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T-bet Promotes Acute Graft-versus-Host Disease by Regulating Recipient Hematopoietic Cells in Mice

Jianing Fu,∗,†,‡ Yongxia Wu,∥Hung Nguyen,‡ Jessica Heinrichs,‡ Steven Schutt,‡ Yuejun Liu,‡ Chen Liu,‡ Junfei Jin,∗,‡,∥ Claudio Anasetti,† and Xue-Zhong Yu‡,*

Beyond its critical role in T cells, T-bet regulates the functions of APCs including dendritic cells and B cells, as well as NK cells. Given that recipient APCs are essential for priming allogeneic T cells and recipient NK or T cells are able to reject allogeneic donor cells, we evaluated the role of T-bet on the host in acute graft-versus-host disease (GVHD) using murine models of allogeneic bone marrow transplantation. T-bet−/− recipients developed significantly milder GVHD than their wild type counterparts in MHC-mismatched or CD4-dependent minor histocompatibility Agmismatched models. Allogeneic donor T cells, in particular, CD4 subset, significantly reduced IFN-γ production, proliferation and migration, and caused less injury in liver and gut of T-bet−/− recipients. We further observed that T-bet on recipient hematopoietic cells was primarily responsible for the donor T cell response and pathology in GVHD. T-bet−/− dendritic cells expressed higher levels of Trail, whereas they produced lower levels of IFN-γ and IL-12/23 p40, as well as chemokine CXCL9, resulting in significantly higher levels of apoptosis, less priming, and infiltration of donor T cells. Meanwhile, NK cells in T-bet−/− hosts partially contribute to the decreased donor T cell proliferation. Furthermore, although T-bet on hematopoietic cells was required for GVHD development, it was largely dispensable for the graft-versus-leukemia effect. Taken together with our previous findings, we propose that T-bet is a potential therapeutic target for the control of GVHD through regulating donor T cells and recipient hematopoietic cells. The Journal of Immunology, 2016, 196: 3168–3179.
In this study, by using several well-defined, clinically relevant murine models of allogeneic bone marrow transplantation (allo-BMT), we found that T-bet deficiency on recipient hematopoietic cells attenuates GVHD. The proliferation and IFN-γ production of allogeneic donor T cells were significantly impaired in T-bet−/− recipients, but more Foxp3+ T regulatory cells (Tregs) were present in their spleens. In addition, T-bet−/− hematopoietic cells, mainly DCs and NK cells, enhanced apoptosis and impaired proliferation of allogeneic donor T cells within lymphoid organs primarily through the Trail-death receptor 5 (DR5) axis, with additional contribution of decreased production of T cell priming cytokines IFN-γ and IL-12/23 p40 and Th1-promoting chemokine CXCL9, leading to decreased T cell activation, infiltration, and tissue damage onto GVHD target organs. Furthermore, allogeneic donor T cells in T-bet−/− recipients largely preserved graft-versus-leukemia (GVL) effect. Our data demonstrate T-bet is a promising therapeutic target for the treatment of GVHD through regulating recipient hematopoietic cell functions.

Materials and Methods

Mice

C57BL/6 (B6; H-2b), congenic B6.Ly5.1+ (CD45.1; H-2b), BALB/c (H-2d), and FVB/N (FVB; H-2q) were purchased from the National Cancer Institute. 129S2/Sv (H-2d) were purchased from Charles River Laboratories. T-bet−/− mice on B6 or BALB/c background and C3H.SW (H-2b) were purchased from The Jackson Laboratory. All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at Moffitt Cancer Center or Medical University of South Carolina (MUSC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida or MUSC.

BMT models

T cell purification from whole spleen and lymph nodes was done by negative depletion using magnetic beads as previously described (21). MHC- and miHA-mismatched (FVB→B6; B6→BALB/c) or MHC-matched but miHA-mismatched (129S2/Sv→B6; C3H.SW→B6) BMT models were used as previously established (20, 33). In brief, different strains of recipient mice were conditioned with a lethal dose of total body irradiation (TBI). Within 24 h postconditioning, recipients were i.v. injected with T cell–depleted (TCD)-BM alone or plus various numbers of T cells purified from donor mice. Recipients were monitored for weight loss and other clinical signs of GVHD twice a week (34). Spleens of DR5−/− mice on B6 background and their wild type (WT) counterparts were kindly provided by Dr. Niklas Finnberg from Penn State Hershey Cancer Institute. H&E staining and a semiquantitative scoring system were used to account for histologic changes consistent with GVHD in the target organs as previously described (20, 21, 35).

Chimeras BMT model

WT-B6 Ly5.1+ or T-bet−/− B6 mice were lethally irradiated with 1000–1200 cGy and infused with TCD-BM cells from syngeneic T-bet−/− B6 or WT-B6 Ly5.1+ donors such that T-bet deficiency is confined to only the hematopoietic cells or the nonhematopoietic cell compartments, respectively. Control chimeras were also established by using lethally irradiated WT-B6 B6.Ly5.1+ or the T-bet−/− B6 mice as recipients, and transferred with TCD-BM cells from syngeneic WT or T-bet−/− B6 donors, respectively. Chimerism analyses of the donor hematopoietic cells were complete donor type (>95%) 2–3 mo after first syngeneic BMT (data not shown). The B6→B6 Ly5.1+, B6 T-bet+/+→B6 T-bet−/−, B6 T-bet−/−→B6 Ly5.1+, and B6 Ly5.1+→B6 T-bet−/− mice were then used as recipients in an allo-BMT. The chimeric mice received 1000–1200 cGy irradiations and were i.v. injected with purified T cells along with TCD-BM cells from allogeneic FVB donors.

GVL model

In the B6→BALB/c model, recipients received 2×10^5 luc/neo plasmid-transduced A20 B cell lymphoma cells (A20-luc) on BALB/c background, whereas in the FVB→B6 model, recipients received 0.1×10^5 C1498 luc/neo plasmid-transduced atypical myeloid leukemia cells (C1498-luc) generated in B6 mice at the time of BMT. Tumor mortality and GVHD mortality were determined by bioluminescence imaging (BLI) signal intensity and clinical manifestation of GVHD (20, 35).

Flow cytometry

Mononuclear cells were isolated from recipient spleen and liver, and stained and analyzed for surface receptors and intracellular cytokines using standard flow cytometry protocols as previously described (21, 35, 36).

In vivo MLR

T cells isolated from donor mice were gently mixed and incubated with 2 μM CFSE in PBS at 37°C for 7 min. The CFSE-labeled T cells were i.v. injected into lethally irradiated recipients. After 3 or 4 d, recipient splenocytes were stained and analyzed using flow cytometry. In some experiments, recipient mice were treated with IgG2a isotype control Ab or anti-NK1.1 Ab (clone: PK136; Bio-X-Cell, NH) at 200 μg/mouse on days −2 and 0 before transplant. In other experiments, donor T cells and TCD-BM cells were i.v. injected into lethally irradiated recipients without labeling with CFSE.

Statistics

For comparison of recipient survival or tumor relapse rate, the log-rank test was used to determine statistical significance (p < 0.05). To compare GVHD clinical scores, pathology scores, weight loss, cytokine levels, as well as in vivo and in vitro T cell proliferation, we used a Student t test.

Results

T-bet expressed on host contributes to GVHD

Multiple cell types on the host affect GVHD (4, 18, 37), and T-bet plays an important role in regulating the immune activity of many of those cells, including DCs, B cells, NK cells, and T cells (38). To determine the effect of T-bet on host in vivo, we first used a fully MHC-mismatched model (FVB→B6) of allog-BMT. Compared with WT, T-bet−/− recipients demonstrated significantly lower mortality from GVHD after allog-BMT (Fig. 1A) with less weight loss (Fig. 1B), and displayed significantly better donor-derived B220+ B cell and CD4 and CD8 T cell reconstitution in spleen 80 d posttransplant (Fig. 1C–F). In recipient BM, however, we did not observe significant differences in donor T and B cell reconstitution (data not shown). Spleen is a major peripheral lymphoid organ and it contains the mature T and B cells developed from the progenitors within BM; thus, it could well reflect the posttransplant reconstitution. Because GVHD impairs donor B and T cell reconstitution, these results reflect that T-bet−/− recipients suffered only mild GVHD. The reduced mortality of T-bet−/− recipients was associated with less severe pathologic injuries in liver and gastrointestinal tract on day 14 posttransplant as compared with their WT counterparts. We did not observe significant difference of skin GVHD between WT and T-bet−/− recipients at this time point in this BMT model. T-bet−/− recipients showed better maintenance of normal cell morphology in GVHD target organs without severe lymphocyte infiltration (Fig. 1G, 1H). Thus, T-bet expression on the host is critical for GVHD development.

T-bet on host determines alloresponse of allogeneic donor T cells

Recipient cells may affect the pathogenicity of donor T cells after allo-BMT in various ways, including alloreactivity, homeostasis, and/or migration. We next evaluated these aspects as the indirect functional readout of recipient cells to understand the cellular mechanisms by which T-bet regulates GVHD through host cells. Donor CD4 and CD8 T cells showed significantly reduced proliferation (CFSE staining) and activation (IFN-γ production) in T-bet−/− as compared with WT allogeneic recipients 4 d posttransplant, in which more defects were observed in CD4 than CD8 T cells (Fig. 2A–C). In those allogeneic recipients, absolute numbers of donor T cells (H-2Kq+) and CD4, but not CD8, T cells were significantly reduced in the T-bet−/− recipients compared with the WT counterparts (Fig. 2D). The impact of T-bet on host
cells specific to T cell proliferation and activation was dependent on allogeneic recognition because such an impact on T cells was not seen in syngeneic recipients (Fig. 2E). Collectively, these data suggest that the deficiency of T-bet on host mitigates allo- geneic T cell proliferation and IFN-γ production.

T-bet expressed on host contributes to GVHD in CD4- but not CD8-dependent miHA-mismatched model

In addition to the MHC-mismatched model, we also evaluated the effect of T-bet on host using more clinically relevant MHC-matched but miHA-mismatched models. Given previous findings that demonstrated that APCs derived from the host, rather than from the donor, are critical in inducing GVHD across miHA mismatch (39), we conditioned WT or T-bet→ mice recipients were analyzed. Expression of H-2Kq, B220, CD4, and CD8 was detected by flow cytometry, and absolute numbers of total splenocytes (C), donor-derived B cells (D), CD4 (E), and CD8 (F) T cells were calculated. Pooled data from three independent experiments are presented. In separate experiments with the same setting as (A)–(F), recipients were euthanized 14 d posttransplant and samples of liver, lung, small intestine (S.I.), large intestine (L.I.), and skin were collected for H&E staining and scored for microscopic GVHD severity by a pathologist blinded to the treatment groups (G). Photomicrographs depict the average disease score morphology from one representative experiment (n = 3–4 per group). Pathological score mean ± SE of GVHD target organs are depicted (H). Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 1. Absence of T-bet expression on host ameliorates GVHD. Lethally irradiated WT or T-bet→ mice on B6 background (n = 9–15 per group) were transplanted with 5 × 10^7/mouse TCD-BM from FVB donors alone, or plus purified FVB T cells at 2 × 10^5/mouse. Recipient mice were monitored throughout the experimental period for survival (A) and weight change (B) posttransplant. Upon completion of the experiment on day 80, phenotypes of the spleen cells from survived recipient mice were analyzed. Expression of H-2Kq, B220, CD4, and CD8 was detected by flow cytometry, and absolute numbers of total splenocytes (C), donor-derived B cells (D), CD4 (E), and CD8 (F) T cells were calculated. Pooled data from three independent experiments are presented. In separate experiments with the same setting as (A)–(F), recipients were euthanized 14 d posttransplant and samples of liver, lung, small intestine (S.I.), large intestine (L.I.), and skin were collected for H&E staining and scored for microscopic GVHD severity by a pathologist blinded to the treatment groups (G). Photomicrographs depict the average disease score morphology from one representative experiment (n = 3–4 per group). Pathological score mean ± SE of GVHD target organs are depicted (H). Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

T-bet on host hematopoietic cells is primarily responsible for promoting GVHD

Given both hematopoietic and nonhematopoietic cells are known to contribute to the development of GVHD (13, 42), we next evaluated the role of T-bet expression on either population in mediating GVHD by using established BM chimeric mice as recipients. Severe GVHD with <20% long-term survival was induced in B6 → B6 Ly5.1+ and B6 Ly5.1+ → B6 T-bet→ chimeras in which T-bet was intact in their hematopoietic system, whereas mild GVHD with >70% long-term survival was observed in B6 T-bet→ → B6 T-bet→ and B6 T-bet→ → B6 Ly5.1+ chimeras in which T-bet was absent in their hematopoietic system (Fig. 4). These results demonstrate that T-bet on recipient hematopoietic cells plays an essential role in initiating GVHD, but with a much lesser effect on nonhematopoietic cells. Although the contribution of T-bet on nonhematopoietic cells cannot be ruled out because a delayed lethality was observed in B6 Ly5.1+ → B6 T-bet→ chimeras as compared with B6 → B6 Ly5.1+ chimeras (Fig. 4).
T-bet expressed on host promotes donor CD4 T cell accumulation in spleen and infiltration to liver

To further exclude the model dependence, we used another MHC-mismatched model where the expression of T-bet is sufficient or absent on BALB/c background. Similarly as we observed in the B6 background model (Fig. 1), we noticed the significant attenuated GVHD in B6→BALB/c setting, reflected by lower mortality (Fig. 5A) and less body weight loss (Fig. 5B) of T-bet$^{-/-}$ recipients. To delineate allogeneic donor T cell expansion and migration capacity, we used congenic marker Ly5.1 to distinguish the donor T cells (Ly5.2+) from donor BM cells (Ly5.1+). We found that, 7 d posttransplant, T-bet$^{-/-}$ recipients had higher numbers of total splenocytes, H-2Kb+, CD4, or CD8 T cells are presented (D); WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and transplanted with $3 \times 10^7$ mouse purified CFSE-labeled T cells from WT B6 donors (n = 3 per group). Profiles of CFSE and IFN-γ in donor CD4 or CD8 T cells are shown from representative recipients in each group 4 d postransplant (E). Asterisks indicate statistical significance: *p < 0.05, **p < 0.001.

### FIGURE 2.
Allogeneic donor T cells significantly reduce IFN-γ production and proliferation in T-bet$^{-/-}$ recipients.

WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and transplanted with $3 \times 10^7$/mouse purified CFSE-labeled T cells from FVB donors (n = 10 per group, pooled from three experiments). Profiles of CFSE and IFN-γ in donor CD4 or CD8 T cells were shown from representative recipients in each group 4 d postransplant (A). Percentages of CFSE$^{low}$ (B) and CFSE$^{low}$ IFN-γ$^{+}$ donor T cells (C) are shown, and the absolute numbers of total splenocytes, H-2Kb+, CD4, or CD8 T cells are presented (D). WT or T-bet$^{-/-}$ B6 mice were lethally irradiated and transplanted with $3 \times 10^7$ mouse purified CFSE-labeled T cells from WT B6 donors (n = 3 per group). Profiles of CFSE and IFN-γ in donor CD4 or CD8 T cells are shown from representative recipients in each group 4 d postransplant (E). Asterisks indicate statistical significance: *p < 0.05, **p < 0.001.

**Trail expression on DCs and NK cells contributes to increased apoptosis and decreased proliferation of allogeneic donor T cells in T-bet$^{-/-}$ host**

In addition to the effect on allogeneic T cell activation, proliferation, and migration, T-bet$^{-/-}$ recipient cells also induced a much higher level of donor T cell death and apoptosis reflected by the percentage of Live/Dead Yellow$^+$ Annexin V$^+$ cells and Live/Dead Yellow$^+$ Annexin V$^-$ cells, respectively (Fig. 6A). We also noticed the increased apoptosis rate in both CD4 and CD8 T cells in T-bet$^{-/-}$ recipients (Fig. 6A, 6B). Trail is one of the ligands that
trigger the DR pathway to induce cell apoptosis (43), and particularly Trail-overexpressing DCs have been shown to attenuate GVHD through inducing donor T cell apoptosis (44). We then detected the expression of Trail on representative types of cells in the recipient, including DCs (CD11c+), NK cells (NK1.1+), and T cells (TCRβ+). Trail expression on T cells was generally very low regardless the irradiation condition or T-bet expression on the host (Fig. 6C, 6D). Although either before or after lethal irradiation, CD11c+ DCs in T-bet2/2 host upregulated Trail compared with their WT counterparts, suggesting likely contribution of Trail in triggering apoptosis of donor T cells (Fig. 6C, 6D). NK1.1+ cells express higher basal level of Trail than DCs or T cells, although there was no significant difference between WT and T-bet2/2 host (Fig. 6C, 6D). Given that T-bet2/2 NK cells present an immature phenotype (31), and immature NK cells preferentially use Trail rather than FasL pathway to exert their cytolytic activity (15), we next tested the possibility of NK involvement by using Ab depletion of NK on recipients before transplant. We found that, as compared with isotype control Ab treatment, anti-NK1.1 Ab treatment decreased the difference in donor CD4 T cell proliferation between WT and T-bet2/2 recipients (Fig. 6E). Thus, Trail expression by T-bet2/2 DCs and/or NK cells likely contributed to increased apoptosis and decreased proliferation of allogeneic donor T cells, which was associated with the ameliorated GVHD in T-bet2/2 recipients (Fig. 5A, 5B).

**Trail/DR5 axis is critical for ameliorated GVHD in T-bet2/2 host**

Trail triggers apoptosis through its respective receptors, including DR4 and DR5 in humans and in mice include only DR5 (43, 45). To test whether Trail/DR5 interaction was responsible for the induction of donor T cell apoptosis (Fig. 6A, 6B) and subsequent reduction of GVHD (Fig. 5A, 5B) in T-bet2/2 recipients, we used DR52/2 mice as donors. We observed that DR52/2 T cells had intact responses to alloantigen stimulation in terms of proliferation and activation in vitro (data not shown), and comparable ability as WT T cells to induce GVHD in WT recipients in vivo (Fig. 7). Although WT T cells induced severe GVHD in WT recipients, they failed to do so in T-bet2/2 recipients, reflected by the overall survival (Fig. 7A) and GVHD clinical scores (Fig. 7B). Conversely, DR52/2 donor T cells were able to induce severe GVHD in the recipients regardless of T-bet expression (Fig. 7). These data strongly suggest that the Trail/DR5 interaction is a major signaling pathway responsible for limiting donor T cell proliferation and...
pathogenicity by T-bet−/− recipient cells, through which the development of GVHD is alleviated.

T-bet−/− DCs produce less T cell priming cytokine and Th1-promoting chemokine

To further investigate the potential mechanism of impaired T cell alloresponse in T-bet−/− host other than apoptosis, we focused on DCs to detect the effect of T-bet expression on their activation, cytokine, and chemokine productions. We found comparable expression levels of MHC-II (I-Ab), costimulatory markers CD86 and CD40, and co-inhibitory marker PD-L1 on ex vivo-isolated WT and T-bet−/− splenic DC11c+ DCs before or 24 h after lethal dose of TBI (data not shown), consistent with previous studies that T-bet does not play an obvious role in DC activation (25). Because our study focused on allogeneic response, we tested how T-bet regulates host DCs by transferring purified WT FVB BM cells and T cells into lethally irradiated WT or T-bet−/− B6 mice (Fig. 8). Four days after cell transfer, we harvested the recipient spleen and found that the absolute number of total splenocytes and CD11c+I-Ab+ DCs was significantly lower in T-bet−/− recipients (Fig. 8A, 8B). There was no difference in terms of MHC-II (I-Ab) and PD-L1 expression (Fig. 8C–E), although we did observe increased level of CD86 expression by T-bet−/− DCs (Fig. 8D). Because upregulated Trail expression by T-bet−/− DCs is a major contributor to allogeneic T cell apoptosis (Figs. 6, 7), we interpret that increased CD86 may strengthen the interaction between T-bet−/− DCs and donor T cells and thus benefit the Trail-DR5 pathway to induce T cell death. Furthermore, T-bet−/− DCs produced significantly lower levels of T cell priming cytokines IFN-γ, IL-12/23 p40, and Th1-promoting chemokine CXCL9 compared with WT DCs (Fig. 8F–K). Taken together, these data support the decreased T cell priming and impaired IFN-γ production by allogeneic donor T cells (Fig. 2) and less T cell infiltration into liver and gut (Figs. 1, 5) in T-bet−/− recipients.

T-bet−/− recipients partially maintain GVL effect

We next evaluated whether T-bet−/− hosts may preserve the T cell–mediated GVL effect. Using A20 B cell lymphoma with a relatively low dose of T cells, we observed that although donor T cells induced less severe GVHD in T-bet−/− recipients (Fig. 9A), as presented earlier (Fig. 1), they mediated a robust GVL effect in either WT or T-bet−/− recipients (p > 0.05; Fig. 9B). It is notable that the T-bet−/− recipients had delayed lymphoma relapse, although a somewhat higher rate in the later stage (Fig. 9B). Using C1498 atypical myeloid leukemia with a relatively high dose of T cells, we found that T-bet−/− recipients had significantly better survival than WT recipients (Fig. 9C), and most of the T-bet−/− recipients were free of leukemia relapse (Fig. 9D, 9E). In fact, leukemia relapse in those recipients did not occur until >70 d after BMT (Fig. 9D, 9E). Taken together, T-bet expression on the host is critical for GVHD induction but much less important for mediating the GVL effect. We further observed that donor CD8 T cells expressed comparable levels of CD107a and granzyme B in T-bet−/− recipients compared with WT recipients (data not shown), which likely contributed to preserved GVL activity.

Discussion

Because allogeneic donor T cells need cross talking with the host cells to survive and get activated, the host factors become an attractive target for GVHD control. After conditioning, the residual host cells form a complex system because of the involvement of multiple cell types and their different sensitivity and availability toward irradiation. Previous studies showed that, unlike murine macrophages that persist in lymphoid tissues >3 d after lethal TBI, the majority of DCs, B, T, and NK cells are quickly eliminated around day 2 (46). Even with such a short period, the residual host APCs are capable of priming allogeneic donor T cells and contributing to GVH reactions (12, 13). Our data demonstrate that T-bet is critical for the activity and function of host hematopoietic cells to induce GVHD in MHC-mismatched models (Figs. 1, 4, 5A, 5B), through regulating donor T cell alloresponse (Fig. 2), migration (Fig. 5C–J), and homeostasis (Fig. 6). T-bet on the host also boosts GVHD mediated by CD4, but not by CD8 T cells in the miHA-mismatched BMT model (Fig. 3). We further validate that deficiency of T-bet on hematopoietic cells, particularly DCs, dampens allogeneic T cell response by inducing apoptosis and limiting T cell priming, proliferation, and migration through regulating Trail/DR5 axis (Fig. 7), reducing IFN-γ, IL-12/23 p40 priming cytokines, and CXCL9/CXCXR3 chemokaxis (Fig. 8).

It is clear that T cell proliferation and activation in syngeneic recipients were intact regardless of T-bet expression (Fig. 2E), indicating that T-bet expression on host cells does not impact T cell homeostatic response. In allogeneic BMT, recipient APCs present alloantigens directly to activate donor T cells, which are essential for GVHD development (6). The fact that T-bet−/− re-
cipients developed only mild GVHD (Figs. 1, 5A, 5B) suggests the crucial role of T-bet in regulating activity of recipient APCs. The first several days posttransplant are considered to be critical in GVHD induction according to the studies of spatial and temporal dynamics of alloreactive T cell activation, proliferation, and tissue distribution (47, 48), and the most actively dividing T cells are specific for recipient alloantigens in this period (49, 50). Thus, the CFSElow population has been referred to as fast-dividing alloreactive T cells and the CFSEhigh population as slow-dividing nonalloreactive T cells (51, 52). In MHC-mismatched BMT (Fig. 2), the frequency of alloreactive T cells among total donor T cells should be the same in WT or T-bet−/− recipients at the time of transplant. A large proportion of CFSElow T cells was observed in WT recipients with high levels of IFN-γ production, whereas in T-bet−/− recipients, the majority of donor T cells are still CFSEhigh and with minimal IFN-γ secretion (Fig. 2), which indicates a severe impairment on donor T cell alloresponse.

T cell migration to GVHD target organs is required for GVHD development, and the interaction of chemokine and chemokine receptors plays an important role in this process (53, 54). CXCL9 is one of the Th1-attracting chemokines (CXCL9-11) produced by Th1-promoting DCs (55) that recruiting CXCR3-expressing T cells migrate to liver and gut (53). We found less infiltrated donor CD4, but not CD8, T cells in the livers of T-bet−/− recipients compared with in WT recipients (Figs. 2A, 6E), although the exact mechanism needs further investigation. Moreover, these results are supported in MHC-matched BMT, where T-bet expression on hosts contributes to the development of GVHD induced by CD4, but not CD8, T cells (Fig. 3). A reduced effect of T-bet−/− host cells on CD8 T cells may account for the preserved GVL effect (Fig. 9). More specifically, we found that CD8 T cells maintained their cytolytic function reflected by preserved CD107a and granzyme B expression (data not shown).

FIGURE 5. Allogeneic donor T cells significantly decrease accumulation in spleen and infiltration to liver in T-bet−/− recipients, consistent with attenuated GVHD. Lethally irradiated WT or T-bet−/− mice on BALB/c background (n = 10 per group, pooled from two experiments) were transplanted with 5 × 10⁶ mouse TCD-BM from WT B6 donor alone, or plus 1 × 10⁶/mouse purified T cells from B6 mice. Recipient mice were monitored throughout the experimental period for survival (A) and weight change (B) posttransplant. Lethally irradiated WT or T-bet−/− mice on BALB/c background (n = 9–10 per group, pooled from two experiments) were transplanted with 5 × 10⁶ mouse TCD-BM from Ly5.1 B6 donor alone, or plus 0.25 × 10⁶/mouse purified Ly5.2 T cells from B6 mice. Seven days posttransplant, mononuclear cells were isolated from recipient spleens and livers. Under the Live/Dead Yellow− gate, percentages of Ly5.2 H-2Kb+ T cells (C) or CD25+ Foxp3+ Tregs (D), and their CD4 and CD8 subpopulation in spleen are shown. Absolute numbers of total live splenocytes, total donor, CD4 or CD8 T cells, and CD25+Foxp3+ Tregs in spleen are presented (E). Absolute numbers of the donor CD4 or CD8 T cells producing IFN-γ (F) or expressing CXCR3 (G) in spleen are presented. Under the Live/Dead Yellow− gate, percentages (H) and absolute number (I) of liver Ly5.2 H-2Kb+ T cells and their CD4 and CD8 subpopulation are shown. Absolute numbers of IFN-γ–producing donor CD4 or CD8 T cells in liver (J) are presented. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.
In general, both hematopoietic and nonhematopoietic cells in the recipient are capable of regulating donor T cells and affect their ability to induce GVHD (4, 12, 13, 57, 58). Our chimeras BMT data demonstrate that deficiency of T-bet, particularly on host hematopoietic compartment, to a much lesser extent on non-hematopoietic compartment, attenuated the severity of GVHD (Fig. 4). In the hematopoietic system, DCs, B cells, and macrophages are classified as APCs because of their ability in priming donor T cells. Given host DCs rather than host B cells are critical in the induction of CD4 or, to a lesser extent, CD8 T cell–dependent GVHD (10), and T-bet does not express on murine macrophages (25), we thus focused on host DCs as the representative cell type of APCs. T-bet has been shown to regulate optimal production of IFN-γ and Ag-specific T cell activation by DCs (25), suggesting that T-bet regulates allostimulatory functions of DCs by promoting a positive feedback loop of IFN-γ between DCs and T cells. We indeed found decreased production of IFN-γ and IL-12/23 p40 by T-bet−/− DCs after allogeneic T cell transfer (Fig. 8). IL-12 and IL-23 produced by APCs including DCs are predominantly responsible for promoting Th1 and Th17 cells, respectively (34, 50), two subtypes of Th cells that contribute to aGVHD induction (59). Fourteen days after allogeneic BMT, very low levels of Th2-related cytokine IL-4 and Th17-related cytokine IL-17A could be detected in either WT or T-bet−/− recipients (data not shown), suggesting that Th1 cells were the main culprit for GVHD induction.

**Figure 6.** Trail expression by host DCs and NK cells likely contributes to increased apoptosis and decreased proliferation of allogeneic donor T cells in T-bet−/− recipients. WT or T-bet−/− BALB/c mice (n = 2 per group) were lethally irradiated and transplanted with 3 × 10^6/mouse purified, CFSE-labeled T cells from WT B6 donors. Expression of Annexin V and Live/Dead Yellow in H-2K^b+ cells and profiles of Annexin V versus CFSE in CD4 and CD8 subpopulation in Live/Dead Yellow− H-2K^b+ donor T cells was shown from representative recipients in each group 4 d posttransplant (A). Apoptosis rate in CFSE−/− CD4 or CD8 T cells was calculated [% CFSE−/− Annexin V+ cells/(% CFSE−/− Annexin V− cells + % CFSE−/− Annexin V− cells) × 100] and shown on gated Live/Dead Yellow− H-2K^b+ cells (B). Splenocytes from WT or T-bet−/− BALB/c mice without irradiation (n = 2 per group) or 24 h after lethal irradiation (n = 3 per group) were stained for Trail expressed on CD11c+ (gating on CD11c+ TCRβ2 cells), NK1.1+ (gating on NK1.1+ TCRβ2 cells), or TCRβ+ (gating on TCRβ+ NK1.1− cells) cells (C). Percentages of Trail-expressing cells in CD11c+, NK1.1+, or TCRβ+ population are presented (D). WT or T-bet−/− BALB/c recipients were i.p. injected with either IgG2a isotype control or anti-NK1.1 (clone: PK136) at 200 μg/mouse on days −2 and 0. Recipients were lethally irradiated and transplanted with 3 × 10^6/mouse purified CFSE-labeled T cells from WT B6 donors on day 0. Three days posttransplant, CFSE dilution profile in Live/Dead Yellow− H-2K^b+ CD4 or CD8 T cells in recipient spleen were shown (E). Asterisks indicate statistical significance: *p < 0.05, **p < 0.01.
Our data further showed that both percentage and absolute numbers of donor Tregs were increased in T-bet−/− recipients (Fig. 5), whereas absolute number of donor CD4 T cells was decreased, suggesting that in the absence of T-bet on the host, naive donor CD4 T cells increased their differentiation toward Tregs, but decreased Th1 differentiation, given the impaired Th1 priming caused by less IFN-γ and IL-12/23 p40 production by T-bet−/− DCs. In addition, less Th1 migration ability because of impaired CXCR3/CXCL9 chemotaxis may trap Th1 cells within the spleen and increase their opportunity to undergo apoptosis through DR5-Trail interaction with T-bet−/− DCs. CD25-depleted donor T cells still fail to induce severe GVHD in T-bet−/− but not WT recipients (data not shown), indicating that the effect of host T-bet expression on GVHD was independent of natural Tregs. The increased Tregs observed in T-bet−/− recipients given pan-T cells (Fig. 5) suggests the enhancement of inducible Treg generation as a consequence of impaired Th1 priming and reduced inflammatory environment, which may additionally contribute to alleviated GVHD through their regulatory effect.

Although the residual host DCs can prime alloreactive T cells within a short period after TBI, remaining host NK and T cells may also contribute to ameliorated GVHD in T-bet−/− recipients because of their ability to reject donor cells (17–19). The apoptosis of donor T cells after allo-BMT appears to be an important process in regulating T cell homeostasis (60, 61). T cell apoptosis generally occurs through two pathways termed active

**FIGURE 7.** Trail-DR5 pathway is critical for ameliorated GVHD in T-bet−/− hosts. Lethally irradiated WT or T-bet−/− BALB/c mice (n = 5 ~ 6 per group) were transplanted with 5 × 10⁶/mouse TCD-BM from WT B6 donor alone, or plus 1 × 10⁶/mouse purified WT or DR5−/− T cells on B6 background. Recipient mice were monitored throughout the experimental period for survival (A) and clinical scores (B) posttransplant. Asterisks indicate statistical significance: *p < 0.05, **p < 0.01.

**FIGURE 8.** T-bet−/− DCs significantly reduce production of CXCL9, IFN-γ, and IL-12/23 p40. WT or T-bet−/− B6 mice were lethally irradiated and transplanted with 1.5 × 10⁶/mouse purified T cells with 4 × 10⁶/mouse TCD-BM from FVB donors (n = 5 per group). Absolute numbers of total splenocytes (A) and CD11c+I-Aβ+ host cells (B) were calculated. Mean florescence intensities (MFIs) of I-Aβ (C), CD86 (D), PD-L1 (E), and CXCL9 (F) expressed by WT or T-bet−/− CD11c+I-Aβ+ host cells were detected by flow cytometry. Representative profile of IFN-γ (H) and IL-12/23 p40 production by WT or T-bet−/− CD11c+I-Aβ+ host cells (G), as well as the percentage (H and J) and absolute numbers (I and K) of those IFN-γ or IL-12/23 p40-producing cells, were shown. Asterisks indicate statistical significance: **p < 0.01, ***p < 0.001.
apoptosis, mediated by DR signaling (62, 63) and passive apoptosis, controlled by bcl-2 family members (64). Active apoptosis has been shown to be one mechanism to limit the survival of both alloreactive and bystander donor T cells in GVHD (65). DRs, including Fas, TNFR, and DR5, are cell-surface receptors that transmit apoptotic signals initiated by specific TNF family ligands such as FasL, TNF-\( \alpha \), and Trail, respectively (63). Our data show that T-bet\(^{-/-}\) CD11c\(^{+}\) DCs upregulated Trail (Fig. 6C) with or without irradiation, to induce allogeneic T cell apoptosis (Fig. 6A, 6B). Our data are in line with a previous study, which showed that genetically modified, Trail-transduced DCs protected mice from GVHD and leukemia relapse through induction of apoptosis of both alloreactive T cells and tumor cells (44). We also found that NK cells express much higher levels of Trail than T cells in either WT or T-bet\(^{-/-}\) recipients (Fig. 6C, 6D). By using Ab depletion of NK1.1\(^{+}\) cells on the recipients before TBI, our data suggested that recipient NK cells partially contribute to the impaired proliferation of allogeneic donor CD4, but not CD8, T cells in T-bet\(^{-/-}\) recipients (Fig. 6E). We interpret that NK cells with immature phenotype in T-bet\(^{-/-}\) host tend to use Trail pathway for their cytolytic function, whereas mature WT NK cells prefer to use FasL pathway, as suggested by previous studies (15, 31, 32). Using donor T cells deficient for DR5 (Fig. 7) further proved our hypothesis that the Trail/DR5 axis significantly contributes to the alleviated GVHD in T-bet\(^{-/-}\) hosts. We reason that host DCs and NK cells are primarily responsible for limiting donor T cell pathogenicity, although contribution through other types of host cells cannot be excluded.

In conclusion, we demonstrated that T-bet plays a critical role in the development of GVHD by regulating the activity of recipient hematopoietic cells, particularly DCs and NK cells. Furthermore, Trial/DR5 pathway plays a critical role in limiting donor T cell activation, proliferation, and subsequent pathogenicity when T-bet is absent in the host after allo-BMT. Impaired T cell priming and Th1 migration promoting ability of T-bet\(^{-/-}\) DCs reflected by reduced IFN-\( \gamma \) and IL-12/23 p40 productions, as well as

**FIGURE 9.** T-bet\(^{-/-}\) recipients partially preserve GVL effect. WT or T-bet\(^{-/-}\) BALB/c mice (n = 11–13 per group) were lethally irradiated and transplanted with 5 \( \times \) 10\(^{5}\)/mouse TCD-BM from WT B6 donors alone, or plus purified T cells from WT B6 donors at 0.25 \( \times \) 10\(^{6}\)/mouse. In addition, recipients were i.v. injected with 2 \( \times \) 10\(^{5}\) A20-luc on BALB/c background at the time of BMT. Recipients were monitored throughout the experimental period for survival (A) and tumor growth by luciferin i.p. injection and whole-body BLI. Percentages of tumor relapse are shown (B). Data pooled from two separate experiments. WT or T-bet\(^{-/-}\) B6 mice (n = 7–8 per group) were lethally irradiated and transplanted with 5 \( \times \) 10\(^{5}\)/mouse TCD-BM from FVB B6 donors alone, or plus 2 \( \times \) 10\(^{6}\)/mouse purified T cells from FVB donors. In addition, recipients were i.v. injected with 1 \( \times \) 10\(^{5}\) C1498-luc generated in B6 mice at the time of BMT. Recipients were monitored throughout the experimental period for survival (C) and tumor growth. Percentages of relapse (D) and recipient BLI image (E) are shown. Tumor mortality and GVHD mortality were distinguished by BLI signal intensity and clinical manifestation of GVHD. Asterisks indicate statistical significance between WT and T-bet\(^{-/-}\) recipients given T cell transfer: **\( p \leq 0.01. $
CXCL9 secretion, also contribute to ameliorated GVHD. The lesser effect of T-bet−/− host cells on donor CD8 T cells may limit the effect on GVHD alveation, but it likely contributes to the largely preserved GVL effect (Fig. 9). This study, together with our previously published work (20), set a strong rationale to pharmacologically inhibit T-bet in vivo for the control of GVHD through targeting both donor T cells and recipient hematopoietic cells synchronously. Further studies are required to translate our findings toward clinical application. The gene-based therapy (52) using small interfering RNA (siRNA) or short hairpin RNA to silence T-bet could be a direct targeting strategy because of the lack of small molecular inhibitor specific for T-bet. Mice transferred with siRNA–T-bet–transfected splenocytes failed to develop EAE, and administration of siRNA–T-bet has been suggested to effectively alleviate established EAE in mice (47). Although currently unavailable, a small molecular inhibitor to pharmacologically inhibit T-bet in vivo for the control of GVHD but not for GVL.

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Disclosures

The authors have no financial conflicts of interest.

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