SUPPLEMENTARY APPENDIX

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

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Methods

Bone Marrow Transplant. Bone marrow (BM) cells from B6 mice were harvested and T-cell depleted [anti-CD90 microbeads (Miltenyi Biotec)]. Recipients (BALB/c) were conditioned with total body irradiation (TBI; 950 cGy) and rescued with 10M T-depleted BM (TDBM) cells plus 2 x 10^6 $CD4^+$ T cells from B6.WT or B6 molecule-deficient mice. At day 14, post-BMT, splenic $CD4^+$T cells were isolated and transferred into B6.*Rag2*$^{-/-}$ recipients (2 x 10^6 cells/mouse). For cell mixing experiments, WT CD4 cells were harvested from CD45.1 congenic B6 mice. For mechanistic Treg induction experiments, $CD4^+$ cells from WT Foxp3^{gfp} and *Tbx21*$^{-/-}$Foxp3^{gfp} were flow sorted at day 14 post-allogeneic BMT for isolation of GFP^{neg} cells that were then adoptively transferred into B6.*Rag2*$^{-/-}$ recipients.

Flow cytometry. After secondary transfer to *Rag2*$^{-/-}$ recipients, splenic T cells were isolated and intracellular (IC) flow cytometry was performed using Ki67 PE (B56) and FoxP3 APC. For IC cytokine measurement, T cells were stimulated for 4h with phorbol 12-myristate 13-acetate and ionomycin (Brefeldin A [GolgiPlug] and Monensin [Golgi stop] added for last 2hrs); cells were fixed, permeabilized and stained with IFN-γ PE (XMG1.2) and FoxP3 APC. Cells were analyzed using LSRII (FlowJo software). All antibodies except anti-FoxP3 were from BD Bioscience.

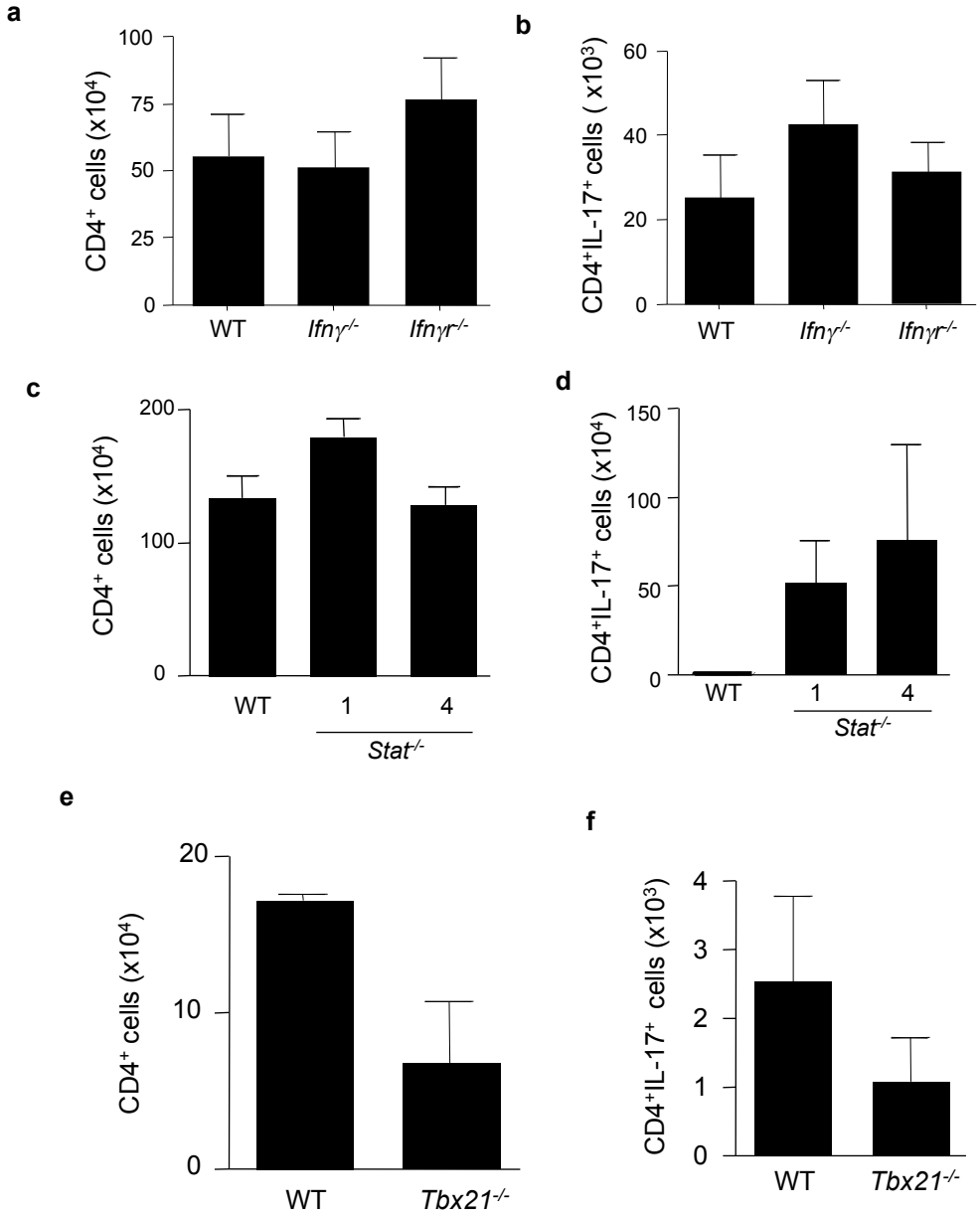
Post-Secondary Transfer Cytokine Secretion Assay. Spleen single cell suspensions were stimulated with syngeneic dendritic cells (DC) at spleen cell to DC ratio of 10:1. DC were obtained by culture of bone marrow cells with rmGM-CSF (1000 IU/ml) and rmIL-4 (1000 IU/ml) for four days; during the last 24 hours of DC generation, LPS (1 μg/ml) was added. After 24 hours of spleen cell and DC co-culture, supernatants were tested for IFN-γ content by multiplex array (eBioscience/ProcartaPlex).

Post-Secondary Transfer Evaluation of In Vivo Tissue RNA Genes. Skin tissue was obtained, and a homogenate was prepared by pulverizing frozen tissue in liquid nitrogen; once tissue became a powder, a working solution was added and samples were stored at -80^{0}C. Assay samples were warmed to 37^{0}C for 30 minutes and dispensed on hybridization plates containing a working bead mix (lysis mixture, blocking reagent, proteinase K, capture beads and probe set) for target hybridization. After hybridization, samples were transferred to magnetic separation plates and washed. Amplifier, label probe solution and streptavidin phycoerythrin were added and signal was detected using Luminex 100 (BioRad). Results were represented as mean intensity fluorescence; sample values were divided by geometric mean for housekeeping genes (HPRT, PPIB, GAPDH) and fold-change was calculated using normalized values for experimental groups (WT, KO and WT:KO) by normalizing values for controls (syngeneic). The panel of target genes included TGFB1, IL6, CLEC4E, IRAK4, TLR7, IFIH1, AIM2, FPR2, TNF, IL2, FOXP3, GATA3, ITGB3, P2RY12, IL1B, IL10, HSPD1, TLR4, IL1A, HPRT, PPIB, TLR2, RORC, PYDC4, TBX21, IFNA1, IFNG, HSPA1B, and CD4.

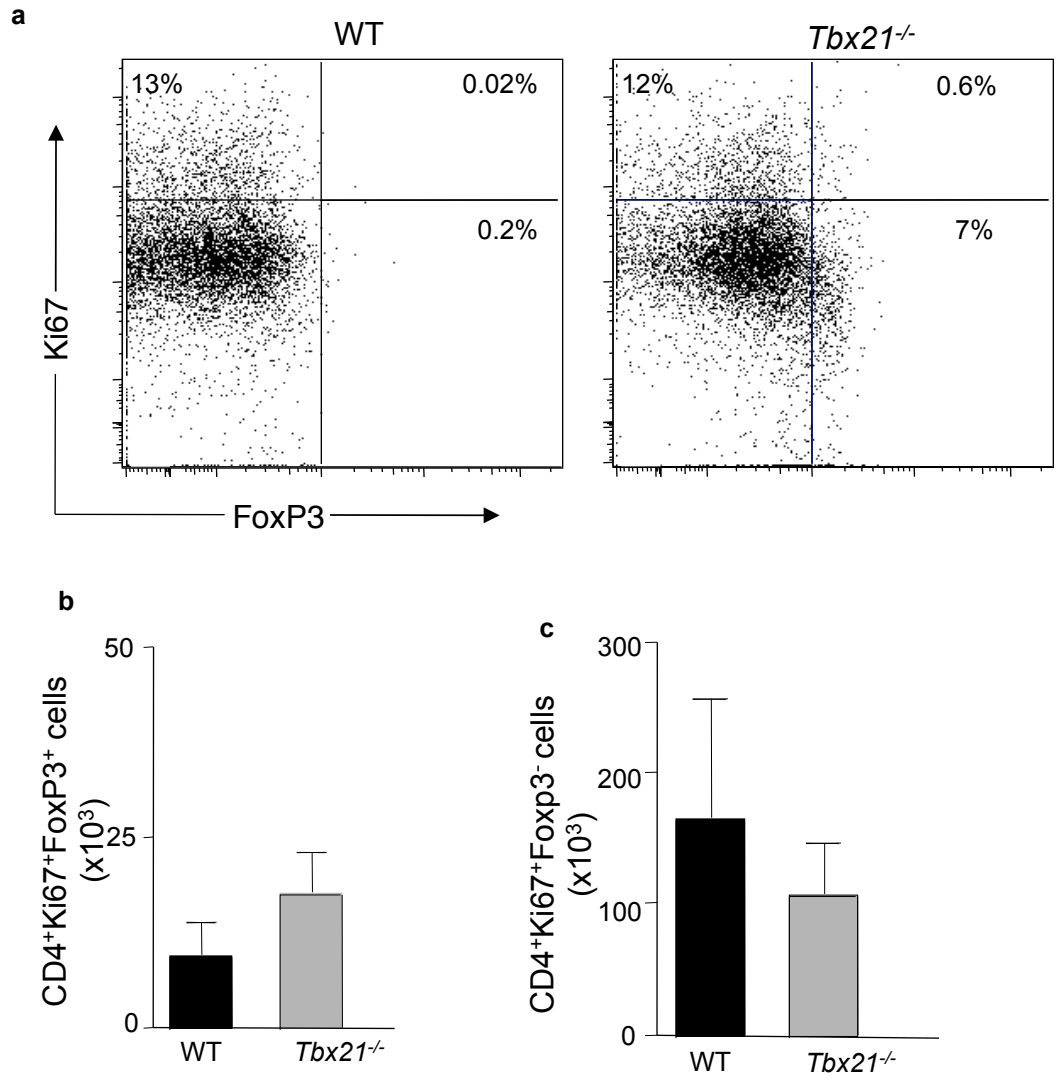
Isolation of T cells from LPL and skin. Isolation of T cells from LPL and skin. Large intestines were extracted and cleared of mesentery and fat; Peyers Patches were cut into pieces and washed in HBSS without calcium and magnesium. Briefly, dorsal skin was removed, minced and incubated with serum free media containing Liberase TL (250μg/ml; Roche) and 0.05% DNase I (Roche) for 1h 45min at 37^{0}C. Single cell suspensions were prepared using a 100μm nylon mesh and sequentially filtered through 70 and 40μm filters.

T cell isolation and differentiation. Naive CD4$^+$ T cells from spleens of 6-8 week old WT B6 or *Tbx21*$^{-/-}$ mice were purified by negative selection and magnetic separation (Miltenyi Biotec). T cells were activated for 3 days with plate bound αCD3 (2C11; eBioscience) and αCD28 (37.51; eBioscience). Cells were polarized to iTreg conditions using IL-2 (100IU/ml) and TGF-β1(5ng/ml) alone or with IL-12 (20ng/ml) and IL-18 (10ng/ml).

CHIP Assay. Naive $CD4^+$ T cells were polarized for 3-4 d, and crosslinked for 10 mins with 1% (vol/vol) formaldehyde. Cells were collected and lysed by sonication. After being precleared with protein A agarose beads (Upstate), cell lysates were immunoprecipitated overnight at 4^0C with antibody to T-bet (Clone H-210, Santa Cruz**).** After washing and elution, crosslinks were reversed for 4h at 65^0C. Eluted DNA was purified and quantitative PCR was used to analyze samples with custom design primers and probes (forward primer: TGCGGGCCCGAAGAT, reverse primer: CGTGCTCAGACTTTCCCACTAA and probe: TAGCAGCAGGAGTATGACA) with a 7500 Real-Time PCR system (all from Applied Biosystems). Each cycling threshold value was normalized to input values.

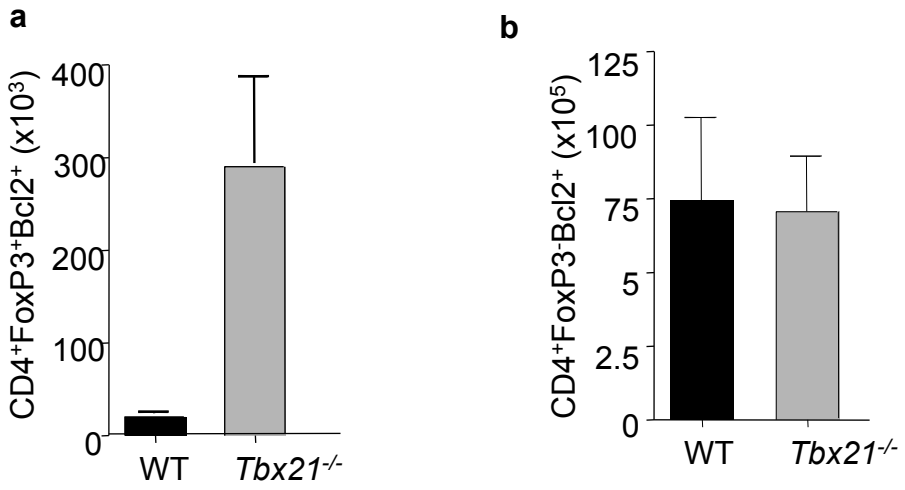


Supplemental Figure 1 : Deficiency in Th1 cytokine, cytokine receptor or transcription factors does not result in increased CD4$^+$ T cell numbers or Th17 phenotype. Host BALB/c mice were subjected to TBI (950cGy) and then reconstituted with allogeneic B6 TDBM (10M) and WT CD4$^+$ T cells (2M). In certain cohorts, mice were reconstituted with BM and T cells deficient in Th1 associated cytokine, cytokine receptor signaling or Th1 transcription factors. B6 *Rag2*$^{-/-}$ recipients were reconstituted with 2M of either alloreactive WT CD4$^+$ T cells or CD4$^+$ T cells deficient in Th1 cytokine or signaling capacity. At day 60-post transplant, splenocytes were harvested. (A) absolute numbers of CD4$^+$ T cells in cohorts that received either WT, *IFNγ*$^{-/-}$ or *IFNγr*$^{-/-}$ T cells. Spelnocytes were stimulated with PMA/ionomycin and then cytokine expression was measure by IC flow cytometry. (B) show absolute numbers of IL-17 producers in the CD4$^+$ compartment. (C) absolute numbers of CD4$^+$ T cells in cohorts that received either WT, *Stat1*$^{-/-}$ or *Stat4*$^{-/-}$ T cells. (D) show absolute numbers of IL-17 producers in the CD4 compartment in the respective cohorts. (E) absolute numbers of CD4$^+$ T cells in cohorts that received either WT, *Tbx21*$^{-/-}$ T cells. (F) show absolute numbers of IL-17 producers in the CD4 compartment in the respective cohorts



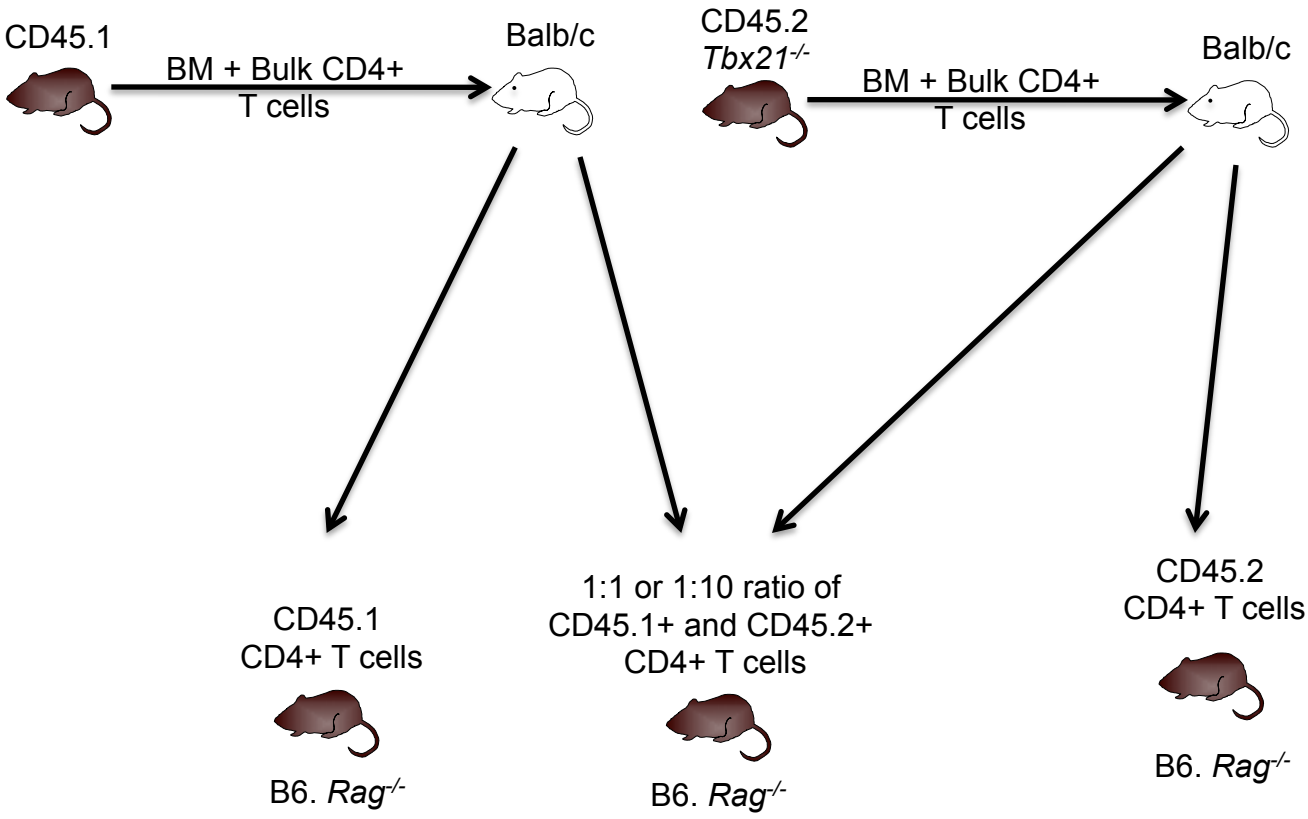
Supplementary Figure 2: Increase in Treg numbers in *Tbx21*$^{-/-}$ cohorts during autoimmune GVHD is not due to differential proliferation phenotype

Balb/c mice were lethally irradiated and then reconstituted with B6 WT or *Tbx21*$^{-/-}$ T depleted bone marrow (10M) and T cells (2M). At day 14 post-transfer, recipients were euthanized and 2M alloreactive CD4^{+}T cells were isolated and adoptively transferred into B6 *Rag2*$^{-/-}$ mice. At day 60-post transplant, spleens were harvested and T cell proliferation phenotype was monitored by flow cytometry. (A) Representative dot plots showing Ki67 and FoxP3 expression in WT and *Tbx21*$^{-/-}$ cohorts is shown. (B) Absolute numbers of FoxP3^{+} cells that are Ki67^{+} are shown in the various groups and (C) shows the absolute numbers of FoxP3^{-} cells that are Ki67^{+} in the various cohorts.

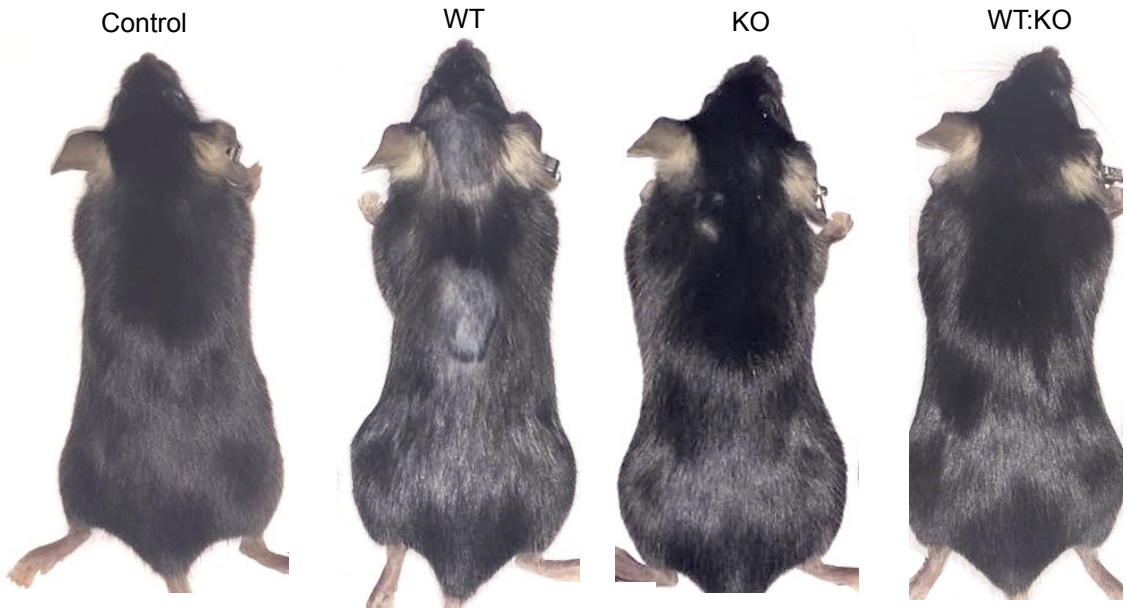


Supplementary Figure 3: WT and *Tbx21*$^{-/-}$ CD4^{+}T cells show similar anti-apoptotic bcl2 expression

Balb/c mice were lethally irradiated and then reconstituted with B6 WT or *Tbx21*$^{-/-}$ TDBM (10M) and CD4^{+}T cells (2M). At day 14 post-transfer, recipients were euthanized and alloreactive T cells (2M) were isolated and adoptively transferred into B6 *Rag2*$^{-/-}$ mice. At day 60-post transplant, spleens were harvested and T cell apoptotic phenotype was monitored by flow cytometry. (A) Absolute numbers of FoxP3^{+} cells that express bcl2 (B) absolute numbers of FoxP3^{-} cells that express bcl2. n=5 animals per cohort



Supplementary Figure 4: Schematic Representation of Co-transfer Experiment
Balb/c mice were lethally irradiated and then reconstituted with WT TDBM and B6 WT (CD45.1) or *Tbx21*$^{-/-}$ (CD45.2) T cells (2x 10^6). At day 14 post-transfer, recipients were euthanized and 2M alloreactive T cells were isolated and adoptively transferred into *B6 Rag2*$^{-/-}$ mice. Alloreactive cells of WT (CD45.1) or *Tbx21*$^{-/-}$ origin (CD45.2) was independently transferred into *B6. Rag*$^{-/-}$ recipients. In some cohorts a 1:1 or 1:10 ratio of WT (CD45.1) and *Tbx21*$^{-/-}$ (CD45.2) was transferred together into *B6.Rag*$^{-/-}$ recipients. At day 60, mice were euthanized and splenocytes were characterized for Foxp3 expression by flow cytometry.



Supplementary Figure 5: Clinical hair loss in murine recipients that received WT or *Tbx21*$^{-/-}$ deficient cells
WT and T-bet deficient T cells were isolated at day 14 after allogeneic BMT and adoptively transferred into B6 *Rag2*$^{-/-}$ recipients either alone or combined in a ratio of 1:1 (WT:*Tbx21*$^{-/-}$). An additional control cohort ("Control") first received a syngeneic transplant with WT cells followed by a secondary transfer into the syngeneic B6 *Rag2*$^{-/-}$ host. Representative hair loss pictures from each cohort is shown.