Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells

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Immunity to intracellular pathogens requires dynamic balance between terminal differentiation of short-lived, cytotoxic effector CD8+ T cells and self-renewal of central–memory CD8+ T cells. We now show that T-bet represses transcription of IL-7Rα and drives differentiation of effector and effector–memory CD8+ T cells at the expense of central–memory cells. We also found T-bet to be overexpressed in CD8+ T cells that differentiated in the absence of CD4+ T cell help, a condition that is associated with defective central–memory formation. Finally, deletion of T-bet corrected the abnormal phenotypic and functional properties of "unhelped" memory CD8+ T cells. T-bet, thus, appears to function as a molecular switch between central– and effector–memory cell differentiation. Antagonism of T-bet may, therefore, represent a novel strategy to offset dysfunctional programming of memory CD8+ T cells.

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Abbreviation used: LCMV, lymphocytic choriomeningitis virus.

When a naive CD8+ T cell encounters antigen, it undergoes vigorous clonal expansion and differentiation into a population comprised primarily of short-lived, cytotoxic effectors that undergo cell death after pathogen is cleared (1). The elimination of the antigen-experienced progeny of a selected T cell clone, however, is generally incomplete, ensuring preservation of a portion of daughter cells to provide a "memory" of the pathogen encounter. At least two functionally distinct classes of memory cells have been described, based on their pattern of tissue homing in the absence of antigen. Effector–memory CD8+ T cells provide protection against reinfection by patrolling peripheral tissues, but have a poor capacity for homeostatic renewal and secondary proliferation. Central–memory CD8+ T cells, in contrast, recapitulate the surveillance behavior of their naive predecessor by migrating through secondary lymphoid organs; they are distinguished by efficient homeostatic renewal and rapid secondary proliferative responses to generate cytotoxic effectors upon reencounter with pathogen (2, 3). It remains controversial whether commitment to the self-renewing,

central–memory lineage occurs before or after adoption of the effector fate (3–6).

The intrinsic molecular program that directs commitment to the effector– versus central–memory CD8+ T cell lineage is not fully understood. We found that deficiency of the transcription factor T-bet resulted in defective generation of effector–memory CD8+ T cells and excessive generation of central–memory CD8+ T cells. The enhanced central–memory formation resulting from T-bet deficiency directly contrasted with the defective central–memory formation occurring in the absence of CD4+ T cell help. Deletion of T-bet prevented the defective phenotypic and functional characteristics of unhelped CD8+ T cells. These results suggest that T-bet may represent a novel target for manipulating the balance between terminal differentiation and self-renewal of pathogen-specific CD8+ T cells.

RESULTS AND DISCUSSION

T-bet represses IL-7Rα in effector CD8+ T cells

During the acute phase of an infection, repression of IL-7Rα marks pathogen-specific CD8+ T cells destined for elimination, whereas the cells that will give rise to self-renewing memory

The online version of this article contains supplemental material.

CD8$^+$ T cells seem to be contained within the IL-7Rα–expressing subset (7, 8). When we examined the lymphocytic choriomeningitis virus (LCMV)–specific CD8$^+$ T cell response in T-bet–deficient mice, we observed defective repression of IL-7Rα at day 8 after infection (Fig. 1 A). Because this finding suggested that T-bet may function as a repressor of IL-7Rα, we tested whether T-bet was sufficient to repress IL-7Rα in T cells. Retroviral-mediated expression of T-bet in CD8$^+$ or CD4$^+$ T cells stimulated in vitro resulted in repression of IL-7Rα mRNA (Fig. 1 B). In addition, ectopic expression of T-bet in developing Th2 cells in which T-bet is not normally expressed resulted in repression of IL-7Rα surface expression (Fig. 1 C).

The identification of T-bet as a repressor of IL-7Rα prompted us to test whether T-bet expression is associated with the IL-7Rα^{lo} subset of effector CD8$^+$ T cells. We sorted LCMV-specific P14 CD8$^+$ T cells 8 d after infection on the basis of IL-7Rα expression and examined T-bet mRNA. T-bet was enriched in the IL-7Rα^{lo} subset of effector CD8$^+$ T cells, whereas the IL-7Rα^{hi} subset had reduced expression of T-bet mRNA (Fig. 1 D) and protein (not depicted). A similar pattern of gene expression was observed in IL-7Rα^{hi} and IL-7Rα^{lo} cells from the endogenous LCMV-specific CD8$^+$ T cell response (not depicted).

In addition to elevated expression of T-bet, the IL-7Rα^{lo} subset of effector CD8$^+$ T cells exhibited substantial enrichment for KLRG1 mRNA (Fig. 1 D) and protein (not depicted). KLRG1 is an NK-like inhibitory receptor that marks replicative senescence in CD8$^+$ T cells (7, 9) and whose expression in NK cells is dependent on T-bet (10). In contrast, IL-7Rα^{lo} effectors had reduced expression of CCR7 mRNA (Fig. 1 D), which is a chemokine receptor that represents a defining feature of central–memory CD8$^+$ T cells (2, 3). In addition, we discovered that the IL-7Rα^{lo} subset exhibited elevated expression of Blimp-1 mRNA (Fig. 1 D), a transcriptional repressor that promotes terminal differentiation of plasma cells (11) and that may also function in T lymphocytes (12, 13). Thus, enhanced expression of T-bet appears to selectively mark IL-7Rα^{lo} effector CD8$^+$ T cells, which exhibit features of terminal differentiation and which have previously been shown to fail to give rise to self-renewing memory cells (7, 8).

Cell transfer experiments have suggested that the IL-7Rα^{lo} subset of effector CD8$^+$ T cells preferentially gives rise to effector–memory cells (7). Consistent with this potential lineage relationship, we found that T-bet was enriched in effector–memory CD8$^+$ T cells (Fig. 1, E and F). The skewing of T-bet expression in effector– versus central–memory cells was also recently reported in human memory CD8$^+$ T cells (14). Given the variety of haploinsufficient phenotypes resulting from hemizygous deletion of T-bet, it is possible that the differences in the amount of T-bet between IL-7Rα^{lo} versus IL-7Rα^{hi} effectors and central– versus effector–memory CD8$^+$ T cells could be functionally relevant (15–19). Expression of Blimp-1 paralleled that of T-bet, with elevated expression in the effector–memory CD8$^+$ T cell subset (Fig. 1, E and F).

Eomes expression, in contrast, did not vary substantially between IL-7Rα^{hi} and IL-7Rα^{lo} (Fig. 1 D) or central– and effector–memory subsets (Fig. 1, E and F).

T–bet deficiency results in enhanced generation of central–memory CD8$^+$ T cells

The expression pattern of T-bet and its role in repressing IL-7Rα suggested that T-bet might negatively regulate the development of central–memory CD8$^+$ T cells. We found that loss of T-bet, in addition to causing derepression of IL-7Rα, resulted in effector CD8$^+$ T cells that acquired several characteristics of central–memory cells, including high expression of CD27, low expression of KLRG1, and robust IL-2 production (Fig. 2, A and C) (16, 20). 30 d after infection, LCMV-specific CD8$^+$ T cells from wild-type mice exhibited substantial heterogeneity (Fig. 2 B), which is consistent with the presence of both central– and effector–memory CD8$^+$ T cells (2, 3, 5). In contrast, T-bet–deficient mice exhibited a predominance of central–memory CD8$^+$ T cells (Fig. 2, B and C). T-bet–deficient memory CD8$^+$ T cells also exhibited a gene expression pattern similar to central–memory cells, with increased CCR7, reduced Blimp-1, and elevated Eomes mRNA (Fig. 2, D and E).

It was previously suggested that T-bet functions as a positive regulator of memory CD8$^+$ T cell development because T-bet–deficient mice were found to have decreased numbers of LCMV-specific memory CD8$^+$ T cells in the blood and spleen (16). In the memory phase of the response (30 and 60 d after infection), we also found that $Tbx21^{-/-}$ mice had fewer LCMV-specific CD8$^+$ T cells in the blood and spleen (Fig. 2 F and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070841/DC1). Despite the deficiency in the blood and spleen, $Tbx21^{-/-}$ mice were found to have increased numbers of memory cells in the lymph nodes. This might be partially attributable to the elevated expression of CCR7 in $Tbx21^{-/-}$ CD8$^+$ T cells (Fig. 2 D). To ensure that the phenotype of T-bet deficiency was CD8$^+$ T cell–intrinsic, we used a transfer system in which the behavior of both wild-type and $Tbx21^{-/-}$ P14 CD8$^+$ T cells could be studied within the same wild-type host (Fig. S2). This system recapitulated the phenotypic, functional, and anatomic characteristics observed in the endogenous CD8$^+$ T cell response of $Tbx21^{-/-}$ mice. Together, these results suggest that T-bet inhibits the formation of lymph node–homing, central–memory CD8$^+$ T cells and positively regulates the development of effector–memory CD8$^+$ T cells.

Two features of central–memory CD8$^+$ T cells are their robust capacity for secondary proliferation and an ability to confer heightened resistance to reinfection. Therefore, we assessed these characteristics in $Tbx21^{-/-}$ memory CD8$^+$ T cells by transferring equal numbers of GP33-specific wild-type or $Tbx21^{-/-}$ memory CD8$^+$ T cells into naive wild-type recipients and challenging with *Listeria monocytogenes* expressing GP$_{33-41}$. T-bet–deficient memory CD8$^+$ T cells exhibited severalfold greater reexpansion than wild-type cells upon rechallenge (Fig. 3 A) and were found to express IL-2
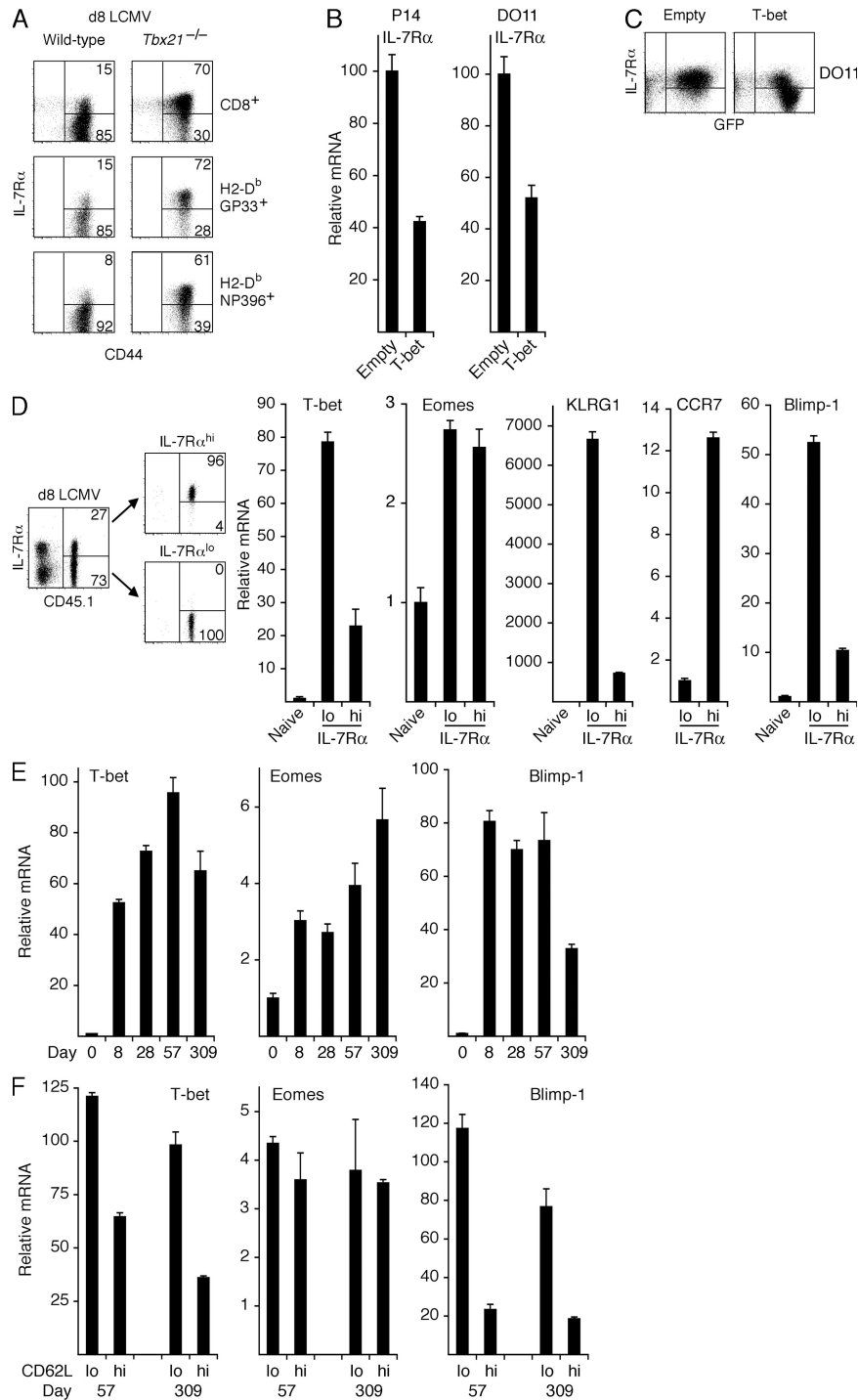
Figure 1. T-bet–mediated repression of IL-7Rα in effector CD8+ T cells. (A) IL-7Rα expression on CD8+ T cells from blood of wild-type or *Tbx21*−/− mice 8 d after LCMV infection. Top row shows CD8+ T cells; middle and bottom rows show H-2D^{b}GP33+ or H-2D^{b}NP396+ T cells, respectively; the percentage of events are indicated. (B) Quantitative RT-PCR (Q-PCR) of IL-7Rα mRNA from P14 CD8+ (left) or DO11.10 CD4+ (right) T cells stimulated with peptide plus APCs and transduced with retrovirus. Cells sorted 5 d after transduction based on GFP. (C) IL-7Rα expression on DO11.10 CD4+ T cells in T$_H$2 conditions stimulated and transduced as in B. Cells stained 5 d after transduction. (D) Q-PCR of P14 CD8+ T cells from spleens 8 d after infection sorted for IL-7Rα^{hi} or IL-7Rα^{lo} expression. Naive (CD44^{lo}) CD8+ T cells are included in some graphs for reference. (E and F) Q-PCR of T-bet, Eomes, or Blimp-1 mRNA in LCMV-specific CD8+ T cells. H-2D^{b}GP33+ plus H-2D^{b}NP396+ CD8+ T cells sorted from spleens of wild-type mice at the indicated day after infection. Unfractionated tetramer-positive cells (E) or tetramer-positive cells fractionated by CD62L^{hi} or CD62L^{lo} expression (F). Values for Q-PCR represent the mean ± the SEM of triplicate determinations, normalized to HPRT. All results are representative of at least three experiments.
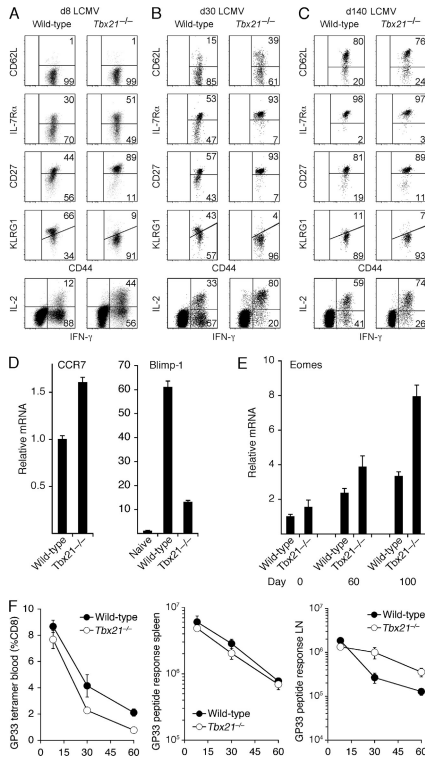
Figure 2. Enhanced generation of central–memory CD8$^+$ T cells in T-bet–deficient mice. (A–C) Phenotype of GP33-specific CD8$^+$ T cells from spleens of wild-type or *Tbx21*$^{-/-}$ mice 8 (A), 30 (B), or 140 d (C) after LCMV infection. Plots display H-2D^{b}GP33$^+$ events; the percentage of events are indicated. For bottom row, splenocytes were stimulated with GP$_{33-41}$ peptide; numbers indicate the percentage producing both IFN-γ and IL-2. Results are representative of three experiments with multiple mice per time point. (D and E) Q-PCR of H-2D^{b}GP33$^+$ plus H-2^{b}NP396$^+$ CD8$^+$ T cells from spleens of wild-type or *Tbx21*$^{-/-}$ mice. (D) CCR7 and Blimp-1 mRNA 60 d after infection. Naive represents CD44^{lo}CD8$^+$ T cells from uninfected wild-type mice. (E) Eomes mRNA 60 and 100 d after infection. Day 0 represents CD44^{lo}CD8$^+$ T cells from uninfected wild-type or *Tbx21*$^{-/-}$ mice. Results are representative of three similar experiments. (F) Quantification of LCMV-specific CD8$^+$ T cells from wild-type or *Tbx21*$^{-/-}$ mice 8, 30, and 60 d after infection. Left graph shows H-2D^{b}GP33$^+$ cells as the percentage of CD8$^+$ in blood. Middle and right graphs show numbers of CD8$^+$ T cells from spleen or lymph node (pooled axillary, inguinal, cervical, paraaortic, and mesenteric), respectively, producing IFN-γ in response to GP$_{33-41}$. Results represent the mean ± the SEM for at least three mice per data point.

at a greater frequency (Fig. 3, A and B). Mice that received *Tbx21*$^{-/-}$ memory CD8$^+$ T cells also showed substantially reduced bacterial burdens in both spleen and liver compared with recipients of wild-type cells (Fig. 3 C). Thus, deletion of T-bet promotes the development of highly functional memory CD8$^+$ T cells. Presently, we are unsure why the defect in effector–memory CD8$^+$ T cells resulting from deficiency of T-bet appears more permanent in this study compared with prior findings (16), although the operational definitions of effector–memory differ (phenotypic/functional versus chemokine receptor) in the two studies.
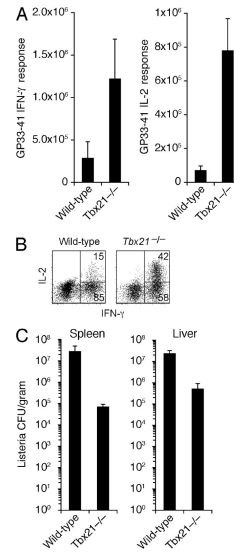

Figure 3. Enhanced secondary expansion and protection of T–bet–deficient memory cells. Equal numbers (2 × 10^5) of wild-type or *Tbx21*$^{-/-}$ GP33-specific CD8$^+$ T cells (Thy1.2$^+$) isolated 45 d after LCMV infection and transferred to naive wild-type recipients (Thy1.1$^+$). 1 d after transfer, recipient mice were infected with 2.5 × 10^5 CFU of *L. monocytogenes*. 4 d after challenge, spleens and livers were harvested to assess CD8$^+$ T cell expansion and perform quantitative bacterial cultures. (A) Wild-type or *Tbx21*$^{-/-}$ GP33-specific CD8$^+$ T cell expansion. Graphs display numbers of transferred cells (Thy1.2$^+$) from spleens of recipient mice producing IFN-γ or IL-2 in response to GP$_{33-41}$. (B) Cytokine production by wild-type or *Tbx21*$^{-/-}$ GP33-specific CD8$^+$ T cells from spleens of recipient mice. Plots display CD8$^+$ events; numbers indicate the percentage of cells producing both IFN-γ and IL-2 in response to GP$_{33-41}$. (C) Bacterial load in mice that received wild-type or *Tbx21*$^{-/-}$ GP33-specific CD8$^+$ T cells. Spleens and livers were homogenized in 1% Triton X-100. Bacteria quantified by limiting dilution culture. Data for A and C represent the mean ± the SEM of six recipients of wild-type and four recipients of *Tbx21*$^{-/-}$ cells. Results are representative of three similar experiments.

Dysregulated T–bet expression and impaired central–memory CD8$^+$ T cell formation in the absence of CD4$^+$ T cell help

A well-characterized model of defective memory CD8$^+$ T cell development involves CD8$^+$ T cell activation in the absence of CD4$^+$ T cell help (21–25). We examined the properties of "unhelped" CD8$^+$ T cells by transfer of P14 CD8$^+$ T cells into *Cd4*$^{-/-}$ recipients (Fig. 4) or recipients depleted of CD4$^+$ T cells using monoclonal antibody injection (Fig. 5, Fig. S3 [available at http://www.jem.org/cgi/content/full/jem.20070841/DC1], and not depicted). 8 d after infection, there was no difference in the properties of P14 CD8$^+$ T cells in wild-type compared with *Cd4*$^{-/-}$ hosts (not depicted). Several weeks after infection, however, P14 CD8$^+$ T cells from *Cd4*$^{-/-}$ hosts began to manifest signs of aberrant memory differentiation, with impaired expression of CD62L, IL-7Rα, CD27, and IL-2, and elevated expression of KLRG1 (Fig. 4 A).

The memory cells arising in the absence of CD4$^+$ T cell help appeared to be more effector–memory–like, thus contrasting with T-bet–deficient memory CD8$^+$ T cells, which
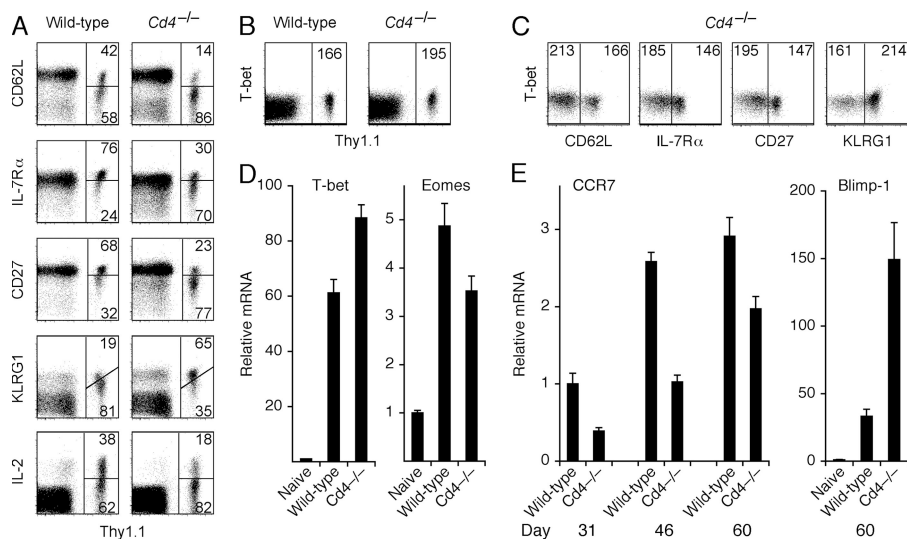
Figure 4. Enhanced T-bet expression in unhelped memory CD8$^+$ T cells. (A) Phenotype of P14 cells from wild-type or $Cd4^{-/-}$ hosts 60 d after infection. For bottom row, splenocytes were stimulated with GP$_{33-41}$. Plots show CD8$^+$ events; numbers indicate the percentage of P14 cells (Thy1.1$^+$) expressing indicated surface marker or cytokine. (B and C) Intranuclear T-bet staining of P14 cells from wild-type or $Cd4^{-/-}$ hosts 75 d after infection. (B) Plots show CD8$^+$ events; numbers indicate MFI of T-bet staining in bulk P14 population (Thy1.1$^+$). (C) Plots show P14 cells (Thy1.1$^+$) from $Cd4^{-/-}$ hosts; numbers represent MFI of T-bet staining in subsets with high or low expression of indicated marker. (D) Q-PCR of T-bet and Eomes mRNA in P14 cells from wild-type or $Cd4^{-/-}$ hosts 60 d after infection. (E) Q-PCR of CCR7 and Blimp1 mRNA in P14 cells from wild-type or $Cd4^{-/-}$ hosts 31, 46, or 60 d after infection. Values for Q-PCR represent the mean ± the SEM of triplicate determinations, normalized to HPRT. All results are representative of at least three experiments.

appear to be more central–memory–like. Using intranuclear staining of the T-bet protein, unhelped memory CD8$^+$ T cells exhibited a detectable increase in expression of T-bet (Fig. 4 B), which specifically localized to the expanded effector–memory–like subset (CD62L^{lo}, IL-7Rα^{lo}, CD27^{lo}, KLRG1^{hi}; Fig. 4 C and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070841/DC1). In addition to exhibiting elevated T-bet mRNA (Fig. 4 D), unhelped memory CD8$^+$ T cells were found to express more Blimp-1 mRNA, but less CCR7 and Eomes mRNA (Fig. 4, D and E). Lack of CD4$^+$ T cell help, thus, appears to impair central–memory and/or promote effector–memory CD8$^+$ T cell development.

T-bet deletion prevents dysfunctional programming of unhelped memory CD8$^+$ T cells

To discriminate whether elevated T-bet expression in unhelped memory CD8$^+$ T cells plays a causal role in, or is simply a consequence of, their altered differentiation, we performed antibody depletion of CD4$^+$ T cells from wild-type or $Tbx21^{-/-}$ mice, followed by infection with LCMV. Again, we found that unhelped memory CD8$^+$ T cells in wild-type mice exhibited a predominance of effector–memory–like cells (CD62L^{lo}, IL-7Rα^{lo}, CD27^{lo}, and KLRG1^{hi}), with impaired production of IL-2 and CD40L (Fig. 5, A and B, and Fig. S3). Deletion of T-bet, however, prevented the phenotypic and functional defects associated with the lack of CD4$^+$ T cell help (Fig. 5, Fig. S3, and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070841/DC1), resulting in enhanced generation of central–memory CD8$^+$ T cells, regardless of the

availability of CD4$^+$ T cell help. The rescue was apparent even when T-bet deficiency was confined to antigen-specific CD8$^+$ T cells (Fig. S5). Moreover, delayed antagonism of T-bet after unhelped memory CD8$^+$ T cell development was capable of reversing several aspects of their dysfunctional phenotype (Fig. S6). Thus, T-bet appears to be required for the aberrant differentiation of memory CD8$^+$ T cells that occurs in the absence of CD4$^+$ T cell help.

How T-bet and CD4$^+$ T cells execute their opposing effects on the balance between central– versus effector–memory CD8$^+$ T cells remains to be determined. In several different systems, transcription factors function as intrinsic modulators of self-renewal or terminal differentiation. In B lymphocytes, the transcriptional repressor Blimp-1 promotes terminal differentiation (plasma cell) at the expense of self-renewal (memory B cell) (11). In the *Drosophila melanogaster* neural stem cells, the homeodomain transcription factor Prospero functions as a similar switch by repressing genes required for self-renewal, such as stem cell and cell-cycle genes, while activating genes involved in terminal differentiation (26). Parallels from other biological systems may help elucidate whether T-bet similarly functions to promote terminal differentiation at the expense of self-renewal, which has been proposed to represent an essential distinction between effector– versus central–memory CD8$^+$ T cells, respectively (2–7, 27).

The potential ability of T-bet to switch between two polar states of differentiation may explain numerous aspects of viral pathogenesis. Clonal deletion, for example, could be regarded as a "self-renewal deficiency state," associated
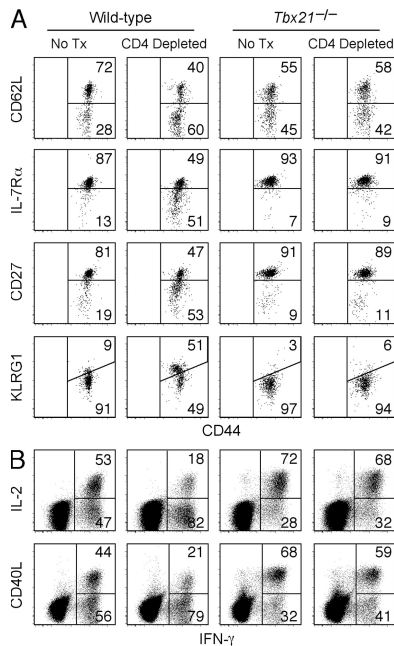
Figure 5. T-bet–dependent dysfunction of unhelped memory CD8+ T cells. (A and B) Wild-type or *Tbx21*−/− mice were left untreated (No Tx) or treated with CD4-depleting antibody (0.2 mg GK1.5 i.p.; CD4 depleted) 1 d before and 1 d after LCMV infection. (A) Surface phenotype of GP33-specific CD8+ T cells from spleens 90 d after infection. Plots show H-2D^{b}GP33$^+$ events; numbers represent the percentage of tetramer-positive cells expressing indicated surface marker. (B) Cytokine production of GP33-specific CD8+ T cells. Plots show CD8+ events; numbers indicate the percentage of cells producing both IFN-γ and IL-2 or CD40L in response to GP$_{33-41}$. Results are representative of two independent experiments.

with extreme excess of T-bet expression resulting from prolonged antigenic stimulation. Likewise, the association of high precursor frequency with enhanced central–memory formation might be considered a "disorder of excessive self-renewal," explained by diminished T-bet expression as a consequence of excessive competition for antigen (5). The potential role of T-bet in these processes is currently being investigated.

The excessive differentiation of effector–memory CD8+ T cells in the absence of CD4+ T cell help could involve direct effects, such as loss of soluble or membrane-bound signals typically delivered by CD4+ T cells to dendritic cells or CD8+ T cells, including IL-2, chemokines, or CD40L (28–30). Alternatively, loss of CD4+ T cell help could act more indirectly, for example, by altering kinetics of pathogen clearance and necessitating prolonged effector function (3). Although the abnormalities arising in the absence of CD4+ T cell help appear to be at least partially T-bet–dependent, it is anticipated there could be T-bet–independent derangements contributing to the aberrant differentiation of unhelped memory CD8+ T cells. Nonetheless, antagonism of T-bet might represent a novel therapeutic approach for preventing the dysfunctional programming of CD8+ T cells in patients with compromised CD4+ T cell function.

MATERIALS AND METHODS

Mice and pathogens. Mice were maintained and used in accordance with the University of Pennsylvania Institutional Animal Care and Use Guidelines. Wild-type C57BL/6, *Tbx21*−/− (18), DO11.10 TCR transgenic, and P14 TCR transgenic mice were housed in specific pathogen-free conditions before use. *Tbx21*−/− P14 TCR transgenic mice were generated by interbreeding *Tbx21*−/− mice with P14 transgenic mice. For analysis of the endogenous CD8+ T cell response, C57BL/6 mice or *Tbx21*−/− were infected with 2 × 10^5 PFU of LCMV Armstrong. H-2D^{b}GP33 or H-2D^{b}NP396 tetramers were used to identify LCMV-specific CD8+ T cells. For P14 transfer experiments, ~5 × 10^4 CD8+ T cells from naive P14 TCR transgenic mice (Thy1.1/1.1) were transferred intravenously into C57BL/6 or *Cd4*−/− recipients (Thy1.2/1.2). For P14 cotransfer experiments, ~5 × 10^4 wild-type P14 cells (Thy1.1/1.1) were mixed with ~5 × 10^4 *Tbx21*−/− P14 cells (Thy1.1/1.2) and transferred to wild-type C57BL/6 recipients (Thy1.2/1.2). 1 d after transfer, recipient mice were infected with LCMV. For CD4 depletion experiments, wild-type or *Tbx21*−/− mice received intraperitoneal injection of GK1.5 antibody (0.2 mg in PBS) 1 d before and 1 d after infection with LCMV. GK1.5 treatment resulted in efficient depletion of CD4+ T cells for >1 wk (not depicted). For *Listeria monocytogenes* experiments, mice were challenged with 2.5 × 10^5 CFU of *L. monocytogenes* with transgenic expression of the LCMV-derived peptide GP$_{33-41}$. 4 d after bacterial challenge, spleens and livers were homogenized in 1% Triton X-100 in PBS, followed by limiting dilution culture on blood heart infusion agar.

Flow cytometry, cell culture, stimulation, and retroviral transduction. Surface staining, intracellular cytokine staining, and flow cytometry were performed as previously described (18). For LCMV-derived peptide stimulations, splenocytes or lymph node cells were stimulated for 6 h with 0.2 μg/ml GP$_{33-41}$ or NP$_{396-404}$ peptide in the presence of 1 μg/ml brefeldin A. Antibodies used for flow cytometry were purchased from BD Biosciences. Intranuclear T-bet staining (Santa Cruz Biotechnology) was performed by fixation with 4% paraformaldehyde in PBS, followed by permeabilization and staining in 0.1% Triton X-100 and 1% FBS in PBS. Splenocytes from DO11.10 or P14 TCR transgenic mice were stimulated and transduced as previously described (18).

Quantitative RT-PCR. RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR were performed as previously described (18). Primer and probe sets used for HPRT and Eomes detection were previously described (18). Presynthesized Taqman Gene Expression Assays (Applied Biosystems) were used to amplify the following sequences (gene symbols and Applied Biosystems primer set numbers in parentheses): Blimp-1 (*Prdm1*; Mm00476128_m1), CCR7 (*Ccr7*; Mm01301785_m1), KLRG1 (*Klrg1*; Mm00516879_m1), and T-bet (*Tbx21*; Mm00450960_m1). 'Test' gene values are expressed relative to that of HPRT, with the lowest experimental value standardized at 1.

Online supplemental material. Fig. S1 shows that T-bet–deficient antigen-specific memory CD8+ T cells preferentially accumulate in lymph nodes. Fig. S2 shows that T-bet represses central–memory development in a CD8+ T cell–intrinsic manner. Fig. S3 shows that deletion of T-bet rescues the phenotype and function of unhelped memory CD8+ T cells. Fig. S4 shows that unhelped memory CD8+ T cells have elevated expression of T-bet. Fig. S5 shows that T-bet deficiency restricted to antigen-specific CD8+ T cells is capable of correcting abnormalities associated with unhelped memory cells. Fig. S6 shows that preexisting dysfunction in unhelped memory CD8+ T cells can be reversed by antagonism of T-bet. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070841/DC1.

REFERENCES

1. Williams, M.A., and M.J. Bevan. 2007. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 25:171–192.

2. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 401:708–712.

3. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.

4. Manjunath, N., P. Shankar, J. Wan, W. Weninger, M.A. Crowley, K. Hieshima, T.A. Springer, X. Fan, H. Shen, J. Lieberman, and U.H. von Andrian. 2001. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J. Clin. Invest.* 108:871–878.

5. Marzo, A.L., K.D. Klonowski, A. Le Bon, P. Borrow, D.F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat. Immunol.* 6:793–799.

6. Chang, J.T., V.R. Palanivel, I. Kinjyo, F. Schambach, A.M. Intlekofer, A. Banerjee, S.A. Longworth, K.E. Vinup, P. Mrass, J. Oliaro, et al. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science.* 315:1687–1691.

7. Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198.

8. Huster, K.M., V. Busch, M. Schiemann, K. Linkemann, K.M. Kerksiek, H. Wagner, and D.H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc. Natl. Acad. Sci. USA.* 101:5610–5615.

9. Voehringer, D., C. Blaser, P. Brawand, D.H. Raulet, T. Hanke, and H. Pircher. 2001. Viral infections induce abundant numbers of senescent CD8 T cells. *J. Immunol.* 167:4838–4843.

10. Robbins, S.H., M.S. Tessmer, L. Van Kaer, and L. Brossay. 2005. Direct effects of T-bet and MHC class I expression, but not STAT1, on peripheral NK cell maturation. *Eur. J. Immunol.* 35:757–765.

11. Lin, Y., K. Wong, and K. Calame. 1997. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. *Science.* 276:596–599.

12. Kallies, A., E.D. Hawkins, G.T. Belz, D. Metcalf, M. Hommel, L.M. Corcoran, P.D. Hodgkin, and S.L. Nutt. 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat. Immunol.* 7:466–474.

13. Martins, G.A., L. Cimmino, M. Shapiro-Shelef, M. Szabolcs, A. Herron, E. Magnusdottir, and K. Calame. 2006. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat. Immunol.* 7:457–465.

14. Chtanova, T., R. Newton, S.M. Liu, L. Weininger, T.R. Young, D.G. Silva, F. Bertoni, A. Rinaldi, S. Chappaz, F. Sallusto, et al. 2005. Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets. *J. Immunol.* 175:7837–7847.

15. Szabo, S.J., B.M. Sullivan, C. Stemmann, A.R. Satoskar, B.P. Sleckman, and L.H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science.* 295:338–342.

16. Juedes, A.E., E. Rodrigo, L. Togher, L.H. Glimcher, and M.G. von Herrath. 2004. T-bet controls autoaggressive CD8 lymphocyte responses in type 1 diabetes. *J. Exp. Med.* 199:1153–1162.

17. Townsend, M.J., A.S. Weinmann, J.L. Matsuda, R. Salomon, P.J. Farnham, C.A. Biron, L. Gapin, and L.H. Glimcher. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity.* 20:477–494.

18. Intlekofer, A.M., N. Takemoto, E.J. Wherry, S.A. Longworth, J.T. Northrup, V.R. Palanivel, A.C. Mullen, C.R. Gasink, S.M. Kaech, J.D. Miller, et al. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat. Immunol.* 6:1236–1244.

19. Takemoto, N., A.M. Intlekofer, J.T. Northrup, E.J. Wherry, and S.L. Reiner. 2006. Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation. *J. Immunol.* 177:7515–7519.

20. Hwang, E.S., J.H. Hong, and L.H. Glimcher. 2005. IL-2 production in developing Th1 cells is regulated by heterodimerization of RelA and T-bet and requires T-bet serine residue 508. *J. Exp. Med.* 202:1289–1300.

21. Bevan, M.J. 2004. Helping the CD8(+) T-cell response. *Nat. Rev. Immunol.* 4:595–602.

22. Janssen, E.M., E.E. Lemmens, T. Wolfe, U. Christen, M.G. von Herrath, and S.P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature.* 421:852–856.

23. Shedlock, D.J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science.* 300:337–339.

24. Sun, J.C., and M.J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science.* 300:339–342.

25. Sun, J.C., M.A. Williams, and M.J. Bevan. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat. Immunol.* 5:927–933.

26. Choksi, S.P., T.D. Southall, T. Bossing, K. Edoff, E. de Wit, B.E. Fischer, B. van Steensel, G. Micklem, and A.H. Brand. 2006. Prospero acts as a binary switch between self-renewal and differentiation in *Drosophila* neural stem cells. *Dev. Cell.* 11:775–789.

27. Luckey, C.J., D. Bhattacharya, A.W. Goldrath, I.L. Weissman, C. Benoist, and D. Mathis. 2006. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA.* 103:3304–3309.

28. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science.* 297:2060–2063.

29. Castellino, F., A.Y. Huang, G. Altan-Bonnet, S. Stoll, C. Scheinecker, and R.N. Germain. 2006. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell–dendritic cell interaction. *Nature.* 440:890–895.

30. Williams, M.A., A.J. Tyznik, and M.J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature.* 441:890–893.