Eomes identifies thymic precursors of self-specific memory-phenotype CD8+ T cells

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Unprimed mice harbor 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. Using deep T cell antigen 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. CD8-MP clones exhibited reactivity to widely expressed self-ligands. T cell precursors expressing CD8-MP TCRs showed upregulation of the transcription factor Eomes during maturation in the thymus, prior to induction of the full memory phenotype, which is suggestive of a unique program triggered by recognition of self-ligands. Moreover, CD8-MP cells infiltrate oncogene-driven prostate tumors and express high densities of PD-1, which suggests potential roles in antitumor immunity and the response to immunotherapy.

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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 (GF) mice that have not been exposed to foreign pathogens, there exists a substantial population of CD8+ T cells that exhibit a CD44hiCD122+ 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-memory1 or innate memory T cells), make up >5% of the CD8+ repertoire in adult mice, and exhibit numerous hallmarks of conventional memory CD8+ T cells that are reactive to agonist ligands. Although the existence of an analogous cell population has been suggested in humans3–5, 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 challenge2,6, and roles in the maintenance of immune homeostasis at steady state7. However, it remains unclear whether these reflect broad functions of all CD8-MP cells, or distinct functions of heterogeneous T cell populations that fall within the CD44hiCD122+ subset. Efforts to elucidate the mechanisms that drive 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 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-major histocompatibility complex class I (pMHC-I) ligands. CD8-MP cells exhibit slightly higher average densities of CD5, 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 thymus9–11, CD5 density cannot be used to assess the intensity of additional TCR signaling events that occur after positive selection. The finding that the phenotype and frequency of CD8-MP cells is not diminished in GF mice and in GF mice fed an elemental diet12 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 CD44hiCD122+CD8+ T cells that are reactive to any foreign pMHC-I complex13,14 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 reticogenic mice15, which indicates 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 idea is largely based on the fact that CD44hiCD122+ cells are first detected in the periphery of neonatal mice12 and are phenotypically similar to ‘lymphopenia-induced memory’ (LIM) T cells, a population of CD44hiCD122+ cells that emerge in the periphery following intravenous transfer of naive-phenotype CD8+ T cells into lymphopenic mice16. 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.
Fig. 1 | The TCR repertoire of memory-phenotype CD8+ T cells is recurrent and distinct from that of naive-phenotype CD8+ T cells. CD8-MP (CD8αCD44hiCD122+) and CD8-Naive (CD8αCD44loCD122−) T cells were purified by FACS from the pooled spleen and lymph nodes of nine-week-old TCRγtg− males and subjected to complete TCRα sequences using the iRepertoire platform. n = 5 for CD8-MP and CD8-Naive samples. TCRα chains were assessed solely on the basis of 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 (log2(fold-change)) in the CD8-MP versus CD8-Naive subsets is shown for the 3,926 recurrently expressed TCRs. n = 5 for CD8-MP and CD8-Naive samples. Comparisons were made using edgeR and were adjusted for multiple comparisons. For the differential testing, the general linear model (GLM) method was used (see Methods). Red dots denote TCRs with FDR < 0.05. The horizontal dashed line indicates FDR cutoff. b, Line indicates FDR cutoff. For the MH index, a value of 1 indicates identity and a value of 0 denotes complete dissimilarity. c, For the ten most prevalent TCRs recurrently expressed by CD8-MP (left plot) and CD8-Naive (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-Naive samples. n = 5 for CD8-MP and CD8-Naive samples. Mean ± s.e.m. is indicated. **P < 0.0079, two-tailed nonparametric Mann–Whitney test.

Here, we address these key questions using an in-depth clonal approach that pairs complete TCR repertoire profiling with studies that enable the developmental arc of individual CD8-MP clones to be assessed at all stages of T cell maturation, and the reactivity of distinct CD8-MP clones to 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.

Results
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 GF mice (Extended Data Fig. 1), 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 diversity, whether it varies stochastically from mouse to mouse, and whether it overlaps with the TCR repertoire expressed by naive-phenotype CD8+ T cells. To comprehensively examine these questions, we sequenced the complete TCR repertoires of naive-phenotype CD8+ T cells (CD8-Naive cells, CD44loCD122−) and memory-phenotype CD8+ T cells (CD8-MP cells, CD44hiCD122+) using previously established methods[4,15]. We focused our analysis on CD8-MP cells from C57BL/6j (B6) mice, in which interleukin 4 (IL-4)-induced CD8+ memory cells[13,14] make minimal contributions to the peripheral CD8-MP pool[12,16]. CD8-Naive and CD8-MP cells from the pooled spleen and lymph nodes were purified by cell sorting from five different nine-week-old male mice expressing a fixed transgenic TCRβ chain on the B6 background. By fixing the TCRβ chain, a complete survey of the TCRαβ repertoire can be obtained by sequencing the endogenous TCRα chains using the iRepertoire platform[4,17]. This approach yielded approximately 6.5 × 10^5 TCRα sequence reads per sample, which provided a broad survey of TCR usage (Supplementary Table 1). Our data revealed the striking finding that the TCR repertoire of CD8-MP cells is largely distinct from that of CD8-Naive cells, and was highly recurrent from mouse to mouse (Fig. 1). Comparative analysis of TCR frequency identified 493 recurrent TCRα chains that were significantly overrepresented in the CD8-MP subset relative to the CD8-Naive subset (Fig. 1a, right arm of volcano plot). Cumulatively, these TCRs accounted for 65% ± 1% of the CD8-MP TCR repertoire, which demonstrates the broad extent of repertoire skewing. The recurrent nature of the
CD8-MP TCR 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 5 different mice revealed a mean MH index of 0.92 ± 0.03 (Fig. 1b), which demonstrates that distinct T cell clones were reproducibly directed into the CD8-MP subset. By contrast, pairwise comparisons of CD8-Naive versus CD8-MP repertoires between the five mice revealed a mean MH index of 0.06 ± 0.01, which is indicative of minimal overlap. The concepts that the CD8-MP TCR repertoires were highly recurrent and distinct from the CD8-Naive TCR repertoire are also illustrated in Fig. 1c, which plots the frequency of the ten most prevalent CD8-MP TCRs (left) and the ten most prevalent CD8-Naive TCRs (right) within the CD8-Naive 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-Naive repertoire (Fig. 1d), with an average complexity of 6,979 TCR complementarity determining region 3 (CDR3) segments for the CD8-MP subset and 24,443 CDR3s for the CD8-Naive subset. Analyses of CDR3 lengths and amino acid composition revealed no consistent differences between the CD8-Naive and CD8-MP TCRs (Extended Data Fig. 2). 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' (TCRg) mice that express 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-Naive TCR' for receptors that are skewed to the CD8-Naive subset. For TCR nomenclature, we depict the amino acid sequence of the CDR3 segment of a given TCR clone using a three-letter code that reflects the amino acids at positions 3–5 of the CDR3ζ. For example, TCRg cells with CDR3ζ sequence AASMNYNQGKLI are denoted 'SMNrg' cells. Our work focused on the study of four recurrent CD8-MP TCRs and four recurrent CD8-Naive TCRs identified in Fig. 1, which were chosen because they span a range of frequencies and utilize distinct V- and J-region segments (Supplementary Table 2). TCRg mice were generated as described previously14. In brief, bone marrow cells from Tcrα–/–; Cd4-Cre–; TCRBg/CD45.2 mice were retrovirally transduced with a vector in which the genes
that encode a TCRα chain of interest and an IRES-Thy1.1 reporter are preceded by a loxP-flanked stop cassette. Transduced bone marrow was engrafted along with wild-type filler bone marrow cells into lethally irradiated 6–8-week-old CD45.1 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 TCRγ cells, which recapitulates the natural kinetics of TCR expression during development. Resulting TCRγ T cells were identified using the Thy1.1 and Cd45.2 markers.

With this approach, we found that a substantial fraction of T cells expressing CD8-MP TCRs adopted the CD44hiCD122+ memory phenotype in the periphery of TCRγ hosts, whereas very few TCRγ cells expressing CD8-Naive TCRs acquired this phenotype (Fig. 2a–c, Extended Data Fig. 3). These findings validate the TCR sequencing and confirm the idea that CD8-MP differentiation is a TCR-instructed process. Notably, in TCRγ mice that express CD8-MP TCRs, we found that not all peripheral TCRγ cells adopted a CD44hiCD122+ phenotype at the time of analysis, and that the fraction of TCRγ CD44hiCD122+ cells varied from mouse to mouse (Fig. 2a–c). A potential explanation for the lack of complete skewing to the CD8-MP phenotype is the existence of limited niches that support CD8-MP differentiation, which may be overloaded in TCRγ mice that harbor large numbers of monoclonal T cells. To define the phenotype and anatomical distribution of select CD8-MP
clones, we generated TCRrg mice that express CD8-MP 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 CD44loCD122+ phenotype (with nearly all cells shifted away from a naive phenotype), whereas TCRrg cells expressing CD8-Naive TCRs did not (Extended Data Fig. 4). These results are consistent with the existence of limited niches that support the differentiation of CD8-MP clones. Furthermore, peripheral CD8-MP TCRrg T 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 (Fig. 2d). These collective data demonstrate that the expression of CD8-MP TCRs in TCRrg hosts recapitulated differentiation into the CD8-MP subset, which validates the TCRrg approach and provides 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. Since CD8-MP cells are abundant in naive mice that have never been exposed to known foreign pathogens, we reasoned that CD8-MP cells may be reactive to endogenous self-antigens. In TCRrg mice, we found that CD8-MP clones were equally distributed in all secondary lymphoid organs examined (Extended Data Fig. 5) and exhibited elevated percentages of proliferative cells as measured by Ki67 staining, which is suggestive of active sensing of ligands at steady state (Fig. 3a–c). To gain insight into the antigen specificity of CD8-MP clones, we assessed the ex vivo reactivity of purified naive-phenotype (CD44loCD122lo) CD8+ T cells isolated from TCRrg mice that express CD8-MP or CD8-Naive TCRs. We purified naive-phenotype CD44loCD122lo T cells from TCRrg mice that express each of the eight TCRs, and used these cells as a probe for antigen in vitro stimulation assays. We found that the four CD8-MP clones underwent proliferation upon coculture with splenic dendritic cells (DCs) and recombinant IL-2, whereas the four CD8-Naive clones did not (Fig. 3d,e). This reactivity was abolished by the addition of anti-CD44 or anti-CD122 blocking antibodies (Fig. 3d,e), which indicates reactivity to ligands displayed by classical MHC-I molecules. Notably, the reactivity of CD8-MP clones was not impaired using splenic DCs isolated from GF mice (Fig. 3d,e). Thus, our data provide direct evidence that four canonical CD8-MP TCRs examined confer overt reactivity to endogenous self-antigens 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-antigens. We next set out to define the stage at which CD8-MP differentiation is triggered. Previous studies report that CD44loCD122+ CD8+ T cells first appear in the periphery of neonatal B6 mice11, which suggests that CD8-MP cells differentiate in the periphery11. Congruent with this idea, we found that CD44loCD122+ cells were not detected among polyclonal GFP+CD8+CD4+ (‘CD8 single-positive’, hereafter referred to as ‘CD8sp’) thymocytes from adult Rag2-green fluorescent protein (GFP) reporter mice (Fig. 4a), in which GFP expression marks newly developing thymocytes that have recently undergone Rag2-dependent TCR rearrangement46.

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. 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 thymus45) (Extended Data Fig. 6a). Consistent with observations for polyclonal cells, we found that TCRrg cells that express CD8-MP TCRs did not adopt a CD44loCD122+ phenotype in the thymus (Extended Data Fig. 6b). However, CD8-MP TCR rg thymocytes exhibited elevated percentages of Kit+ cells and increased densities of CD5 when compared to CD8-Naive TCRrg thymocytes, which is suggestive of elevated TCR signaling in the thymus (Extended Data Fig. 6c–f). Consistent with this observation, we found that CD11c+ DCs isolated from the thymus stimulated the in vitro proliferation of TCRrg cells that express CD8-MP, but not that of CD8-Naive TCRs (Fig. 4b), which indicates 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 CD4loCD8hi stage displayed reduced densities of the CD4 and CD8 co-receptors (Extended Data Fig. 6g), which...
is a potential indicator of clonal deletion\textsuperscript{22}. However, the findings that CD8-MP clones exhibited negligible staining for cleaved caspase 3 (a marker of ongoing apoptosis; Extended Data Fig. 6g) 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. Thus, we considered the possibility that CD8-MP differentiation is triggered by the recognition of self-ligands in the thymus, and that the upregulation of the CD44\textsuperscript{hi}CD122\textsuperscript{+} 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\textsuperscript{23}, and promotes CD122
Fig. 5 | Eomes identifies polyclonal thymic precursors of CD8-MP T cells. a–c, FACS-purified Eomes-GFP+ or Eomes-GFP− mature (CD73+CD69−) CD8SP thymocytes (1×10⁶) from the thymi of 4-week-old Eomes-GFP reporter mice were transferred intravenously into congenically disparate CD45.1+ recipients. Three 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 versus 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. SLOs, secondary lymphoid organs. b, Summary plot of pooled data from a showing the frequency of recovered TCRβ+CD8β+CD45.2+ cells that exhibited a CD44+CD122+ phenotype for the indicated donor cells. Each symbol represents an individual mouse. n = 7, Eomes-GFP+CD8β+; n = 10, Eomes-GFP−CD8β+. Mean ± s.e.m. 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 that exhibited a CD44+CD122+ phenotype for the indicated donor cells. Each symbol represents an individual mouse. n = 7, Eomes-GFP+CD8β+; n = 10, Eomes-GFP−CD8β+. Mean ± s.e.m. is indicated. **P = 0.0020, two-tailed nonparametric Mann-Whitney test. Data are pooled from three independent experiments. d–i, Five-week-old B6 mice were treated with 7 mg kg⁻¹ FTY720 or PBS control intraperitoneally every other day for 5 d. On day six, the thymocytes of the mice were assessed. d, Representative flow cytometric analysis of Eomes expression by CD73+TCRβ+CD8β+ thymocytes in B6 mice that received 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 CD44+CD122+ thymocytes that exhibited an Eomes+ phenotype in B6 mice that received the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± s.e.m. 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+TCRβ+CD8β+ thymocytes that exhibited an Eomes+ phenotype in B6 mice that received the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± s.e.m. 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 versus CD122 expression by CD73+TCRβ+CD8β+Eomes+ thymocytes in B6 mice that received 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 CD44+CD122+ Eomes+ thymocytes that exhibited a CD44+CD122+ phenotype in B6 mice that received the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± s.e.m. is indicated. n.s., not significant, P = 0.9951, 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+TCRβ+CD8β+Eomes+ thymocytes that exhibited a CD44+CD122+ phenotype in B6 mice that received the indicated treatments. Each symbol represents an individual mouse. n = 8, control; n = 8, FTY720. Mean ± s.e.m. is indicated. *P = 0.0148, two-tailed nonparametric Mann-Whitney test. Data are pooled from two independent experiments.
upregulation by direct binding to the Il2rb promoter. Strikingly, in TCRg mice expressing CD8-MP TCRs, a fraction of TCRg CD8\(^{\beta+}\) thymocytes upregulated expression of Eomes, whereas Eomes upregulation was not observed for TCRg thymocytes expressing CD8-Naive TCRs (Fig. 4c–e). 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 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. To do this, we generated a series of low frequency TCRg 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 TCRg thymocytes expressing Eomes increased with decreasing clonal frequency (peaking at \(>90\%\), Fig. 4f), 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-Naive TCR (Fig. 4f), 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, in which post-selection TCR\(^{\beta+}\)CCR\(^{+}\) thymocytes are subdivided into cells of progressing maturational stage, ranging from semi-mature (CD69\(^{hi}\)MHC-I\(^{-}\), mature-1 (CD69\(^{hi}\)MHC-I\(^{-}\)), and mature-2 (CD69\(^{hi}\)MHC-I\(^{-}\)) (Fig. 4h). Notably, we found that for developing polyclonal T cells and the CD8-MP SAVrg clone, Eomes\(^{+}\) cells primarily exhibited a CD69\(^{hi}\)MHC-I\(^{-}\) mature-2 phenotype (Fig. 4h), 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 reasoned that Eomes-expressing CD8\(^{\beta+}\) 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\(^{-}\) mature (CD73\(^{-}\)CD69\(^{+}\)CD8\(^{\beta+}\) thymocytes from four-week-old Eomes-GFP reporter mice, and transferred these cells separately into the periphery of congenically disparate wild-type hosts via intravenous injection. Three weeks post transfer, we found that \(\sim 47\%\) of the Eomes-GFP\(^{+}\) donor cells recovered from recipient mice exhibited a CD44\(^{hi}\)CD122\(^{+}\) phenotype, compared to \(\sim 7\%\) of Eomes-GFP\(^{-}\) donor cells (Fig. 5a–c), which indicates that Eomes-expressing CD8\(^{\beta+}\) thymocytes are enriched for thymic intermediates with the potential to upregulate the CD8-MP phenotype in the periphery. To determine whether Eomes\(^{CD8\beta}\) thymocytes upregulate CD44 and CD122 when thymic egress is pharmacologically delayed, we used the immunomodulatory agent FTY720 to block the sphingosine 1-phosphate receptor-1 (SIPR1) and prevent thymic egress. We treated B6 mice with FTY720 or PBS control for 5 d and assessed the phenotype of CD8\(^{\beta+}\) thymocytes at day 6. This treatment induced a marked increase in the percentage and number of Eomes-expressing CD8\(^{\beta+}\) thymocytes (Fig. 5d–f), but did not induce an increase in the fraction of Eomes\(^{+}\) thymocytes that exhibit a CD44\(^{hi}\)CD122\(^{+}\) phenotype (Fig. 5g–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 cotransferred congenically disparate polyclonal CD8-Naive and polyclonal CD8-MP cells into two-month-old transgenic adenocarcinoma of mouse prostate (TRAMP) male mice, in which transgenic expression of a model oncogene drives the development of prostatic adenocarcinoma with high penetrance. 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, which ranged from 2–17% of intratumoral CD8+ T cells (Fig. 6a–f). Strikingly, the majority of donor CD8-MP cells expressed high densities of the inhibitory receptor PD-1 (Fig. 6a–f).

To further address the role of CD8-MP cells in antitumor immunity, we used our TCR sequencing approach to determine the extent to which CD8-MP-skewed clones contribute to the pool of tumor-infiltrating lymphocytes in TRAMP mice. To do this, we isolated CD8+ T cells from the prostate tumors of 5 male 27-week-old TRAMP+/– mice that expressed the fixed TCRβtg chain, and subjected these samples to deep Tcra sequencing (Supplementary Table 1). The survey identified numerous CD8+ T cell clones that are recurrently enriched in TRAMP prostate tumors (Fig. 6g, 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 (Fig. 6g, red boxes), and that the eight 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 that is 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, which suggests that intratumoral CD8-MP cells may functionally impact autotumor 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 frequencies20,26, and involves a two-step process marked by an initial TCR-dependent triggering step followed by a second phase of consolidation10,24. These findings challenge a common idea that CD8-MP cells represent LIM T cells that differentiate from naive CD8+ T cells in the periphery1,24. The finding that hundreds of recurrent CD8+ T cell clones, that constitute ~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 that coordinate the differentiation of these cells. Given our data which shows that many CD8-MP clones exhibit overt reactivity to self-ligands, we propose that the robust segregation of CD8-MP-biased clones to the CD8-MP subset is critical for the removal of overtly self-reactive CD8+ T cells from the naive compartment. Lastly, although TCR-independent factors such as IL-15 contribute to CD8-MP differentiation or survival20,23, 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 GF mice. Despite this, CD8-MP clones readily populate the peripheral repertoire and are not substantially impacted by clonal deletion. This suggests either that the ligand-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+CD122+ phenotype24 and exhibit innate-like effector functions in the early stages of pathogen challenge25. In view of our findings presented here, we suggest that foreign pMHC-I-specific CD8-MP cells in unprimed mice probably represent CD8-MP clones that were selected on self-pMHC-I ligands, but that cross-react with foreign pMHC-I ligands due to the plasticity that is inherent in TCR-pMHC ligand recognition24. 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 response26.

Seminal studies of CD8+ T cell memory differentiation and survival examined the biology of LIM cells, a population of CD44+CD122+ T cells that emerge following the intravenous transfer of mature naive-phenotype CD8+ T cells into lymphopenic hosts27. Given 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, which suggests that the forces that drive 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 probably depleted of the relevant TCRs that are naturally expressed by CD8-MP cells, and implies 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+ Eomes+ phenotype within tumors. Previously, the contribution of CD8-MP cells to the antitumor immune response in human or murine cancers had been largely undefined owing 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 cells20–23, and that only a minor fraction of such cells are overtly reactive to tumor-expressed antigens20,23. Given our findings presented here, we propose 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 that target 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, as well as the development of loss-of-function approaches for the selective depletion of CD8-MP cells.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0653-1.

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Methods

Mice. The following mice were purchased from The Jackson Laboratory, and were bred and maintained at the University of Chicago under SPF conditions: B6 mice, CD4^+^ (B6.SJL-Ppic^−^Pep^−^BoyJ) mice, Rag^−^ (B6.129Rag1tm1Mom) mice, B6.Ly5.2^−^ (B6;Ly5.2^−^Eorm6^−^Eorm7^−^) mice, CD4^+^C57BL/6.Cg-Tcratm1Mom/CJ (B6C3F1) mice, and TRAMP (C57BL/6.Tg(TCP8247Ng)J) mice. TCRβ^+^ mice expressing a fixed TCR were generated as described previously. All mice were bred and maintained in accordance with the animal care and use regulations of the University of Chicago. GF B6 mice were housed at the University of Chicago gnotobiotic facility under strict GF conditions. Both male and female mice were used across individual experiments. Mice were not randomized for assignment to experimental groups and experiments were not conducted in a blinded fashion.

Antibodies, flow cytometry, and fluorescence-activated cell sorting. 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 parentheses): active caspase 3 (392–405), CCR7 (AB12), CD4 (GK1.5, RM4–1 or RM4–5), CD8 (53–73.5), CD80 (YTS156.7.7), CD44 (IM7), CD69 (H1.2F3), CD73 (OX-7). Cells were stained for 20 min on ice in staining buffer (PBS with 2% FCS, 0.02% NaN3, 1% BSA; Sigma) with antibodies to Thy1.1 (AF6–88.5), Ki67 (SolA15), PD-1 (RMP1–30), TCR (H57–597) and CD8^α^ (R3–58.5). Cells were washed for 5–10 min on ice using staining buffer (PBS with 2% FCS, 0.02% NaN3, 1% BSA; Sigma) and were analyzed on a FACSCanto II or FACSCalibur (BD Biosciences). 10^5 TCR sequence reads were obtained per sample. TCRs were analyzed as described previously. TCRβ^+^ cells were labeled with CellTrace Violet (Thermo Fisher Scientific) according to the manufacturer’s instructions, with slight modifications. Briefly, cells were pelleted, resuspended in CellTrace Violet (CTV) at 1:10 dilution and incubated for 20 min at 37°C. The reaction was quenched by the addition of 10 ml of complete culture media. To isolate dendritic cells, spleens were isolated from 6-week-old SPF or GF B6 mice and thymi were isolated from 3 to 4-week-old SPF B6 mice. Spleens or thymi were injected and digested with Liberase TL (400 μg ml^−1^, Roche) and DNase (800 μg ml^−1^, Roche) in RPMI for 30 min at 37°C. For isolation of thymic antigen presenting cells, EDTA (10 mM) was added to freshly digested thymocytes to stop the digestion. Cells were enriched by layering digested thymocytes on top of a discontinuous Percoll gradient (GE Healthcare) at 1.115 g ml^−1^ in PBS, followed by centrifugation at 1,350 g for 30 min; cells that settled at the Percoll interface were isolated. Antigen presenting cells were enriched from spleens or thymi for CD11c^+^ cells by MACs-based positive selection. CTV-labeled T cells (1×10^6^) were co-cultured with CD11c^+^ antigen presenting cells and 100 μl of the supernatant was harvested at 24 h.

Statistical analysis. Data were analyzed using Prism software (GraphPad). Statistical testing on CDR3 length and GRAVY analysis section for script details). Statistical testing on CDR3 length and GRAVY

Thymocyte adoptive transfer experiments and FTY720 treatment. Thymus were isolated from 4-week-old CD45.2^+^ Eomes-GFP^+^ mice and CD4^+^ thymocytes were depleted by incubating thymi with anti-mouse CD4 antibody conjugated to biotin (clone RM4–5, Bio X Cell, clone M1/42.3.9.8) or rat IgG2a isotype control antibody (Bio X Cell, clone 2A3) was added to indicated cultures at a final concentration of 500 μg ml^−1^. 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.

Lymphocyte isolation from TRAMP prostate tumors. Whole male gonadectomy tracts were isolated and prostate lobes (anterior, dorsolateral and ventral) were separated by microdissection from 24-week-old TRAMP^−/^ mice for 24 h, followed by incubation with streptavidin beads (Stemcell Technologies) for 3 min at 37°C. Cells were then placed on the EasySep magnet (Stemcell Technologies) for 2 min and the supernatant was isolated. CD4 CD73 CD69 mature CD45^−^Eomes^−^CD4^+^IBD8^−^CD11c^+^ thymocytes were purified by MACs and 1×10^6^ cells from GFP^+^ or GFP^−^ cell suspensions were transferred intraperitoneally into CD45.1^+^ B6.SJL hosts. Three 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 and the phenotype of transferred cells was analyzed by flow cytometry. To prevent thymic egress of developing T cells, B6 mice were injected intraperitoneally every other day for 5 d with 140 μg FTY720 (Sigma) in 50 μl deionized H2O or PBS control. The phenotype of lymphocytes was analyzed by flow cytometry on day 6.

Retrovirus production, infection and generation of TCRg mice. Retrovirus production, infection and generation of TCRg mice.
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.

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Author contributions
C.H.M. designed the study, performed experiments, interpreted data and wrote the manuscript; D.E.J.K. performed experiments; S.Z. performed computational and statistical analysis of TCR sequence data; V.L. performed experiments and provided technical and conceptual advice; N.D.S. performed computational and statistical analysis of TCR sequence data; P.A.S. designed the study, interpreted data and wrote the manuscript. All authors contributed to discussion.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | 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 B6 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 CD44hiCD122+ 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).
Extended Data Fig. 2 | CD8-MP and CD8-Naive CDR3α chain hydrophobicity and length analysis. CD8-MP (CD8β+ CD44hiCD122+) and CD8-Naive (CD8β+ CD44loCD122–) 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-Naive 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-Naive (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 Kolmogorov-Smirnov test (K-S). Mouse 1 (CD8-MP CDR3 n = 11999, CD8-Naive CDR3 n = 29048), p = 0.2449 (W) and p = 1 (K-S); Mouse 2 (CD8-MP CDR3 n = 3327, CD8-Naive CDR3 n = 14736), p = 0.0511 (W) and p = 1 (K-S); Mouse 3 (CD8-MP CDR3 n = 2159, CD8-Naive CDR3 n = 26649), **p = 0.0017 (W) and p = 1 (K-S); Mouse 4 (CD8-MP CDR3 n = 2848, CD8-Naive CDR3 n = 24501), ***p < 0.0001 (W) and p = 1 (K-S); Mouse 5 (CD8-MP CDR3 n = 13524, CD8-Naive 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-Naive (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 Kolmogorov-Smirnov test (K-S). Mouse 1 (CD8-MP CDR3 n = 11999, CD8-Naive CDR3 n = 29048), p = 0.2031 (W) and p = 1 (K-S); Mouse 2 (CD8-MP CDR3 n = 3327, CD8-Naive CDR3 n = 14736), p = 0.2031 (W) and p = 1 (K-S); Mouse 3 (CD8-MP CDR3 n = 2159, CD8-Naive CDR3 n = 26649), **p = 0.0017 (W) and p = 1 (K-S); Mouse 4 (CD8-MP CDR3 n = 2848, CD8-Naive CDR3 n = 24501), **p = 0.0010 (W) and p = 1 (K-S). (n.s. not significant).
**Extended Data Fig. 3 | Phenotype of TCRrg and filler CD8^+ T cell populations.** (Top) Representative flow cytometric analysis of Thy1.1 vs. CD45.1 expression by TCRβ^+ CD8β^+ 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.
Extended Data Fig. 4 | A greater fraction of CD8-MP TCRg cells adopt the CD44hiCD122+ 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” TCRg mice expressing the indicated TCRs, assessed 7 weeks after bone marrow reconstitution. It should be noted that the expression of the Thy11 reporter varies in different TCRg 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.
CD8-MP TCRs

Extended Data Fig. 5 | CD8-MP TCRg cells are broadly distributed across lymphoid sites. Summary plots of the frequency of Thy1.1 expressing TCRg 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” TCRg mice. Frequencies at different lymphoid sites were normalized to the spleen to control for varying engraftment of TCRg 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 TCRg mouse. n = 3, GSSrg; n = 4, DTGrg; n = 4, SAVrg; n = 4, SATrg; n = 3, SMNrg; n = 3, RDTrg; n = 4, LNNrg; n = 4, DYQrg. Mean ± SEM is indicated. Data is pooled from four independent experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Thymocytes expressing CD8-MP-skewed TCRs exhibit hallmarks of elevated TCR signaling. a. 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. b. 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. c. Representative flow-cytometric analysis of Ki67 expression by TCRβ+ CD8β+ Thy1.1+ TCRrg thymocytes 6 weeks after bone marrow reconstitution. The percentage of cells in the indicated gates is denoted. Data are representative of five independent experiments. d. Left: Summary plot of pooled data from (c) 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-Naive TCRs. Each symbol represents an individual TCRrg mouse. n = 11, CD8-MP TCRrg mice; n = 10, CD8-Naive TCRrg mice. Mean ± SEM is indicated. **p = 0.0017, two-tailed nonparametric Mann–Whitney test. Data are pooled from five independent experiments. e. Representative flow cytometric analysis of CD5 expression by TCRβ+ CD8β+ Thy1.1+ thymocytes expressing the indicated CD8-MP and CD8-Naive 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. f. Left: Summary plot of pooled data from (e) showing the normalized mean florescence intensity (MFI) of CD5 in TCRrg thymocytes compared to CD8SP thymocytes from a B6 thymus. Right: Data from the left panel were pooled from the CD8-MP TCRs and the CD8-Naive TCRs. Each symbol represents an individual TCRrg mouse. n = 13, CD8-MP TCRrg mice; n = 12, CD8-Naive TCRrg mice. Mean ± SEM is indicated. ***p < 0.0001, two-tailed nonparametric Mann–Whitney test. Data are pooled from six independent experiments. g. Representative flow cytometric analysis of CD4 vs. CD8, PD-1 vs. CD69, and cleaved Caspase3 expression by TCRβ+ CD8β+ Thy1.1+ thymocytes from the thymi of TCRrg mice expressing the indicated CD8-MP TCRs and CD8-Naive 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.
Extended Data Fig. 7 | Gating strategy to analyze TCRγδ cells. Lymphocytes were gated on forward and side scatter and doublets were removed by gating on FSC-H by FSC-A. TCRβ⁺ T cells were then gated for the expression of CD8β⁺ and CD4⁻. CD8β⁺ T cells were then gated for the expression of Thy1.1. This TCRγδ cell population was used for subsequent phenotyping stains used throughout the paper. The percentage of cells in the indicated gates is denoted. Data are representative of six independent experiments.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection

- Complete TCRa sequence data was obtained using the Amp2Seq service from iRepertoire, a platform based on semi-quantitative multiplex PCR coupled with Illuma sequencing. Flow cytometry was performed on an LSR Fortessa and FACSFlow software was used in collection. FACS was performed using a FACSaria (BD Biosciences).

Data analysis

- GraphPad Prism [v6]
- Flowjo v10.2
- R version 3.5.1

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon request. 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.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose these points even when the disclosure is negative.

- **Sample size**: Power analysis reveals that in order to detect a difference of two standard deviations with a power of 80% and a = 0.05, 8 mice per group are required. We generally analyze 3 mice per group in a given experiment, and pool results from at least 3 experiments to attain sufficient group sizes and ensure reproducibility.

- **Data exclusions**: All collected data were included in the analyses.

- **Replication**: Experiments in each primary figure were replicated at least two times. All attempts at data replication were successful.

- **Randomization**: Whenever possible, littermate controls were used to control for age and potential effects of the microbiota and rare polymorphisms in various mouse lines on the B6 background.

- **Blinding**: Investigators were not blinded to group allocation during data collection and analysis. Mouse ages and genotypes were previously determined and used to allocate different experimental groups.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                             | n/a     |
| ☑ Antibodies                    | ☑ Involved in the study |
| ☑ Eukaryotic cell lines         | ☑ ChiP-seq |
| ☑ Palaeontology                 | ☑ Flow cytometry |
| ☑ Animals and other organisms   | ☑ MRI-based neuroimaging |
| ☑ Human research participants   |         |
| ☑ Clinical data                 |         |

**Antibodies**

Biologend antibodies: CCR7 (clone 4B12, Cat# 120108), CD4 (clones G1.1.5, RM4-4, or RM4-5, Cat#: 100508, 116006, 100548, 100442), CD8 (clone 53-7.3, Cat# 100606), CD25 (clone YTS169.7, Cat# 126610), CD44 (clone IV7, Cat# 103006, 103030, 103062), CD45.1 (clone A20, Cat# 110724, 110708, 110739), CD45.2 (clone 104, Cat# 109814), CD49d (clone R1-2, Cat# 103622), CD62L (clone MEL-14, Cat# 104424), CD69 (clone H1.2F3, Cat# 104506, 104508, 104512), CD73 (clone 1Y11.1, Cat# 127210, 127215), CD112 (clone TM-b1, Cat# 123210, 123216, 123214), CD127 (clone A7R24, Cat# 135009), H-2Kd (clone A6-88.1, Cat# 116520), PD-1 (clone RMP1-30, Cat# 109910), TCRb (clone H57-597, Cat# 109222, 109219), and Thy1.1 (clone OX-7, Cat# 202526, 202529, 202537, 202524).

Fisher Scientific antibodies: Active caspase-3 (clone C92-605, Cat# BDB54094), Eomes (clone Dan11mag, Cat# 50-107-94), Ki67 (clone SolA15, Cat# 50-112-4170).

**Validation**

All antibodies are commercially available.

**Eukaryotic cell lines**

Policy information about [cell lines](#)

**Cell line source(s)**

Plat-E Retroviral Packaging Cell Line (Cell Biolabs)

**Authentication**

Transfection of cell line was confirmed via fluorescent microscopy of GFP expression.
Mycoplasma contamination

Cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines

(See ICTAC register)

No commonly misidentified cell lines were used in this study.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Strains used: C57BL/6J (B6), CD45.1/1 (B6.SJL-Ptprca Pepcb/BoyJ), Rag1/-/- (B6.129577 Rag1tm1Mom/J), TCRa/-/- (B6.12952-Tcratm1Mom/J), CD4-Cre (B6.Cg-Tg(CD4-Cre)1Cwi/Jflu), “TCRbg” mice expressing a fixed TCRb chain of sequence TRBV26-ASLGSSYEQY, Rag2-GFP (C57BL/6-Tg(Rag2-EGFP)1Mn2/J), Eomes-GFP (C57BL/6-Eomesem1.1Twa/Cnbc), TRAMP C57BL/6-Tg(TRAMP)8247Ng/J.

All animals were used at 3 to 24 weeks of age. Both male and female mice were used.

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve samples collected from the field.

Ethics oversight

All mice were bred and maintained in accordance with the animal care and use regulations of the University of Chicago.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells from the lymph nodes and spleen were stained for 20 minutes on ice in staining buffer (phosphate-buffered-saline with 2% FCS, 0.1% NaN3, 5% normal rat serum, 5% normal mouse serum, 5% normal rabbit serum (all sera from Jackson Labs), and 10 ug/ml 2.4G2 antibody). Intracellular staining for Eomes and Ki67 was performed using fixation and permeabilization buffers from ebioscience. Cells being stained for CCR7 were incubated for 30 minutes at room temperature with surface stain antibodies in 2% FCS in PBS.

Instrument

Flow cytometry was performed on an LSR Fortessa [BD Biosciences]

Software

FACSDiva software was used for data collection and data was analyzed using FlowJo software.

Cell population abundance

The purity of T cell sorted subsets was >99% and was determined by purity check on the sorter.

Gating strategy

Cells were gated through a lymphocyte gate with a subsequent doublet discriminating gate. The gating strategy for each experiment is indicated in parentheses under each figure.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.