

247. R. M. Welsh, L. K. Selin, E. Szomolanyi-Tsuda, Immunological memory to viral infections. *Annu. Rev. Immunol.* **22**, 711–43 (2004).

248. V. Vezys, *et al.*, Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457**, 196–199 (2009).

249. A. Zaid, *et al.*, Persistence of skin-resident memory T cells within an epidermal niche. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5307–12 (2014).

250. M. D. Buck, R. T. Sowell, S. M. Kaech, E. L. Pearce, Metabolic Instruction of Immunity. *Cell* **169**, 570–586 (2017).

251. G. Cui, *et al.*, IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity. *Cell* **161**, 750–61 (2015).

252. D. O'Sullivan, *et al.*, Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity* **41**, 75–88 (2014).

253. A. T. Phan, *et al.*, Constitutive Glycolytic Metabolism Supports CD8+ T Cell Effector Memory Differentiation during Viral Infection. *Immunity* **45**, 1024–1037 (2016).

254. Y. Pan, *et al.*, Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature* **543**, 252–256 (2017).

255. H. Frizzell, *et al.*, Organ-specific isoform selection of fatty acid-binding proteins in tissue-resident lymphocytes. *Sci. Immunol.* **5** (2020).

256. L. M. Wakim, J. Waithman, N. van Rooijen, W. R. Heath, F. R. Carbone, Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science* **319**, 198–202 (2008).

257. T. Shiohara, N. Moriya, Epidermal T cells: Their functional role and disease relevance for dermatologists. *J. Invest. Dermatol.* **109**, 271–275 (1997).

258. Y. Mizukawa, *et al.*, Direct evidence for interferon-gamma production by effector-memory-type intraepidermal T cells residing at an effector site of immunopathology in fixed drug eruption. *Am. J. Pathol.* **161**, 1337–47 (2002).

259. B. D. Hondowicz, *et al.*, Interleukin-2-Dependent Allergen-Specific Tissue-Resident Memory Cells Drive Asthma. *Immunity* **44**, 155–166 (2016).

260. B. Bošnjak, S. Kazemi, L. M. Altenburger, G. Mokrović, M. M. Epstein, Th2-TRMs Maintain Life-Long Allergic Memory in Experimental Asthma in Mice. *Front. Immunol.* **10**, 840 (2019).

261. D. Hijnen, *et al.*, CD8+ T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-γ, IL-13, IL-17, and IL-22. *J. Invest. Dermatol.* **133**, 973–979 (2013).

262. B. T. Malik, *et al.*, Resident memory T cells in the skin mediate durable immunity to melanoma. *Sci. Immunol.* **2**, eaam6346 (2017).

263. S. Cheuk, *et al.*, Epidermal Th22 and Tc17 cells form a localized disease memory in clinically healed psoriasis. *J. Immunol.* **192**, 3111–20 (2014).

264. K. Steinbach, *et al.*, Brain-resident memory T cells generated early in life predispose to autoimmune disease in mice. *Sci. Transl. Med.* **11**, 1–14 (2019).

265. J. H. Jung, *et al.*, Synovial fluid CD69+CD8+ T cells with tissue-resident phenotype mediate perforin-dependent citrullination in rheumatoid arthritis. *Clin. Transl. Immunol.* **9**, 1–15 (2020).

266. J. Machado-Santos, *et al.*, The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* **141**, 2066–2082 (2018).

267. N. D. Glennie, S. W. Volk, P. Scott, Skin-resident CD4+ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes. *PLoS Pathog.* **13**, e1006349 (2017).

268. C. O. Park, *et al.*, Staged development of long-lived T-cell receptor αβ TH17 resident memory T-cell population to Candida albicans after skin infection. *J. Allergy Clin. Immunol.* **142**, 647–662 (2018).

269. P. A. Szabo, M. Miron, D. L. Farber, Location, location, location: Tissue resident memory T cells in mice and humans. *Sci. Immunol.* **4**, 1–12 (2019).

270. P. Savas, *et al.*, Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nat. Med.* **24**, 986–993 (2018).

271. S. L. Park, *et al.*, Tissue-resident memory CD8+ T cells promote melanoma-immune equilibrium in skin. *Nature* **565**, 366–371 (2019).

272. R. Purwar, *et al.*, Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* **6**, e16245 (2011).

273. J. J. C. Thome, *et al.*, Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat. Med.*

22, 72–7 (2016).

274. M. E. Snyder, *et al.*, Generation and persistence of human tissue-resident memory T cells in lung transplantation. *Sci. Immunol.* **4**, eaav5581 (2019).

275. R. Bartolomé-Casado, *et al.*, Resident memory CD8 T cells persist for years in human small intestine. *J. Exp. Med.* **216**, jem.20190414 (2019).

276. K. de Leur, *et al.*, Characterization of donor and recipient CD8+ tissue-resident memory T cells in transplant nephrectomies. *Sci. Rep.* **9**, 1–12 (2019).

277. L. J. Pallett, *et al.*, Longevity and replenishment of human liver-resident memory T cells and mononuclear phagocytes. *J. Exp. Med.* **217** (2020).

278. R. Bartolomé-Casado, *et al.*, CD4+ T cells persist for years in the human small intestine and display a TH1 cytokine profile. *Mucosal Immunol.*, 1–9 (2020).

279. L. K. Beura, P. C. Rosato, D. Masopust, Implications of Resident Memory T Cells for Transplantation. *Am. J. Transplant* **17**, 1167–1175 (2017).

280. L. K. Beura, *et al.*, Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* **532**, 512–6 (2016).

281. B. Polic, D. Kunkel, A. Scheffold, K. Rajewsky, How alpha beta T cells deal with induced TCR alpha ablation. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8744–9 (2001).

282. A. G. Levine, A. Arvey, W. Jin, A. Y. Rudensky, Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* **15**, 1070–8 (2014).

283. Y. Ruzankina, *et al.*, Deletion of the Developmentally Essential Gene ATR in Adult Mice Leads to Age-Related Phenotypes and Stem Cell Loss. *Cell Stem Cell* **1**, 113–126 (2007).

284. J. Schindelin, *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–82 (2012).

285. K. G. Anderson, *et al.*, Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat. Protoc.* **9**, 209–22 (2014).

286. M. Guilliams, *et al.*, Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity* **45**, 669–684 (2016).

287. T. Höfer, K. Busch, K. Klapproth, H.-R. Rodewald, Fate Mapping and Quantitation of Hematopoiesis In Vivo. *Annu. Rev. Immunol.* **34**, 449–478 (2016).

288. C. A. Janeway, *et al.*, Modes of cell:cell communication in the immune system. *J. Immunol.* **135**, 739s-742s (1985).

289. H. Qi, W. Kastenmüller, R. N. Germain, Spatiotemporal basis of innate and adaptive immunity in secondary lymphoid tissue. *Annu. Rev. Cell Dev. Biol.* **30**, 141–67 (2014).

290. S. K. Bromley, *et al.*, The immunological synapse. *Annu. Rev. Immunol.* **19**, 375–96 (2001).

291. S. N. Mueller, L. K. Mackay, Tissue-resident memory T cells: local specialists in immune defence. *Nat. Rev. Immunol.* **16**, 79–89 (2016).

292. R. N. Germain, Y. Huang, ILC2s - resident lymphocytes pre-adapted to a specific tissue or migratory effectors that adapt to where they move? *Curr. Opin. Immunol.* **56**, 76–81 (2019).

293. M. M. Klicznik, *et al.*, Human CD4+CD103+ cutaneous resident memory T cells are found in the circulation of healthy individuals. *Sci. Immunol.* **4**, eaav8995 (2019).

294. F. R. Carbone, T. Gebhardt, Should I stay or should I go-Reconciling clashing perspectives on CD4+ tissue-resident memory T cells. *Sci. Immunol.* **4**, 8–10 (2019).

295. B. Stockinger, T. Barthlott, G. Kassiotis, The concept of space and competition in immune regulation. *Immunology* **111**, 241–247 (2004).

296. K. M. Huster, *et al.*, Cutting edge: memory CD8 T cell compartment grows in size with immunological experience but nevertheless can lose function. *J. Immunol.* **183**, 6898–902 (2009).

297. A. Iwasaki, Exploiting Mucosal Immunity for Antiviral Vaccines. *Annu. Rev. Immunol.* **34**, 575–608 (2016).

298. D. Amsen, K. P. J. M. van Gisbergen, P. Hombrink, R. A. W. van Lier, Tissue-resident memory T cells at the center of immunity to solid tumors. *Nat. Immunol.* **19**, 538–546 (2018).

299. R. van Furth, Z. A. Cohn, The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128**, 415–435 (1968).

300. I. L. Weissman, Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100**, 157–68 (2000).

301. S. J. Im, *et al.*, Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* **537**, 417–421 (2016).

302. H. Geiger, G. de Haan, M. C. Florian, The ageing haematopoietic stem cell compartment. *Nat. Rev. Immunol.* **13**, 376–89 (2013).

303. R. A. Nicoll, A Brief History of Long-Term Potentiation. *Neuron* **93**, 281–290 (2017).

304. A. I. Basbaum, D. M. Bautista, G. Scherrer, D. Julius, Cellular and Molecular Mechanisms of Pain. *Cell* **139**, 267–284 (2009).

305. V. E. Abraira, D. D. Ginty, The sensory neurons of touch. *Neuron* **79**, 618–639 (2013).

306. K. Früh, L. Picker, CD8+ T cell programming by cytomegalovirus vectors: applications in prophylactic and therapeutic vaccination. *Curr. Opin. Immunol.* **47**, 52–56 (2017).

307. P. A. Darrah, *et al.*, Prevention of tuberculosis in macaques after intravenous BCG immunization. *Nature* **577**, 95–102 (2020).

308. J. L. Gowans, J. W. Uhr, The carriage of immunological memory by small lymphocytes in the rat. *J. Exp. Med.* **124**, 1017–1030 (1966).

309. D. D. McGregor, J. L. Gowans, The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. Exp. Med.* **117**, 303–320 (1963).

310. R. van Furth, *et al.*, The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull. World Health Organ.* **46**, 845–852 (1972).

311. M. Merad, *et al.*, Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat. Immunol.* **3**, 1135–41 (2002).

312. F. Ginhoux, *et al.*, Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841–5 (2010).

313. M. Guilliams, G. R. Thierry, J. Bonnardel, M. Bajenoff, Establishment and Maintenance of the Macrophage Niche. *Immunity* **52**, 434–451 (2020).

314. A. I. Lim, *et al.*, Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation. *Cell* **168**, 1086-1100.e10 (2017).

315. G. Gasteiger, X. Fan, S. Dikiy, S. Y. Lee, A. Y. Rudensky, Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* **350**, 981–5 (2015).

316. Y. Huang, *et al.*, S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* **359**, 114–119 (2018).

317. A. Teijeira, *et al.*, T Cell Migration from Inflamed Skin to Draining Lymph Nodes Requires Intralymphatic Crawling Supported by ICAM-1/LFA-1 Interactions. *Cell Rep.* **18**, 857–865 (2017).

318. S. Jennrich, M. H. Lee, R. C. Lynn, K. Dewberry, G. F. Debes, Tissue exit: a novel control point in the accumulation of antigen-specific CD8 T cells in the influenza a virus-infected lung. *J. Virol.* **86**, 3436–45 (2012).

319. J. M. Stolley, *et al.*, Retrograde migration supplies resident memory T cells to lung-draining LN after influenza infection. **217** (2020).

320. G. F. Debes, *et al.*, Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat. Immunol.* **6**, 889–94 (2005).

321. C. R. Mackay, W. L. Marston, L. Dudler, Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171**, 801–17 (1990).

322. S. F. Gilbert, *Developmental Biology*, 6th Ed. (Sinauer Associates, 2000).

323. R. He, *et al.*, Follicular CXCR5- expressing CD8(+) T cells curtail chronic viral infection. *Nature* **537**, 412–428 (2016).

324. R. Ahmed, M. J. Bevan, S. L. Reiner, D. T. Fearon, The precursors of memory: models and controversies. *Nat. Rev. Immunol.* **9**, 662–8 (2009).

325. J. A. Bluestone, C. R. Mackay, J. J. O'Shea, B. Stockinger, The functional plasticity of T cell subsets. *Nat. Rev. Immunol.* **9**, 811–6 (2009).

326. C. Lucas, *et al.*, Longitudinal immunological analyses reveal inflammatory misfiring in severe COVID-19 patients. *medRxiv*, 2020.06.23.20138289 (2020).