

# A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy

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**T-bet plays a crucial role in Th1 development. We investigated the role of T-bet in the development of allograft rejection in an established MHC class II–mismatched (bm12 into B6) model of chronic allograft vasculopathy (CAV). Intriguingly, and in contrast to IFN- $\gamma$ <sup>-/-</sup> mice that are protected from CAV, T-bet<sup>-/-</sup> recipients develop markedly accelerated allograft rejection accompanied by early severe vascular inflammation and vasculopathy, and infiltration by predominantly IL-17–producing CD4 T cells. Concurrently, T-bet<sup>-/-</sup> mice exhibit a T helper type 1 (Th1)–deficient environment characterized by profound IFN- $\gamma$  deficiency, a Th2 switch characterized by increased production of interleukin (IL) 4, IL-5, IL-10, and IL-13 cytokines, as well as increased production of the proinflammatory cytokines IL-6, IL-12p40, and IL-17. Neutralization of IL-17 inhibits accelerated allograft rejection and vasculopathy in T-bet<sup>-/-</sup> mice. Interestingly, CD4 but not CD8 T cell deficiency in T-bet<sup>-/-</sup> mice affords dramatic protection from vasculopathy and facilitates long-term graft acceptance. This is the first study establishing that in the absence of Th1-mediated alloimmune responses, CD4 Th17 cells mediate an aggressive proinflammatory response culminating in severe accelerated allograft rejection and vasculopathy. These results have important implications for the development of novel therapies to target this intractable problem in clinical solid organ transplantation.**

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Abbreviations used: CAV, chronic allograft vasculopathy; DKO, double KO; EAE, experimental autoimmune encephalomyelitis; H&E, hematoxylin and eosin; MST, median survival time.

Until recently, primed CD4 Th cells were considered to segregate into two distinct populations, each producing its own set of cytokines and mediating separate effector functions (1). Th1 cells produce IFN- $\gamma$ , and mediate activation of macrophages and induction of delayed-type hypersensitivity responses. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which provide help for B cell function and are important in IgG switching, IgE production, and eosinophilic inflammation (2).

T-bet is a Th1-specific T-box transcription factor that plays a crucial role in Th1 development, in part via the induction of IFN- $\gamma$ . T-bet not only induces Th1 development but also actively suppresses Th2 differentiation, and more remarkably, introduction of T-bet into fully Th2-differentiated CD4 T cells triggers conversion to a Th1 phenotype. In the absence of T-bet, CD4 T cells fail to differentiate into the Th1

lineage and default to a Th2 fate. Mice with homozygous deletion of T-bet show normal lymphoid development but fail to generate functional Th1 responses, have impaired regulation of antibody isotype class switching, and are protected from several T cell–mediated autoimmune diseases but show evidence of heightened allergic (Th2) responses (3).

It has been proposed that a Th1 phenotype promotes allograft rejection via IFN- $\gamma$ –induced activation of macrophage function and up-regulation of MHC class II antigen expression that favors T cell allosensitization. In contrast, the Th2 phenotype has been proposed to favor long-term graft survival in some models (4). Thus, in

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the transplant setting, it was widely believed that allograft rejection is predominantly a Th1-mediated immune response, although this conclusion was challenged by data in cytokine-deficient mice that indicated that rejection could proceed in the absence of IL-2 or IFN- $\gamma$ . Mice deficient in the hallmark Th1 cytokine IFN- $\gamma$  rapidly reject fully mismatched cardiac allografts, as do mice lacking STAT4, a transcription factor associated with the development of Th1 responses (5–7). Th2 cytokines, on the other hand, have also been noted in grafts undergoing acute vascular rejection (8, 9). As a result, the validity of the Th1/Th2 paradigm in transplantation has been questioned (10). Nevertheless, in MHC class II-mismatched cardiac allograft models, transplanted hearts are protected from chronic allograft vasculopathy (CAV) in both STAT4- and IFN- $\gamma$ -deficient mice, suggesting a key role for IFN- $\gamma$  and/or Th1 CD4 T cells in the development of subacute immune-mediated vascular injury and CAV (11, 12).

We hypothesized that in the absence of T-bet, a Th2-predominant environment in combination with impaired CD8 effector function would result in inhibition of destructive alloimmune responses and could promote transplantation tolerance and prevent the development of CAV. This hypothesis is supported by studies in Th1-mediated autoimmune disease models in which T-bet deficiency confers protection (13–15), as well as earlier studies in chronic rejection models in which STAT4- and IFN- $\gamma$ -deficient mice are protected from CAV (11, 12). However, in contrast to our a priori hypothesis, we now report for the first time the role of T-bet in alloimmunity, and show that T-bet deficiency contributes to the development of aggressive CD4 Th17 cell-mediated inflammatory alloimmune responses that ultimately culminate in accelerated rejection and vasculopathy in an established model of chronic rejection.

## RESULTS

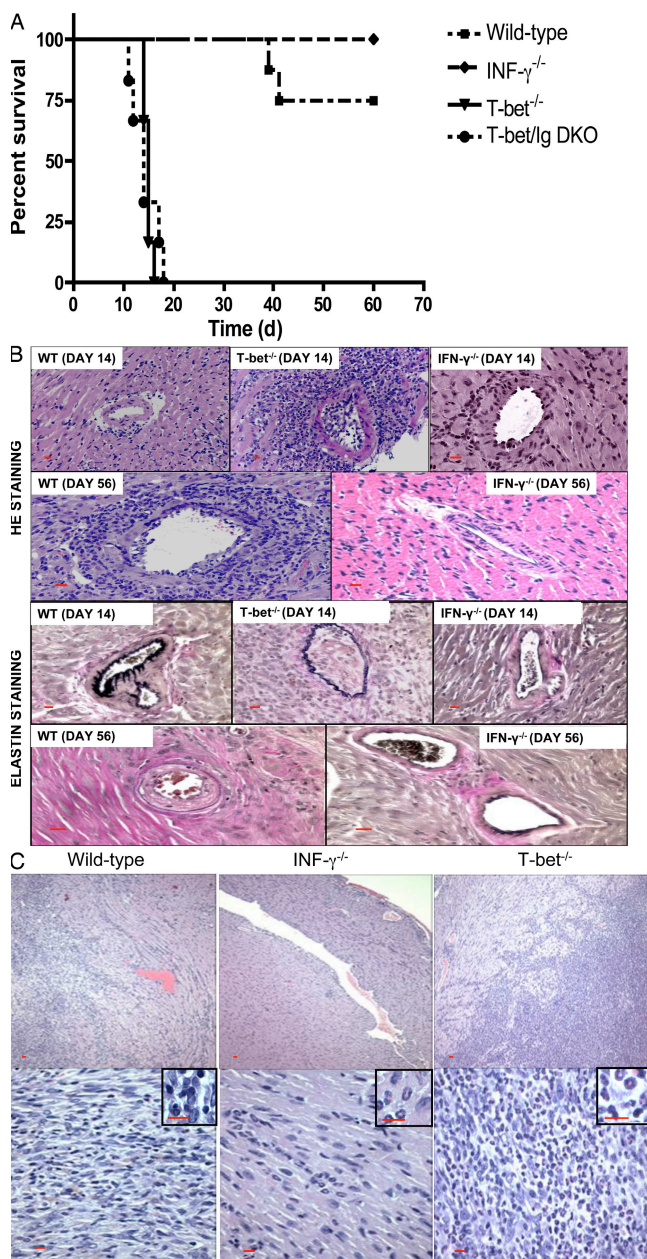
### T-bet deficiency accelerates cardiac allograft rejection in a model of CAV

T-bet expression appears to be critical for the development of a variety of inflammatory autoimmune diseases classically considered related to Th1 immunity, such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis, Crohn's disease, and type 1 diabetes (13–15). Allograft rejection is also considered a predominantly Th1 immune response. Moreover, IFN- $\gamma$  is a key regulatory cytokine associated with chronic allograft rejection and is thought to play a critical role in the pathogenesis of CAV (11). Therefore, we investigated the role of T-bet in alloimmune responses and the development of CAV in an established MHC class II-mismatched model (bm12 into C57BL/6) of CAV, where vascularized cardiac allografts survive long term without immunosuppression but develop CAV (16, 17). Unexpectedly, T-bet<sup>-/-</sup> mice ( $n = 6$ ) rejected their grafts with a median survival time (MST) of 15 d compared with WT ( $n = 8$ ) and IFN- $\gamma$ <sup>-/-</sup> ( $n = 6$ ) mice in which 75% (two out of eight grafts were rejected at days 39 and 41) and 100% of grafts survived for >60 d, respectively ( $P < 0.002$  compared with both

groups; Fig. 1 A). Further, grafts harvested from T-bet<sup>-/-</sup> recipients 2 wk after transplantation were notable for histological signs of severe vascular inflammation and vasculopathy, characterized by perivascular and intraluminal leukocytic infiltration, that were entirely absent in the case of IFN- $\gamma$ <sup>-/-</sup> recipients (Fig. 1 B). Interestingly, grafts from the T-bet<sup>-/-</sup> recipients were heavily infiltrated with PMNs (including eosinophils and neutrophils) compared with a predominantly mononuclear infiltration in the case of the WT recipients (Fig. 1 C and Table I). Because the PMN-dominant infiltrate is also consistent with antibody-mediated rejection, we performed bm12 heart transplants in T-bet/Ig double KO (DKO) recipients that lacked both T-bet and mature B cells. Interestingly, T-bet/Ig DKO recipients also rejected the bm12 heart grafts with the same tempo as T-bet<sup>-/-</sup> recipients (MST of 15 d in the T-bet<sup>-/-</sup> group vs. 14 d in the T-bet/Ig DKO group;  $n = 6$  in each group;  $P = 0.8676$ ; Fig. 1 A), and the pathology, including PMN infiltration, of the grafts harvested from T-bet/Ig DKO mice was very similar to those harvested from T-bet<sup>-/-</sup> recipients, indicating no role of antibodies or B cells in this model (not depicted). 8 wk after transplantation, grafts from the IFN- $\gamma$ <sup>-/-</sup> recipients demonstrated an intermediate pattern of infiltration, with more PMNs and fewer mononuclear cells compared with WT recipients. As previously reported (11), we also noted that IFN- $\gamma$ <sup>-/-</sup> mice demonstrated no evidence of allograft vasculopathy up to 8 wk after engraftment, in contrast to WT recipients in which occlusive CAV was noted in 50% of the vessels examined (Fig. 1 B).

These observations were at first somewhat surprising in that T-bet<sup>-/-</sup> mice rejected the grafts despite exhibiting a Th1-deficient environment, characterized by profound IFN- $\gamma$  deficiency and increased Th2 cytokines, whereas many Th1-dominated diseases are repressed in T-bet deficiency (13–15). Further, STAT4<sup>-/-</sup> mice, which like T-bet<sup>-/-</sup> mice exhibit a Th1-deficient environment, are in contrast protected from CAV (12). However, paradoxical results have been obtained involving the STAT1–T-bet–STAT4–IFN- $\gamma$  axis in various models of auto- and alloimmunity. For example, STAT1<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice are susceptible to EAE, whereas T-bet<sup>-/-</sup> and STAT4<sup>-/-</sup> mice are resistant (15, 18). Some of these inconsistencies are now beginning to be unraveled by the appreciation of a novel, IL-17-secreting Th cell lineage termed Th17 as a mediator of tissue inflammation. In view of this and the peculiar pattern of graft-infiltrating leukocytes consisting of predominantly PMNs, we examined the grafts showing severe vascular inflammation for the presence of IL-17-producing cells. 2 wk after transplantation, when the grafts from T-bet<sup>-/-</sup> recipients had undergone rejection, there was infiltration of PMNs along with CD4 and CD8 T cells with severe vascular inflammation and vasculopathy that were entirely absent in IFN- $\gamma$ <sup>-/-</sup> recipients. Besides intense PMN infiltration observed by light microscopy (Fig. 1 C), immunofluorescence staining of the grafts indicated that both CD4 and CD8 T cells were present, but the majority of the graft-infiltrating lymphocytes in T-bet<sup>-/-</sup> recipients were

IL-17-producing CD4 T cells (Fig. 2). There were hardly any and no IL-17-producing cells in grafts from WT and IFN- $\gamma^{-/-}$  recipients, respectively. Further, there were no NK cells and all of the T cells expressed the  $\alpha\beta$ TCR, indicating that there were no  $\gamma\delta$ T cells infiltrating the grafts from T-bet $^{-/-}$  recipients (not depicted). Collectively these results suggest that T-bet negatively regulates the development of Th17 cells that mediate the vascular inflammation and acute rejection in a model of CAV.



**Figure 1. T-bet deficiency accelerates cardiac allograft rejection characterized by severe vasculopathic changes and PMN infiltration.** (A) MHC class II-mismatched cardiac allograft survival (bm12 into C57BL/6) in WT, T-bet $^{-/-}$ , IFN- $\gamma^{-/-}$ , and T-bet/Ig DKO recipients ( $n = 8$  in WT, and  $n = 6$  in T-bet $^{-/-}$ , IFN- $\gamma^{-/-}$ , and T-bet/Ig DKO groups). Survival

### T-bet deficiency promotes CD4 and CD8 T cell effector memory differentiation and proliferation during an alloimmune response

To investigate whether T-bet deficiency, in addition to promoting Th17 cells, had any effect on CD4 and CD8 effector memory T cell ( $T_{EM}$ ) populations and proliferation in response to alloantigens, we first evaluated the frequency of T cells bearing the  $T_{EM}$  phenotype (CD44<sup>hi</sup>CD62L<sup>low</sup>) in the peripheral blood of bm12 graft recipients 2 wk after transplantation. We found that the activation of CD4 T cells in T-bet $^{-/-}$  and IFN- $\gamma^{-/-}$  recipients ( $11.8 \pm 0.2\%$  and  $13.3 \pm 3.4\%$ , respectively) was slightly but significantly higher than in WT recipients ( $9 \pm 0.2\%$ ;  $P < 0.05$ ). Further, the percentage of CD8 T cells with  $T_{EM}$  phenotype in T-bet $^{-/-}$  recipients ( $32.3 \pm 2.4\%$ ) was markedly higher than in IFN- $\gamma^{-/-}$  and WT recipients ( $9.56 \pm 3.1\%$  and  $4.9 \pm 1.5\%$ , respectively;  $P < 0.001$ ), with the majority of the CD8 T cells in T-bet $^{-/-}$  recipients expressing low levels of CD62L (Fig. 3 A). These findings are consistent with recent reports of increased CD8-central/memory T cell generation in T-bet $^{-/-}$  mice (19).

Next, to investigate the impact of T-bet deficiency on T cell proliferation in vivo in response to alloantigen, we adoptively transferred T-bet $^{-/-}$  T cells into irradiated bm12 mice, and compared their proliferation and apoptosis with that of WT and IFN- $\gamma^{-/-}$  T cells. T-bet $^{-/-}$  CD4 T cells exhibited enhanced in vivo proliferation ( $24.6 \pm 4.6\%$  in T-bet $^{-/-}$  vs.  $11.6 \pm 1.7\%$  and  $12.7 \pm 2\%$  in the case of WT and IFN- $\gamma^{-/-}$  T cells;  $P < 0.05$  for both groups) and were somewhat more resistant to apoptosis ( $44.9 \pm 2.2\%$  in T-bet $^{-/-}$  vs.  $59.7 \pm 4.2\%$  and  $64.4\% \pm 2.3$  in WT and IFN- $\gamma^{-/-}$  T cells;  $P < 0.005$  for both groups). T-bet $^{-/-}$  CD8 T cell proliferation was also more pronounced ( $42.3 \pm 4.4\%$  vs.  $19.8 \pm 3\%$  and  $14.8 \pm 2.4\%$  in T-bet $^{-/-}$  vs. WT and IFN- $\gamma^{-/-}$  T cells;  $P < 0.001$  for both groups). However, more of the proliferating T-bet $^{-/-}$  CD8 T cells underwent apoptosis than WT and IFN- $\gamma^{-/-}$  CD8 T cells ( $57.3 \pm 5.2\%$  vs.  $45.1 \pm 1.4\%$  and  $45.3 \pm 2.2\%$ , respectively;  $P < 0.05$  for both groups), explaining the lower absolute numbers of CD8 T cells in T-bet $^{-/-}$  recipients (unpublished data).

A reciprocal relationship between the development of pathogenic Th17 cells and regulatory Foxp3<sup>+</sup> T reg cells

data are presented as a Kaplan-Meier plot. (B) Pathology (top, H&E staining; bottom, elastin staining) of bm12 cardiac allografts from WT, T-bet $^{-/-}$ , and IFN- $\gamma^{-/-}$  recipients at 2 and 8 wk after transplantation. Note the severe vascular inflammation in the T-bet $^{-/-}$  recipient and the normal vascular integrity in the IFN- $\gamma^{-/-}$  recipient in contrast to that of the WT recipient even at 8 wk after engraftment. Bars, 20  $\mu$ m. (C) Pathology (H&E staining) of bm12 cardiac allografts from WT, T-bet $^{-/-}$ , and IFN- $\gamma^{-/-}$  recipients at 2 wk after transplantation (top, low magnification; bottom, high magnification) showing severe PMN infiltration (both neutrophils and eosinophils) in the T-bet $^{-/-}$  recipients and minimal PMN (predominantly eosinophilic) infiltration in IFN- $\gamma^{-/-}$  recipients, in contrast to modest mononuclear infiltration in WT recipients. Insets show infiltrating cell morphology. Histology results presented are from one experiment and are representative of three independent experiments. Bars, 20  $\mu$ m.

orchestrated by IL-6 and TGF- $\beta$  has been recently demonstrated (20). To investigate whether a decrease in Foxp3<sup>+</sup> T reg cells is responsible for the increase in CD4 and CD8 T cells bearing the T<sub>EM</sub> phenotype, we examined the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T reg cell population in the peripheral blood 2 wk after transplantation. No significant difference was found among WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients ( $6.8 \pm 1.4\%$ ,  $6.25 \pm 2.6\%$ , and  $7.7 \pm 1.8\%$ , respectively;  $P > 0.05$  among all groups; Fig. 3 B). These observations are consistent with a previous report that there was no defect in the numbers of T reg cells in the absence of IFN- $\gamma$  or T-bet (21). Collectively, an alloimmune response in the setting of T-bet deficiency is characterized by an increased proliferation of T cells and an expansion of CD4 and CD8 T<sub>EM</sub> cells. This, coupled with unaltered T reg cell numbers, results in a net increase in T cells of the T<sub>EM</sub> phenotype that may contribute to the accelerated rejection and pathological changes in the graft in this model of CAV.

#### Up-regulation of Th2 and Th17 cytokine production by T-bet<sup>-/-</sup> recipients

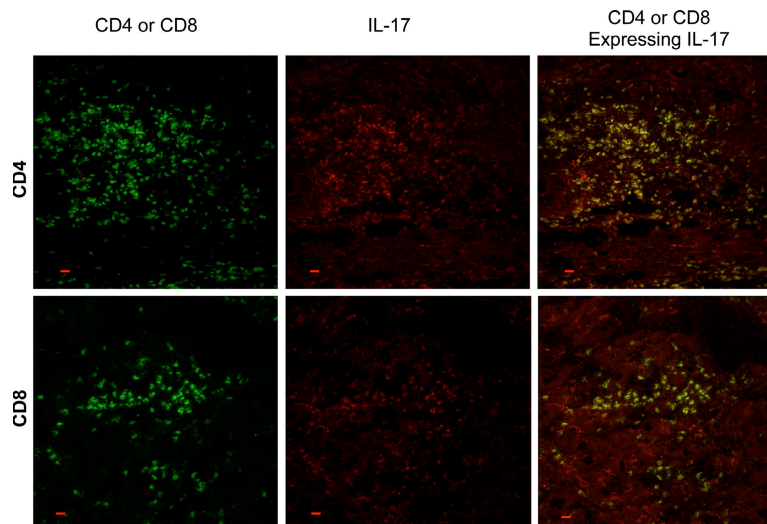
T-bet controls Th cell polarization toward a Th1 phenotype (3). In EAE studies, clinical improvement correlated with reduced T-bet expression, reduced IFN- $\gamma$  production, and a reciprocal increase in the Th2 transcription factor GATA3 in splenocytes (15). Recently, it has been noted that both IFN- $\gamma$  and T-bet repress the differentiation of Th17 cells (18, 22). Further, T-bet expression has been reported to be a critical determinant of Th1 versus Th17 cell fate (23). Moreover, T-bet appears to negatively regulate certain autoimmune diseases by suppressing IL-17 production (21, 24). As shown below, we noted that the majority of the graft-infiltrating lympho-

**Table I.** Phenotype of graft-infiltrating leukocytes in WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients

Infiltrating cells	WT (day 60)	IFN- $\gamma$ <sup>-/-</sup> (day 60)	T-bet <sup>-/-</sup> (day 14)
Lymphocytes	57.2 $\pm$ 13.1	2.7 $\pm$ 1.3	17.9 $\pm$ 4.2
Macrophages	24.8 $\pm$ 6.1	6.8 $\pm$ 1.6	22.3 $\pm$ 7.2
Neutrophils	3.9 $\pm$ 3.3	0.5 $\pm$ 0.7	16.7 $\pm$ 4.2
Eosinophils	0	13.3 $\pm$ 4.4	88.1 $\pm$ 11
Total	85.9 $\pm$ 15.5	23.3 $\pm$ 4.9	145 $\pm$ 17.6

Five high-power fields were counted per slide, with one slide each from five to six heart grafts per group. Results are presented as mean numbers of cells  $\pm$  SD.

cytes in T-bet<sup>-/-</sup> recipients were IL-17-producing CD4 T cells (Fig. 2). We therefore examined alloantigen-specific production of Th1 (IFN- $\gamma$ ), Th2 (IL-4, IL-5, IL-10, and IL-13), and the proinflammatory cytokines IL-2, TNF- $\alpha$ , IL-6, IL-17, IL-12p40, and IL-12p70 in WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients of bm12 cardiac allografts. Consistent with previous observations, T-bet<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> splenocytes produced significantly less Th1 cytokine IFN- $\gamma$  and more Th2 cytokines IL-4, IL-5, IL-10, and IL-13 (Fig. 4). Interestingly, splenocytes from T-bet<sup>-/-</sup> recipients compared with those from WT or IFN- $\gamma$ <sup>-/-</sup> recipients produced significantly higher amounts of proinflammatory cytokines IL-6 ( $339 \pm 10.5$  vs.  $75.3 \pm 25.9$  and  $208 \pm 44.5$  pg/ml, respectively;  $P < 0.01$ ), IL-17 ( $337 \pm 94.4$  vs.  $138.4 \pm 68.3$  and  $95.8 \pm 29.8$  pg/ml, respectively;  $P < 0.05$ ), and IL-12p40 ( $18,383.33 \pm 1,522.38$  vs.  $11,335 \pm 1,391.89$  and  $12,200 \pm 433.59$  pg/ml, respectively;  $P < 0.002$ ). Notably, IL-12p70 (representing IL-12, a heterodimer of IL-12p35 and IL-12p40) levels were not significantly different between the three groups (unpublished data),



**Figure 2.** IL-17-expressing graft-infiltrating lymphocytes in T-bet<sup>-/-</sup> recipients. Immunofluorescence staining and confocal microscopy of heart allografts harvested 2 wk after transplantation for IL-17 expression by CD4 and CD8 T cells. IL-17 expression by most of the CD4 (top) and some of the CD8 (bottom) graft-infiltrating T cells in the T-bet<sup>-/-</sup> recipients is seen in contrast to WT recipients (not depicted), where only a few of the infiltrating T cells express IL-17 and no IL-17-expressing cells were seen in grafts from IFN- $\gamma$ <sup>-/-</sup> recipients (not depicted). Results presented are from one experiment and are representative of three independent experiments. Bars, 20  $\mu$ m.

indicating that the differences in levels of IL-12p40 could be caused by differences in IL-23 (a heterodimer of IL-12p40 and IL-23p19) production (25), consistent with previous reports of increased IL-17 and IL-23 production in autoimmune disease models in T-bet<sup>-/-</sup> mice (21, 24). However, on direct measurement of IL-23 by ELISA we noted that IL-23 production by splenocytes harvested from bm12 heart allograft recipients was generally low and not different between T-bet<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> recipients ( $2.67 \pm 2.31$ ,  $5.47 \pm 7.49$ , and  $5.7 \pm 7.35$  pg/ml in WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients, respectively;  $P = \text{NS}$  between all groups). Collectively, in the presence of increased IL-6 and in the absence of Th1-polarizing conditions and the loss of direct negative regulatory effect of T-bet on Th17 cells in the T-bet<sup>-/-</sup> recipients, a positive feedback loop for the generation of Th17 cells is established, ultimately resulting in a heavily skewed immune response toward the Th17 phenotype.

### Proinflammatory cytokine production and accelerated cardiac allograft rejection in a model of CAV in T-bet<sup>-/-</sup> mice is dependent on CD4 T cells

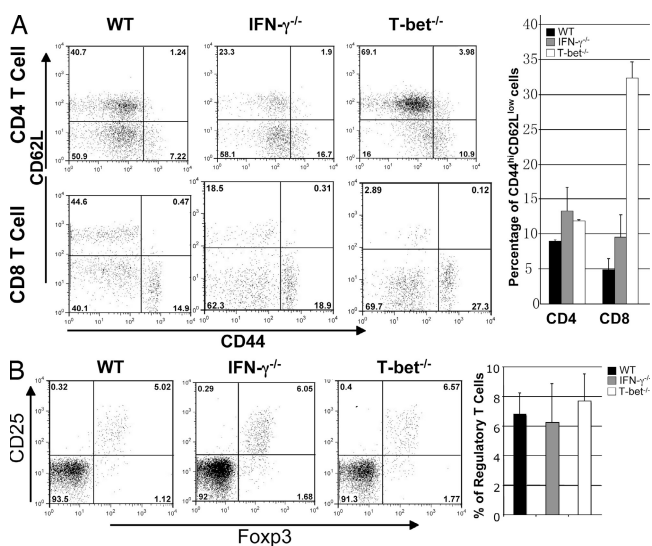
Both CD4 and CD8 lymphocytes contribute to CAV development via secretion of IFN- $\gamma$ , a known mediator of CAV in this model (26). Recently, it has been suggested that T-bet-expressing CD8 T cells mediate suppression of experimental autoimmune myocarditis (24). To evaluate the relative importance of T-bet deficiency in CD4 or CD8 T cell compartments in mediating the accelerated rejection, we injected CD4 or CD8 depleting mAb into T-bet<sup>-/-</sup> recipients of bm12 hearts. CD8 depletion resulted in a slight prolongation of MST from 15 to 21 d ( $P < 0.001$ ; Fig. 5 A). In contrast, CD4 depletion resulted in a dramatic prolongation of allograft survival ( $>60$  d) in T-bet<sup>-/-</sup> recipients (Fig. 5 A). In addition, we transplanted bm12 hearts into CD4/T-bet DKO and CD8/T-bet DKO recipients. CD4/T-bet DKO recipients, similar to the CD4-depleted T-bet<sup>-/-</sup> recipients above, also accepted the grafts for  $>60$  d, whereas CD8/T-bet DKO mice rejected the heart grafts with an MST of 19 d (Fig. 5 A). Further, pathological examination of the grafts from CD4-depleted T-bet<sup>-/-</sup> and CD4/T-bet DKO recipients revealed only mild vasculopathy in 20 and 12.5% of the vessels, respectively, 8 wk after transplantation, whereas grafts from CD8-depleted T-bet<sup>-/-</sup> or CD8/T-bet DKO recipients showed severe vasculopathy 3 wk after transplantation (Fig. 5 B). These data are in keeping with the observation that the majority of the IL-17-producing graft-infiltrating lymphocytes in T-bet<sup>-/-</sup> recipients were CD4 T cells (Fig. 2) and indicate that T-bet expressed in the CD4 rather than CD8 T cell compartment prevents accelerated rejection in this model of CAV.

Further, splenocytes from T-bet/CD4 DKO recipients compared with those from T-bet<sup>-/-</sup> and T-bet/CD8 DKO recipients produced significantly less IL-6 ( $12.9 \pm 3.8$  vs.  $940.7 \pm 176.3$  and  $1,240.5 \pm 215.6$  pg/ml, respectively;  $P < 0.01$ ) and IL-17 ( $7.8 \pm 4.6$  vs.  $210.2 \pm 73.4$  and  $113.1 \pm 61.2$  pg/ml, respectively;  $P < 0.01$ ) cytokines. These data indicate that both of these cytokines are predominantly produced by

the CD4 cell population and are consistent with the observation that CD4 but not CD8 T cell deficiency or depletion in T-bet<sup>-/-</sup> recipients affords dramatic protection from vasculopathy and facilitates long-term acceptance of heart grafts (Fig. 5 C). Collectively, these data indicate that T-bet in CD4 cells plays a critical role in determining the fate and type of the alloimmune response.

### Anti-IL-17 antibody inhibits accelerated cardiac allograft rejection in a model of CAV in T-bet<sup>-/-</sup> recipients

From the foregoing, it is reasonable to consider that IL-17-producing Th17 cells play a critical role in mediating alloimmune responses. Further, blocking IL-17 with anti-IL-17 antibody resulted in markedly reduced skin inflammation in a model of systemic autoimmunity (21). We therefore administered anti-IL-17 mAb or control Ig to T-bet<sup>-/-</sup> recipients of bm12 heart grafts and observed for graft survival to directly determine whether IL-17 neutralization can inhibit accelerated cardiac allograft rejection in this model of CAV. Neutralization of IL-17 with anti-IL-17 mAb indeed resulted in significant prolongation of allograft survival in T-bet<sup>-/-</sup> recipients compared with the group that received control IgG (MST of 38 vs. 15 d, respectively;  $P < 0.01$ ; Fig. 6 A). The protection from acute rejection, although significant, was not complete and may have been caused by the relatively brief duration of anti-IL-17 mAb administration. We then, as above, examined alloantigen-specific production of Th1, Th2,



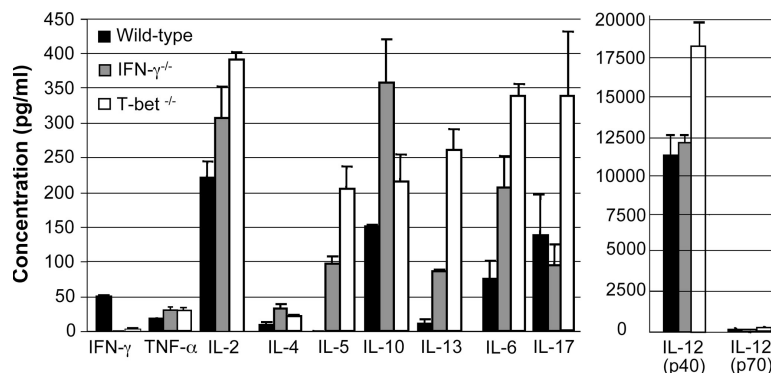
**Figure 3. T-bet deficiency promotes CD4 and CD8 T cell effector/memory differentiation and proliferation in MHC class II-mismatched cardiac allograft recipients.** (A) FACS analysis of lymphocytes in the peripheral blood of WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients bearing a CD44<sup>hi</sup>CD62L<sup>low</sup> effector/memory phenotype gated on either CD4 (top) or CD8 (bottom) T cell populations. Percentages of cells are shown. (B) CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory CD4 T cells in the MHC class II-mismatched cardiac allograft recipients. Gates were set on the CD4<sup>+</sup> population, and percentages of cells are shown. Bar graphs show means  $\pm$  SD. All results are representative of at least three different sets of experiments.

and proinflammatory Th17 cytokines in the anti-IL-17 mAb or control Ig-treated T-bet<sup>-/-</sup> recipients of bm12 cardiac allografts 14 or 40 d after transplantation. Consistent with our earlier observations, splenocytes from T-bet<sup>-/-</sup> recipients at each time point produced very little Th1 cytokine IFN- $\gamma$  (Fig. 6 B). Further, at days 14 and 40, splenocytes from T-bet<sup>-/-</sup> recipients treated with anti-IL-17 mAb in comparison to those from control Ig-treated T-bet<sup>-/-</sup> recipients at day 14 produced significantly less of the proinflammatory cytokines IL-17 ( $13.6 \pm 0.43$  and  $72.3 \pm 81.12$  vs.  $337 \pm 111.85$  pg/ml, respectively;  $P < 0.01$ ) and IL-12p40 ( $3.42 \pm 1.12 \pm 0.43$  vs.  $18,433 \pm 152.75$  pg/ml, respectively;  $P < 0.001$ ). Notably again, IL-12p70 (representing IL-12, a heterodimer of IL-12p35 and IL-12p40) levels were not significantly different between the three groups (unpublished data). Interestingly, splenocytes from T-bet<sup>-/-</sup> recipients treated with anti-IL-17 mAb in comparison to those from control Ig-treated T-bet<sup>-/-</sup> recipients produced significantly higher amounts of the Th2 cytokines IL-4 ( $174.83 \pm 22.12$  and  $200.8 \pm 22.2 \pm 33.5$  pg/ml, respectively;  $P < 0.01$ ) and IL-5 ( $2,448.33 \pm 675.79$  and  $641 \pm 36.11$  vs.  $204.7 \pm 19$  pg/ml, respectively;  $P < 0.001$ ). IL-10 levels were not significantly different between the three groups; however, IL-13 production was initially increased at day 14 and then diminished at day 40 in anti-IL-17-treated groups (Fig. 6 B). IL-6 is upstream of IL-17 in the Th17 differentiation pathway (27); consequently, IL-6 production is markedly elevated in anti-IL-17-treated groups ( $1,405.2 \pm 203.44$  and  $726 \pm 116.9$  vs.  $262 \pm 12.12$  pg/ml, respectively;  $P < 0.001$ ). Pathological examination of heart allografts from T-bet<sup>-/-</sup> recipients treated with anti-IL-17 mAb at day 14 revealed minimal vascular inflammation and no changes of CAV (Fig. 6 C and Table II). However, examination at day 40, when the anti-IL-17-treated T-bet<sup>-/-</sup> recipients had rejected the heart allografts, revealed considerable leukocytic infiltration in the parenchyma but minimal CAV (Fig. 6 C and Table II). These data are consistent with previous reports of reduced skin inflammation with IL-17 neutralization in a model of systemic autoimmunity (21). Collectively, with the increased

IL-17 cytokine production in T-bet<sup>-/-</sup> recipients and infiltration of vascular lesions with IL-17-producing CD4 T cells, we conclude that CD4 Th17 cells are the main cells responsible for mediating the accelerated rejection and CAV in T-bet<sup>-/-</sup> recipients.

## DISCUSSION

Since the original description of Th lymphocyte subsets (Th1 and Th2) and their divergent effector functions, each has been linked with the development of specific diseases or immune responses, including transplant rejection or tolerance (28, 29). It has been postulated that Th1 cells are critically involved in the development of allograft rejection, whereas Th2 cells are involved in promoting transplantation tolerance (29). T-bet is believed to be crucial for the development of a variety of inflammatory diseases by virtue of being the master regulator of Th1 differentiation (30). T-bet<sup>-/-</sup> mice are resistant to EAE (13), and silencing T-bet by small interfering RNA specific for T-bet at the time of EAE induction reduced the incidence and severity of disease, indicating that T-bet is critical for the differentiation of autoreactive T cells. Further, silencing T-bet after disease onset significantly improves the clinical course of established EAE (15). Intriguingly, we found that T-bet deficiency in cardiac allograft recipients, on the contrary, accelerates rejection in a model of CAV despite exhibiting a Th1-deficient environment characterized by a profound deficiency of IFN- $\gamma$  and increased Th2 cytokines. This was particularly surprising because IFN- $\gamma$  is thought to play a critical role in the pathogenesis of CAV, and either STAT4 or IFN- $\gamma$  deficiency confers protection from CAV in this model (11, 12), raising questions about the notion that IFN- $\gamma$  is the key regulatory cytokine associated with chronic allograft rejection. However, intriguing and paradoxical results have been obtained involving the STAT1-T-bet-STAT4-IFN- $\gamma$  axis in various models of auto- and alloimmunity. For example, STAT1<sup>-/-</sup> mice and IFN- $\gamma$ <sup>-/-</sup> mice are susceptible to EAE, whereas T-bet<sup>-/-</sup> and STAT4<sup>-/-</sup> mice are resistant (15, 18). IFN- $\gamma$  has been implicated in the acute rejection process in

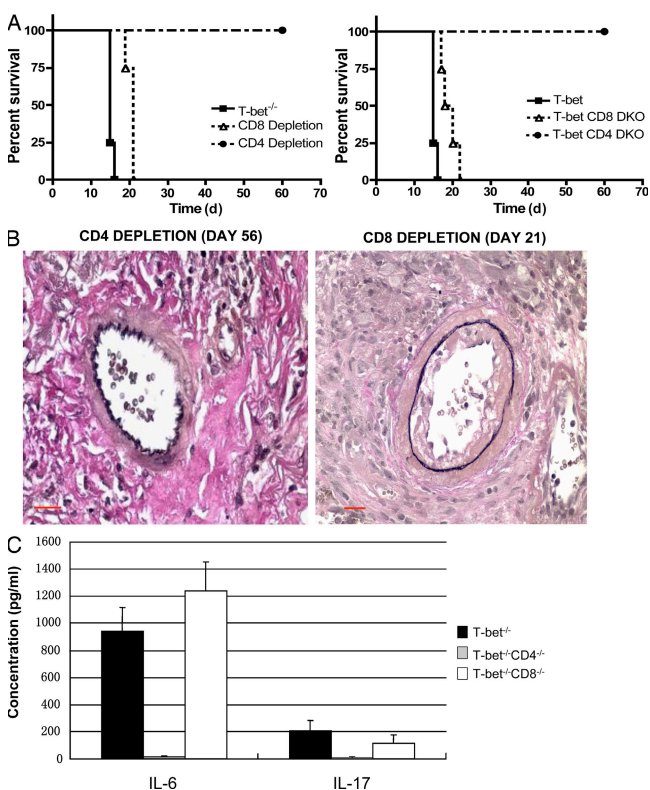


**Figure 4. Up-regulation of Th2 and Th17 proinflammatory cytokine production by T-bet<sup>-/-</sup> allograft recipients of bm12 hearts.** Th1, Th2, and Th17 proinflammatory cytokine production by the splenocytes of WT, IFN- $\gamma$ <sup>-/-</sup>, and T-bet<sup>-/-</sup> recipients of bm12 heart grafts assessed by the Luminex assay. IL-12 data are presented separately because of the need to use a different scale. Results presented are means  $\pm$  SD and are representative of at least three independent experiments.

recipients of allografts, whereas IFN- $\gamma$  deficiency has also been reported to result in severe vascular inflammation leading to acute rejection and to interfere with tolerance induction (31). The differences seen in Th17 response after targeting T-bet with small interfering RNA in the EAE model (15) versus the CAV model in our studies are likely related to the differences in the model, mode of priming, precursor T cell frequency, strength of TCR stimulation, and partial versus complete T-bet deficiency. Interestingly, recent studies have shown that T-bet acts as a negative regulator of Th17-mediated immune responses in two different models of autoimmunity (21, 24). In a model of experimental autoimmune myocarditis, heightened disease severity in T-bet $^{-/-}$  mice was suggested to be caused by increased CD4 T cell-mediated IL-17 production in the heart (24). Lohr et al., in another model of systemic autoimmune disease, reported worsening of disease in T-bet $^{-/-}$  mice, suggesting a reciprocal relationship between Th1 responses and IL-17 production indicating that IL-17 is an important cytokine in tissue inflammation (21). Our observation of accelerated rejection in T-bet $^{-/-}$  recipients associated with severe vascular inflammation characterized by PMNs and IL-17-producing T cell infiltration are in keeping with these previous reports of T-bet as a negative regulator of Th17-mediated immune responses in vivo. These results suggest that T-bet negatively regulates the development of Th17 cells that mediate the vascular inflammation and acute rejection in a model of CAV.

Interestingly, the accelerated allograft rejection and vasculopathy are associated with increased production of the proinflammatory cytokines IL-6, IL-17, and IL-12p40. These observations are consistent with previous reports of increased IL-17 production in autoimmune disease models in T-bet $^{-/-}$  mice (21, 24). T-bet decreases antigen receptor-induced IL-17 production (23). IL-23, on the other hand, promotes IL-17 expression and the proliferation of IL-17-producing cells from a pool of memory T cells, thus playing an important role in maintaining Th17 effector function (32, 33). It has been proposed that in an inflammatory milieu, IL-6 and TGF- $\beta$  together induce Th17 cells and up-regulate IL-23R, thereby allowing IL-23 to strengthen and sustain the Th17 phenotype (34). However, in this model of CAV, although IL-12p40 levels were elevated, IL-23 production in vitro by splenocytes from allograft recipients was minimal, indicating that IL-23-independent mechanisms may be responsible for the maintenance of the Th17 response. IL-12p40 is believed to have opposing immunomodulatory effects in different experimental conditions; for example, IL-12p40 specifically inhibited the effects of IL-12 and IL-23 heterodimers (35, 36), whereas IL-12p40 homodimers exacerbated cardiac allograft rejection by enhancing alloantigen-specific Th2 development (37). However the latter study predated the discovery of Th17 cells, and the aggressive alloimmune responses were attributed to skewing of the immune response toward a Th2 phenotype and Th17 responses were not examined. Further, in lung transplantation, enhanced epithelial production of IL-12p40 homodimers was associated with progressive chronic

allograft dysfunction independent of IL-12p70 or IL-23 (38). Collectively, in the presence of increased IL-6 and in the absence of Th1-polarizing conditions and the loss of a direct negative regulatory effect of T-bet on Th17 cells in the T-bet $^{-/-}$  recipients, a positive feedback loop for the generation of Th17 cells is established, ultimately resulting in a heavily skewed immune response toward the Th17 phenotype. Interestingly, IL-17 neutralization in T-bet $^{-/-}$  recipients inhibited accelerated cardiac allograft rejection and was associated with markedly reduced IL-17 and IL-12p40 production (Fig. 6, A and B). However, production of IL-6 and Th2 cytokines, including IL-13, was actually increased with IL-17 neutralization, indicating that IL-17 is downstream of IL-6 and that IL-17 is critical for mediating cardiac allograft rejection and vasculopathy. In an antigen-induced allergic airway inflammation



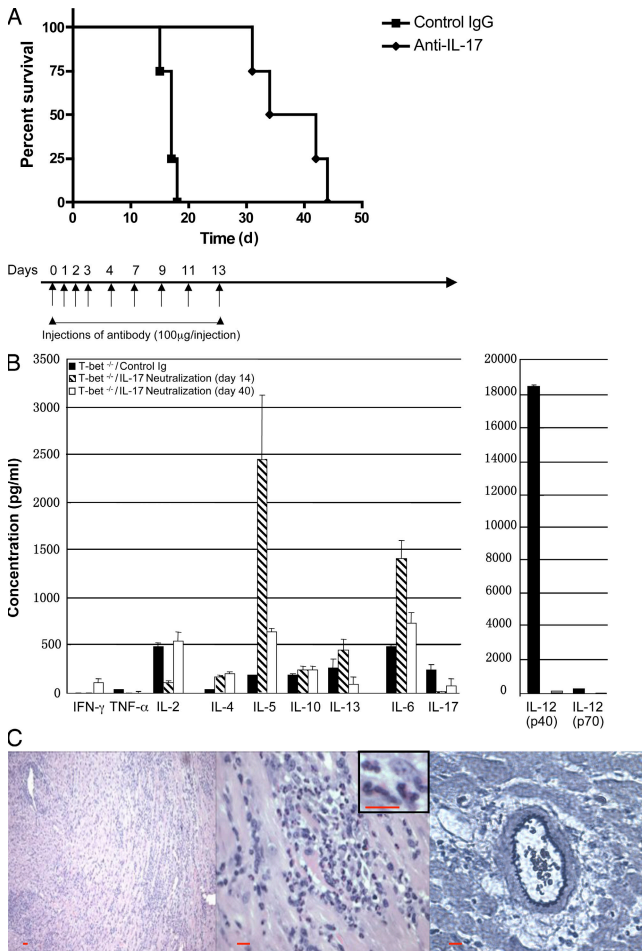
**Figure 5. T-bet $^{-/-}$  CD4 T cells produce proinflammatory cytokines and mediate accelerated cardiac allograft rejection in the MHC class II-mismatched cardiac allograft model of CAV.** (A) Survival of allogeneic bm12 cardiac graft in CD4- or CD8-depleted (left) and CD4/T-bet or CD8/T-bet DKO (right) recipients ( $n = 4$  in each group). Survival data are presented as Kaplan-Meier plots. (B) Pathology (elastin staining) of bm12 cardiac allografts from CD4- or CD8-depleted T-bet $^{-/-}$  recipients at 8 and 3 wk, respectively, after transplantation. Note the significant vasculopathy in the CD8-depleted T-bet $^{-/-}$  recipient and, in contrast, the relatively normal vascular integrity in the CD4-depleted T-bet $^{-/-}$  recipient even at 8 wk after engraftment. Bars, 20  $\mu$ m. (C) IL-6 and IL-17 proinflammatory cytokine production by splenocytes of T-bet $^{-/-}$ , CD4/T-bet DKO, and CD8/T-bet DKO recipients of bm12 heart grafts assessed by the Luminex assay. Bar graphs show means  $\pm$  SD. Results presented are from one experiment and are representative of three independent experiments.

model, T-bet inhibited differentiation of both Th17 and IL-13-producing Th2 cells (39). In addition, IL-13 appears to down-regulate Th17 differentiation in models of pulmonary and CNS inflammation (40, 41). Therefore, targeting IL-13 may not be beneficial in the prevention of Th17 immunity. On the other hand, IL-6 blockade inhibited the development of EAE, indicating that IL-6 targeting prevents differentiation of Th17 cells but does not inhibit committed Th17 cells (42). Therefore, IL-6 blockade, particularly early in the allo-

immune response, appears to be an attractive strategy for the inhibition of Th17 differentiation.

IL-17 is a potent proinflammatory cytokine that induces chemokine expression and leukocyte infiltration and mediates tissue inflammation (18). IL-17 induces granulopoiesis via G-CSF production and regulates neutrophil recruitment via CXC chemokine induction by epithelial cells (43). Homozygous deletion of IL-17RA abrogates the increase in splenic neutrophil progenitors induced by overexpression of IL-17A (43). Further, in a model of acute trinitrobenzenesulfonic acid-induced colitis, mice deficient in IL-17RA expression were significantly protected from trinitrobenzenesulfonic acid-induced weight loss and colonic inflammation and substantially reduced PMN immigration into the colon (44, 45). Neutralization of IL-17A homodimers strongly inhibits neutrophilic airway inflammation mediated by adoptive transfer of ovalbumin-specific polarized Th17 cells and airway challenge with antigen (46). In keeping with these, we noted intense PMN infiltration within the lesions of vascular inflammation in grafts from T-bet<sup>-/-</sup> recipients; interestingly, however, the PMN infiltration consisted of both neutrophils and eosinophils, perhaps as a result of markedly increased Th2 cytokines along with a Th17 immune response. Eosinophilic infiltration is well described in cardiac allografts associated with a Th2 immune response (47). Further, neutralization of IL-17 in T-bet<sup>-/-</sup> recipients markedly reduced the vascular inflammation, particularly neutrophil infiltration, suggesting a link between IL-17 and neutrophil recruitment to the graft (Fig. 6 C and Table II).

Despite the predominantly PMN infiltration, CD4 cells appear to be critical for the development of CAV, because CD4 depletion or deficiency in T-bet<sup>-/-</sup> recipients affords complete protection from CAV and long-term allograft survival (Fig. 5, A and B). Further, IL-6 and IL-17 production is markedly diminished in CD4/T-bet DKO recipients (Fig. 5 C), indicating the CD4 cells are critical for the initiation of the aggressive Th17 alloimmune response. Moreover, there is an increase in proliferation of T cells and an expansion of CD4 and CD8 T<sub>EM</sub> cells in T-bet<sup>-/-</sup> recipients (Fig. 3 A). These findings are consistent with recent reports of increased CD8-central/memory T cell generation in T-bet<sup>-/-</sup> mice (19). Further, we examined whether this net increase in T cells of the T<sub>EM</sub> phenotype is caused by a deficiency in Foxp3<sup>+</sup> T reg cells, particularly because a reciprocal relationship between the development of pathogenic Th17 cells and Foxp3<sup>+</sup> T reg cells orchestrated by IL-6 and TGF-β has been recently demonstrated (20). In addition, IL-6, together with IL-1 and STAT3, induces genetic reprogramming of Foxp3<sup>+</sup> T reg cells (48). However, we did not find a decrease in Foxp3<sup>+</sup> T reg cells in T-bet<sup>-/-</sup> recipients (Fig. 3 B). These observations are consistent with a previous report that there was no defect in numbers of T reg cells in the absence of IFN-γ or T-bet (21). Whether the function of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T reg cells in T-bet<sup>-/-</sup> mice is also unaltered remains to be investigated. Further, the inflammatory milieu within the grafts in T-bet<sup>-/-</sup> recipients may promote the conversion of T reg



**Figure 6. IL-17 neutralization inhibits accelerated cardiac allograft rejection in a model of CAV in T-bet<sup>-/-</sup> mice.** (A) MHC class II-mismatched cardiac allograft survival (bm12 into C57BL/6) in T-bet<sup>-/-</sup> recipients treated with anti-IL-17 mAb (n = 4) or control IgG (n = 4). Survival data are presented as a Kaplan-Meier plot. (B) Th1, Th2, and Th17 proinflammatory cytokine production, assessed by the Luminex assay at days 14 or 40 after transplantation, by the splenocytes of T-bet<sup>-/-</sup> recipients of bm12 heart grafts treated with control Ig or anti-IL-17 mAb. IL-12 data are presented separately because of the need to use a different scale. Results presented are means ± SD and are representative of three independent experiments. (C) Pathology of bm12 cardiac allografts from anti-IL-17 mAb-treated T-bet<sup>-/-</sup> recipients at 14 d after transplantation (left and middle, H&E staining; inset, infiltrating cell morphology), showing a decrease in PMN infiltration (particularly neutrophils), and at 40 d after transplantation (right, elastin staining), showing minimal CAV. Bars, 20 μm.



**Table II.** Phenotype of graft-infiltrating leukocytes in T-bet<sup>-/-</sup> recipients with and without IL-17 neutralization

Infiltrating cells	T-bet <sup>-/-</sup> control Ig (day 14)	T-bet <sup>-/-</sup> IL-17 neutralization (day 14)	T-bet <sup>-/-</sup> IL-17 neutralization (day 40)
Lymphocytes	15.4 ± 2.1	3.2 ± 1.3	10.3 ± 3
Macrophages	21.6 ± 4.3	4.7 ± 1.8	13.6 ± 3
Neutrophils	18.4 ± 5.6	1.2 ± 0.3	4.6 ± 1.9
Eosinophils	96.1 ± 13.5	26.4 ± 6.5	71.7 ± 7.9
Total	151.1 ± 18.3	34.1 ± 6.9	100.3 ± 13.7

Five high-power fields were counted per slide, with one slide each from three to four heart grafts per group. Results are presented as mean numbers of cells ± SD.

cells to Th17 phenotype in an IL-6–dependent manner, as has been recently demonstrated in the context of priming with activated DCs (49). Whether this is the case in our model needs further investigation. CD4 Th17 cells have been implicated in many autoimmune diseases (50), and collagen type V–specific Th17 cells contribute to obliterative bronchiolitis in human lung transplants (51). Therefore, it is conceivable that the cardiac allograft rejection in T-bet<sup>-/-</sup> recipients is mediated by Th17 cells reacting against self-antigens exposed in the ischemically injured heart allografts and needs further investigation. However, we believe that this is unlikely because the strong Th17 recall response of the splenocytes from T-bet<sup>-/-</sup> recipients is in response to alloantigen, and in general, the frequency of alloreactive T cells is significantly higher than that of autoreactive T cells.

In another model of vascular rejection, however, IL-17 antagonism attenuated acute rather than chronic rejection (52). It has been reported that an initial vascular inflammation, characterized by perivascular and intraluminal lymphocytic infiltration, precedes development of the classical fibroproliferative arteriosclerotic lesions of CAV (11). This initial vascular inflammation likely represents a counterpart of acute rejection in clinical transplantation and is a harbinger of pathological changes of impending CAV or clinical chronic rejection. In our experiments with T-bet<sup>-/-</sup> recipients, this early vascular inflammation is so severe that acute rejection ensues, precluding assessment of the impact of T-bet deficiency on CAV. However, in WT recipients, vascular inflammation of the grafts becomes apparent by 2 wk after transplantation and progresses to typical lesions of CAV by 8 wk. Indeed, IL-17 neutralization in our model of CAV in T-bet<sup>-/-</sup> recipients resulted in a marked decrease in initial vascular inflammation (Fig. 6 C and Table II) and, consequently, prolongation of graft survival (Fig. 6 A). Interestingly, IL-17 neutralization converts the phenotype (in relation to CAV) of the T-bet<sup>-/-</sup> recipients to that of IFN- $\gamma$ <sup>-/-</sup> recipients, and the grafts undergo delayed parenchymal rejection with minimal changes of CAV (Fig. 6 C), similar to a previous report in IFN- $\gamma$ <sup>-/-</sup> recipients in this model (11). This suggests that early vascular inflammation is mediated by CD4 Th17 cells and contributes to the eventual development of CAV, and that even a brief antagonism of CD4 Th17 cells by IL-17 neutralization affords protection from CAV.

In summary, recent elucidation of a novel lineage of T-helper Th17 cells (18, 22, 25) has shed light on some of the

seemingly inconsistent findings in models of Th1-mediated autoimmune inflammation such as EAE and collagen-induced arthritis in IFN- $\gamma$ <sup>-/-</sup> or STAT1<sup>-/-</sup> mice (13). Th17 immunity has been implicated in various models of immune-mediated tissue injury, particularly in the absence of a Th1 environment (21, 53, 54). Our studies have extended this novel paradigm to the transplant setting, enabling us to explain transplant rejection by inflammatory processes mediated by Th17 cells in the absence of Th1-dominant immune responses. Indeed, IL-17 has been implicated in both experimental and human renal and lung allograft rejection (51, 55–57). Further, IL-17 promotes secretion of inflammatory cytokines by stromal cells and macrophages and promotes DC maturation. IL-17R:Fc administration promoted cardiac allograft survival by inhibiting maturation of DC progenitors (58), and local expression of soluble IL-17R by gene therapy prolonged cardiac allograft survival (59). From the data presented in this paper, it is clear that CD4 Th17 cells are the major mediators of vascular inflammation, thereby setting the stage for the progression of these lesions to CAV. We have shown that T-bet negatively regulates this vascular inflammation and suppresses the development of CAV. Further, neutralization of IL-17 prevents vascular inflammation and suppresses CAV. In conclusion, IL-17–producing CD4 Th17 cells are important in allograft rejection and may serve as a novel target of strategies for the prevention of transplant rejection and graft vasculopathy.

#### MATERIALS AND METHODS

**Animals.** WT C57BL/6, B6.C-H2<sup>bmi12</sup> (bm12), IFN- $\gamma$ <sup>-/-</sup>, CD4<sup>-/-</sup>, Ig<sup>-/-</sup> (B-less), and T-bet<sup>-/-</sup> mice, all on a C57BL/6 background, were purchased from the Jackson Laboratory. Mice deficient in both T-bet and CD4, CD8, or B cells were generated by crossbreeding. Genotyping was used to confirm homozygous deletion of the T-bet and CD4, CD8, or Ig genes. Experiments were approved by the Institutional Animal Care and Use Committee at the Harvard Medical School Center for Animal Resources and Comparative Medicine.

**Heart transplantation and antibody treatment.** In the MHC class II mismatch model, bm12 mice were used as donors, and C57BL/6 WT, IFN- $\gamma$ <sup>-/-</sup>, T-bet<sup>-/-</sup>, T-bet<sup>-/-</sup>/CD4<sup>-/-</sup>, T-bet<sup>-/-</sup>/CD8<sup>-/-</sup>, or T-bet<sup>-/-</sup>/Ig<sup>-/-</sup>, all on C57BL/6 background, were used as recipients. Vascularized heart grafts were transplanted using a microsurgical technique as previously described (16). Depleting anti-CD4 (GK1.5) and –CD8 (2.43; both from BioExpress) were administered i.p. at a dose of 0.1 mg on days –6, –3, and –1. Neutralizing IL-17 mAb (MAB421; R&D Systems) was administered i.p. at a dose of 100  $\mu$ g daily on days 0–3, followed by every other day until day 13 after transplantation. Graft function was assessed by daily palpation of the abdomen. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization.

**Histology.** Grafts harvested at 2 or 8 wk or at the time of rejection were sectioned transversely, fixed in 10% buffered formalin, and stained by hematoxylin and eosin (H&E), and elastin stains and the severity of parenchymal rejection and CAV were scored as previously described (16). For the phenotype of graft-infiltrating leukocytes, five high-power fields were counted per slide, with one slide each from five to six heart grafts per group. Results are presented as mean numbers of cells  $\pm$  SD. The remaining portions of the graft were used for frozen sections for immunofluorescence staining using goat anti-mouse IL-17 (R&D Systems) and rat anti-mouse CD4 and CD8 (both from BioExpress) as primary antibodies. Secondary detection was performed using Cy2-conjugated donkey anti-rat IgG and Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories). Images were captured using a confocal laser scanning microscope (C1 Plus; Nikon).

**Cytokine analysis by Luminex and ELISA assay.** For cytokine analysis, splenocytes harvested at 14 d after transplantation from recipients of bm12 heart allografts were restimulated by irradiated donor spleen cells. The cell-free supernatants of individual wells were removed after 48 h and analyzed by a multiplexed cytokine bead-based immunoassay using a preconfigured 21-plex mouse cytokine detection kit (Millipore) according to the manufacturer's instructions. In brief, the assay is based on conventional sandwich assay technology. The antibody specific to each cytokine was covalently coupled to Luminex microspheres uniquely labeled with a fluorescent dye. The 25- $\mu$ l microspheres were incubated overnight at 4°C with standards, controls, and samples (50  $\mu$ l) in a 96-well microtiter filter plate. The plate was washed to remove excess reagent after incubation, and a detection antibody (25  $\mu$ l biotin; Beadlyte Mouse 21-plex Multi-Cytokine kit; Millipore) was added in the form of a mixture containing each of the antibodies. After 30 min of incubation at room temperature, streptavidin-phycoerythrin was added for an additional 30 min. After a final wash step, the beads were resuspended in buffer and analyzed on the Luminex 100 instrument to determine the concentration of the cytokine of interest. IL-23 was measured by a specific ELISA kit from eBioscience according to the manufacturer's instructions. All samples were tested in triplicate wells.

**Flow cytometry.** Splenocytes from naive and posttransplant C57BL/6, T-bet<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice were stained using fluorochrome-conjugated mouse-specific mAbs against CD4, CD8, CD44, CD62L, CD25 (all from BD), and Foxp3 (eBioscience). Cytometry was performed on a cytometer (FACSCalibur; BD), and data were analyzed using FlowJo software (Tree Star, Inc.).

**Adoptive transfer model.** 40 million CFSE-labeled lymphocytes from WT, T-bet<sup>-/-</sup>, or IFN- $\gamma$ <sup>-/-</sup> mice were injected i.v. into lethally irradiated (1,000 rads) bm12 mice in 0.25 ml of sterile PBS. Recipients were killed 3 d later, and spleen and lymph nodes were harvested. The proliferation of CFSE-labeled donor cells in the host spleen and lymph nodes was examined by flow cytometry based on the dilution profile of the CFSE dye. Apoptosis was evaluated by Annexin V and 7-amino-actinomycin D staining.

**Statistics.** For the graft survival, Kaplan-Meier graphs were constructed and log-rank comparison of the groups was used to calculate p-values. For the cytokine levels by Luminex, data are presented as means  $\pm$  SD, and comparisons between the values were performed using the two-tailed Student's *t* test. For all statistical analyses, the level of significance was set at a probability of *P* < 0.05. All experiments were repeated at least three to five times.

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