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T-bet Is Critical for the Development of Acute Graft-versus-Host Disease through Controlling T Cell Differentiation and Function

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T-bet is a master regulator for IFN-γ production and Th1 differentiation. We evaluated the roles of T-bet and IFN-γ in T cell responses in acute graft-versus-host disease (GVHD) and found that T-bet+/− T cells induced significantly less GVHD compared with wild-type or IFN-γ−/− counterparts in both MHC-mismatched and MHC-matched but minor histocompatibility Ag–mismatched models driven by CD4 T cells. T-bet−/−, but not IFN-γ−/−, CD4 T cells had a markedly reduced ability to cause tissue damage in liver and gut. This distinct outcome is reflected by the differential gene expression on donor CD4 T cells deficient for T-bet or IFN-γ. At mRNA and protein levels, we defined several T-bet–dependent molecules that may account for the impaired ability of T-bet−/− T cells to migrate into target organs and to produce Th1-related cytokines. Moreover, these molecules were independent of either endogenous IFN-γ, such as CXCR3 and programmed death-1, or systematic IFN-γ, such as NKG2D, I-Aβ, and granzyme B. Although both T-bet−/− and IFN-γ−/− CD4 T cells are prone to differentiate into Th17 cells, polarized Th17 cells deficient for T-bet but not for IFN-γ had a significantly reduced ability to cause GVHD. Finally, T-bet−/− T cells had a compromised graft-versus-leukemia effect, which could be essentially reversed by neutralization of IL-17 in the recipients. We conclude that T-bet is required for Th1 differentiation and migration, as well as for optimal function of Th17 cells. Thus, targeting T-bet or regulating its downstream effectors independent of IFN-γ may be a promising strategy to control GVHD in the clinic. The Journal of Immunology, 2015, 194: 000–000.

Graft-versus-host disease (GVHD) is a major limitation for the efficacy of allogeneic hematopoietic stem cell transplantation in the treatment of hematologic malignancies because it leads to significant morbidity and mortality (1). The cytokine storm caused by conditioning and Th1 cytokine cytokines produced by allogeneic T cells are the driving forces for the initiation and development of GVHD (2–5). Paradoxically, the principal Th1 cytokine, IFN-γ, plays a dispensable role for GVHD development in some experimental murine bone marrow transplants (BMT) models (6–11), where exacerbated GVHD was observed in hosts receiving IFN-γ−/− grafts (7–9, 11) or after IFN-γ neutralization (7) following lethal irradiation. Alternatively, administration of recombinant IFN-γ showed a protective effect for CD4 T cell–mediated GVHD (10).

T-bet, the T-box transcription factor, has a unique role in the differentiation of all three subsets (Th1, Th2, Th17) of CD4+ helper T cells by promoting Th1 differentiation while simultaneously suppressing the development of Th2 and Th17 (12). T-bet target genes have been identified in primary human T cells, which show that T-bet is associated with genes of various functions in Th1 cells, including those with roles in transcriptional regulation, chemotaxis, and adhesion (13). T-bet is a transcriptional activator of IFN-γ (14) and orchestrates the cell-migratory program by directly controlling expression of the chemokine receptors CXCR3 and CCR5, as well as the chemokines CCL3 and CCL4 (13, 15). T-bet also has cooperative and partially redundant functions with comesdermin (Eomes), another T-box transcription factor, to control CD8 T cell cytotoxicity and IFN-γ production (16, 17).

Previously, we observed that T cells deficient for T-bet are impaired in the induction of acute GVHD (18). However, the effect and mechanism of T-bet on T cells to induce GVHD and mediate the graft-versus-leukemia (GVL) effect has not been thoroughly studied, particularly the reason for the paradoxical outcomes of GVHD caused by T-bet−/− or IFN-γ−/− T cells. We therefore used T cells from T-bet−/− or IFN-γ−/− mice as donors and tested whether T-bet could be a potential target for preventing GVHD after allogeneic BMT (allo-BMT). We then elucidated the underlying mechanisms by which T-bet or IFN-γ differentially regulates allogeneic T cell response after allo-BMT. We identified several molecules that depend on T-bet, but not on endogenous IFN-γ produced by donor T cells or systematic IFN-γ produced by
any type of cell, which may be responsible for T cell pathogenicity in GVHD induction. Furthermore, we define the role of T-bet in Th17 function related to GVHD and its impact on the GVL effect. Our study provides new biological insight on T-bet, as well as the rationale to target T-bet or its downstream effectors, to control GVHD after allo-BMT.

**Materials and Methods**

**Mice**

C57BL/6 (B6; H-2<sup>b</sup>, CD45.2), B6.129.P2-Pten<sup>tm1</sup> (CD45.1), and BALB/c (H-2<sup>b</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c mice were bred at the H. Lee Moffitt Cancer Center (Tampa, FL). All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at the H. Lee Moffitt Cancer Center or the Medical University of South Carolina (Charleston, SC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of University of South Florida (Tampa, FL) or the Medical University of South Carolina.

**BMT models**

T cell purification from whole spleen and lymph nodes was done by negative depletion using magnetic beads as previously described (18, 25). Heat maps were generated by using Cluster 3.0 and Java TreeView. Tonome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Array images were analyzed using FACSDiva software, LSR II (BD Biosciences, San Jose, CA) at the H. Lee Moffitt Cancer Center, or the University of South Carolina (Charleston, SC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of University of South Florida (Tampa, FL) or the Medical University of South Carolina.

**Flow cytometry and serum cytokine detection**

Mononuclear cells were isolated from recipient spleen or liver as previously described (18, 21, 22) and stained for surface receptors and intracellular cytokines using standard flow cytometric protocols. Stained cells were analyzed using FACSVerse software, LSR II (BD Biosciences, San Jose, CA), and FlowJo (Tree Star, Ashland, OR). Cytokine levels in recipient serum were quantified using a cytokine bead assay (BD Biosciences).

**Microarray and real-time PCR**

Lethally irradiated BALB/c mice were transplanted with wild-type (WT) Ly5.1<sup>+</sup> B6 TCD-BM (5 × 10<sup>6</sup>/mouse) plus WT, T-bet<sup>−/−</sup>, or IFN-γ<sup>−/−</sup> naive CD4<sup>+</sup>Ly5.1<sup>+</sup> T cells (1 × 10<sup>6</sup>/mouse). Recipients were euthanized on day 7. Donor-derived T cells (CD4<sup>+</sup>Ly5.2<sup>+</sup>Ly5.1<sup>+</sup> DAPI<sup>−</sup>) in recipient splenocytes were isolated by cell sorting (BD FACSARia II cell sorter) and lysed in TRizol (Life Technologies, Grand Island, NY) to extract total RNAs. Microarray analysis was performed using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA). Array images were analyzed and processed by the robust multiarray average procedure (24, 25). Heat maps were generated by using Cluster 3.0 and Java TreeView. To identify potential genes significantly changed (either increased or decreased) in the T-bet<sup>−/−</sup> versus WT group, but not shared by the IFN-γ<sup>−/−</sup> group, three sets of genes were selected: 1) signal fold change of T-bet<sup>−/−</sup> versus WT > 2; 2) signal fold change of T-bet<sup>−/−</sup> versus IFN-γ<sup>−/−</sup> > 2; and 3) signal fold change of IFN-γ<sup>−/−</sup> versus WT > 2. Fold change calculation was based on the mean value of individual genes from three independent experiments. The genes that overlapped in set 1 and set 2 were further selected, and genes from set 3 were excluded from those selected genes. The signal value < 500 was considered as background noise. Thus, the primary pool of T-bet–dependent but endogenous IFN-γ–independent genes was established. Additional criteria were added when heat map analysis was performed. When selecting T-bet positively regulated genes, the signal fold change of T-bet<sup>−/−</sup> versus WT, T-bet<sup>−/−</sup> versus IFN-γ<sup>−/−</sup>, and IFN-γ<sup>−/−</sup> versus WT is >4, >3, and <2, respectively, and the lowest signal value limit of WT group is set at 1675 (2<sup>10.75</sup>). When selecting T-bet negatively regulated genes, the signal fold change of T-bet<sup>−/−</sup> versus WT, T-bet<sup>−/−</sup> versus IFN-γ<sup>−/−</sup>, and IFN-γ<sup>−/−</sup> versus WT is >2, >2, and <2, respectively, and the lowest signal value limit of T-bet<sup>−/−</sup> group is set at 1024 (2<sup>10.1</sup>). In heat map scale, a signal value of 1024 (2<sup>10.1</sup>) was termed “0.00” and is represented by black, a signal value of 8192 (2<sup>13.0</sup>) was termed “3.00” and is represented by red, and a signal value of 128 (2<sup>7</sup>) was termed “−3.00” and is represented by green. Mouse array data can be accessed at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2198.

To quantify mRNA using real-time PCR, extracted total RNA was reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit. All PCR experiments were performed using TaqMan gene expression assays (Life Technologies). Relative gene expression levels were calculated using the comparative method for relative quantitation upon normalization to the internal GAPDH expression control.

**In vitro generation of Th17 cells and Th17-mediated GVHD model**

CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from WT, T-bet<sup>−/−</sup>, or IFN-γ<sup>−/−</sup> mice on B6 background were stimulated in the presence of syngeneic APCs with 2 μg/ml anti-CD3 mAb, 5 ng/ml TGF-β, 10 ng/ml IL-6, and 5 μg/ml anti–IFN-γ mAb. On day 3, 50 U/ml murine IL-2 was added. Cell phenotype (CD4<sup>+</sup>IL-17<sup>+</sup> percentage) was confirmed on day 4 by intracellular cytokine staining (IFN-γ and IL-17). On day 5, polarized T cells were collected and cultured. Cells were removed using Ficoll separation. Equal numbers of live CD4<sup>+</sup>IL-17<sup>+</sup> cells derived from WT, T-bet<sup>−/−</sup>, or IFN-γ<sup>−/−</sup> T cells were used to induce GVHD to compare pathogenicity.

**Statistical analysis**

For comparison of recipient survival among groups in GVHD experiments, the log-rank test was used to determine statistical significance. To compare GVHD clinical scores, pathology scores, weight loss, cytokine levels, and gene expression levels, a Student t test was used.

**Results**

T-bet is required for CD4<sup>+</sup> T cell–mediated acute GVHD

Our previous study indicated that T-bet<sup>−/−</sup> total T cells are less pathogenic than their WT counterparts in inducing GVHD in fully MHC-mismatched BMT models (18). In contrast, IFN-γ, a major cytokine regulated by T-bet, is dispensable or even protective against GVHD development (6–11). Considering that Eomes is preferentially expressed on CD8<sup>+</sup> T cells (16, 26) and CD4<sup>+</sup> T cell differentiation is better defined (5, 27), we first focused our studies on CD4<sup>+</sup>-mediated GVHD and aimed to further understand how T-bet and IFN-γ differentially affect the development of acute GVHD. As shown in Fig. 1, IFN-γ<sup>−/−</sup> CD4<sup>+</sup> T cells mediated comparable GVHD to WT CD4<sup>+</sup> T cells, whereas T-bet<sup>−/−</sup> CD4<sup>+</sup> T cells caused significantly milder GVHD. Recipients of T-bet<sup>−/−</sup> donor T cells showed significantly longer-term survival rates (Fig. 1A), less weight loss (Fig. 1B), and lower GVHD clinical scores after 4 and 8 wk following allo-BMT (Fig. 1C, 1D) compared with the recipients of either WT or IFN-γ<sup>−/−</sup> CD4<sup>+</sup> T cells.
Alleviated GVHD caused by T-bet−/− CD4 T cells was also supported by significantly lower pathological scores of those recipients 14 d after allo-BMT. Recipients of T-bet−/− CD4 T cells had markedly reduced T cell infiltration and tissue damage in GVHD target organs, including the liver, gut, and skin (Fig. 1E, 1F). However, T-bet−/− CD4 T cells induced similar severity of pulmonary GVHD as did WT CD4 T cells (Fig. 1E, 1F), consistent with the previous report that pulmonary GVHD is not associated with Th1-mediated response (28). Serum cytokines detected 14 d posttransplant revealed that T-bet−/− CD4 T cells produced significantly lower levels of IFN-γ compared with WT CD4 T cells (Fig. 1G). Consistent with the pathological scores, T-bet−/− CD4 T cells also produced lower levels of TNF-α but higher levels of IL-10 and IL-6 compared with either WT or IFN-γ−/− CD4 T cells (Fig. 1G). Furthermore, intracellular IL-17 expression was significantly higher in T-bet−/− or IFN-γ−/− donor T cells than in WT donor T cells in recipient spleens and livers (Fig. 1G). Altogether, T-bet-deficient CD4 T cells produced lower levels of inflammatory cytokines and induced less damage in the recipient liver, gut, and skin, indicating that T-bet, not IFN-γ, is required for CD4 T cell-mediated acute GVHD.

T-bet is important for GVHD induction in miHA-mismatched model

miHA mismatches play a critical role in clinical GVHD development in HLA-identical BMT conditions (29). Given that the effect of T-bet−/− or IFN-γ−/− total T cells in fully MHC-mismatched models has been reported previously by us and others (8, 18, 30), we used a clinically relevant MHC-matched, multiple miHA–mismatched CD4-dependent BMT model, B6→BALB.B, and found that the recipients that received splenocytes from T-bet−/− mice demonstrated prolonged survival with decreased GVHD severity compared with those that received WT or IFN-γ−/− splenocytes (Fig. 2). These results suggested that T-bet plays a critical role in the development of acute GVHD regardless of the BMT model.

Differential gene profiles of T-bet−/− and IFN-γ−/− CD4 T cells after allo-BMT

To determine the mechanism of how T-bet affects T cell pathogenicity in the induction of GVHD, we hypothesized that the molecules that are regulated by T-bet but not required for IFN-γ production are responsible for GVHD development. To identify
these molecules, we isolated donor CD4 T cells from spleens of recipients at 7 d following allo-BMT (B6→BALB/c) and analyzed the gene profiles by microarray. Internal controls, Tbx21 (gene encoding T-bet) and Ifng (gene encoding IFN-γ), were essentially absent in T-bet−/− CD4 T cells and IFN-γ−/− CD4 T cells, respectively (Fig. 3A, 3B). The microarray heat map showed that a total of 28 genes were downregulated and 8 genes were upregulated only in T-bet−/− but not in IFN-γ−/− CD4 T cells compared with WT CD4 T cells (Fig. 3A). Among them, we chose to present those genes potentially relevant to GVHD in a bar graph, including Cxcr3, Ccr5, Ccl3, Ccl4, Klrc1, Klrd1, Nkg7, and Pdcd1 that were positively regulated, whereas H2-Aa and H2-Ab1 were negatively regulated by T-bet independent of endogenous IFN-γ (Fig. 3B). Changes in representative genes (Hopx, Slanf1, Serpinb9, Iqf2r, Klrd1, Klrc1, H2-Aa, H2-Ab1, and Retnla) were confirmed by real-time PCR (Fig. 3C). We reason that at least some of those genes we identified are responsible for the compromised ability of T-bet−/−, not IFN-γ−/−, CD4 T cells in the induction of GVHD.

**T-bet regulates T cell pathogenicity through IFN-γ–independent manners**

Our microarray data provide potential explanations for why T-bet−/− and IFN-γ−/− T cells induce distinct outcomes of GVHD at the gene level. However, protein expression is typically better correlated with biological function. To confirm our microarray study...
and further investigate the underlying mechanism, we used an MHC-mismatched BMT model (B6→BALB/c) and examined the expression of several molecules that represent multiple aspects of allogeneic T cell function, that is, activation (I-A^B and NKG2D), migration (CXCR3), cytotoxicity (CD94 and granzyme B [GZMB]), and exhaustion (programmed death-1 [PD-1]), of donor T cells by flow cytometry 7 d after allo-BMT. We observed that T-bet^−/− donor T cells significantly impaired proliferation in secondary lymphoid organs (spleen) and migration into target organs (liver) when compared with their WT counterparts (Fig. 4A). This was consistent with their significantly decreased IFN-γ production, but increased IL-17 and IL-10 secretion (Fig. 4B). T-bet^−/− donor T cells also showed significantly less infiltration into the liver compared with T-bet^+/+ donor T cells in the spleen is mostly displayed in the CD8 but not CD4 subpopulation (Fig. 4A), which indicated that absence of T-bet affects multiple aspects of CD4 T cell function independent of cell expansion. Moreover, in contrast to WT or IFN-γ^−/− T cells, T-bet^−/− T cells significantly decreased the expression of CXCR3, NKG2D, PD-1, and CD94, but increased the expression of I-A^B and GZMB in CD4 or CD8 constituent subpopulations (Supplemental Table I) or both (Fig. 4C).

Given that IFN-γ can be produced by other cell types besides donor T cells, we aimed to distinguish the dependence of endogenous or systematic IFN-γ by including IFN-γR^−/− T cells as additional controls, which are able to produce IFN-γ (Fig. 4B) but are unable to respond to IFN-γ. We found that CXCR3 and PD-1 were expressed in similarly low levels on the T cells deficient for T-bet or IFN-γR, but not IFN-γ. This suggests that their protein expression depends on T-bet, not endogenous IFN-γ, although it can be regulated by IFN-γ produced by other types of cells in the recipient. Alternatively, the expression of NKG2D, CD94, I-A^B, and GZMB depends on T-bet, but not systematic IFN-γ, because their protein profiles are distinct on the T cells deficient for T-bet versus either IFN-γ or IFN-γR. Therefore, we have identified several T-bet–dependent but endogenous or systematic IFN-γ–independent molecules that likely contributed to the impaired target organ migration and Th1 cytokine production by T-bet^−/− T cells.

**T-bet controls the optimal function of Th17 cells in GVHD induction**

It is known that Th1 differentiation antagonizes Th17 differentiation (31, 32). Our data show that either T-bet^−/− or IFN-γ^−/− T cells are prone to differentiate into Th17 cells both in vitro (Supplemental Fig. 1) and in vivo (Fig. 1H), which is consistent with previous reports (18, 33). Given that the Th17 subset per se is capable of causing GVHD (34, 35), we hypothesized that optimal activity of Th17 cells may require T-bet but not IFN-γ, which may be attributed to a distinct pathogenicity of T-bet versus IFN-γ in GVHD induction. Therefore, we examined the role of T-bet or IFN-γ in the pathogenicity of Th17 cells to cause GVHD using optimized Th17-polarizing culture conditions. We generated >70% CD4^+IL-17^+ cells from naive CD4 T cells of WT, T-bet^−/−, or IFN-γ^−/− B6 mice (Fig. 5A) and then transferred equal numbers of these T cells into lethally irradiated BALB/c recipients. We found that T-bet^−/−, not IFN-γ^−/−, Th17 cells had a significantly reduced ability to induce GVHD. This is reflected by ameliorated morbidity and mortality of the recipients (Fig. 5B, 5C). Because bone marrow stromal

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**FIGURE 4.** T-bet regulates T cell migration and cytokine production through IFN-γ–independent manners. Lethally irradiated BALB/c mice were transplanted with 5 × 10^4/mouse TCD-BM from WT Ly5.1^+ B6 donor plus WT, T-bet^−/−, IFN-γ^−/−, or IFN-γR^−/− naive CD4^+Ly5.1^+ T cells at 1 × 10^7/mouse (n = 3–4/group). Recipients were euthanized 7 d posttransplant and their spleens and livers were collected and absolute numbers of donor total T cells, CD4^+ T cells, or CD8^+ T cell are shown (A). Production of IFN-γ, IL-17, and IL-10, as well as expression of CXCR3, NKG2D, I-A^b, PD-1, CD94, and GZMB, by WT, T-bet^−/−, IFN-γ^−/−, or IFN-γR^−/− donor T cell in recipient spleen are shown in bar graphs (B) and histograms (C). Data from one representative experiment are presented. *p < 0.05, **p < 0.01, ***p < 0.001.
niches is considered a sensitive target of allogeneic T cells, and the defective donor bone marrow–derived B lymphopoiesis is correlated with GVHD severity (36), we examined the percentage of donor-derived B cells at the end of observation periods (around day 85 after allo-BMT), which was found to be significantly higher in the recipients of T-bet−/− Th17 cells than those of WT or IFN-γ−/− Th17 cells (Fig. 5E), despite a similar number of total splenocytes among these groups (Fig. 5D). These data strongly suggest T-bet also affects the pathogenicity of Th17 cells in GVHD induction. Moreover, Th17 pathogenic and nonpathogenic signature molecules are previously defined in autoimmune models, such as experimental autoimmune encephalomyelitis (EAE) (37–39). Based on the similar requirement of Th1 and/or Th17 cells and on T-bet expression in the induction of EAE and GVHD (18, 40), we used the genes identified in EAE model as a reference to indirectly reflect the effects of T-bet on regulating Th17 pathogenicity in allo-BMT settings. Our microarray results indicate that T-bet positively regulated Th17 pathogenic signature genes on CD4 T cells after allo-BMT, such as Lmp2, Ccl5, ICOS, Stat4, Lgals3, Malt1, and GM-CSF, while negatively regulating Th17 nonpathogenic signature genes such as IL-10 (Supplemental Fig. 1). The observation is in line with our hypothesis that T-bet contributes to the optimal function of Th17 cells after allo-BMT.

**T-bet−/− donor T cells largely preserve the GVL effect by neutralizing IL-17 in allo-BMT recipients**

The ultimate goal of allogeneic hematopoietic stem cell transplantation is to prevent GVHD while preserving the GVL effect. Cytotoxic CD8 T cells are known to play a predominant role in mediating GVL effects (41, 42). The GVL effects of IFN-γ−/− T cells have been shown to correlate inversely with their GVHD-inducing activity in a CD8-dependent model (11). We hypothesized that T-bet−/− T cells may maintain their GVL activity owing to the presence of Eomes, which preserves CD8 T cell cytotoxic activity (16, 17). We tested the expression of Eomes and IFN-γ on CD4 and CD8 T cells in the presence or absence of T-bet and found that Eomes was mainly expressed on CD8 T cells compared with CD4 T cells. T-bet−/− CD8 T cells highly expressed Eomes and maintained IFN-γ production (Supplemental Fig. 3). To determine whether T-bet is required for the T cell–mediated GVL effect, we used a B6→BALB/c BMT model and infused a low dose of A20-luc B cell lymphoma on the day of allo-BMT, which mimics clinical setting where a small number of malignant cells survive in patients after preconditioning regimen. We found that WT T cells at 0.25–0.5 × 10^6/mouse could effectively reject tumor, but most recipients (79%) quickly died of GVHD (Fig. 6A–C). However, the recipients of 0.25–0.5 × 10^6 T-bet−/− T cells showed delayed tumor growth compared with those of BM alone, but eventually most of these recipients (86%) died of tumor relapse (Fig. 6A–C). These data suggest that although T-bet–deficient T cells still express Eomes and had partially preserved the ability to produce IFN-γ, their GVL activity was largely compromised.

T cells deficient for T-bet produce elevated levels of IL-17 (Figs. 1H, 4B). Substantial evidence supports that IL-17 enhances tumor resistance to anti–angiogenesis therapy (43), promoting tumor progression through an IL-6/stat3 signaling pathway (44, 45). Our previous study indicates that additional retinoic acid–related orphan receptor (ROr)γt (transcription factor of Th17 cells) deficiency reverses the enhanced ability of T-bet–deficient T cells to produce IL-17. Furthermore, T-bet/ RORγt–deficient T cells have largely preserved GVL activity (18). Therefore, we further hypothesized that elevated IL-17 contributed to the compromised GVL effect of T-bet−/− T cells. To test
In this study we show that T-bet--deficient donor T cells, in contrast to IFN-γ--deficient donor T cells, are impaired in their ability to induce acute GVHD in allogeneic recipients in fully MHC-mismatched (Figs. 1, 5) or MHC-matched but miHA-mismatched (Fig. 2) murine BMT models. We identified distinct genetic profiles of T cells deficient for T-bet or IFN-γ to account for this difference (Fig. 3) and further elucidated that several T-bet downstream molecules, independent of either endogenous or systematic IFN-γ, contributed to GVHD pathogenicity (Fig. 4). Furthermore, we found that T-bet--deficient donor T cells have preserved GVL activity when the resulting increased IL-17 is neutralized (Fig. 6).

Acute GVHD has been considered a Th1-type disease dominated by cytotoxic T cell–mediated pathology and increased production of Th1-type cytokines, including IFN-γ (2–4). Our data indicate that T-bet is required for CD4 T cell–mediated GVHD by controlling the differentiation and migration of Th1 cells (Fig. 1) and the optimal function of Th17 cells (Fig. 5). T-bet--/− CD4 T cells produced significantly lower levels of the pathogenic cytokine TNF-α, but higher levels of the anti-inflammatory cytokine IL-10 in recipient sera after adoptive transfer, which led to reduced tissue damage in the liver and gut, compared with either WT or IFN-γ--/− CD4 T cells (Fig. 1). Conversely, the Th17-related cytokine IL-6 was elevated in recipient sera when the donor CD4 T cells were T-bet--/− (Fig. 1G). Additionally, increased IL-17 production by T-bet--/− donor CD4 T cells was present in recipient spleens and livers (Fig. 1H). Our previous study also indicates that T-bet--/− CD4 T cells produced higher levels of Th2-related cytokines, IL-4 and IL-5, in recipient spleens, livers, and lungs (18). Consistent with previous reports, donor CD4 T cells can reciprocally differentiate into Th1, Th2, and Th17 cells, and each Th subset contributes to specific GVHD target organ tissue damage. Liver and gut are the primary target organs for Th1 cells, and skin is the primary target organ for Th17 cells, whereas pulmonary GVHD is mainly mediated by Th17 and Th2 cells (33). We observed that T-bet--/− CD4 T cells induced severe pulmonary GVHD similar to WT counterparts (Fig. 1E, 1F). We attribute the severe lung pathology mediated by T-bet--/− CD4 T cells to the augmented Th2 and Th17 cells and to the minimal

*FIGURE 6. T-bet--/− donor T cells largely preserve GVL effect upon neutralizing IL-17 in allo-BMT recipients. BALB/c mice were lethally irradiated and transplanted with 5 × 10^6/mouse TCD-BM from WT B6 donor alone or plus WT or T-bet--/− purified T cells at 0.25–0.5 × 10^6/mouse. Additionally, recipients were i.v. injected with 2 × 10^7/mouse A20 luciferase-transduced lymphoma cells at the time of BMT. Recipients were i.p. injected with anti-IL-17 mAb (500 μg/mouse for the first dose and 200 μg/mouse for the following doses) or PBS control for 4 wk (at days 0, 3, 7, 10, 14, 18, 21, 25, and 28) and were monitored throughout the experimental period for survival (A), weight change (B), and tumor growth by luciferin i.p. injection and whole-body BLI (n = 4–7/group/experiment). Recipient BLI image (C) represents one of the two replicated experiments, and average radiance intensity of BLI (D) pooled from all experiments is shown.*
IFN-γ production, which leads to decreased PD-1 ligand expression on lung parenchyma (33). Moreover, owing to the direct cytotoxicity of IFN-γ to the gastrointestinal tract (6), the absence of T-bet or IFN-γ in donor CD4 T cells induced similar severity of colon GVHD but was less than that induced by WT CD4 T cells (Fig. 1E, 1F).

Multiple preclinical studies have investigated a paradoxical protective role of IFN-γ in GVHD development under lethal conditioning (6–11). In our present settings, IFN-γ−/− T cells induced comparable, not more severe, GVHD as did their WT counterparts, which is likely due to the type and dose of donor T cells given that were different from other studies (6–11). Taken together, the consensus is that IFN-γ produced by donor T cells is not required for the development of acute GVHD after lethal total body irradiation and allo-BMT. However, in the present study we showed that T-bet, a transcriptional activator of IFN-γ, was required for GVHD induction. To further define the underlying mechanisms, we identified the differential gene profiles of donor T cells deficient for T-bet or IFN-γ after allo-BMT. The potential key mediators include but are not limited to the following genes: Cxcr3, Ccr5, Ccl3, Ccl4, Klrk1, Krdl1, Nkg7, Pdcd1, H2-Aa, and H2-Ab1 (Fig. 3). These targets are either positively or negatively regulated by T-bet and represent different aspects of allogeneic T cell activity: activation (H2-Aa and H2-Ab1), migration (Cxcr3, Ccr5, Ccl3, and Ccl4), cytotoxic function (Klrk1, Krdl1, and Nkg7), or exhaustion (Pdcd1).

It is interesting that expression of MHC class II (MHC-II) genes H2-Au and H2-Ab1 were increased in T-bet−/− CD4 T cells. The protein level of I-Aα (gene H2-Aa) was also significantly increased in T-bet−/− T cells compared with either WT, IFN-γ−/−, or IFN-γR−/− T cells (Fig. 4C, Supplemental Table 1), which we classified as a T-bet–dependent but systematic IFN-γ–independent molecule. Given that transfer of MHC molecules from APCs to T cells does exist, and the MHC-II–expressing T cells can engage in T–T interactions leading to increased apoptosis and hyporesponsiveness (46, 47), it is possible that overexpression of MHC-II molecules (such as I-Aα) on T-bet−/− donor T cells may result in increased cell death due to fratricide of allogeneic T cells. Alternatively, those MHC-II–expressing T-bet−/− T cells may compete with professional dendritic cells to present Ag, but not effectively induce T cell proliferation due to lack of costimulatory signals, the regulatory mechanism used by T-bet Nkp46 RORγt+ innate lymphoid cells as demonstrated in a recent report (48).

Chemokine receptors and chemokines play important roles in T cell migration to GVHD target organs (49, 50). CXCRR3 is a direct target of T-bet (13), and targeting CXCRR3 using its mAb can inhibit CD8-mediated GVHD in murine allo-BMT models (51). Targeting CCR5 using a small molecule inhibitor (marcan) inhibits CD8-mediated GVHD in murine allo-BMT models (49, 50). CXCR3 is expressed significantly lower levels of Th1-related chemokine receptor genes (Cxcr3 and Ccr5) and chemokine genes (Ccl3 and Ccl4), as well as the CXCRR3 protein. This impedes the migration and infiltration of allogeneic T cells to GVHD target organs (Figs. 1, 4).

Administration of an agonistic mAb of NKG2A inhibited donor T cell expansion and ameliorated acute GVHD in mice (54). However, we show that T-bet−/− donor T cells express extremely low levels of CD94/NKG2A genes (Klrk1 and Krdl1, respectively) (Fig. 3), as well as CD94 protein (Fig. 4), in concert with ameliorated GVHD, suggesting that CD94/NKG2A could be biomarkers to positively predict GVHD severity. Lower expression levels of inhibitory molecules CD94, NKG2A, and the T cell exhaustion marker PD-1 (Figs. 3, 4), alternatively, may suggest that T-bet−/− T cells can still preserve their cytotoxic function to overcome tumor growth, which was additionally supported by enhanced production of GZMB by T cells deficient for T-bet but not IFN-γ (Fig. 4C). Nkg7, a promoter of the T and NK cell surface cytotoxic molecule (55), has been considered as a Th1 cell–specific gene of which expression was previously shown to be regulated by T-bet (13, 56), and it is positively correlated to cytotoxic T cell destruction of epidermal cells in human GVHD (57).

In murine EAE models, deficiency in IFN-γ leads to exacerbated disease (58, 59). However, T-bet–deficient mice are protected from developing EAE (40, 60). We have observed similar results in GVHD models, which suggest that silencing Th2x2 has therapeutic potential. Because transcription factors are difficult to be pharmacologically targeted, an alternative approach is targeting T-bet downstream molecules that are independent of endogenous or systematic IFN-γ (Fig. 4), such as NKG2D, which is expressed on a variety of immune cells and plays a costimulatory role in activating CD8 T cells (61). Indeed, we classified NKG2D as a T-bet–dependent but systematic IFN-γ–independent molecule (Fig. 4), consistent with the unpublished data from us and others (62), which indicate that blockade of NKG2D alleviated GVHD induced by CD8 or total T cells.

Th17 cells are capable of inducing GVHD in mice (34, 35). Both T-bet−/− and IFN-γ−/− CD4 T cells are prone to Th17 differentiation with high levels of IL-17 production (Supplemental Fig. 1). In contrast to in vitro–polarized WT or IFN-γ−/− Th17 T cells, T-bet−/− Th17 cells caused very mild GVHD (Fig. 5B–E). Consistently, our microarray data reveal that T-bet controls the optimal function of Th17 cells in GVHD possibly through regulating the expression of multiple genes representing Th17 pathogenic or nonpathogenic signatures (Supplemental Fig. 2). Our study showed that T-bet−/− and IFN-γ−/− CD4 T cells have similar expression patterns of IFN-γ and IL-17 but opposing GVHD severities, which indicated that the culprit for GVHD development may not be IFN-γ or IL-17, but likely the downstream effectors of T-bet we identified.

Although IL-17 seems to have less effect on GVHD development, it influences the tumor microenvironment, especially when shielding the GVL response of T-bet–deficient donor T cells (Fig. 6). T-bet−/− T cells produce more IL-17 than do WT T cells (Fig. 1H, Supplemental Fig. 1), and IL-17 promotes tumor progression in a variety of tumors (43–45). Consistently, neutralizing IL-17 benefits overall survival of recipients transplanted with T-bet−/−, not WT, T cells. However, the GVL preservation of T-bet−/− T cells is reduced after stopping anti–IL-17 treatment. The tumor gradually relapsed, which further supports the detrimental role of IL-17 against preserved GVL effects of T-bet−/− T cells. We conclude that T-bet–deficient T cells induce less GVHD and may still preserve GVL effects when the elevated levels of IL-17 are reversed. This is also supported by our previous findings that double deficiency of T-bet and RORγt prevents GVHD while sparing the GVL effect (18). Targeting T-bet itself is still far from translational application due to a lack of a specific
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inhibitor. Recently, the small molecule inhibitors for other transcription factors, such as c-Rel and RORγt, have been developed and showed promising results in alleviating GVHD (63) or cutaneous inflammatory disorders (64), respectively. The c-Rel inhibitor also permits the maintenance of the GVL effect. Once T-bet inhibitors are developed, the mechanism we define in the present study together with our previous report (18) will become applicable, for example, a combinational treatment of T-bet inhibitor and anti–IL-17 mAb may be beneficial in controlling GVHD while maintaining the GVL effect.

Collectively, using genetic knockout mice, we prove that T-bet is critical for the development of acute GVHD through controlling the differentiation and migration of Th1 cells as well as the pathogenicity of Th17 cells. Given that T-bet is a transcription factor that is unavailable to be pharmacologically targeted currently, we identify potential molecular targets downstream of T-bet and define the underlying mechanisms accounting for the distinct GVHD outcomes caused by T-bet– versus IFN-γ–deficient donor T cells. This provides the rationale to target those T-bet–dependent but endogenous or systemic IFN-γ–independent molecules for the control of acute GVHD in clinical settings.

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References


T-bet IS ESSENTIAL FOR T CELLS TO INDUCE ACUTE GVHD


