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Materials and Methods
Table S1

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Control of Effector CD8$^+$ T Cell Function by the Transcription Factor *Eomesodermin*

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Activated CD8$^+$ T cells play a critical role in host defense against viruses, intracellular microbes, and tumors. It is not clear if a key regulatory transcription factor unites the effector functions of CD8$^+$ T cells. We now show that *Eomesodermin* (*Eomes*), a paralogue of *T-bet*, is induced in effector CD8$^+$ T cells in vitro and in vivo. Ectopic expression of Eomes was sufficient to invoke attributes of effector CD8$^+$ T cells, including interferon-γ (IFN-γ), perforin, and granzyme B. Loss-of-function analysis suggests Eomes may also be necessary for full effector differentiation of CD8$^+$ T cells. We suggest that *Eomesodermin* is likely to complement the actions of *T-bet* and act as a key regulatory gene in the development of cell-mediated immunity.

Emerging evidence suggests that the functional development of distinct T cell lineages is directed by key, or "master," regulatory transcription factors, which have the capacity to impart the defining attributes of a lineage (*1*–*8*). *T-bet* is a member of the T-box family of genes responsible for controlling lineage commitment in T helper 1 (T_H1) cells (*2*–*5*). CD4$^+$ T cells and natural killer (NK) cells from T-bet–deficient mice are defective in their ability to express IFN-γ, although CD8$^+$ T cells from T-bet–deficient mice reportedly can exhibit normal IFN-γ induction and cytolytic function (*3*). This implies the existence of a T-bet–independent pathway for effector differentiation of CD8$^+$ T cells.

To further examine this, we tested the effect of a dominant negative form of T-bet (DN T-bet) on the differentiation of CD8$^+$ T cells. The DN T-bet construct, which contains the *Drosophila* engrailed repression domain in place of the endogenous transactivation domain, inhibits Th1 differentiation (*5*, *9*) in a manner similar to *T-bet* gene deletion (*3*). In light of the reported T-bet–independent expression of IFN-γ from CD8$^+$ T cells (*3*), the profound defect in IFN-γ expression resulting from introduction of DN T-bet retrovirus (RV) into activated CD8$^+$ T cells was unexpected (Fig. 1A). Consistent with prior studies of T-bet deficiency (*3*), we found that CD8$^+$ T cells from a newly generated line of T-bet–deficient mice (*10*) also exhibit only minimal defect in their ability to express IFN-γ (Fig. 1A). Despite the absence of detectable T-bet activity in cells of *T-bet*$^{-/-}$ mice, DN T-bet was nevertheless able to antagonize the expression of IFN-γ in CD8$^+$ T cells from these animals (Fig. 1A). These results reveal a T-bet–independent, but DN T-bet–sensitive, pathway for IFN-γ induction in CD8$^+$ T cells.

The apparent antagonism of IFN-γ induction by DN T-bet, in the absence of T-bet, suggested the existence of an additional T-box factor in CD8$^+$ T cells that might control the expression of IFN-γ. We, therefore, used degenerate oligonucleotides complementary to a highly conserved portion of the T-box (DNA binding) region to amplify cDNA from activated CD8$^+$ T cells (*9*). Of 18 cloned and sequenced amplicons, 8 encoded *T-bet*; the other 10 encoded *Eomesodermin* (Fig. 1B). Eomesodermin (Eomes) is a T-box factor that plays a key regulatory role in initiating mesodermal cell fate in most vertebrates (*11*) and has an additional role in mammalian trophoblast differentiation (*12*). *Eomes*$^{-/-}$ mice die early in embryogenesis (*12*). Phylogenetic and sequence analysis has placed Eomes and T-bet within the same subfamily of T-box factors (*13*, *14*), and amino acid alignment (*9*) reveals that Eomes and T-bet are 74% identical in their T-box regions (Fig. 1B).

Because it was unknown whether Eomes is expressed in the immune system (*12*), we first examined expression of Eomes and T-bet in activated T cell subsets. Using sequences from their divergent amino termini (Fig. 1B), we developed specific molecular probes that could distinguish the related Eomes and T-bet transcripts (*9*). Although both activated CD4$^+$ and CD8$^+$ T cells expressed T-bet (Fig. 1C), as previously reported (*2*, *3*), significant induction of Eomes mRNA was restricted to activated CD8$^+$ T cells. Low, but detectable, amounts of Eomes mRNA were evident in naive CD8$^+$ T cells from normal mice (Fig. 1C) and in purely enriched naive populations obtained from recombination-deficient T cell receptor (TCR) transgenic T cells (*15*). To further explore the function of Eomes within the CD8$^+$ population, we constructed a DN Eomes RV, in a manner analogous to DN T-bet, by replacing the carboxy-terminal trans-activation domain of Eomes with the *Drosophila* engrailed repression domain (*9*). DN Eomes displayed equivalent effects to DN T-bet in inhibiting IFN-γ induction in wild-type (Fig. 1D) and T-bet–deficient (*9*) CD8$^+$ T cells. Specificity was further confirmed by the findings that the most closely related T-box factor to Eomes and T-bet is not expressed in CD8$^+$ T cells and that a DN form

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of a more distantly related T-box factor had little effect on IFN-γ induction in CD8$^+$ T cells (*9*). Together, these findings suggested that Eomes may be required for T-bet–independent IFN-γ induction in CD8$^+$ T cells.

To further evaluate the function of Eomes in differentiation, we constructed a RV containing the entire coding region of the gene (*9*). When introduced in developing T_H2 cells, which are nonpermissive for expression of T-bet (*2*, *4*) or Eomes (*15*), both T-bet and Eomes RV mediated substantial induction of IFN-γ (Fig. 1E). Under these conditions, Eomes and T-bet RV did not appear to repress interleukin 4 (IL-4) expression (*15*). The apparent rescue of IFN-γ expression by either T-box factor was also evident when cross-linking CD3-specific antibody was used for restimulation instead of chemical mitogens (*9*). We also found that Eomes RV was sufficient to induce effector function independently of T-bet, by rescuing IFN-γ expression in *T-bet*$^{-/-}$ CD4$^+$ T cells (*9*). Finally, the full-length Eomes RV also allowed us to test our original hypothesis that

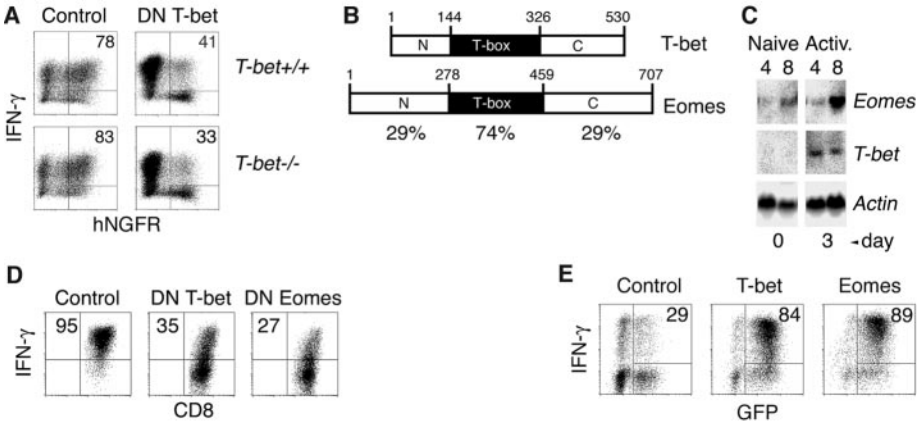

Fig. 1. Eomesodermin mediates T-bet–independent IFN-γ induction. (**A**) DN T-bet antagonizes IFN-γ expression in CD8$^+$ T cells. CD4-depleted splenocytes from *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ B6 × 129 mice were stimulated for 24 hours and then transduced with either control (empty) human nerve growth factor receptor (hNGFR) RV or DN T-bet hNGFR RV (*9*). Cells were recultured for an additional 3 days before analysis of CD8, hNGFR, and IFN-γ expression by flow cytometry. Only CD8$^+$ events are shown. Numbers indicate percentage of transduced, CD8$^+$ cells expressing IFN-γ. (**B**) Major domains of the paralogues, T-bet and Eomes. Amino-terminus (N), DNA-binding domain (T-box), and carboxy-terminal trans-activation domain (C), plus percent identities are indicated. (**C**) Eomes is expressed in activated CD8$^+$ T cells. Naive CD4$^+$ ("4") and CD8$^+$ ("8") T cells from the spleens of normal mice were examined directly (left) or activated for 3 days (right) before sequential Northern analysis with specific probes for Eomes, T-bet, and β-actin. (**D**) DN Eomes antagonizes IFN-γ expression in CD8$^+$ T cells. CD4-depleted splenocytes from wild-type (*T-bet*$^{+/+}$) B6 × 129 mice were stimulated for 24 hours, and then transduced with designated green fluorescent protein (GFP) RV. Cells were recultured for an additional 6 days before analysis of CD8, GFP, and IFN-γ expression by flow cytometry. Only GFP$^+$CD8$^+$ events are shown. Numbers indicate percentage of transduced, CD8$^+$ cells expressing IFN-γ. Identical results were obtained with *T-bet*$^{-/-}$ mice (*9*). (**E**) Eomes is sufficient to induce IFN-γ expression in developing T_H2 cells. CD8-depleted BALB/c splenocytes were stimulated in T_H2 conditions for 24 hours, and then transduced with control GFP RV, T-bet GFP RV, or Eomes GFP RV. Each group was co-transduced with control (empty) hNGFR RV. Cells were recultured in T_H2 conditions for an additional 8 days before analysis of hNGFR, GFP, and IFN-γ expression. Numbers indicate percentage of transduced cells expressing IFN-γ. All results are representative of at least two separate experiments.

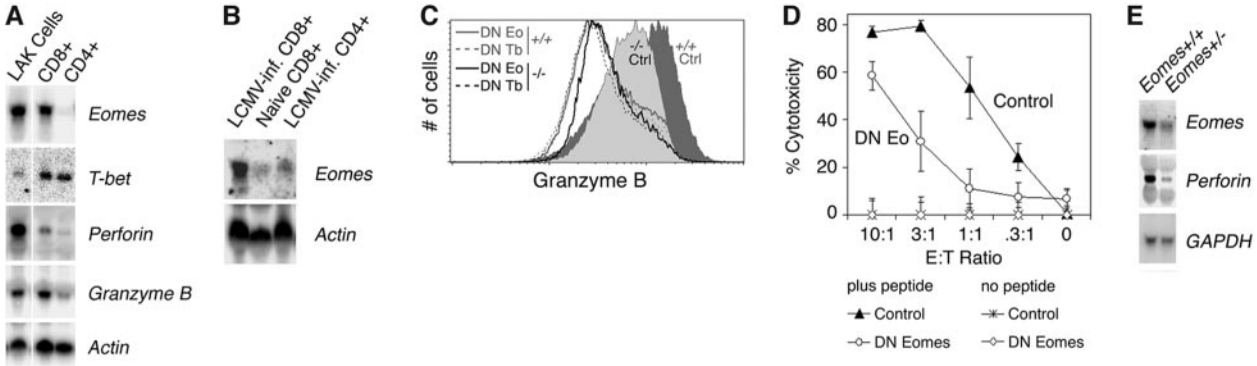

Fig. 2. Eomesodermin may be critical for cytolytic gene expression and function. (**A**) Eomes is coordinately regulated with lytic gene expression in lymphokine-activated natural killer (LAK) cells and cytotoxic T cells. LAK cells were prepared from the bone marrow of C57BL/6 lymphocyte-deficient mice by using human recombinant IL-2 (*9*) and compared with C57BL/6 CD4$^+$ and CD8$^+$ T cells that were activated for 3 days before RNA extraction and sequential Northern analysis with specific probes for Eomes, T-bet, perforin, granzyme B, and β-actin. (**B**) Eomes is expressed in activated CD8$^+$ T cells as part of the antiviral response in vivo. C57BL/6 mice were infected intraperitoneally with 2 × 10^5 plaque-forming units Armstrong strain of LCMV. On day 6 postinfection, CD4$^+$ and CD8$^+$ T cells were isolated before RNA extraction and sequential Northern analysis with specific probes for Eomes and β-actin. Groups from infected mice are compared with CD8$^+$ T cells from unchallenged controls. (**C**) DN Eomes antagonizes T-bet–dependent and T-bet–independent granzyme B expression. CD4-depleted splenocytes from *T-bet*$^{+/+}$ (dark gray filled plot and line plots) and *T-bet*$^{-/-}$ (light gray filled plot and black line plots) B6 × 129 mice were stimulated for 24 hours, and then transduced with control (Ctrl) GFP RV (filled histograms), DN Eomes (Eo) GFP RV (solid lines), or DN T-bet (Tb) GFP RV (dashed lines). Cells were recultured for an additional 5 days before analysis of CD8, GFP, and granzyme B expression by flow cytometry. Only CD8$^+$GFP$^+$ events are shown. Relative to *T-bet*$^{+/+}$ cells with control RV, the reduction in mean fluorescence intensity for each group (genotype and RV) was: −/− Ctrl, 1.4-fold; +/+ DN Eo, 2.0-fold; −/− DN Eo, 2.2-fold; +/+ DN Tb, 2.1-fold; −/− DN Tb, 2.4-fold. (**D**) DN Eomes antagonizes cytolytic function. Transgenic T cells specific for LCMV were transduced with control GFP or DN Eomes GFP RV 24 hours after stimulation with peptide and antigen-presenting cells. Cells were recultured for an additional 5 days and sorted for expression of GFP and CD8. After overnight rest, these effector cells were used at the indicated ratios with peptide-pulsed or unpulsed targets. Percent cytotoxicity was measured with a standard methodology (*9*). (**E**) Eomes may be necessary for full effector CD8$^+$ T cell development. Splenocytes from *Eomes*$^{+/+}$ and *Eomes*$^{+/-}$ littermates were stimulated for 3 days before RNA extraction and sequential Northern analysis with specific probes for Eomes, perforin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

DN T-bet might promiscuously coinhibit Eomes (Fig. 1A), because the specificity of T-box factors partly resides in the T-box domain (*11*, *16*). We found DN T-bet RV equally, albeit incompletely, antagonized the ability of full-length T-bet or Eomes RV to induce IFN-γ in developing T_H2 cells (*9*). Thus, Eomes appears to complement T-bet in mediating induction of IFN-γ in T cells and may play a key role in the effector differentiation of $CD8^+$ T cells.

In addition to secretion of IFN-γ, the other major effector function of $CD8^+$ T cells is the ability to lyse target cells by using granules containing perforin and granzymes. We found Eomes, perforin, and granzyme B to be coordinately induced in activated NK cells and $CD8^+$ T cells (Fig. 2A), two lineages that have been suggested to exhibit substantial cytotoxic function in the absence of T-bet (*3*). By contrast, activated $CD4^+$ T cells express lower levels of Eomes, perforin, and granzyme B. We also detected substantial Eomes expression in vivo. $CD8^+$ T cells from mice infected for 6 days with lymphocytic choriomeningitis virus (LCMV), a potent inducer of cytolytic effector cells (*17*–*19*), expressed high levels of Eomes mRNA compared with $CD8^+$ T cells from uninfected mice, or $CD4^+$ T cells from the infected animals (Fig. 2B). Thus, Eomes appeared to be expressed in cells undergoing cytolytic effector $CD8^+$ T cell development, in vitro and in vivo.

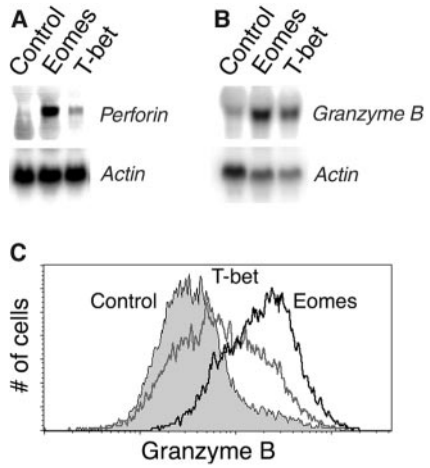

Fig. 3. Eomesodermin is sufficient to invoke cytotoxic T cell gene expression. (**A** and **B**) CD8-depeleted BALB/c splenocytes were stimulated in T_H2 conditions for 24 hours, and then transduced with control GFP RV, T-bet GFP RV, or Eomes GFP RV. Cells were recultured in T_H2 conditions for 4 days before sorting for GFP and CD4 expression. RNA was extracted from sorted cells and analyzed by Northern blotting with specific probes for perforin (A), granzyme B (B), and β-actin (A and B). (**C**) Eomes is sufficient to induce granzyme B protein expression in developing T_H2 cells. CD8-depeleted BALB/c splenocytes were stimulated in T_H2 conditions for 24 hours, and then transduced with control GFP RV, T-bet GFP RV, or Eomes GFP RV. Cells were recultured in T_H2 conditions for 4 days before analysis of GFP, CD4, and granzyme B expression by flow cytometry. Granzyme B staining among $CD4^+GFP^+$ events is shown. Filled gray histogram designates control RV, open gray histogram designates T-bet RV, and open black histogram designates Eomes RV. Relative to control, the increase in mean fluorescence intensity induced by T-bet and Eomes was 1.8-fold and 3.8-fold, respectively.

To determine whether Eomes plays a causal role in T-bet–independent cytolytic effector differentiation, we analyzed activated $CD8^+$ T cells from *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice. We found that T-bet deficiency resulted in a modest but reproducible defect in granzyme B induction, as assessed by antibody staining and flow cytometry (Fig. 2C). Introduction of DN Eomes or DN T-bet RV into either *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ cells led to a significantly greater defect in granzyme B expression than simple *T-bet* gene deletion (Fig. 2C). In addition, we introduced DN Eomes RV into differentiating effector $CD8^+$ T cells from LCMV peptide-specific TCR transgenic mice (*20*). Effector cells transduced with DN Eomes exhibited a substantial defect in killing of peptide-pulsed target cells compared with cells transduced with control RV (Fig. 2D).

These findings suggested that Eomes, perhaps in concert with T-bet, was required for the lytic effector program seen in $CD8^+$ T cells. Although *Eomes*$^{-/-}$ mice die in embryogenesis (*12*, *21*), we attempted to explore the necessity of Eomes in effector differentiation by examining haplo-insufficient mice, whose peripheral T cell composition appears normal (*9*). Splenocytes from littermate *Eomes*$^{+}$ and *Eomes*$^{+/-}$ mice (*21*) were stimulated via the TCR for 3 days. Cells from *Eomes*$^{+}$ mice indeed exhibited reduction in Eomes mRNA (Fig. 2E), and this partial knockdown of Eomes levels was accompanied by a substantial reduction in perforin mRNA (Fig. 2E). A similar defect was observed when purified $CD8^+$ T cells were used (*15*). We detected only minimal loss of IFN-γ expression in $CD8^+$ T cells from *Eomes*$^{+}$ mice, possibly owing to the partial knockdown and redundancy from the actions of T-bet (*15*).

To further establish specific causality between Eomes and the induction of cytolytic genes, we examined the effect of overexpression of Eomes or T-bet in developing T_H2 cells. Eomes and, to a lesser extent, T-bet RV were each sufficient to induce perforin (Fig. 3A) and granzyme B (Fig. 3B) mRNA, as well as granzyme B protein (Fig. 3C). By contrast to the lytic genes, T-bet appeared to be superior to Eomes at induction of IL-12Rβ2 (*9*), which suggests that each factor had some level of specificity in its target genes. Some caution must of course be used in interpreting the effects achieved with overexpression, because amounts of RV mRNA may exceed endogenous levels (*9*). Nonetheless, the collective results presented here suggest that Eomes is a critical factor in directing lytic effector differentiation of $CD8^+$ T cells.

Elegant genetic studies have recently illustrated that the transcriptional control of cellular immunity, at least partially, transcends the functions of T-bet (*3*, *22*). By virtue of its homology to T-bet, its pattern of expression and, in particular, the results of genetic manipulation, Eomesodermin appears to be a key component of this elusive pathway. The general agreement between the results of dominant negative experiments, partial knockdown of Eomes, and ectopic overexpression suggests that Eomes is not simply an inducer of IFN-γ but also appears to be critical for invoking the characteristics of the cytolytic effector lineage. Eomes, thus, appears to complement the role of T-bet in governing cellular immunity by providing redundancy and, quite likely, cooperativity in the induction of effector genes of T cells and NK cells. Future experiments, such as conditional gene deletion, should help address the issue of relative necessity of Eomes and T-bet in various in vivo immune responses or determine whether further redundancy and robustness of such pathways underlie our evolutionary success in mediating immunity to infection and tumors.

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Supporting Online Material

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REPORTS: "Control of effector CD8$^+$ T cell function by the transcription factor *Eomesodermin*" by E. L. Pearce *et al.* (7 Nov. 2003, p. 1041). On p. 1043, in the third paragraph of the middle column, superscript symbols were removed, which changed the meaning of three sentences. The correct sentences are as follows: "Splenocytes from littermate *Eomes*$^{+/+}$ and *Eomes*$^{+/-}$ mice (*21*) were stimulated via the TCR for 3 days. Cells from *Eomes*$^{+/-}$ mice indeed exhibited reduction in Eomes mRNA (Fig. 2E), and this partial knockdown of Eomes levels was accompanied by a substantial reduction in perforin mRNA (Fig. 2E)... We detected only minimal loss of IFN-γ expression in CD8$^+$ T cells from *Eomes*$^{+/-}$ mice, possibly owing to the partial knockdown and redundancy from the actions of T-bet (*15*)."

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Editor's Summary