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DEVELOPMENT AND FUNCTION OF SELF-SPECIFIC MEMORY-PHENOTYPE $CD8^+$ T CELLS

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To my parents.

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ABSTRACT

Classically, memory T cells arise from naive T cells after a productive immune response to a foreign pathogen in the periphery, and are poised to respond rapidly upon repeated pathogen challenge. However, in mice that have never encountered foreign pathogens, there is a substantial population of "memory-phenotype" $CD8^+$ T cells (CD8-MP cells) that exhibit hallmarks of activation and innate-like functional properties. Due to the lack of faithful markers to distinguish CD8-MP cells from bona fide $CD8^+$ memory T cells, the developmental origins and antigen specificities of CD8-MP cells remain incompletely defined. In this study, we used a clonal approach pairing complete TCR repertoire profiling with in-depth analysis of individual CD8-MP clones. Our findings reveal that CD8-MP cells are a distinct subset of self-reactive T cells, whose differentiation parallels the development of $Foxp3^+$ $CD4^+$ Treg cells in several respects. Specifically, CD8-MP differentiation is a TCR-instructed process that is triggered by the recognition of self-ligands in the thymus, occurs optimally at low clonal frequencies, and involves a multi-step process marked by an initial TCR-dependent triggering step followed by a second phase of consolidation. Developmentally, precursors expressing CD8-MP TCRs upregulate the transcription factor Eomes during thymic maturation, prior to upregulation of the full memory phenotype, providing a molecular flag for the identification of thymic CD8-MP precursors. We also used adoptive transfer and comparative TCR profiling to demonstrate that CD8-MP cells readily infiltrate oncogene-driven mouse prostate tumors and express high densities of PD-1, suggesting a previously unanticipated role for CD8-MP cells in the tumor context. Collectively, our findings challenge the common notion that CD8-MP cells differentiate from naive T cells in

the periphery in lymphopenic settings, instead demonstrating that CD8-MP differentiation is a robust TCR-instructed process triggered in the thymus.

INTRODUCTION[a]

Overview of adaptive immunity and T cell antigen recognition

The mammalian immune system has evolved to protect the host from injurious pathogen infection. It is composed of two interconnected branches that work together to respond to infection: the innate and adaptive immune systems. The innate immune system is the more evolutionary ancient form of host defense, which relies on germline encoded sensors that recognize conserved triggers that are associated with perturbations to immune homeostasis or host physiology, including both pathogen-associated and danger-associated molecular patterns[1,2]. Upon sensing these signals through their innate receptors, cellular constituents of the innate response are rapidly recruited to the site of infection to assist in clearing the infection both by engulfing the invading pathogen as well as producing pro-inflammatory chemokines and cytokines to recruit and activate the cellular components of the adaptive immune system[3,4]. The adaptive immune system is composed of B and T lymphocytes that have rearranged their antigen receptors during development. Although adaptive immunity exerts its effector functions more slowly than innate immunity (days versus minutes), it has the advantage of sensing an enormous diversity of antigens that cannot all be encoded in germline, and conferring specificity to host defense. An additional feature of the adaptive immune system is the formation of immunological memory, the ability of T and B cells to quickly and potently respond to a secondary encounter with the same pathogen.

[a] Parts of this section are reproduced from Savage P.A., Klawon D.E.J., and Miller C.H. (2020) Regulatory T Cell Development. *Annual Review of Immunology* 38:421-453

The adaptive immune system has evolved to recognize an enormous repertoire of antigens. T cells recognize short peptides bound to major histocompatibility complex expressed on the surfaces of host cells. The T cell pool is comprised of ~10^{11} T cells in humans and ~10^{8} T cells in mice. Within that enormous population of naïve T cells, 1-100 cells per million in both humans and mice are specific for a particular peptide-MHC (pMHC) ligand[5]. Such massive diversity cannot be encoded in the germline DNA of the host. Instead, V(D)J recombination achieves combinatorial diversity by mixing and matching V, D, and J segments to form distinct TCR antigen receptors. This unique process takes place within developing T cells in the thymus, and is mediated by recombination-activating gene (RAG) proteins[6]. V(D)J recombination has the capacity to produce on the order of 10^{15} possible TCR combinations, however it is limited to 10^{11} T cells in humans and 10^{8} T cells in mice. Since the entire possible TCR repertoire cannot be represented in one individual, a subset of TCRs must be selected. The main filtering criteria for TCR selection in the thymus is the requirement of a TCR expressed on a $CD8^{+}$ or $CD4^{+}$ T cell to recognize peptide antigens presented on MHC-I or MHC-II respectively. The ability of the T cell repertoire to recognize a great diversity of antigens, initiate effector functions, and form memory achieves host protection against harmful foreign agents.

Thymic development and potential fates of self-specific T cells

To undergo development, T cell precursors migrate through the bloodstream from the bone marrow into the thymus. The purpose of the thymus is to "train" developing T cells. The thymus must generate a diverse array of specialized T cell populations to promote host defense against foreign pathogens. At the same time, the thymus must confer tolerance to prevent immune attack

of self-tissues. Anatomically, the thymus is composed of an outer cortex and an inner medulla, which provide specialized environments for the various stages of T cell development.

T cell precursors begin their development by entering the cortico-medullary junction of the thymus. These "double-negative" T cells lacking expression of the CD4 and CD8 co-receptors (DN; $CD4^{-}$ $CD8^{-}$) migrate through the cortex of the thymus as they mature through the DN1 ($CD44^{+}$ $CD25^{-}$), DN2 ($CD44^{+}$ $CD25^{+}$) and DN3 ($CD44^{-}$ $CD25^{+}$) stages of development. During the DN1 stage, the thymocyte precursor expresses CD44, which acts as an adhesion molecule. During the DN2 phase, the T cell precursor upregulates CD25, the IL-2 receptor which facilitates IL-2 signaling. The DN1 and DN2 phases prepare the T cell precursor for V(D)J recombination. At the DN3 stage, rearrangement of the TCR γ, δ, and β chains occurs. At this point, two developmental pathways of T cells diverge: $\gamma\delta$ versus $\alpha\beta$ T cell lineages. For $\alpha\beta$ T cells, after the rearranged TCRβ chain pairs with a pre-TCRα chain, the DN3 thymocyte proceeds to the DN4 stage, where rearrangement of the TCRα chain occurs. At this point, the thymocyte upregulates the CD4 and CD8 co-receptors and becomes a double-positive thymocyte (DP). When the TCR of the DP thymocyte binds productively with peptide-MHC expressed by cortical epithelial cells, the thymocyte downregulates either CD4 or CD8, transits to the medulla of the thymus and becomes a single-positive (SP) thymocyte[7].

During thymic development of an $\alpha\beta$ T cell, there are several checkpoints a thymocyte must pass in order to egress from the thymus and join the repertoire of peripheral T cells. Of all the TCRs that are rearranged at the DP stage of development, only 3-5% make it into the periphery[5,8]. In a process termed positive selection, a rearranged TCR is first tested at the DP stage of development: the TCR must bind with sufficient affinity to pMHC, this ensures that the T cell repertoire has the capacity to recognize peptides displayed by host MHC molecules in the

periphery. If the TCR fails to productively bind pMHC, then the T cell dies from lack of survival signaling (known as “death by neglect”). V(D)J recombination has evolved for T cells to recognize a great diversity of antigens; however, many TCRs confer strong reactivity to self-pMHC complexes. These self-specific T cells have the capacity to cause autoimmunity if left unchecked, however healthy humans and mice do not direct their immune responses to self-tissues. A classic paradigm suggests that if a rearranged TCR binds pMHC with a high affinity beyond a given signaling threshold, the T cell expressing the self-reactive TCR will die by apoptosis. This process, termed clonal deletion or negative selection, is thought to have evolved to remove overtly self-reactive cells that could initiate harmful autoimmune responses.

Although a plethora of evidence exists to support the general model of clonal deletion, many molecular details are poorly defined, likely due to the fact that most studies examining clonal deletion have utilized non-physiological experimental systems which can lead to conflicting findings over mechanistic details. For example, clonal deletion has been shown to occur at the DN, DP, and SP phases in the thymic cortex and medulla, and it is unknown whether deletion does indeed occur at all stages of T cell development, or whether these disparate results are a reflection of the system used in a particular experiment. In early studies demonstrating clonal deletion, the authors found that T cells bearing the Vβ17a receptor were absent in mouse strains that expressed the MHC class II molecule I-E, but present in mice that lacked I-E. In this study, the authors concluded that $V\beta17a^+$ thymocytes were purged from the T cell repertoire at the DP to SP transition in the thymic medulla[9]. However, this deletion effect was shown to be mediated by bacterial-derived "super-antigen" proteins, rather than self-pMHC complexes, raising questions about the relevance of these findings to tolerance to self-pMHC ligands. In contrast, subsequent studies examining negative selection for $CD4^+$ and $CD8^+$ T cells demonstrated that deletion

occurred at the DN to DP transition. Many of these later studies utilized systems in which both the TCR of interest and its cognate self-antigen were expressed in mice as transgenes. For example, one study compared male and female HY-transgenic mice, which express an MHC-I-restricted TCR that recognizes a peptide derived from the Y chromosome. In male mice, which express the HY antigen, the HY-transgenic T cells were deleted at the DN to DP transition in the cortex[10]. In general, when utilizing TCR transgenics, the observed site of deletion heavily depends on the model used. TCR transgenic models where the T cell expresses TCR inappropriately early (prior to the DP stage) and are reactive to a ubiquitous self-peptide demonstrate deletion events as early as the DN stage, whereas TCR transgenic models where self-peptide expression is restricted to the medulla demonstrate that deletion occurs at the SP phase in the medulla[11]. In addition, many experimental systems have studied clonal deletion of T cell progenitors present at high clonal frequencies. As discussed later in this thesis, introduction of precursors at supraphysiological clonal frequencies may profoundly affect the fate of developing thymocytes. Although these studies utilizing model antigens and TCRtg systems demonstrate that deletion can be induced at different maturation stages, studies of clonal deletion in a polyclonal repertoire suggest that clonal deletion occurs primarily at the DP stage in the cortex[12,13]. Recent work aimed to use a physiological approach to study clonal deletion in wild-type (WT) mice by using cleaved caspase-3 to identify apoptotic cells and CD5 expression to distinguish cells that have signaled through their TCR versus cells that have undergone "death by neglect." In this work, the authors found that the majority of clonal deletion events (78%) occur at the DP phase in the cortex and few clonal deletion events occur at the SP phase when cells are found in the medulla[13]. In addition to overtly self-reactive thymocytes being targeted for clonal deletion, a recent study screened pre-selection TCRs via cloning and retroviral expression of individual TCRs and found that αβ TCRs which

were cross-reactive to multiple MHC haplotypes were the primary targets of negative selection at the DP phase in the thymic cortex[14]. These later studies highlight the need for new approaches to study long-standing notions, since the methodology used in a given study can strongly dictate the result.

Although many overtly self-reactive T cells are eliminated from the repertoire by clonal deletion (studies report 3-30% of thymocytes are purged during negative selection[5]), substantial evidence demonstrates that many autoreactive αβ T cell clones are diverted into alternative lineages including Foxp3$^+$ CD4 regulatory T (Treg) cells, invariant natural killer T (NKT) cells, CD8αα$^+$ intraepithelial lymphocytes (IEL), and MR1-restricted mucosal associated invariant T (MAIT) cells[15]. In addition to these unique lineages of self-specific T cells in the endogenous repertoire, there are self-specific conventional CD4$^+$ T cells that remain in the repertoire and remain dangerous to the host, as they are unleashed and cause fatal autoimmunity when Foxp3$^+$ Tregs are depleted[16]. As detailed below, a common theme amongst self-specific T cell clones that are diverted into these lineages is that clonal deletion may be a common alternate fate for some lineages. These concepts highlight fascinating unanswered questions. First, why are some self-reactive T cells purged by clonal deletion, whereas others are directed into alternative innate-like T cell lineages? Second, what are the immunological forces driving these alternate fates, and what are the consequences when these processes are dysregulated? Third, for self-specific cells directed to alternate lineages, do these cells serve unique non-redundant functional roles in health and disease?

These questions have been studied most extensively for MHC-II-restricted CD4$^+$ T cells. T cells exhibiting overt reactivity to self-pMHC-II ligand have at least three potential fates: establishment in the peripheral repertoire as a naive conventional T cell, elimination via clonal

deletion, or differentiation into Foxp3-expressing Treg cells. Foxp3-expressing $CD4^+$ regulatory T (Treg) cells are a unique lineage of $CD4^+$ T cells that play key roles in the prevention of autoimmunity and the maintenance of immune homeostasis, and represent a major barrier to the induction of robust anti-tumor immune responses. Treg cells perform these roles by suppressing the activation and differentiation of self-reactive conventional T cells that have escaped clonal deletion. Treg cell development largely follows the conventional αβ T cell developmental pathway, utilizing a variety of V and J segments, however the Treg lineage program is triggered due to TCR-dependent recognition of self-peptide presented on MHC-II expressed by thymic epithelial cells and APCs of hematopoietic origin. TCR sequencing studies revealed that the TCR repertoire expressed by peripheral Treg cells is largely distinct from that of conventional $CD4^+$ T cells, with some degree of overlap[17–19], suggesting that Treg cell differentiation is a TCR-directed process. The paradigm that thymus-derived Treg cells exhibit specificity for endogenous self-ligands stemmed from a substantial body of indirect evidence, including experiments showing that TCR-transduced cells expressing Treg-biased TCRs undergo proliferation in lymphopenic hosts, and that many Treg cells are proliferative at steady state[19] and express high densities of the Nur77-GFP reporter, a surrogate readout of TCR signal strength[20]. Early studies examining the antigenic signals driving Treg cell development utilized engineered systems in which model antigens were transgenically expressed in mice, and the developmental fate of antigen-specific TCR transgenic T cells was assessed. These studies showed that the recognition of strong agonist ligands in the thymus promoted Treg cell differentiation[21–29]. Notably, a universal feature of these studies was that expression of these engineered antigens also induced clonal deletion of a major fraction of TCR transgenic cells, with a minor fraction of surviving cells exhibiting a $CD25^+$ $Foxp3^+$ phenotype. This led to the notion that recognition of MHC-II-restricted agonist ligands in the

thymus induces both clonal deletion and Treg cell development. A major question lies in understanding the factors that determine whether a self-reactive MHC-II-restricted thymocyte will undergo Treg cell differentiation or clonal deletion. Some potential factors, illustrated in Figure 1, are the TCR-pMHC binding properties, ligand density, APC type, duration of TCR signaling, maturational stage, and possible age-dependent effects[30,31].

Although studies utilizing TCR transgenic systems have been useful in elucidating the nature of antigenic signals required for Treg development, a complete understanding of Treg cell specificity requires identification and characterization of endogenous Treg cell ligands and naturally occurring Treg cell clones reactive to such ligands. In contrast to studies of Treg cells reactive to model antigens, studies of Treg cells reactive to natural self-ligands have yet to reveal evidence of concurrent clonal deletion[32]. Recent work by our group identified two endogenous self-peptides recognized by recurrent Treg cell clones by focusing on Aire-dependent Treg cell specificities reactive to prostate-associated antigens[33]. Notably, the two peptides were derived from a single prostatic protein, Tcaf3, which was previously identified as a major auto-antigen targeted by antibodies under settings of immune dysregulation[34]. In recently submitted work, our group found that clonal deletion does not impact the developmental trajectory of T cell clones reactive to Tcaf3-derived peptides, in contrast to many of the studies utilizing model antigens[35]. More work is required to understand whether expression of model antigens accurately mimics expression of natural endogenous ligands driving Treg cell selection, and whether clonal deletion is a common alternate fate for other Treg cell-biased specificities.

Invariant natural killer T (NKT) cells represent another distinct lineage of self-reactive αβ T cells that arise in the thymus from developing αβ T cells. In contrast to conventional T cells and Treg cells, NKT cells express NK cell markers (NK1.1, DX5, and NKG2D) and a semi-invariant

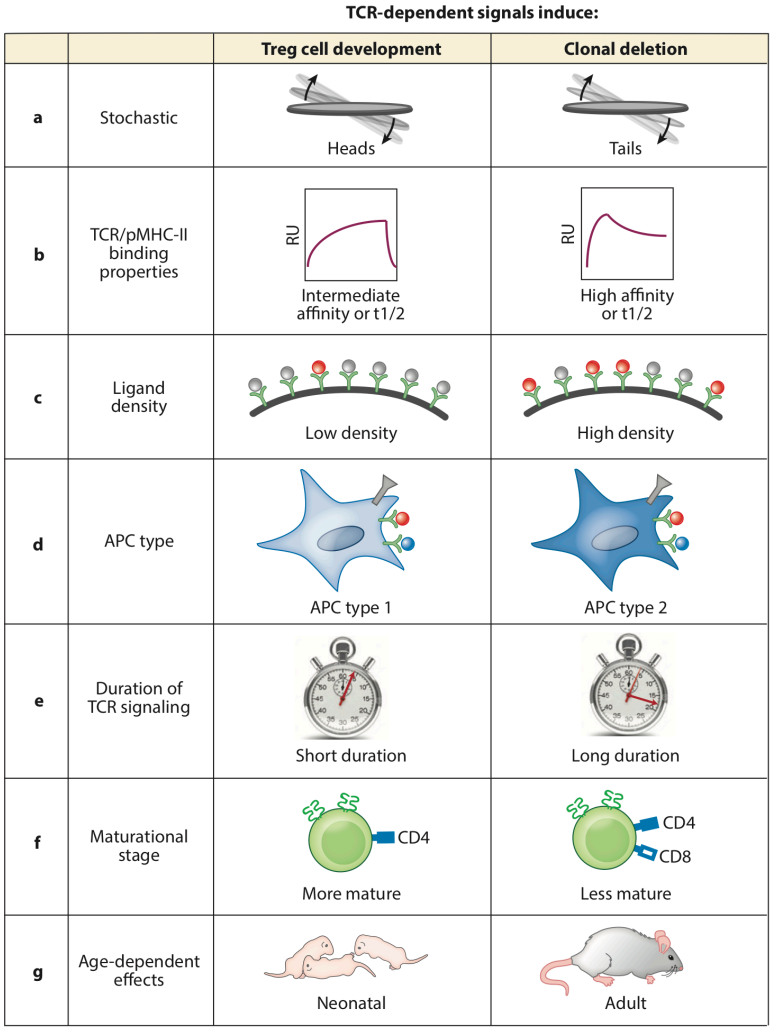

Figure 1. Potential factors driving the alternate fates of CD4$^+$ Treg cell development vs. clonal deletion.

During maturation in the thymus, thymocytes exhibiting overt reactivity to self-pMHC-II ligands can undergo Treg cell differentiation or clonal deletion. The factors that determine these alternate fates remain incompletely defined. The figure illustrates six potential factors which are not mutually exclusive and are potentially interrelated. **a**, Stochastic. Cell fate is determined stochastically, with some cells undergoing Treg cell development and others removed by deletion. **b**, TCR / pMHC-II binding properties. Outcome is determined by the affinity or half-life of TCR binding to pMHC-II. For example, modest interactions could favor Treg cell development whereas stronger interactions could favor deletion. **c**, Ligand density. Fate is impacted by the density of pMHC-II ligands displayed by the collective population of thymic APCs, with low-density ligands promoting Treg cell selection and high-density ligands triggering deletion. **d**, APC type. The identity of the APC displaying pMHC-II and accessory

Figure 1, continued
factors impacts outcome, with a given APC type promoting Treg cell selection and a different APC type driving deletion. **e**, Duration of TCR signaling. The duration with which a thymocyte senses TCR-dependent signals, impacted by the factors described in panels **b-d**, determines outcome. For example, transient TCR signaling triggered by engagement of sparse ligands could promote Treg cell differentiation, whereas sustained TCR signaling induced by widely presented, high-density ligands could favor deletion. **f**, Maturational stage. The outcome of encounter with pMHC-II ligands may be impacted by the maturational state of the thymocyte. For example, less mature thymocytes at the $CD4^+CD8^+$ stage could be prone to deletion, whereas more mature $CD4^+CD8^-$ thymocytes could be more likely to undergo Treg cell differentiation. **g**, Age-dependent effects. The collective impact of factors **a-f** may change throughout life. For example, the early neonatal period may be permissive for Treg cell selection, whereas later periods may favor deletion.

TCR, utilizing mostly Vα14-Jα18/Vβ8, Vβ7, and Vβ2 in mice and Vα24-Jα18/Vβ11 in humans. Instead of recognizing peptide antigens presented on classical MHC molecules, NKT cells recognize lipid antigens presented on a non-classical MHC-I molecule CD1d, expressed in the thymic cortex[36]. In parallel with Treg cells, NKT cells express high densities of Nur77 in the thymus, suggestive of high reactivity towards self-antigens[20]. Although NKT cells are selected on self-lipids in the thymus, their TCRs have the property of promiscuous recognition, and in the periphery their TCRs have the capacity to recognize a microbial antigen which initiates innate-like cytokine and chemokine release for host protection[36]. Interestingly, over-expression of CD1d agonist in the thymic cortex can lead to negative selection of NKT cells[15,37], suggestive that clonal deletion is an alternate fate for some NKT TCR specificities, similarly to Treg cells.

CD8αα$^+$ intraepithelial lymphocytes (IELs) represent a second class of innate-like αβ T cells that differentiate in response to agonist signals in the thymus. IELs are characterized by expression of CD8αα homodimers, comprise a substantial fraction of T cells in the gut[15]. Unlike NKT cells, which express conserved semi-invariant TCRs, IEL T cells express a diverse TCR repertoire. Studies using a TCR transgenic approach found that CD8αα$^+$ IEL differentiation is correlated with TCR dependent interactions with agonist ligands in the thymus[15,38]. In one study, the authors cloned and expressed TCRs isolated from unconventional iIEL and found IEL precursors exhibit elevated TCR signaling in the thymus and undergo either clonal deletion or differentiation into IEL that survive, upregulate α4β7, and home to the gut epithelium. Taking a reciprocal approach, the authors found that when TCRs were isolated from autoreactive thymocytes, which are preferentially clonally deleted, T cells bearing those TCRs selectively differentiated into the IEL subset[39]. Collectively, these results suggest that there is a shared TCR repertoire between autoreactive T cells that are selected into the IEL subset and those which are

negatively selected, implying that there is some alternate factor, other than specificity, that directs a given self-specific TCR into the IEL lineage versus undergoing apoptosis.

Lastly, MR1-restricted mucosal associated invariant T (MAIT) cells are a large population of unconventional αβ T cells that are CD4$^-$ CD8$^-$ but express an invariant TCR (Vα19−Jα33 in mice and Vα7.2−Jα33 in humans). MAIT cells recognize intermediates in the vitamin B synthesis pathway that are presented on MR1, a non-classical MHC-I-like molecule. MAIT cells are positively selected in the thymus, and are dependent on MR1-restricted ligand presentation by DP thymocytes. Thymic output of MAIT cells is relatively small compared to that of conventional T cells, but MAIT cells expand greatly in the periphery due to commensal microbes. MAIT cells have innate-like functions, recognizing bacterial vitamin B antigens and producing effector cytokines to protect the host from bacterial infection[40]. Although MR1-restricted MAIT cells were originally thought to respond exclusively to microbial antigen presentation, recent work has found some populations of MR1-restricted T cells that respond to self-antigens in the absence of microbial ligands[41]. Along the same line, a recent study identified an MR1-restricted T cell clone that could recognize and kill multiple types of human cancer cells, but not healthy cells, and demonstrated that this killing was independent of presentation of microbial antigens, implying reactivity to a self-antigen[42]. It is unknown if these populations of self-reactive MR1-restricted T cells are a subset of MAIT cells or whether they are a unique self-reactive MR1-restricted T cell population.

Thus, the display of self-ligands in the thymus directs antigen-reactive thymocytes into a diverse array of T cell lineages that serve numerous functions in homeostasis and host defense. Within the thymus, self-specific αβ T cells mature through well-defined stages of development, until their TCR binds pMHC with an avidity that exceeds a threshold that triggers cell fate

decisions. At this stage, a self-specific T cell is either clonally deleted or diverted into some alternative lineage. As detailed above, some TCR specificities can direct cells to multiple fates, suggesting that there are other factors besides specificity that direct fate decisions. The factors outlined in Figure 1 for Treg cells may also impact fate decisions for other self-reactive thymocytes; understanding the contributions of these parameters to self-specific T cell differentiation will be essential for understanding how the immune system harnesses these self-reactive cells for host protection while ensuring robust tolerance to self-constituents. Additionally, new experimental approaches are needed to better model differentiation of self-specific T cells, since current evidence demonstrates that the experimental system used can heavily influence the observed result. Specifically, TCR transgenic model antigen systems may not appropriately model self-reactive T cell populations, because they are generated at inappropriately high clonal frequencies and expressed with inappropriate timing. It is possible that the observation of a single specificity being diverted down multiple fate decisions is an artefact of the expression systems used to model T cell development and differentiation and not a true, physiological outcome of self-specific T cells. Moving forward, the study of naturally occurring self-specific T cell specificities at low clonal frequency with the correct developmental timing has the potential to yield key insight into the principles of agonist-selected T cell differentiation.

Conventional memory T cell differentiation

When a mature T cell leave the thymus, it enters the lymphatic system as a naïve cell expressing CD62L and CCR7, trafficking molecules that retain naïve T cells within the lymph and circulation. Naive T cells constantly circulate through the host anticipating cognate antigen encounter and activation. Peripheral tissues, primarily the skin and the mucosa, are the major sites

of pathogen entry. Upon pathogen entry into these peripheral tissues, cellular components of the innate system are activated and local dendritic cells (DCs) become activated and uptake invading pathogens and their associated antigens. These DCs then migrate to the secondary lymphoid organs, where they present pathogen-associated antigens to naïve antigen-specific T cells. In a complementary pathway, antigens drain through the lymphatics and are acquired, processed, and presented by lymph node-resident DCs and macrophages. Naïve T cells are primed upon sensing their cognate antigen with the proper co-stimulatory signals from DCs. Activated antigen specific T cells proliferate and differentiate into effector T cells, characterized by acquisition of effector functions such as production of cytokines (IFNγ and TNFα) and cytolytic capacity. Importantly, these activated T cells alter their expression of homing markers, downregulating CD62L and CCR7 in order to leave the secondary lymphoid tissues and enter the site of infection in the peripheral tissue.

During an acute viral or intracellular bacterial infection, antigen-specific cytotoxic $CD8^+$ T cells rapidly proliferate and expand. Studies examining antigen-specific $CD8^+$ T cells reactive to LCMV have estimated there to be ~10^2 naïve antigen-specific T cells per mouse at homeostasis, but during an acute infection this population can expand to greater than 10^7 effector antigen-specific $CD8^+$ T cells[43]. During this expansion, these T cells differentiate and undergo extensive transcriptional changes to provide T cells with the functional capacity to eliminate the pathogen with key effector functions. After the pathogen is cleared, a small fraction (5 – 10%) of the antigen-specific $CD8^+$ T cells remain and differentiate into various subsets of memory $CD8^+$ T cells, which are poised to respond rapidly upon repeated pathogen infection and protect the host from the harmful effects of infection[44].

There are thought to be three major subsets of antigen-specific $CD8^+$ memory T cells that arise after an acute pathogen infection: central memory T cells, effector memory T cells, and resident memory T cells[45]. Although the precise phenotypes and relationships between these subsets remain an active area of research, each subset is defined by an array of canonical properties with respect to markers, localization, and functions. Central memory T cells are characterized by high expression of CD62L, CCR7, and Eomes, elevated proliferative capacity, and circulation through the secondary lymphoid organs. Effector memory T cells are characterized by their low expression of CD62L and CCR7, but high expression of Tbet. They display constitutive cytotoxicity and are predominantly found in non-lymphoid tissues, but can also circulate. Resident memory T cells have high expression of CD69, CD103, and Hobit and low expression of CD62L, Eomes, and Tbet. Resident memory T cells are found in non-lymphoid tissues but, unlike effector memory T cells, parabiosis experiments revealed that resident memory T cells are largely retained at non-lymphoid sites. These three antigen-specific memory subsets are thought to coordinate in order to rapidly generate a burst of effector T cells upon secondary infection. Effector and resident memory T cells are poised at the site of pathogen entry to provide an immediate effector response, whereas central memory T cells can rapidly proliferate in the secondary lymphoid organs to control pathogens that infringe beyond the site of pathogen entry. Collectively, these memory T cells are better equipped than naïve T cells to clear a pathogen upon secondary infection and provide host protection.

During an acute infection, antigen-specific T cells that will give rise to long-lived memory T cells can be identified by their high expression of IL-7Rα (CD127) and Bcl2[46] and lower expression of effector molecules, such as Klrg1. After clearance of an acute infection, fully differentiated antigen-specific memory T cells are maintained in the periphery in an antigen-

independent manner via homeostatic turnover dependent on survival cytokines such as IL-7 and IL-15[47]. Memory T cells express CD44, an activation marker that distinguishes memory and effector T cells from their naïve counterparts. Memory T cells also express enhanced levels of cytokine receptors, such as CD122 (also known as IL-15Rβ and IL-2Rβ), which facilitate memory T cell homeostatic turnover. These memory T cells are maintained at an elevated precursor frequency and have a unique transcriptional program that allow them to respond more rapidly and robustly than naïve T cells upon repeat exposure to antigen, making memory T cells a critical feature of the adaptive immune response.

Endogenous memory phenotype $CD8^+$ T cells

Classically, as outlined above, memory T cells arise after a productive immune response to a foreign pathogen in the periphery, and are poised to respond rapidly upon repeated pathogen challenge. However, in mice that have never encountered foreign pathogens, including specific pathogen free (SPF), germ-free (GF), and antigen-free (AF) mice fed an elemental diet[48,49], there is a major subset of $CD8^+$ T cells that express phenotypic markers of immunological memory (e.g. $CD44^{hi}$ $CD122^+$). Endogenous $CD8^+$ memory-phenotype (hereafter referred to as CD8-MP) populations found in the periphery of WT mice are often referred to as virtual memory cells in the literature, however for clarity I will refer to them as endogenous CD8-MP cells. As detailed below, these CD8-MP cells acquire their phenotype in the absence of known foreign stimuli and have been shown to exhibit memory-like functions, including robust IFNγ production and ex vivo cytotoxic killing capacity. The current literature is muddied by the heterogenous nomenclature of four separate fields: (1) those that study endogenous CD8-MP cells (virtual memory cells), (2) those that study lymphopenia-induced memory cells, (3) those that study IL-4 dependent innate

memory cells, and (4) those that study Qa-1-restricted $CD8^+$ regulatory T (Treg) cells[48–51] (Table 1). Although it has been proposed that endogenous CD8-MP cells are some combination of naturally occurring lymphopenia-induced memory, IL-4 dependent innate memory cells, and Qa-1-restricted $CD8^+$ Tregs[50,51], it is unknown whether these populations represent distinct subsets of endogenous CD8-MP cells, or whether they overlap and are somehow inter-related. A key gap in knowledge is understanding how these various subsets contribute to the peripheral CD8-MP T cell pool in mice and humans.

A common paradigm suggests that endogenous CD8-MP cells arise in the periphery of mice from naïve cell homeostatic proliferation due to lymphopenia. In line with this, a study examining the kinetics of CD44 and CD122 expressing T cells in B6 mice found that $CD44^{hi}$ $CD122^+$ cells arise in the periphery of B6 mice at 2 weeks of age during the neonatal period, when mice are known to have a lymphopenic environment[52]. Furthermore, cells of this phenotype were not found in the thymus until 3 weeks, suggesting that endogenous CD8-MP cells originate in the periphery and then recirculate back to the thymus[52]. Consistent with the lymphopenia-induced proliferation hypothesis, studies of mice with genetic deficiencies of key transcription factors involved in T cell development have suggested that cells of this phenotype are partially dependent on cytokines IL-4[50] and IL-15[53], both known to be important in homeostatic-driven proliferation. Additionally, endogenous CD8-MP cells have slightly elevated levels of CD5 expression[54] and there is association with CD5 expression levels and T cells that undergo lymphopenia-induced proliferation. CD5 is a surface protein that acts as a negative regulator of TCR signaling and is upregulated by developing thymocytes undergoing selection. Its expression correlates with the intensity of TCR signaling by self-peptide/ MHC during thymic development. However, CD5

	Cell population analyzed	Cytokine dependence	Proposed site of differentiation	Role of TCR signaling	References
Virtual memory (Endogenous CD8-MP)	Polyclonal $CD44^{hi}$ $CD122^{+}$ $CD8^{+}$ T cells in periphery of WT mice	IL-15, IL-4	Periphery	Differentiation potential correlates with CD5 expression density	48, 52 - 54, 65, 68, 71
Lymphopenia - induced memory	Naïve polyclonal and TCRtg monoclonal $CD8^{+}$ T cell adoptive transfer into lymphopenic host	Common γ chain cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21)	Periphery	Differentiation potential correlates with CD5 expression density	49, 58 - 64
IL-4 dependent innate memory	Polyclonal T cells in gene-targeted mice with altered NKT cell compartment	IL-4, IL-15	Thymus	Unknown	48, 50, 66, 67
Qa-1-restricted $CD8^{+}$ Tregs	Polyclonal $CD44^{hi}$ $CD122^{+}$ $Ly49^{+}$ $CD8^{+}$ T cells in wild-type and Qa-1 mutant mice	IL-15	Periphery	Unknown	51, 69, 70

Table 1. Heterogeneous nomenclature of endogenous memory phenotype $CD8^{+}$ T cells

Current literature has described four populations of endogenous $CD44^{hi}$ $CD122^{+}$ $CD8^{+}$ T cells found in mice: virtual memory cells, lymphopenia-induced memory, IL-4 dependent innate memory, and Qa-1-restricted $CD8^{+}$ Tregs[48–51]. These CD8-MP populations acquire a $CD44^{hi}$ $CD122^{+}$ phenotype and have been shown to acquire memory-like functions in the absence of known foreign stimuli. A key gap in knowledge is understanding how these different subsets contribute to the peripheral T cell pool and determining whether these populations represent four distinct subsets of endogenous CD8-MP cells, or whether they overlap.

densities are determined by reactivity to the low-potency self-ligands supporting positive selection, and are thought to be "hard-wired" at the earlier stages of thymic development. Thus, CD5 density cannot be used to assess the intensity of additional TCR signaling events occurring after positive selection[55–57]. Thus, although it is tempting to suggest that $CD8^+$ T cells with high self-reactivity may be more prone to undergo lymphopenia-induced proliferation and become endogenous CD8-MP cells, this hypothesis has not been tested directly. Moving forward, understanding the specificity of CD8-MP cells will require studies examining the reactivity of T cell clones expressing CD8-MP TCRs, as well as biochemical analyses of CD8-MP TCR binding to natural agonist ligands.

As alluded to above, there are many parallels between endogenous CD8-MP cells and cells that undergo lymphopenia-induced memory cell differentiation. Early studies demonstrated that a fraction of naïve $CD8^+$ T cells could acquire a memory phenotype upon transfer into a lymphopenic host. Multiple studies utilized monoclonal TCR transgenic (TCRtg) $CD8^+$ T cells with known cognate foreign antigen specificity; when naïve ($CD44^{lo}$) TCRtg $CD8^+$ T cells were adoptively transferred into irradiated host mice, they robustly proliferated and acquired classical markers of memory T cells, including CD44 and CD122 among others. Strikingly, these lymphopenia-induced memory (LIM) cells also acquired effector functions, such as robust IFNγ production and ex vivo cytotoxic killing capacity. Notably, the acquisition of these memory-like characteristics and functions following transfer to lymphopenic hosts was comparable to TCRtg cells stimulated with their cognate antigen plus co-stimulatory signals[58–63], and these LIM cells exhibited a similar gene expression program to that of "true memory" cells that differentiated during a foreign antigen response[64]. Various stochastic cues have been shown to play important roles during lymphopenia-induced proliferation[50]. Cytokines, specifically the common γ chain

cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), are critical for $CD8^+$ T cells to undergo lymphopenia-induced proliferation. In addition, evidence suggests that only certain TCRs with higher self-reactivity will undergo lymphopenia-induced proliferation, demonstrating a role for TCR specificity in LIM cell generation[50]. For example, OT-I, P14, and 2C $CD8^+$ TCRtg cells will undergo lymphopenia-induced proliferation, whereas H-Y and pmel-1 $CD8^+$ TCRtg cells will not. In addition, $CD8^+$ T cells expressing higher densities of CD5 have a greater propensity to undergo LIM cell differentiation, similarly to endogenous CD8-MP cells. Although the physiological relevance of classical LIM cell experiments, whereby TCRtg cells are adoptively transferred into the periphery of irradiated hosts, is not immediately clear, neonatal mice are naturally lymphopenic. Thus, one hypothesis on the origins of naturally occurring CD8-MP cells in unprimed mice is that naïve T cells acquire their memory phenotype in response to homeostatic proliferation in the peripheral neonatal environment.

To probe the ontogeny of $CD8^+$ T cells subsets more directly, recent work has taken a "timestamp" approach to mark the thymic $CD8^+$ T cells that arise at various ages in mice[65]. This study demonstrated that endogenous CD8-MP cells are enriched in $CD8^+$ T cells that develop during the neonatal period. Although this work may first appear to support the neonatal homeostatic proliferation hypothesis, thymic grafting experiments demonstrated that T cell acquisition of the memory markers is T cell intrinsic, which suggests that development of the memory phenotype is not necessarily due to the lymphopenic environment, but instead due to some intrinsic property of T cells that arise from fetal liver HSC progenitors during this period. Importantly, this finding is inconsistent with the hypothesis that endogenous CD8-MP cells arise due to naïve cell homeostatic proliferation. If endogenous CD8-MP cells indeed arise from naïve cell homeostatic proliferation, the acquisition of the memory phenotype by neonatal T cells would

be T cell extrinsic, not intrinsic, in these timestamping experiments. Based on this work, it is important to re-consider the relevance of classical LIM experiments performed in adult mice to the understanding of endogenous CD8-MP cells.

IL-4 induced innate memory cells are thought to be another subset of endogenous CD8-MP cells found in mice[50]. They are often distinguished from endogenous CD8-MP cells because IL-4 induced innate memory cells are thought to arise in the thymus, not the periphery. The presence of such cells was first revealed in gene-deficient mouse models in which enhanced IL-4 secretion by thymic NKT cells led to bystander $CD8^+$ T cell acquisition of memory phenotype markers[50]. Interestingly, similar to LIM cells, these IL-4 dependent innate memory T cells can rapidly produce IFNγ upon TCR restimulation, suggestive of memory-like function[66]. The study of IL-4 dependent innate memory cells was extended beyond gene-deficient animals by comparing various inbred stains of wild-type (WT) mice that naturally varied in the number of thymic IL-4 producing NKT cells[67]. Balb/c mice have a much larger population of IL-4 producing NKT cells in their thymi than C57BL/6J (B6) mice. It was demonstrated that CD8 single-positive (SP) thymocytes in Balb/c, but not B6, mice contained a population of CD8-MP cells that expressed Eomes, CD44, and CD122. Importantly, the authors demonstrated that this population was largely dependent on IL-4, because that thymic CD8-MP population was greatly diminished in *Il4*$^{-/-}$ mice on a Balb/c background, and were comparable to wild-type B6 mice[67]. Although the dependence on IL-4 and NKT cells has been demonstrated for these thymic innate memory cells, it remains unknown whether generation of these IL-4 dependent innate memory T cells is a TCR-instructed process, or is driven by stochastic forces downstream of IL-4 exposure. Although IL4-induced innate memory cells are rare in the thymi of B6 mice, genetic deficiencies of factors involved in innate memory cell generation (IL-4R, IL-4, PLZF, or NKT cells) lead to a reduction

in the frequency of CD44^{hi} CD122^{+} CD8-MP cells found in the periphery of B6 mice, suggesting some contribution of IL-4 and NKT cells to peripheral CD8-MP cell generation[50]. It is possible that in B6 mice, IL4-dependent innate memory cells arise at some other IL-4 rich site outside of the thymus.

Studies have aimed to characterize the specificity and function of CD8-MP cells using pMHC-I tetramer-based approaches to characterize foreign pathogen reactive CD8^{+} T cells. In the pool of foreign pMHC reactive T cells, there exists a minor fraction of tetramer-positive T cells that exhibit a CD44^{hi} CD122^{+} phenotype, even in mice that have never been exposed to those antigens[52,68]. These studies have led some investigators to classify CD8-MP cells as "antigen inexperienced" memory cells that differentiate in response to TCR-independent signals[48]. It is important to note that examining such a limited repertoire of CD8-MP T cells can lead to observation bias. It is tempting to presume that all tetramer-binding cells are reactive to a single foreign epitope, and thus the CD44^{hi} CD122^{+} cells that bind are "antigen inexperienced." However, it is possible that the small number of CD44^{hi} CD122^{+} cells that bind to a given foreign pMHC tetramer are cross-reactive to self-ligands that support their differentiation and homeostasis.

Qa-1-restricted CD8^{+} regulatory T (Treg) cells are another population of CD44^{hi} CD122^{+} memory phenotype T cell population found in mice[51]. These CD8^{+} Treg cells are characterized phenotypically by their expression of CD44, CD122, and Ly49. CD44^{hi} CD122^{+} Ly49^{+} cells comprise 3-5% of all CD8^{+} T cells, and, as has been described for other populations of peripheral CD8-MP cells, their survival and/ or maintenance is dependent on IL-15[69]. This small population of CD8-MP cells is thought to suppress autoimmune reactions by killing CD4^{+} T follicular helper cells that present self-peptide on Qa-1, a non-classical MHC-I molecule[70]. The nature of the TCRs within this subset and the self-antigens they recognize is currently unknown.

It is currently unclear what the true relationship is between endogenous CD8-MP, lymphopenia-induced memory, IL-4 dependent innate memory, and Qa-1-restricted $CD8^+$ T cells and it is unknown how these populations contribute to the endogenous population of CD8-MP cells in WT mice. It is likely that the endogenous population of $CD8^+$ $CD44^{hi}$ $CD122^+$ T cells is a heterogenous group of cells that have diverse origins and divergent immune characteristics. Alternatively, CD8-MP cells may represent a single subset and the various groups studying these cells may be creating false distinctions between them. Work defining the antigen specificities of CD8-MP cells has been limited to the study of rare CD8-MP cells that stain with peptide/MHC tetramers bearing foreign peptides, yet little is known about the antigen specificities are of the thousands of CD8-MP cells in mice. Furthermore, work on the developmental origins of CD8-MP cells has led to conflicting results, and has led the field to describe four different subsets of cells with the same phenotype. In general, studies to date have been limited by a reliance on TCRtg and pMHC tetramer models reactive to foreign antigens and the study of bulk polyclonal CD8-MP populations. The potential heterogeneity within the bulk CD8-MP cell subset highlights a critical need to identify distinct CD8-MP clones and to study the biology of these T cells at the clonal level. A deeper understanding of the origins and antigen specificities of CD8-MP cells will require two types of studies: 1) TCR sequencing to characterize the nature of TCRs expressed by naturally occurring CD8-MP cells, and to identify naturally occurring CD8-MP specificities; 2) Clonal analysis in which the reactivity and developmental trajectories of specific clones can be tracked in the thymus and periphery.

Potential functions of CD8-MP cells

A key question lies in understanding the function of naturally occurring CD8-MP cells in host defense and immune homeostasis. As described above, the CD8-MP subset has been described as a heterogenous group of cells, composed of T cells with diverse origins. This heterogeneity extends to published studies of CD8-MP cell function, which have suggested divergent functions including enhanced early innate-like functions during infection and roles in the maintenance of immune homeostasis as $CD8^+$ "suppressor" T cells. It is unknown if these diverse functions are the true functions of the entire CD8-MP subset, or whether there are distinct functions of heterogenous cell populations that share the $CD44^{hi}$ $CD122^+$ phenotype.

As alluded to in the above section, CD8-MP cells appear to share many functional traits with true memory cells, including rapid proliferation, robust IFNγ production and ex vivo cytotoxic killing capacity upon encounter with cognate antigen. RNA sequencing analysis of CD8-MP cells and naïve cells from WT mice have demonstrated that CD8-MP cells have enhanced cytokine responsiveness, which is thought to contribute to their memory-like functions[54]. Using a tetramer-based approach, one group found that OVA-specific CD8-MP cells rapidly produce IFNγ and can proliferate faster than their naïve counterparts upon infection with *Listeria monocytogenes* that expressed OVA (*Lm*-OVA). An important question was whether these enhanced responses led to protective immunity. *Lm*-OVA infected recipient mice that received OVA-specific CD8-MP cells had significantly reduced bacterial load in their spleens and livers relative to mice that received OVA-specific naïve T cells[71]. Although the above data suggest that rare foreign-antigen-reactive CD8-MP cells can respond to early phases of pathogen infection, it remains unclear whether the remaining >99% of endogenous CD8-MP cells that do no cross-react with pathogen-derived ligands contribute to host defense in these contexts. Moving forward, there is a critical

need for loss-of-function studies to examine the requirement of CD8-MP cells in the immune response to pathogens.

Beyond the infection studies described above, additional work has suggested a role for CD8-MP cells in the maintenance of immune homeostasis. It was hypothesized that $CD8^+$ $CD122^+$ T cells are regulatory T cells that are analogous to their $CD4^+$ $CD25^+$ $Foxp3^+$ T cell counterparts, because CD122 is the β subunit of the IL-2 receptor and CD25 is the α subunit[72]. Early work testing this hypothesis utilized an adoptive transfer approach, in which total $CD8^+$ T cells or CD122-depleted $CD8^+$ T cells were transferred into *Rag2*$^{-/-}$ lymphopenic recipients. Mice that received $CD8^+$ cells depleted of $CD122^+$ T cells died starting at 5 weeks after transfer and these mice had highly activated T cells that infiltrated the bone marrow of recipient mice[73]. Subsequent mechanistic studies revealed that this effect was mediated by Fas/FasL-dependent killing of activated T cells by $CD8^+$ $CD122^+$ T cells, and not through IL-10[74]. Although intriguing, the Suzuki studies have not been corroborated by other labs, including our own, indicating that further evidence is needed to support the notion that $CD8^+$ $CD122^+$ T cells have unique roles in maintaining immune homeostasis.

Another group examined the suppressive potential of CD8-MP cells by examining a small fraction of CD8-MP cells that are restricted to a non-classical MHC-I molecule, Qa-1. This study utilized targeted mice harboring a point mutation in the Qa-1 molecule (B6 Qa-1(D227K)), that interfered with TCR binding to Qa-1, but not with NK cell binding. Controlling for NK cell binding was important, since NK cell receptor NKG2A can also bind Qa-1. The B6 Qa-1(D227K) mouse model allowed the authors to directly probe the function of Qa-1-restricted $CD8^+$ T cells in vivo, without interfering with NK cell function. It was shown that B6 Qa-1(D227K) mice developed spontaneous manifestations of autoimmunity, characterized by autoantibody production and a

lupus-like disease. In WT mice, Qa-1-restricted CD8-MP cells were shown to bind Qa-1 on T_{FH} cells and suppress their autoantibody production. Thus, when the Qa-1 point mutation was introduced in B6 Qa-1(D227K) mice, the Qa-1-restricted CD8-MP cells failed to suppress autoreactive T_{FH}, leading to autoimmunity[70]. The limitation of these studies is that these Qa-1-restricted $CD8^+$ cells have yet to be directly identified and enumerated, and are likely to be dwarfed by CD8-MP cells restricted to classical MHC-I. Thus, it is unknown if the Qa-1-restricted suppressor cells in these studies are related to the suppressor function attributed to $CD8^+$ $CD122^+$ T cells in the earlier adoptive transfer experiments.

For the divergent functions attributed to CD8-MP cells, it is unknown whether these reflect broad functions of all CD8-MP cells, or distinct functions of heterogeneous T cell populations falling within the $CD44^{hi}$ $CD122^+$ subset. In addition, the functional studies that have been reported have been limited by the lack of available markers to distinguish endogenous CD8-MP cells from "true memory" $CD8^+$ T cells, especially in the context of immune activation. In this regard, although the existence of an analogous cell population has been suggested in humans[54,75,76], the lack of validated markers has limited the ability to study CD8-MP cells in human samples. Lastly, efforts to elucidate the function of CD8-MP cells in the context of homeostasis, host defense, inflammation, and cancer have been hampered by the lack of experimental approaches that enable the constitutive or inducible depletion of CD8-MP cells without impacting conventional effector and memory $CD8^+$ T cell populations.

Role of $CD8^+$ T cells in tumor-associated immune responses

Immune checkpoint blockade therapy for cancer has demonstrated positive clinical effects, demonstrating that the immune system can be manipulated for clinical benefit[77]. Despite the

importance of endogenous $CD8^+$ T cells in anti-tumor immunity, the antigen specificities of most tumor-infiltrating $CD8^+$ T cells remain incompletely defined. A substantial body of work from pre-clinical mouse models and recent clinical trials from human patients suggests that $CD8^+$ T cells reactive to mutated tumor-specific "neo-antigens" may play an important role in anti-tumor immunity[78–81]. However neo-antigen-reactive T cells comprise only a minor fraction of the tumor-infiltrating lymphocyte (TIL) population in any given tumor, as shown via tetramer staining of TIL or ex vivo culture assays[82]. In addition, a large body of evidence demonstrates that T cells recognizing non-mutated self-epitopes[83] can be detected ex vivo, but that these cells are typically present at low frequencies. One group used exome sequencing and multiplexed tetramer approaches to demonstrate that $CD8^+$ T cells reactive to mutated neo-antigens or non-mutated tumor-expressed antigens are rare or undetectable in human lung and colorectal cancers. Instead, tumors were largely infiltrated by "bystander" cells that were phenotypically distinct from rare tumor-reactive T cells[84]. More recently, another group demonstrated that only a minor fraction of prominent $CD8^+$ T cell clones from ovarian or colorectal cancers exhibited overt reactivity to autologous tumor cells[85]. These and other findings suggest that in many human cancers, the majority of $CD8^+$ TILs are not reactive to tumor-expressed antigens. Additionally, recent single-cell RNA sequencing and mass cytometry studies demonstrate that $CD8^+$ T cells from any given tumor occupy multiple transcriptional or phenotypic states, indicative of substantial heterogeneity[86–90]. Thus, cumulative evidence suggests that tumors are infiltrated by a phenotypically diverse array of $CD8^+$ T cells, and that only a minor fraction of such cells are overtly reactive to tumor-expressed antigens.

Although CD8-MP cells make up 5-10% of the endogenous $CD8^+$ T cell repertoire, there are no studies that have examined the contribution of the CD8-MP subset to the tumor infiltrating

$CD8^+$ T cell repertoire. Given the significant size of this population, coupled with putative innate-like functional properties, it is possible that CD8-MP cells are readily recruited into the tumor environment. However, if CD8-MP cells did indeed contribute to the tumor environment, it is unclear what role these infiltrating CD8-MP cells would play. It is possible that infiltrating CD8-MP cells could play an anti-tumorigenic role, rapidly producing IFNγ and acting as early cytotoxic effectors against tumor cells. Alternatively, CD8-MP cells could act as suppressor cells, promoting tumor progression by suppressing anti-tumor immune responses.

Aims of this thesis: Critical gaps in knowledge that will be addressed

As introduced above, fundamental aspects of the biology of CD8-MP cells remain undefined. Prior work on CD8-MP cells has primarily relied on the study of bulk populations and the use of TCRtg and pMHC tetramer models reactive to foreign antigens. While these studies have been useful in revealing some unique properties of CD8-MP cells, they have not been sufficient to fully elucidate the nature of antigens recognized by these cells, the forces driving their differentiation, and the function of these cells at homeostasis and in the tumor environment. This thesis aims to gain a deeper understanding of these principles via TCR profiling of the thousands of CD8-MP specificities in the endogenous mouse repertoire coupled with the analysis of the differentiation and antigen specificity of individual CD8-MP clones. Using this approach, several gaps in knowledge will be addressed.

Previous work has demonstrated the importance of IL-15 in the maintenance of CD8-MP cells. Additionally, work utilizing tetramer based approaches has found that in the pool of foreign pMHC reactive T cells, there exists a minor fraction of tetramer-positive T cells that exhibit markers of antigen experience, even in mice that have never been exposed to those antigens[52,68].

Collectively, these results have led CD8-MP cells to be classified as "antigen inexperienced" memory cells that differentiate in response to TCR-independent signals[48]. However, it is unknown what role TCR signaling plays in CD8-MP differentiation. Using TCR repertoire profiling, our work will characterize the TCR repertoire of endogenous CD8-MP cells present in B6 mice, relative to the TCR repertoire expressed by naive $CD8^+$ T cells. Coupled with a clonal approach, we will determine whether CD8-MP differentiation is driven by stochastic TCR-independent signals, or whether CD8-MP differentiation is a TCR-instructed process. Determining whether CD8-MP differentiation is TCR-instructed is a key question, because TCR dependency would suggest that CD8-MP cells represent an agonist-selected T cell that may have non-redundant functional roles in homeostasis or host defense. We will directly probe the reactivity of individual CD8-MP clones in vivo and in vitro and determine whether they are overtly self-reactive. Evidence of such reactivity would place CD8-MP cells on an expanding list of other known self-reactive T cell lineages, such as $Foxp3^+$ CD4 regulatory T (Treg) cells, invariant natural killer T (NKT) cells, $CD8\alpha\alpha^+$ intraepithelial lymphocytes (IEL), and MR1-restricted mucosal-associated invariant T (MAIT) cells.

This work will also aim to determine whether CD8-MP differentiation is initiated in the thymus or the periphery, by tracking the developmental trajectory of individual CD8-MP clones at different stages of maturation. The current literature has described four possibly inter-related subsets of CD8-MP cells: endogenous CD8-MP (virtual memory) cells, lymphopenia-induced memory cells, IL-4 dependent innate memory cells, and Qa-1-restricted $CD8^+$ Treg cells. It is currently unknown how each of these populations contribute to the endogenous population of CD8-MP cells in WT mice. Based on the separate work performed utilizing various models of CD8-MP cells, it is unclear whether CD8-MP cells arise in the thymus, or whether they arise in

the periphery due to homeostatic proliferation in the neonatal period. Understanding the site at which CD8-MP differentiation is triggered will provide key insight into the biology of these cells. If CD8-MP differentiation occurs in the thymus, then it implies that the machinery driving this process is inherently different from the machinery driving the differentiation of true memory $CD8^+$ T cells in the periphery. Thus, even though CD8-MP cells "masquerade" as true memory cells, they may have unique properties that are imparted during thymic differentiation. Additionally, thymic differentiation would suggest that CD8-MP differentiation represents an alternate fate of self-specific $CD8^+$ T cells restricted to classical MHC-I molecules, demonstrating that clonal deletion is not the sole fate of self-reactive $CD8^+$ thymocytes. Such a finding would fundamentally alter how self-reactive $CD8^+$ T cells are viewed, and highlight the need for new research to understand the forces driving alternate cell fates.

Finally, defining the nature of antigenic-ligands recognized by tumor-infiltrating T cells has been a challenging problem that has restricted progress in the field. Ligands recognized by TILs have been identified using a number of strategies, including characterization of T cells lines reactive to autologous tumor cells, computational neo-epitope prediction, and yeast-display of pMHC libraries[78–81,83]. However, pMHC tetramer staining has revealed that T cells reactive to defined antigens are present at low frequency in TIL infiltrates when analyzed directly ex vivo[82] suggesting that the vast majority of TILs are reactive to undefined antigens. Our work will leverage TCR fingerprinting and cell transfer approaches to define the contribution of CD8-MP cells to the tumor infiltrate in a murine prostate cancer model, and define the phenotypes of tumor-infiltrating cells. A finding that CD8-MP cells make measurable contributions to the tumor infiltrate in this model would suggest that CD8-MP cells may account for many of the "orphan" CD8+ T cells of

undefined specificity in human and murine cancers, and open new areas of inquiry that were not previously appreciated.

MATERIALS AND METHODS[b]

Mice

The following mice were purchased from the Jackson Laboratory, and bred and maintained at the University of Chicago under specific-pathogen free conditions: C57BL/6J (B6), CD45.1/.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ), *Rag1*$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}/J), *Tcra*$^{-/-}$ (B6.129S2-*Tcra*^{tm1Mom}/J), CD4-Cre (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ), D$^{b-/-}$K$^{b-/-}$ (B6.129P2-*H2-K1*^{tm1Bpe}*H2-D1*^{tm1Bpe}/DcrJ), and TRAMP (C57BL/6-Tg(TRAMP)8247Ng/J). "TCRβtg" mice expressing a fixed TCRβ chain of sequence TRBV26-ASSLGSSYEQY were generated as described previously[91]. *Rag2*-GFP (C57BL/6-Tg(Rag2-EGFP)1Mnz/J) mice were received from the laboratory of M. Nussenzweig at Rockefeller University[92]. Eomes-GFP (C57BL/6-Eomes$^{tm1.1Twa}$/Cnbc) mice were received from the laboratory of T. Walzer at Inserm[93]. All mice were bred and maintained in accordance with the animal care and use regulations of the University of Chicago. Germ free C57BL/6 mice were housed at the University of Chicago gnotobiotic facility under strict germ-free conditions. Both male and female mice were used across individual experiments. Mice were not randomized for assignment to experimental group and experiments were not conducted in a blinded fashion.

Antibodies, flow cytometry, and FACs

All antibodies used were purchased from BioLegend or Fisher Scientific. Cells were stained with conjugated antibodies specific for the following proteins (with clone name in

[b] Much of this section is reproduced, with some modification, from Miller, C.H., Klawon, D.E.J., Zeng, S., Lee, V., Socci, N.D., and Savage, P.A. (2020). Eomes identifies thymic precursors of self-specific memory-phenotype CD8^{+} T cells. Nature Immunology *21*, 567–577.

parentheses): Active caspase-3 (C92-605), CCR7 (4B12), CD4 (GK1.5, RM4-4, or RM4-5), CD5 (53-7.3), CD8β (YTS156.7.7), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD49d (R1-2), CD62L (MEL-14), CD69 (H1.2F3), CD73 (TY/11.8), CD122 (TM-β1), CD127 (A7R24), Eomes (Dan11mag), H-2K^{b} (AF6-88.5), Ki67 (SolA15), PD-1 (RMP1-30), TCRβ (H57-597), and Thy1.1 (OX-7). Cells were stained for 20 min on ice in staining buffer (phosphate-buffered-saline with 2% FCS, 0.1% NaN_3, 5% normal rat serum, 5% normal mouse serum, 5% normal rabbit serum (all sera from Jackson Labs), and 10 μg/mL 2.4G2 antibody). Intracellular staining for Eomes, Ki67, and active Caspase3 was performed using fixation and permeabilization buffers from eBioscience. Cells being stained for CCR7 were incubated for 30 min at 23°C with surface stain antibodies in 2% FCS in PBS. Flow cytometry was performed on an LSR Fortessa (BD Biosciences) and data was analyzed using FlowJo software (Tree Star). FACS was performed using a FACSAria (BD Biosciences).

TCR sequence analysis

CD8-Naïve (CD44^{lo}CD122$^{–}$) and CD8-MP (CD44^{hi}CD122^{+}) T cells were purified by FACS from 9-week-old TCRβTg males. To identify recurrent, tumor-infiltrating CD8^{+} T cell clones, CD8^{+} T cells were purified by FACs from the prostate tumors of 27-week-old TRAMP$^{+/–}$ TCRβTg males. RNA was extracted from purified subsets and subjected to complete *Tcra* sequencing using the Amp2Seq service from iRepertoire, a platform based on semi-quantitative multiplex PCR coupled with Illumina sequencing. This approach allows analysis of the complete TCRα repertoire, regardless of variable-region usage. Typically, >6.5 × 10^{5} TCR sequence reads were obtained per sample. TCRs were analyzed solely based on the predicted CDR3 sequence,

regardless of V-region usage. We first filtered for TCRs with CDR3 segments between 7 and 17 amino acids in length. In order to focus on recurrent TCRs, we then removed those TCRs with counts that were less than ten in N_s - 1 samples where N_s was the size of the smaller group. The differential analysis was done using R/Bioconductor *edgeR* package. The standard *edgeR* normalization function calcNormFactors was called. For the differential testing the *GLM* method was used. Since the data consisted of paired samples from each of several mice a paired test was done with the following design:

design ← model.matrix(~ mouse + group)

where group is the "treatment" grouping variable and mouse is the mouse id. We then processed the data using the GLM version of the dispersion estimators:

d ← estimateGLMCommonDisp(d,design)

d ← estimateGLMTrendedDisp(d,design)

d ← estimateGLMTagwiseDisp(d,design)

and then for differential testing:

fit ← glmFit(d,design)

results ← glmLRT(fit,coef=group.cont)

where group.cont is the contrast that selects for comparisons between groups. For the purposes of computing average expression in a natural scale, the normalized counts were rescaled to the original size of the dataset. This was done by setting a scaling factor that is equal to the geometric mean of the total sample counts for the samples. Significant CDR3s were filtered to an FDR less than 0.05. To analyze TCR repertoire similarity, we also analyzed CDR3 elements using the Morisita-Horn similarity index[94]**.** The grand average of hydropathy (GRAVY) value (http://www.gravy-calculator.de) for CDR3 regions was calculated by taking the sum of

hydropathy values of all amino acids divided by the protein length. The TCR sequence data are available at the Gene Expression Omnibus (GEO) repository under accession number GSE145365. The script used for TCR sequence analysis is available at https://github.com/soccin/MILLER_SAVAGE_CD8MP.

Retrovirus production, transduction, and generation of TCRrg mice

TCRrg mice were generated as described previously[39]. Briefly, *Tcra* sequences encoding TCRα chains of interest were cloned into a modified retroviral construct[39,95]. Plat-E cells[96] were used to generate retrovirus. *Tcra*$^{-/-}$ *CD4*-Cre^{+} TCRβtg^{+} mice on a C57BL/6 background were injected with 5-fluorouracil (APP Pharmaceuticals) 3 days prior to bone marrow harvest. Bone marrow cells were cultured for 2 days in X-Vivo 10 (Lonza) containing 15% FCS, 1% penicillin/streptomycin, mouse SCF, mouse IL-3 and mouse IL-6 (BioLegend). Cells were infected with retrovirus by spinfection in the presence of 6 μg/mL polybrene (EMD Millipore) and cultured for an additional 24 h (TCRrg bone marrow). TCRrg bone marrow from one mouse was then mixed with 5 × 10^6 freshly harvested bone marrow "filler" cells from *Rag1*$^{-/-}$ mice and injected intravenously into one lethally irradiated (800 rad) CD45$^{.1/.1}$ B6.SJL recipient mouse. “Low frequency” TCRrg mice were generated by mixing 12-30% cultured TCRrg bone marrow with 70-88% CD45$^{.1/.1}$ B6.SJL bone marrow. 5 × 10^6 bone marrow cells were injected intravenously into one sub-lethally irradiated (500 rad) B6.SJL recipient mouse. TCRrg and “low frequency” TCRrg mice were analyzed 6-11 weeks after bone marrow reconstitution.

In vitro T cell stimulation assay

CD8^{+} T cells from pooled spleen and lymph nodes (axillary, brachial, cervical, inguinal,

mesenteric, and periaortic) from TCRrg mice were enriched for $CD8^+$ T cells by MACS (Miltenyi Biotech), and $Thy1.1^+$ $CD44^{lo}CD122^-$ $CD8^+$ T cells were purified by FACs. $Thy1.1^+$ $CD44^{lo}CD122^-$ $CD8^+$ TCRrg cells were CellTrace-Violet (ThermoFisher) labeled per manufacturer instructions with slight modification. Briefly, cells were pelleted, resuspended in CellTrace-Violet (CTV) at 1:1000 dilution and incubated for 20 min at 37°C. The reaction was quenched by the addition of 13 mL of complete culture media. To isolate dendritic cells, spleens were isolated from 6-week-old SPF or GF C57BL/6 mice and thymi were isolated from 3 to 4-week-old SPF C57BL/6 mice. Spleens or thymi were injected and digested with Liberase TL (400 μg/mL, Roche) and DNase (800 μg/mL, Roche) in RPMI for 30 min at 37°C. For thymic APC isolation, EDTA (10 mM) was added to digests and enriched by layering digested thymocytes on top of a discontinuous Percoll gradient (GE Healthcare) at 1.115 g/mL in PBS, followed by centrifugation at 1,350 × g for 30 min and isolation of cells settling at the Percoll interface[97]. APCs were enriched from spleens or thymi for $CD11c^+$ cells by MACS-based (Miltenyi Biotech) positive selection. 1×10^4 CTV-labeled T cells were co-cultured with 5×10^4 $CD11c^+$ APCs and 100 U/mL recombinant mouse interleukin-2 (IL-2). Where indicated, anti-MHC-I (H2) blocking antibody (clone M1/42.3.9.8, BioXCell) or rat IgG2a isotype control antibody (clone 2A3, BioXCell) was added to indicated cultures at a final concentration of 500 μg/mL. Cell cultures were set up in 384-well ultra-low attachment, round-bottom plates (Corning). Dilution of CTV was assessed by flow cytometry on day 5.

TCRrg cell adoptive transfer

$CD8^+$ T cells from pooled spleen and lymph nodes (axillary, brachial, cervical, inguinal, and periaortic) from 6-8-week-old TCRrg mice were enriched for $CD8^+$ T cells by MACS (Miltenyi)

and Thy1.1^{+} CD8^{+} CD44^{hi}CD122^{+} cells and Thy1.1^{+} CD8^{+} CD44^{lo}CD122$^{—}$ cells were sorted by FACS. 2.5 x 10^5 purified cells were transferred intravenously into congenically disparate CD45.1^{+} B6.SJL recipients. 3-weeks post-transfer, the fate of the donor Thy1.1^{+} cells was assessed in the spleens of recipient mice.

Thymocyte adoptive transfer and FTY720 treatment

Thymi were isolated from 4-week-old CD45.2^{+} Eomes-GFP^{+} mice and CD4^{+} thymocytes cells were depleted by incubating thymi with anti-mouse CD4 antibody conjugated to Biotin (clone RM4-5, BioLegend) for 10 min at 23°C, followed by incubation with Streptavidin beads (StemCell) for 3 min at 23°C. Cells were then placed on the EasySep magnet (StemCell) for 2 min and the supernatant was isolated. CD4$^{–}$ CD73$^{–}$ CD69$^{–}$ mature CD8β^{+} Eomes-GFP^{+} and Eomes-GFP$^{–}$ thymocytes cells were purified by FACs and 1 × 10^5 GFP^{+} or GFP$^{–}$ cell suspensions were transferred intravenously into CD45.1^{+} B6.SJL hosts. 3 weeks later, CD8^{+} T cells from pooled spleen and lymph nodes (axillary, brachial, cervical, inguinal, and periaortic) from host mice were enriched for CD8^{+} T cells by MACS (Miltenyi Biotech) and the phenotype of transferred cells was analyzed by flow cytometry. To prevent thymic egress of developing T cells, B6 mice were injected I.P. every other day for 5 days with 140 μg FTY720 (Sigma) in 50 μL diH_20 or PBS control. The phenotype of thymocytes was analyzed by flow cytometry on day 6.

Lymphocyte isolation from TRAMP prostate tumors

Whole male genitourinary tracts were isolated and prostate lobes (anterior, dorsolateral, and ventral) were separated by microdissection from 24-week-old TRAMP$^{+/+}$ or 27-week-old TRAMP$^{+/–}$ TCRβTg males. Prostate lobes were injected and digested with Liberase TL (10

mg/mL, Roche) and DNase (20 mg/mL, Roche) in RPMI for 30 min at 37°C. Digested tissue was mechanically disrupted with frosted microscope slides and viable lymphocytes were enriched using Histopaque 1119 (Sigma). $CD8^+$ T cells were analyzed by flow cytometry or purified by FACs.

Statistical analysis

Data were analyzed using Prism software (GraphPad). Significance testing was performed using the nonparametric Mann-Whitney test (two-tailed) or one-way ANOVA with Bonferroni's multiple comparisons test (two-tailed). Statistical analysis of TCR sequencing data was performing with R (The R Project for Statistical Computing) using EdgeR-based methods (see "TCR sequence analysis" section for script details). Statistical testing on CDR3 length and GRAVY distributions was performed using R. Significance testing was performed using both the paired Wilcoxon signed-rank test (two-tailed), which tests for distribution shift, and the Kolmolgorov-Smirnov test, which tests for differences in distribution shape.

RESULTS—EOMES IDENTIFIES THYMIC PRECURSORS OF SELF-SPECIFIC MEMORY-PHENOTYPE CD8$^+$ T CELLS—*NATURE IMMUNOLOGY* 2020[c]

Introduction

Classically, memory T cells arise after an immune response to a foreign pathogen in the periphery, and are poised to respond more rapidly upon repeated pathogen challenge. However, in conventionally housed mice and germ-free mice that have not been exposed to foreign pathogens, there exists a substantial population of CD8$^+$ αβ T cells that exhibit a CD44^{hi} CD122$^+$ memory phenotype, suggestive of previous encounter with agonist ligands. This population, termed "memory-phenotype" CD8$^+$ T cells (CD8-MP cells, also referred to as "virtual-memory"[48,68] or "innate memory"[50] T cells), make up >5% of the CD8$^+$ repertoire in adult mice, and exhibit numerous hallmarks of conventional memory CD8$^+$ T cells reactive to foreign ligands. Although the existence of an analogous cell population has been suggested in humans[54,75,76], the lack of validated markers has limited the ability to study CD8-MP cells in human samples. To date, diverse and dichotomous functions have been attributed to CD8-MP cells, including innate-like effector functions in the early stages of pathogen challenge[68,98], and roles in the maintenance of immune homeostasis at steady state[73]. However, it remains unclear whether these reflect broad functions of all CD8-MP cells, or distinct functions of heterogeneous T cell populations falling within the CD44^{hi} CD122$^+$ subset. Efforts to elucidate the mechanisms driving CD8-MP differentiation and the function of CD8-MP cells in the context of homeostasis, host defense, inflammation, and cancer have been hampered by the lack of available markers to directly identify CD8-MP cells and

[c] Much of this section is reproduced, with some modification, from Miller, C.H., Klawon, D.E.J., Zeng, S., Lee, V., Socci, N.D., and Savage, P.A. (2020). Eomes identifies thymic precursors of self-specific memory-phenotype CD8$^+$ T cells. Nature Immunology *21*, 567–577.

their precursors, especially in the context of immune activation. Thus, fundamental aspects of the biology of CD8-MP cells remain incompletely defined, including the nature of antigens recognized by these cells, the mechanisms driving their differentiation, and the functions of CD8-MP cells at steady state and in inflammatory contexts.

A long-standing question is whether CD8-MP differentiation is a T cell antigen receptor (TCR)-independent process driven by cytokines or accessory factors, or a TCR-instructed process triggered by the recognition of peptide/MHC-I ligands. CD8-MP cells exhibit slightly higher average densities of CD5[54], a surrogate marker of reactivity to positively selecting ligands. However, given that CD5 densities are thought to be "hard-wired" following positive selection in the thymus[55–57], CD5 density cannot be used to assess the intensity of additional TCR signaling events occurring after positive selection. The finding that the phenotype and frequency of CD8-MP cells is not diminished in germ-free mice and germ-free mice fed an elemental diet[48,50], indicates that the absence of microbial and dietary antigens does not impact CD8-MP cells, and suggests that CD8-MP differentiation is either triggered by the recognition of endogenous self-ligands, or is driven by TCR-independent cues. In this regard, the observation that the endogenous repertoire harbors a small number of $CD44^{hi}$ $CD122^{+}$ $CD8^{+}$ T cells reactive to any foreign peptide/MHC-I complex[52,68] suggested that CD8-MP differentiation can occur in the absence of known agonist ligands, and that CD8-MP differentiation may be triggered by TCR-independent signals. However, a recent study identified two CD8-MP-biased TCRs, differing by one amino acid, that promoted CD8-MP differentiation when expressed in TCR retrogenic mice[99] indicating that CD8-MP differentiation of this clonotype is TCR-directed. Thus, it is currently unknown whether TCR-instructed differentiation applies to the thousands of individual CD8-MP

specificities within the endogenous T cell repertoire, and whether CD8-MP differentiation is a robust, orchestrated process that occurs reproducibly.

Regarding the developmental origins of CD8-MP cells, common thought suggests that CD8-MP differentiation occurs in the periphery. This notion is largely based on the fact that $CD44^{hi}$ $CD122^{+}$ cells are first detected in the periphery of neonatal mice[52] and are phenotypically similar to "lymphopenia-induced memory" (LIM) T cells, a population of $CD44^{hi}$ $CD122^{+}$ cells that emerge in the periphery following intravenous transfer of naive-phenotype $CD8^{+}$ T cells into lymphopenic mice[48,50]. However, it is unclear whether peripherally induced LIM cells are representative of endogenous CD8-MP cells found in wild-type mice. Thus, there is a critical need for developmental studies in which the differentiation trajectories of individual CD8-MP clones can be tracked in the thymus and periphery.

Here, we address these key questions using an in-depth clonal approach, pairing complete TCR repertoire profiling with studies whereby the developmental arc of individual CD8-MP clones can be assessed at all stages of T cell maturation, and the reactivity of distinct CD8-MP clones can be directly assessed ex vivo. Our findings demonstrate that CD8-MP differentiation parallels the thymic differentiation of $Foxp3^{+}$ regulatory T (Treg) cells in many respects, and reveal a previously unanticipated role for CD8-MP cells in the setting of cancer.

The CD8-MP TCR repertoire is distinct and recurrent

Despite comprising >5% of the $CD8^{+}$ T cell repertoire in unprimed specific pathogen free (SPF) and germ-free (GF) mice (Figure 2), little is known about the array of TCRs expressed by CD8-MP cells. In particular, it is unknown whether the CD8-MP TCR repertoire has limited

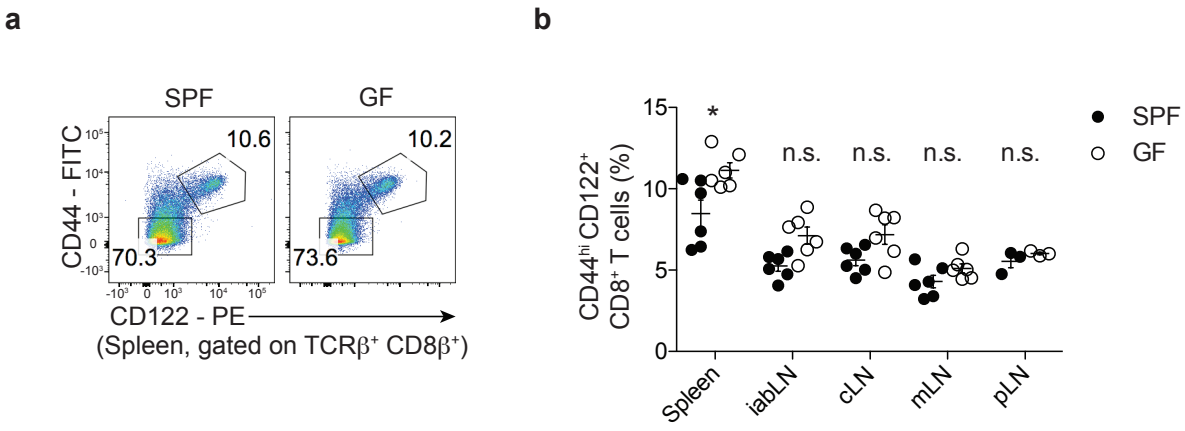

Figure 2. The frequency of CD8-MP cells is not diminished in germ-free mice.

a, Representative flow-cytometric analysis of CD44 vs. CD122 expression by CD8β$^+$ T cells from the spleens of 16-week-old C57BL/6 specific pathogen free (SPF) and germ-free (GF) mice. The percentage of cells falling in the indicated gates is denoted. Data are representative of two independent experiments. **b**, Summary plot of the frequency of CD44^{hi} CD122$^+$ expressing TCRβ$^+$ CD8β$^+$ T cells from the indicated lymphoid sites in 6 to 16-week-old SPF or GF mice. iabLN: inguinal, axial, brachial lymph nodes; cLN: cervical lymph nodes; mLN: mesenteric lymph nodes; pLN: periaortic lymph nodes. Each symbol represents an individual mouse. Mean ± SEM is indicated. n = 6, Spleen, iabLN, cLN, mLN; n = 3, pLN. At each lymphoid site, the frequency of CD8-MP cells was compared between the SPF and GF mice using one-way ANOVA with Bonferroni post-test analysis, comparing all pairs of columns (ANOVA $p < 0.0001$, $F = 17.43$, df = 53). Adjusted p-values from the Bonferroni post-test are depicted: Spleen, *$p = 0.0133$; iabLN, n.s. $p = 0.3669$; cLN, n.s. $p > 0.9999$; mLN, n.s. $p > 0.9999$; pLN, n.s. $p > 0.9999$. Data are pooled from two independent experiments. (n.s., not significant)

diversity, whether it varies stochastically from mouse to mouse, and whether it overlaps with the TCR repertoire expressed by naïve-phenotype $CD8^+$ T cells. To comprehensively examine these questions, we sequenced the complete TCR repertoires of naïve-phenotype $CD8^+$ T cells (CD8-Naïve cells, $CD44^{lo}$ $CD122^-$) and memory-phenotype $CD8^+$ T cells (CD8-MP cells, $CD44^{hi}$ $CD122^+$) using previously established methods[100,101]. We focused our analysis on CD8-MP cells from C57BL/6 mice, in which thymic interleukin 4 (IL-4)-induced $CD8^+$ memory cells[48,50,102] make minimal contributions to the peripheral CD8-MP pool[52,102]. CD8-Naïve and CD8-MP cells from the pooled spleen and lymph nodes were purified by cell sorting from five different 9-week-old male mice expressing a fixed transgenic TCRβ chain on the C57BL/6J (B6) background. By fixing the TCRβ chain, a complete survey of the TCRαβ repertoire can be obtained by sequencing the endogenous *Tcra* chains using the iRepertoire platform[100,103]. This approach yielded approximately 6.5×10^5 *Tcra* sequence reads per sample, providing a broad survey of TCR usage. Our data revealed the striking finding that the TCR repertoire of CD8-MP cells is largely distinct from that of CD8-Naïve cells, and was highly recurrent from mouse to mouse (Figure 3). Comparative analysis of TCR frequency identified 493 recurrent *Tcra* chains that were significantly overrepresented in the CD8-MP subset relative to the CD8-Naïve subset (Figure 3a, right arm of volcano plot). Cumulatively, these TCRs accounted for 65 +/- 1% of the CD8-MP TCR repertoire, demonstrating the broad extent of repertoire skewing. The recurrent nature of the CD8-MP repertoire was further illustrated using the Morisita-Horn (MH) similarity index, for which a value of 1 denotes identity and a value of 0 indicates complete dissimilarity. Pairwise comparison of the CD8-MP TCR repertoires from five different mice revealed a mean MH index of 0.92 +/- 0.03 (Figure 3b), demonstrating that distinct T cell clones were reproducibly directed

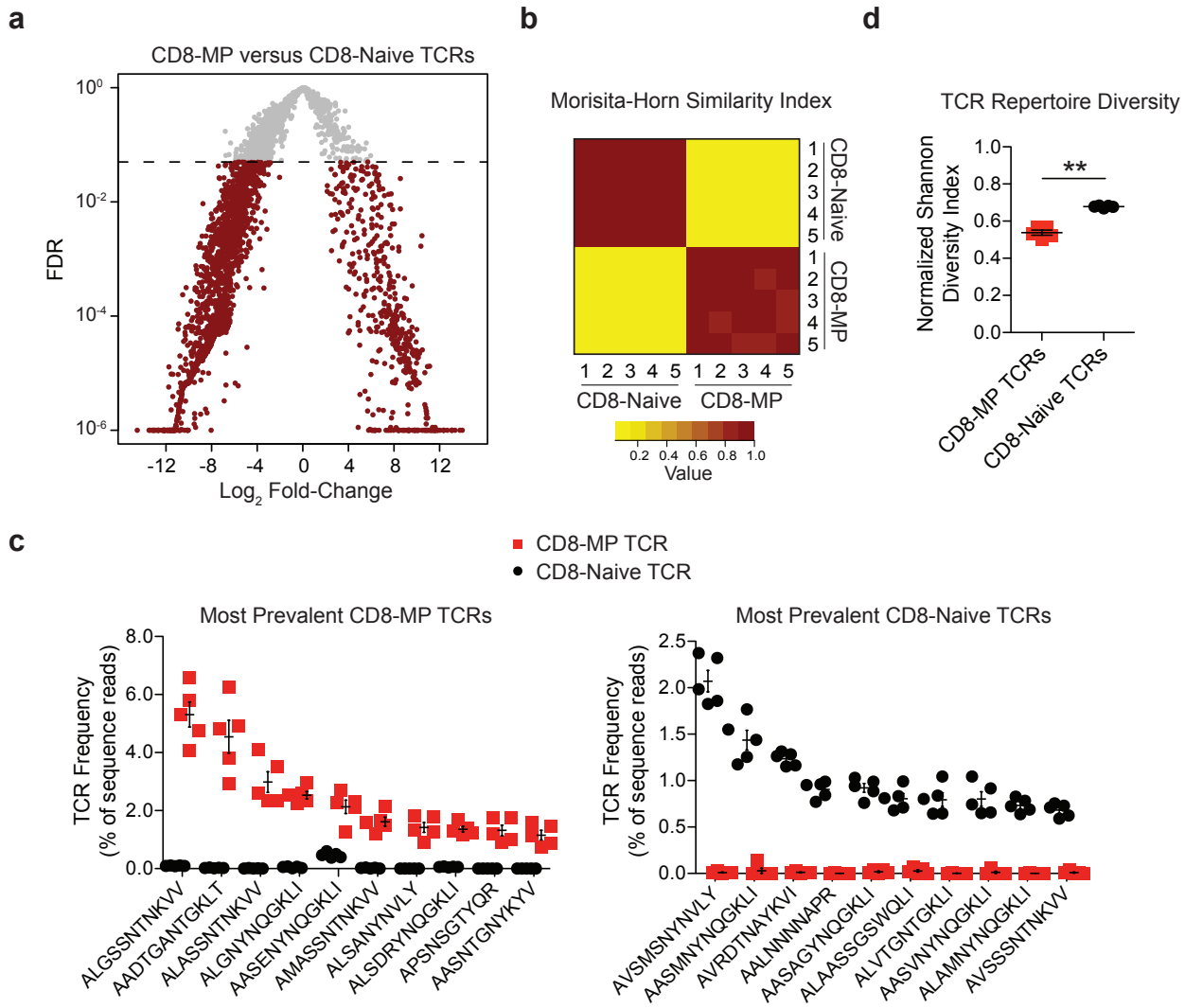

Figure 3. The TCR repertoire of memory-phenotype CD8$^+$ T cells is recurrent and distinct from that of naïve-phenotype CD8$^+$ T cells.

CD8-MP (CD8$^+$ CD44^{hi} CD122$^+$) and CD8-Naïve (CD8$^+$ CD44^{lo} CD122$^-$) T cells were purified by FACS from the pooled spleen and lymph nodes of 9-week-old TCRβtg$^+$ males and subjected to complete TCRα sequencing using the iRepertoire platform. N = 5 for CD8-MP and CD8-Naïve samples. TCRα chains were assessed solely based on their predicted CDR3 segment, regardless of V-region usage **a,** For the TCRα chain sequences, a volcano plot of false discovery rate (FDR) versus differential TCR representation (log_2 fold-change) in the CD8-MP vs. CD8-Naïve subsets is shown for the 3,926 recurrently expressed TCRs. n = 5 for CD8-MP and CD8-Naïve samples. Comparisons were made using EdgeR and were adjusted for multiple comparisons. Red dots denote TCRs with FDR < 0.05. The horizontal dashed line indicates FDR cutoff. **b,** Heat map of the Morisita-Horn (MH) similarity index (see Methods), which is a measure of repertoire overlap, for the five CD8-MP and five CD8-Naïve samples. For the MH index, a value of 1 indicates identity and a value of 0 denotes complete dissimilarity. **c,** For the 10 most prevalent TCRs

Figure 3, continued
recurrently expressed by CD8-MP (left plot) and CD8-Naïve (right plot) subsets, a summary plot of the frequency of these TCRs in individual mice is depicted, with the TCRα CDR3 sequences listed below. Red squares denote frequencies in CD8-MP samples, whereas black circles denote frequencies in CD8-Naïve samples. n = 5 for CD8-MP and CD8-Naïve samples. Mean ± SEM is indicated. **d,** Summary plot of normalized Shannon diversity index for CD8-MP and CD8-Naïve subsets. The normalized index ranges from 0 (no diversity) to 1 (maximal diversity). Each symbol represents an individual mouse. n = 5 for CD8-MP and CD8-Naïve samples. Mean ± SEM is indicated. **p = 0.0079, two-tailed nonparametric Mann-Whitney test.

into the CD8-MP subset. By contrast, pairwise comparisons of CD8-Naïve vs. CD8-MP repertoires between the five mice revealed a mean MH index of 0.06 +/- 0.01, indicative of minimal overlap. The concepts that the CD8-MP TCR repertoires were highly recurrent and distinct from the CD8-Naïve TCR repertoire are also illustrated in Figure 3c, which plots the frequency of the 10 most prevalent CD8-MP TCRs (left) and 10 most prevalent CD8-Naïve TCRs (right) within the CD8-Naïve and CD8-MP subsets in individual mice. Lastly, analysis of repertoire complexity using the Shannon diversity index demonstrated that the CD8-MP TCR repertoire was less diverse than the CD8-Naïve repertoire (Figure 3d), with an average complexity of 6,979 TCR complementarity determining regions 3 (CDR3) segments for the CD8-MP subset and 24,443 CDR3s for the CD8-Naive subset. Analyses of CDR3α hydrophobicity and amino acid length revealed no consistent differences between the CD8-Naïve and CD8-MP TCRs (Figure 4). Collectively, these data suggest that CD8-MP differentiation is a robust TCR-directed process that occurs in a highly reproducible, orchestrated fashion.

CD8-MP differentiation is a TCR-directed process

To study representative CD8-MP-biased specificities at the clonal level, we generated a series of monoclonal TCR "retrogenic" (TCRrg) mice expressing a single TCR of interest, and used T cells from these mice to perform both in vivo and ex vivo studies. In this study, we use the term "CD8-MP TCR" to define a TCR that is preferentially expressed by CD8-MP cells, and "CD8-Naïve TCR" for receptors skewed to the CD8-Naïve subset. For TCR nomenclature, we depict the amino acid sequence of the CDR3 segment of a given TCRα chain (Figure 3c), and denote a TCR clone using a three-letter code reflecting the amino acids at positions 3-5 of the

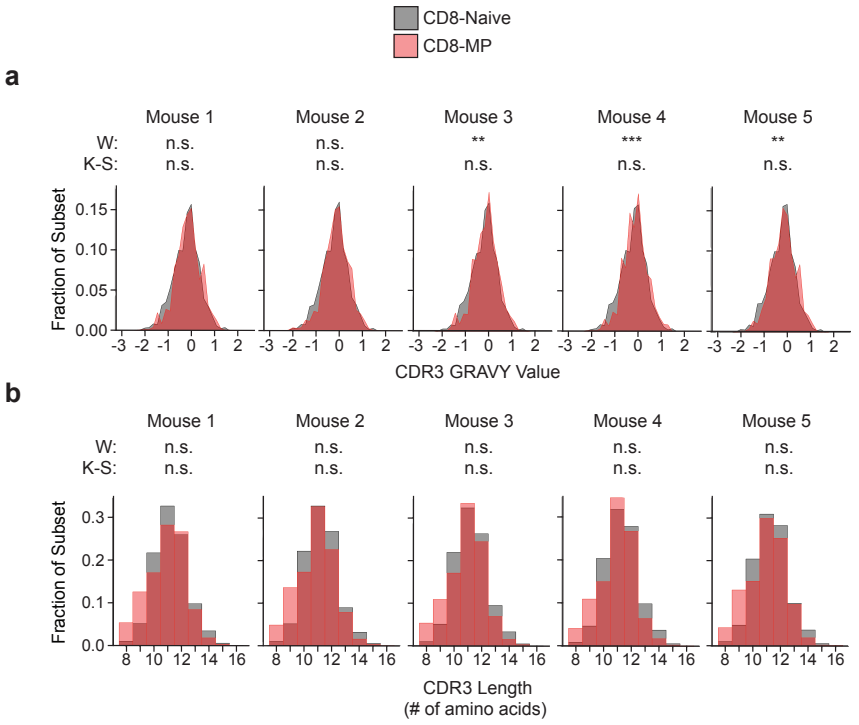

Figure 4. CD8-MP and CD8-Naïve CDR3α chain hydrophobicity and length analysis.

CD8-MP (CD8β^{+} CD44^{hi}CD122^{+}) and CD8-Naïve (CD8β^{+} CD44^{lo}CD122^{-}) T cells were purified by FACS from the pooled spleen and lymph nodes of 9-week-old TCRβtg males and subjected to complete TCRα sequencing using the iRepertoire platform. N = 5 for CD8-MP and CD8-Naïve samples. TCRα chains were assessed solely based on their predicted CDR3 segment, regardless of V-region usage. **a,** Histograms depicting grand average of hydropathy (GRAVY) values for CDR3 regions of the CD8-MP (red) and CD8-Naïve (black) subsets for each mouse, n = 5 mice. Significance testing was performed with the paired, two-tailed Wilcoxon signed-rank test (W) and the paired, two-tailed Kolmolgorov-Smirnov test (K-S). Mouse 1 (CD8-MP CDR3 n = 11999, CD8-Naïve CDR3 n = 29048), p = 0.2449 (W) and p = 1 (K-S); Mouse 2 (CD8-MP CDR3 n = 3327, CD8-Naïve CDR3 n = 14736), p = 0.0511 (W) and p = 1 (K-S); Mouse 3 (CD8-MP CDR3 n = 2159, CD8-Naïve CDR3 n = 26649), **p = 0.0017 (W) and p = 1 (K-S); Mouse 4 (CD8-MP CDR3 n = 2848, CD8-Naïve CDR3 n = 24501), ***p < 0.0001 (W) and p = 1 (K-S); Mouse 5 (CD8-MP CDR3 n = 13524, CD8-Naïve CDR3 n = 25367), **p = 0.0010 (W) and p = 1 (K-S). (n.s. not significant). **b,** Histograms depicting CDR3 lengths of the CD8-MP (red) and CD8-Naïve (black) subsets for each mouse, n = 5 mice. Significance testing was performed with the paired, two-tailed Wilcoxon signed-rank test (W) and the paired, two-tailed Kolmolgorov-Smirnov test (K-S). Mouse 1 (CD8-MP CDR3 n = 11999, CD8-Naïve CDR3 n = 29048), p = 0.2031 (W) and p = 1 (K-S); Mouse 2 (CD8-MP CDR3 n = 3327, CD8-Naïve CDR3 n = 14736), p = 0.2031 (W) and p = 1 (K-S); Mouse 3 (CD8-MP CDR3 n = 2159, CD8-Naïve CDR3 n = 26649), p = 0.4258 (W) and p = 1 (K-S); Mouse 4 (CD8-MP CDR3 n = 2848, CD8-Naïve CDR3 n = 24501), p = 1 (W) and p = 1 (K-S); Mouse 5 (CD8-MP CDR3 n = 13524, CD8-Naïve CDR3 n = 25367), p = 0.9102 (W) and p = 1 (K-S). (n.s. not significant).

CDR3α. For example, TCRrg cells with CDR3α sequence AASMNYNQGKLI are denoted "SMNrg" cells. Our work focused on the study of four recurrent CD8-MP and four recurrent CD8-Naïve TCRs identified in Figure 3, which were chosen because they span a range of frequencies and utilize distinct V and J-region segments (Table 2). TCRrg mice were generated as previously described[39]. Briefly, bone marrow (BM) cells from *Tcra*$^{-/-}$ *Cd4*-Cre^{+} TCRβtg^{+} CD45.2/.2 mice were retrovirally transduced with a vector in which the genes encoding a TCRα chain of interest and an IRES-Thy1.1 reporter are preceded by a loxP-flanked stop cassette[95]. Transduced BM was engrafted along with wild-type filler BM cells into lethally irradiated 6-8-week-old CD45.1/.1 B6.SJL hosts, and mice were analyzed >6 weeks post-engraftment. Expression of *Cd4*-Cre at the CD4^{+} CD8^{+} stage of thymic maturation induces TCRα expression by TCRrg cells, recapitulating the natural kinetics of TCR expression during development. Resulting TCRrg T cells were identified using the Thy1.1 and CD45.2 markers.

Using this approach, we found that a substantial fraction of T cells expressing CD8-MP TCRs adopted the CD44^{hi} CD122^{+} memory phenotype in the periphery of TCRrg hosts, whereas very few TCRrg cells expressing CD8-Naïve TCRs acquired this phenotype (Figure 5a-c, Figure 6). These findings validate the TCR sequencing and confirm the notion that CD8-MP differentiation is a TCR-instructed process. Notably, in TCRrg mice expressing CD8-MP TCRs, we found that not all peripheral TCRrg cells adopted a CD44^{hi} CD122^{+} phenotype at the time of analysis, and that the fraction of TCRrg CD44^{hi} CD122^{+} cells varied from mouse to mouse (Figure 5a-c). A potential explanation for the lack of complete skewing to the CD8-MP phenotype is the existence of limited niches supporting CD8-MP differentiation, which may be overloaded in TCRrg mice harboring large numbers of monoclonal T cells. To define the phenotype and anatomical distribution of select CD8-MP clones, we generated TCRrg mice expressing CD8-MP

Selected CD8-MP TCRs

CDR3α	FDR	log_2FC	Mean Frequency in CD8-MP subset	V-region	J-region
ALGSSNTNKVV	4.52E-11	6.11	4.58	mTRAV13D-1	mTRAJ34
AADTGANTGKLT	9.61E-15	7.96	3.97	mTRAV14D-3/DV8	mTRAJ52
AVSAVGSNNRIF	1.46E-10	13.36	0.64	mTRAV9D-4	mTRAJ31
AASATNAYKVI	7.72E-10	11.16	0.25	mTRAV14D-1	mTRAJ30

Selected CD8-Naïve TCRs

CDR3α	FDR	log_2FC	Mean Frequency in CD8-Naive subset	V-region	J-region
AASMNYNQGKLI	3.84E-07	-10.14	1.85	mTRAV14D-3/DV8	mTRAJ23
AVRDTNAYKVI	3.27E-06	-6.90	1.57	mTRAV3D-3	mTRAJ30
AALNNNNAPR	1.91E-08	-13.49	1.17	mTRAV4D-3	mTRAJ43
AIDYQGGRALI	2.69E-05	-9.59	0.33	mTRAV13-2	mTRAJ15

Table 2. Selected CD8-MP and CD8-Naïve TCRs for retrogenic mouse generation.

TCRs were selected from the top 50 most prevalent, differentially expressed (FDR < 0.05) CD8-MP and CD8-Naive clones from n = 5 mice. Comparisons were made using EdgeR and were adjusted for multiple comparisons. From these lists, we sorted CDR3 sequences by TRAV usage. From each TRAV group, we selected the clone with the highest mean frequency. The selected TCR clones represent a range of frequencies (0.2 – 5.0% of the subset) and a diversity in TRAV and TRAJ usages, giving us broad representation of each $CD8^+$ T cell repertoire. CDR3α, complementary determining region 3, alpha chain; FDR, false discovery rate; log_2FC, log_2 fold-change.

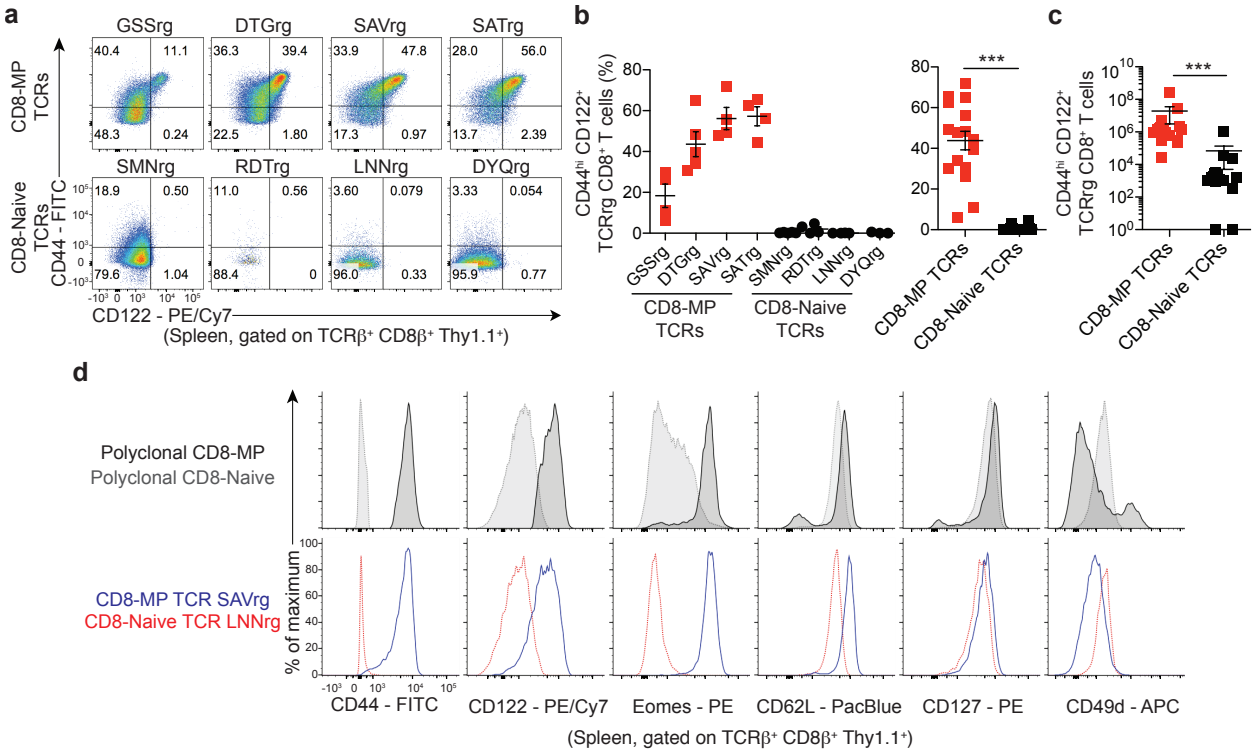

Figure 5. CD8-MP differentiation is a TCR-directed process.

Four representative CD8-MP (top) and four CD8-Naïve (bottom) TCRs defined in Fig. 1 were cloned and expressed in TCR retrogenic (TCRrg) mice (see Methods). 7-11 weeks after bone marrow reconstitution, the phenotype of TCRrg T cells was assessed. **a**, Representative flow cytometric analysis of CD44 vs. CD122 expression by TCRrg cells from the spleen, gated on TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells. The percentage of cells falling within the respective gates is indicated. Data are representative of six independent experiments. **b**, Left: Summary plot of pooled data from (a) showing the frequency of TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells exhibiting a CD44^{hi}CD122$^+$ phenotype for the listed TCRs. Right: Data from the left plot were pooled from the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 17, CD8-MP TCRrg mice; n = 16, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from six independent experiments. **c**, Summary plot of pooled data from (a) showing the absolute number of TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells exhibiting a CD44^{hi}CD122$^+$ phenotype for the CD8-MP TCRs and CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 17, CD8-MP TCRrg mice; n = 16, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from six independent experiments. **d**, Representative flow cytometric analysis of CD44, CD122, Eomes, CD62L, CD127, and CD49d expression by splenic polyclonal TCRβ$^+$ CD8β$^+$ CD8-MP (CD44^{hi}CD122$^+$) and CD8-Naïve (CD44^{lo}CD122$^-$) cells from a 7-week-old C57BL/6 mouse (top panels) and monoclonal TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ SAVrg (CD8-MP TCR) and LNNrg (CD8-Naïve TCR) T cells 7 weeks after bone marrow reconstitution in "low frequency" TCRrg mice (bottom panels). Data are representative of three independent experiments.

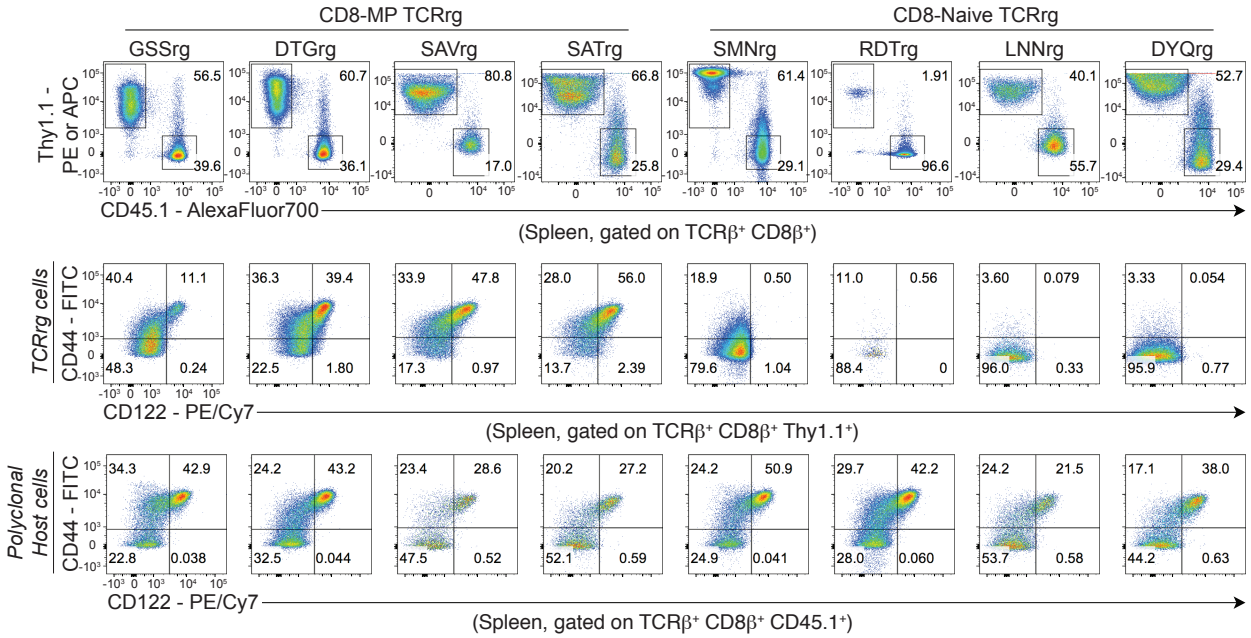

Figure 6. Phenotype of TCRrg and filler CD8$^+$ T cell populations.

(Top) Representative flow cytometric analysis of Thy1.1 vs. CD45.1 expression by TCRβ$^+$ CDβ$^+$ cells from the spleens of indicated TCRrg mice. The percentage of cells falling in the indicated gates is denoted. (Bottom) Representative flow cytometric analysis of CD44 vs. CD122 expression by Thy1.1$^+$ TCRrg or CD45.1$^+$ "filler" TCRβ$^+$ CD8β$^+$ cells from the spleens of indicated TCRrg mice. The percentage of cells falling in the indicated gates is denoted. Data are representative of six independent experiments.

TCRs at low clonal frequency ("low frequency" TCRrg mice; see Methods). In such mice, when TCRrg cells were present at frequencies of <15% of $CD8^+$ T cells, the majority (>65%) of TCRrg cells expressing a CD8-MP TCR exhibited the $CD44^{hi}$ $CD122^+$ phenotype (with nearly all cells shifted away from a naive phenotype), whereas TCRrg cells expressing CD8-Naive TCRs did not (Figure 7). These results are consistent with the existence of limited niches supporting the differentiation of CD8-MP clones. Furthermore, peripheral CD8-MP TCRrg cells from these mice adopted a central memory-like phenotype characteristic of polyclonal CD8-MP cells, including high expression of CD44, CD122, CD62L, CD127, and Eomes, and low expression of CD49d (Figure 5d). These collective data demonstrate that the expression of CD8-MP TCRs in TCRrg hosts recapitulated differentiation into the CD8-MP subset, validating the TCRrg approach and providing further evidence that CD8-MP differentiation is a TCR-directed process.

CD8-MP T cell clones exhibit self-reactivity

Our TCR repertoire and TCRrg mouse analyses suggest that CD8-MP differentiation is antigen driven. We first set out to determine the MHC restriction of the endogenous CD8-MP repertoire to narrow the set of potential ligands CD8-MP cells are reactive to. Previous work indicated that some undefined fraction of CD8-MP cells are restricted to the non-classical MHC-I molecule Qa-1[70]. To determine whether Qa-1-restricted or other non-classical MHC-I-restricted CD8-MP cells represent a large portion of the CD8-MP subset, we analyzed the number of CD8-MP cells found in $K^{b-/-}D^{b-/-}$ mice, which lack the classical MHC-I molecules H2-K^b and H2-D^b. In $K^{b-/-}D^{b-/-}$ mice, the number of CD8-MP cells is greatly reduced, indicating that a substantial number of CD8-MP cells are restricted to classical MHC-I molecules (Figure 8). Since CD8-MP cells are abundant in naïve mice that have never been exposed to known foreign pathogens, we

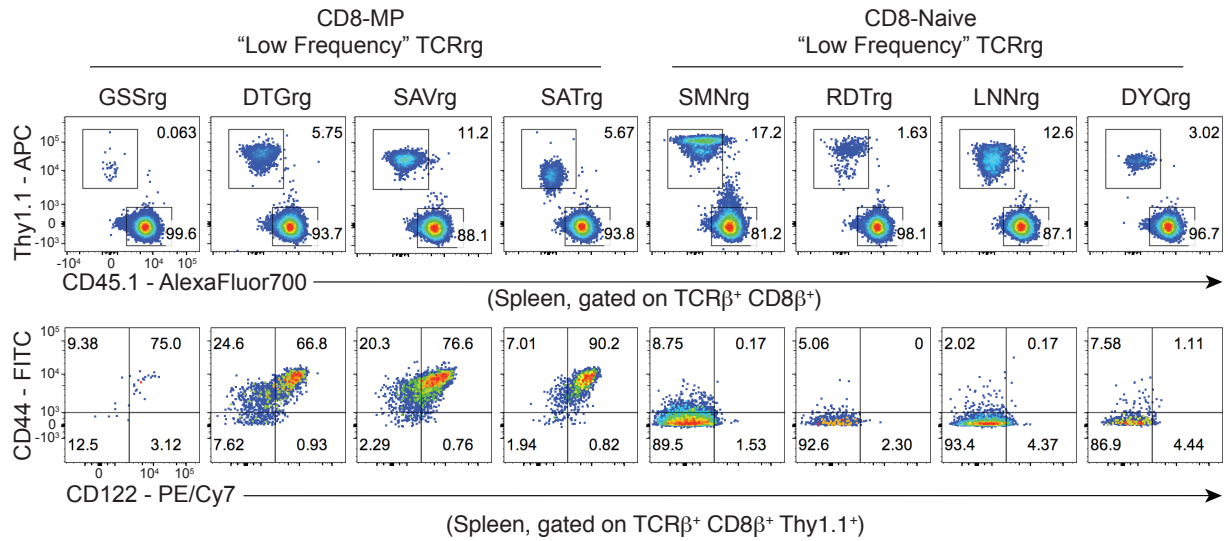

Figure 7. A greater fraction of CD8-MP TCRrg cells adopts the CD44^{hi}CD122^{+} phenotype at lower clonal frequencies.

(Top) Representative flow cytometric analysis of Thy1.1 vs. CD45.1 expression by TCRβ^{+} CD8^{+} cells and (Bottom) CD44 vs. CD122 expression by TCRβ^{+} CD8^{+} Thy1.1^{+} cells from "low frequency" TCRrg mice expressing the indicated TCRs, assessed 7 weeks after bone marrow reconstitution. It should be noted that the expression of the Thy1.1 reporter varies in different TCRrg mice, but the expression of TCRβ is uniform and comparable to that of endogenous cells (not shown). The percentage of cells falling in the indicated gates is denoted. Data are representative of four independent experiments.

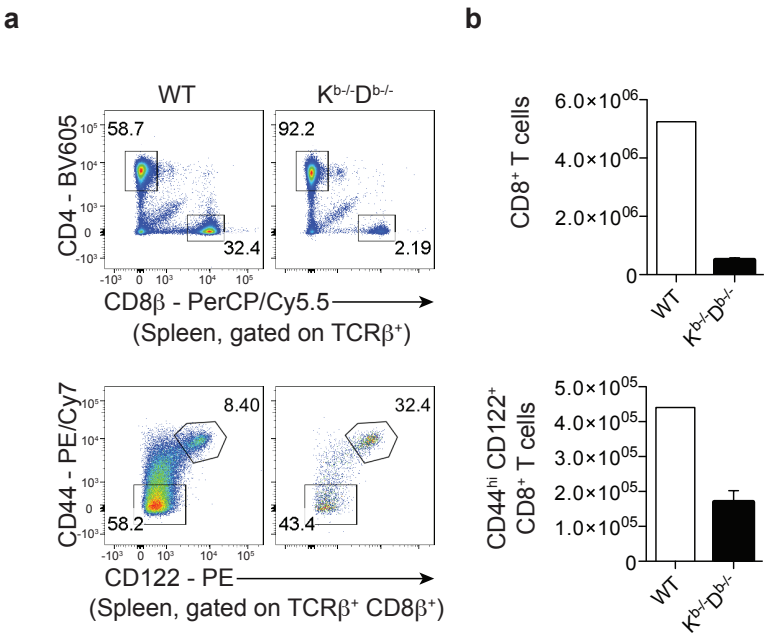

Figure 8. The number of CD8-MP cells is substantially reduced in $K^{b-/-}D^{b-/-}$ mice.

a, Representative flow-cytometric analysis of CD4 vs. CD8β expression by T cells (top) or CD44 vs. CD122 expression by $CD8\beta^+$ T cells (bottom) from the spleens of 6-week-old C57BL/6 (WT) and $K^{b-/-}D^{b-/-}$ mice. The percentage of cells falling in the indicated gates is denoted. **b**, Summary plot of the absolute number of $CD8^+$ T cells (top) or $CD44^{hi}$ $CD122^+$ expressing $TCR\beta^+$ $CD8\beta^+$ T cells (bottom) from spleens of the indicated 6-week-old mice. Mean ± SEM is indicated.

hypothesized that endogenous CD8-MP cells are reactive to endogenous self-ligands presented on classical MHC-I molecules. In TCRrg mice, we found that CD8-MP clones were equally distributed in all secondary lymphoid organs examined (Figure 9) and exhibited elevated percentages of proliferative cells as measured by Ki67 staining, suggestive of active sensing of ligands at steady state (Figure 10a-c). To gain insight into the antigen specificity of CD8-MP clones, we assessed the *ex vivo* reactivity of purified naïve-phenotype ($CD44^{lo}$ $CD122^{-}$) $CD8^{+}$ T cells isolated from TCRrg mice expressing CD8-MP or CD8-Naïve TCRs. We purified naïve-phenotype $CD44^{lo}$ $CD122^{-}$ from TCRrg mice expressing each of the eight TCRs, and used these cells as a probe for antigen in *in vitro* stimulation assays. We found that the four CD8-MP clones underwent proliferation upon co-culture with splenic dendritic cells (DCs) and recombinant IL-2, whereas the four CD8-Naïve clones did not (Figure 10d,e). This reactivity was abolished by the addition of anti-MHC-I blocking antibodies (Figure 10d,e), indicative of reactivity to ligands displayed by classical MHC-I molecules. Notably, the reactivity of CD8-MP clones was not impaired using splenic DCs isolated from germ-free mice (Figure 10d,e). Thus, our data provide direct evidence that four canonical CD8-MP TCRs examined confer overt reactivity to endogenous self-ligands presented by splenic DCs in the context of classical MHC-I molecules.

Our cumulative data suggest that CD8-MP differentiation is an orchestrated TCR-dependent process driven by reactivity to self-ligands. We next set out to define the stage at which CD8-MP differentiation is triggered. Previous studies report that $CD44^{hi}$ $CD122^{+}$ $CD8^{+}$ T cells first appear in the periphery of neonatal B6 mice[52], suggesting that CD8-MP cells differentiate in the periphery[48,50]. Congruent with this idea, we found that $CD44^{hi}$ $CD122^{+}$ cells were not detected amongst polyclonal GFP^{+} $CD8^{+}$ $CD4^{-}$ ("CD8 single-positive", hereafter referred to as "CD8SP") thymocytes from adult *Rag2*-green fluorescent protein (GFP) reporter mice (Figure 11a), in which

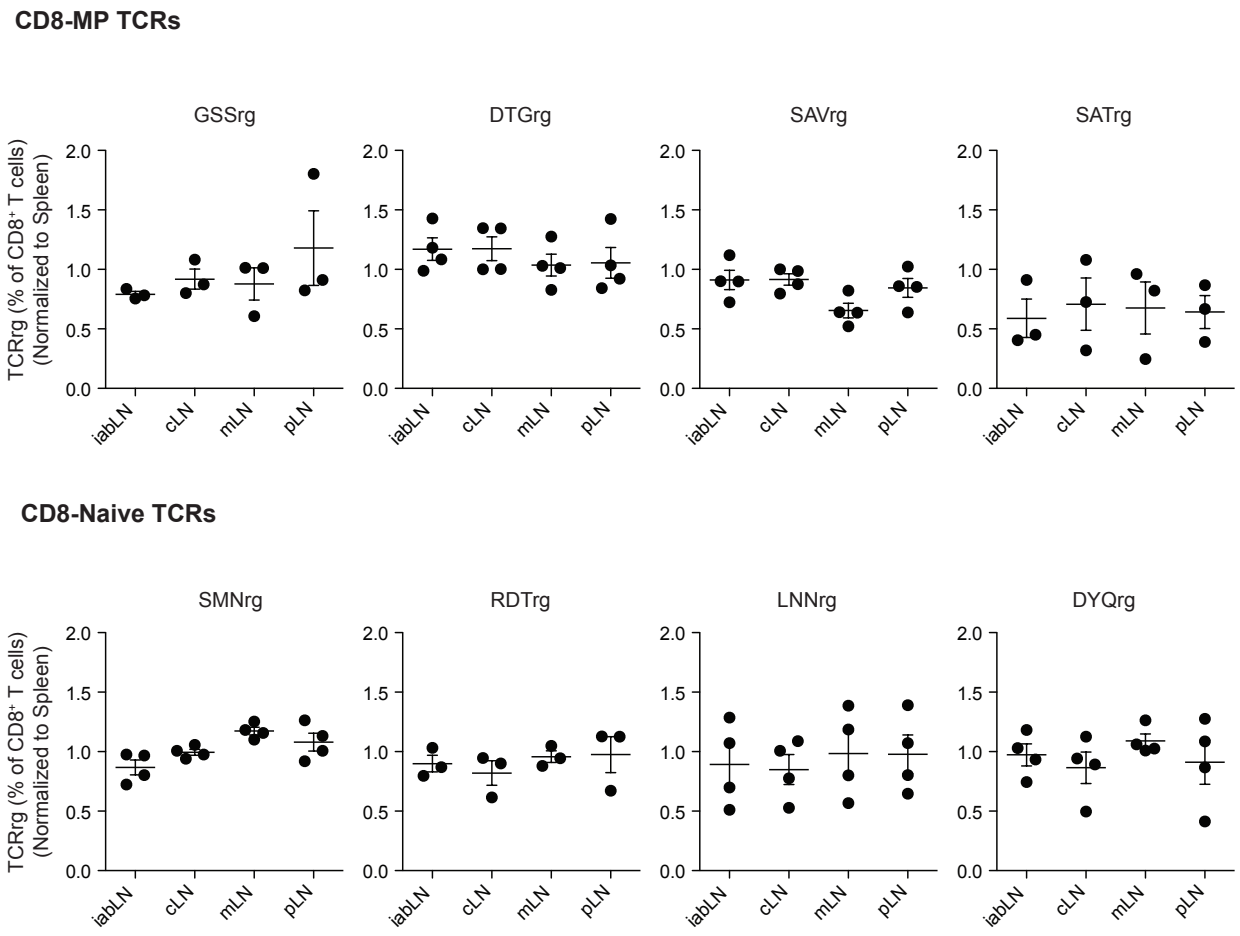

Figure 9. CD8-MP TCRrg cells are broadly distributed across lymphoid sites.

Summary plots of the frequency of Thy1.1 expressing TCRrg cells of TCRβ$^+$ CD8β$^+$ T cells, normalized to the spleen from the indicated lymphoid sites 6-7 weeks after bone marrow reconstitution of the indicated "low frequency" TCRrg mice. Frequencies at different lymphoid sites were normalized to the spleen to control for varying engraftment of TCRrg bone marrow across different mice. iabLN: inguinal, axial, brachial lymph nodes; cLN: cervical lymph nodes; mLN: mesenteric lymph nodes; pLN: periaortic lymph nodes. Each symbol represents an individual TCRrg mouse. n = 3, GSSrg; n = 4, DTGrg; n = 4, SAVrg; n = 3, SATrg; n = 4, SMNrg; n = 3, RDTrg; n = 4, LNNrg; n = 4, DYQrg. Mean ± SEM is indicated. Data is pooled from four independent experiments.

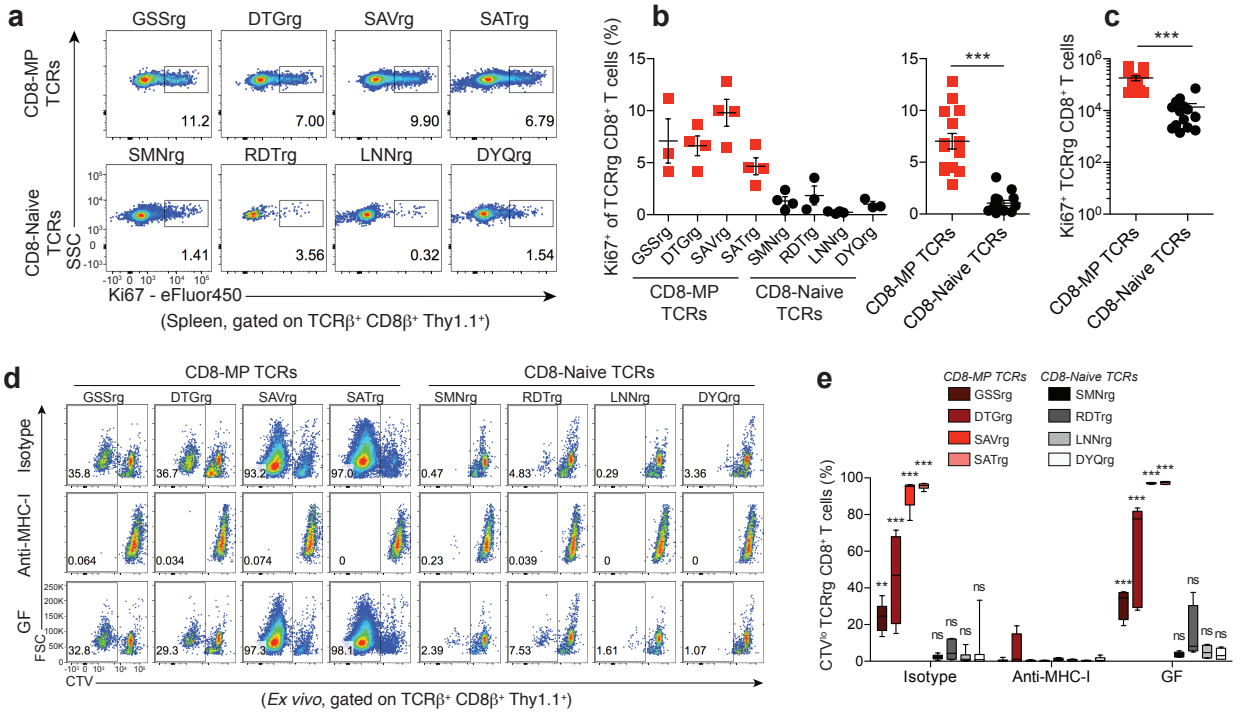

Figure 10. CD8-MP T cell clones exhibit reactivity to MHC class-I restricted self-ligands presented by splenic dendritic cells.

a, Representative flow cytometric analysis of Ki67 expression by TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells from the spleens of TCRrg mice expressing the indicated CD8-MP TCRs (top) and CD8-Naïve TCRs (bottom), analyzed 6 weeks after bone marrow reconstitution. The percentage of cells in the indicated gates is denoted. Data are representative of five independent experiments. **b**, Left: Summary plot of pooled data from (a) showing the frequency of TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells that are positive for Ki67 staining for the listed TCRs. Right: Data from the left panel were pooled from the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 15, CD8-MP TCRrg mice; n = 14, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from five independent experiments. **c**, Summary plot of pooled data from (a) showing the absolute number of TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells that are positive for Ki67 staining for the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 15, CD8-MP TCRrg mice; n = 14, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from five independent experiments. **d**, CD8$^+$ naïve-phenotype (CD44^{lo}CD122^{neg}) T cells were sorted from TCRrg mice expressing the indicated TCRs, labeled with CellTrace-Violet (CTV) and used as a probe for antigen. 1 × 10^4 TCRrg cells were cultured with 5 × 10^4 CD11c$^+$ cells isolated from the spleens of SPF or GF C57BL/6 mice plus rmIL-2. Dilution of CTV was assessed by flow cytometry on day 5. Where indicated, cells were cultured with isotype control antibody or anti-MHC-I blocking antibody. The percentage of cells within the indicated gates is denoted. Data are representative of six independent experiments. **e,** Summary plot of pooled data showing the frequency of divided cells in the indicated co-cultures

Figure 10, continued
from (d). Each sample represents an individual co-culture. The median, upper quartile, and lower quartile are indicated by the box plot and the whiskers indicate the minimum and maximum. GSSrg (n = 6 isotype, n = 6 anti-MHC-I, n = 4 GF), DTGrg (n = 4 isotype, n = 4 anti-MHC-I, n = 7 GF), SAVrg (n = 9 isotype, n = 9 anti-MHC-I, n = 4 GF), SATrg (n = 6 isotype, n = 6 anti-MHC-I, n = 4 GF), SMNrg (n = 6 isotype, n = 6 anti-MHC-I, n = 7 GF), RDTrg (n = 6 isotype, n = 6 anti-MHC-I, n = 4 GF), LNNrg (n = 9 isotype, n = 9 anti-MHC-I, n = 4 GF), DYQrg (n = 7 isotype, n = 7 anti-MHC-I, n = 4 GF). For each TCR, the frequency of divided cells in the isotype control condition (or GF condition) was compared to the proliferation in the anti-MHC-I condition using one-way ANOVA with Bonferroni post-test analysis, comparing all pairs of columns (ANOVA $p < 0.0001$, $F = 103.1$, $df = 143$). Adjusted p-values from the Bonferroni post-test are depicted: n.s., not significant, $p > 0.9999$; **$p = 0.0014$; ***$p < 0.0001$, Data are pooled from six independent experiments.

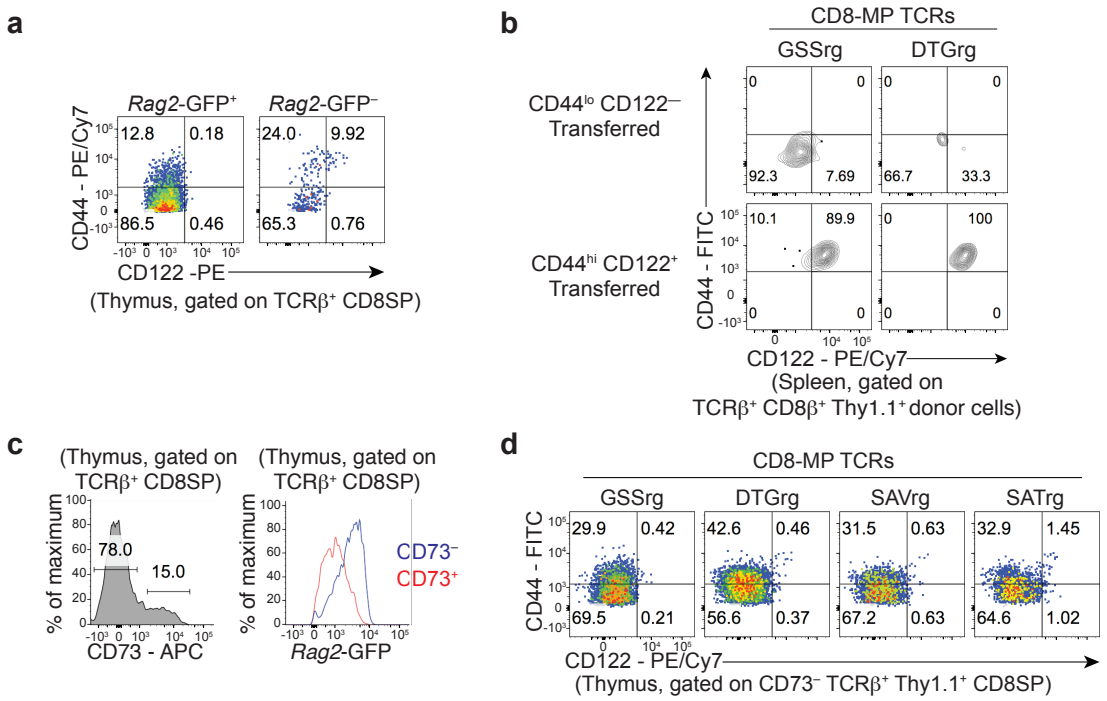

Figure 11. CD8-MP TCRrg cells with a naïve-phenotype do not adopt the CD44^{hi} CD122^{+} phenotype upon transfer to the periphery of wild-type hosts nor do CD8-MP TCRrg cells acquire the CD44^{hi} CD122^{+} phenotype in the thymus.

a, CD44^{hi} CD122^{+} cells are not detected amongst newly developing thymocytes. Representative flow cytometric analysis of CD44 vs. CD122 expression by GFP^{+} (left) or GFP^{-} (right) CD8^{+} T cells isolated from the thymus of a 7-week-old *Rag2*-GFP mouse. *Rag2*-GFP^{+} cells represent newly developing T cells that have recently rearranged their antigen receptors. Cells are gated on TCRβ^{+} CD8SP thymocytes. The percentage of cells within the indicated gates is denoted. Data are representative of five independent experiments. **b**, 2.5 × 10^{5} FACS-purified CD44^{lo} CD122^{-} or CD44^{hi} CD122^{+} CD8^{+} T cells from indicated CD8-MP TCRrg donors were transferred intravenously into congenically disparate CD45.1^{+} recipients. 3-weeks post-transfer, the fate of the donor Thy1.1^{+} cells was assessed in the spleens of recipient mice. Data are representative flow cytometric analysis of CD44 vs. CD122 expression by donor Thy1.1^{+} T cells recovered after transfer. The percentage of cells falling in the indicated gates is denoted. Data are representative of two independent experiments. **c**, Left: Representative flow cytometric analysis of CD73 expression by TCRβ^{+} CD8SP thymocytes from a 7-week-old *Rag2*-GFP mouse. Right: Expression of *Rag2*-GFP on the CD73^{-} and CD73^{+} CD8SP thymocyte populations. The percentage of cells falling in the indicated gates is denoted. Data are representative of two independent experiments. **d**, Representative flow-cytometric analysis of CD44 and CD122 expression by indicated CD73^{-} TCRβ^{+} Thy1.1^{+} CD8SP CD8-MP TCRrg thymocytes 6 weeks after bone marrow reconstitution. The percentage of cells falling in the indicated gates is denoted. Data are representative of five independent experiments.

GFP expression marks newly developing thymocytes that have recently undergone *Rag2*-dependent TCR rearrangement[92].

To address this question at the clonal level, we utilized our TCRrg approach to track the developmental trajectories of distinct $CD8^+$ T cell clones that are destined to adopt a CD8-MP phenotype. To determine whether $CD8^+$ T cell clones acquire the $CD44^{hi}$ $CD122^+$ phenotype in the periphery, we took advantage of the fact that some TCRrg cells expressing CD8-MP TCRs remain phenotypically naive in primary hosts and we purified naïve-phenotype ($CD44^{lo}$ $CD122^-$) and memory-phenotype ($CD44^{hi}$ $CD122^+$) $CD8^+$ T cells isolated from TCRrg mice expressing CD8-MP TCRs and adoptively transferred these cells separately into the periphery of congenically disparate wild-type hosts via intravenous injection. 3 weeks post-transfer, we found that >90% of transferred $CD44^{hi}$ $CD122^+$ TCRrg cells maintained their phenotype, but transferred naïve-phenotype cells failed to upregulate CD44 and CD122 (Figure 11b). Contrary to previously published work[48,50], these results suggest that CD8-MP cells do not differentiate in the periphery, and instead may require some thymic signal for their differentiation. We next considered the possibility that rare thymic-derived CD8-MP cells expand when they arrive in the periphery. For our thymic analyses of TCRrg mice, we used CD73-negativity as a surrogate marker for *Rag2*-GFP^+ cells, as >97% of $CD73^-$ cells are *Rag2*-GFP^+ in the thymus[104] (Figure 11c). Consistent with observations for polyclonal cells, we found that TCRrg cells expressing CD8-MP TCRs did not adopt a $CD44^{hi}$ $CD122^+$ phenotype in the thymus (Figure 11d). However, CD8-MP TCRrg thymocytes exhibited elevated percentages of $Ki67^+$ cells and increased densities of CD5 when compared to CD8-Naïve TCRrg thymocytes, suggestive of elevated TCR signaling in the thymus (Figure 12a-d). Consistent with this observation, we found that $CD11c^+$ DCs isolated from the thymus stimulated the in vitro proliferation of TCRrg cells expressing CD8-MP, but not CD8-

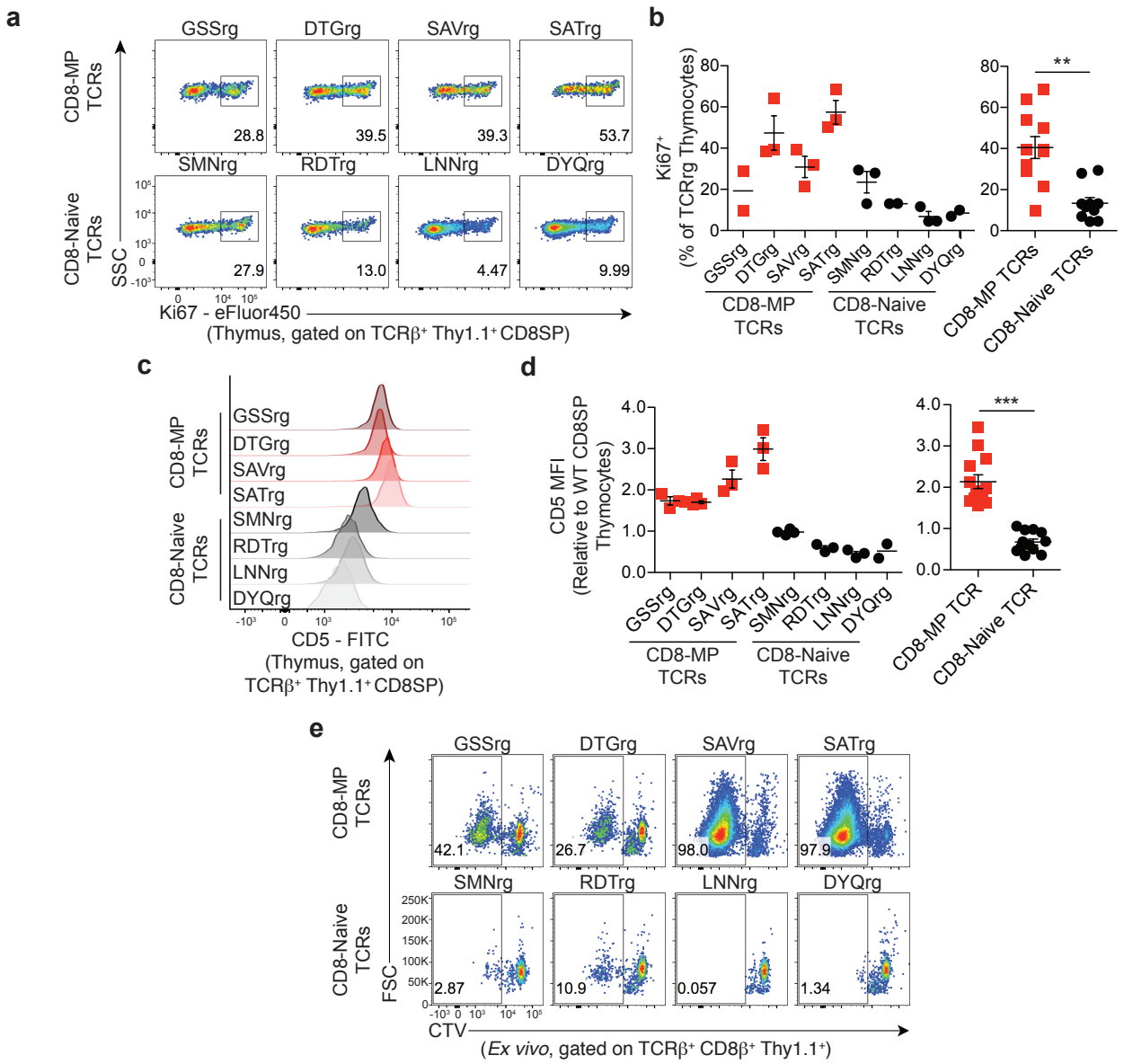

Figure 12. Thymocytes expressing CD8-MP-skewed TCRs exhibit hallmarks of elevated TCR signaling.

a, Representative flow cytometric analysis of Ki67 expression by TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells from the thymi of TCRrg mice expressing the indicated CD8-MP TCRs (top) and CD8-Naïve TCRs (bottom), analyzed 6 weeks after bone marrow reconstitution. The percentage of cells in the indicated gates is denoted. Data are representative of five independent experiments. **b**, Left: Summary plot of pooled data from (a) showing the frequency of TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells that are positive for Ki67 staining for the listed T cell clone. Right: Data from the left panel were pooled from the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 11, CD8-MP TCRrg mice; n = 10, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. **p = 0.0017, two-tailed nonparametric Mann-Whitney test. Data are pooled from five

Figure 12, continued
independent experiments. **c**, Representative flow cytometric analysis of CD5 expression by TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells from the thymi of TCRrg mice expressing the indicated CD8-MP and CD8-Naïve TCRs, analyzed 6 weeks after bone marrow reconstitution. The percentage of cells in the indicated gates is denoted. Data are representative of six independent experiments. **d**, Left: Summary plot of pooled data from (c) showing the normalized mean florescence intensity (MFI) of CD5 in TCRrg thymocytes compared to CD8SP thymocytes from a C57BL/6 thymus. Right: Data from the left panel were pooled from the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 13, CD8-MP TCRrg mice; n = 12, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***$p < 0.0001$, two-tailed nonparametric Mann-Whitney test. Data are pooled from six independent experiments. **e**, CD8-MP clones exhibit reactivity to thymic dendritic cells. CD8$^+$ naïve-phenotype (CD44^{lo}CD122$^-$) T cells were sorted from TCRrg mice expressing the indicated TCRs, labeled with CellTrace-Violet (CTV) and used as a probe for antigen. 1×10^4 TCRrg cells were cultured with 5×10^4 CD11c$^+$ cells isolated from the thymi of SPF C57BL/6 mice plus rmIL-2. Dilution of CTV was assessed by flow cytometry on day 5. At analysis, cells are gated on TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ TCRrg cells. The percentage of cells within the indicated gates is denoted. Data are representative of three independent experiments.

Naïve TCRs (Figure 12e), indicating that ligands recognized by CD8-MP clones are displayed by DCs in the thymus. Comparative analysis of TCRrg thymocytes revealed that CD8-MP clones at the $CD4^+$ $CD8^+$ stage displayed reduced densities of the CD4 and CD8 co-receptors (Figure 13), a potential indicator of clonal deletion[105]. However, the findings that CD8-MP clones exhibited negligible staining for cleaved Caspase 3 (a marker of ongoing apoptosis, Figure 13) and were readily detected within polyclonal and monoclonal repertoires suggests that CD8-MP clones are not substantially impacted by clonal deletion.

CD8-MP clones upregulate Eomes in the thymus

Collectively, the above results suggest that CD8-MP cells encounter endogenous self-ligands in the thymus and that naïve CD8-MP cells do not acquire their phenotype in the periphery. Thus, we considered the possibility that CD8-MP differentiation is triggered by the recognition of self-ligands in the thymus, but the upregulation of the $CD44^{hi}$ $CD122^+$ phenotype is delayed until cells emigrate to the periphery. To test this idea, we looked for early hallmarks of CD8-MP differentiation that lie upstream of CD122 upregulation. Our analysis focused on Eomes, a transcription factor that is highly expressed by peripheral CD8-MP cells, is required for the differentiation and/or survival of CD8-MP cells[53], and promotes CD122 upregulation by direct binding to the *Il2rb* promoter[106]. Strikingly, in TCRrg mice expressing CD8-MP TCRs, a fraction of TCRrg CD8SP thymocytes upregulated expression of Eomes, whereas Eomes upregulation was not observed for TCRrg thymocytes expressing CD8-Naïve TCRs (Figure 14a-c). These findings suggest that the differentiation of many CD8-MP clones is triggered during T cell maturation in the thymus, prior to upregulation of the $CD44^{hi}$ $CD122^+$ phenotype in the periphery. To examine

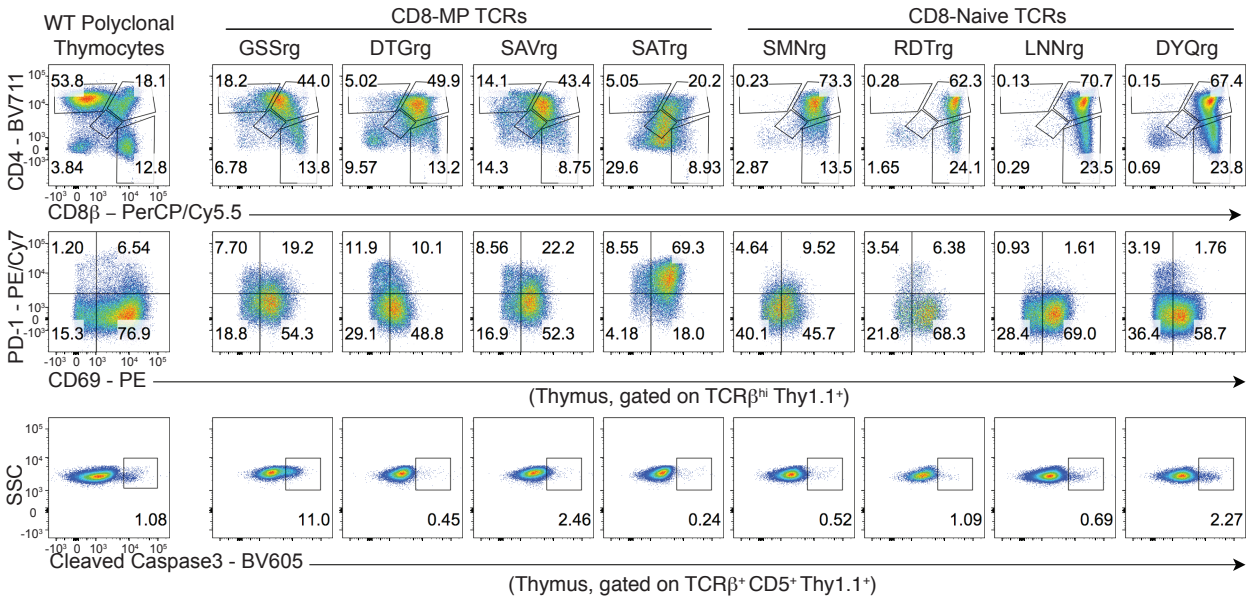

Figure 13. CD8-MP TCRrg cells do not exhibit hallmarks of clonal deletion.

Representative flow cytometric analysis of CD4 vs. CD8, PD-1 vs. CD69, and cleaved Caspase3 expression by TCRβ$^+$ CD8β$^+$ Thy1.1$^+$ cells from the thymi of TCRrg mice expressing the indicated CD8-MP TCRs and CD8-Naïve TCRs, analyzed 6 weeks after bone marrow reconstitution. The percentage of cells in the indicated gates is denoted. Data are representative of three independent experiments.

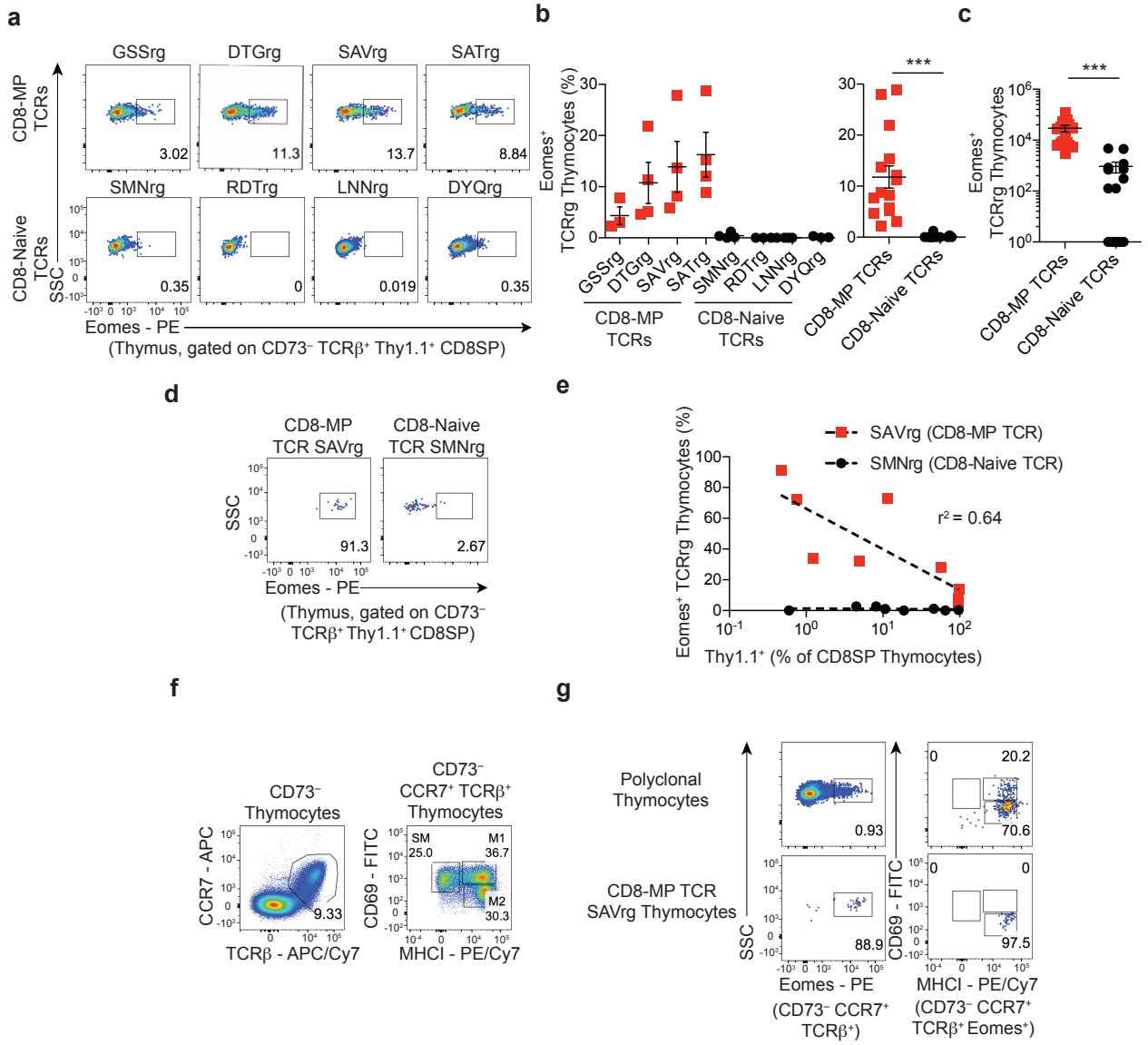

Figure 14. Thymocytes expressing CD8-MP TCRs upregulate Eomes during thymic maturation.

a, Representative flow cytometric analysis of Eomes expression by CD73⁻ TCRβ⁺ Thy1.1⁺ CD8SP TCRrg thymocytes expressing the indicated TCRs, analyzed 6 weeks after bone marrow reconstitution. The percentage of cells within the indicated gates is denoted. Data are representative of five independent experiments. **b**, Left: Summary plot of pooled data from (a) showing the frequency of TCRrg CD8SP thymocytes expressing Eomes for the indicated TCRs. Right: Data from the left panel were pooled from the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse. n = 15, CD8-MP TCRrg mice; n = 14, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from five independent experiments. **c**, Summary plot of pooled data from (a) showing the absolute number of TCRrg CD8SP thymocytes expressing Eomes for the CD8-MP TCRs and the CD8-Naïve TCRs. Each symbol represents an individual TCRrg mouse.

Figure 14, continued
n = 15, CD8-MP TCRrg mice; n = 14, CD8-Naïve TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from five independent experiments. **d**, Representative flow cytometric analysis of Eomes expression by $CD73^{-}$ $TCR\beta^{+}$ $Thy1.1^{+}$ CD8SP TCRrg cells from "low frequency" TCRrg mice expressing the indicated TCRs, assessed 6 weeks after bone marrow reconstitution. The percentage of cells within the indicated gates is denoted. Data are representative of three independent experiments. **e**, Summary plot of multiple TCRrg mice from (d) demonstrating a "niche effect" of Eomes expression in developing TCRrg thymocytes. For the SAV and SMN TCRs, the percentage of TCRrg cells that express Eomes is plotted versus the frequency of $Thy1.1^{+}$ TCRrg thymocytes as a percentage of all CD8SP thymocytes. Dashed lines indicate best-fit semi-log curves. Data are pooled from nine independent experiments. **f**, Representative flow cytometric plots of CD69 vs. MHCI expression for $CD73^{-}$ $CCR7^{+}$ $TCR\beta^{+}$ thymocytes demonstrating the gating strategy to identify three subsets of medullary thymocytes, as described previously[108]. SM, semi-mature; M1, mature 1; M2, mature 2. The percentage of cells within the indicated gates is denoted. Data are representative of two independent experiments. **g**, Left: Representative flow cytometric analysis of Eomes expression by $CD73^{-}$ $CCR7^{+}$ $TCR\beta^{+}$ polyclonal (top) or monoclonal CD8-MP SAVrg (bottom) thymocytes. Right: Representative analysis of CD69 vs. MHCI expression by Eomes expressing cells. The percentage of cells within the indicated gates is denoted. Data are representative of two independent experiments.

this phenomenon further, we determined whether there are saturable niches driving Eomes upregulation by select CD8-MP clones during thymic maturation, as has been described for Foxp3$^+$ regulatory T cells[32,107]. To do this, we generated a series of low-frequency TCRrg mice expressing the SAV CD8-MP TCR, and calculated the extent of Eomes upregulation at varying clonal frequencies. This approach demonstrated that the fraction of TCRrg thymocytes expressing Eomes increased with decreasing clonal frequency (peaking at >90%, Figure 14d,e), suggesting the existence of a saturable niche supporting Eomes upregulation by the SAV CD8-MP clone. In contrast, we did not observe this phenomenon with the control SMN CD8-Naïve TCR (Figure 14d,e), further highlighting the TCR-dependency of Eomes upregulation.

Next, we performed additional phenotypic analyses to examine hallmarks of antigen sensing at different stages of thymic maturation. To do this, we used a staging approach defined previously[108], in which post-selection TCRβ^{hi} CCR7$^+$ thymocytes are sub-divided into cells of progressing maturational stage, ranging from semi-mature (CD69$^+$ MHC-I$^–$), mature-1 (CD69$^+$ MHC-I$^+$), and mature-2 (CD69$^–$ MHC-I$^+$) (Figure 14f). Notably, we found that for developing polyclonal T cells and the CD8-MP SAVrg clone, Eomes$^+$ cells primarily exhibited a CD69$^–$ MHC-I$^+$ mature-2 phenotype (Figure 14g), suggesting that Eomes expression is induced in the latest stages of thymic maturation, when cells are known to reside in the medulla.

Eomes identifies polyclonal thymic precursors of CD8-MP T cells

Next, we aimed to determine whether the principles observed using our clonal approach extended to polyclonal T cell populations in wild-type mice. Specifically, we hypothesized that Eomes-expressing CD8SP thymocytes represent thymic intermediates that are destined to upregulate the CD44^{hi} CD122$^+$ CD8-MP phenotype following emigration to the periphery. To test

this hypothesis, we purified Eomes-GFP^{+} or Eomes-GFP^{-} CD73^{-} CD69^{-} CD8SP thymocytes from 4-week-old Eomes-GFP reporter mice[93], and transferred these cells separately into the periphery of congenically disparate wild-type hosts via intravenous injection. 3 weeks post-transfer, we found that ~47% of the Eomes-GFP^{+} donor cells recovered from recipient mice exhibited a CD44^{hi} CD122^{+} phenotype, compared to ~7% of Eomes-GFP^{-} donor cells (Figure 15a-c), indicating that Eomes-expressing CD8SP thymocytes are enriched for thymic intermediates with the potential to upregulate the CD8-MP phenotype in the periphery. To determine whether Eomes^{+} CD8SP thymocytes upregulate CD44 and CD122 when thymic egress is pharmacologically delayed, we utilized FTY720 to block sphingosine 1-phosphate receptor-1 (S1PR1) and prevent thymic egress. We treated B6 mice with FTY720 or PBS control for 5 days and assessed the phenotype of CD8SP thymocytes at day 6. This treatment induced a marked increase in the percentage and number of Eomes-expressing CD8SP thymocytes (Figure 15d-f), but did not induce an increase in the fraction of Eomes^{+} thymocytes that exhibit a CD44^{hi}CD122^{+} phenotype (Figure 15g-i). This finding is consistent with a model in which CD8-MP differentiation is triggered in the thymus, but requires a subsequent consolidation phase that can only be conferred in the periphery.

CD8-MP cells infiltrate murine prostate tumors

As introduced above, there remains a lack of available markers to distinguish CD8-MP cells from conventional CD8^{+} effector or memory T cells in the context of immune activation. Thus, the contribution of CD8-MP cells to the immune response to human and murine cancers is undefined. To examine this question from a unique perspective, we co-transferred congenically disparate polyclonal CD8-Naive and polyclonal CD8-MP cells into 2-month-old TRAMP males, in which transgenic expression of a model oncogene drives the development of prostatic

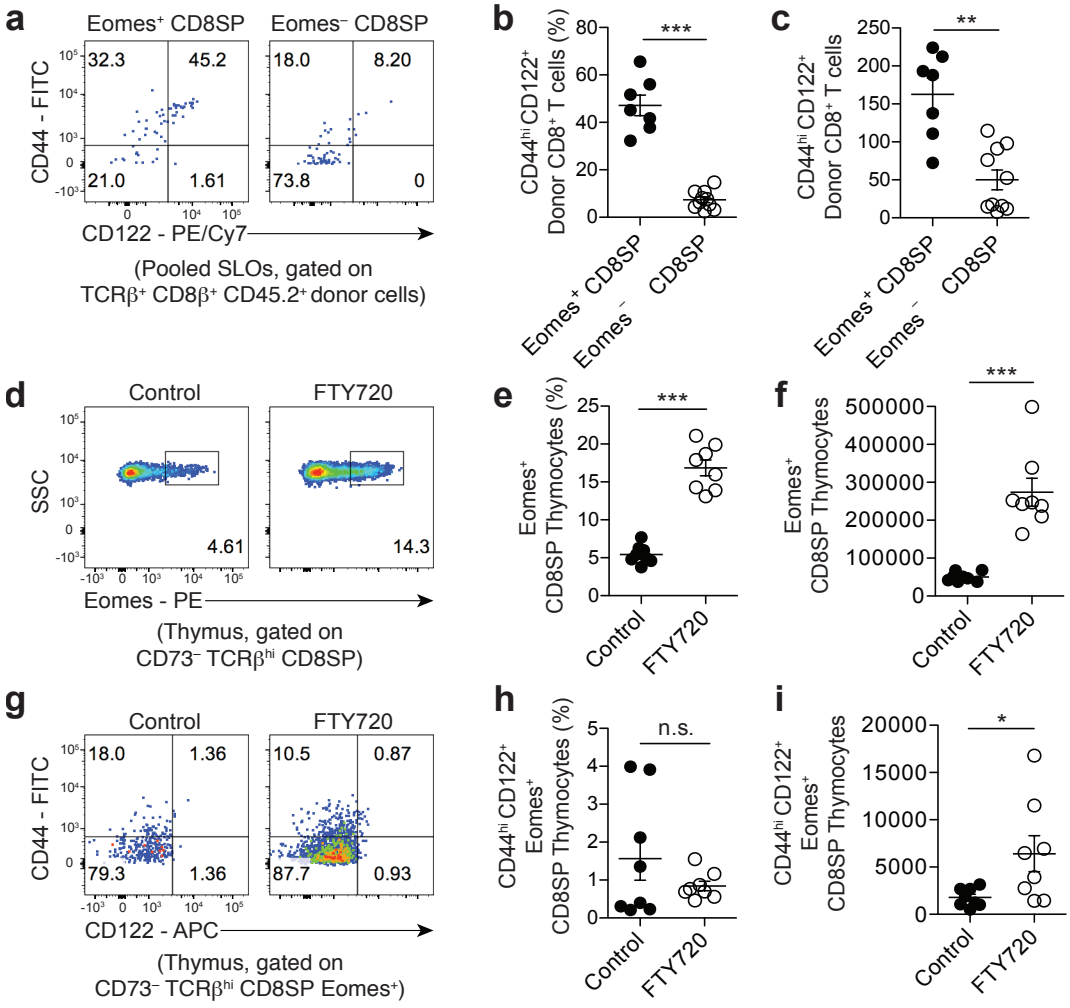

Figure 15. Eomes identifies polyclonal thymic precursors of CD8-MP T cells.

a-c, 1 × 10^5 FACS-purified Eomes-GFP$^+$ or Eomes-GFP$^-$ CD73$^-$ CD69$^-$ mature CD8SP thymocytes from the thymi of 4-week-old Eomes-GFP reporter mice were transferred intravenously into congenically disparate CD45.1$^+$ recipients. 3-weeks post-transfer, the fate of the donor CD45.2$^+$ cells was assessed in the pooled spleen and lymph nodes of recipient mice.
a, Representative flow cytometric analysis of CD44 vs. CD122 expression by donor CD45.2$^+$ T cells recovered after transfer. The percentage of cells falling in the indicated gates is denoted. Data are representative of three independent experiments. **b**, Summary plot of pooled data from (a) showing the frequency of recovered TCRβ$^+$ CD8β$^+$ CD45.2$^+$ cells exhibiting a CD44^{hi}CD122$^+$ phenotype for the indicated donor cells. Each symbol represents an individual mouse. n = 7, Eomes-GFP$^+$ CD8SP; n = 10, Eomes-GFP$^-$ CD8SP. Mean ± SEM is indicated. ***p = 0.0001, two-tailed nonparametric Mann-Whitney test. Data are pooled from three independent experiments. **c**, Summary plot of pooled data from (a) showing the absolute number of recovered TCRβ$^+$ CD8β$^+$ CD45.2$^+$ cells exhibiting a CD44^{hi}CD122$^+$ phenotype for the indicated donor cells. Each symbol represents an individual mouse. n = 7, Eomes-GFP$^+$ CD8SP; n = 10, Eomes-GFP$^-$

Figure 15, continued
CD8SP. Mean ± SEM is indicated. **p = 0.0020, two-tailed nonparametric Mann-Whitney test. Data are pooled from three independent experiments.
d-i, 5-week-old B6 mice were treated with 7 mg/ kg FTY720 or PBS control I.P. every other day for 5 days. On day 6 the thymocytes of the mice were assessed.
d, Representative flow cytometric analysis of Eomes expression by $CD73^{-}$ $TCR\beta^{+}$ CD8SP thymocytes in B6 mice receiving the indicated treatments. The percentage of cells falling in the indicated gates is denoted. Data are representative of two independent experiments. **e**, Summary plot of pooled data from (d) showing the frequency of $CD73^{-}$ $TCR\beta^{+}$ CD8SP thymocytes exhibiting an $Eomes^{+}$ phenotype in B6 mice receiving the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± SEM is indicated. ***p = 0.0002, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **f**, Summary plot of pooled data from (d) showing the absolute number of $CD73^{-}$ $TCR\beta^{+}$ CD8SP thymocytes exhibiting an $Eomes^{+}$ phenotype in B6 mice receiving the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± SEM is indicated. ***p = 0.0002, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **g**, Representative flow cytometric analysis of CD44 vs. CD122 expression by $CD73^{-}$ $TCR\beta^{+}$ CD8SP $Eomes^{+}$ thymocytes in B6 mice receiving the indicated treatments. The percentage of cells falling in the indicated gates is denoted. Data are representative of two independent experiments. **h**, Summary plot of pooled data from (g) showing the frequency of $CD73^{-}$ $TCR\beta^{+}$ CD8SP $Eomes^{+}$ thymocytes exhibiting a $CD44^{hi}CD122^{+}$ phenotype in B6 mice receiving the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± SEM is indicated. n.s., not significant, p = 0.9591, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **i**, Summary plot of pooled data from (g) showing the absolute number of $CD73^{-}$ $TCR\beta^{+}$ CD8SP $Eomes^{+}$ thymocytes exhibiting a $CD44^{hi}CD122^{+}$ phenotype in B6 mice receiving the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± SEM is indicated. *p = 0.0148, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments.

adenocarcinoma with high penetrance[109]. Four months later, when the mice developed advanced prostate tumors, we analyzed the fate of the transferred cells. We found that donor CD8-MP cells constituted a substantial fraction of the tumor-infiltrating $CD8^+$ T cells, ranging from 2-17% of intratumoral $CD8^+$ T cells (Figure16a-f). Strikingly, the majority of donor CD8-MP cells expressed high densities of the inhibitory receptor PD-1 (Figure 16a-f). To further address the role of CD8-MP cells in anti-tumor immunity, we utilized our TCR sequencing approach to determine the extent to which CD8-MP-skewed clones contribute to the pool of tumor-infiltrating lymphocytes (TILs) in TRAMP mice. To do this, we isolated $CD8^+$ T cells from the prostate tumors of five 27-week-old $TRAMP^{+/-}$ males expressing the fixed TCRβtg chain, and subjected these samples to deep *Tcra* sequencing. The survey identified numerous $CD8^+$ T cell clones that are recurrently enriched in TRAMP prostate tumors (Figure 16g, bottom). In addition, by examining the frequency of these intratumoral clones in the CD8-MP and CD8-Naive TCR data sets derived from the secondary lymphoid organs of tumor-free mice, we found that two of the ten most prevalent intratumoral clones were skewed to the CD8-MP subset (Figure 16g, red boxes), and that the 8 remaining clones were rare within both the CD8-MP and CD8-Naive data sets. Notably, one of these CD8-MP-skewed clones was the "SAT" (AASATNAYKVI) clone examined elsewhere in this study. Thus, the use of comparative TCR profiling revealed that TRAMP prostate tumors drive the recurrent enrichment of self-specific "tumor-associated" CD8-MP clones that are uncommon in the periphery but are selectively enriched in prostate tumors. These collective findings demonstrate that recurrent CD8-MP clones make measurable contributions to the tumor-infiltrating T cell pool in TRAMP mice and express high densities of PD-1, suggesting that intratumoral CD8-MP cells may functionally impact anti-tumor immunity and may be directly impacted by anti-PD-1 or anti-PD-L1 checkpoint blockade antibodies.

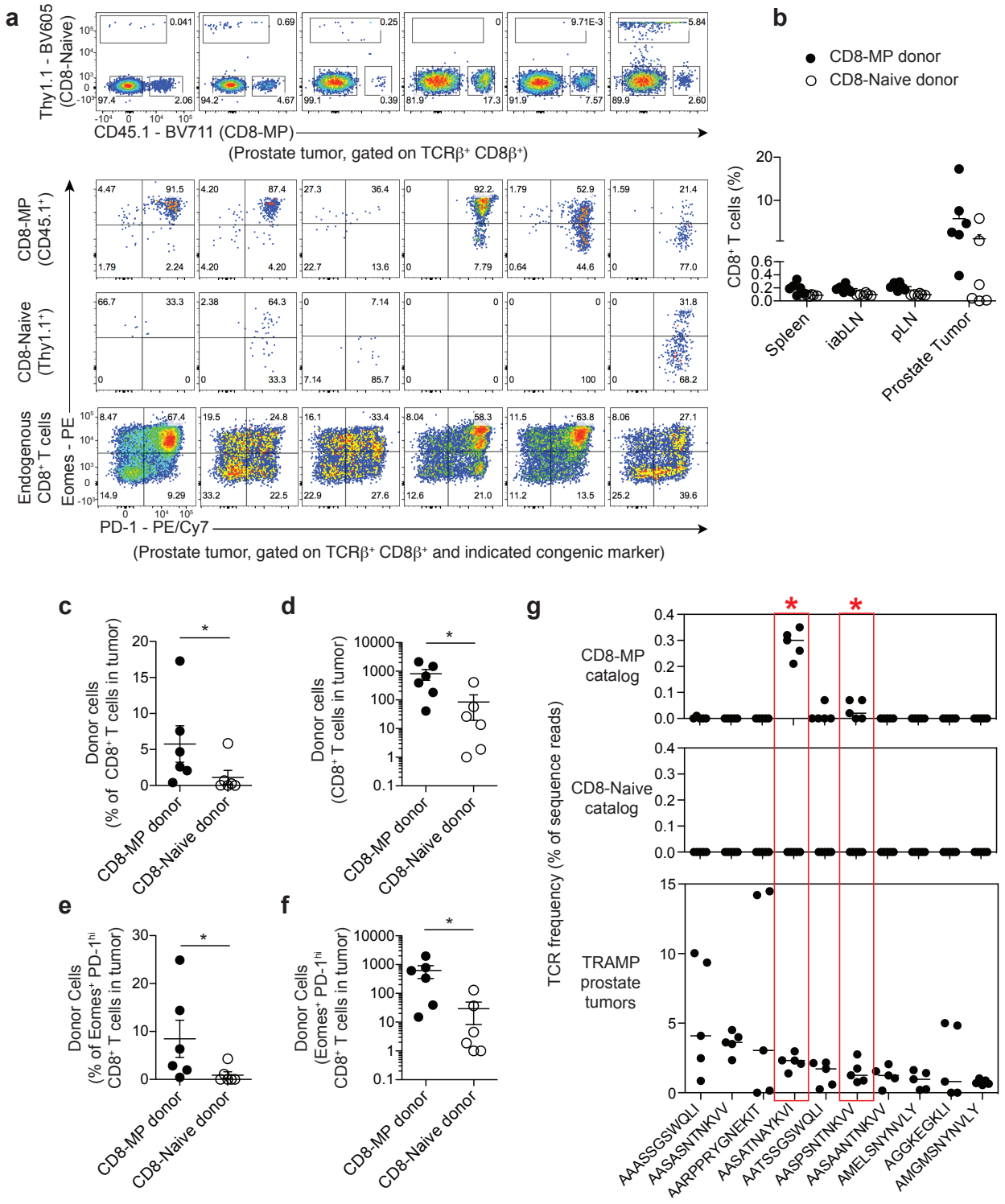

Figure 16. CD8-MP cells infiltrate TRAMP prostate tumors and express high densities of PD-1.

Figure 16, continued

a-f, 1 × 10^6 FACS-purified Thy1.1$^+$ CD8-Naïve (CD44^{lo}CD122$^-$ Eomes-GFP$^-$) and 1 × 10^6 CD45.1$^+$ CD8-MP (CD44^{hi}CD122$^+$) CD8$^+$ T cells from pooled spleen and lymph nodes of 2-month-old Thy1.1$^+$ Eomes-GFP reporter mice or CD45.1$^+$ B6.SJL mice were co-transferred intravenously into congenically disparate CD45.2$^+$ TRAMP$^{+/+}$ male recipients. 4 months later, the fate of the donor cells was assessed in the prostate tumors of recipient mice.

a, (Top) Flow cytometric analysis of congenic marker (Thy1.1 vs. CD45.1) expression by CD8$^+$ T cells infiltrating the six prostate tumors. The percentage of cells falling in the indicated gates is denoted. (Bottom) Flow cytometric analysis of Eomes vs. PD-1 expression by TCRβ$^+$ CD8β$^+$ T cells from indicated congenically marked populations infiltrating the six prostate tumors. The percentage of cells falling in the indicated gates is denoted. Data are pooled from two independent experiments. **b**, Summary plot of pooled data of the frequency of CD8-MP (CD45.1$^+$) and CD8-Naïve (Thy1.1$^+$) TCRβ$^+$ CD8β$^+$ T cells from indicated lymphoid sites of TRAMP$^{+/+}$ recipients. Each symbol represents an individual mouse, n = 6. Mean ± SEM is indicated. Data are pooled from two independent experiments. **c**, Summary plot of pooled data of the frequency of CD8-MP (CD45.1$^+$) and CD8-Naïve (Thy1.1$^+$) TCRβ$^+$ CD8β$^+$ T cells infiltrating prostate tumors. Each symbol represents an individual mouse, n = 6. Mean ± SEM is indicated. *p = 0.0411, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **d**, Summary plot of pooled data of the absolute number of CD8-MP (CD45.1$^+$) and CD8-Naïve (Thy1.1$^+$) TCRβ$^+$ CD8β$^+$ T cells infiltrating prostate tumors. Each symbol represents an individual mouse, n = 6. Mean ± SEM is indicated. *p = 0.0260, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **e**, Summary plot of pooled data of the frequency of CD8-MP (CD45.1$^+$) and CD8-Naïve (Thy1.1$^+$) amongst the Eomes$^+$ PD-1$^+$ TCRβ$^+$ CD8$^+$ T cells infiltrating prostate tumors. Each symbol represents an individual mouse, n = 6. Mean ± SEM is indicated. *p = 0.0295, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **f**, Summary plot of pooled data of the absolute number of CD8-MP (CD45.1$^+$) and CD8-Naïve (Thy1.1$^+$) Eomes$^+$ PD-1$^+$ TCRβ$^+$ CD8β$^+$ T cells infiltrating prostate tumors. Each symbol represents an individual mouse, n = 6. Mean ± SEM is indicated. *p = 0.0200, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments. **g**, (Bottom) CD8$^+$ T cells were isolated from the prostate tumors of N = 5 27-week-old TRAMP$^{+/-}$ males expressing a fixed TCRβ chain (TCRβtg), and subjected to complete TCRα sequencing using the iRepertoire platform. Summary plots of the frequencies of the 10 most prevalent recurrent clones. The predicted CDR3α amino acid sequence of each clone is shown. (Top and middle) For the top 10 recurrent intratumoral clones listed at the bottom, the top and middle plots depict the frequencies of these clones in the CD8-MP and CD8-Naive TCR data sets derived from the secondary lymphoid organs of tumor-free mice (Figure 3). Median is indicated. The asterisks and red boxes highlight the "SAT" and "SPS" clones, which represent clones that are significantly skewed to the CD8-MP subset in tumor-free mice (FDR < 0.05, defined in Figure 3) and recurrently enriched in prostate tumors. Statistical analysis of differential representation in CD8-MP and CD8-Naive subsets was done using EdgeR-based methods.

To further address the role of CD8-MP cells in anti-tumor immunity, we utilized our TCR sequencing approach to determine the extent to which CD8-MP-skewed clones contribute to the pool of tumor-infiltrating lymphocytes (TILs) in TRAMP mice. To do this, we isolated $CD8^+$ T cells from the prostate tumors of five 27-week-old $TRAMP^{+/-}$ males expressing the fixed TCRβtg chain, and subjected these samples to deep *Tcra* sequencing. The survey identified numerous $CD8^+$ T cell clones that are recurrently enriched in TRAMP prostate tumors (Figure 16g, bottom). In addition, by examining the frequency of these intratumoral clones in the CD8-MP and CD8-Naive TCR data sets derived from the secondary lymphoid organs of tumor-free mice, we found that two of the ten most prevalent intratumoral clones were skewed to the CD8-MP subset (Figure 16g, red boxes), and that the 8 remaining clones were rare within both the CD8-MP and CD8-Naive data sets. Notably, one of these CD8-MP-skewed clones was the "SAT" (AASATNAYKVI) clone examined elsewhere in this study. Thus, the use of comparative TCR profiling revealed that TRAMP prostate tumors drive the recurrent enrichment of self-specific "tumor-associated" CD8-MP clones that are uncommon in the periphery but are selectively enriched in prostate tumors. These collective findings demonstrate that recurrent CD8-MP clones make measurable contributions to the tumor-infiltrating T cell pool in TRAMP mice and express high densities of PD-1, suggesting that intratumoral CD8-MP cells may functionally impact anti-tumor immunity and may be directly impacted by anti-PD-1 or anti-PD-L1 checkpoint blockade antibodies.

Taken together, our findings demonstrate that CD8-MP differentiation is a robust TCR-directed process that is triggered by the recognition of self-ligands in the thymus, that Eomes identifies thymic precursors with a propensity to upregulate the CD8-MP phenotype in the periphery, and that CD8-MP cells make measurable contributions to the immune infiltrate of autochthonous prostate tumors.

Discussion

Our cumulative findings reveal the unexpected finding that CD8-MP differentiation parallels the development of Foxp3$^+$ Treg cells in several respects. Specifically, CD8-MP differentiation is a TCR-instructed process that is triggered by the recognition of self-ligands in the thymus, occurs optimally at low clonal frequencies[32,107], and involves a two-step process marked by an initial TCR-dependent triggering step followed by a second phase of consolidation[110,111]. These findings challenge a common notion that CD8-MP cells represent "lymphopenia-induced memory" T cells that differentiate from naive CD8$^+$ T cells in the periphery[48,50]. The finding that hundreds of recurrent CD8$^+$ T cell clones, constituting ~65% of the CD8-MP repertoire, strongly and reproducibly segregate to the CD8-MP subset demonstrates that TCR-dependent differentiation is a broad principle of most CD8-MP cells in B6 mice, and implies the existence of dedicated mechanisms coordinating the differentiation of these cells. Given our data showing that many CD8-MP clones exhibit overt reactivity to self-ligands, we hypothesize that the robust segregation of CD8-MP-biased clones to the CD8-MP subset is critical for removing overtly self-reactive CD8$^+$ T cells from the naive compartment. Lastly, while TCR-independent factors such as IL-15 contribute to CD8-MP differentiation or survival[53,54], they are not sufficient to direct CD8-MP differentiation in unprimed mice, as only select CD8$^+$ T cell clones will adopt the CD8-MP phenotype within the endogenous repertoire.

Our data demonstrate that some CD8-MP clones display overt reactivity to classical MHC-I-restricted ligands displayed by both thymic DCs and splenic DCs from germ-free mice. Despite this, CD8-MP clones readily populate the peripheral repertoire and are not substantially impacted

by clonal deletion. This suggests either that the TCR/pMHC-I binding properties that trigger CD8-MP differentiation are distinct from the properties that drive clonal deletion, or that unique contextual cues (such as anatomical region, identity of the antigen presenting cell, or thymocyte maturational stage) trigger CD8-MP differentiation without inducing extensive deletion.

Previous studies using pMHC-I tetramer enrichment assays demonstrated that in unprimed mice, a percentage of $CD8^+$ T cells reactive to any given foreign pMHC-I complex display a $CD44^{hi}CD122^+$ phenotype[52,68], and exhibit innate-like effector functions in the early stages of pathogen challenge[68,98]. Based on our findings presented here, we suggest that foreign pMHC-I-specific CD8-MP cells in unprimed mice likely represent CD8-MP clones that were selected on self-pMHC-I ligands, but cross-react with foreign pMHC-I ligands due to the plasticity inherent in TCR-pMHC ligand recognition[112]. Thus, we suggest that selection on endogenous self-ligands plays a critical role in poising CD8-MP cells for rapid recruitment into the early phases of an immune response[68,98].

Seminal studies of $CD8^+$ T cell memory differentiation and survival examined the biology of lymphopenia-induced memory (LIM) cells, a population of $CD44^{hi}CD122^+$ T cells that emerge following the intravenous transfer of mature naive-phenotype $CD8^+$ T cells into lymphopenic hosts[113]. Based on the fact that the phenotypic and functional properties of CD8-MP cells are similar to those of LIM cells, it has been tempting to equate these populations. However, two pieces of evidence presented here suggest that naturally occurring CD8-MP cells in B6 mice are distinct from LIM cells. First, we find that the differentiation of many CD8-MP clones is initiated in the thymus, suggesting that the forces driving LIM cell differentiation in the periphery may not be relevant for many naturally occurring CD8-MP clones. Second, our finding that CD8-MP differentiation is a TCR-instructed process, with hundreds of CD8-MP-biased clones exhibiting

robust segregation to the CD8-MP subset, suggests that the naive-phenotype donor cells used in studies of LIM cell differentiation are likely depleted of the relevant TCRs that are naturally expressed by CD8-MP cells, implying that the antigen specificities of CD8-MP cells and LIM cells are inherently distinct.

Lastly, our transfer experiments and comparative TCR profiling approach revealed that CD8-MP cells are recurrently enriched in autochthonous prostate tumors in TRAMP mice and adopt a PD-1^{hi} $Eomes^{+}$ phenotype within tumors. Previously, the contribution of CD8-MP cells to the anti-tumor immune response in human or murine cancers had been largely undefined due to the lack of available markers or signatures to identify CD8-MP cells and distinguish them from bona fide tumor-specific $CD8^{+}$ T cells. In this regard, an expanding body of evidence suggests that human tumors are infiltrated by a phenotypically diverse array of $CD8^{+}$ T cells[86–90], and that only a minor fraction of such cells are overtly reactive to tumor-expressed antigens[84,85]. Given our findings presented here, we hypothesize that self-specific CD8-MP cells, selected on endogenous self-ligands in the thymus, make substantial contributions to the infiltrate of human and murine cancers and may be directly impacted by checkpoint blockade antibodies targeting the PD-1 axis. Elucidation of the functional role of CD8-MP cells in the tumor context will require the identification of unique markers that are exclusively expressed by tumor-associated CD8-MP cells, plus the development of loss-of-function approaches for the selective depletion of CD8-MP cells.

DISCUSSION

Overview

In this work, we examined the TCR repertoire of endogenous CD8-MP cells in B6 mice with a fixed TCRβ chain and found that the TCR repertoire of CD8-MP cells is distinct from the repertoire of CD8-Naïve cells and recurrent across mice. Using a TCR retrogenic approach, in which TCR chains of interest are retrovirally expressed to track T cell clones of interest, we found that CD8-MP differentiation is a TCR directed process triggered by overt reactivity to self-antigens in the thymus. Taking this clonal approach, we found that CD8-MP differentiation is a tightly controlled, multi-step process, triggered in the thymic medulla where CD8-MP precursors can be identified by their Eomes expression, and completed in the periphery with upregulation of classical memory markers CD44 and CD122.

Despite their overt reactivity to self-ligands, the CD8-MP clones examined thus far do not exhibit overt hallmarks of clonal deletion in the thymus and they readily populate the peripheral repertoire at high frequency. Despite their autoimmune potential, T cells bearing CD8-MP TCRs do not readily infiltrate non-lymphoid organs at homeostasis and are $CD62L^{hi}$. We speculate that CD8-MP differentiation protects the host by shunting autoreactive $CD8^{+}$ T cells into the CD8-MP program. This CD8-MP lineage is defined by markers of central memory, which retains these cells in the lymphoid tissues and prevents these cells from entering non-lymphoid organs during homeostasis. In contrast to their seemingly inert role during homeostasis, CD8-MP cells are selectively recruited into inflammatory murine prostate tumors and express high densities of the inhibitory receptor PD-1, suggestive of a possible role in immunotherapies that utilize checkpoint blockade.

Endogenous CD8-MP cells as a distinct lineage of self-reactive T cells

The finding that the TCR repertoire expressed by peripheral CD8-MP cells is largely distinct from that of CD8-Naïve cells, coupled with our data showing that CD8-MP-biased TCRs facilitate CD8-MP differentiation in TCRrg mice, demonstrates that the differentiation of many CD8-MP clones is a TCR-directed process driven by ligand recognition. Thus, while TCR-independent factors such as IL-15 contribute to CD8-MP differentiation or survival[53,54], they are not sufficient to direct CD8-MP differentiation in unprimed mice, as only select $CD8^+$ T cell clones will adopt the CD8-MP phenotype within the endogenous repertoire. TCR profiling also revealed that the segregation of distinct clones into the CD8-MP subset is both highly reproducible from mouse to mouse, and is robust, with complete or near-complete skewing observed for hundreds of CD8-MP-biased clones. Thus, the differentiation of T cells into the CD8-MP subset does not represent the sporadic expansion of varying clones, but instead reflects the orchestrated differentiation of hundreds of $CD8^+$ T cell clones into the CD8-MP subset, suggestive of the existence of strong immunological mechanisms coordinating CD8-MP differentiation.

The finding that CD8-MP differentiation is a robust TCR-dependent differentiation process that begins in the thymus, at the earliest point in the life of a T cell, suggests that CD8-MP differentiation is an important evolutionary adaptation. We hypothesize that the robust segregation of CD8-MP biased clones to the CD8-MP subset is due to their overt reactivity to self-antigens. Prior to this work, little was known about the nature of ligands recognized by CD8-MP cells. Early evidence suggested that CD8-MP cells may exhibit some degree of reactivity to endogenous ligands, based on observations that the frequency of CD8-MP cells is not diminished in germ-free mice, and that CD8-MP cells express slightly higher average densities of CD5 relative to CD8-Naïve cells[54]. However, given that CD5 expression is largely determined by reactivity to low-

potency ligands during positive selection[55–57], and that the CD5 expression levels of polyclonal CD8-MP and CD8-Naïve cells overlap substantially[54], CD5 is not a reliable measure of overt self-reactivity by endogenous CD8-MP cells. Here we present direct evidence that multiple CD8-MP clones exhibit overt reactivity to widespread classical MHC-I-restricted ligands displayed by both thymic DCs and splenic DCs from germ-free mice. Linking these observations with our TCR profiling data, we hypothesize that the robust segregation of CD8-MP biased clones to the CD8-MP subset is critical for removing overtly self-reactive $CD8^+$ T cells from the naive compartment. If this indeed proves to be the case, dysregulation or perturbation of CD8-MP differentiation in could potentially predispose humans to the development of autoimmunity.

Future work will aim to identify the self-ligands of CD8-MP cells and the impact of disrupting proper CD8-MP differentiation. In an ongoing collaboration, we are working with the Joglekar lab and using their signaling and antigen-presenting bifunctional receptor (SABR) platform[114] to identify bona fide self-antigens recognized by our canonical CD8-MP TCRrg clones. Once specific peptides are identified, we can genetically delete these antigens using a CRISPR/Cas9 approach and assess the impact on the development and function of individual CD8-MP clones, and develop peptide/MHC-I tetramer reagents to characterize endogenous CD8-MP populations in context of health and disease.

Future work will aim to test directly whether the CD8-MP differentiation program is required to prevent overtly self-reactive $CD8^+$ T cells from causing autoimmunity and whether dysregulation of CD8-MP differentiation predisposes an individual to autoimmunity. This hypothesis can be tested by examining the consequences of disrupting or disabling CD8-MP differentiation. Targeted disruption of CD8-MP differentiation would require identification of specific factors required for CD8-MP differentiation. For example, is there a “master regulator” of

CD8-MP cells, akin to Foxp3 in $CD4^+$ regulatory T cells? Previous work examining the transcriptional program of bulk polyclonal CD8-MP cells has found that their program lies somewhere in between naïve $CD8^+$ T cells and conventional memory $CD8^+$ T cells and there are no unique markers that distinguish CD8-MP cells from conventional memory cells[54]. Our identification of a thymic precursor of CD8-MP cells provides a path forward in discovery of unique factors that are specific for CD8-MP cells of thymic origin. In future work, we will generate low frequency TCRrg mice of a known CD8-MP clone and perform RNA-Seq and ATAC-Seq on purified congenically marked TCRrg cells at the various maturation stages during thymic development, from the preselection DN stage and DP stages, and the post-selection semi-mature, mature-1 and mature-2 stages[108]. We hypothesize that this unique approach will more fully define each step of the transcriptional and epigenetic program of thymic CD8-MP cells. This future work will aim to identify a potential "master regulator" of thymic CD8-MP cells and define regions of the *Eomes* locus that are accessible in CD8-MP cells, but not in true memory $CD8^+$ T cells or NK cells, which also express Eomes. It is also likely that there are regions in loci other than *Eomes* that are uniquely accessible in CD8-MP cells. Once identified, these elements could be genetically deleted using CRISPR/Cas9 approaches, and the consequences of specifically disrupting CD8-MP differentiation could be assessed. We hypothesize that disabling CD8-MP differentiation would result in the emergence of these self-reactive specificities in the conventional $CD8^+$ T cell compartment and would result in lethal autoimmunity.

Our studies demonstrates that CD8-MP cells are overtly self-reactive, but do not cause autoimmunity at homeostasis in healthy mice. We hypothesize that there is some aspect of the CD8-MP program that renders these self-reactive cells innocuous to the host, but able to perform other select functions, particularly in inflammatory contexts such as the tumor environment.

Although CD8-MP cells appear to be innocuous at homeostasis in healthy mice, there may be some diseases in which in which self-reactive CD8-MP cells contribute to pathogenesis. For example, type I diabetes is an autoimmune disease characterized by $CD8^+$ T cell destruction of pancreatic β cells. In humans, $CD8^+$ T cells infiltrate the pancreas in type I diabetic patients and certain MHC-I alleles are associated with type I diabetes[115]. It is possible that aberrant regulation of self-specific CD8-MP cells could contribute to this devastating autoimmune disease. For most autoimmune diseases in humans (including rheumatoid arthritis, celiac disease, psoriasis, ankylosing spondylitis, systemic lupus erythematosus, type 1 diabetes, multiple sclerosis, Crohn's disease, ulcerative colitis, and dermatomyositis) there are genetic associations with both MHC class I and MHC class II, with MHC class II and $CD4^+$ T cells thought to be the drivers of most autoimmune diseases[116]. Although $CD4^+$ T cells are thought to be the drivers of many autoimmune diseases, self-specific CD8-MP cells, if aberrantly regulated, could contribute to autoimmune destruction, especially given their cytolytic capacity.

Future work on CD8-MP cells in mice can provide a path forward for the study of CD8-MP cells in human disease. Currently, there are no validated markers that can distinguish endogenous self-specific CD8-MP cells from conventional foreign antigen-specific memory cells. As described above, future work will aim to identify specific cell surface markers or a specific transcriptional or epigenetic signature of thymic derived CD8-MP cells that is distinct from conventional memory cells that differentiate in the periphery due to foreign pathogens. This signature could then be used in human studies, to identify bona fide CD8-MP cells in healthy people and in patients with autoimmune diseases. Additionally, discovery of endogenous ligands recognized by CD8-MP clones in humans would enable tetramer-based analysis to identify and study antigen-specific CD8-MP cells in various human disease contexts. Lastly, foreign-pathogen

cross-reactive CD8-MP cells can be studied in humans who have never been exposed to that pathogen, an approach that has yielded insights regarding human $CD4^+$ memory phenotype cells[117].

Thymic origins of endogenous CD8-MP cells

Based on our collective findings, we propose that the recognition of distinct self-ligands in the thymic medulla triggers the CD8-MP differentiation program, which is marked by Eomes expression in the thymus and delayed upregulation of the $CD44^{hi}$ $CD122^+$ phenotype following egress to the periphery. We propose a model of CD8-MP differentiation whereby TCR recognition of self-antigen enables a subsequent cytokine-dependent signaling step to complete CD8-MP differentiation (Figure 17). This is analogous to "two-step" $Foxp3^+$ Treg cell development, which requires an initial TCR-instructive phase followed by an IL-2-dependent consolidation phase[110,111]. Our finding that CD8-MP differentiation is initiated in the thymus due to TCR recognition of self-ligands challenges common thought which, prior to this work, suggested that CD8-MP cells differentiate from naive T cell precursors in the periphery following homeostatic proliferation. This prior notion was founded on evidence demonstrating that $CD8^+$ T cells exhibiting a $CD44^{hi}$ $CD122^+$ T cells first appear in the periphery of neonatal mice[118] and that CD8-MP cells share some phenotypic and functional properties with lymphopenia-induced memory (LIM cells), a population of $CD44^{hi}$ $CD122^+$ T cells that emerge following the intravenous transfer of mature naive-phenotype $CD8^+$ T cells into lymphopenic hosts[49]. Based on the fact that several properties of CD8-MP cells are similar to those of LIM cells, it has been tempting to equate these populations. However, two pieces of evidence presented here suggest that naturally occurring CD8-MP cells in B6 mice are distinct from LIM cells. First, we find that the differentiation of many CD8-MP clones

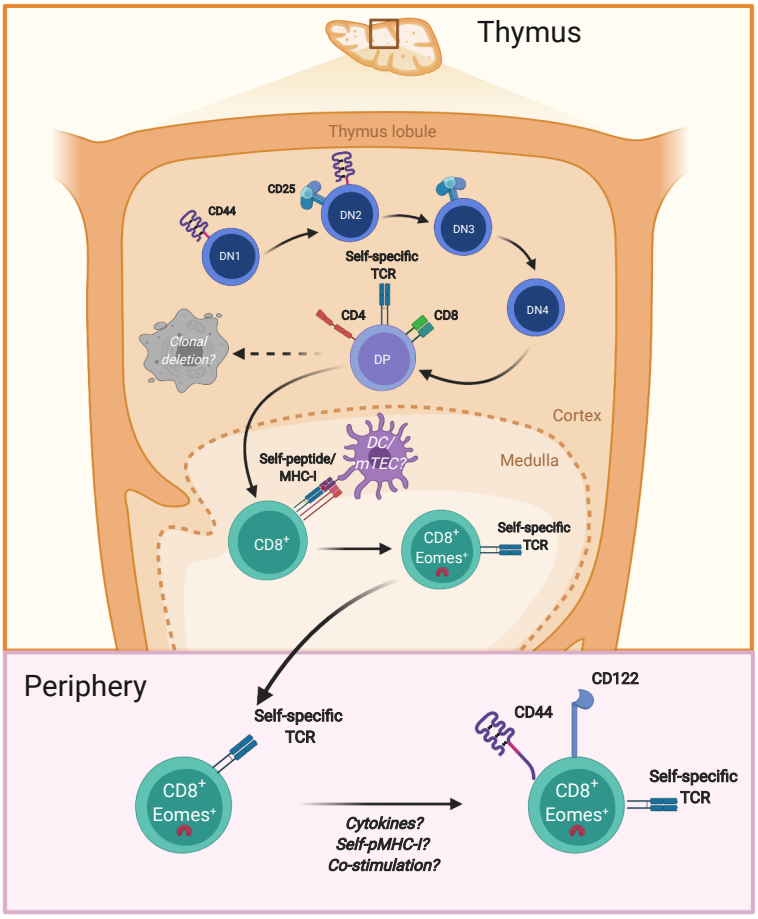

Figure 17. Model of thymic development of self-specific CD8-MP cells.

Collectively, our results reveal new insight into the origins of CD8-MP cells. A thymocyte expressing a TCR exhibiting overt reactivity to self-peptide/MHC-I ligands escapes clonal deletion and enters the thymic medulla. There, antigen presenting cells display self-peptide on MHC-I to developing CD8-MP precursors, which initiate a differentiation program characterized by upregulation of the transcription factor Eomes. Upon egress from the thymus, the Eomes^{+} CD8-MP precursor encounters additional (undefined) signals in the periphery, which direct maturation to the full CD8-MP phenotype, including expression of classical markers of T cell memory, CD44 and CD122.

is initiated in the thymus, suggesting that the forces driving LIM cell differentiation in the periphery may not be relevant for many naturally occurring CD8-MP clones. Second, our finding that CD8-MP differentiation is a TCR-instructive process, with hundreds of CD8-MP-biased clones exhibiting robust segregation to the CD8-MP subset, suggests that the naive-phenotype donor cells used in studies of LIM cell differentiation are likely depleted of the relevant TCRs that are naturally expressed by CD8-MP cells, implying that the antigen specificities of CD8-MP cells and LIM cells are inherently distinct.

In both monoclonal and polyclonal settings, Eomes-expressing CD8SP thymocytes are predominantly found at the $CD69^{neg}$ MHC-I^{+} "mature 2" stage[108], indicating that Eomes is upregulated at the latest stages of thymic maturation, when cells are localized in the medulla. This suggests that CD8-MP differentiation may be triggered by encounter with self-ligands presented in the medulla, which are expected to include a mixture of widely expressed antigens and promiscuously expressed tissue-restricted antigens[119]. Expression of Aire by medullary thymic epithelial cells (mTECs) leads to promiscuous expression of many tissue restricted antigens. Previous work by our lab has demonstrated that expression of Aire in the thymic medulla is required for the proper differentiation of hundreds of $Foxp3^{+}$ $CD4^{+}$ Treg specificities[100]. In Aire-deficient mice, $CD4^{+}$ T cell specificities that would normally be directed into the $Foxp3^{+}$ Treg lineage in WT mice instead become $CD4^{+}$ conventional T cells, and cause rampant autoimmunity. Given the many parallels between CD8-MP differentiation and $CD4^{+}$ $Foxp3^{+}$ Treg differentiation, especially CD8-MP cell upregulation of Eomes in the thymic medulla, perhaps there is a role for Aire expression in the development of CD8-MP specificities. To probe this question in the future, a sequencing approach can be utilized to determine the impact of Aire on the development of CD8-

MP specificities can be assessed. This approach will reveal whether there are CD8-MP specificities that are Aire-dependent and identify another key role of Aire in shaping the T cell repertoire.

The results presented here demonstrate that although $CD8^+$ T cells do not upregulate CD44 and CD122 until egress into the periphery, differentiation of many CD8-MP clones is triggered in the thymus via a TCR-directed process and can be identified by expression of Eomes. This observation was made possible through analysis of developmental trajectories of individual CD8-MP-biased clones, paired with analysis of the expression of Eomes, a transcription factor that is required for CD8-MP differentiation[53] and promotes CD122 expression by direct binding to the *Il2rb* promoter[106]. Although our studies focus on CD8-MP cells in B6 mice, in which IL-4-dependent memory cells are infrequent[102,118], the concept of TCR-instructive thymic differentiation has not been previously demonstrated for either IL-4-dependent or IL-4-independent memory cells. Future work will aim to define the extent to which thymic differentiation of CD8-MP cells is IL-4 dependent. First, we will assess the impact of IL-4 deficiency on Eomes-expressing thymocytes. Then we will use our canonical CD8-MP clones and generate low frequency CD8-MP TCRrg mice in hosts lacking IL-4 or NKT cells, the primary source of IL-4 in the thymus[67]. It is possible that Eomes expression and/ or subsequent upregulation of CD44 and CD122 by CD8-MP clones are dependent on IL-4 sensing in the thymus or periphery. Alternatively, it is possible that some CD8-MP clones are IL-4 dependent, whereas others are not, which would indicate that there are distinct subsets of thymus-derived CD8-MP cells.

Although our work demonstrates a role of TCR specificity in thymic CD8-MP development, the other thymic and peripheral signals required for CD8-MP differentiation remain incompletely defined. Based on our data demonstrating that that thymic DCs induce the in vitro proliferation of CD8-MP clones and that CD28 signaling is required for the differentiation of

conventional memory CD8$^+$ T cells[120,121], we hypothesize that classical DCs (cDC) direct CD8-MP differentiation in the thymic medulla. The thymus contains numerous antigen presenting cell (APC) types, including cortical thymic epithelial cells (cTECs), medullary TECs (mTECs), CD8α^+ and Sirpα^+ cDCs, plasmacytoid DCs (pDCs), B cells, and macrophages. To varying degrees, these cells types can present self-peptides derived from exogenous or endogenous proteins to developing T cells. Due to the fact that CD8-MP differentiation was previously thought to occur in the periphery, nothing is known about the nature of APCs that trigger CD8- MP differentiation in the thymus. Future work will aim to characterize the thymic APC populations required for CD8-MP differentiation.

In this work, we demonstrate that CD8-MP differentiation is initiated in the thymus due to TCR-dependent signals and completed in the periphery, where CD8-MP clones upregulate CD44 and CD122. Previous work has demonstrated a clear requirement of IL-15 signaling in CD8-MP differentiation[53,54], however it is unknown whether IL-15 is required for establishment of the CD8-MP program or is simply required for survival and fitness. In addition, it is unknown what other accessory signals, besides IL-15, are required for proper CD8-MP differentiation. Our identification of distinct CD8-MP clones provides us with a unique tool to dissect the cellular and molecular requirements of CD8-MP differentiation. Specifically, future work will combine our TCRrg approach and mixed bone marrow chimera experiments to identify the accessory signals and APC types that direct CD8-MP differentiation in the thymus and in the periphery.

We hypothesize that CD8-MP differentiation is a multi-step process requiring CD28-dependent signals and the recognition of select pMHC-I ligands presented by cDCs in the thymic medulla, followed by the sensing of IL-15 or IL-4 in the periphery. In this way, CD8-MP differentiation would closely mirror the differentiation of Foxp3$^+$ Treg cells in many respects[122].

First, CD8-MP differentiation is a robust TCR-directed process. Second, CD8-MP clones exhibit overt reactivity to self-ligands. Third, CD8-MP differentiation is triggered in the thymus by limited niches. Fourth, CD8-MP differentiation is first evident in the medulla. Finally, CD8-MP differentiation is a multi-step process characterized by a TCR dependent step in the thymus followed by a second step in the periphery. These many parallels between Foxp3-expressing $CD4^+$ Tregs and CD8-MP cells highlight thymic CD8-MP cells as a unique subset of self-specific $CD8^+$ T cells.

The concept that the differentiation of self-specific CD8-MP cells is triggered in the thymus would place CD8-MP cells on an expanding list of αβ T cell subsets for which differentiation is induced by recognition of self-ligands in the thymus, including $Foxp3^+$ regulatory T cells, invariant natural killer T cells, $CD8\alpha\alpha^+$ intraepithelial lymphocytes, and MR1-restricted mucosal associated invariant T cells. Our data indicate that CD8-MP clones exhibit hallmarks of antigen encounter in the thymus yet readily populate the peripheral repertoire, suggesting that such clones are not substantially impacted by clonal deletion. This suggests either that the TCR-pMHC-I binding properties promoting CD8-MP differentiation are distinct from the properties driving clonal deletion, or that unique contextual cues (such as anatomical region, identity of the antigen presenting cell, or thymocyte maturational stage, illustrated in Figure 1) trigger CD8-MP differentiation without inducing extensive deletion. Future work will aim to determine which of these properties drive a self-reactive MHC-I restricted thymocyte to undergo clonal deletion or CD8-MP differentiation. One possibility is the MHC restriction of a self-reactive $CD8^+$ T cell. The work presented here demonstrates that many canonical CD8-MP clones are restricted to classical MHC-I molecules H2-K^b and H2-D^b and that the number of CD8-MP cells is substantially reduced in $K^{b-/-}D^{b-/-}$ mice. Work by McDonald et. al.[14] suggests that many T cell clones that undergo clonal

deletion are cross-reactive to multiple MHC haplotypes, suggestive of direct reactivity to MHC (independent of peptide). This study also demonstrated that a fraction of T cells expressing cross-reactive TCRs are shunted into the intestinal CD8αα iIEL compartment[14,39]. Thus, one possibility is that self-reactive $CD8^+$ T cells that are classically MHC-I restricted differentiate into CD8-MP cells, whereas self-reactive $CD8^+$ T cells that are cross-reactive to multiple MHC haplotypes differentiate into iIELs or are purged from the repertoire. An alternative model posits that the fate of self-reactive thymocytes is dictated by the maturational stage at which agonist ligand is encountered. For example, encounter of agonist self-peptide/MHC-I complexes at the double-positive stage (when cells are in the cortex) may trigger deletion, whereas encounter at the CD8-SP stage (when cells are in the medulla) may trigger CD8-MP differentiation. Further work is required to determine the properties that drive particular self-reactive $CD8^+$ T cell clones down certain T cell lineages.

In this body of work, we demonstrate that multiple CD8-MP clones display overt reactivity to classical MHC-I-restricted ligands displayed by both thymic DCs and splenic DCs from germ-free mice, and are broadly distributed in the secondary lymphoid organs, suggesting that at least some CD8-MP clones are reactive to self-ligands that are widely presented. In addition, analysis of the thymic differentiation of the SAV CD8-MP-biased clone revealed that robust Eomes expression is only observed at low clonal frequencies, suggesting the existence of limited antigenic niches supporting the differentiation of this clone, similar to the saturable niches described for Treg cell clones[32,107]. In addition, analysis of CD44 and CD122 upregulation by CD8-MP clones in the periphery of low frequency TCRrg mice demonstrates that limited niches exist for many CD8-MP clones. Moving forward, a broader survey of CD8-MP-biased clones will be needed to determine whether all CD8-MP clones exhibit reactivity to widespread ligands, or whether some

clones are reactive to tissue-restricted ligands. Additionally, TCR repertoire profiling of CD8-MP cells isolated from distinct lymph nodes may identify CD8-MP clones that are asymmetrically distributed, suggestive of reactivity to regional tissue-restricted antigens.

A recent report from Smith et al.[65] showed that thymocytes maturing in the neonatal period are more likely to give rise to CD8-MP cells, relative to cells maturing in adult mice. Intriguingly, this work demonstrated that this effect was T cell intrinsic, and not due to the neonatal lymphopenic environment. Coupled with our data, which demonstrates that the TCR repertoire of CD8-MP cells is distinct from that of CD8-Naïve cells, these results suggest that there is a unique TCR repertoire that arises during the neonatal period when T cell precursors are derived from fetal liver HSCs, instead of the bone marrow. Our current study demonstrates that the CD8-MP clones examined can readily differentiate into CD8-MP cells in adult mice, suggesting that the neonatal period is not uniquely permissive to the generation of CD8-MP cells. In addition, our analysis of TCR CDR3α lengths and hydrophobicity revealed no significant differences between CD8-MP and CD8-Naive-biased T cells isolated from adult mice. Regardless, it remains possible that fetal HSC progenitors preferentially give rise to CD8-MP T cells, and that some T cell-intrinsic property impacts the nature of the TCR. Moving forward, it will be critical to determine the extent to which the CD8-MP TCR repertoire changes with age, and to define the impact of terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes the addition of nucleotides at TCR junctional regions and has limited activity during TCR rearrangement early in life[123], on CD8-MP differentiation and reactivity to self-ligands.

The role of self-reactive CD8-MP cells in foreign pathogen responses

Studies using foreign pMHC-I tetramer enrichment assays demonstrated that in unprimed mice, a fraction of $CD8^+$ T cells reactive to any given foreign pMHC-I complex display a $CD44^{hi}$ $CD122^+$ phenotype[52,68]. This evidence suggested that CD8-MP differentiation might be triggered by TCR-independent signals, and led to their classification as "antigen-inexperienced" memory-phenotype T cells. In contrast to this notion, we demonstrate that CD8-MP cells represent bona fide antigen-experienced cells, whose differentiation is a TCR-directed process due to self-ligand encounter. Thus, CD8-MP cells in unprimed mice exhibiting reactivity to foreign pMHC-I ligands likely represent CD8-MP clones that were selected on self-pMHC-I ligands, but cross-react with foreign pMHC-I ligands due to the plasticity inherent in TCR-pMHC ligand recognition[112]. From a functional perspective, our findings reveal that the innate memory-like properties of CD8-MP cells are imparted by robust immunological mechanisms involving the TCR-instructive recognition of endogenous self-ligands in the thymus, and that the ligands recognized by some CD8-MP clones are presented by dendritic cells in the periphery. Thus, we suggest that selection on endogenous self-ligands plays a critical role in poising CD8-MP cells for rapid recruitment into the early phases of the immune response to pathogen infection[68,71]. To study this moving forward, we will take a sequencing approach and compare the CD8-MP clones that are recruited into the pathogen response to those clones that are abundant at steady state. If CD8-MP clones are recruited into foreign pathogen responses in an antigen-independent manner, then the hierarchy of CD8-MP clonal frequency at sites of infection may be similar to that of the hierarchy at baseline in the secondary lymphoid organs. Alternatively, if the hierarchy is found to change, it would be suggestive of antigen-dependent CD8-MP enrichment, driven by recognition of either pathogen-derived peptides or self-peptides that are readily displayed at the site of infection.

Although previous studies have demonstrated that a small fraction of self-specific CD8-MP cells cross-react to foreign peptides and can be recruited into foreign pathogen responses, these studies utilized a foreign-pMHC-I tetramer approach which is associated with inherent observation bias, as such cells represent less than 1% of all CD8-MP cells. With this in mind, it remains to be determined whether CD8-MP innate-like recruitment into inflammatory sites represents a true function of the remaining 99% of CD8-MP cells that do not cross-react with a given foreign antigen. To study this moving forward, we must take an unbiased approach to determine the true functions of CD8-MP cells. Utilizing T cell deficient mice reconstituted with congenically marked CD8-MP cells or reconstituted mice in which CD8-MP cells are withheld, we can examine how CD8-MP cells are recruited into immune responses to pathogens, tumors, and sites of organ-specific autoimmunity. In addition to reconstitution approaches, which can lead to T cell homeostatic proliferation, there is a critical need to develop new approaches to inducibly deplete CD8-MP cells. As described in the above sections, we aim to identify unique surface markers, transcription factors, or accessible loci that are unique in thymic derived CD8-MP cells that can be used to specifically track and ablate these cells in models of pathogens, tumors, and auto-immunity.

The role of endogenous CD8-MP cells in the tumor environment

Lastly, our transfer experiments and comparative TCR profiling approach revealed that CD8-MP cells are recurrently enriched in autochthonous prostate tumors in TRAMP mice and adopt a PD-1^{hi} $Eomes^{+}$ phenotype within tumors (Figure 18). Previously, the contribution of CD8-MP cells to the anti-tumor immune response in human or murine cancers had been largely

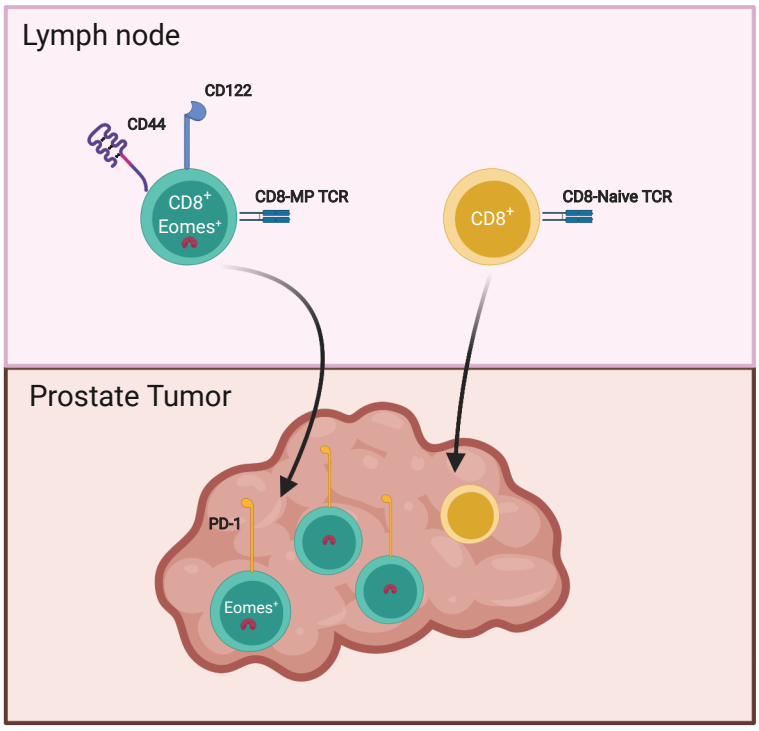

Figure 18. Model of CD8-MP cell recruitment into murine prostate tumors

Self-specific CD8-MP cells, whose differentiation is triggered due to self-antigen recognition in thymus, are selectively recruited into murine prostate tumors in a TCR dependent manner. These CD8-MP cells downregulate CD122 and upregulate PD-1, suggesting a change in their transcriptional program within the tumor microenvironment. Our data indicating that distinct CD8-MP clones are selectively enriched within prostate tumors, suggestive of antigen-driven recruitment or retention. It remains unknown whether CD8-MP clones recognize ligands displayed by tumor cells or ligands displayed by intratumoral stromal cells.

undefined, due to the lack of available markers or signatures to identify CD8-MP cells and distinguish them from bona fide tumor-specific $CD8^+$ T cells. In this regard, an expanding body of evidence suggests that $CD8^+$ T cells reactive to tumor-expressed antigens are rare in many human cancers. For example, using exome sequencing and multiplexed tetramer approaches, Simoni et al. found that $CD8^+$ T cells reactive to mutated neo-antigens or non-mutated tumor antigens are rare or undetectable in human lung and colorectal cancers[84]. Instead, tumors were largely infiltrated by "bystander" cells that were phenotypically distinct from rare tumor-reactive T cells. More recently, Scheper et al. demonstrated that only a minor fraction of prominent $CD8^+$ T cell clones from ovarian or colorectal cancers exhibited overt reactivity to autologous tumor cells[85]. These and other findings suggest that in many human cancers, the majority of $CD8^+$ TILs are not reactive to tumor-expressed antigens. Additionally, recent single-cell RNA sequencing and mass cytometry studies demonstrate that $CD8^+$ T cells from any given tumor occupy multiple transcriptional or phenotypic states, indicative of substantial heterogeneity[86–90]. Thus, cumulative evidence suggests that tumors are infiltrated by a phenotypically diverse array of $CD8^+$ T cells, and that only a minor fraction of such cells are overtly reactive to tumor-expressed antigens. Given our findings presented here, we hypothesize that self-specific CD8-MP cells, selected on endogenous self-ligands in the thymus, make substantial contributions to the infiltrate of human and murine cancers and may be directly impacted by checkpoint blockade antibodies targeting the PD-1 axis. Our TCR profiling data demonstrate that CD8-MP cell recruitment into the tumor environment is TCR and antigen dependent. Future work will aim to determine the nature of antigens that drive recruitment of CD8-MP cells into the tumor environment. Given the studies that demonstrate that the majority of $CD8^+$ TILs are not reactive to tumor-expressed antigens, it is

a great interest to determine the antigens that CD8-MP cells are responding to and which cell types (DCs, tumor cells, stromal cells, etc.) are presenting those antigens.

Although our data demonstrate that CD8-MP cells make substantial contributions to the tumor infiltrate in the TRAMP model, it is currently unknown what the functional contributions of these CD8-MP clones are to the tumor-associated immune response. For example, evidence showing that CD8-MP cells exhibit rapid expansion and IFNγ production in the early phases of infection[71,124] suggests that tumor-infiltrating CD8-MP cells may exhibit similar innate-like effector functions in the tumor context. This suggests that CD8-MP may augment anti-tumor immune responses by catalyzing broader immune cell infiltration or activating intratumoral immune cells via IFN-γ production. Alternatively, given evidence suggesting that $CD8^+$ $CD122^+$ cells play a critical role in the maintenance of immune homeostasis[73], plus recent evidence showing that oligoclonal $CD8^+$ T cell populations suppress pathogenic $CD4^+$ T cells in experimental autoimmune encephalomyelitis[125], it is also feasible that tumor-infiltrating CD8-MP cells exhibit some form of suppressor function. Our ongoing studies aim to elucidate these questions through reconstitution experiments, whereby T cell deficient TRAMP mice are reconstituted with congenically distinct $CD4^+$ and $CD8^+$ T cell populations. Such an approach may help define how loss of CD8-MP cells impacts the prostate tumor environment. Furthermore, the approach can be used to define the kinetics of CD8-MP infiltration into the tumor and determine what role these cells play in response to anti-PD-1 immunotherapy. Given the high density of PD-1 on infiltrating CD8-MP cells, we hypothesize that these cells will be impacted by immunotherapies that target the PD-1 axis. In addition to studies on CD8-MP cells in the TRAMP tumor model, similar approaches can be taken to study the impact of CD8-MP cells in other tumor models such as B16 melanoma.

An intriguing finding is that CD8-MP cells are recruited into the inflammatory tumor environment, but are largely maintained in lymphoid organs and do not cause autoimmunity at steady state. As discussed above, we hypothesize that there is some aspect of the CD8-MP program that renders these self-reactive cells innocuous to the host, but able to perform other select functions, particularly in inflammatory contexts, such as the tumor environment, to which CD8-MP cells are selectively recruited. Future work will aim to study the positioning of CD8-MP cells within the secondary lymphoid organs at homeostasis and how their positioning changes following bacterial infection or in tumor-bearing hosts. In addition to defining the epigenetic and transcriptional program of CD8-MP cells at homeostasis, we aim to identify unique transcriptional programs expressed by tumor-associated CD8-MP cells, compared to tumor-infiltrating CD8+ cells derived from naive T cell precursors. Identification of unique markers that are exclusively expressed by tumor-associated CD8-MP cells will enable the direct characterization of intratumoral CD8-MP cells in murine cancer models, and facilitate the development of loss-of-function approaches enabling the selective depletion of CD8-MP cells, leaving conventional memory and effector T cells untouched. Furthermore, identification of unique CD8-MP markers in mice may provide a path forward for human studies, as analagous markers and programs may allow delineation of CD8-MP cells in human cancers.

Conclusion

Prior to this work, the origins, antigen specificities, and functional characteristics of endogenous CD8-MP cells have remained largely undefined. Using deep T cell receptor (TCR) sequencing, we found that the TCRs expressed by CD8-MP cells are highly recurrent and distinct from the TCRs expressed by naive-phenotype $CD8^+$ T cells. Taking a unique clonal approach, we

demonstrated that CD8-MP cells represent a previously unappreciated self-specific $CD8^+$ T cell subset, whose differentiation program is triggered by self-antigen recognition in the thymus. These results starkly contrast the current paradigm in the field, which has described CD8-MP cells as "antigen-inexperienced" cells that acquire the memory phenotype due to naïve T cell homeostatic proliferation in the periphery. Our results demonstrate that although self-specific CD8-MP cells share a phenotype with foreign-reactive conventional memory cells, their differentiation program is distinct in that it is initiated in the thymus due to self-antigens, not due to foreign antigen encounter. Furthermore, we demonstrate that these self-specific thymic CD8-MP cells are selectively recruited into autochthonous cancers in mice and they express high densities of the co-inhibitory receptor PD-1, suggesting a role of CD8-MP cells in anti-tumor immunity and response to immunotherapy.

Future Issues

1. What are the unique surface markers, transcription factors, or epigenetic marks of thymic derived CD8-MP cells? With these markers, develop an approach in which CD8-MP cells can be consitutively or inducibly deleted, leaving true memory cells untouched.
2. What are the cellular and molecular requirements of CD8-MP differentiation in the thymus and periphery? What APCs coordinate CD8-MP differentiation?
3. Do CD8-MP cells provide feedback signals to activate DCs?
4. What are the self-peptides recognized by naturally occurring CD8-MP clones?
5. Are there CD8-MP clones reactive to tissue restricted antigens? Are there CD8-MP clones that are Aire dependent?

6. What is the functional role of CD8-MP cells in anti-tumor immunity and in response to checkpoint blockade therapy?
7. Where are CD8-MP cells positioned within secondary lymphoid organs and tumors? Does their positioning change during infection?
8. Do CD8-MP cells require continued antigen recognition in the periphery?
9. What are the Qa-1-restricted CD8-MP clones? How do they exert their suppressive function?

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