



EUROPEAN  
HEMATOLOGY  
ASSOCIATION



Ferrata Storti  
Foundation

# Tbet is a critical modulator of FoxP3 expression in autoimmune graft-versus-host disease

Shoba Amarnath,<sup>1</sup> Arian Laurence,<sup>1</sup> Nathaniel Zhu,<sup>2</sup> Renato Cunha,<sup>2</sup> Michael A. Eckhaus,<sup>3</sup> Samuel Taylor,<sup>2</sup> Jason E. Foley,<sup>2</sup> Monalisa Ghosh,<sup>2</sup> Tania C. Felizardo<sup>2</sup> and Daniel H. Fowler<sup>2</sup>

<sup>1</sup>Institute of Cellular Medicine, Newcastle University, Newcastle Upon Tyne, UK,

<sup>2</sup>Experimental Transplantation Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA and <sup>3</sup>Division of Veterinary Resources, Office of Research Services, Bethesda, MD, USA

Haematologica 2017  
Volume 102(8):1446-1456

## ABSTRACT

CD4<sup>+</sup> T-helper subsets drive autoimmune chronic graft-versus-host disease, a major complication after allogeneic bone marrow transplantation. However, it remains unclear how specific T-helper subsets contribute to chronic graft-versus-host disease. T-helper type 1 cells are one of the major disease-mediating T-cell subsets and require interferon- $\gamma$  signaling and Tbet expression for their function. Regulatory T cells on the other hand can inhibit T-helper type 1 cell-mediated responses. Using an established murine model that isolates the autoimmune component of graft-versus-host disease, we hypothesized that T-helper type 1 cells would restrict FoxP3-driven regulatory T cells. Upon transfer into immune-deficient syngeneic hosts, alloreactive *Tbx21*<sup>-/-</sup> CD4<sup>+</sup> T cells led to marked increases in FoxP3<sup>+</sup> cells and reduced clinical evidence of autoimmunity. To evaluate whether peripheral induction contributed to regulatory T-cell predominance, we adoptively transferred *Tbx21*<sup>-/-</sup> T cells that consisted of fate mapping for FoxP3: recipients of flow-purified effector cells that were Foxp3<sup>-</sup> and *Tbx21*<sup>-/-</sup> had enhanced T-regulatory-cell predominance during autoimmune graft-versus-host disease. These data directly demonstrated that peripheral T-regulatory-cell induction was inhibited by Tbet. Finally, *Tbx21*<sup>-/-</sup> T-regulatory cells cross-regulated autoimmune wild-type T-effector-cell cytokine production *in vivo*. The Tbet pathway therefore directly impairs T-regulatory-cell reconstitution and is consequently a feasible target in efforts to prevent autoimmune graft-versus-host disease.

## Correspondence:

shoba.amarnath@ncl.ac.uk

Received: September 3, 2016

Accepted: May 3, 2017.

Pre-published: May 4, 2017.

doi:10.3324/haematol.2016.155879

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: [www.haematologica.org/content/102/8/1446](http://www.haematologica.org/content/102/8/1446)

©2017 Ferrata Storti Foundation

Material published in Haematologica is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>,

sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



## Introduction

T-helper (Th) 1, Th2, and Th17 cells mediate distinct acute graft-versus-host disease (GvHD) syndromes.<sup>1</sup> In contrast, T-regulatory (Treg) cells prevent acute GvHD<sup>2</sup> and are associated with reduced clinical GvHD. T-helper subsets are primarily driven by cross-regulatory transcription factors, namely: deficiency of Th1- and Th17-driving transcription factors allows FoxP3<sup>+</sup> Treg reconstitution and prevents acute GvHD.<sup>3,4</sup> However, transcription factor cross-regulation is less clear for chronic GvHD, which is distinct from acute GvHD in part due to an autoimmune mechanism.<sup>5</sup>

Chronic GvHD is propagated by donor T cells that recognize host peptides presented by donor antigen-presenting cells. The pathological manifestations of chronic GvHD therefore resemble those of autoimmunity.<sup>6,9</sup> The mechanism by which autoimmunity arises from alloimmunity remains unresolved.<sup>10</sup> In animal models, a decrease in Treg cells occurs along with an expansion of Th1 and Th17 cells,<sup>11</sup> which leads to autoimmune pathology. During alloimmunity, donor T cells respond to shared antigens, thereby resulting in repertoire skewing and recognition of non-polymorphic antigens.<sup>12</sup> There are no data indicating whether this process results in prevention of the induction of Treg cells thereby resulting in long-term loss in immune regulation. While animal models of chronic GvHD exist,<sup>13</sup> there is a pauci-

ty of data reproducing the break in T-cell regulation. Hence, we utilized a chronic GvHD model<sup>11,12,14</sup> that captures these two processes and allows understanding of the specific regulatory signals that prevent immune tolerance of alloreactive donor T cells and consequently cause autoimmunity.

Chronic GvHD autoimmunity can be experimentally dissected from acute GvHD by sequential allogeneic and syngeneic T-cell transfer. In this secondary transfer autoimmune chronic GvHD, Th1 and Th17 subsets predominate with a relative deficiency of Treg cells;<sup>11</sup> a similar immune imbalance was also observed in a sclerodermatous chronic GvHD model.<sup>15</sup> However, the underlying molecular mechanism by which such Treg insufficiency occurs in chronic GvHD is still unknown but therapies that enhance Treg numbers in patients with chronic GvHD are promising.<sup>16</sup> The existence of a Th1 component in the pathogenesis of chronic GvHD diverges from initial models that emphasized a Th2-dominant disease<sup>17</sup> but is not inconsistent with the current understanding of Th1-mediated autoimmunity<sup>18</sup> and knowledge that type I immunity drives lethality in genetic Treg deficiency.<sup>19</sup> Taken together, Th1-driving signaling molecules may restrict peripheral Treg generation.

We hypothesized that Th1-driving signals inhibit Treg generation during chronic GvHD. Because interferon (IFN)- $\gamma$  signaling induces Tbet,<sup>20</sup> which propagates Th1-mediated inflammation, sequential deletion of IFN- $\gamma$ R followed by Tbet may identify checkpoints that inhibit peripheral Treg generation. We evaluated key Th1 transcription factors, including STAT1 and STAT4, in modulating chronic GvHD.<sup>11</sup>

## Methods

### Mice

Female C57BL/6 (B6, H<sub>2</sub>K<sup>b</sup>) and BALB/c (H2kd) mice 8- to 10-weeks old were obtained from Frederick Cancer Research Facility. *Rag2*<sup>-/-</sup>, *Tbx21*<sup>-/-</sup>, *Ifn $\gamma$*  and *Ifn $\gamma$* <sup>-/-</sup> animals were from Jackson Laboratories. B6.*Tbx21*<sup>-/-</sup>*Foxp3*-GFP mice were generated by crossing B6.*Tbx21*<sup>-/-</sup> with B6.*Foxp3*GFP; B6.*FoxP3*GFP littermate controls were simultaneously maintained. Mice were maintained in a specific pathogen-free facility at the National Institutes of Health. Drinking water was supplemented with ciprofloxacin from day -1 to day +14 after bone marrow transplantation. Experiments were carried out in accordance with National Institutes of Health animal health and safety guidelines and approved by the Animal Care and Use Committee, National Cancer Institute, National Institutes of Health.

### Bone marrow transplant

Bone marrow experiments were performed as outlined in the *Online Supplementary Methods*.

### Flow cytometry

On day 14 after allogeneic bone marrow transplantation, splenocytes were stained with CD4 PE-Cy5 (H129.19), H<sub>2</sub>K<sup>b</sup> PE (AF6.88.5), CD3 FITC (145-2C11) and FoxP3 APC (FJK.16s; eBioscience). After secondary transfer to *Rag2*<sup>-/-</sup> recipients, splenic T cells were isolated and intracellular flow cytometry was performed as outlined in the *Online Supplementary Methods*.

### Cytokine secretion assay after secondary transfer

On the indicated day after secondary transfer into syngeneic

hosts, spleens were harvested and single cell suspensions were obtained to test for autoreactivity, as outlined in the *Online Supplementary Methods*.

### Evaluation of *in vivo* tissue RNA genes after secondary transfer

On the indicated days, recipients' skin tissue was evaluated for chronic GvHD biomarkers as outlined in the *Online Supplementary Methods*.

### Isolation of T cells from lamina propria lymphocytes and skin

Intraepithelial lymphocytes were removed and lamina propria lymphocytes were separated.<sup>21</sup> Lymphocytes were extracted from the skin as previously described and outlined in the *Online Supplementary Methods*.<sup>22</sup>

### Chromatin immunoprecipitation

Chromatin was immunoprecipitated as previously described and outlined in the *Online Supplementary Methods*.<sup>23</sup>

### Histopathology

Tissue from *Rag2*<sup>-/-</sup> recipients (colon, skin) was fixed in 10% (vol/vol) formyl saline and embedded in paraffin blocks. Tissue sections were stained with eosin and hematoxylin and evaluated by a pathologist (MAE). Skin GvHD scores were either 0 (normal) or 1 (acanthosis, hyperkeratosis); skin stages 2, 3, and 4, which include mononuclear infiltrates and epidermal loss, were not observed. Intestinal GvHD was scored (0 to 4) according to degree of mononuclear cell infiltration and crypt destruction (stage 1, focal and mild; stage 2, diffuse and mild; stage 3, diffuse and moderate; stage 4, diffuse and severe).

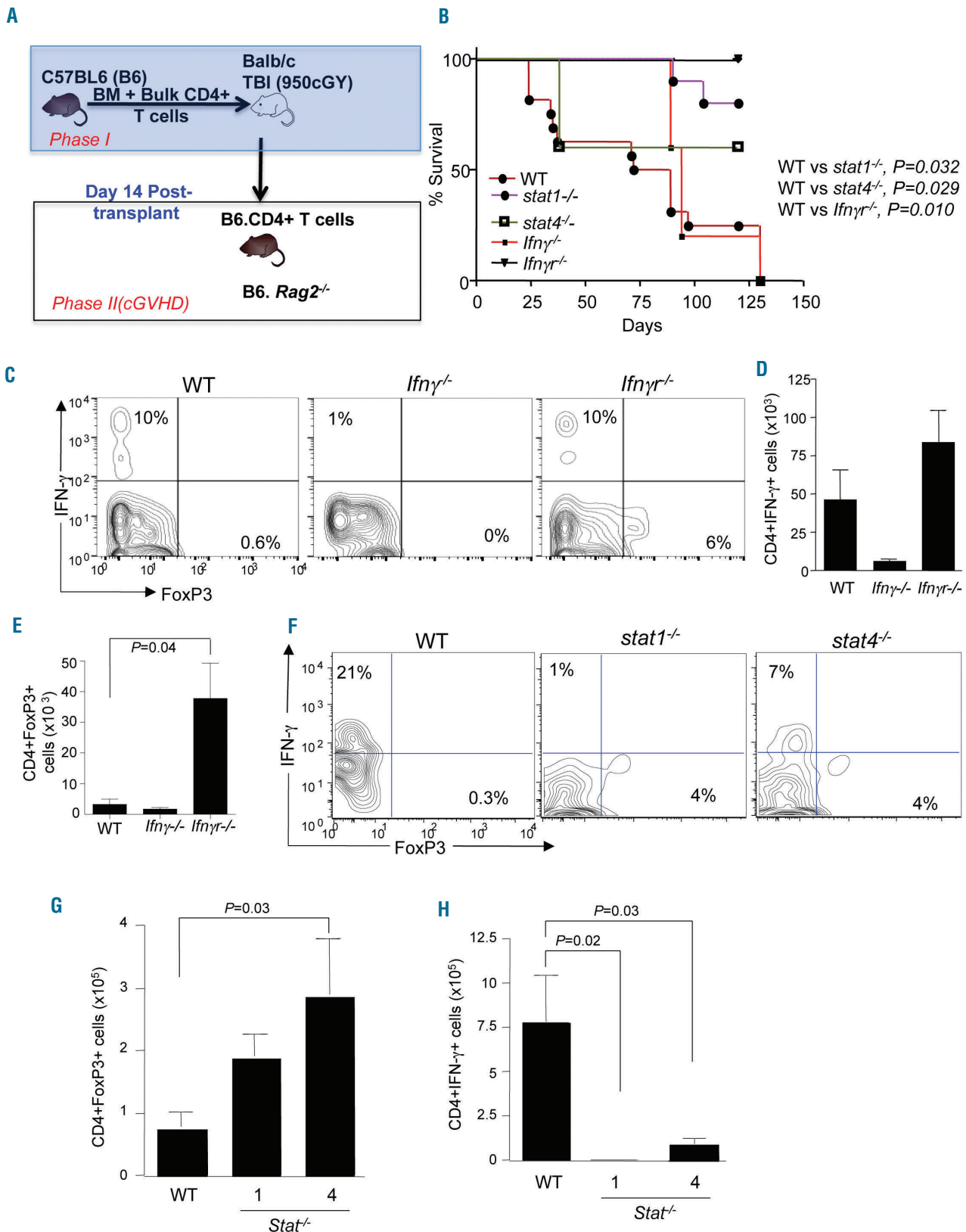
### Statistics

Kaplan-Maier survival analysis was performed and survival curves were compared using log-rank testing. Statistical significance was determined for normally distributed data using a two-tailed Student *t* test or one-way analysis of variance (ANOVA) followed by Dunn post-hoc tests. For statistical analysis of histology, a Mann-Whitney-U test was performed. *P* values <0.05 were considered statistically significant.

## Results

### Type I signaling is important for autoimmune chronic graft-versus-host disease

Experiments were designed to identify whether Th1 cells contributed to autoimmune chronic GvHD. A brief schema of the chronic GvHD model is shown in Figure 1A. Wild-type (WT), *IFN- $\gamma$* <sup>-/-</sup>, *IFN $\gamma$* R<sup>-/-</sup>, *STAT1*<sup>-/-</sup>, or *STAT4*<sup>-/-</sup> CD4<sup>+</sup> T cells were transferred into *Rag2*<sup>-/-</sup> recipients in the post-alloreactive phase. Contradicting prior reports suggesting that chronic GvHD primarily resembles a Th2 process,<sup>17,24</sup> *IFN $\gamma$* R<sup>-/-</sup> CD4<sup>+</sup> T cells abrogated chronic GvHD pathogenesis (Figure 1B); recipients had increased Treg-cell numbers but no difference in IFN $\gamma$ -producing T-effector cells; [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^5$ ); mean $\pm$ SEM, WT versus *IFN $\gamma$* <sup>-/-</sup> versus *IFN $\gamma$* R<sup>-/-</sup>; 3.35 $\pm$ 1.5 versus 1.78 $\pm$ 4.8 versus 37.8 $\pm$ 11.4; #CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> ( $\times 10^5$ ); mean $\pm$ SEM, WT versus *IFN $\gamma$* <sup>-/-</sup> versus *IFN $\gamma$* R<sup>-/-</sup>; 46 $\pm$ 19.7 versus 6 $\pm$ 9 versus 83.7 $\pm$ 20) (Figure 1C-E). Recipients of *STAT1*<sup>-/-</sup> or *STAT4*<sup>-/-</sup> T cells had increased Treg cells, decreased cytokine expression [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^5$ ); mean $\pm$ SEM, WT versus *STAT1*<sup>-/-</sup> ver-



**Figure 1. Lack of Th1 signaling in T cells alleviates autoimmune graft-versus-host disease.** Schematic representation of the experimental murine model for autoimmune chronic GvHD. (A) BALB/c mice were subjected to lethal total body irradiation (TBI) and then reconstituted with T-depleted (TD) bone marrow (BM) and CD4<sup>+</sup> T cells from C57BL/6 (B6) mice. On day 14 after transplant, murine recipients were euthanized and B6.CD4<sup>+</sup> T cells were isolated and adoptively transferred into B6.*Rag2*<sup>-/-</sup> mice. The occurrence of autoimmune GvHD was monitored over a period of 60 days. Host BALB/c mice were subjected to TBI (950 cGy) and then reconstituted with allogeneic WT B6 TDBM (10M) and CD4<sup>+</sup> T cells (2M). In certain cohorts, mice were reconstituted with BM and T cells deficient in IFN- $\gamma$ , STAT1, STAT4, or IFN- $\gamma$ . On day 14 after allogeneic bone marrow transplantation, splenic CD4<sup>+</sup> T cells from WT or KO recipients were isolated and adoptively transferred into B6 *Rag2*<sup>-/-</sup> mice. (B) Survival curves after secondary transfer of either WT or KO T cells ( $n=8-10$  per cohort). (C-H) Representative flow image and absolute numbers of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells and IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in the WT and KO cohorts ( $n=10$  per cohort).

*versus STAT4<sup>-/-</sup>*;  $0.64 \pm 0.13$  versus  $1.87 \pm 0.3$  versus  $3 \pm 0.8$ ; #CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> ( $\times 10^5$ ); mean $\pm$ SEM, WT versus *STAT1<sup>-/-</sup>* versus *STAT4<sup>-/-</sup>*;  $7.7 \pm 2.6$  versus  $0.2 \pm 0.2$  versus  $0.9 \pm 0.3$ ) (Figure 1F,H), and reduced lethality (Figure 1B).

These results suggested that deficiency of Th1-cell signaling (IFN- $\gamma$ R) or transcription factors (STAT1, STAT4) directly impaired chronic GvHD. However, such deficiencies may have reduced chronic GvHD indirectly, namely, via reduction in Th1 cytokines. To address this, we evaluated a transplant cohort that received IFN- $\gamma$ -deficient T cells: such recipients had low IFN- $\gamma$  and reduced Treg cells (Figure 1C-E) and chronic GvHD lethality similar to that of WT controls (Figure 1B). Recipients of *IFN $\gamma$ R<sup>-/-</sup>* T cells, even though protected against chronic GvHD lethality, had similar IFN- $\gamma$  production to that of WT controls (Figure 1E). Recipients of *IFN $\gamma$ R<sup>-/-</sup>*, *STAT1<sup>-/-</sup>*, and *STAT4<sup>-/-</sup>* T cells had similar numbers of CD4<sup>+</sup> T cells as the WT cohort [#CD4<sup>+</sup> ( $\times 10^4$ ); mean $\pm$ SEM, WT versus *IFN $\gamma$ <sup>-/-</sup>* versus *IFN $\gamma$ R<sup>-/-</sup>*;  $55.1 \pm 16.5$  versus  $50.8 \pm 14.3$  versus  $76.59 \pm 15.9$ ; WT versus *STAT1<sup>-/-</sup>* versus *STAT4<sup>-/-</sup>*;  $133.8 \pm 18.4$  versus  $180.7 \pm 14.1$  versus  $129.4 \pm 14.6$ ] (Online Supplementary Figure S1A,C) and similar interleukin-17 (IL17) secretion [#CD4<sup>+</sup>IL17<sup>+</sup> ( $\times 10^6$ ); mean $\pm$ SEM, WT versus *IFN $\gamma$ <sup>-/-</sup>* versus *IFN $\gamma$ R<sup>-/-</sup>*;  $25 \pm 10.6$  versus  $42.5 \pm 10.7$  versus  $31 \pm 7.6$ ; WT versus *STAT1<sup>-/-</sup>* versus *STAT4<sup>-/-</sup>*;  $0.51 \pm 0.005$  versus  $51.5 \pm 25$  versus  $75.9 \pm 54.4$ ] (Online Supplementary Figure S1B,D). These data indicate that the reduction in chronic GvHD was primarily attributable to a deficiency in Th1 cell signaling and Th1 cell transcription factors rather than a secondary deficiency in Th1 cytokines.

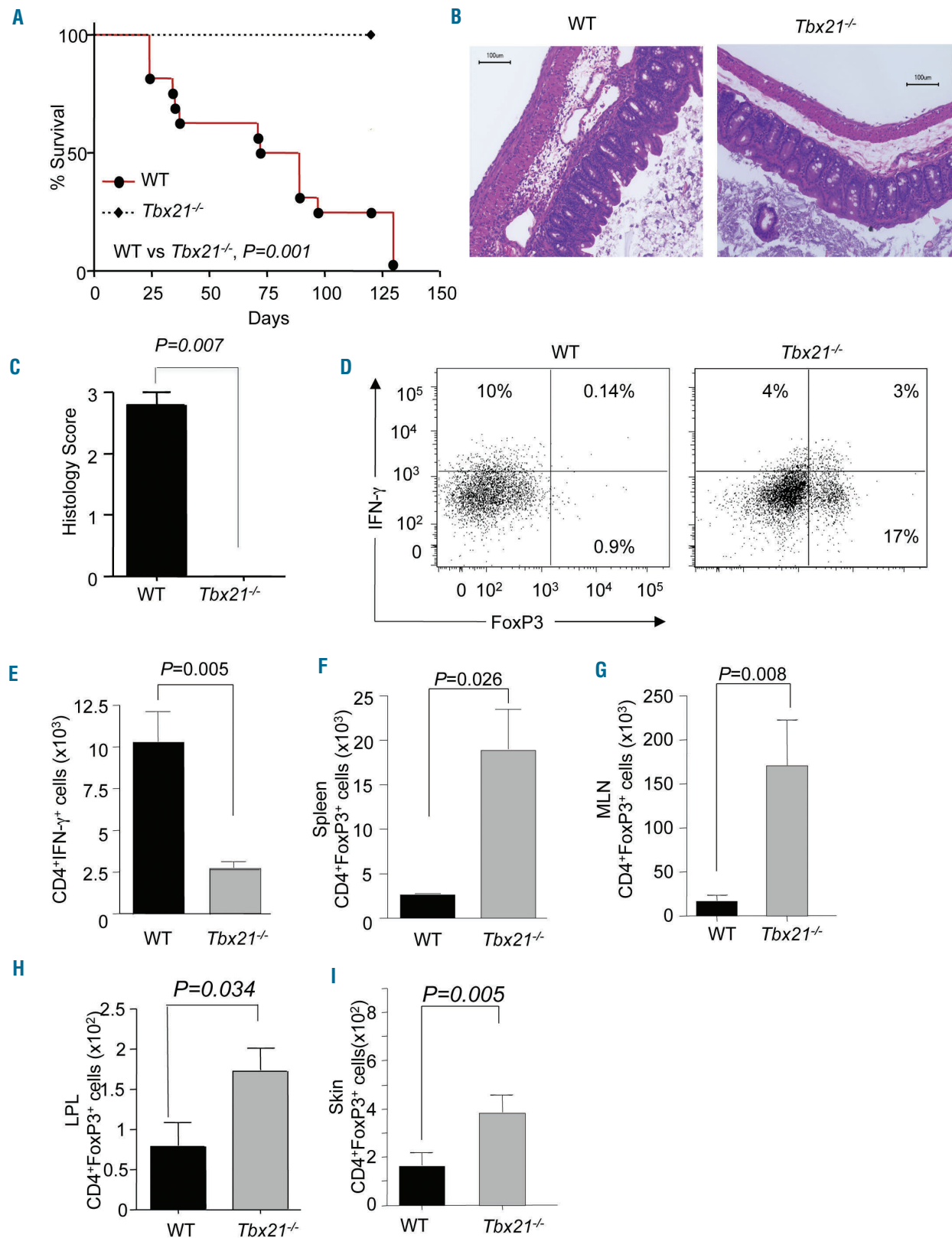
### Autoimmune graft-versus-host disease requires T-cell Tbx21

Increased Treg numbers in *IFN $\gamma$ R<sup>-/-</sup>* and *STAT1<sup>-/-</sup>* cohorts suggested an inhibitory mechanism by which Th1 signaling molecules prevented peripheral Treg generation. Because an increased number of Treg cells may be beneficial in decreasing chronic GvHD,<sup>25,29</sup> we characterized the mechanism by which Treg cells were inhibited during chronic GvHD. Experiments were performed with CD4<sup>+</sup> T-effector cells from *Tbx21<sup>-/-</sup>* mice (Tbet). Tbet is a master regulator of Th1 cells; lack of Tbet results in Th1-cell deficiency. To study the role of Tbet in chronic GvHD, we utilized *Tbx21<sup>-/-</sup>* T cells.<sup>30</sup> Consistent with published results,<sup>3</sup> WT CD4<sup>+</sup> T cells caused acute GvHD in the alloreactive phase; in contrast, recipients of *Tbx21<sup>-/-</sup>* T cells were partially protected against acute GvHD [WT: n=10/10 succumbed to acute GvHD; knockout (KO): n=5/10 succumbed to acute GvHD]. At the time of splenic T-cell harvest after allogeneic bone marrow transplantation, the frequencies of Treg cells in WT and Tbet-deficient recipients were similar (0.40% versus 0.17%, respectively;  $P=NS$ ). However, upon secondary transfer to *Rag2<sup>-/-</sup>* recipients, *Tbx21<sup>-/-</sup>* CD4<sup>+</sup> T cells did not mediate lethality (Figure 2A) or cause autoimmune pathology (Figure 2B, representative result; Figure 2C, pooled results). To evaluate potential cellular mechanisms, subsequent cohorts were euthanized prior to lethality (day 60 after secondary transfer). Protection against autoimmune GvHD was not associated with reduced numbers of CD4<sup>+</sup> T cells [#CD4<sup>+</sup> ( $\times 10^4$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $17.2 \pm 0.5$  versus  $6.8 \pm 4.1$ ] (Online Supplementary Figure S1E) or Th17 effectors [#CD4<sup>+</sup>IL17<sup>+</sup> ( $\times 10^6$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $2.5 \pm 1.3$  versus  $1.1 \pm 0.7$ ] (Online Supplementary Figure S1F) but was associated with reduced CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells [#CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> ( $\times 10^4$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $102.8 \pm 18.4$  versus  $27.1 \pm 4$ ] (Figure 2D representative result,

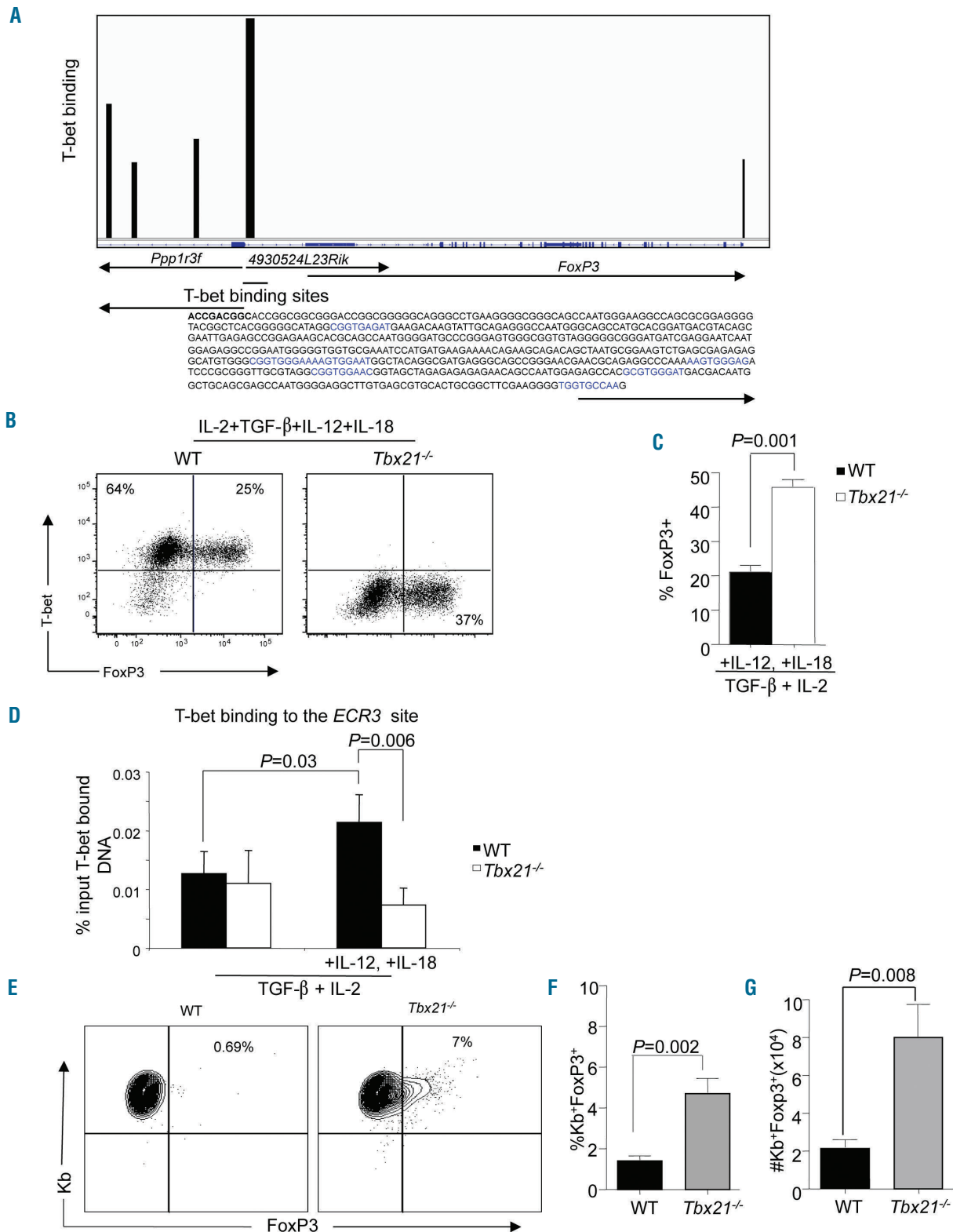
2E pooled results). Next, we evaluated the number of Treg cells in WT and *Tbx21<sup>-/-</sup>* cohorts. We reasoned that any survival advantage might be attributable to defective homing of T cells to target tissues, thereby limiting GvHD-mediated pathology.<sup>31</sup> Homing defects can be directly attributed to Tbet deficiency which is activated downstream of IFN- $\gamma$ R signaling.<sup>20,32</sup> However, contrary to this reasoning, we found increased FoxP3<sup>+</sup>Treg cells in spleen [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $2.7 \pm 0.1$  versus  $18.8 \pm 4.6$ ] (Figure 2F), mesenteric lymph nodes [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $16.9 \pm 7.1$  versus  $169.9 \pm 52.8$ ] (Figure 2G), lamina propria [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $0.78 \pm 0.3$  versus  $1.72 \pm 0.2$ ] (Figure 2H), and skin [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $1.6 \pm 0.5$  versus  $3.8 \pm 0.7$ ] (Figure 2I). STAT1 deficiency has been associated with enhanced Treg proliferation;<sup>33</sup> however, a similar biology was not operational in our model, as Tbet deficiency did not increase the Treg proliferative phenotype [#CD4<sup>+</sup>Ki67<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $9.5 \pm 4.5$  versus  $17.7 \pm 5.6$ ; #CD4<sup>+</sup>Ki67<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $165.7 \pm 92.8$  versus  $106.6 \pm 42.7$ ] (Online Supplementary Figure S2). Furthermore, Tbet deficiency did not alter Treg cell Bcl-2 expression [#CD4<sup>+</sup>bcl2<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $36 \pm 4.3$  versus  $228.1 \pm 101.5$ ; mean $\pm$ SEM, #CD4<sup>+</sup>bcl2<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^3$ ); mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $73.9 \pm 42.7$  versus  $70.9 \pm 41.5$ ] (Online Supplementary Figure S3). Therefore, *Tbx21<sup>-/-</sup>* Treg cells are similar to WT Treg cells with respect to their proliferation and apoptotic tendency during chronic GvHD.

### Tbet restricts CD4<sup>+</sup> peripheral T-regulatory-cell generation

Tbet cross-regulation of FoxP3 might occur through several mechanisms, one of which involves the ability of Tbet to inhibit FoxP3 expression and subsequent peripheral Treg generation.<sup>34</sup> We considered the possibility that *bona fide* effector CD4<sup>+</sup> T cells might be more amenable to FoxP3 expression and acquiring a Treg phenotype in the absence of Tbet. To elucidate the intrinsic mechanistic implications of Tbet deficiency in FoxP3 expression, we determined whether direct Tbet inhibition of FoxP3 occurs. In light of the report by Eckerstorfer *et al.*,<sup>35</sup> we sought to identify whether Tbet regulates FoxP3 expression and has binding sites in the evolutionary conserved region (ECR) upstream of the *Foxp3* promoter site. ECR1, 2 and 3 induce *Foxp3* promoter activity in human cells by luciferase assays. In particular, ECR3, which is located in close proximity to *ppp1r3f*, enhanced *Foxp3* promoter activity with negligible *ppp1r3f* activity. Using chromatin immunoprecipitation sequencing analysis in Th1 polarized cells (GSM836124),<sup>36</sup> we determined that Tbet has a binding site in the ECR3 region upstream of the *Foxp3* promoter (Figure 3A). To validate this site, naive CD4<sup>+</sup> T cells from WT and *Tbx21<sup>-/-</sup>* mice were polarized with IL-2, TGF- $\beta$ 1, IL-12 and IL-18 (Figure 3B) prior to chromatin immunoprecipitation analysis. Lack of Tbet preferentially increased *Foxp3* expression in induced Treg (iTreg) cells that were exposed to Th1 cytokines [% CD4<sup>+</sup>FoxP3<sup>+</sup>; mean $\pm$ SEM, WT versus *Tbx21<sup>-/-</sup>*;  $21 \pm 2.0$  versus  $45.7 \pm 2.2$ ] (Figure 3C). In contrast, FoxP3 expression in iTreg cells generated from WT and *Tbx21<sup>-/-</sup>* cohorts were 61% versus 59%, respectively. WT iTreg cells cultured with IL-12 and IL-18 had significant Tbet co-expression with FoxP3 and enhanced binding to the ECR locus of *Foxp3* [%Tbet



**Figure 2. Lack of Tbet alleviates autoimmune graft-versus-host disease.** Host BALB/c mice were subjected to total body irradiation (950 cGy) and then reconstituted with allogeneic B6 T-cell-depleted (TD) bone marrow (BM) (10M) and CD4<sup>+</sup> T cells (2M). In certain cohorts, mice were reconstituted with BM and T cells deficient in Tbet. On day 14 after allogeneic bone marrow transplantation, splenic CD4<sup>+</sup> T cells from WT or *Tbx21*<sup>-/-</sup> recipients were isolated and adoptively transferred into B6 *Rag2*<sup>-/-</sup> mice. (A) Survival curve after secondary transfer (n=10 per cohort). (B) Representative pictures of colon at day 60 after secondary transfer of WT (left panel) or *Tbx21*<sup>-/-</sup> (right panel) T cells. (C) Summary of histological scores (n=5 per cohort). Immune cell phenotype was also evaluated at day 60 after secondary transfer. (D) Representative flow plots showing frequency of IFN $\gamma$ <sup>+</sup> and FoxP3<sup>+</sup> T cells in the WT and *Tbx21*<sup>-/-</sup> cohorts. (E) Pooled results (n=10 per cohort) for the absolute numbers of splenic CD4<sup>+</sup> T cells secreting IFN $\gamma$  and (F) absolute number of splenic CD4<sup>+</sup> FoxP3<sup>+</sup> T cells. (G) Absolute numbers of mesenteric lymph node CD4<sup>+</sup> FoxP3<sup>+</sup> T cells. (H) Absolute numbers of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells from the lamina propria (n=10 per cohort). (I) Absolute numbers of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells from the skin (n=5 per cohort).



**Figure 3. Peripheral Treg numbers are increased in the absence of *Tbx21*.** Chromatin immunoprecipitation (ChIP) sequencing analysis of naïve CD4<sup>+</sup> T cells stimulated for 3 days under Th1 conditions. (A) Peaks denote regions of DNA associated with Tbet binding. The contralateral DNA sequence upstream of the first exon of *ppp1r3f* is shown below, with potential Tbet binding sites indicated in blue. (B) Naïve CD4<sup>+</sup> T cells from WT and *Tbx21*<sup>-/-</sup> mice were isolated and polarized under iTreg conditions [ $\alpha$ CD3,  $\alpha$ CD28, IL-2 (100 IU) and TGF- $\beta$ 1 (5 ng/mL)] in the presence of rIL-12 and rIL-18 (10 ng/mL). On day 3, cells were characterized using intracellular flow cytometry for co-expression of Tbet and FoxP3. (C) Percentage of FoxP3<sup>+</sup> iTreg cells in WT and *Tbx21*<sup>-/-</sup> mice following stimulation with Th1 conditions. (D) Histograms denote percentage of input binding of Tbet to the ECR3 locus upstream of the *Foxp3* promoter in WT or *Tbx21*<sup>-/-</sup> iTreg polarized in the presence or absence of rIL-12 and IL-18 measured by ChIP. Data shown are replicates of three experiments. Bone marrow transplantation experiments were carried out with either WT T cells (*B6.Foxp3<sup>fl/fl</sup>*) or T cells deficient in Tbet (*B6.Tbx21<sup>-/-</sup>Foxp3<sup>fl/fl</sup>*) to allow flow sorting for FoxP3<sup>+</sup> T cells for secondary transfer. Splenocytes were harvested 60 days after transplant and then flow cytometry was performed in unstimulated cells to detect FoxP3 frequency. (E) Representative flow cytometry plot of peripheral Treg induction in WT and KO cohorts. (F) Pooled analysis of frequency of Treg induction in WT and KO (n=5 per cohort). (G) Absolute numbers of induced Treg cells in WT and KO cohorts during autoimmune GvHD.

bound to DNA; WT iTreg *versus* WT iTreg + Th1 cytokines;  $0.015 \pm 0.001$  *versus*  $0.025 \pm 0.002$ ] relative to control *Tbx21*<sup>-/-</sup> iTreg cells (Figure 3C). Allogeneic bone marrow transplantation was performed using CD4<sup>+</sup> T cells harvested from *B6.Tbx21*<sup>-/-</sup>*FoxP3*<sup>GFP</sup> mice; then, 14 days after the transplant, effector T cells were purified by flow cytometry (CD4<sup>+</sup>GFP<sup>+</sup>) and transferred into *Rag2*<sup>-/-</sup> recipients. At day 60 after transfer, recipients of Tbet-deficient T cells were devoid of clinical autoimmune GvHD and had increased Treg cells [%CD4<sup>+</sup>FoxP3<sup>+</sup>, mean±SEM,  $1.4 \pm 0.3$  *versus*  $4.8 \pm 0.6$ ; # CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^4$ ), mean±SEM,  $2.1 \pm 0.5$  *versus*  $8 \pm 1.7$ ] (Figure 3E representative data; 3F,G pooled data). Tbet is, therefore, a critical checkpoint and prevents peripheral Treg generation during ongoing autoimmune GvHD. These results stand in contrast to those of studies showing the importance of Tbet<sup>32</sup> or GATA3<sup>37,38</sup> expression in FoxP3<sup>+</sup> Treg cells. However, there is emerging literature indicating that this may not be the case in autoimmune syndromes in which acquisition of Tbet generates dysfunctional Treg cells.<sup>39</sup> As such, these data illustrate that Tbet can bind to the ECR3 locus of the FoxP3 promoter *in vitro* and demonstrate that lack of Tbet positively regulates peripheral Treg generation during chronic GvHD.

### Tbet-deficient T-regulatory cells cross-regulate pathogenic T cells

Infectious disease models suggest that Treg cells that do not express Tbet have limited functional capacity.<sup>32,40</sup> Such functional Treg defects have been attributed to homing defects that occur in the absence of Tbet rather than to a Treg suppressor defect.<sup>41,42</sup> We thus sought to identify whether Treg cells generated in the absence of Tbet were functional. Cell mixing studies incorporating congenic donor cells were performed to evaluate whether induced peripheral Treg cells from Tbet-deficient CD4<sup>+</sup> cells might inhibit otherwise pathogenic WT CD4<sup>+</sup> effectors (see experimental design, *Online Supplementary Figure S4A*). Recipients of a 1:1 mix of WT and Tbet-deficient T cells had decreased CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells in the WT compartment as compared to the WT cohort alone [% CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>, mean±SEM; WT *versus* *Tbx21*<sup>-/-</sup> *versus* WT (1:1) *versus* WT (1:10) *versus* *Tbx21*<sup>-/-</sup> (1:1) *versus* *Tbx21*<sup>-/-</sup> (1:10);  $41.3 \pm 2.9$  *versus*  $3.8 \pm 0.6$  *versus*  $21.4 \pm 0.8$  *versus*  $28.2 \pm 4.1$  *versus*  $1.9 \pm 0.6$  *versus*  $2.5 \pm 0.8$ ] (Figure 4A,B). Therefore, Tbet-deficient Treg cells dampened WT cytokine secretion *in vivo* thereby cross-regulating otherwise pathogenic effectors. Also, although increased FoxP3 frequency was noted in the WT compartment in the presence of Tbet-deficient cells [% CD4<sup>+</sup>FoxP3<sup>+</sup>, mean±SEM; WT *versus* *Tbx21*<sup>-/-</sup> *versus* WT (1:1) *versus* WT (1:10) *versus* *Tbx21*<sup>-/-</sup> (1:1) *versus* *Tbx21*<sup>-/-</sup> (1:10);  $1.7 \pm 0.3$  *versus*  $4.2 \pm 0.5$  *versus*  $4.5 \pm 1.1$  *versus*  $1.5 \pm 0.5$  *versus*  $3.7 \pm 0.8$  *versus*  $1.9 \pm 0.8$ ] (Figure 4C,D), this was not reflected in absolute numbers of FoxP3 cells [#CD4<sup>+</sup>FoxP3<sup>+</sup> ( $\times 10^5$ ), mean±SEM; WT *versus* *Tbx21*<sup>-/-</sup> *versus* WT (1:1) *versus* WT (1:10) *versus* *Tbx21*<sup>-/-</sup> (1:1) *versus* *Tbx21*<sup>-/-</sup> (1:10);  $14.4 \pm 5$  *versus*  $51.9 \pm 24$  *versus*  $35.8 \pm 3.6$  *versus*  $17.1 \pm 8.8$  *versus*  $59.6 \pm 23.6$  *versus*  $24.7 \pm 13.2$ ] (Figure 4E); this result is consistent with a model in which there is a cell-intrinsic regulation of FoxP3 by Tbet.

### Tbet-deficient T-regulatory cells modulate clinical graft-versus-host disease manifestations in the presence of wild-type pathogenic cells

An additional experiment was performed to confirm Tbet-deficient cell cross-regulation of pathogenic WT

effectors and to further characterize the resultant modulation of chronic GvHD. Consistent with the results shown in Figure 4, we found that: (i) recipients of WT T cells had reduced absolute numbers of Treg cells relative to recipients of non-alloreactive T cells during chronic GvHD; (ii) recipients of Tbet-deficient alloreactive T cells had increased Treg cells during the autoimmune phase relative to recipients of alloreactive WT cells; and (iii) Tbet-deficient T cells cross-regulate pathogenic WT cells, as evidenced by increased Treg cells (Figure 5A). Furthermore, recipients of Tbet-deficient T cells and recipients of the mix of Tbet-deficient and WT T cells had reduced secretion of IFN- $\gamma$  in response to syngeneic dendritic cells relative to WT T-cell recipients (Figure 5B). Syngeneic recipients of WT T cells had increased weight loss at day 17 after transfer (Figure 5C) relative to both recipients of Tbet-deficient T cells ( $P=0.0012$ ) and recipients of the 1:1 mix ( $P=0.0003$ ); however, weight of WT cell recipients recovered to values similar to that of other cohorts. Nonetheless, recipients of WT T cells later developed extensive hair loss, primarily across the back (see photographs, *Online Supplementary Figure S5*); the other three cohorts did not have clinical hair loss. To characterize the apparent cutaneous autoimmune chronic GvHD, histology was performed. In syngeneic recipients of WT cells, there was mild acanthosis and mild hyperkeratosis (Figure 5D; stage 1 of 4); in contrast, there was no acanthosis or hyperkeratosis in syngeneic recipients of Tbet-deficient cells or recipients of both WT and Tbet-deficient cells. These results were observed consistently (Figure 5E). The histological evidence of chronic skin GvHD was observed in clinically affected and non-affected skin samples in WT recipients; this latter result indicates that histological evidence of chronic GvHD is a more sensitive parameter than the clinical sign of hair loss. In this experiment, in which the clinical presentation was mild relative to that of previous experiments, we did not observe evidence of intestinal GvHD by histology. In parallel with histological studies, we performed RNA quantification of molecules associated with autoimmunity. As shown in Figure 5F, recipients of Tbet-deficient cells or the mix of WT plus Tbet-deficient cells had similar expression of CD4 RNA relative to WT recipients but had reduced expression of TGF- $\beta$ 1; of note, TGF- $\beta$  represents an effector molecule for cutaneous chronic GvHD.<sup>43</sup> In addition, relative to recipients of WT cells, skin samples from recipients of Tbet-deficient cells or a mix of Tbet-deficient cells plus WT cells had reduced RNA expression of inflammatory mediators, including IL-6 (WT *versus* KO:  $P=0.013$ ; WT *versus* Mix:  $P=0.006$ ), TLR2 (WT *versus* KO:  $P=0.004$ ; WT *versus* Mix:  $P=0.004$ ), and TLR7 (WT *versus* KO:  $P=0.0006$ ; WT *versus* Mix:  $P=0.0002$ ). These data indicate that Tbet-deficient cells, even when administered with otherwise pathogenic WT cells, increase Treg cells and decrease the molecular, pathological, and clinical features of autoimmune chronic GvHD.

## Discussion

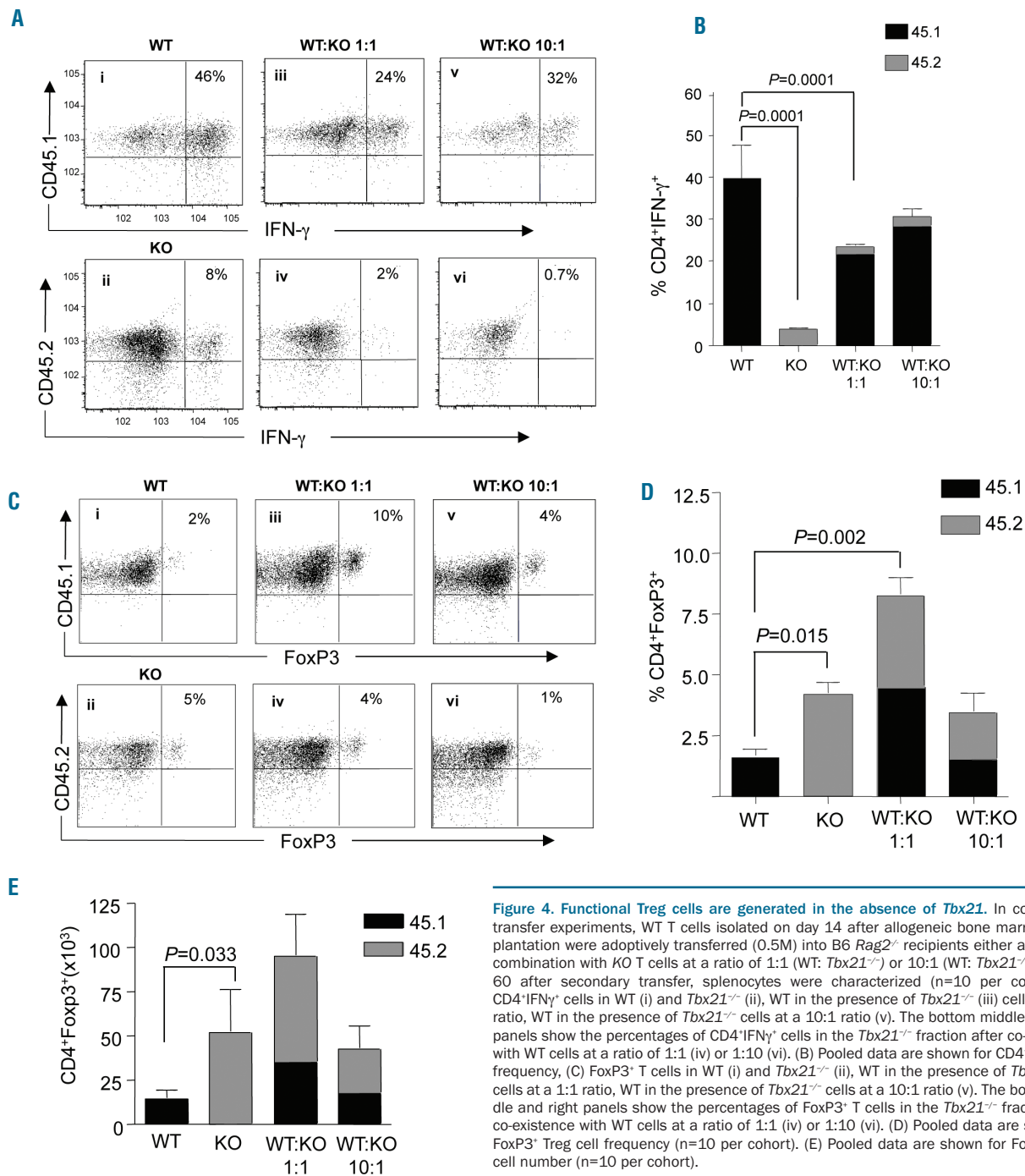
We used alloreactive T-cell transfer into a syngeneic murine host model to study the autoimmune manifestations of chronic GvHD. Development of autoimmunity was dependent on intact Th1 signaling in the transferred T cells. The ability of the cells to secrete IFN- $\gamma$  did not

affect the disease course, and we did not find any differences in the ability of the cells to proliferate or traffic to target organs in the absence of IFN- $\gamma$  signaling. By contrast, the absence of IFN- $\gamma$  signaling and deletion of Tbet was associated with the generation of peripheral Treg cells from CD4<sup>+</sup> T cells that had been primed in an alloreactive environment.

By comparing IFN- $\gamma$ -deficient with IFN- $\gamma$  signaling-deficient T cells, we dissected the critical role of Th1 signaling molecules from the less contributory role of the Th1 cytokine IFN- $\gamma$ . The inflammatory syndrome associated with alloimmunity and autoimmunity has historically

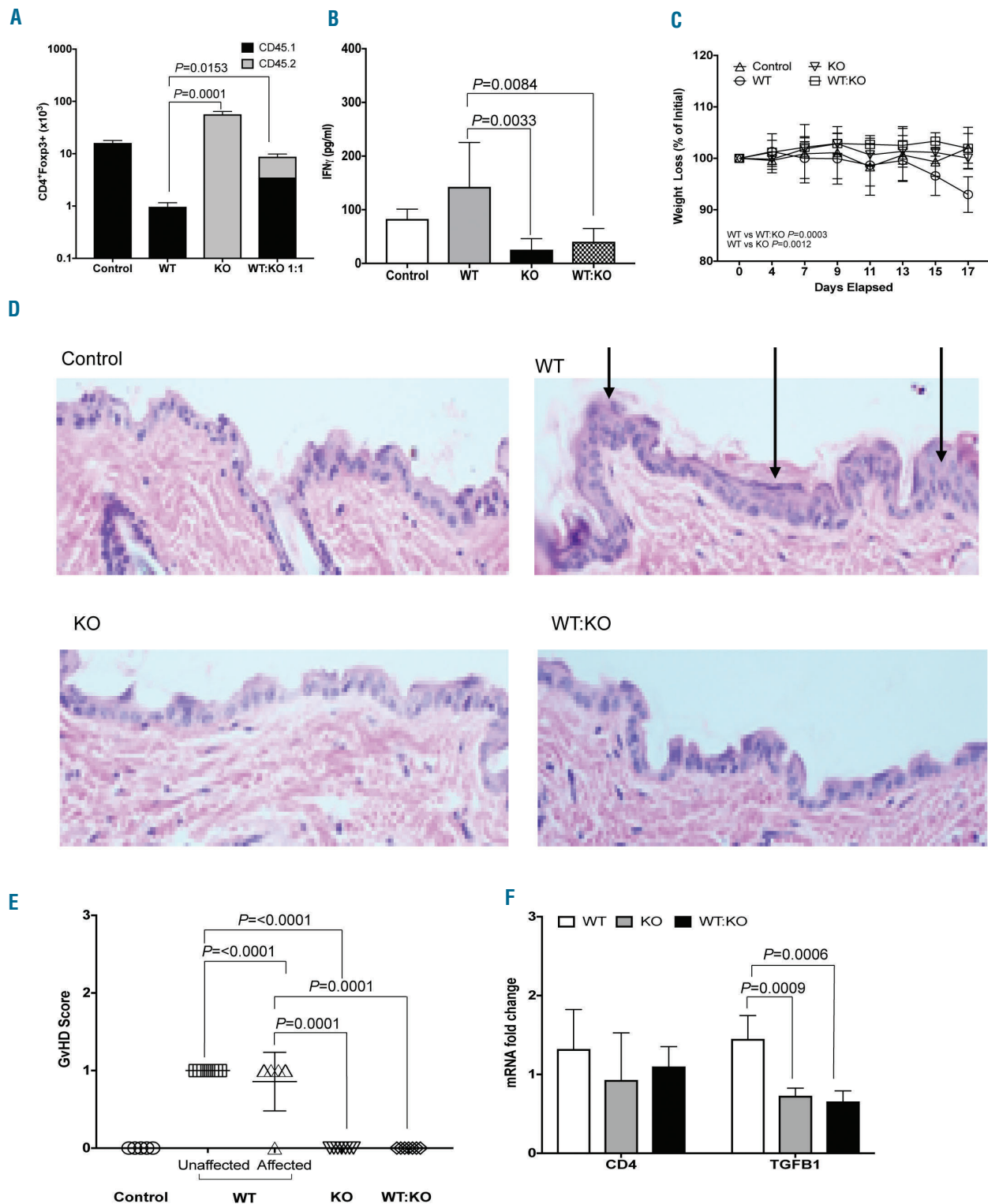
been attributed to the presence of Th1 and Th17 cells.<sup>14,44</sup> Moreover, IFN- $\gamma$  is pivotal to Treg function during alloimmunity,<sup>45</sup> while Treg cells expressing IFN- $\gamma$  are dysfunctional in autoimmunity.<sup>39</sup> Here, we show that the complete abrogation of Th1 differentiation factors rather than the absence of the Th1 cytokine IFN- $\gamma$  reduces autoimmune chronic GvHD.

We identified a novel regulatory mechanism by which Tbet modulates peripheral Treg generation, namely, that Tbet binds to the ECR3 locus of the *Foxp3* promoter. There is a paucity of data regarding the functionality of the ECR regions upstream of the *Foxp3* promoter. There



**Figure 4. Functional Treg cells are generated in the absence of *Tbx21*.** In co-adoptive transfer experiments, WT T cells isolated on day 14 after allogeneic bone marrow transplantation were adoptively transferred (0.5M) into B6 *Rag2*<sup>-/-</sup> recipients either alone or in combination with KO T cells at a ratio of 1:1 (WT: *Tbx21*<sup>-/-</sup>) or 10:1 (WT: *Tbx21*<sup>-/-</sup>). On day 60 after secondary transfer, splenocytes were characterized (n=10 per cohort). (A) CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in WT (i) and *Tbx21*<sup>-/-</sup> (ii), WT in the presence of *Tbx21*<sup>-/-</sup> (iii) cells at a 1:1 ratio, WT in the presence of *Tbx21*<sup>-/-</sup> cells at a 10:1 ratio (v). The bottom middle and right panels show the percentages of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in the *Tbx21*<sup>-/-</sup> fraction after co-existence with WT cells at a ratio of 1:1 (iv) or 10:1 (vi). (B) Pooled data are shown for CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cell frequency. (C) FoxP3<sup>+</sup> T cells in WT (i) and *Tbx21*<sup>-/-</sup> (ii), WT in the presence of *Tbx21*<sup>-/-</sup> (iii) cells at a 1:1 ratio, WT in the presence of *Tbx21*<sup>-/-</sup> cells at a 10:1 ratio (v). The bottom middle and right panels show the percentages of FoxP3<sup>+</sup> T cells in the *Tbx21*<sup>-/-</sup> fraction after co-existence with WT cells at a ratio of 1:1 (iv) or 10:1 (vi). (D) Pooled data are shown for FoxP3<sup>+</sup> Treg cell frequency (n=10 per cohort). (E) Pooled data are shown for FoxP3<sup>+</sup> Treg cell number (n=10 per cohort).





**Figure 5.** Tbet-deficient cells have reduced chronic graft-versus-host disease potential and modulate otherwise pathogenic WT cell-induced chronic graft-versus-host disease. WT and Tbet-deficient T cells were isolated on day 14 after allogeneic bone marrow transplantation and adoptively transferred into B6 *Rag2*<sup>-/-</sup> recipients either alone or combined in a ratio of 1:1 (WT:*Tbx21*<sup>-/-</sup>). An additional control cohort ("Control") first received a syngeneic transplant with WT cells followed by a secondary transfer into the syngeneic B6 *Rag2*<sup>-/-</sup> host. (A) On day 50 after secondary transfer, recipients were euthanized, and splenic T cells were evaluated by flow cytometry and the absolute number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells derived from the WT or *Tbx21*<sup>-/-</sup> inoculum was calculated. (B) Post-transfer splenic T cells were also stimulated *ex vivo* with syngeneic dendritic cells and the 24 h supernatant was evaluated for IFN- $\gamma$  content. (C) After transfer into B6 *Rag2*<sup>-/-</sup> hosts, the four cohorts were monitored for weight loss. (D) On day 50 after syngeneic transfer, recipients were killed and skin samples were evaluated for histological evidence of cutaneous chronic GvHD by hematoxylin and eosin staining; as illustrated in these representative photomicrographs, there was mild acanthosis and hyperkeratosis in recipients of the WT cells (affected areas indicated by arrows). (E) Cumulative data from the cohorts indicate the consistency of the histology findings; samples in the WT cohort were evaluated from both clinically affected and non-affected regions of the skin. (F) RNA expression of inflammatory molecules was evaluated in the skin tissues using QuantiGene Plex assay; results for control CD4 expression and TGF- $\beta$ 1 expression are shown. For these experiments, there were five replicates in the control cohort and seven replicates in the other cohorts; however, for the RNA studies, there were five replicates in each cohort. Results shown are mean values  $\pm$  SEM.

are three highly conserved ECR regions (ECR1, 2 and 3) which are located upstream of the transcriptional start site.<sup>35</sup> ECR3, which lies proximal to the *pppr13f* gene, surprisingly, had transcriptional activity in the direction of FoxP3. While no specific role for ECR3 has been defined by our experiments, we show for the first time that the ECR3 region upstream of the *Foxp3* gene possesses Tbet binding sites. The importance of Tbet binding to this locus is reflected in the *in vivo* experiments in which lack of Tbet allowed for the generation of peripheral Treg cells, thereby abrogating the typical Treg deficiency during chronic GvHD. Hence both the chromatin immunoprecipitation and *in vivo* data are consistent with a model whereby Tbet possesses a vital regulatory function in FoxP3 expression in the context of chronic GvHD.

Tbet is vital for the development of acute GvHD,<sup>3</sup> while its role during chronic GvHD is less clear. Here, for the first time, Tbet has been shown to be critical not only in the pathogenesis of chronic GvHD but also in limiting Treg cells. Indeed, a cumulative increase in Treg cells was noted in the secondary lymphoid organs and GvHD target tissues in *Tbx21*<sup>-/-</sup> recipients, which correlated with decreased pathogenesis. Our observations are consistent with previous reports according to which: (i) Treg cells in chronic GvHD patients with a favorable prognosis failed to express Th1 chemokine factors;<sup>46</sup> and (ii) expression of the Th1 phenotype in Treg cells from patients with multiple sclerosis was associated with diminished Treg function.<sup>39</sup> Our results elucidate the specific role of Th1 transcription factors in Treg function during chronic GvHD. Although the presence of Th1 transcription factors within Treg cells allows these cells to combat Th1-mediated damage during infectious disease,<sup>32,47</sup> our data suggest that in protracted autoimmune disorders such as chronic GvHD, Tbet becomes a negative regulator of FoxP3 expression in Treg cells.

Co-transfer experiments using WT and *Tbx21*<sup>-/-</sup> alloreactive cells confirmed the generation of functional Treg cells in the absence of Tbet. Such co-adoptive transfer decreased IFN $\gamma$ <sup>+</sup> T cells in the WT compartment, increased Treg cells during autoimmune chronic GvHD, and reduced the molecular, pathological, and clinical evidence of chronic GvHD. Our experiments also show that the GvHD autoimmune model that we utilized can result in a diversity of clinical presentations, the variability of which is not currently known but may involve factors such as host microbial status. Tbet-deficient cells, including when used in combination with otherwise pathogenic WT cells, had the potential to reduce both manifestations such as autoimmune colitis and resultant lethality and more protracted manifestations such as cutaneous chronic GvHD. We focused on cytokine-mediated events in autoimmune chronic GvHD, namely the role of IFN- $\gamma$ , which we found was relatively non-contributory given the lack of disease modulation in IFN- $\gamma$  KO recipients. It should be noted that cytolytic pathways, namely perforin/granzyme, fas ligand, and TNF- $\alpha$  contribute to GvHD, although these path-

ways are less well characterized in chronic GvHD models. Our experiments did not evaluate the role of these cytolytic pathways, and as such, it is possible that deficiency in these pathways might ameliorate autoimmune GvHD and perhaps likely that Tbet-deficiency would reduce these cytolytic effector mechanisms for amelioration of disease. However, the capacity of the Tbet-deficient cells to down-regulate the otherwise pathogenic WT cells in cell mixing studies provides evidence for a cross-regulatory mechanism to the Tbet-deficiency finding rather than a more direct mechanism involving lack of cytotoxic effector molecule expression.

We further clarified the immunopathology of autoimmunity associated with GvHD. Early reports suggested that both Th1 and Th2 cells have specific roles in causing acute GvHD and chronic GvHD<sup>24</sup> but not until recently has the immunopathology of chronic GvHD been widely investigated. The autoimmune manifestations in GvHD are primarily thought to be of Th1 origin<sup>11,14,48,49</sup> with minimal involvement of Th17 cells.<sup>14,50</sup> Here, we found that autoimmune chronic GvHD is indeed caused by CD4<sup>+</sup> cells of Th1 origin. Our data are also consistent with clinical observations in chronic GvHD patients where poor prognosis is correlated to decreased Tregs.<sup>16,29</sup>

In summary, our study identifies a novel molecular mechanism that controls the T-bet/FoxP3 axis in the context of chronic GvHD. The data presented here suggest that adoptive Treg cell therapy strategies currently being pursued to treat chronic GvHD post-BMT may not be fully effective until methods to control Th1 signaling can be harnessed. Inhibition of transcription factors such as Tbet and STAT4 represent key molecular targets for the treatment of autoimmune GvHD. In addition, our work specifically delineated the IFN $\gamma$  signaling pathway in preventing autoimmunity. To boost anti-viral responses, an intact type I IFN $\gamma$  signaling and STAT1 activation is critical. The data presented here suggest that by specifically inhibiting type II IFN $\gamma$  receptor signaling, one might be able to augment Treg cells during chronic GvHD while maintaining type I mediated anti-viral responses. We envision that use of antagonists against IFN $\gamma$  type II receptor might be particularly beneficial for therapy of autoimmune chronic GvHD. Therefore, the Tbet pathway, including the IFN- $\gamma$  receptor and STAT1/STAT4 as upstream pathway members, drives experimental autoimmune GvHD. Interventions that restrict the Tbet pathway might either be used alone or in combination with adoptive Treg-cell therapy for treatment of autoimmune chronic GvHD.

### Funding

*This study was funded by the Intramural Research Program, National Cancer Institute, National Institutes of Health, USA and Newcastle University Research Fellowship, Newcastle University, UK. AL is supported by the Crohns and Colitis Foundation of America (CCFA).*

### References

1. Yi T, Chen Y, Wang L, et al. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. *Blood*. 2009;114(14):3101-3112.
2. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood*. 2002;99(10):3493-3499.
3. Yu Y, Wang D, Liu C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and ROR $\gamma$ mat in mice. *Blood*. 2011;118(18):5011-5020.
4. Laurence A, Amarnath S, Mariotti J, et al. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-

- host disease. *Immunity*. 2012;37(2):209-222.
5. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. 2012;12(6):443-458.
  6. Sugerman PB, Savage NW, Walsh LJ, et al. The pathogenesis of oral lichen planus. *Crit Rev Oral Biol Med*. 2002;13(4):350-365.
  7. Snyder LD, Palmer SM. Immune mechanisms of lung allograft rejection. *Semin Respir Crit Care Med*. 2006;27(5):534-543.
  8. Bharat A, Fields RC, Steward N, Trulock EP, Patterson GA, Mohanakumar T. CD4+25+ regulatory T cells limit Th1-autoimmunity by inducing IL-10 producing T cells following human lung transplantation. *Am J Transplant*. 2006;6(8):1799-1808.
  9. Regauer S. Immune dysregulation in lichen sclerosus. *Eur J Cell Biol*. 2005;84(2-3):273-277.
  10. Shlomchik WD, Lee SJ, Couriel D, Pavletic SZ. Transplantation's greatest challenges: advances in chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2007;13(1 Suppl 1):2-10.
  11. Chen X, Vodanovic-Jankovic S, Johnson B, Keller M, Komorowski R, Drobyski WR. Absence of regulatory T-cell control of TH1 and TH17 cells is responsible for the autoimmune-mediated pathology in chronic graft-versus-host disease. *Blood*. 2007;110(10):3804-3813.
  12. Rangarajan H, Yassai M, Subramanian H, et al. Emergence of T cells that recognize non-polymorphic antigens during graft-versus-host disease. *Blood*. 2012;119(26):6354-6364.
  13. Zeiser R, Blazar BR. Preclinical models of acute and chronic graft-versus-host disease: how predictive are they for a successful clinical translation? *Blood*. 2016;127(25):3117-3126.
  14. Chen X, Das R, Komorowski R, van Snick J, Uyttenhove C, Drobyski WR. Interleukin 17 is not required for autoimmune-mediated pathologic damage during chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2010;16(1):123-128.
  15. Radojic V, Pletneva MA, Yen HR, et al. STAT3 signaling in CD4+ T cells is critical for the pathogenesis of chronic sclerodermatous graft-versus-host disease in a murine model. *J Immunol*. 2010;184(2):764-774.
  16. Koreth J, Matsuoka K, Kim HT, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. 2011;365(22):2055-2066.
  17. Zhang C, Todorov I, Zhang Z, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood*. 2006;107(7):2993-3001.
  18. Rosenblum MD, Remedios KA, Abbas AK. Mechanisms of human autoimmunity. *J Clin Invest*. 2015;125(6):2228-2233.
  19. Suscovich TJ, Perdue NR, Campbell DJ. Type-1 immunity drives early lethality in scurfy mice. *Eur J Immunol*. 2012;42(9):2305-2310.
  20. Afkarian M, Sedy JR, Yang J, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol*. 2002;3(6):549-557.
  21. Yang XP, Jiang K, Hirahara K, et al. EZH2 is crucial for both differentiation of regulatory T cells and T effector cell expansion. *Sci Rep*. 2015;5:10643.
  22. Hwang J, Kita R, Kwon HS, et al. Epidermal ablation of Dlx3 is linked to IL-17-associated skin inflammation. *Proc Natl Acad Sci USA*. 2011;108(28):11566-11571.
  23. Yang XP, Ghoreschi K, Steward-Tharp SM, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol*. 2011;12(3):247-254.
  24. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. *J Clin Invest*. 2000;105(9):1289-1298.
  25. Nguyen VH, Zeiser R, Dasilva DL, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood*. 2007;109(6):2649-2656.
  26. Di Biaso I, Di Maio L, Bugarin C, et al. Regulatory T cells and extracorporeal photochemotherapy: correlation with clinical response and decreased frequency of proinflammatory T cells. *Transplantation*. 2009;87(9):1422-1425.
  27. Giorgini A, Noble A. Blockade of chronic graft-versus-host disease by alloantigen-induced CD4+CD25+Foxp3+ regulatory T cells in nonlymphopenic hosts. *J Leukoc Biol*. 2007;82(5):1053-1061.
  28. Rieger K, Loddenkemper C, Maul J, et al. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GVHD. *Blood*. 2006;107(4):1717-1723.
  29. Zorn E, Kim HT, Lee SJ, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood*. 2005;106(8):2903-2911.
  30. Finotto S, Neurath MF, Glickman JN, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science*. 2002;295(5553):336-338.
  31. Choi J, Ziga ED, Ritchey J, et al. IFN $\gamma$  signaling mediates alloreactive T cell trafficking and GVHD. *Blood*. 2012;120(19):4093-4103.
  32. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*. 2009;10(6):595-602.
  33. Ma H, Lu C, Ziegler J, et al. Absence of Stat1 in donor CD4(+) T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice. *J Clin Invest*. 2011;121(7):2554-2569.
  34. Wei J, Duramad O, Peng OA, Reiner SL, Liu YJ, Qin FX. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proc Natl Acad Sci USA*. 2007;104(46):18169-18174.
  35. Eckerstorfer P, Novy M, Burgstaller-Muehlbacher S, et al. Proximal human FOXP3 promoter transactivated by NF-kappaB and negatively controlled by feedback loop and SP3. *Mol Immunol*. 2010;47(11-12):2094-2102.
  36. Nakayamada S, Kanno Y, Takahashi H, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity*. 2011;35(6):919-931.
  37. Wohlfert EA, Grainger JR, Bouladoux N, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest*. 2011;121(11):4503-4515.
  38. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity*. 2011;35(3):337-348.
  39. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat Med*. 2011;17(6):673-675.
  40. Koch MA, Thomas KR, Perdue NR, Smigiel KS, Srivastava S, Campbell DJ. T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. *Immunity*. 2012;37(3):501-510.
  41. Yu F, Sharma S, Edwards J, Feigenbaum L, Zhu J. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. *Nat Immunol*. 2015;16(2):197-206.
  42. McPherson RC, Turner DG, Mair I, O'Connor RA, Anderton SM. T-bet expression by Foxp3(+) T regulatory cells is not essential for their suppressive function in CNS autoimmune disease or colitis. *Front Immunol*. 2015;6:69.
  43. Du J, Paz K, Flynn R, et al. Pirfenidone ameliorates murine chronic GVHD through inhibition of macrophage infiltration and TGF-beta production. *Blood*. 2017;129(18):2570-2580.
  44. Amarnath S, Mangus CW, Wang JC, et al. The PDL1-PD1 axis converts human TH1 cells into regulatory T cells. *Sci Transl Med*. 2011;3(111):111ra120.
  45. Koenecke C, Lee CW, Thamm K, et al. IFN-gamma production by allogeneic Foxp3+ regulatory T cells is essential for preventing experimental graft-versus-host disease. *J Immunol*. 2012;189(6):2890-2896.
  46. Croudace JE, Inman CF, Abbotts BE, et al. Chemokine-mediated tissue recruitment of CXCR3+ CD4+ T-cells plays a major role in the pathogenesis of chronic graft versus host disease. *Blood*. 2012;120(20):4246-4255.
  47. Hall AO, Beiting DP, Tato C, et al. The cytokines interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology. *immunity*. 2012;37(3): 511-523.
  48. Broady R, Yu J, Chow V, et al. Cutaneous GVHD is associated with the expansion of tissue-localized Th1 and not Th17 cells. *Blood*. 2010;116(25):5748-5751.
  49. Imanguli MM, Swaim WD, League SC, Gress RE, Pavletic SZ, Hakim FT. Increased T-bet+ cytotoxic effectors and type I interferon-mediated processes in chronic graft-versus-host disease of the oral mucosa. *Blood*. 2009;113(15):3620-3630.
  50. Nishimori H, Maeda Y, Teshima T, et al. Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17. *Blood*. 2012;119(1):285-295.