Protective Role of T-bet and Th1 Cytokines in Pulmonary Graft-versus-Host Disease and Peribronchiolar Fibrosis

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T-box expressed in T cells (T-bet) is a critical transcription factor for T helper (Th) 1 responses. Although Th1 cells are thought to contribute to certain alloimmune responses, their role in pulmonary graft-versus-host disease (GVHD) is uncertain. We have established a murine model of acute pulmonary GVHD after hematopoietic cell transplant (HCT) and inhaled LPS exposure. We tested the hypothesis that pulmonary GVHD can occur independent of Th1 cells using T-bet–deficient donors. B10.BR($H2^k$) mice underwent allogeneic (Allo) or syngeneic (Syn) HCT with cells from either C57Bl/6J($H2^b$) mice (Allo wild-type [WT] or SynWT) or C57Bl/6J mice lacking T-bet ($AlloTbet^{-/-}$ or $SynTbet^{-/-}$). After HCT, mice were exposed daily to aerosolized LPS and subsequently bronchoalveolar lavage and lung tissue were analyzed for cytokines, lymphocytic inflammation, pathology, and fibrosis. Independent of LPS exposure, $AlloTbet^{-/-}$ mice developed pulmonary GVHD manifested by lymphocytic inflammation. Furthermore, $AlloTbet^{-/-}$ mice developed features of chronic pulmonary GVHD, including increased peribronchiolar fibrosis and collagen content. LPS exposure increased neutrophil recruitment and decreased static compliance in $AlloTbet^{-/-}$ mice as compared with LPS-exposed AlloWT mice or LPS-exposed $SynTbet^{-/-}$ mice. In addition, LPS-exposed $AlloTbet^{-/-}$ mice had increased pulmonary IL-17, IL-13, and Th17 cells, and diminished regulatory T cells compared with the other groups. Our results demonstrate that Th1 cytokines are dispensable in pulmonary GVHD. In the absence of T-bet, there is increased production of Th17 and Th2 cytokines that is associated with peribronchiolar fibrosis and is further enhanced by LPS. These results suggest that the interplay between local innate immunity and non-Th1 T cell subsets contribute to chronic pulmonary GVHD.

Keywords: pulmonary graft-versus-host disease; T helper cell type 1; IL-17; LPS; peribronchiolar fibrosis

CLINICAL RELEVANCE

Alloimmune lung injury in the form of pulmonary graft-versus-host disease (GVHD) is increasingly recognized, and causes significant morbidity and mortality among hematopoietic cell transplant recipients. Our results demonstrate that T helper (Th) type 1 cytokines are dispensable in pulmonary GVHD and, in the absence of T-box expressed in T cells, there is increased production of Th17 and Th2 cytokines that is associated with peribronchiolar fibrosis and is further enhanced by LPS. These results suggest that the interplay between local innate immunity and non-Th1 T cell subsets contributes to chronic pulmonary GVHD.

Hematopoietic cell transplant (HCT) is widely used as a treatment for hematologic malignancies, nonmalignant hematologic diseases, and autoimmune diseases (reviewed in Ref. 1). However, a common complication after HCT is graft-versus-host disease (GVHD), wherein donor immune cells attack mucosal organs, such as the skin, gastrointestinal tract, liver, and lungs (2, 3). Alloimmune lung injury in the form of pulmonary GVHD is increasingly recognized, and causes significant morbidity and mortality among HCT recipients (4–6). Acute pulmonary GVHD occurs early after transplantation, and is characterized by the influx of donor lymphocytes into the lungs (7), whereas chronic pulmonary GVHD is a later manifestation after HCT with features of progressive airflow obstruction and small airway fibrosis (8, 9).

Despite the importance of pulmonary GVHD, little is known regarding the T cell subsets responsible for mediating this disease. T helper (Th) 1 cytokines, such as IFN-γ, TNF-α, and IL-2, are produced by multiple cell types in response to many innate stimuli, including LPS and can regulate both innate and adaptive immune responses (reviewed in Ref. 10). Previous studies have noted that lung injury in recipients after allogeneic (Allo) HCT is associated with increased levels of TNF-α, IFN-γ, and alloreactive T cells in the bronchoalveolar lavage (BAL) (11) and, thus, Th1 cells are generally thought to play a prominent role in the development of pulmonary GVHD (12). However, increasingly complex families of T cell subset are now recognized, including Th2 cells, Th17 cells, regulatory T cells (Tregs), and Th22 cells (13). The importance of these different T cell subsets in pulmonary GVHD relative to Th1 cells remains uncertain.

To further investigate the importance of Th1 cells in pulmonary GVHD, we used a model of pulmonary GVHD established in our laboratory, which involves daily, subacute inhaled LPS exposures. The LPS exposure replicates clinically relevant environmental lung exposures after Allo HCT (14, 15). We hypothesized that Th1 cells were dispensable in the development of pulmonary GVHD. T-box expressed in T cells (T-bet) is a transcription factor that is critical in the polarization of naive T cells toward Th1 development. Mice deficient in T-bet ($Tbet^{-/-}$) fail to generate Th1 cytokines, such as IFN-γ, but have normal numbers of $CD4^+$ and $CD8^+$ lymphocytes and recruitment (16). Therefore, to test our hypothesis and determine if Th1 polarization is critical for the development of environmentally induced pulmonary GVHD, we performed HCT using $Tbet^{-/-}$ donor mice in this model system. Furthermore, we sought to

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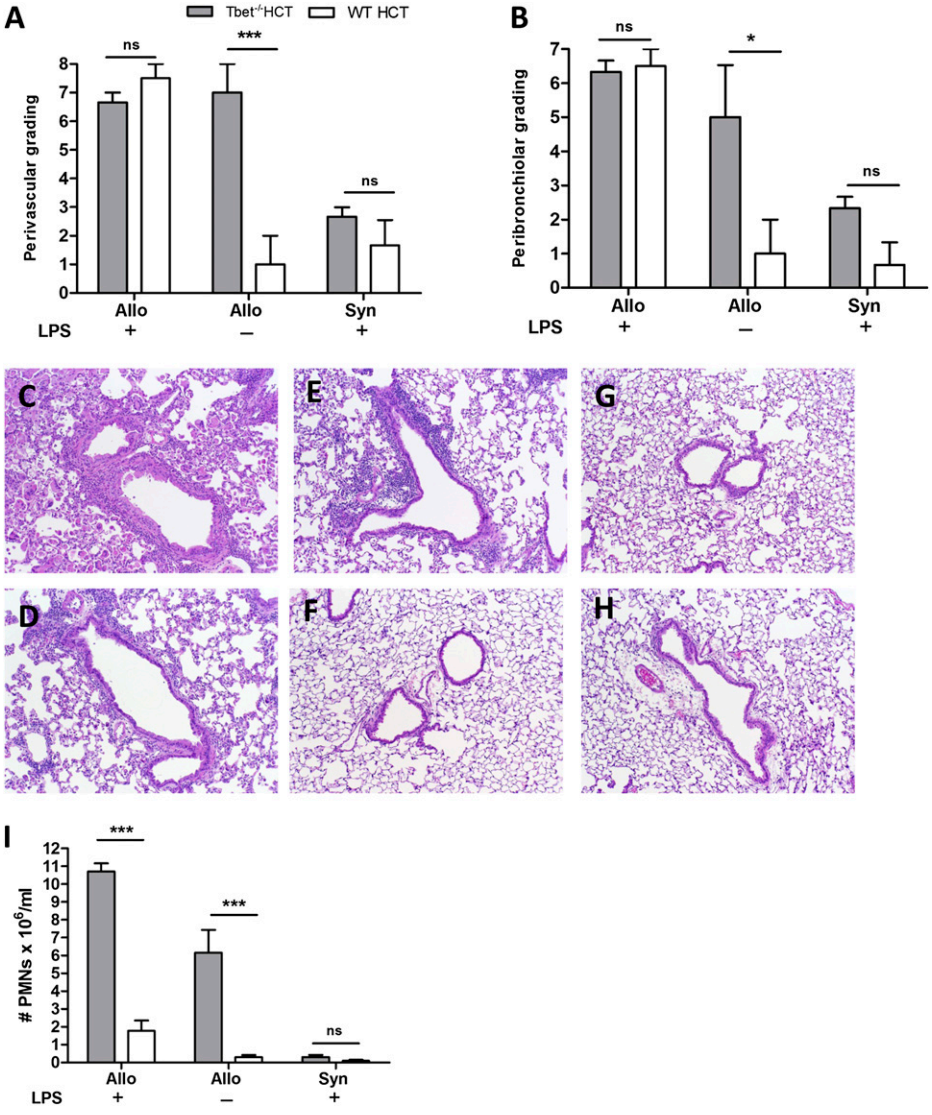

Figure 1. Pulmonary graft-versus-host disease (GVHD) is increased in allogeneic (Allo) hematopoietic cell transplant (HCT) mice reconstituted with donor cells deficient in T-box expressed in T cells (T-bet). Allo HCT (AlloHCT) or syngeneic (Syn) HCT (SynHCT) mice reconstituted with cells from Tbet$^{-/-}$ or wild-type (WT) control animals were exposed to subacute levels of inhaled LPS for 5 days or unexposed and killed 72 hours after the last exposure. Lung pathology was evaluated and pathology grades were determined in a blinded fashion using a semiquantitative scoring system based on the thickness of perivascular and peribronchiolar inflammation, as well as the overall percentage of lung involved. (*A*) Lymphocytic perivascular and (*B*) peribronchiolar inflammation. (*C–H*) Representative histology sections are shown for all groups (hematoxylin and eosin stain, 100× magnification): (*C*) AlloTbet$^{-/-}$ + LPS; (*D*) AlloTbet$^{-/-}$; (*E*) AlloWT + LPS; (*F*) AlloWT; (*G*) SynTbet$^{-/-}$ + LPS; and (*H*) SynWT +LPS. (*I*) Absolute numbers of neutrophils in bronchoalveolar; lavage (BAL). (n = 3–8/group; data replicated in two independent experiments; graph is representative of one experiment). *$P < 0.05$; ***$P < 0.0001$; ns, not significant.

determine if Th2 or Th17 responses predominate in the absence of T-bet and to assess the extent to which this T cell subset polarization affects the biological, pathological, and physiological features of pulmonary GVHD.

MATERIALS AND METHODS

Mice

Experiments were approved by the Institutional Animal Care and Use Committee. Male 8-week-old C57BL/6J(H2^b), B10.BR-*H2^{k}H2-T18^a*/SgSnJ(H2^k), and Tbet$^{-/-}$ mice were purchased (Jackson Laboratories, Bar Harbor, ME) and housed on LPS-free bedding (Shepherd Papers, Kalamazoo, MI) with irradiated food (Purina, Richmond, IN) and antibiotic water (Sulfamethoxazole/Trimethoprim 1.2/0.24 mg/ml).

Murine Hematopoietic Cell Transplantation

Donor mice (C57BL/6, or Tbet$^{-/-}$) were killed using CO_2. Bone marrow and splenocytes were resuspended in FBS-containing media. Recipient B10.BR mice were lethally irradiated using a cesium irradiator (8 Gy) and injected retro-orbitally with 4×10^6 bone marrow cells and 1×10^6 splenocytes. Engraftment was evaluated 4 weeks posttransplantation using anti–H2D^b-FITC and anti–H2K^k-R-phycoerythrin (BD Biosciences, San Jose, CA). Mice were more than 95% engrafted.

LPS Exposures

LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) was aerosolized by a six-jet atomizer 9,306 (TSI Inc., Shoreview, MN) at 35 psi, flow rate of 3.3 L/minute, for a final concentration of 4.5 μg/m^3. Mice were exposed to LPS (2.5 h/d, 5 d) for 5 weeks after HCT, and were killed 72 hours after last exposure.

BAL and Analysis

Lungs were lavaged with 0.9% saline, and supernatant was analyzed using an IL-17 ELISA kit (R&D systems, Minneapolis, MN).

Lung Tissue Analysis

The accessory lobe of the right lung was preserved in RNAlater (Ambion/Applied Biosystems, Austin, TX). RNA was extracted, reverse transcribed, and used for RT-PCR for FoxP3 (Mm00475162_m1), IL-17a (Mm00439618_m1), procollagen 1 (Mm00483403_g1), IL-4 (Mm00445258_g1), IFN-γ (Mm99999071_m1), IL-13 (Mm00434204_m1), and endogenous β-actin control (4352933) (Applied Biosystems, Foster City, CA). Ct values were determined using ABI 7,500 Real-Time PCR System (Applied Biosystems). Change in expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

The right lung was processed for hydroxyproline assay (17). The left lung was fixed in 10% buffered formalin and paraffin embedded.

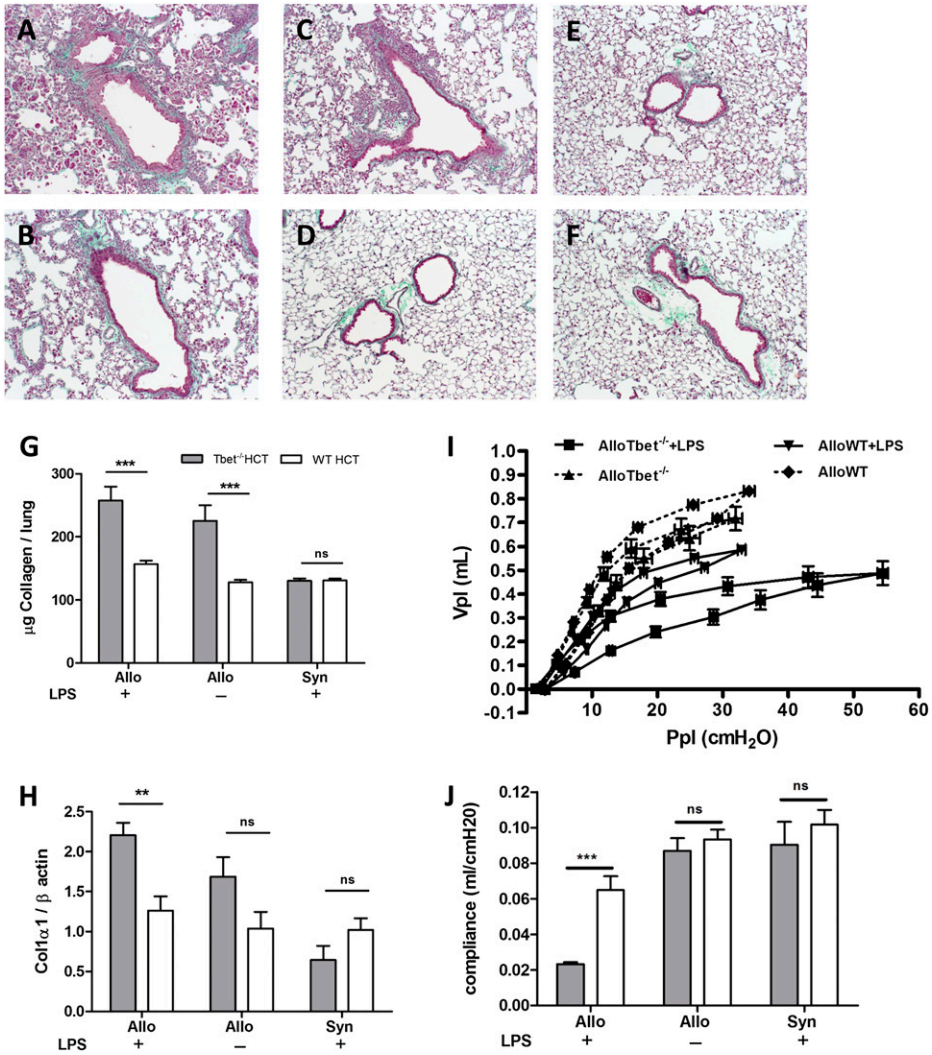

Figure 2. T-bet deficiency leads to features of chronic pulmonary GVHD after AlloHCT. After AlloHCT or SynHCT and exposure to subacute levels of inhaled LPS, lung tissue from mice reconstituted with cells from Tbet$^{-/-}$ or WT control animals was collected and assayed for collagen content using a conventional hydroxyproline assay and analyzed by RT-PCR. Pulmonary function was assessed in all experimental groups by *flexiVent*. (*A–F*) Representative histology sections are shown for all groups (Masson trichrome stain, 100× magnification): (*A*) AlloTbet$^{-/-}$ + LPS; (*B*) AlloTbet$^{-/-}$; (*C*) AlloWT + LPS; (*D*) AlloWT; (*E*) SynTbet$^{-/-}$ + LPS; and (*F*) SynWT +LPS. (*G*) Collagen content in lung tissue measured by hydroxyproline assay. (*H*) RNA from lung tissue was assayed for procollagen 1 (Col1a1) transcripts by real-time PCR. (*I*) Pressure–volume loops were measured directly *in vivo* to determine any changes in lung compliance. (*J*) Static lung compliance was calculated from pressure–volume curves and compared between treatment groups (n = 3–8/group; data replicated in two independent experiments; graph represents data of one experiment). *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$; ns, not significant; Ppl, pressure plateau pressure; Vpl, volume plateau pressure.

Sections (5 μm) were stained with hematoxylin and eosin and Masson trichrome. Perivascular and peribronchial lymphocytic inflammation was graded as described previously (14).

BAL Flow Cytometry

BAL cells were labeled with anti–CD3–FITC, anti–CD8–PE–Cy7, anti–CD11b–Allophycocyanin–Cy7, and anti–CD4–PE–Cy5 (eBioscience, San Diego, CA). For intracellular staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) and incubated with GolgiStop (BD Biosciences) before fixing, permeabilizing, and staining with anti–IL–17A–PE (eBioscience) and anti–IFN-γ–Allophycocyanin (eBioscience). For FoxP3 staining, cells were labeled with anti–FoxP3–PE (eBioscience). FlowJo software (Tree Star Inc., Ashland, OR) was used for analysis. Cell percentages were converted to absolute numbers by multiplying by live cell counts.

Pressure–Volume Curves

Mice were anesthetized (60 mg/kg pentobarbital) and placed on a computer-controlled ventilator (flexiVent; SCIREQ, Montreal, PQ, Canada) at a tidal volume of 7.5 ml/kg and a peak expiratory pressure of 3 cm H_2O. Mice then received neuromuscular blockade (0.8 mg/kg pancuronium bromide) and allowed to adjust to the ventilator. Compliance was monitored from pressure and volume data that was generated by applying a 2-second sine wave volume perturbation with an amplitude of 0.2 ml and a frequency of 2.5 Hz. Pressure–volume curves were generated by step-wise (seven equal steps) inflation and deflation of the lungs (18).

Statistical Analysis

Data are expressed as mean (±SEM). Two-tailed Student's t tests were performed to determine if Tbet$^{-/-}$ HCT mice were significantly different from wild-type (WT) HCT mice in LPS-exposed Allo, unexposed Allo, and LPS-exposed Syn mice. Data were replicated in two independent experiments.

RESULTS

AlloTbet$^{-/-}$ Mice Develop Pulmonary GVHD after HCT Independent of LPS

B10.BR recipient mice that had undergone Allo HCT (Allo) with donor cells from T-bet$^{-/-}$ mice (AlloTbet$^{-/-}$) and were exposed to 5 days of subacute inhaled LPS (2.5 h/d) had significant perivascular and peribronchiolar lymphocytic inflammation indicative of alloimmune lung injury (Figures 1A–1C). However, unexposed AlloTbet$^{-/-}$ mice also had significantly increased perivascular and peribronchiolar pathology (Figures 1A, 1B, and 1D) that was more severe than unexposed AlloWT (Figures 1A, 1B, and 1E) and comparable to LPS-exposed AlloWT (Figures 1A, 1B, and 1F). Pathology seen in AlloTbet$^{-/-}$ exposed or

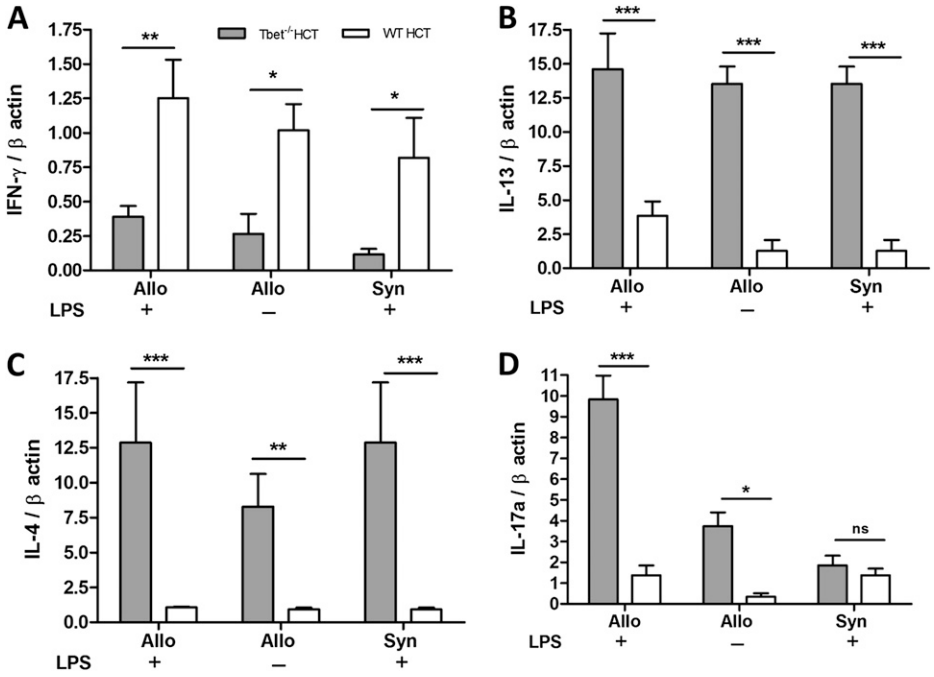

Figure 3. T-bet deficiency after AlloHCT promotes the up-regulation of Th17 and Th2 cytokines over Th1 cytokines in chronic pulmonary GVHD. After HCT and exposure to subacute levels of inhaled LPS, lung tissue was analyzed by real-time PCR for cytokine expression. (*A*) IFN-γ (*B*) IL-4 (*C*) IL-13, and (*D*) IL-17a transcripts ($n = 3$–8/group; data replicated in two independent experiments; graph represents data of one experiment). $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; ns, not significant.

unexposed had a significant accumulation of intra-alveolar macrophages and neutrophils that was not noted in AlloWT. Minimal pathology was seen with mice that had undergone syngeneic (Syn) HCT and had been exposed to LPS (SynTbet$^{-/-}$ or SynWT) (Figures 1A, 1B, 1G, and 1H). At 72 hours after LPS exposure, the neutrophil influx in AlloHCT usually has resolved in our model of acute pulmonary GVHD. However, BAL differential cell counts confirmed that AlloTbet$^{-/-}$ mice had a significant increase in neutrophil influx in the BAL that was enhanced with LPS exposure when compared with AlloWT and Syn control animals (Figure 1I). The pulmonary pathology seen in AlloTbet$^{-/-}$ mice did not have any systemic effects, as reflected by no significant differences in body weights after HCT (data not shown). Mice that had not undergone an HCT (nontransplanted [NT]), whether deficient (Tbet$^{-/-}$) or sufficient (WT) in T-bet, did not develop any significant pathological changes or neutrophil recruitment in BAL after 1 week of LPS when measured 72 hours after the last exposure (*see* Figure E1 in the online supplement).

AlloTbet$^{-/-}$ Mice Have Features of Chronic Pulmonary GVHD

AlloTbet$^{-/-}$ mice had significantly increased collagen deposition around the airways as well as intraalveolar macrophage accumulation consistent with features of chronic pulmonary GVHD (Figure 2B). However, when exposed to LPS, peribronchiolar fibrosis was significantly augmented in AlloTbet$^{-/-}$ mice (Figure 2A). Masson trichrome staining confirmed increased collagen in AlloTbet$^{-/-}$ mice exposed to LPS or unexposed, localized around airways with little to no staining seen in the interstitial spaces (Figures 2A and 2B). These features of chronic pulmonary GVHD were not seen in AlloWT mice whether exposed to LPS or unexposed (Figures 2C and 2D). Peribronchiolar fibrosis occurred only in an alloimmune environment, as no collagen deposition was seen in SynTbet$^{-/-}$ or SynWT mice whether exposed to LPS (Figures 2E and 2G) or unexposed (data not shown).

To quantify this peribronchiolar fibrosis, lung tissue was used for hydroxyproline assay and procollagen 1 transcript analysis (Figures 2G and 2H). AlloTbet$^{-/-}$ mice exposed to LPS and those unexposed had a significant increase in collagen content and procollagen 1 mRNA when compared with AlloWT control animals (Figures 2G and 2H). AlloWT, SynTbet$^{-/-}$, and SynWT mice had no increase in collagen content or increase in markers associated with increased collagen regardless of exposure (Figures 2G and 2H).

AlloTbet$^{-/-}$ Mice Exposed to LPS Have Decreased Lung Compliance

To analyze the physiological consequences of increased peribronchiolar fibrosis, pressure–volume curves and static lung compliance were directly measured *in vivo* under anesthesia (Figures 2I and 2J). After Allo or Syn HCT with WT donor cells, static lung compliance was normal. However, AlloTbet$^{-/-}$ mice exposed to LPS had a robust shift in the pressure–volume curve indicative of a decrease in lung compliance. This physiological change was reflected in a significant decrease in static compliance in AlloTbet$^{-/-}$ mice exposed to LPS (Figure 2J). These physiological changes were not seen in Syn HCT or NT Tbet$^{-/-}$ mice (Figures 2I and 2J and Figure E2).

AlloTbet$^{-/-}$ Mice Have Increased Pulmonary Th17 and Th2 Cytokine Expression

To identify mediators that may potentiate the features of chronic pulmonary GVHD seen in AlloTbet$^{-/-}$ mice with or without LPS exposure, whole-lung RNA was isolated and transcript levels of Th1, Th2, and Th17 cytokines were analyzed (Figure 3). AlloWT mice exposed to LPS had increased expression of the Th1 cytokine, IFN-γ, compared with unexposed AlloWT mice (Figure 3A). However, AlloTbet$^{-/-}$ and SynTbet$^{-/-}$ mice had significantly less IFN-γ expression when compared with WT control animals (Figure 3A). AlloTbet$^{-/-}$ and SynTbet$^{-/-}$ mice had a significant increase in the expression of Th2 cytokines IL-13 and IL-4 compared with WT control animals, regardless of exposure to LPS (Figures 3B and 3C). Pulmonary IL-17a expression was significantly increased and unique to AlloTbet$^{-/-}$ mice, and was exacerbated by LPS exposure when compared with AlloWT and Syn control animals (Figure 3D).

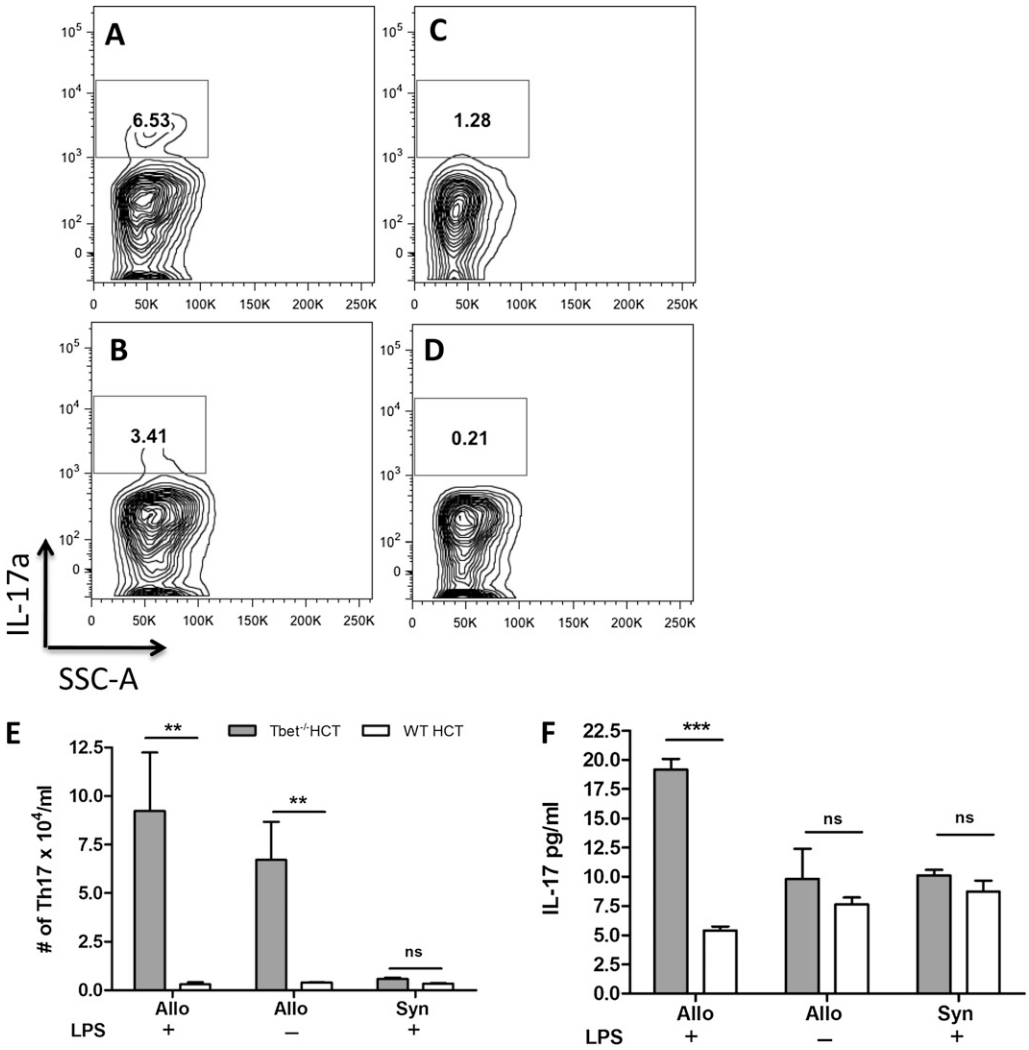

Figure 4. T-bet deficiency promotes Th17 cell recruitment and IL-17 production after AlloHCT. After HCT and exposure to subacute levels of inhaled LPS, BAL cells were analyzed by flow cytometry. (*A–D*) Representative flow cytometric plots show $CD3^+CD4^+IL\text{-}17^+$ (Th17) populations for each experimental group: (*A*) AlloTbet$^{-/-}$ + LPS; (*B*) AlloTbet$^{-/-}$; (*C*) AlloWT + LPS; and (*D*) AlloWT. (*E*) Absolute numbers of Th17 cells. (*F*) BAL cytokine analysis for IL-17 protein levels in BAL fluid (n = 3–8/group; data replicated in two independent experiments; graph represents data of one experiment). $^{**}P < 0.001$; $^{***}P < 0.0001$; ns, not significant.

AlloTbet$^{-/-}$ Mice Have Increased Pulmonary Th17 Cells that Are Further Augmented by LPS

AlloWT mice, whether exposed to LPS or unexposed, had no Th17 cells ($CD3^+CD4^+IL\text{-}17a^+$) in the BAL (Figures 4C and 4D). However, after Allo HCT with Tbet$^{-/-}$ donor cells, a population of Th17 cells was identified in the BAL (~3% of $CD3^+CD4^+$ cells) that was increased with LPS exposure (~6% of $CD3^+CD4^+$ cells) (Figures 4A and 4B). This population of T cells was only observed in the lung, not in the spleen, and was unique to the $CD4^+$ population, as no $CD8^+$ production was seen (Figures E4C–E4F). These trends were also reflected in the absolute numbers of Th17 cells in the BAL (Figure 4E). IL-17 production in BAL was also verified by ELISA and followed a similar trend as intracellular staining, with AlloTbet$^{-/-}$ mice exposed to LPS having significantly more IL-17 than AlloWT control animals (Figure 4F). SynTbet$^{-/-}$ and SynWT mice exposed to LPS did not exhibit Th17 cell recruitment or IL-17 production in the BAL (Figures 4E and 4F). Th17 cell recruitment and IL-17 production was not seen in NT Tbet$^{-/-}$ when compared with NT WT mice (Figure E3).

AlloTbet$^{-/-}$ Mice Have Decreased Tregs

Similar numbers of CD4 and CD8 T cells were seen in the lungs of alloTbet-sufficient and -deficient mice with or without LPS exposures (Figures E4A and E4B). However, in AlloWT mice, approximately 9% of $CD3^+$ T cells in BAL were Tregs ($CD4^+CD25^+Foxp3^+$) that increased to approximately 15% with LPS exposure (Figures 5C and 5D). However, in AlloTbet$^{-/-}$ mice with or without LPS exposure, the Treg population in the BAL was decreased to approximately 50% of the levels seen in WT control animals (Figures 5A and 5B). No differences in Treg numbers were noted in the spleen (data not shown). This decrease in Tregs was not seen in the absolute numbers of Tregs in BAL with SynTbet$^{-/-}$ mice exposed to LPS when compared with SynWT control animals (Figure 5E). To further validate the decrease in Tregs in the lungs of AlloTbet$^{-/-}$ mice, lung RNA was examined for FoxP3 expression by real-time PCR (Figure 5F). FoxP3 mRNA was significantly less abundant in lung tissue of AlloTbet$^{-/-}$ mice exposed to LPS when compared with AlloWT control animals.

DISCUSSION

GVHD pathophysiology is complex and not well understood, but is critically dependent on the differentiation of donor-derived T cells into subsets (19). Furthermore, which T cell subsets are necessary for the development of pulmonary GVHD has not been fully characterized, although Th1 cells have been implicated in many features of GVHD (12). Recently, a protective role for the Th1 cytokine, IFN-γ, has also been discovered (20). The current study presents novel data that shows that Th1 cytokines

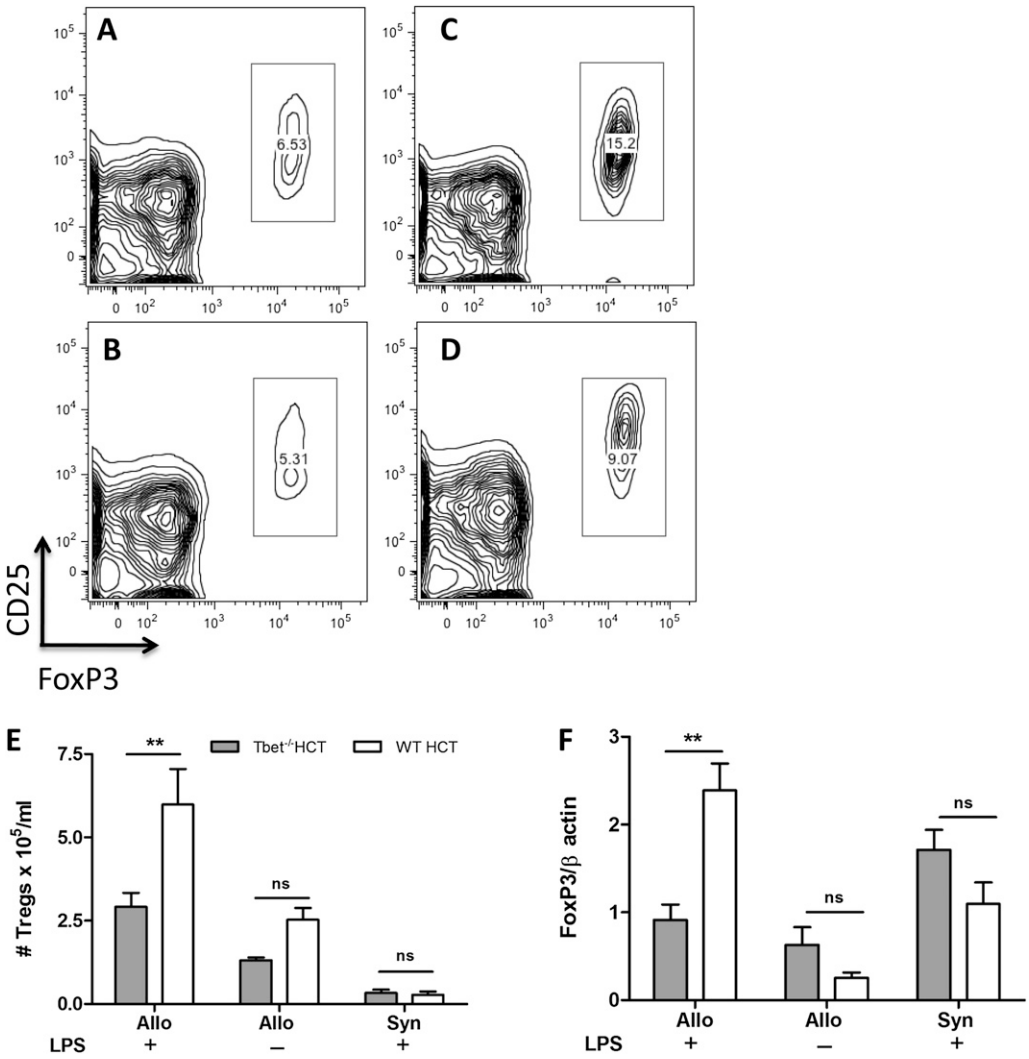

Figure 5. T-bet deficiency in AlloHCT decreases regulatory T cell differentiation during pulmonary GVHD. AlloHCT mice were reconstituted with Tbet$^{-/-}$ or sufficient WT hematopoietic cells and subsequent exposure to subacute levels of inhaled LPS. BAL cells and lung tissue were analyzed by flow cytometry and RT-PCR. (*A–D*) Representative flow cytometric plots show CD3^{+} CD4^{+}CD25^{+}FoxP3^{+} (regulatory T cell [Treg]) populations in BAL cells for each experimental group: (*A*) AlloTbet$^{-/-}$ + LPS; (*B*) AlloTbet$^{-/-}$; (*C*) AlloWT + LPS; and (*D*) AlloWT. (*E*) Absolute numbers of Tregs in BAL. (*F*) mRNA expression of FoxP3 in lung tissue ($n = 3$–8/group; data replicated in two independent experiments; graph represents data of one experiment). $^{**}P < 0.001$; $^{***}P < 0.0001$; ns, not significant; SSC-A, side scatter area.

are dispensable in the development of pulmonary GVHD, and highlights the potential importance of Th17 or Th2 cytokines in the development of chronic pulmonary GVHD and associated airway fibrosis.

Pulmonary complications after HCT are increasingly recognized as a significant cause of morbidity and mortality (21). However, the mechanisms behind pulmonary GVHD have yet to be fully elucidated, and there are no established effective treatments for chronic graft rejection. This, in part, reflects the lack of an adequate animal model, particularly one that replicates features of chronic pulmonary GVHD (22–24). Previous reports by our laboratory and others have been able to model components of acute pulmonary GVHD, such as idiopathic pneumonia syndrome and lymphocytic bronchiolitis, but only a single report reproduced features of chronic pulmonary GVHD in mice (25). In that study, recipient mice were first conditioned with a chemotherapeutic agent before lethal irradiation, and then reconstituted with Allo T cell–depleted bone marrow and splenocytes. This resulted in airway obstruction, peribronchiolar and perivascular lymphocytic inflammation, pulmonary epithelial changes, and prominent inflammatory cytokines in the BAL. However, penetration was variable, and not all mice developed chronic pathological changes of chronic pulmonary GVHD. Furthermore, the extent to which various T cell subsets participate in the development of chronic pathology in that model was not assessed.

Our current results provide *in vivo* evidence that the production of Th1 cytokines by donor cells may have a protective role in pulmonary GVHD and airway fibrosis. The extent to which this finding applies to other forms of GVHD is uncertain, as our current model replicates features of pulmonary, not systemic, GVHD. Consistent with this point, in our current study no significant changes in body weight were noted after HCT, regardless of T-bet deficiency. Although we did not specifically examine extrapulmonary tissues in this study, our previous work suggests that minimal extrapulmonary GVHD occurs in this model (14, 26). Contrary to earlier reports that linked IFN-γ production to the severity of GVHD (27), more recent studies have implicated a protective role for this Th1 cytokine in GVHD and models of lung fibrosis (28, 29). In the context of GVHD, there appears to be a dual role for IFN-γ, as previous studies have shown that production of this Th1 cytokine is a key mediator in amplifying T cell activation (reviewed in Ref. 30). In contrast, the absence of IFN-γ is also associated with enhanced GVHD (27), and treatment with IFN-γ has been shown to decrease the morbidity associated GVHD (31). In models of lung fibrosis, increased levels of IFN-γ have been shown to reduce bleomycin-induced pulmonary fibrosis (29) and inhibit

the growth of normal human lung fibroblasts and fibroblast proliferation after bleomycin (32, 33). Our results are consistent with the more recent studies that demonstrate that IFN-γ has a beneficial effect and can limit certain types of T cell–driven inflammation in pulmonary GVHD. Regardless of whether IFN-γ acts directly on fibroblasts or T cells, its presence appears to play a protective role in chronic pulmonary GVHD-associated peribronchiolar fibrosis in the current studies.

Although we demonstrate that Th1 cytokines are dispensable in the development of pulmonary GVHD, it remains to be determined which of the remaining T cell subsets contributes to the development of disease in the absence of donor T-bet. Th2 T cells have been associated with chronic lung transplant rejection, a process that is immunologically similar to chronic pulmonary GVHD. In a recent study, BAL from patients with chronic lung transplant rejection had elevated levels of IL-13, and mice that had undergone an Allo heterotopic tracheal transplant exhibited elevated levels of IL-13 and its receptors (34). Overexpression of IL-13 has also been implicated in bleomycin-induced lung fibrosis (34, 35). Consistent with these other models, our data suggest that Th2 cytokine production represents a potential mechanism leading to airway fibrosis in the setting of chronic pulmonary GVHD.

T-bet not only induces Th1 development, but influences the development of Th17 T cells by inhibiting transcription of RAR-related orphan receptor gamma (36, 37). Our results demonstrate that the lack of T-bet in Allo donor cells promotes the differentiation of Th17 cells and subsequent pulmonary fibrosis that includes a phenotypic decrease in lung compliance. This effect was unique to the Allo lung setting, as Th17 cells were not noted systemically or in Syn control animals. Recent studies have implicated Th17 cells as an important T cell subset in transplant-related lung disease (38–40) and models of pulmonary fibrosis (41, 42). *In vitro*–differentiated Th17 cells have also been associated with severe acute pulmonary GVHD (38). This finding is unique to the $CD4^+$ T cell population in our model, as no $CD8^+$ T cell production was noted (Figure E4). This is consistent with a previous study of chronic cardiac transplant rejection (37) where CD4 T cell depletion prevented IL-17 production and the development of rejection in $Tbet^{-/-}$ mice. In contrast, in another cardiac rejection model involving acute rejection, $CD8^+$ T cell production of IL-17 that was responsible for the allograft rejection (43). Further studies to determine the precise role of CD4 versus CD8 cells in our model would be useful to further elucidate the mechanisms of pulmonary graft versus host disease in Allo HCT.

In the current study, the increase in Th17 cells in the lungs of $AlloTbet^{-/-}$ mice was augmented by exposure to the Toll-like receptor 4 ligand, LPS, resulting in a decrease in static compliance. This is consistent with recent studies that have reported that LPS can induce IL-17 production *in vitro* by promoting macrophage production of polarizing cytokines, such as IL-23, IL-6, and TNF-α (44, 45). Although not quantified through pathology grading, there was an increase in myeloid cell accumulation in the alveolar spaces of $AlloTbet^{-/-}$ mice exposed to LPS. These cells could be the source of the proinflammatory cytokines responsible for increased Th17 cells and decreased compliance in $AlloTbet^{-/-}$ mice exposed to LPS. Previous studies have also shown that increased neutrophil infiltration into the alveolar spaces, and IL-17 production can have a detrimental effect and lead to a decrease in pulmonary compliance (18, 46). The elevated number of Th17 cells in the $AlloTbet^{-/-}$ mice exposed to LPS when compared with unexposed $AlloTbet^{-/-}$ mice could be explained by enhanced Th17 cell survival due to proinflammatory cytokine production by activation of Toll-like receptor 4 on antigen-presenting cells, which recruits myeloid cells into the interstitial spaces causing decreased pulmonary compliance.

The transcription factor RAR-related orphan receptor gamma that is responsible for Th17 polarization has previously been reported to block the induction of Tregs (reviewed in Ref. 47). Our data mirror the counteractive balance of Th17 and Tregs with a significant reduction of Tregs in the BAL of AlloHCT mice given donor cells deficient in T-bet. Previous studies have demonstrated that freshly isolated or *ex vivo*–cultured donor Tregs are able to delay or prevent GVHD (48–50). Consistent with these data, depletion of Tregs increases the incidence and severity of acute GVHD *in vivo* (51). Therefore, the presence of Tregs appears to be critical for immune tolerance in preventing pulmonary GVHD. The decrease in the presence of Tregs in the BAL in our data demonstrates an additional potential cellular mechanism by which pulmonary GVHD is exacerbated.

In conclusion, our findings in this study demonstrate that Th1 cytokine production by donor cells is not only dispensable in the development of pulmonary GVHD, but also serves to protect the lung from chronic GVHD-related airway fibrosis. Although our current study did not determine the precise mechanisms that lead to chronic pulmonary GVHD, we have identified increased Th17 and Th2 cytokines in the lung fluid and tissues in association with allotransplant peribronchiolar fibrosis, suggesting a potential role for these T cell subsets in mediating chronic disease. Blocking IL-17 and/or IL-13 presents viable approaches to pursue in future studies in an attempt to develop new avenues for treating patients and reducing the burden of chronic pulmonary GVHD after HCT.

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