Cutting Edge: The Transcription Factor Eomesodermin Enables CD8^+ T Cells To Compete for the Memory Cell Niche

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CD8+ T cells responding to intracellular infection give rise to cellular progeny that become terminally differentiated effector cells and self-renewing memory cells. T-bet and eomesodermin (Eomes) are key transcription factors of cytotoxic lymphocyte lineages. We show in this study that CD8+ T cells lacking Eomes compete poorly in contributing to the pool of Ag-specific central memory cells. Eomes-deficient CD8+ T cells undergo primary clonal expansion but are defective in long-term survival, populating the bone marrow niche and reexpanding postrechallenge. The phenotype of Eomes-deficient CD8+ T cells supports the hypothesis that T-bet and Eomes can act redundantly to induce effector functions, but can also act to reciprocally promote terminal differentiation versus self-renewal of Ag-specific memory cells. The Journal of Immunology, 2010, 185: 4988–4992.

Memory CD8+ T cells can be categorized into at least two groups, effector memory and central memory, distinguishable by cell surface marker expression, anatomic location, and functional properties (1, 2). Effector memory CD8+ T cells are more prevalent in peripheral, nonlymphoid tissues, rapidly exert effector functions upon Ag encounter, and have limited proliferative capacity. Central memory cells retain greater capacity for secondary re-expansion and greater long-term persistence, enabled by efficient homeostatic self-renewal. Central memory CD8+ T cells are more prevalent in lymphoid tissues including the bone marrow, spleen, and lymph nodes. Of these tissues, the bone marrow is thought to provide a niche that supports homeostatic self-renewal and acts as a reservoir for memory CD8+ T cells (3–6).

Prior work supports critical roles for two members of the T-box transcription factor family, T-bet and eomesodermin (Eomes), in the formation of CD8+ T cell effector and memory subsets (7–9). The observation of enhanced central-memory differentiation in CD8+ T cells lacking T-bet suggests that T-box factors may serve as regulators of CD8+ T cell propensity for terminal effector differentiation versus persistence as long-lived memory cells (10, 11). In this study, we have evaluated terminal differentiation versus memory cell development in CD8+ T cells lacking Eomes. We observe diminished capacity to compete for the Ag-specific memory compartment in CD8+ T cells lacking Eomes. Eomes-deficient memory CD8+ T cells have defects in long-term persistence and secondary expansion postrechallenge, two hallmark properties of central memory CD8+ T cells. We further observe diminished ability to compete effectively for the bone marrow memory niche in memory CD8+ T cells lacking Eomes. These results suggest that Eomes confers competitive fitness to memory cells and support a role for Eomes in promoting persistence as long-lived memory versus terminal effector differentiation of Ag-specific CD8+ T cells.

Materials and Methods

Mice

Mice were used in accordance with the University of Pennsylvania Institutional Animal Care and Use Guidelines (Philadelphia, PA). C57BL/6 mice, P14 TCR-transgenic mice, Tbx21−/− (T-bet knockout [KO]) mice, Eomesfl/fl CD4-Cre (Eomes KO) mice (12), Thy1.1 mice, CD45.1 mice, and P14 TCR-transgenic mice, Tbx21−/− (T-bet knockout [KO]) mice, Eomesfl/fl CD4-Cre (Eomes KO) mice (12), Thy1.1 mice, CD45.1 mice, and RAG1−/− mice were backcrossed to C57BL/6 for at least 10 generations. For adoptive transfer experiments, 5 × 105 Thy1.1 P14 CD8+ T cells were mixed with 5 × 105 Eomes KO P14 CD8+ T cells transferred i.v. into CD45.1 recipients, with viral infection the following day.

Infections

Mice were infected with 2 × 105 PFU (initial challenge) or 1 × 106 PFU (rechallenge) lymphocytic choriomeningitis virus (LCMV) Armstrong strain by i.p. injection. For rechallenges, 5 × 10^5 Listeria monocytogenes-expressing GP33–41 (GP33) were injected i.v.

RAG1−/− bone marrow chimeras

Recipient RAG1−/− mice were subjected to sublethal irradiation (400 rad) and injected i.v. with 5 × 10^5 Thy1.1 bone marrow cells mixed with 5 × 10^5 Eomes KO bone marrow cells harvested on the same day. Eight to 10 wk posttransplant, peripheral blood from each recipient was analyzed for presence and relative numbers of CD8+ T cells, CD4+ T cells, and B cells derived from each background.

Abbreviations used in this paper: Eomes, eomesodermin; GP33, GP33–41; KO, knockout; LCMV, lymphocytic choriomeningitis virus; NP396, NP396–404; WT, wild-type.

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Flow cytometry and T cell stimulation

Surface staining, peptide stimulations, intracellular cytokine staining, H-2D<sub>b</sub>, GP33, and NP<sub>396–404</sub> (NP396) tetramer staining and flow cytometry were done as described (12). Abs used for flow cytometry were purchased from BD Biosciences (CD44, CD62L, CD122, CD127, CD27, IFN-γ, KLRC1, Thyl.1.2, and B220; San Jose, CA) or eBioscience (Eomes, CXCR4, integrin α<sub>4</sub>, and integrin β1; San Diego, CA).

BrdU incorporation

Mice received 0.2 ml PBS containing 2 mg BrdU by i.p. injection daily for 7 d preanalysis.

Quantitative real-time PCR

The quantitative real-time PCR primer and probe set used for hypoxanthine phosphoribosyltransferase was previously described (10). Presynthesized TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to amplify CXCR4, CXCR3, and Bcl-2. Sample gene values are expressed relative to hypoxanthine phosphoribosyltransferase, with the lowest value standardized at 1.

Results and Discussion

Diminished central memory CD8<sup>+</sup> T cell population in the absence of Eomes

To evaluate the expression of Eomes at the single-cell level in CD8<sup>+</sup> T cells responding to infection, we infected C57BL/6 mice with LCMV Armstrong and analyzed the expression of Eomes using intracellular flow cytometry in LCMV-specific CD8<sup>+</sup> T cells. Specificity of Eomes staining was validated using Eomes KO cells (Supplemental Fig. 1). Compared to CD8<sup>+</sup> T cells 8 d after LCMV infection, the vast majority of which are effector cells, LCMV specific memory CD8<sup>+</sup> cells 90 d after initial infection express higher levels of Eomes (Fig. 1A). Our results support a correlation between Eomes protein expression and preservation as LCMV-specific memory.

We infected Eomes KO mice and Eomes-proficient control mice (hereafter referred to as wild-type [WT]) with LCMV. Sixty days postinfection, we observed a modest deficit in memory CD8<sup>+</sup> T cells specific to the LCMV epitope GP33 in Eomes KO mice (Fig. 1B). We found that Eomes KO LCMV GP33-specific memory CD8<sup>+</sup> T cells have a substantial reduction in percentage of cells that are CD62L<sup>hi</sup> compared with WT, suggesting that fewer Ag-specific CD8<sup>+</sup> T cells differentiate into central memory CD8<sup>+</sup> T cells in the absence of Eomes (Fig. 1C).

We next examined the effector memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and central memory (CD44<sup>hi</sup>CD62L<sup>hi</sup>) CD8<sup>+</sup> T cell compartments in Eomes KO mice compared with T-bet KO and WT mice that were aged at least 6 mo and unchallenged (no LCMV infection) (Fig. 1D). In contrast to the increased central memory population in the absence of T-bet, central memory CD8<sup>+</sup> T cells are less abundant in Eomes KO mice compared with WT.

To assess the ability of Eomes-deficient CD8<sup>+</sup> T cells to mount a secondary memory response, we challenged Eomes KO or WT mice that had been infected with LCMV 100 d prior with 10 times the LD<sub>50</sub> of Listeria monocytogenes expressing GP33. Eomes KO mice had a mild defect in clearance of bacteria from the liver, but no defect in the spleen, suggesting preserved functional protective capacity in Eomes KO memory CD8<sup>+</sup> T cells (Fig. 1E). Overall, our findings suggest that there may not be an absolute requirement for Eomes in central memory CD8<sup>+</sup> T cell development.

Nonetheless, the data are compatible with a role for Eomes in promoting central memory-like cell persistence.

Eomes-deficient CD8<sup>+</sup> T cells contribute poorly to the memory cell pool when competing with normal cells

The relative defect in LCMV-specific CD8<sup>+</sup> T cell memory in Eomes KO mice indicates that although Eomes is not essential in memory development, its expression in individual CD8<sup>+</sup>
Eomes KO CD8+ T cells may provide a competitive advantage to persist as a memory cell. We employed a mixed bone marrow chimeric system to assess memory development in Eomes KO cells in direct competition with WT CD8+ T cells. Sublethally irradiated RAG1-deficient mice were reconstituted with bone marrow from an Eomes KO (Thy1.2+) mouse mixed in equal proportion with Thy1.1+ CD45 Cre+ (hereafter referred to as WT) bone marrow. Eomes deficiency did not appear to confer an advantage or defect in naive T cell homeostasis, as relative numbers of naive CD8+ T cells from Eomes KO and WT backgrounds were roughly equivalent (not shown). We infected these chimeras (Eomes KO/WT BM chimeras) with LCMV and followed the development of GP33- and NP396-specific memory CD8+ T cells derived from each genotype (Fig. 2A).

Eomes KO CD8+ T cells responded comparably to control CD8+ T cells after acute LCMV infection (day 8) (Fig. 2A), but at time points post viral clearance (days 30 and 70), the number of Eomes KO GP33-specific and NP396-specific CD8+ T cells declined relative to WT. On reinfection with LCMV on day 70 after primary infection, GP33- and NP396-specific CD8+ T cells derived from WT bone marrow underwent more robust re-expansion than those derived from Eomes KO bone marrow (Fig. 2A). Although most subsequent results are shown in GP33-specific populations, similar findings were found in NP396-specific CD8+ T cells. Eomes KO GP33-specific memory CD8+ T cells were not defective in the expression of IFN-γ following activation with GP33 peptide (Fig. 2B).

To reduce variability resulting from TCR heterogeneity, we also employed a competitive adoptive transfer model using TCR transgenic T cells. We combined naive WT P14 (LCMV-TCR transgenic T cells. We combined naive WT P14 (LCMV-TCR transgenic T cells. We combined naive WT P14 (LCMV-TCR transgenic T cells.
GP33-specific) TCR-transgenic CD8+ T cells (hereafter referred to as WT P14 and marked by Thy1.1) with an equal number of Eomes KO P14 CD8+ T cells (marked by Thy1.2) and injected the combination into CD45.1 non–TCR-transgenic recipients. We infected recipient animals with LCMV and followed the relative abundance of Eomes KO P14 and WT P14 CD8+ T cells. We found no consistent differences between Eomes KO and WT effector CD8+ T cells expansion 8 d postinfection (Fig. 2C). After viral clearance (day 45), the relative percentage of memory Eomes KO P14 cells declined precipitously (Fig. 2C). We analyzed the relative prevalence of Eomes KO versus WT GP33-specific memory cells in the blood, lymph nodes, spleen, and bone marrow of Eomes KO/WT bone marrow chimeric mice 60 d postinfection with LCMV (Fig. 2D). We observed a consistent hierarchy among different tissues, with the least amount of skewing in the blood, modestly increased skewing in the spleen and lymph nodes, and markedly higher skewing in the bone marrow (Fig. 2D). Although much of the data shown are derived from blood, similar observations were made in almost all lymphoid organs (not shown). In the setting of acute infection, we observed a 2.3-fold defect in bone marrow localization in Eomes KO P14 cells 8 d after LCMV infection in the competitive adoptive transfer model (Fig. 2E), suggesting a role for Eomes in bone marrow localization in both effector and memory CD8+ T cells.

Upon reinfection of immune mice 45 d after initial infection, WT P14 cells underwent re-expansion, whereas Eomes KO P14 cells underwent virtually no expansion and remained barely detectable (Fig. 2F). The defect in Eomes KO memory CD8+ T cell re-expansion when competing with WT cells was more severe than differences observed in nonchimeric Eomes KO and control mice rechallenged with LCMV 90 d after initial infection (Fig. 2F, 2G). The results suggest that Eomes enables CD8+ T cells to compete for niches and signals that promote memory differentiation rather than being an absolute regulator of memory differentiation.

The results raise the possibility of a relationship between Eomes expression and the development of memory-precursor (KLRG1hi CD127hi) CD8+ T cells (11). In the setting of acute LCMV infection, we observed similar levels of Eomes in memory precursors (KLRG1hi CD127hi) and short-lived effector cells (KLRG1hi CD127hi) (Fig. 2H). Similarly, 8 d postinfection, short-lived effector and memory-precursor population frequencies do not significantly differ within Eomes-deficient and -sufficient populations in a competitive setting (Fig. 2I). Several months after viral clearance, we observed significantly higher levels of Eomes in central memory (CD44hi CD62Llo) relative to effector memory (CD44hi CD62Lhi) CD8+ T cells (Fig. 2J). Taken together, these data do not support a role for Eomes in promoting the memory-precursor fate. Instead, the results provide evidence that Eomes is involved in bone marrow localization, long-term persistence, and re-expansion capacity of memory cells.

Defective population of the bone marrow niche by Eomes KO memory CD8+ T cells

The different ratios of WT to Eomes KO memory CD8+ T cells in different lymphoid tissues raise possibilities of altered bone marrow survival, proliferation, or trafficking in CD8+ T cells lacking Eomes. Molecules associated with memory T cell bone marrow localization include the chemokine receptor CXCR4 and VLA-4, which is composed of integrin α4 and integrin β1 (5). We observed diminished expression of CXCR4, but neither integrin α4 nor integrin β1, in Eomes KO GP33-specific memory CD8+ T cells from Eomes/WT bone marrow chimeric mice compared with WT 60 d after LCMV infection (Fig. 3A). Sorted Eomes KO central memory (CD44hi CD62Lhi) CD8+ T cells from Eomes/WT bone marrow chimeric mice 60 d after LCMV infection were found to have less CXCR4 and CXCR3 mRNA compared with their WT counterparts (Fig. 3B). Currently, there does not appear to be a known role for CXCR3 in bone marrow homing.

Diminished persistence of Eomes KO memory CD8+ T cells could be related to defects in homeostatic proliferation, survival, or both. The highest rate of homeostatic proliferation of memory CD8+ T cells is found in the bone marrow, and in bone marrow memory CD8+ T cells, we observed a modest reduction in proliferation in Eomes KO cells compared with WT as measured by BrdU incorporation (Fig. 3C). In addition to their proliferation defect, a survival disadvantage is suggested by our observation of reduced expression of Bcl-2 mRNA in Eomes KO memory CD8+ T cells (Fig. 3D). Proliferation and survival of memory CD8+ T cells are supported by cytokine signals, including IL-15 and IL-7, as well as CD27–CD70 interactions (1). We found a modest but reproducible defect in CD122 (IL-15Rβ) expression but no deficit in expression of CD127 (IL-7Rα) or CD27 on Eomes KO memory CD8+ T cells (Fig. 3E, Supplemental Fig. 2). These data suggest that reduced proliferation and survival might both be involved in the defective persistence of Eomes KO memory CD8+ T cells.

In summary, we provide evidence that CD8+ T cells lacking Eomes are less fit for preservation as memory and population
of the bone marrow memory niche. These observations are in keeping with a model in which the relative expression levels of Eomes and T-bet may contribute to the adoption of a memory fate versus terminal effector differentiation in CD8+ T cells. Given the ability to manipulate the relative expression of T-bet and Eomes with agents including rapamycin and IL-12 (13, 14), the data provide rationale for novel approaches to generating long-lasting immunity.

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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. Eomes expression in Wild-type versus Eomes KO CD8⁺ T cells. Splenocytes from wild-type or Eomes KO mice were stained with fluorochrome conjugated antibodies to CD8, CD44 and Eomes. CD8⁺ cells are shown. Data is representative of three independent experiments.
Figure S2. Reduced CD122 expression in Eomes KO memory CD8⁺ T cells.

(A) CD122 expression in wild-type versus Eomes KO memory CD8⁺ T cells in a competitive setting. Splenocytes from four individual Eomes KO / WT bone marrow chimeras 30 days after LCMV infection were stained with antibodies to CD8, CD122, and H2-D₀ GP33 tetramer. Graph shows average mean fluorescence intensity of CD122 in CD8⁺ GP33⁺ population with error bar representing SEM. *P<.01; Student's two tailed T test.

(B) CD122 expression in wild-type versus Eomes KO memory CD8⁺ T cells. Splenocytes from wild-type or Eomes KO mice 60 days after LCMV infection were stained with antibodies to CD8, CD122, and H2-D₀ GP33 tetramer. Graph shows average mean fluorescence intensity of CD122 in CD8⁺ GP33⁺ population with error bar representing SEM. Data is representative of two experiments, each with four Eomes KO mice and four C57BL/6 mice. *P<.01; Student's two-tailed T test.