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CD8\(^+\) Th17 Mediate Costimulation Blockade-Resistant Allograft Rejection in T-bet-Deficient Mice\(^1\)

Bryna E. Burrell,\(^2\)* Keri Csencsits,\(^2\)* Guanyi Lu,\(*\) Svetlana Grabauskiene,\(*\) and D. Keith Bishop\(^3\)*†

While studying Th responses induced by cardiac transplantation, we observed that mice deficient in the Th1 transcription factor T-bet (T-bet\(^{−/−}\)) mount both Th1 and Th17 responses, whereas wild-type recipients mount only Th1 responses. Cells producing both IFN-γ and IL-17 were readily detectable within the rejecting graft of T-bet\(^{−/−}\) recipients, but were absent from the spleen, indicating that the in vivo microenvironment influences Th function. In addition, disrupting CD40-CD40L costimulatory interactions was highly effective at prolonging allograft survival in WT mice, but ineffective in T-bet\(^{−/−}\) recipients. In this study, we report that CD8\(^+\) Th17 mediate costimulation blockade-resistant rejection in T-bet\(^{−/−}\) allograft recipients. Depleting CD8\(^+\) cells or neutralizing IL-17 or the Th17-inducing cytokine IL-6 ablated the Th17 response and reversed costimulation blockade-resistant graft rejection. Neutralizing IL-4 in IFN-γ\(^{−/−}\) allograft recipients did not induce Th17, suggesting that T-bet, rather than IL-4 and IFN-γ (known inhibitors of Th17), plays a critical role in negatively regulating Th17 in the transplant setting. The Journal of Immunology, 2008, 181: 3906–3914.

Multiple effector mechanisms of allograft rejection exist, and the roles of distinct Th subsets have not been completely defined (reviewed in Refs. 1–3). The well-established Th1-Th2 paradigm (4) has been extensively investigated in the transplant setting, yet the roles of Th1 and Th2 cytokines in allograft rejection vs acceptance remain controversial (1). In wild-type (WT)\(^∗\) mice, cardiac allograft rejection is characterized by a dominant Th1 response and mononuclear cell infiltration of the transplant (5–7). These IFN-γ-producing Th1 are predominantly CD8\(^+\) and require CD4\(^+\) help (7–9) and the CD40-CD40L costimulatory pathway (7, 10) for their generation. The transcription factor T-bet is a positive regulator of the Th1 response and is expressed by CD4\(^+\) and CD8\(^+\) T cells, NK cells, dendritic cells, and B cells (reviewed in Ref. 11). T-bet induces and supports IFN-γ production (reviewed in Ref. 11) by both remodeling the IFN-γ gene (12) and inducing expression of the IL-12Rβ2 chain of the IL-12R (13). Although T-bet is critical for CD4\(^+\) T cell commitment to a Th1 lineage, CD8\(^+\) T cells may express the T-bet homologue cemoderomin (14) as an alternative Th1 transcription factor resulting in IFN-γ production. Although IFN-γ has been historically associated with allograft rejection, other studies suggest that this cytokine may be required for graft acceptance (7, 15). Hence, this study was initiated to explore the requirement for T-bet in the generation of graft-reactive Th1 and its role in allograft rejection.

IL-4-producing Th2 were initially believed to play a protective role in the transplant setting due to their ability to antagonize Th1 responses (reviewed in Ref. 1). TCR and IL-4R signaling up-regulate the Th2 transcription factor GATA3, which drives remodeling of the Th2 cytokine cluster and Th2 cytokine gene expression (16). In addition, GATA3 inhibits Th1 differentiation by suppressing STAT4 and IL-12Rβ2 expression (17). Despite this Th1-antagonizing activity, Th2 responses have been associated with an alternate form of rejection that is characterized by eosinophil and neutrophil infiltration of the graft (5, 18). Hence, the role of Th2 in transplant rejection vs acceptance remains controversial.

The role of Th17 in the transplant setting has not been explored in depth, and the few reports have been associative in nature (reviewed in Ref. 19). However, IL-17-producing Th17 have been implicated in a number of chronic inflammatory diseases (20, 21) including autoimmune myocarditis (22), allergic asthma (19), autoimmune arthritis (19), and mycobacterial infection (23). Th17-induced pathologies are characterized by granulocytic infiltration of the tissues (21). The transcription factor RORγ\(^T\) and the cytokines TGF-β and IL-6 induce IL-17 secretion, whereas IL-23 perpetuates the Th17 response (21). Negative regulators of Th17 include IFN-γ (20, 21), IL-4 (20, 21), IL-2 (24), and, interestingly, T-bet (25).

In many transplant models, disrupting CD40-CD40L costimulatory interactions is a highly effective means of preventing graft-reactive T cell priming and promoting allograft acceptance (reviewed in Ref. 26). However, this form of costimulatory blockade is less effective under certain conditions (reviewed in Refs. 27 and 28). Costimulatory blockade-resistant rejection has been attributed to redundancies in costimulatory molecules (29), a high frequency of graft-reactive T cells (30), heterologous memory cells (28), IL-2 (31), and the absence of IFN-γ (7, 15). In this study, we describe a novel form of costimulatory blockade-resistant rejection that is mediated by CD8\(^+\) Th17 in T-bet\(^{−/−}\) allograft recipients.

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\(^{4}\)Abbreviations used in this paper: WT, wild type; EAE, experimental autoimmune encephalomyelitis; EAM, experimental autoimmune myocarditis; GIC, graft-infiltrating cell.

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Materials and Methods

Culture medium

Culture medium consists of the following: RPMI 1640 supplemented with 2% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µM streptomycin, 1.6 mM L-glutamine, 10 mM HEPES buffer (all from Invitrogen), 0.27 mM t-asparagine, 1.4 mM L-arginine HCl, 14 µM folic acid, and 50 µM 2-ME (all from Sigma-Aldrich).

Mice

Female C57BL/6 WT and IFN-γ−/− (H-2b) mice, and BALB/c (H-2b) mice were purchased from The Jackson Laboratory. Breeder pairs of T-bet−/− mice (32) on the C57BL/6 background were provided by K. McDonagh (University of Michigan, Ann Arbor, MI) with permission from L. Glimcher (Harvard School of Public Health, Boston, MA). Colonies of T-bet−/− mice were established, and all mice were housed under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. Mice used were between 6 and 12 wk of age. These experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Heterotopic cardiac transplantation

C57BL/6 WT, T-bet−/−, or IFN-γ−/− mice were transplanted with intact BALB/c cardiac allografts, as described (33). In this model, the donor heart is anastomosed to the great vessels of the abdomen and perfused with the recipient mouse’s blood, and resums contraction. Transplant function was monitored by abdominal palpation.

In vivo Ab treatment

Hybridomas secreting anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), anti-IL-4 (clone 11B11), and anti-IL-6 (clone MP5-20F3) were obtained from American Type Culture Collection. The hybridoma-secreting anti-CD40L (clone MR1) was provided by R. Noelle (Dartmouth, Lebanon, NH). Anti-CD4, anti-CD8, anti-IL-4, anti-IL-6, and anti-CD40L mAb were purified and resuspended in PBS by BioExpress. Neutralizing anti-IL-17 mAb (clone 50104) was obtained from R&D Systems. Mice received 1 mg of anti-CD8, anti-CD4, and detection Abs specific for IFN-γ ELISPOT assays were performed as previously described (35). Capture ELISPOT assay for cytokine-producing cells

Allografts were recovered at the times indicated posttransplantation, fixed, and stained with Wright stain. Viability of leukocytes were enumerated by Trypan blue exclusion. For differential enumeration, GIC were placed on slides with a cytocentrifuge and stained with Wright stain. Production of cytokines was analyzed by ELISPOT assay.

In vitro depletion of T cell subsets

CD4+ or CD8+ T cells were depleted using Dynabeads (Invitrogen) as per manufacturer’s directions. Single-cell suspensions of splenocytes were incubated with anti-CD4- or anti-CD8-coated beads (5 beads to 1 target cell) for 30 min. Bead-bound cells were removed magnetically, and unbound cells were confirmed to be either CD4+ or CD8+ T cell depleted by flow cytometry (~1% contamination; FACSCalibur, BD Biosciences). Cells were then suspended at 5 × 10^6/ml in RPMI 1640 for use in ELISPOT assays.

Intracellular cytokine staining

Splenocytes and GIC from T-bet−/− allograft recipients were enriched for CD8+ cells by depleting splenocytes of B220+ and CD4+ cells and depleting GIC of Gr-1+ cells with Dynal beads. Resulting splenocyte and GIC populations were ~85 and 50% CD4+ respectively. Enriched populations were stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) and cultured with brefeldin A (BD Biosciences). Cells were then washed and permeabilized, and intracellular cytokine staining was performed using the BD Cytofix/Cytoperm fix/permeabilization kit (with BD GolgiPlug protein transport inhibitor containing brefeldin A), as per manufacturer’s instructions (BD Biosciences). Cells were dual stained with PE-conjugated anti-IL-17 and FITC-conjugated anti-IFN-γ. Cell analysis was performed on a BD Biosciences FACSCalibur.

RNA isolation and RT-PCR

Cardiac allografts were homogenized in 1 ml of TRIzol (Invitrogen Life Technologies) and RNA was isolated as per manufacturer’s protocol. Five micrograms of total RNA was reverse transcribed using 10X PCR buffer (Roche), 10 mM dNTPs, oligo(dT), Moloney murine leukemia virus reverse transcriptase (all from Invitrogen), and RNASin (Promega). Products were then cleaned with 1:1 phenol/chloroform/isoamyl alcohol (25:24:1) and reprecipitated with 2 M NaOAc in pure EtOH overnight at ~80°C.

Real-time PCR was performed on cDNA using a Rotor-Gene 3000 (Corbett Life Science). Primer binding to DNA was detected by SYBR Green I dye (BioRad). Relative expression of the gene of interest was expressed as the comparative concentration of the gene product compared with CycA product as calculated by accompanying Rotor-Gene software. Significance was determined with unpaired Student’s t test with Welch’s correction. Primer sequences are as follows: IL-7 sense, 5'-GGACTTCTCCACCCGAGTAAAG-3’; and antisense, 5’-GACCATAGCTTCCTGTTG-3’. IL-4 sense, 5’-AAGGGTGGTGACTTTACACGC-3’; and antisense, 5’-GACCATAGCTTCCTGTTG-3’. Statistical analysis

Data were analyzed with GraphPad Prism 4.0c software using unpaired Student’s t tests with Welch’s correction or log rank tests, as appropriate. Values of p < 0.05 were considered statistically significant.

Results

Unconventional rejection in T-bet−/− allograft recipients

WT and T-bet−/− mice were transplanted with cardiac allografts to study the impact of T-bet on cytokine production and allograft rejection. Allografts were acutely rejected by day 10 posttransplantation.
in both groups of recipients (Fig. 1A). However, the histopathology and composition of the GIC were strikingly different (Fig. 1, B and C). In WT recipients, rejection was characterized by a diffuse mononuclear cell infiltrate (Fig. 1B) that was composed primarily of lymphocytes and macrophages, with few granulocytes (Fig. 1C). In contrast, rejection in T-bet<sup>−/−</sup> recipients was associated with massive infiltration of the graft by neutrophils. Because granulocytic infiltration of the graft has been reported to be associated with costimulation blockade-resistant rejection (7), we treated WT and T-bet<sup>−/−</sup> allograft recipients with the anti-CD40L mAb MR1 to disrupt the CD40-CD40L costimulatory pathway. As previously reported (7, 10, 26, 36), disrupting CD40-CD40L interactions was highly effective in preventing rejection (Fig. 1A) and graft infiltration (Fig. 1B) in WT recipients. However, anti-CD40L treatment was completely ineffective at preventing acute rejection and granulocytic infiltration of the graft in T-bet<sup>−/−</sup> mice.

**Donor-reactive Th1, Th2, and Th17 responses in WT and T-bet<sup>−/−</sup> allograft recipients**

Mononuclear cell infiltration of the graft has been associated with a dominant Th1 response in this transplant model (5–8). We and others have reported that granulocytic infiltration of allografts may be associated with the induction of Th2 responses (5, 7, 18). However, Th17 responses have also been implicated in pathologies that are characterized by neutrophil infiltration of tissues (21). Hence, we used ELISPOT assays (35) to quantify in vivo primed, donor-reactive Th1 (IFN-γ), Th2 (IL-4), and Th17 (IL-17) responses by splenocytes and GIC of WT and T-bet<sup>−/−</sup> mice (Fig. 2). Allograft recipients were either unmodified or treated with anti-CD40L mAb. As expected, high numbers of donor-reactive Th1 (Fig. 2A, right panel) were present in the spleens and grafts of unmodified WT recipients. Interestingly, Th1 responses were also observed in unmodified T-bet<sup>−/−</sup> recipients, most likely due to the presence of the alternate Th1 transcription factor eomesodermin in CD8<sup>+</sup> cells (14). Indeed, depleting CD8<sup>+</sup> cells, but not CD4<sup>+</sup> cells, before addition to the ELISPOT cultures eliminated the Th1 response in T-bet<sup>−/−</sup> mice (Fig. 4A). Anti-CD40L mAb treatment of either WT or T-bet<sup>−/−</sup> allograft recipients eliminated the Th1 response (Fig. 2A, right panel).

Donor-reactive Th2 responses (Fig. 2B) were unremarkable in both WT and T-bet<sup>−/−</sup> recipients, suggesting that the aggressive neutrophil infiltration of grafts in T-bet<sup>−/−</sup> recipients (Fig. 1, B and C) was not due to the induction of Th2. However, the Th17 response (Fig. 2C) was markedly different in WT and T-bet<sup>−/−</sup> mice. Although the Th17 response was negligible in unmodified WT recipients, strong Th17 responses were observed in both the spleens and allografts of unmodified T-bet<sup>−/−</sup> mice. Of particular note, anti-CD40L treatment of T-bet<sup>−/−</sup> recipients did not eliminate the Th17 response within the allograft. Hence, costimulation blockade-resistant allograft rejection in T-bet<sup>−/−</sup> mice was associated with the presence of donor-reactive Th17 in the transplant.
IFN-γ allograft recipients do not mount Th17 responses.

We have reported that costimulation blockade-resistant rejection also occurs in IFN-γ allograft recipients and is associated with donor-reactive Th2 responses and granulocytic infiltration of the transplant. Because IFN-γ is inhibitory to Th17 (20, 21), we asked whether the absence of IFN-γ would promote donor-reactive Th17 development (Fig. 3A). Although donor-reactive Th2 were readily detectable by ELISPOT, Th17 were rare in IFN-γ mice. Because IL-4 has also been reported to be inhibitory to Th17 (20, 21), we treated IFN-γ recipients with neutralizing anti-IL-4 mAb using a dosing schedule with proven in vivo bioactivity (7). However, Th17 were also rare in this setting (Fig. 3B). To verify that sera of these mice contained adequate levels of neutralizing anti-IL-4 mAb, we used a competitive IL-4 ELISA technique (7) (Fig. 3C). This analysis revealed that a 1/10,000 dilution of sera from anti-IL-4-treated mice contained at least enough anti-IL-4 mAb to neutralize 5 ng/ml rIL-4. Hence, eliminating IFN-γ and IL-4, both known inhibitors of Th17, did not promote the

FIGURE 2. T-bet−/−, but not WT allograft recipients mount donor-reactive Th17 responses. WT and T-bet−/− allograft recipients were left either untreated or treated with anti-CD40L mAb. At the time of rejection, splenocytes (closed symbols) and GIC (open symbols) were harvested and processed for ELISPOT assays to quantify primed, donor-reactive IFN-γ (A), IL-4 (B), or IL-17 (C)-producing cells. Cells from WT recipients that were treated with anti-CD40L mAb and did not reject their allografts were harvested on day 7 posttransplant. Data points for splenocytes represent responses for individual allograft recipients. GIC from three individual mice were pooled to ensure enough cells for analysis. An insufficient number of GIC was obtained from WT recipients treated with anti-CD40L for ELISPOT analysis to be performed. Horizontal bars represent the mean + SEM IL-4-neutralizing capacity of 6 anti-IL-4-treated allograft recipients is depicted.

FIGURE 3. Eliminating IFN-γ and IL-4 does not induce donor-reactive Th17. IFN-γ−/− allograft recipients were either left untreated (A) or treated with neutralizing anti-IL-4 mAb (B). At the time of rejection (day 7 posttransplant), splenocytes were processed for ELISPOT analysis to quantify primed donor-reactive IFN-γ-, IL-4-, and IL-17-producing cells. C. To verify that adequate levels of neutralizing anti-IL-4 mAb were present in the circulation of anti-IL-4-treated mice, dilutions of sera were assessed for their ability to neutralize 2.5 (open bars) or 5 (shaded bars) ng/ml rIL-4 in a competitive ELISA. For reference, the IL-4-neutralizing capacity of diluted sera was compared with the IL-4-neutralizing capacity of 1, 0.1, and 0.01 μg/mL of purified anti-IL-4 mAb. The mean + SEM IL-4-neutralizing capacity of 6 anti-IL-4-treated allograft recipients is depicted.
emergence of Th17 in this transplant system. Because IFN-γ−/− mice express T-bet (Fig. 6B), these observations emphasize the importance of T-bet as an inhibitor of Th17 in transplantation.

CD8+ cells produce IFN-γ and IL-17 in T-bet−/− allograft recipients

To identify the T cell subset(s) that produces IFN-γ and IL-17 in T-bet−/− allograft recipients, splenocytes were harvested on day 7 posttransplant and were left intact or were depleted of CD4+ or CD8+ cells before addition to ELISPOT assays to quantify primed, donor-reactive IFN-γ- or IL-17-producing cells. Responses of individual allograft recipients are depicted, and bars represent the mean response of experimental groups. C. Splenocytes and GIC from T-bet−/− allograft recipients were enriched for CD8+ cells by depleting splenocytes of B220+ and CD4+ cells and depleting GIC of Gr-1+ cells. Enriched populations were stained for intracellular IFN-γ and IL-17. Representative flow cytometric analyses of splenocytes (n = 7) and GIC (n = 3) are depicted. Note that GIC, but not splenocytes, contain an appreciable population of cells expressing both IFN-γ and IL-17.

FIGURE 4. CD8+ T cells produce IFN-γ and IL-17 in T-bet−/− allograft recipients. Splenocytes were harvested from T-bet−/− allograft recipients on day 7 posttransplant. Splenocytes were either left untreated (■) or depleted of CD4+ (○) or CD8+ (▲) cells before addition to ELISPOT assays to quantify primed, donor-reactive IFN-γ (A)- or IL-17 (B)-producing cells. Responses of individual allograft recipients are depicted, and bars represent the mean response of experimental groups. C. Splenocytes and GIC from T-bet−/− allograft recipients were enriched for CD8+ cells by depleting splenocytes of B220+ and CD4+ cells and depleting GIC of Gr-1+ cells. Enriched populations were stained for intracellular IFN-γ and IL-17. Representative flow cytometric analyses of splenocytes (n = 7) and GIC (n = 3) are depicted. Note that GIC, but not splenocytes, contain an appreciable population of cells expressing both IFN-γ and IL-17.

FIGURE 5. CD8+ cells mediate costimulatory blockade-resistant rejection in T-bet−/− allograft recipients. WT (A) or T-bet−/− (B) allograft recipients were depleted of CD4+ (▲) or CD8+ (■) cells by i.p. injections with anti-CD4 or anti-CD8 mAb. In addition, T-bet−/− recipients were depleted of both CD4+ and CD8+ cells (▲). C. T-bet−/− recipients were treated with anti-CD40L mAb and were either initially depleted of CD4+ (▲) or CD8+ cells as above (transient depletion; ▲), or were given weekly injections of anti-CD8 mAb (continued depletion; □) to assess the contribution of CD8+ cells to costimulation blockade-resistant rejection.

Intracellular cytokine staining (Fig. 4C). Although CD8+ splenocytes produced either IFN-γ or IL-17, an appreciable population of GIC produced both cytokines.

CD8+ cells mediate costimulation blockade-resistant rejection and are the principal source of IL-17 in vivo

To identify the T cell subset responsible for costimulation blockade-resistant rejection, we depleted cardiac allograft recipients of CD4+ or CD8+ cells in vivo by perioperative injections of anti-CD4 or anti-CD8 mAb (Fig. 5). For comparison, Fig. 5A depicts T
cell subset requirements for costimulation blockade-sensitive rejection in WT mice. As previously reported (5, 7, 8), acute rejection in WT mice is dependent upon the presence of CD4+ cells, but not CD8+ T cells.

In contrast, T-bet-/- mice rejected allografts regardless of whether CD4+ or CD8+ cells were depleted (Fig. 5B), indicating that effector CD8+ cells emerge independent of CD4+ cell help. When both T cell subsets were depleted, allografts continued to function for at least 30 days posttransplant. To test whether CD8+ cells were responsible for costimulation blockade-resistant rejection, T-bet-/- allograft recipients were depleted of CD8+ cells and treated with anti-CD40L mAb (Fig. 5C). Although anti-CD40L mAb significantly prolonged graft survival in T-bet-/- recipients that were initially depleted of CD8+ cells, allografts were rejected as CD8+ cells repopulated the periphery (■). However, if CD8+ cells were continually depleted by weekly injections of anti-CD8 mAb (□), anti-CD40L therapy was effective at preventing rejection. Anti-CD40L therapy had no impact on survival when used in conjunction with CD4+ T cell depletion (●). These data indicate that CD8+ cells are responsible for costimulation blockade-resistant rejection in T-bet-/- mice.

Because ELISPOT revealed that anti-CD40L treatment did not eliminate the Th17 response within the grafts of T-bet-/- mice (Fig. 2C), we used quantitative real-time PCR to assess the level of IL-17 expression in the grafts of mice under various conditions (Fig. 6A). As expected, IL-17 was not expressed in the grafts of unmodified WT mice. In contrast, IL-17 expression was readily detectable in the grafts of WT mice depleted of CD8+ cells (T-bet-/-), indicating that CD8+ T cells are responsible for costimulation blockade-resistant rejection in WT mice.

We next asked whether CD4+ T cells were required for IL-17 expression in the grafts of T-bet-/- mice. To determine this, we treated allograft recipients with anti-CD40L mAb and either neutralizing anti-IL-17 mAb (n = 5), anti-IL-6 (n = 9), or control rat IgG (n = 7). At day 10 posttransplant, allografts were processed for H&E staining and histologic analysis (A), and RNA was isolated to assess intragraft IL-17 expression by real-time RT-PCR (B). An additional six anti-IL-6-treated recipients were harvested on day 14 posttransplant. At the termination of the experiment, all allografts placed in anti-IL-17- or anti-IL-6-treated recipients continued to function normally, whereas three of the recipients treated with control rat IgG rejected their allografts and the remaining four control allografts exhibited deteriorating function.

Neutralizing IL-17 or IL-6 reverses costimulation blockade-resistant rejection. T-bet-/- allograft recipients were treated with anti-CD40L mAb and either neutralizing anti-IL-17 mAb (n = 5), anti-IL-6 (n = 9), or control rat IgG (n = 7). At day 10 posttransplant, allografts were processed for H&E staining and histologic analysis (A), and RNA was isolated to assess intragraft IL-17 expression by real-time RT-PCR (B). An additional six anti-IL-6-treated recipients were harvested on day 14 posttransplant. At the termination of the experiment, all allografts placed in anti-IL-17- or anti-IL-6-treated recipients continued to function normally, whereas three of the recipients treated with control rat IgG rejected their allografts and the remaining four control allografts exhibited deteriorating function.
We also assessed the intragraft expression of the Th17 transcription factor RORγT (21) relative to the expression of T-bet in these mice (Fig. 6B). In this analysis, a value of 1 indicates that RORγT and T-bet are equivalently expressed relative to the housekeeping gene cyclophilin A. It should be noted that T-bet−/− mice have a nonsense transcript of the T-bet gene, thereby allowing RT-PCR amplification of the nonsense T-bet cDNA (32, 37). This analysis revealed that the intragraft RORγT:T-bet ratio mirrored the expression of IL-17 in that T-bet−/− recipients expressed higher levels of RORγT, unless they were depleted of CD8+ cells. Collectively, the data presented in Figs. 5 and 6 indicate a strong correlation between the expression of IL-17 by CD8+ cells and costimulation blockade-resistant rejection in T-bet−/− allograft recipients.

**In vivo neutralization of IL-17 or IL-6 reverses costimulation blockade-resistant rejection**

To confirm a role for IL-17 in costimulation blockade-resistant rejection, T-bet−/− allograft recipients were treated with anti-CD40L mAb and either control rat IgG or neutralizing anti-IL-17 mAb. Anti-IL-17 mAb was given i.p. at 0.2 mg every other day, similar to a dosing regimen that is effective at inhibiting IL-17-mediated experimental autoimmune encephalomyelitis (EAE) (34). Allografts continued to function normally until the termination of the experiment (day 10 posttransplant) in anti-IL-17-treated mice, whereas grafts were undergoing acute rejection in the rat IgG-treated controls. As shown in Fig. 7A, neutralizing IL-17 prevented allograft infiltration by neutrophils and preserved myocardial architecture of the graft in T-bet−/− recipients that were treated with anti-CD40L mAb. Fig. 7B revealed that neutralizing IL-17 reduced intragraft IL-17 expression relative to treatment with control rat IgG, reflecting the absence of graft infiltration. Because IL-6 is critical for the development of the Th17 lineage (21), we treated T-bet−/− allograft recipients with anti-CD40L and neutralizing anti-IL-6 mAb. Allografts continued to function normally until the termination of the experiment on day 10 (n = 3) or day 14 (n = 6) posttransplant. As was the case with anti-IL-17 treatment, neutralizing IL-6 prevented allograft infiltration, intragraft IL-17 expression, and IL-17-associated graft pathology (Fig. 7). Indeed, continued neutralization of IL-6 in anti-CD40L-treated T-bet−/− recipients prevented graft infiltration, inhibited Th17 responses, and prevented rejection for at least 50 days posttransplant (n = 5, data not shown). Thus, neutralizing IL-17, or the Th17-inducing cytokine IL-6, reversed costimulation blockade-resistant allograft rejection in T-bet−/− mice.

**Discussion**

Both Th1 and Th2 responses have been implicated in acute allograft rejection, albeit with distinct pathologies (1, 3). Th1-mediated rejection is generally associated with mononuclear cell infiltration of the graft (5–7) (Fig. 1, B and C) and CD8+ T cells are the principal source of IFN-γ in unmodified rejection. Th2-mediated rejection is associated with graft infiltration by eosinophils (5, 7, 18) and emerges under conditions in which IFN-γ is limited. The contributions of Th17 to allograft rejection have not been rigorously investigated. Although several associative studies indicate that Th17 may contribute to acute rejection (reviewed in Ref. 19), elements that regulate graft-reactive Th17 development have not been defined.

While studying the requirement for the Th1 transcription factor T-bet in graft-reactive Th1 responses, we observed that rejection in T-bet−/− recipients was characterized by heavy infiltration of the graft by neutrophils (Fig. 1, B and C). Because neutrophil-rich infiltrates are characteristic of Th17-induced pathologies (21) and T-bet has been reported to be a negative regulator of Th17 responses (25), we assessed Th1, Th2, and Th17 responses in WT and T-bet−/− allograft recipients (Fig. 2). Although the Th2 response was not remarkable in either group, strong Th17 responses were observed in T-bet−/− recipients, but were not induced in WT recipients (Fig. 2C). As expected, WT recipients mounted strong Th1 responses (Fig. 2A). T-bet−/− recipients also mounted donor-reactive Th1 responses, albeit to a lesser degree than WT mice. This is not surprising, because CD8+ cells were the principal source of IFN-γ in T-bet−/− recipients (Fig. 4A), and these cells express the alternate Th1 transcription factor eomesodermin (14). Indeed, real-time RT-PCR revealed that eomesodermin was expressed within the allografts of both WT and T-bet−/− recipients (data not shown). Interestingly, CD8+ cells were also the source of IL-17 in this system (Figs. 4B and 6A). Regarding CD4+ cells, Th1 and Th17 are believed to represent distinct lineages (20). To determine whether the same CD8+ cells produced both IFN-γ and IL-17, or if each cytokine was produced by distinct populations of CD8+ cells, we used dual intracellular cytokine staining (Fig. 4C). An appreciable number of GIC, but not splenocytes, produced both IFN-γ and IL-17. Similar observations have been reported by Korn et al. (38) in an EAE model in which IFN-γ- and IL-17-expressing cells are detectable in the inflamed CNS, but not in the draining lymph nodes. Hence, Th1 and Th17 do not necessarily represent distinct Th cell lineages. Importantly, the plasticity of these Th subsets is in part dependent on the in vivo microenvironment.

It is not clear whether CD8+ cells that produce IFN-γ and/or IL-17 are comparable to their CD4+ counterparts. We have reported that CD8+ cells are “hard wired” to produce IFN-γ, in that they do not require IL-12 to acquire a Th1 phenotype (9). Indeed, alloantigen-reactive CD4+ and CD8+ cells differ in several additional aspects, including their susceptibility to the suppressive activities of TGF-β and their relative contribution to costimulation blockade-resistant rejection (reviewed in Ref. 39).

The absence of a Th17 response in WT allograft recipients could be attributed to the overwhelming Th1 response in these mice, since IFN-γ inhibits IL-17 production (20). Indeed, in experimental autoimmune myocarditis (EAM), IFN-γ-producing CD8+ heart-infiltrating cells inhibit Th17 responses and protect against severe EAM (40). Furthermore, T-bet−/− CD8+ heart-infiltrating cells lose the capacity to release IFN-γ within the heart in EAM. Hence, it is possible that the relatively reduced Th1 response in T-bet−/− recipients (compared with WT; Fig. 2A) may result in insufficient levels of IFN-γ to inhibit Th17 induction. To this end, we assessed Th17 responses in IFN-γ−/− allograft recipients (Fig. 3). However, Th17 were not induced in IFN-γ−/− recipients, although IL-4-producing Th2 were detectable. Because IL-4 is also antagonistic to Th17 (20), we treated IFN-γ−/− allograft recipients with neutralizing anti-IL-4 mAb (Fig. 3B). However, neutralizing IL-4 failed to induce Th17 in IFN-γ−/− recipients, suggesting that T-bet, rather than IFN-γ or IL-4, negatively regulates Th17 in the transplant setting. It should be noted that Komiya et al. (41) reported that Th17 were detectable in IFN-γ−/− mice with EAE. This apparent difference from our findings may be due to the relative dominance of CD8+ (cardiac allograft rejection) vs CD4+ (EAE) cells in these distinct experimental models.

The mechanism(s) by which T-bet represses graft-reactive CD8+ Th17 responses in vivo is not yet known. In vitro, T-bet has been shown to interfere with IL-23-stimulated CD4+ Th17 development by diverting these cells into the Th1 lineage (25). IL-23 plays an important role in amplifying and/or stabilizing the Th17 phenotype (reviewed in Refs. 21 and 42). In addition, T-bet represses IL-21 transcription by inhibiting NF-ATc2.
binding to the IL-21 promoter in CD4+ cells (43). IL-21, which is produced primarily by Th17, cooperates with TGF-β in the absence of IL-6 to promote Th17 development, and therefore may serve as an autocrine amplification factor for Th17 (38). However, it is not known whether CD4+ and CD8+ Th17 have the same cytokine requirements for their development, amplification, and maintenance. The vast majority of information regarding Th17 biology has been generated in CD4+ cells (reviewed in Refs. 21, 42, and 44), whereas reports documenting CD8+ Th17 are limited (i.e., Refs. 45–48).

To our knowledge, a role for Th17 in costimulation blockade-resistant rejection has not been reported. It is unlikely that Th17-mediated costimulation blockade-resistant rejection is due to heterogeneous memory cells (28, 49), in that the mice used in these studies were maintained under pathogen-free conditions and were not subjected to overt antigenic challenge before transplantation. Indeed, primed alloantigen-reactive Th1, Th2, and Th17 are not detectable in nontransplanted T-bet−/− mice (data not shown). Furthermore, it is unlikely that resistance to costimulatory blockade is due to the absence of IFN-γ (7, 15) because graft-reactive Th1 were readily detectable in T-bet−/− allograft recipients (Fig. 2A). It should be noted that Th17 may be poorly susceptible to regulation by regulatory T cells (50), and regulatory T cells are believed to contribute to allograft acceptance following costimulatory blockade (26).

In summary, these data reveal a novel relationship between T-bet, CD8+ Th17, and costimulatory blockade-resistant allograft rejection. These findings have implications for tolerance induction in the transplant setting, and provide further insight into the complexity of Th17 biology and Th17-associated pathologies.

Disclosures

The authors have no financial conflict of interest.

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