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T-bet and Eomesodermin Are Required for T Cell-Mediated Antitumor Immune Responses

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Cell-mediated adaptive immunity is very important in tumor immune surveillance and tumor vaccination. However, the genetic program underlying an effective adaptive antitumor immunity is elusive. T-bet and Eomesodermin (Eomes) have been suggested to be master regulators of TH1 cells and CD8+ T cells. However, whether they are important for T cell-mediated antitumor immunity is controversial. In this paper, we show that the combined germline deletion of T-bet and T cell-specific deletion of Eomes resulted in profound defects in adaptive antitumor immune responses. T-bet and Eomes drive Tc1 differentiation by preventing alternative CD8+ T cell differentiation to Tc17 or Tc2 cells. Surprisingly, T-bet and Eomes are not critical for the generation of systemic CTL activities against cancer cells. Instead, T-bet and Eomes are crucial for tumor infiltration by CD8+ T cells. This study defines T-bet and Eomes as critical regulators of T cell-mediated immune responses against tumor. The Journal of Immunology, 2010, 185: 3174–3183.

The transcription factors T-bet and Eomesodermin (Eomes) are master transcription factors of the TH1 phenotype. T-bet is highly expressed in type 1 immune cells such as TH1, Tc1, γδ T, NKT, and NK cells and is critical for the effector function of TH1 cells and NK cells but is only partially required for CD8+ Tc1 cells. Eomes was found to be highly expressed in CD8+ T cells but weakly in CD4+ T cells and is proposed to play a key role in the effector function of CD8+ T cells (9). In addition, T-bet and Eomes have been implicated in anticancer responses, and higher expression of T-bet is associated with a favorable outcome of cancer patients (7). The lack of T-bet leads to increased cancer metastasis (10); however, the role of T-bet in cancer is attributed to its regulation of innate immunity, in particular, NK cell function (11). Overall, the role of T-bet and Eomes in adaptive immunity against cancer is unclear.

To study the role of T-bet and Eomes in tumor vaccination, we generated mice with the germline deletion of T-bet and T cell-specific deletion of Eomes. Unexpectedly, we have found that T-bet and Eomes are involved but not crucial for IFN-γ production by CD8+ T cells. In contrast, T-bet and Eomes are necessary for suppressing alternative fates of CD8+ T cells such as Tc17 and Tc2. More surprisingly, T-bet and Eomes are not required for the generation of systemic CTLs against a dominant tumor Ag after tumor vaccination. Nevertheless, T-bet and Eomes are critical for inhibiting tumor growth after tumor vaccination. The lack of both T-bet and Eomes results in a lower expression of CXC3R1 in T cells and a drastic decrease in the number of tumor-infiltrating T cells. Consistent with this, CXC3R1-deficient mice showed compromised antitumor immune responses against tumor vaccination. In addition, T-bet/Eomes (T/E)-deficient tumor-infiltrating CD8+ T cells produced much more IL-17 and less IFN-γ. Therefore, our study establishes T-bet and Eomes as key regulators of adaptive cell-mediated immunity against cancer through promoting migration of CD8+ T cells to the tumor tissue, enhancing IFN-γ production, and suppressing inflammatory IL-17 production.

Materials and Methods

Mice

The bacteria artificial chromosome containing Eomes was obtained from P. J. de Jong (Children’s Hospital, Oakland, CA). The targeting vector was constructed using the recombineering technique (12). Details of vector construction will be available upon request. This vector was used to insert two loxP sites that flank the second exon of Eomes, which encodes a critical region for DNA binding. The vector was electroporated into SW9.5 ES cells, a gift from R. Chaillet (University of Pittsburgh, Pittsburgh, PA). Drug-resistant mouse embryonic stem cell clones were screened by Southern blot analysis.

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Abbreviations used in this paper: DKO, double knockout; EKO, Eomes−/−; Eomes, Eomesodermin; i.d., intradermally; IP-10, inflammatory protein 10; KO, knockout; T/E, T-bet/Eomesodermin; TIL, tumor-infiltrating lymphocyte; TKO, T-bet−/−; TRP, tyrosinase; VAC, vaccination; WT, wild-type.

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analysis of genomic DNA digested with XmnI, with either 3′ or 5′ probes. Two independent correctly targeted embryonic stem cell clones were injected into C57BL/6 blastocysts, and germline transmission was obtained from each clone. The neo gene was removed by breeding F1 mice with a strain of actin promoter-driven Flpase transgenic mice (JAX). These mice were then bred with T-bet−/− (TKO) mice on the C57BL/6 background for eight generations to generate TKO Eomes/fl+ mice. Then, TKO Eomes/fl+ mice were crossed with CD4-cre on the C57BL/6 background to generate CD4-cre-TKO Eomes/fl+ mice. These mice were intercrossed to generate CD4-cre-TKOEomes/fl mice. Age- and gender-matched C57BL/6 mice (JAX) were used as wild-type (WT) controls. All animals were maintained under specific pathogen-free conditions. CXCR3−/− mice were provided to us by Dr. F. Lakakis (University of Pittsburgh) with permission from Dr. C. Gerard (Harvard University, Cambridge, MA). All animal work has been approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. For adoptive transfer experiment, C57BL/6 WT recipient mice were lethally irradiated at 1000 rad and then adoptively transferred with different donor bone marrow cells (WT, TKO, Eomes−/− [EKO], or double knockout [DKO]) within 24 h of irradiation. The reconstituted mice were used for experiments 2–3 mo later.

**Cell lines**

B16F0 melanoma cells were provided by Dr. Z. Yin (Yale University, School of Medicine, New Haven, CT). B16-GM-CSF cells were a gift from Dr. W. Storkus. B16F0 and B16-GM-CSF cells were grown in DMEM plus 10% FCS.

**In vitro CD8+ T cells differentiation**

Spleen and lymph node were collected from C57BL/6 WT, TKO, EKO, and T/E DKO mice. Naïve CD62L+ CD44low CD8+ T cells were purified by FACS and cultured in Tc1, Tc0, Tc2, and Tc17 condition as indicated. Cells were stimulated with 5 μg/ml plate-bound anti-CD3 and 5 μg/ml plate-bound anti-CD28 mAbs in the presence of anti–IL-2Rα (10 μg/ml) and IL-12 (3.4 ng/ml) for 48 h. Cells were replated to new wells without anti-CD3 and IL-2 (20 U/ml) and IL-12 (3.4 ng/ml) plus anti–IL-4 (10 μg/ml) and IL-2 (20 U/ml) for another 48 h. Cells were replated to new wells without anti-CD3 and anti-CD28 and with freshly added IL-2 (20 U/ml) for another 48 h.

**Biosystems**

The Journal of Immunology 3175-3189.

**Flow cytometric analysis**

CD4 (GK1.5), CD8 (53-6.7), CD44 (IM/T), CD122 (5H4), CD62L (MEL-14), CD127 (ATR34), CD25 (PC6.2), Foxp3 (FJK-16s), B220 (RA3-6B2), NK1.1 (PK136), pan-NK (DX5), and TCRβ (H5-579) were all purchased from eBioscience (San Diego, CA). Flow cytometry was performed using a FACS flow cytometer (BD Biosciences, San Jose, CA).

For intracellular cytokine staining, naïve CD62LhighCD44lowCD8+ T cells purified from TKO, EKO, T/E DKO, or C57BL/6 WT mice were stimulated with plate-bound anti-CD28 (5 μg/ml) plus anti-CD3 (5 μg/ml) in Tc1 or Tc17 conditions for 96 h. Cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 4 h and incubated for the last 3 h with brefeldin A (10 μg/ml). Cells were re-fixed and labeled for a V-bottom plate, stained with anti-CD8 in HBSS (containing 1% FCS), then fixed with 2% formaldehyde, which was followed by permeabilization with 0.5% saponin. The cells were subsequently stained with anti–IL-17 (eBio17B7; eBioscience) and anti–IFN-γ Ab (clone XM1G2; eBioscience). Cells producing IFN-γ and IL-17 were examined with flow cytometry.

**Pentamer staining**

Allophycocyanin-labeled Pro5 MHC Class I Pentamer H-2K from ProlImmune (Bradenton, FL). Splenocytes were harvested from T/E DKO or WT-vaccinated mice and allocated (1, 2) × 10^6 splenocytes per staining condition. Allophycocyanin-labeled Pentamer (10 μl) was added to the cells and incubated at room temperature for 10 min, after which the cells were washed, and incubated with anti-CD8 and anti-B220 Abs on ice for 20 min before flow cytometric analysis.

**In vivo CTL killing assay**

T/E DKO mice or WT mice were vaccinated with anti-CD25 and 5 × 10^5 irradiated B16-GM-CSF cells as described before. Eight days later, splenocytes from a syngeneic mouse were isolated, pulsed with tyrosinase (TRP)-2180–188 (SYYDFFVWL) (10 μg/ml), and labeled using a high concentration of CFSE (5 μM, CFSEhigh); splenocytes from a syngeneic mouse without TRP-2 peptide were labeled using a low concentration of CFSE (0.5 μM, CFSElow) as an internal control. The mixture of the two CFSE-labeled splenic populations (5 × 10^6 cells each) was injected into host DKO mice or WT mice. After 5 h, the relative abundance of CFSEhigh and CFSElow cells in peripheral blood was determined by flow cytometry.

**Harvest of tumor-infiltrating lymphocytes**

Tumor masses were removed, minced, and digested with collagenase and hyaluronidase digestion solution (2.5 mg/ml collagenase I, 1 mg/ml collagenase IV, 0.25 mg/ml hyaluronidase IV-S, 300 μg/ml DNase I, and 0.01% HEPES in RPMI 1640 medium) at 37°C for 2 h. The pieces were then gently pressed between the frosted edges of two sterile glass slides, and the cell suspension was filtered through a 40-μm cell strainer (BD Biosciences) to remove debris and separate cell clumping. Tumor-infiltrating lymphocytes (TILs) were further purified by using the gradient as per manufacturer protocol, washed, and resuspended in HBSS for analysis.

**Chemotaxis assays**

Naïve CD62LhighCD44lowCD8+ T cells purified from TKO, EKO, T/E DKO, or C57BL/6 WT mice were stimulated in Th1 conditions for 96 h. Then live cells were loaded into the top chamber of Transwell inserts (5-μm pore size, Costar, Cambridge, MA); RPMI 1640 medium containing inflammatory protein 10 (IP-10) (200 ng/ml) were added to the bottom well. Plates were incubated at 37°C for 2 h. A chemotaxis index was calculated by dividing the number of cells migrating in response to chemokines by the number of cells migrating in wells with medium alone.

**Statistical analysis**

We used the Mann-Whitney statistics or two-tailed unpaired Student t test as indicated. We considered p values <0.05 as significant.
Results

T-bet and Eomes are required for maintaining CD44+ T cells

Both T-bet and Eomes are expressed in CD8+ T cells and seem to have overlapping functions (9, 13–16). To study the combined function of T-bet and Eomes in CD8+ T cells, we generated Eomes conditional-deficient mice with CD4-cre transgene (Supplemental Fig. 1) and crossed them to TKO mice to generate T/E DKO mice. T/E DKO mice showed normal thymocyte development (Supplemental Fig. 2) as well as normal proportions of CD4+ and CD8+ T cells in the periphery in young mice. We then examined effector/memory T cell markers and found that CD44+ effector/memory T cells are reduced in both CD4+ and CD8+ T cells from T/E DKO mice compared with WT mice (Fig. 1A). Interestingly, compared with WT T cells (∼47% CD44+), TKO CD4+ T cells showed a decrease in CD44+ cells (29%) similar to T/E DKO CD4+ T cells (29%), and EKO CD4+ T cells showed a modest decrease in the CD44+ population (∼35%). In contrast, EKO CD8+ T cells showed a great decrease in the CD44+ population (∼16%), but TKO CD8+ T cells showed no decrease in the CD44+ population (∼35%) compared with WT (∼30%) (Fig. 1A, Supplemental Fig. 3A). These data suggest a dichotomy in the function of T-bet and Eomes in maintaining CD44+ populations in CD4+ and CD8+ T cells in the peripheral lymphoid system. Consistent with a previous publication (13), we also found that CD122 is reduced in T/E DKO CD8+ T cells (Supplemental Fig. 4).

The diminished number of CD44high cells in the T/E DKO mice prompted us to examine whether T-bet and Eomes are required for the activation of CD8+ T cells. We isolated naive CD8+ T cells from WT and T/E DKO mice and assessed their capacity to proliferate upon activation by anti-CD3 using the CFSE assay. There was no difference in proliferation observed between WT and T/E DKO CD8+ T cells after 3 d of culture (Supplemental Fig. 5). Therefore, T-bet and Eomes do not seem to affect clonal expansion of CD8+ T cells in vitro.

We then examined the effector/memory surface markers on WT and T/E DKO CD8+ T cells during activation. We found a 2- to 3-fold reduction of CD44 expression in T/E DKO T cells compared with WT controls (Fig. 1B, Supplemental Fig. 3B). On the basis of these results, it appears that T-bet and Eomes are important for regulating the expression of this T cell activation marker without affecting T cell clonal expansion.

T-bet and Eomes are not essential for the IFN-γ production by effector CD8+ T cells and are required for suppressing alternative CD8+ T cell fates

Both T-bet and Eomes have been implicated in regulating the production of IFN-γ. Reports indicate that T-bet is critical for IFN-γ production by Th1 cells (17). Overexpression of Eomes enhanced IFN-γ production in both CD4+ and CD8+ T cells (9). However, T-bet–deficient CD8+ T cells and WT CD8+ T cells produced similar levels of IFN-γ (17). The expression of Eomes in CD8+...
T cells has been suspected to be responsible for IFN-γ production in T-bet-deficient CD8+ T cells (9). We therefore decided to determine whether T-bet and Eomes together are required for IFN-γ production in CD8+ T cells. T/E DKO CD8+ T cells and WT controls were cultured in Th1 conditions for 4 d and subsequently restimulated. The frequency of CD8+ T cells producing IFN-γ was assayed by flow cytometry. To our surprise, similar numbers of IFN-γ producers were observed in WT and EKO effector CD8+ T cells (Fig. 1C). TKO CD8+ T cells and T/E DKO CD8+ T cells showed a slight reduction (~10%) in IFN-γ cells (Fig. 1C). IFN-γ production was reduced 2- to 3-fold in T/E DKO CD8+ T cells compared with WT control as measured by ELISA (Fig. 1E). Therefore, T-bet and Eomes are required but not crucial for IFN-γ production by CD8+ T cells. Runx3 was shown to be regulated by T-bet in Th1 cells (16). However, Runx-1,2,3 were expressed at similar levels between T/E DKO CD8+ T cells and WT controls (our unpublished observations). Therefore, the high-level IFN-γ production in T/E DKO CD8+ T cells might be driven by Runx factors. It is likely that additional transcription factors such as STAT-4 are involved in regulating IFN-γ production in effector CD8+ T cells (16).

Perforin and granzymes are key molecules for the cytolytic function of CD8+ T cells. We examined the expression of perforin and granzyme B genes in WT and T/E DKO effector CD8+ T cells by real-time RT-PCR. Deletion of T-bet or Eomes alone resulted in ~2-fold reduction of perforin and granzyme B mRNA (Supplemental Fig. 6A). Deletion of both T-bet and Eomes led to a small additional decrease (Supplemental Fig. 6A). These data suggest that T-bet and Eomes are involved in regulating the cytolytic function of effector CD8+ T cells. However, there are likely additional factors involved in regulating cytolytic functions.

CD8+ T cells also produce large amounts of cytokines and can be divided into various subsets based on the cytokines they produce. T-bet was shown to antagonize Th17 and Th2 differentiation in CD4+ T cells. In addition, T/E DKO mice developed wasting diseases during lymphocytic choriomeningitis virus infection because of the generation of a large number of Tc17 cells (15). We therefore investigated whether T-bet and Eomes affect the differentiation of CD8+ T cells into IL-17 producers. When CD8+ T cells were cultured in Th17-polarizing conditions, ~2- to 3-fold more T-bet–deficient CD8+ T cells became Th17 cells (18). In the same culture condition, ~2- to 3-fold more T-bet–deficient CD8+ T cells
became IL-17 producers. Eomes deletion itself did not greatly affect Th17 differentiation. In contrast, ∼3-fold more T/E DKO CD8+ T cells became IL-17 producers when compared with WT controls (Fig. 1D). These data demonstrate that T-bet and Eomes synergistically inhibit Tc17 differentiation. We have found similar results when CD8+ T cells were cultured in the Tc2 condition. Our results indicate that ∼2- to 3-fold more IL-4 was induced in T/E DKO CD8+ T cells when compared with WT cells (Supplemental Fig. 6B), suggesting that T-bet and Eomes are also required to inhibit Tc2 differentiation.

**T-bet and Eomes mediate adaptive immune responses against cancer**

The presence of tumor-infiltrating T cells has been associated with overall survival of cancer patients (7). In addition, the presence of high levels of infiltrating memory CD45RO+ cells has been correlated with the absence of signs that signified early metastatic invasion, a less advanced pathological stage, and increased survival (19). This suggests that the quality of T cells present within tumors is important for immune surveillance. Moreover, T-bet and Eomes were shown to be expressed in TILs of colorectal cancers, and the higher expression levels of these factors are correlated with better survival (7, 20). T-bet-deficient mice are susceptible to prostate cancer metastasis (10). The role of T-bet and Eomes in adaptive antitumor responses is, however, not known. We therefore decided to investigate whether T-bet and Eomes are required for adaptive immunity against cancer. WT, TKO, EKO, and T/E DKO mice were vaccinated with irradiated B16 melanoma cells expressing GM-CSF. To further boost adaptive immune response to tumor cells, we depleted peripheral regulatory T cells by anti-CD25 Abs. We then challenged these mice with live B16 cells. In unvaccinated WT mice, the challenged B16 cells quickly grew and formed tumor nodules. However, no tumor nodules were observed in WT mice that have been vaccinated (Fig. 2B, 2C). Interestingly, no tumor nodules formed in vaccinated T-bet-deficient mice, suggesting T-bet is not critical for antitumor immunity in this experimental setting. In contrast, ∼30% of Eomes-deficient mice allowed tumor growth despite the vaccination (Fig. 2B, 2C). More strikingly, ∼60% of the vaccinated T/E DKO mice grew tumors (Fig. 2B, 2C). Therefore, T-bet and Eomes are both required to mediate antitumor responses.

To further clarify that the antitumor immunity is mainly mediated by lymphocytes rather than tissue cells, we generated bone marrow chimeric mice. We transfused bone marrow cells from WT, Eomes-deficient, T-bet-deficient, and T/E DKO mice to WT C57BL/6 mice, which were lethally irradiated. The transferred stem cells were able to reconstitute all major immune subsets regardless of their genotypes after 2–3 mo. We then performed vaccination experiments on these mice. The chimeric mice reconstituted with WT and T-bet-deficient bone marrow cells generated effective antitumor responses and denied the growth of transplanted B16 melanoma cells. In contrast, ∼50% of mice reconstituted with Eomes-deficient bone marrow cells succumbed to tumor growth. Moreover, 100% of mice, which were adoptively transferred with T/E DKO bone marrow cells, developed tumors (Fig. 2D, 2E). These data further demonstrate that T-bet and Eomes are both required for adaptive immunity against tumor growth.

**T-bet and Eomes are required for therapeutic cancer vaccination**

In a clinical setting, it has long been attempted to elicit an adaptive immune attack against established tumors (21). Therapeutic vaccines have achieved some success in animal models as long as the tumor size is within a certain limit (21). However, the molecular mechanisms mediating antitumor responses remain unclear. We then decided to examine whether T-bet and Eomes are important in the context of a therapeutic vaccination. Live B16 melanoma cells were inoculated i.d., and therapeutic vaccination was started at the same day of tumor cell inoculation (Fig. 3A). Inoculated B16 melanoma cells were able to grow and form nodules in WT mice. We observed that the growth of tumor was stopped in WT mice around day 15, suggesting that the vaccine was effective (Fig. 3B). In contrast, tumor growth was not inhibited by the vaccine in T/E DKO mice (Fig. 3B).

**FIGURE 3.** T-bet and Eomes are required for therapeutic cancer vaccination. A, TKO, EKO, DKO, or WT mice were challenged with 2 × 10^5 B16F0 cells i.d. At the same day, treatment was initiated by injecting anti-CD25 (350 μg, i.p.), and the anti-CD25 injection was repeated 7 d later. Treatment with 5 × 10^5 irradiated B16-GM-CSF cells was administrated s.c. 4 and 7 d after the inoculation of live B16 cells. B, Mice were monitored for tumor growth every other day. At least six mice were used in each group, and data are representative of three independent experiments. *p < 0.05; determined by Mann-Whitney statistics. Comparison was made between WT and DKO.
Tumor growth rate in vaccinated T/E DKO mice was similar to that in unvaccinated mice. In contrast, tumor growth rate in vaccinated EKO mice was similar to that in WT control mice. More interestingly, tumor growth rate in TKO mice was even slightly slower than that in WT control mice, although the data did not reach statistical significance in this experiment (Fig. 3B). Taken together, our data indicates that T-bet and Eomes are crucial for the efficacy of a therapeutic tumor vaccine.

**T-bet and Eomes control migration of antitumor T cells to the tumor site through regulating chemokine receptors**

Because our results suggest that T-bet and Eomes are important for the expression of perforin and granzyme B, we decided to examine whether the lack of antitumor responses was due to a failure to elicit CTL activities against cancer cells. We first examined the frequency of antitumor T cells by staining spleen cells with TRP-2180–188/H-2Kb pentamer. We found that the frequency of tumor-specific CD8+ T cells was similar between T/E DKO mice and WT controls at 8 d after tumor vaccination (Fig. 4A). This is consistent with our in vitro studies, which showed no difference in clonal expansion of CTLs between these mice (Supplemental Fig. 5). To determine whether deletion of T-bet and Eomes affects the generation of functional CTLs, in vivo CTL assays were performed (Fig. 4B). Surprisingly, we did not detect any difference in CTL activities between vaccinated T/E DKO mice and WT mice. These data suggest that T-bet and Eomes are not critical to generate antitumor CTL activities in the peripheral lymphoid system. We also performed the same experiment 30 d after vaccination. The frequency of pentamer-positive T cells was below the detection limit. The in vivo CTL activities were minimal at this later time point, consistent with contraction of tumor Ag-specific T cells in all mice.

We then examined CD8+ T cells in the tumors obtained from the therapeutic experiments. We found that many CD8+ T cells infiltrated the tumor mass in WT mice (Fig. 5A, 5B). We also found that the number of CD8+ T cells within tumor parenchyma in TKO mice is similar to that of WT mice (Fig. 5A, 5B). A modest reduction in tumor-infiltrating CD8+ T cells was observed in tumors from EKO mice compared with WT control mice. In contrast, a drastically reduced number of CD8+ T cells were found in the tumors from T/E DKO mice. Besides the imaging analysis, we also used flow cytometry to quantify the leukocytes that were associated with tumors in our experiments. The percentage of leukocytes (CD45+) was similar in tumors isolated from mice of all genotypes (data not shown). There was a slight decrease in CD4+ T cells in T/E DKO mice but not in EKO or TKO mice compared with WT control mice. In contrast, proportions of tumor-associated CD8+ T cells were reduced in the tumors isolated from EKO mice (13.6%) compared with WT mice (27%) (Fig. 5C, Supplemental Fig. 7). Tumor-infiltrating CD8+ T cells were greatly reduced in T/E DKO mice (2.6%) compared with WT mice (27%) (Fig. 5C, Supplemental Fig. 7). Besides a reduction in number, DKO CD8+ TILs produced reduced levels of IFN-γ and increased levels of IL-17 (Fig. 5D). No such change was observed in TKO or EKO CD8+ TILs (Fig. 5D).

**FIGURE 5.** T-bet and Eomes control migration and cytokine production in TILs. A, TKO, EKO, DKO, or WT mice were vaccinated with anti-CD25 and 5 × 10^5 irradiated B16-GM-CSF cells as described before. Tumors were resected around day 20. Sections were stained with anti-mouse CD8. Images were examined using a fluorescence microscope (original magnification ×400). a, WT; b, TKO; c, EKO; d, DKO. B, Quantification of data from A shows the number of CD8+ T cells per 600 cells in cancer tissues. C, TILs were harvested from tumor-bearing mice. Cells were then stained with CD45, CD4, and CD8. The expression of CD4 and CD8 were analyzed by flow cytometry. Data are representative of data obtained from three mice of each group. D, Analysis of cytokine production; TILs were then stimulated with PMA and ionomycin for 4 h and incubated for the last 3 h with brefeldin A. Cells producing IFN-γ and IL-17 were examined. All plots are gated on CD45+CD8+ cells.
One potential explanation for the decrease in tumor-infiltrating CD8+ T cells in T/E DKO mice is a lack of migration of antitumor T cells in these mice. We then examined whether T-bet and Eomes are required for expression of the important Th1 chemokine receptor CXCR3, which was shown to be regulated by T-bet in CD4+ T cells (22). We harvested and analyzed the spleen cells and found that CXCR3 was expressed on ~6% CD4+ T cells and 14% CD8+ T cells in WT mice (Fig. 6A). In contrast, CXCR3 was expressed on ~3% of EKO CD4+ T cells and was absent in TKO and T/E DKO CD4+ T cells. Its level was normal on TKO CD8+ T cells but dropped to ~5% in EKO CD8+ T cells and <1% of DKO CD8+ T cells (Fig. 6A, lower panel). We then examined CXCR3 expression on in vitro-differentiated Th1 and Tc1 cells. More than 70% of WT Th1 and Tc1 cells expressed CXCR3 (Fig. 6B). Both TKO and EKO Th1 and Tc1 cells decreased their CXCR3 expression. Less than 6% DKO cells expressed CXCR3 (Fig. 6B). To determine whether the difference in CXCR3 levels is functionally significant, we performed chemotaxis assay. We found that WT Th1 and Tc1 cell readily migrated toward IP-10 (Fig. 6C). T-bet knockout (KO) Th1 and Tc1 cells showed a modest decrease in migration. Similarly, Eomes KO Th1 and Tc1 cells showed a small decrease in migration. In contrast, DKO Th1 and Tc1 cells showed total lack of migration toward IP-10 (Fig. 6C). We also examined the expression of CXCR3 on TILs and found WT but not DKO CD8+ TILs expressed CXCR3 on their surfaces (Supplemental Fig. 8). Therefore, T-bet and Eomes are both required for CXCR3 expression and function in Th1 and Tc1 cells.

To examine whether CXCR3 plays any role in antitumor immune responses, we vaccinated CXCR3−/− mice along with WT and T/E DKO mice. Tumor growth was significantly faster in CXCR3−/− mice compared with WT control mice, although it was slightly slower than in T/E DKO mice (Fig. 6D). Consistently with this result, the proportion of CD8+ TILs was reduced ~3-fold in CXCR3−/− mice compared with WT controls (Fig. 6E). Therefore, the lack of CXCR3 expression on T cells might contribute to the lack of antitumor responses in T/E DKO mice.

T-bet and Eomes are critical for T cell function during antitumor immune responses

T-bet deficiency has been associated with increased metastatic disease in a murine prostate cancer model (10). This lack of immune surveillance of cancer metastasis was attributed to NK cell defects in T-bet–deficient mice (11). However, T-bet deficiency did not alter adaptive antitumor responses, likely as a result of the ex-

**FIGURE 6.** T-bet and Eomes are required for migration of antitumor T cells to the tumor site through regulating CXCR3. A. Splenocytes were harvested from TKO, EKO, DKO, or WT mice and stained for CD4, CD8, and CXCR3. Data are representative of three independent experiments. B. Naive T cells purified from TKO, EKO, DKO, or WT mice were cultured in Th1 condition for 96 h and stained for CD4, CD8, and CXCR3. CXCR3 expression on CD4+ T cells of each group is 79.7% (WT), 20.4% (TKO), 33.5% (EKO), and 5.5% (DKO), respectively. CXCR3 expression on CD8+ T cells of each group is 78.4% (WT), 20.5% (TKO), 19.7% (EKO), and 3.9% (DKO), respectively. Data are representative of three independent experiments. C. Naive T cells purified from TKO, EKO, DKO, or WT mice were stimulated with plate-bound anti-CD28 plus anti-CD3 in Th1 condition. Then live cells were placed in the upper wells of Transwell inserts, and their chemotactic responses to IP-10 (200 ng/ml) were determined. Bars represent means of three samples ± SEM. *p < 0.05; **p < 0.001; determined by two-tailed unpaired Student t test. Two independent experiments were performed with similar results. D. CXCR3−/−, T/E DKO, or WT mice were challenged and treated as described in Fig. 3. Mice were monitored for tumor growth every other day. *p < 0.05; determined by Mann-Whitney statistics. Comparison made between WT and CXCR3−/−. E. TILs were harvested from tumor-bearing mice. The expression of CD8 was analyzed by flow cytometry.
pression of Eomes in T cells (11). Because T-bet is also expressed in NK cells, DCs and macrophages (11, 23–25), we used an adoptive transfer system to determine whether T-bet and Eomes expression in T cells is required for adaptive antitumor immune responses. T cells from WT and T/E DKO mice were isolated and mixed with WT T cell-depleted splenocytes. The mixed cells were then transferred to nonlethally irradiated mice. The reconstituted mice had an immune system composed mainly of normal non-T cells and either WT or T/E DKO T cells. These mice were then subjected to therapeutic vaccination as in Fig. 3. Tumor growth was delayed and eventually controlled by vaccination in WT control mice (Fig. 7). In contrast, tumor growth in mice reconstituted with T/E DKO T cells and normal T cell-depleted splenocytes was nonstopable and was similar to that in irradiated mice without any transferred cells (Fig. 7). Therefore, the function of T-bet and Eomes is T cell intrinsic.

**Discussion**

In this study, we used TKO, EKO, and Tbet/Eomes doubly deficient mice to examine the importance of each of these transcription factors in T cell-mediated antitumor immune responses. T-bet and Eomes synergistically regulate multiple steps of Th1/Tc1 differentiation from activation to tissue trafficking. Taken together, these transcription factors take a two-handed approach. On the one hand, they are critical in turning on genes with important functions in cell-mediated immune responses such as effector/memory marker CD44, antitumor cytokine IFN-γ, and chemokine receptors that allow T cells to migrate to cancer tissues. On the other hand, these transcription factors also prevent CD8+ T cell differentiation to alternative subsets such as Tc17 and Tc2. The lack of both T-bet and Eomes in T cells severely perturbed these mechanisms, resulting in profound defects in antitumor immunity and dysregulated T cell differentiation. Moreover, we showed that the regulation of antitumor immune responses by T-bet and Eomes is T cell intrinsic. Collectively, we have identified the Th1-associated transcription factors T-bet and Eomes as key regulators of T cell-mediated antitumor immune responses.

Interestingly, we have found that antitumor CTL activities are not significantly affected in the peripheral lymphoid organs in T/E DKO mice. These data are consistent with our in vitro finding that levels of perforin and granzymes were only modestly reduced in T/E DKO T cells compared with WT. The frequency of anticancer CD8+ T cells was also similar between WT and T/E DKO mice, which aligns with our in vitro data that T-bet and Eomes are not required for clonal expansion. These data suggest that the antitumor CTL activity on a per-cell basis is not significantly different between the vaccinated WT and DKO mice. Other transcription factors such as STAT-4 and Runx-1,2,3 might be involved in regulating CTL activities in effector CD8+ T cells in the periphery (16). In CD8+ T cells, the expression levels of these factors are not regulated by T-bet and Eomes. Whether or not these transcription factors synergize with T-bet and Eomes in regulating CTL activities needs to be further studied. In addition, whether the CTL activities are affected within tumor tissues remains to be explored.

Although T-bet and Eomes were not required for systemic antitumor activities, most vaccinated DKO mice still allowed the growth of s.c. challenged tumor cells. The deficiency in antitumor immune responses seems to be attributed to the lack of tumor infiltration by T cells. We have further shown that chemokine receptor CXCR3 is critically regulated by T-bet and Eomes. In addition, CXCR3−/− mice showed compromised antitumor immune responses. Therefore, T-bet and Eomes seem to be important for T cell migration and retention in the tumor site. This might be partly mediated by their regulation of CXCR3. This finding is significant in light of the fact that many tumor vaccines have failed to improve survival despite data showing that they have elicited immune responses in cancer patients. This is possibly because immune responses have been measured by CTL and cytokine assays done on peripheral blood cells. Our results challenge the utility of these assays, because the CTL activities measured in the blood do not tell us whether antitumor T cells are able to infiltrate and destroy tumors. Therefore, further study on the T/E regulates genes in tumor Ag-specific T cells might provide useful biomarkers for monitoring tumor vaccine trials.

CXCR3 was shown to be regulated by Eomes in thymic CD8+ T cells when IL-4 is overexpressed as a result of deletion of KLF2 (26). Whether Eomes regulates CXCR3 in peripheral CD8+ T cells and whether it is essential for CXCR3 expression are still not known. T-bet was also shown to regulate T cell migration to heart during inflammation partly via its regulation of CXCR3 on CD4+ T cells (22). The role of T-bet in CD8+ T cell migration is not clear, although it has been shown that T-bet positively regulates CXCR3 in CD8+ T cells (27). In addition, the tumor microenvironment is known for its unique immune suppressive characteristic, which differs greatly from autoimmune and infection-driven inflammatory environment. Therefore, it is important to determine whether T-bet and Eomes affect T cell migration to tumor sites. Indeed, we showed that T-bet and Eomes are individually dispensable for T cell migration to tumor. But deletion of both T-bet and Eomes renders CD8+ T cell unable to infiltrate tumors. This finding correlated with our data showing that T-bet and Eomes are crucial for CXCR3 expression on the surface of CD8+ T cells. In addition, we showed that CXCR3−/− mice were partially compromised in their antitumor immune responses. Besides CXCR3, additional factors might further contribute to the lack of CD8+ TILs in T/E DKO mice. For example, it has been reported that T-bet regulates cell adhesion in CD4+ T cells through tyrosine sulfation of P-selectin glycoprotein ligand 1. It was further attributed to regulation of mRNA levels of Tp72 by T-bet in Th1 cells (22). However, no difference in the expression of Tp71 or Tp72 was detected between WT and T/E DKO CD8+ T cells (data not shown). It is also possible that the survival of TILs is affected, because it has been shown that T-bet make cell survive better because of its regulation.
of CD122 (13, 28). We have shown that T-bet and Eomes regulate CD44. The lack of CD44 was shown to reduce migration of TILs within tumor (29). These possibilities and others remain to be tested. Regardless, our data provide a definitive answer that T-bet and Eomes are required for the presence of CD8+ T cells in tumor.

Despite many data support an anticancer role of T-bet and Eomes, there is a report that T-bet is involved in the progression of carcinoma in Helicobacter felis-induced gastric cancer (30). This might be because H. felis-induced gastric cancer progression is associated with Th1-type inflammatory response (30). T-bet likely plays a role in tissue damages that lead to this cancer. Therefore, different types of cancer are associated with and promoted by different types of inflammation. The role of T-bet and Eomes in a cancer is dictated by the type of inflammation that is associated with that particular type of cancer. Indeed, T-bet deficiency in dendritic cells leads to colitis-associated colorectal cancer (25), supporting its role in suppressing this cancer. In contrast, T-bet deficiency inhibited Th1-driven inflammatory bowel disease (31), and we would speculate that T-bet deficiency likely will prevent cancer progression driven by this Th1-mediated colitis.

Previous studies have suggested a minimal role for T-bet in adaptive immunity against cancer (11). The lack of impact of T-bet deletion on adaptive immune responses against cancer is likely because Eomes is also expressed in CD8+ T cells and Th1 cells. Using T/E DKO mice, we have found that T-bet and Eomes are indeed required for the effector phase of the adaptive immunity against cancer. It has also been reported that IL-21 inhibits Eomes expression and is potent in adaptive T cell therapy against cancer (32). Shutting down Eomes during cell culture is important to achieve survival of transferred T cells, likely because Eomes and T-bet might drive more apoptosis in cultured T cells (our unpublished observation). However, our study demonstrates that the expression of T-bet and Eomes is important to allow antitumor T cells to eventually reach and be retained in the tumor site and fulfill their anticancer mission. IL-12 is a hallmark cytokine for inducing Th1 responses and was shown to change the tumor microenvironment to favor tumor surveillance and eradication. Paraadoxically, IL-12 suppresses Eomes expression in CD8+ T cells (14, 26). Our data clearly established that T-bet and Eomes are synergistic in eliminating cancer cells. Therefore, Eomes may use signals other than IL-12, such as IL-2, IL-15, or IL-4 (32), to enhance antitumor responses. We have shown in this study that in optimal Th1 culture condition, the frequency of IFN-γ producers was modestly affected after both T-bet and Eomes were deleted in CD8+ T cells. Runx3 was shown to be regulated by T-bet in Th1 cells (33). However, in CD8+ T cells, we have found it is not regulated by T-bet or Eomes (data not shown). Therefore, Runx3 and perhaps Stat-4 are all also involved in regulating Th1 phenotype in CD8+ T cells (11). Regardless, IFN-γ production in vivo is clearly reduced in tumor sites. Interestingly, previous studies have shown that during infection, there is a clear lack of development of IFN-γ-producing CD8+ T cells (9, 17). The reason for this difference might be because during infection, the T/E DKO CD8+ T cells predominantly differentiate to Tc17 cells (15). Therefore, T-bet and Eomes control balance of inflammation during immune responses.

We have shown here that T-bet and Eomes also regulate memory cells marker CD44. The T/E DKO mice showed a reduction of CD44+ effector/memory T cells. Therefore, it is possible that T-bet and Eomes are important for the generation of memory T cells. This is in line with a prior study, which showed that T-bet and Eomes are required for the expression of CD122 (34), an IL-15R that is important for the homeostasis of memory T cells. Our study further showed that CD122+ CD44+ memory T cells are also regulated by T/E. Therefore, T-bet and Eomes might regulate effector/memory pools by CD122-independent mechanisms. It has been proposed that optimal T-bet expression by CTLs promotes the generation of long-term memory T cells, but high levels of T-bet drives T cell differentiation to short-lived effector cells (35). Our data demonstrate that T-bet is dispensable for the effector function of antitumor immune responses. We further showed that the efficacy of tumor vaccination is dependent on both T-bet and Eomes. Our data clearly established that both T-bet and Eomes are required for the effector stage of T cell responses against tumor. Whether T-bet, Eomes, or both are required for the generation of central and effector memory cells will be the focus for future studies.

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Disclosures
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