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TECHNICAL ADVANCE

Technical Advance: Fluorescent reporter reveals insights into eomesodermin biology in cytotoxic lymphocytes

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ABSTRACT

The T-box transcription factor Eomes is expressed in cytotoxic immune cells and plays an important role in development, maintenance, and function of these cell types. Identification and separation of cells with differential Eomes expression would allow for better understanding of the transcriptional program governing these cytotoxic lymphocytes. Here, we report the use of an Eomes^{gfp}-targeted mouse allele that displays robust fidelity to Eomes protein expression in NK and T cells. Use of this reporter mouse revealed that Eomes expression in antiviral effector cells did not correlate with enhanced cytotoxicity but rather was associated with more efficient central memory differentiation. Weakening of reporter activity in Eomes-deficient CD8$^+$ T cells revealed a critical role for Eomes protein in maintaining central memory cells that have activated the *Eomes* locus. Characterization of reporter activity in NK lineage cells also permitted identification of a novel intermediate of NK cell maturation. Thus, the murine Eomes^{gfp}-targeted allele provides a novel opportunity to explore Eomes biology in cytotoxic lymphocytes.
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Introduction

Cytotoxic lymphocytes play important roles in antiviral and antitumor immune responses. Two major effector functions of NK and CD8$^+$ T cells are the release of proinflammatory cytokines and the killing of virally infected or transformed target cells via the perforin and granzyme pathway. These pathways are regulated by several transcription factors, including Eomes. However, Eomes has also been reported to regulate other essential features of cytotoxic cells. As a result, the relative importance of Eomes activity on effector functions versus differentiation remains obscure.

Eomes supports a variety of differentiation events, ranging from mesoderm formation in the developing embryo [1] to lymphocyte differentiation in response to infection [2–4]. In the hematopoetic system, Eomes has been reported to direct the differentiation of multiple lymphocyte subsets. For example, Eomes plays an important role in formation and maintenance of mature TRAIL$^-$DX5$^+$ NK cells [2, 5, 6]. In CD8$^+$ T cells, Eomes has pleiotropic roles, supporting the developmental program of effector [3] and memory [2, 7] T cells.

Eomes was identified in cytotoxic lymphocytes, owing to its role in regulating expression of several effector molecules, e.g., IFN-γ, perforin, and granzyme B [8]. Correspondingly, Eomes expression has been associated with enhanced CD8$^+$ T cell cytotoxicity [2, 9–14] and antiviral [3] and antitumor [9–11, 14–16] responses. Eomes expression has also been reported in cytotoxic CD4$^+$ T cells and is essential for expression of granzyme B in these cells [17]. Thus, Eomes is thought to be integral to the program of cytotoxic gene expression in multiple lymphocyte lineages and may provide an opportunity for therapeutic targeting.

Consequently, CD8$^+$ T cells that are induced or programmed to express high levels of Eomes would be predicted to have increased expression of effector molecules and enhanced lytic potential. Indeed, Eomes expression is heterogeneous within CD8$^+$ T cell populations, displaying a large dynamic range [7, 18]. Whether CD8$^+$ T cells with high Eomes expression produce more cytotoxic molecules and therefore, exhibit higher cytolytic capacity than Eomes nonexpressors has not been examined.

Abbreviations: ALPS=autoimmune and lymphoproliferative syndrome, CD62L=CD62 ligand, DN=double-negative, Eomes=eomesodermin, KLRG1=killer cell lectin-like receptor subfamily G member 1, LCMV=lymphocytic choriomeningitis virus, MFI=mean fluorescence intensity, NP=nucleoprotein, Tcm=central memory CD8$^+$ T cell, Tem=effector memory CD8$^+$ T cell

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Eomes expression is also particularly important for the maintenance of central memory T cells (Tcm) [7], in part, through its transcriptional regulation of the gene encoding IL-15Rβ (CD122) that enhances IL-15 responsiveness [2]. However, whether expression of Eomes during the expansion phase of the $CD8^+$ T cell response is associated with improved formation or survival of central memory cells has not been determined. The initial Eomes reporter mouse, with lacZ targeting the Eomes locus, required the addition of substrate to generate transient fluorescence [1, 2]. A subsequent construct contained transgenic Eomes control elements driving GFP expression correlated with Eomes expression in the developing embryo [19] yet displayed incomplete fidelity in the developing brain [19, 20]. A novel Eomes fluorescent reporter mouse, however, where GFP is expressed from the *Eomes* locus in lieu of Eomes more effectively paralleled Eomes expression [20]. Here, we report the validation and use of this $Eomes^{gfp}$-targeted allele in the study of Eomes gene expression in NK and $CD8^+$ T cells.

In $CD8^+$ T cells, we were able to separate Eomes expressors (GFP^+) from Eomes nonexpressors (GFP^-) by flow cytometry and address specific cytotoxic capacity of these subsets. Unexpectedly, Eomes expression was not associated with enhanced lytic potential in effector $CD8^+$ T cells following acute viral infection; however, early Eomes expression did correlate with improved central memory formation. Furthermore, examination of $Eomes^{gfp}$ expression in the absence of Eomes protein suggested that $Eomes^+$ Tcm may be dependent on Eomes expression for persistence. Lastly, reporter activity in Eomes-deficient NK cells allowed for the identification of putative intermediates in NK cell development, which are primed for full maturation into $TRAIL^-DX5^+$ NK cells. Thus, the $Eomes^{gfp}$-targeted allele should provide a novel opportunity to further understand the role of Eomes in cytotoxic lymphocytes.

MATERIALS AND METHODS

Mice and infection

All animals were housed at the University of Pennsylvania (Philadelphia, PA, USA). Experiments were performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. $Eomes^{gfp/+}$ mice have been described previously [20]. To study Eomes GFP reporter activity during Fas deficiency, $Eomes^{gfp/+}$ mice were mated with $Fas^{lpr/lpr}$mice. To study Eomes GFP reporter activity in the absence of Eomes protein, $Eomes^{gfp/+}$ mice were mated with $Eomes^{flox/flox}$ mice, with or without CD4-Cre or Vav-Cre. To study $Eomes^{gfp}$ expression during antiviral responses, mice were infected with 2×10^5 PFU of LCMV Armstrong strain by i.p. injection as described [3]. For some experiments, $Eomes^{gfp/+}$ mice were also mated with P14 TCR-transgenic mice to generate P14 $Eomes^{gfp/+}$ mice. $Eomes^{gfp/+}$ P14 $CD8^+$ T cells (CD45.1; 5×10^4) were transferred to WT B6 mice (CD45.2), 1 day prior to infection with LCMV Armstrong.

Flow cytometry, division dye-labeling, and real-time PCR

Surface and intracellular staining were performed as described previously [3, 18]. Antibodies used for flow cytometry were purchased from BD Biosciences (San Jose, CA, USA; CD3, CD4, CD8, CD19, CD27, CD44, CD62L, CD122, DX5, NK1.1) or eBioscience (San Diego, CA, USA; CD45.1, CD45.2, CD90.1, CD107a, CD127, Eomes, KLRG1, TRAIL). Data were collected on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Cell sorting was performed using a BD Aria II (BD Biosciences). For indicated experiments, sorted cells were subsequently labeled with CellTracker Violet (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantitative RT-PCR was carried out as described previously [3]. Target gene probes were purchased from Applied Biosystems (Foster City, CA, USA).

Cytotoxicity assay

Splenocytes from WT CD45.1 mice were labeled with two different concentrations (50 nM="dim" or 2 μM="bright") of CFSE (Invitrogen). $CFSE^{dim}$ target splenocytes were incubated with LCMV-derived NP396–404 peptide, whereas $CFSE^{bright}$ control splenocytes were incubated with OVA-derived SIINFEKL peptide for 1 h at 37°C. Target cells (5×10^3) were mixed with 5×10^3 control cells. GFP^+ and GFP^- $CD8^+$ T cells from Day 8 LCMV-infected mice were separated by cell sorting, and percentages of GFP^+ and GFP^- NP396-specifc $CD8^+$ T cells were determined by flow cytometry after tetramer staining. Increasing doses of GFP^+ and GFP^- NP396-specifc $CD8^+$ T cells were added to wells containing target and control cells. Unlabeled, WT CD45.2 CD57BL/6 splenocytes were used to normalize cell concentrations in each well. After 6 or 24 h incubation at 37°C, target cell destruction was assessed by flow cytometry. Percent-specific lysis was calculated as $100 \times [1-(\%$ target cell remaining/% control cell remaining)].

Statistical analysis

Student's *t*-test (unpaired), Pearson correlation, and Wilcoxon matched pairs test were performed using Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Expression of $Eomes^{gfp}$ is largely restricted to cytotoxic lineages and correlates with Eomes protein expression

Eomes expression has been reported in a variety of cell types during different stages of lymphocyte differentiation. We first validated whether expression of $Eomes^{gfp}$ faithfully matched Eomes expression by correlating GFP expression with cell types and properties previously reported to be associated with Eomes mRNA or protein.

In the hematopoietic system, Eomes is expressed predominantly in cytotoxic lymphocytes [8]. We therefore determined whether $Eomes^{gfp}$ expression followed a similar distribution within leukocyte lineages. In $Eomes^{gfp/+}$ mice, NK and $CD8^+$ T cells contained the highest frequency of GFP^+ cells, whereas GFP expression in $CD4^+$ T cells, B cells, and other lymphocyte lineages remained low or undetectable (**Fig. 1A**). The small population of GFP^+ $CD4^+$ T cells may represent a recently described subset of memory T_H2 cells [21]. In other blood cells, such as monocyte and granulocyte populations, GFP expression was undetectable (data not shown).

As GFP expression was heterogeneous in NK and $CD8^+$ T cells (Fig. 1A), we examined whether $Eomes^{gfp}$ activity matched previous reports of Eomes protein expression in different $CD8^+$ T cell subsets. We have recently identified an important role for Eomes in the generation of Tcm [7]. Consistent with Eomes protein expression [7], Tcm phenotype ($CD44^{hi}CD62L^{hi}$) from unmanipulated mice exhibited a high frequency of GFP expression, whereas naïve ($CD44^{lo}$) and

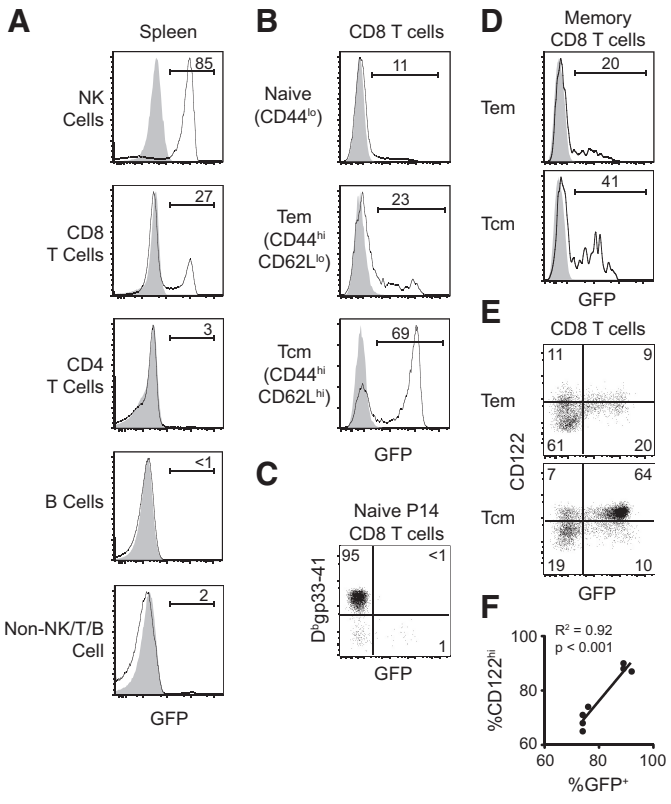

Figure 1. Expression of Eomes^{gfp} is largely restricted to cytotoxic lineages and correlates with Eomes protein expression. Flow cytometry of GFP expression in indicated subpopulations of (A) mature splenocytes and (B) splenic CD8$^+$ T cells from unmanipulated Eomes$^{+/+}$ (filled histograms) and Eomes$^{gfp/+}$(open histograms) mice. (C) Antigen-inexperienced CD8$^+$ T cells lack GFP expression. Flow cytometry of D^{b}gp33-41 binding versus GFP expression in CD8$^+$ T cells from the blood of a naïve P14 mouse. (D) GFP expression in Tcm and Tem from the spleens of LCMV-immune mice (Day 60 postinfection). (E) GFP expression positively correlates with a target of Eomes, CD122, in Tcm. Flow cytometry of CD122 versus GFP expression in Tem and Tcm phenotypes. Numbers denote frequency within the gated population. Results are representative of three to five independent experiments. (F) Graph displays %CD122^{hi} versus %GFP$^+$ of Tcm phenotype (Pearson correlation). Mice are aggregated across four independent experiments.

Tem phenotype (CD44^{hi}CD62L^{lo}) had a lower frequency of GFP expression (Fig. 1B). In addition, CD8$^+$ T cells from naïve P14 TCR transgenic mice specific for the LCMV D^{b}gp33-41 epitope remained GFP$^-$ (Fig. 1C), suggesting minimal Eomes expression in antigen-inexperienced CD8$^+$ T cells.

To examine GFP expression after antigen activation and differentiation, we infected mice with LCMV Armstrong. Examination of virus-specific cells again revealed elevated GFP expression in Tcm compared with Tem (Fig. 1D). Notably, a fraction of Tcm phenotype remained GFP$^-$. We therefore hypothesized that Eomes might regulate a subset of Tcm phenotype.

The β-chain for the IL-15R (CD122), a direct target of Eomes [2], is important for homeostasis of memory CD8$^+$ T cells by supporting basal homeostatic proliferation [22–24]. We therefore examined whether Eomes^{gfp} expression correlated with a known Eomes transcriptional target in memory CD8$^+$ T cells. Tem phenotype expressed low levels of GFP and CD122. In contrast, Tcm phenotype expressed high levels of CD122 and GFP, with a high frequency of GFP and CD122 coexpression (Fig. 1E). Accordingly, the frequency of CD122^{hi} cells correlated with the frequency of GFP expression within the central memory phenotype pool (Fig. 1F). Thus, the Eomes^{gfp}-targeted allele matches Eomes protein expression and transactivation by Eomes.

GFP expression quantitatively parallels Eomes protein expression

We next addressed whether activity of the Eomes^{gfp} locus matched quantitative changes in Eomes protein expression. Mice and humans with mutations in Fas or Fas ligand suffer from ALPS. A major feature of ALPS is lymphadenopathy and splenomegaly as a result of the accumulation of $\alpha\beta$ TCR-bearing CD4$^-$CD8$^-$ (DN) T cells (**Fig. 2A**). These DN T cells express substantial levels of Eomes, and Eomes expression is critical for their accumulation [25]. This model of Fas deficiency allowed us to examine whether activity of the Eomes^{gfp} locus would quantitatively match enhanced Eomes protein expression. Examination of NK, CD4$^+$, CD8$^+$, and DN T cells from Fas$^{WT/WT}$ Eomes$^{gfp/+}$ mice revealed that GFP$^+$ cells in each population expressed similar amounts of GFP/cell, as measured by MFI of GFP$^+$ cells (Fig. 2B). In Fas$^{lpr/lpr}$ Eomes$^{gfp/+}$ mice, however, CD4$^+$ and CD8$^+$ T cells exhibited a 1.5-fold enhancement of GFP expression over NK cells, whereas DN T cells demonstrated a greater than two-fold increase in GFP expression (Fig. 2B). The fold increase of GFP expression paralleled increases in Eomes protein expression (Fig. 2C). Of note, the enhanced frequency Eomes expression in CD8$^+$ T cells was associated with cells having an effector or effector-memory phenotype (CD44^{hi} CD62L^{lo}; Fig. 2D). Thus, the dynamic range of Eomes expression in DN T cells during ALPS is primarily regulated at the transcriptional level. In addition, the activity of the Eomes^{gfp} locus can discriminate between quantitative changes in Eomes protein levels.

To further confirm that Eomes^{gfp} expression correlates with Eomes protein expression in lymphocytes, we directly compared Eomes and GFP protein levels in NK cells. GFP fluorescence is lost upon fixation and permeabilization for Eomes protein staining as a result of direct quenching of fluorescence, loss of GFP protein from the cell, or a combination thereof. Therefore, we measured GFP expression by directly staining for GFP protein using an anti-GFP antibody, followed by an Alexa Flour 488-conjugated secondary antibody. Intracellular staining revealed a positive correlation between Eomes and GFP protein expression, as all GFP$^+$ cells were Eomes$^+$ (Fig. 2E). Of note, a small fraction of cells expressed Eomes protein without immunologic detection of GFP protein (Fig. 2E). Thus, Eomes^{gfp} locus activity appears to be a specific reporter of Eomes protein expression in lymphocyte populations.

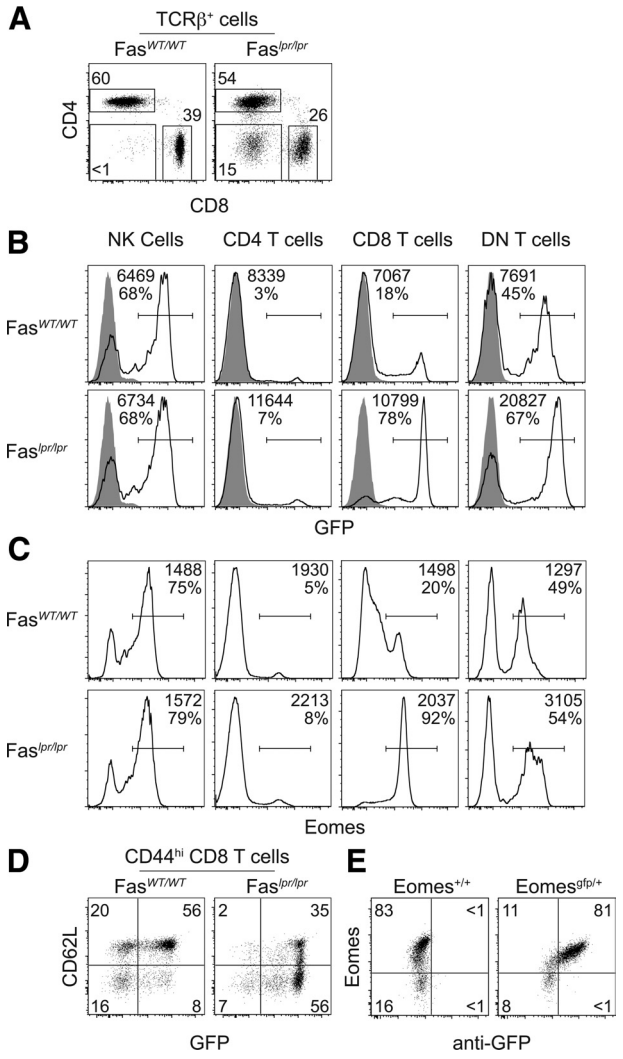

Figure 2. GFP expression quantitatively matches Eomes protein expression. (A) Frequency of $CD4^+$, $CD8^+$, and DN T cells from mice with an indicated Fas genotype. Flow cytometry of (B) GFP or (C) Eomes protein expression in indicated splenic subpopulations from $Eomes^{+/+}$ (filled histograms) and $Eomes^{gfp/+}$ (open histograms) mice with indicated Fas genotype. Numbers denote frequency and GFP MFI and Eomes MFI of GFP^+ and $Eomes^+$ cells, respectively. (D) The increased frequency of Eomes expression in $CD8^+$ T cells from $Fas^{lpr/lpr}$ mice is associated with a $CD44^{hi}$ $CD62L^{lo}$ population. Plots display CD62L and GFP expression in $CD44^{hi}$ $CD8^+$ T cells from mice with indicated Fas genotype. (E) GFP expression is specific for Eomes expression in lymphocytes. Flow cytometry of intracellular staining for Eomes and GFP protein in NK cells from $Eomes^{+/+}$ and $Eomes^{gfp/+}$ mice. Results are representative of two to three independent experiments.

Equivalent cytotoxic potential of effector $CD8^+$ T cells with differential Eomes expression

Eomes has been reported to transcriptionally activate several genes associated with cytotoxic capacity, such as those encoding perforin and granzyme B [8, 13]. Furthermore, in vitro culture conditions that induce heightened expression of Eomes in $CD8^+$ T cells are associated with more potent cytotoxicity than those conditions resulting in lower Eomes [13]. However, direct analysis of whether Eomes expression leads to enhanced cytotoxic potential is lacking. To this end, $Eomes^{gfp/+}$ reporter mice were infected with LCMV Armstrong to assess whether Eomes expression correlates with enhanced cytotoxicity in effector $CD8^+$ T cells.

We have previously reported that Eomes protein is equally distributed between memory precursors and terminal effectors [7]. Therefore, we first confirmed that the $Eomes^{gfp}$ reporter had a similar pattern of expression in these two subsets. Consistent with our previous observations for Eomes expression, virus-specific GFP^+ and GFP^- $CD8^+$ T cells displayed equal expression of terminal effector markers, such as KLRG1, and memory markers, such as CD127 or CD27 (**Fig. 3A**).

To determine whether Eomes expression conferred an enhanced cytolytic potential, we assessed whether Eomes expres-

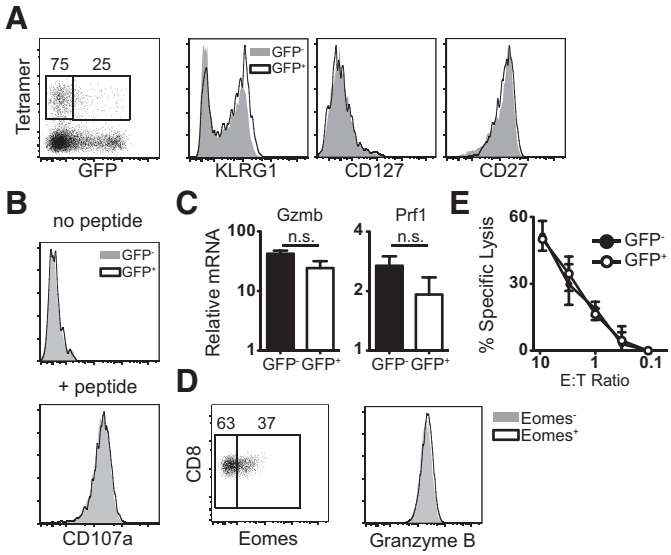

Figure 3. Eomes expression does not correlate with enhanced cytotoxicity. Endogenous or P14 $Eomes^{gfp/+}$ $CD8^+$ T cell responses were assessed after LCMV infection. (A) GFP expression in $CD8^+$ T cells is not biased toward terminal effector or memory precursor cells. GFP^+ and GFP^- NP396-specific $CD8^+$ T cells were assessed for KLRG1, CD127, and CD27 expression, 8 days postinfection. (B) Eomes expression does not correlate with enhanced degranulation. $Eomes^{gfp/+}$ P14 $CD8^+$ T cells were stimulated with or without D^bgp33-41 peptide, 7 days postinfection. Degranulation was assessed by CD107a staining. (C) Eomes expression does not correlate with enhanced transcription of cytotoxic molecules. $Eomes^{gfp/+}$ P14 $CD8^+$ T cells were sorted based on GFP expression, 7 days postinfection, and assessed for perforin (Prf1) and granzyme B (Gzmb) mRNA expression. mRNA levels were normalized to hypoxanthine guanine phosphoribosyl transferase and are reported relative to naïve cells (n=3; Wilcoxon matched pairs test). (D) $Eomes^+$ and $Eomes^-$ NP396-specific $CD8^+$ T cells were assessed for granzyme B protein expression. (E) Cytolytic activity of GFP^+ and GFP^- $CD8^+$ T cells. Sorted GFP^+ and GFP^- $CD8^+$ T cells were equalized for NP396-specific $CD8^+$ T cells and added to target cells pulsed with LCMV-derived peptide NP396–404. Percent-specific lysis was determined by the loss of target cells compared with control cells. Graphs display mean ± SD (n=3). Results are representative of two to four independent experiments.

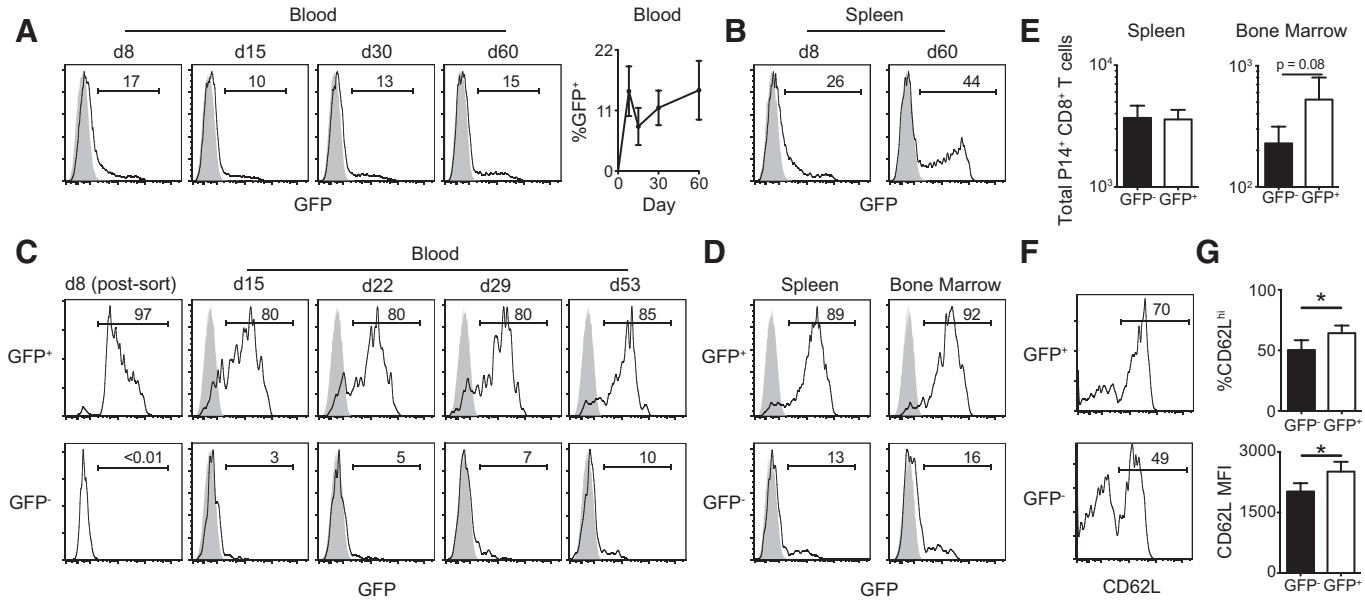

Figure 4. Progressive enrichment of Eomes expression in memory CD8$^+$ T cells. Endogenous or P14 Eomes$^{gfp/+}$ CD8$^+$ T cell responses were assessed after LCMV infection. (A) Induction of Eomes expression is biphasic. Longitudinal GFP expression in gp33-specific CD8$^+$ T cells from the blood of Eomes$^{gfp/+}$ mice at indicated days (d) postinfection (n=4). (B) GFP expression of NP396-specific CD8$^+$ T cells from the spleen of Eomes$^{gfp/+}$ mice at indicated days postinfection. (C) Early expression of Eomes is predominantly stable and is associated with more efficient central memory generation. Eomes$^{gfp/+}$ P14 CD8$^+$ T cells were sorted based on GFP expression, 8 days postinfection, and transferred into infection-matched controls. Plots display GFP expression of sorted populations in the blood at indicated days postinfection. (D) GFP expression of sorted populations recovered from the spleen and bone marrow, 45 days post-transfer (53 days postinfection). (E) Total P14 Eomes$^{gfp/+}$ CD8$^+$ T cells recovered in C (n=4; unpaired t-test). (F) CD62L expression of sorted populations from D. (G) Quantification of percent CD62L^{hi} and CD62L MFI of CD62L^{hi} cells from F (n=4; *P<0.05, unpaired t-test). Numbers indicate frequencies within gated populations. Graphs display mean ± SD. Results are representative of two to four independent experiments.

sion was associated with enhanced degranulation or increased expression of cytotoxic molecules. As peptide stimulation leads to TCR down-regulation and loss of tetramer staining, we used cells from P14 TCR transgenic mice, where we could track virus-specific cells by congenic markers. Eomes$^{gfp/+}$ P14 CD8$^+$ T cells were isolated 1 week postinfection and stimulated with cognate peptide. GFP$^+$ and GFP$^-$ P14 CD8$^+$ T cells displayed equal levels of degranulation, as measured by CD107a (lysosome-associated membrane protein 1) staining (Fig. 3B). We next evaluated whether Eomes expression led to enhanced transcription of cytotoxic molecules. Eomes$^{gfp/+}$ P14 CD8$^+$ T cells from LCMV-infected mice were sorted based on GFP expression. Comparison of GFP$^+$ and GFP$^-$ effector CD8$^+$ T cells revealed a modest reduction in the expression of granzyme B and perforin mRNA in GFP$^+$ cells compared with GFP$^-$ cells, although this trend was not statistically significant (Fig. 3C). We next determined whether these modest differences in mRNA expression led to differences in protein expression. CD8$^+$ T cells, with and without Eomes expression, however, contained equivalent granzyme B protein expression (Fig. 3D), suggesting that Eomes expression does not correlate with differential cytotoxic molecule expression in effector CD8$^+$ T cells following LCMV Armstrong infection.

Last, we determined whether Eomes expression influenced the cytotoxic potential of effector CD8$^+$ T cells. Sorted GFP$^+$ and GFP$^-$ CD8$^+$ T cells displayed equivalent cytotoxicity on a per-cell basis in an in vitro killing assay (Fig. 3E). Thus, whereas Eomes may activate genes encoding cytotoxic molecules early after T cell activation in vitro, Eomes expression does not correlate with enhanced cytotoxicity in fully differentiated in vivo-generated effector T cells. Consequently, robust killing may be independent of Eomes expression at this stage of the CD8$^+$ T cell response following acute infection.

Progressive enrichment of Eomes expression in memory CD8$^+$ T cells

Compared with effector CD8$^+$ T cells at the peak of cellular expansion, memory CD8$^+$ T cells have elevated Eomes expression [2, 7]. Whether this elevated expression is a result of selection of Eomes$^+$ memory CD8$^+$ T cells, gradual induction of Eomes expression in Eomes$^-$ memory CD8$^+$ T cells, or a combination of the two is unclear. To assess whether Eomes enrichment is a result of selection or induction, we first examined GFP expression longitudinally in virus-specific Eomes$^{gfp/+}$ CD8$^+$ T cells after infection with LCMV.

As noted above, GFP expression was elicited in virus-specific CD8$^+$ T cells at the peak of expansion (Day 8). During the contraction phase, however, a consistent reduction in GFP expression was observed (Days 8–15; **Fig. 4A**). Nevertheless, as the memory population matured (Days 15–60), GFP expression was progressively enriched (Fig. 4A and B),

consistent with elevated Eomes expression in late virus-specific memory [2, 7].

As Eomes expression is important for Tcm [7], it is possible that expression of Eomes early after activation would lead to enhanced memory formation [14]; however, this hypothesis has not been formally examined. To determine whether early Eomes expression correlates with the formation of long-lived Eomes$^+$ CD8$^+$ T cells, P14 Eomes$^{gfp/+}$ CD8$^+$ T cells were sorted based on relative GFP expression 8 days postinfection. Sorted GFP$^+$ and GFP$^-$ were transferred into congenic infection-matched controls to confer sufficient competition from an endogenous LCMV-specific response. Whereas analysis of GFP expression in donor cells revealed a modest down-regulation of Eomes in the 1st week after viral clearance, 80–90% of GFP$^+$ cells retained Eomes expression (Fig. 4C and D). Similarly, GFP$^-$ cells largely remained GFP$^-$; however, some accrual of GFP expression was observed (Fig. 4C and D). Thus, whereas some induction of Eomes may occur during the memory maturation, the majority of Eomes expression in the memory population appears to arise during the initial phase of the antiviral response.

We next tested whether early Eomes expression was associated with improved memory differentiation or a quantitative advantage in populating the memory pool. P14 Eomes$^{gfp/+}$ CD8$^+$ T cells, which were separated by GFP expression at Day 8 postinfection, gave rise to equal numbers of memory cells, with a modest trend toward enhanced homing to the bone marrow for GFP$^+$ donor cells (Fig. 4E). The CD8$^+$ T cell population with early Eomes expression, however, did mature more rapidly to central memory, as measured by the expression of CD62L (Fig. 4F and G). Thus, whereas early Eomes expression may not be associated with enhanced survival into the memory phase, early induction of the *Eomes* locus correlates with more efficient central memory formation.

Expression of the Eomes locus is reduced in the absence of Eomes protein

We next evaluated whether CD8$^+$ T cells stably transcribe the Eomes locus in the absence of Eomes protein. As the Eomes^{gfp} knock-in allele creates a null homozygous lethality, we generated Eomes$^{gfp/flox}$ mice, with or without Cre recombinase, under the control of the CD4 promoter (CD4-Cre) that would delete Eomes at the double-positive stage of thymocyte development.

Eomes$^{gfp/flox}$ mice contain a high frequency of CD8$^+$ T cells with a phenotype of long-lived, self-renewing central memory, e.g., expressing L-selectin (CD62L) and components of the IL-15R (CD122) and IL-7R (CD127; **Fig. 5A**, left column). Consistent with a role for Eomes in the support of Tcm differentiation [7], a majority of these Tcm expresses GFP (Fig. 5A, left column). Deletion of Eomes led to reduced expression of all three markers of long-lived memory CD8$^+$ T cells and to a reduced frequency of GFP$^+$ cells (Fig. 5A, right column). In particular, less than one-quarter of CD62L^{hi}, CD122^{hi}, or CD127^{hi} CD8$^+$ T cells maintained GFP expression in the absence of Eomes protein (Fig. 5A, right column). Quantification of GFP$^+$ and GFP$^-$ Tcm phenotype suggested that the lower GFP expression was a result of specific loss of the Eomes$^+$ subset rather than reduced *Eomes* locus activity (Fig. 5B).

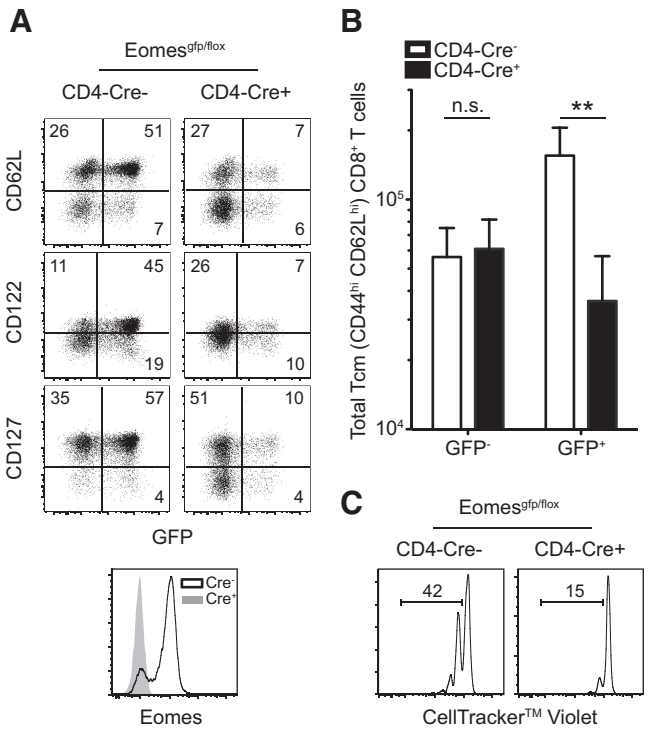

Figure 5. Central memory cells with an active *Eomes* locus are reduced in the absence of Eomes protein. (A) Flow cytometry of CD62L, CD122, and CD127 versus GFP expression in splenic CD44^{hi} CD8$^+$ T cells from mice with an indicated genotype. Numbers denote frequency of cells in each quadrant. Eomes protein expression is also shown for the Cre-negative (open) and Cre-positive (filled) populations. (B) Total GFP$^-$ and GFP$^+$ central memory (CD44^{hi} CD62L^{hi}) cells in the presence (Cre-negative; open) or absence (Cre-positive; filled) of Eomes protein (n=4; **P<0.01, unpaired t-test). Graph displays mean ± SD. (C) Sorted GFP$^+$ central memory (CD44^{hi} CD62L^{hi}) cells from indicated mice were labeled with a cell division dye (CellTracker Violet) and transferred into CD45.1 congenic recipients. Histograms display dilution of CellTracker Violet after 4 weeks in vivo. Results are representative of two to four independent experiments.

In the absence of Eomes, Tcm fail to maintain a long-lived, stable population, perhaps as a result of decreased homeostatic proliferation because of reduced bone marrow homing [7]. To determine whether the specific loss of GFP$^+$ Tcm phenotype resulted from poor homeostatic proliferation, we sorted GFP$^+$ CD44^{hi} CD62L^{hi} CD8$^+$ T cells from Eomes$^{gfp/flox}$ mice, with or without CD4-Cre, labeled the cells with a cell-proliferation dye (CellTracker Violet), and transferred them into congenically disparate hosts. Analysis of cell division after 1 month in vivo demonstrated reduced homeostatic proliferation in Eomes-deficient CD8$^+$ T cells (Fig. 5C). Thus, Tcm that activate Eomes transcription may also become dependent on Eomes protein for persistence.

Eomes^{gfp} allows for detection of putative intermediates of NK cell development

TRAIL$^+$DX5$^-$ NK cells appear to represent developmental intermediates of NK cell maturation. Adoptive transfer of

TRAIL$^+$DX5$^-$ NK cells has been shown to generate TRAIL$^-$DX5$^+$ NK cells in recipient mice [6, 26]. Deletion of Eomes, however, prevents NK cells from progressing past the TRAIL$^+$DX5$^-$ precursor stage [6]. Whether TRAIL$^+$DX5$^-$ NK cells activate Eomes expression prior to the maturation into TRAIL$^-$DX5$^+$ NK cells is not known. To investigate whether Eomes transcriptional activation occurs prior to maturation, we examined Eomes^{gfp} activity in NK cells in the presence or absence of Eomes protein through the expression of Cre recombinase under the control of the Vav regulatory elements (Vav-Cre), which leads to the elimination of Eomes in all hematopoetic lineages [6].

We first determined that GFP expression correlated with markers of NK maturation. In Eomes$^{gfp/+}$ mice, GFP expression was restricted to TRAIL$^-$DX5$^+$ NK cells (**Fig. 6A**), consistent with Eomes protein expression [6]. In the absence of Cre, Eomes$^{gfp/flox}$ NK cells are able to express one functional copy of Eomes and appropriately generate a high-frequency population of TRAIL$^-$DX5$^+$ NK cells that express GFP and Eomes protein (Fig. 6B, left column). As expected, NK cells from Eomes$^{gfp/flox}$ mice harboring Cre had a severe reduction in the frequency of mature TRAIL$^-$DX5$^+$ NK cells and a selective enrichment for TRAIL$^+$DX5$^-$ precursors (Fig. 6B, right column). Nevertheless, Eomes-deficient NK cells contained a small population of cells that expressed high levels of GFP and TRAIL (Fig. 6B, upper). The dual expression of GFP and TRAIL suggests that a population of TRAIL$^+$DX5$^-$ precursors receives differentiation signals and activate Eomes transcription in the initial stages of the development of mature NK cells.

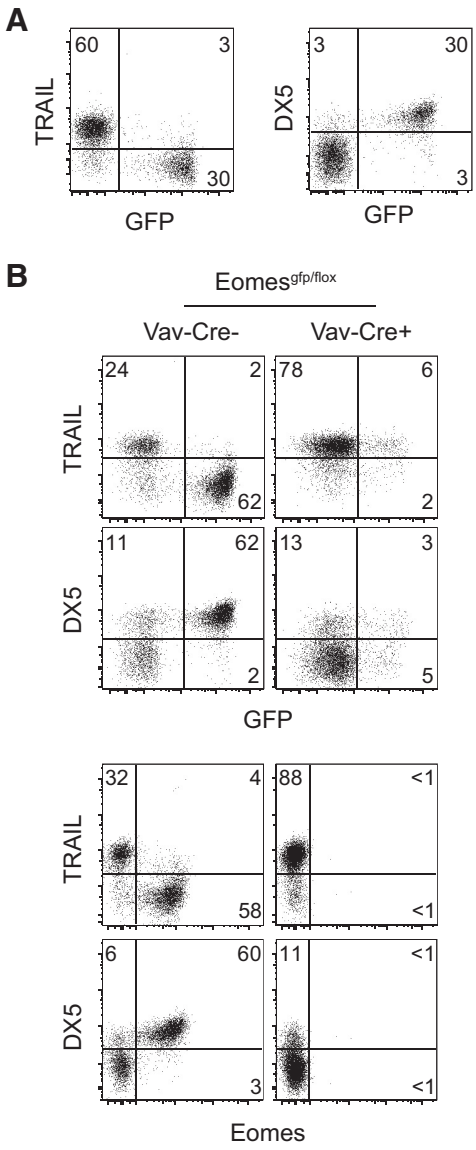

Figure 6. Eomes^{gfp} allows for detection of putative intermediates of NK cell development. (A) GFP is predominantly expressed in mature TRAIL$^-$DX5$^+$ NK cells. Flow cytometry of TRAIL, DX5, and GFP expression in liver NK cells. (B) Flow cytometry of TRAIL or DX5 versus GFP or Eomes protein expression in liver NK cells from mice with indicated genotype. Numbers denote frequency of cells in each quadrant. Results are representative of two independent experiments.

DISCUSSION

In this study, we described the GFP expression of an Eomes^{gfp}-targeted allele in the adult leukocyte compartment and matched Eomes and GFP expression in mature lymphocytes. Whereas Eomes expression did not segregate with differential cytotoxicity in effector CD8$^+$ T cells, early Eomes expression did lead to more efficient central memory formation. In addition, Tcm that activate the *Eomes* locus may become dependent on Eomes protein for persistence and homeostatic proliferation. Lastly, GFP expression in Eomes-deficient NK cells enabled the identification of a putative intermediate of NK cell development.

Retroviral-based overexpression of Eomes appears to initiate a cytotoxic transcriptional program, activating effector genes, such as IFN-γ, granzyme B, and perforin [8, 13, 27]. Furthermore, in cultures with high IL-2 concentration, higher expression of Eomes correlates with CD8$^+$ T cell-killing capacity [11, 13]. However, whether the enhanced killing capacity is a direct result of increased Eomes expression was not addressed. With the availability of an Eomes^{gfp} reporter, we were able to separate cells with high Eomes expression from cells with negative or low Eomes expression within the same population. We found that higher Eomes expression did not correlate with higher expression of the cytotoxic molecules granzyme B and perforin or with enhanced cytotoxicity in fully differentiated effector CD8$^+$ T cells. One possible resolution to this paradox is that Eomes may play a more important role earlier in acquiring cytotoxic potential rather than in exerting cytotoxic function. Alternatively, the equivalent cytotoxic molecule expression and cytotoxicity of GFP$^+$ and GFP$^-$ subsets may be a result of the redundancy of other transcription factors at this stage of the CD8$^+$ T cell response. In particular, the T-box factor T-bet plays a redundant role with Eomes in the induction of the cytotoxic program in effector CD8$^+$ T cells [3]. Indeed, previous work indicates that deletion of Eomes did not appreciably affect the killing capacity of effector CD8$^+$ T cells [3].

Other transcription factors may also contribute to cytotoxic gene expression in the absence of Eomes. Notch [28] and Runx3 [27] can both bind to the granzyme B and perforin loci and activate their transcription. In addition, differential signaling through Akt can modulate perforin expression without altering T-bet or Eomes expression [29], suggesting T-box factor-independent pathways for cytotoxic gene regulation. As a result, multiple transcription factors may compensate for lack of Eomes in activating cytotoxic genes in effector $CD8^+$ T cells.

We have recently reported a role for Eomes in the generation of Tcm in response to viral infection [7]. As the homologous T-box factor T-bet drives the terminal differentiation of effector $CD8^+$ T cells [30, 31], one might hypothesize that Eomes would conversely drive formation of memory precursors. Eomes expression, however, did not correlate with markers of memory precursor cells nor did deletion of Eomes impair the generation of memory precursors [7]. Nevertheless, Eomes expression at the peak of cellular expansion was associated with more rapid accumulation of Tcm and modest quantitative advantage in the bone marrow compartment. Whereas previous findings may predict that early Eomes expression would correlate with competitive survival of memory cells, the moderate differences seen between GFP^- and GFP^+ cells may have been limited by heterozygosity of the Eomes locus. Alternatively, expression of Eomes, other than at the peak of cellular expansion, may be associated with improved survival of memory cells. Irrespective of these modest quantitative differences, Eomes likely supports memory differentiation after the stage of memory cell-fate commitment. When Eomes begins to contribute to the program of memory differentiation, however, remains to be determined. In addition, it will be important to determine whether Eomes is associated with enhanced secondary expansion and when that association emerges.

After viral infection, the memory pool has heterogeneous expression of many proteins that contribute to the function or longevity of memory cells. For example, an early division of the memory pool separated cells into effector memory and central memory populations [32]. However, both of these populations contain further heterogeneity, specifically with regard to the expression of Eomes. Thus, Eomes transcriptional activity may only regulate a fraction of central memory cells. In $CD8^+$ T cells expressing markers of long-lived, self-renewing central memory, Eomes protein expression was important for maintaining cells with stable expression of the *Eomes* locus. This failed persistence of $CD8^+$ T cells that would normally express *Eomes* transcripts in the absence of Eomes protein appeared to be a result of the impaired homeostatic proliferation. Thus, $Eomes^-$ Tcm may contain a distinct program for survival compared with $Eomes^+$ central memory cells.

We [6] and others [26] have reported the development of mature $TRAIL^-DX5^+$ NK cells from $TRAIL^+DX5^-$ NK cells. However, the signals that initiate full maturation of $TRAIL^+DX5^-$ precursors are unknown. Furthermore, the transcriptional events initiating the developmental program of NK maturation are similarly obscure. By examining Eomes-deficient NK cells carrying an $Eomes^{gfp}$ reporter allele, we were able to identify a small population of $TRAIL^+DX5^-$ precursors that had activated Eomes transcription and potentially initiated subsequent steps in NK cell maturation. However, in the absence of Eomes protein, these GFP^+TRAIL^+ cells did not accumulate. These data suggest that Eomes does not initiate this developmental step but rather, that Eomes is critical for full enactment of this differentiation cascade. Future studies will be needed to determine whether these cells fail to persist in the absence of Eomes protein as they progress through NK cell development or whether they return to the $TRAIL^+DX5^-$ precursor program and repress Eomes expression. Further exploration of GFP^+ $TRAIL^+DX5^-$ precursors offers a novel opportunity to define the factors influencing NK cell maturation.

The ability to separate lymphocytes based on graded transcription factor expression is restricted to the identification of correlative surface markers [31] or the generation of reporter mice. This need is especially compelling in the case of transcription factors that exhibit a large dynamic range of expression within a single population. Thus, the generation of an $Eomes^{gfp/+}$ reporter that exhibits robust fidelity to Eomes protein expression in cytotoxic lymphocytes should prove to be a beneficial tool in elucidating how heterogeneous Eomes expression results in differential fate or function.

AUTHORSHIP

M.A.P. and S.M.G. performed and interpreted experiments. E.K.B. and E.J.R. generated the $Eomes^{gfp}$ mouse and helped draft the manuscript. M.A.P., E.J.W., and S.L.R. designed the research and wrote the manuscript.

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