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TCF-1 negatively regulates the suppressive ability of canonical and noncanonical Tregs

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Abstract

Regulatory T cells are suppressive immune cells used in various clinical and therapeutic applications. Canonical regulatory T cells express CD4, FOXP3, and CD25, which are considered definitive markers of their regulatory T-cell status when expressed together. However, a subset of noncanonical regulatory T cells expressing only CD4 and FOXP3 have recently been described in some infection contexts.

Using a unique mouse model for the first time demonstrated that the TCF-1 regulation of regulatory T-cell suppressive function is not limited to the thymus during development. Our data showed that TCF-1 also regulated regulatory T cells' suppressive ability in secondary organs and graft-vs-host disease target organs as well as upregulating noncanonical regulatory T cells. Our data demonstrated that TCF-1 regulates the suppressive function of regulatory T cells through critical molecules like GITR and PD-1, specifically by means of noncanonical regulatory T cells. Our in vitro approaches show that TCF-1 regulates the regulatory T-cell effector-phenotype and the molecules critical for regulatory T-cell migration to the site of inflammation.

Using in vivo models, we show that both canonical and noncanonical regulatory T cells from TCF-1 cKO mice have a superior suppressive function, as shown by their ability to control conventional T-cell proliferation, avert acute graft-vs-host disease, and limit tissue damage. Thus,

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Author contributions

M.M. and M.K. designed the experiments, analyzed the data, wrote the manuscript, and performed all experiments. J.M.S. provided TCF-1 cKO mice. L.S. analyzed the pathology of the GVHD target organs. M.K. assisted with scientific and technical design and discussion, provided funding, and wrote the manuscript.

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Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

for the first time, we provide evidence that TCF-1 negatively regulates the suppressive ability of canonical and noncanonical regulatory T cells. These findings provide evidence that TCF-1 is a novel target for developing strategies to treat alloimmune disorders.

Keywords

T cells; TCF-1; canonical Tregs ($CD25^+$, $FOXP3^+$); noncanonical Tregs ($CD25^-$, $FOXP3^+$) Treg; suppressive function

1 Introduction

Regulatory T cells (Tregs) play a key role in regulating immune lymphocytes and in the promotion of immunologic tolerance.[1] Tregs have been used as immunotherapy for several autoimmune disorders, including bone marrow and solid organ transplantation, autoimmune diseases, and allergy.[2] The importance of Tregs in protection against autoimmunity is exemplified by the systemic fatal autoimmunity that develops in Scurfy mice[3,4] that are deficient in Tregs, as well as in patients with immune dysregulation, polyendocrinopathy, and X-linked (IPEX) syndrome.[5] Scurfy mice and patients with IPEX syndrome harbor a mutation in FOXP3,[3–5] which is essential for Treg development and function.[6] The low number of Tregs in peripheral blood is considered one of the challenges to using Tregs as immunotherapy.[7] Several lines of evidence show that in vitro expansion of Tregs causes the loss of the master regulatory transcription factor for Tregs. Thus, in vitro generation of functional Tregs has hampered their translation into the clinic.[8–10]

Several lines of evidence have been used to classify Tregs into canonical Tregs (canTregs), which are identified by expression of FOXP3 along with CD25, and a noncanonical population of Tregs (ncTregs) that are $FOXP3^+CD25^-$.[11–13] Unlike developing cells in the thymus, which can also express FOXP3 but not CD25, these ncTregs are found to lack the expression of CD25 in the periphery. These ncTregs are suppressive in several experimental models, including autoimmune encephalitis, inflammatory bowel disease, allergy, and diabetes.[13–18] However, $CD25^-$-deficient mice are unable to generate suppressive Tregs, suggesting that CD25 expression is required at some point for the suppressive ability of $CD25^-$ Tregs, but this is later lost in these cells.[14] Even though these cells have been observed in many contexts, the transcription factors that drive production of ncTregs were unknown.

Tregs are an essential part of the T-cell population, and T-cell factor 1 (TCF-1) was recently shown to play a role in Treg survival when it is expressed in combination with the transcription factor LEF-1.[19] TCF-1 has also been recently identified as an inhibitor of FOXP3 expression, as it binds to the promoter region of FOXP3 to prevent its aberrant expression in conventional T cells.[20] A recent report showed that overexpression of TCF-1 in T cells leads to the suppression of FOXP3.[20] In addition, global TCF-1 deficiency causes an increased frequency of $CD25^-FOXP3^{int}$ T cells and that FOXP3 expression was also increased among CD8 T cells.[20] However, TCF-1 is a critical T-cell transcription factor for T-cell development, CD4/CD8 lineage maintenance, and responses to infection,[21–28] so the global deficiency of this factor drastically influences T-cell development and function.[29]

It is unknown whether TCF-1 deficiency within mature T cells has a similar effect on FOXP3. We used a unique mouse strain that has a deletion of TCF-1 only in mature T cells rather than having a global deletion.[30] This TCF-1 flox/flox × CD4cre mouse experiences deletion of TCF-1 in all T cells at the double-positive phase of development, when all T cells express CD4.[25] This allows us to overcome the severe T-cell developmental defect that occurs with the global TCF-1 deletion, as TCF-1 is critical for the double-negative stage of development.[19] Our studies instead utilized a T-cell–specific deletion of TCF-1[31] to investigate the effects of TCF-1 loss in mature cells on canTregs ($CD25^+FOXP3^+$) and ncTregs ($CD25^-FOXP3^+$). We found that loss of TCF-1 in mature T cells led to an increased frequency and number of $CD25^-FOXP3^+$ ncTregs compared to ncTregs from wild-type (WT) mice. This effect was cell intrinsic and unaffected by changes to Eomesodermin (Eomes) and T-box transcription factor TBX21 (T-bet), which are altered when TCF-1 is lost. Eomes and T-bet are downstream of TCF-1 and play critical roles in maintaining T-cell lineage.[28,32,33] Of critical importance, when WT cells were present in the same microenvironment as TCF-1–deficient Tregs (in a chimeric mouse),[34] TCF-1 cKO T cells induced elevated production of canTregs and ncTregs from WT donor cells. Therefore, TCF-1 cKO T cells can promote canTreg and ncTreg fate among nearby cells, despite the WT phenotype of these neighboring cells. These $CD25^-FOXP3^+$ Tregs were found in multiple tissues and not expanded due to aberrant expression of FOXP3, because other functional Treg markers (CTLA-4 and IL-10)[13,33,35–39] were also expressed in these cells. CD8 T cells expressing FOXP3 without CD25 were not increased by loss of TCF-1. Thus, our results show that while TCF-1 does appear to control FOXP3 expression, loss of TCF-1 in mature T cells does not simply cause FOXP3 to be expressed aberrantly. Our observation that TCF-1–deficient T cells induce the ncTreg phenotype in WT cells within the same microenvironment is also novel. Our data for the first time show that the lack of TCF-1 peripheral Tregs, both canTregs $CD25^-FOXP3^+$ and ncTregs $CD25^+FOXP3^+$ Tregs, show significantly less expression of glucocorticoid-induced TNFR-related protein (GITR), which has been shown to reduce Tregs' suppressive function.[35,36] The expression of PD-1 has been known to negatively regulate Tregs' suppressive function.[37,38] Our data demonstrated that ncTregs from TCF-1 cKO mice express significantly less PD-1. Our data also show that canTregs from TCF-1 cKO mice express significantly more CXCR3, which is critical for Treg localization.[10,39] Effector-phenotype Treg cells are heterogeneous in terms of their ability to localize to specific tissues and suppress particular types of immune responses.[40,41] We also show that the loss of TCF-1 on mature T cells significantly increases effector-phenotype Tregs. We also show that ncTregs from TCF-1 cKO mice downregulate ICOS expression, which is important in the maintenance and survival of the effector Tregs. These findings highlight a critical role of TCF-1 in eTreg phenotype production. These findings also suggest that the loss of TCF-1 may increase the potency of noncanonical Tregs' suppressive functions by downregulating PD-1 and GITR expression and by upregulating the naive phenotype, which is important for long-term survival and maintenance. Overall, these findings shows that expression of TCF-1 in Treg limits their suppressive activity. Finally, by using an in vivo allogenic transplant model, we demonstrate that ncTregs and canTregs from TCF-1 cKO can suppress conventional CD8 T cells. Furthermore, ncTregs and canTregs from TCF-1 cKO suppress T-cell–mediated graft-vs-host disease (GVHD) and

ameliorate GVHD-induced organ damage. Novel strategies could be utilized for modulating these molecules to be used for patients with T-cell–mediated diseases.

2 Materials and methods

2.1 Mice

Thy1.1 (B6.PL-Thy1a/CyJ, 000406), B6-Ly5 (CD45.1^{+}, AKA "WT" or B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ, 002014), and BALB/c mice (CR:028) were purchased from Charles River or Jackson Laboratory. TCF-1 cKO mice (Tcf7 flox/flox cross bred with CD4cre)[31] were obtained from Dr. Jyoti Misra Sen at the National Institutes of Health and bred in our facilities. CD4cre (022071), Eomes$^{flox/flox}$ (017293), and T-bet flox/flox (022741) mice were purchased from Jackson Laboratories. CD4cre mice were bred in our facilities with Eomes or T-bet$^{flox/flox}$ mice to produce Eomes cKO or T-bet cKO mice, respectively. WT-expressing red florescent protein specifically on FOXP3 (WT C57BL/6-FOXP3^{RFP}) mice were provided by the August laboratory and were previously generated as described before.[42] We bred TCF-1 cKO with WT C57BL/6- FOXP3^{RFP} mice to generate TCF-1 cKO C57BL/6- FOXP3^{RFP} mice. Using genomic PCR, we confirmed that our newly generated mice are TCF-1$^{flox/flox\ +}$, CD4 cre$^{+/+}$ and RFP^{+}. Eight- to 12-wk-old age- and sex-matched mice were used for all experiments. Animal maintenance and experimentation were approved by the Upstate Medical University Institutional Animal Care and Use Committee (IACUC) committee (IACUC #433). All mice used for transplants were female, and flow cytometry experiments were done with both male and female mice.

2.2 Flow cytometry

To analyze expression of Treg markers, lymphocytes were collected and stained for flow cytometry. Lymphocytes were obtained from organs, filtered through a 70-μm filter, and treated with RBC Lysis Buffer to remove red blood cells (RBCs). The cells were then washed with ice-cold MACS buffer (1× PBS with EDTA and 4 g/L BSA) and plated in a 96-well V-bottom plate.[13] Antibody cocktails were prepared in 1× PBS or MACS buffer and added to each well. The cells were stained for 30 min on ice and covered to protect from light. The cells were then spun to remove antibodies and washed 1 to 2 times with ice-cold 1× PBS or MACS. Cells were fixed overnight at 4 °C in 200 μL fixative (Fix/Perm Concentrate and Fixation Diluent from FOXP3 Transcription Factor Staining Buffer Set; eBioscience cat. 00-5523-00). The next day, stained cells were permeabilized by washing twice with permeabilization buffer (eBioscience cat. 00-5523-00) and then stained for intracellular markers for 30 min at room temperature with antibody in permeabilization buffer and covered to shield them from light. Cells were then washed 1 to 2 times with permeabilization buffer, resuspended in 200 to 400 μL FACS buffer (eBioscience cat. 00-4222-26), and transferred to flow tubes. Data were collected on a BD Fortessa cytometer (BD Biosciences) and analyzed using FlowJo v9 (BD Bioscience).[13,43,44]

2.3 Antibodies

All antibodies were purchased from eBiosciences, Biolegend, or BD Biosciences. Antibodies used included anti-CD4 (BV785, FITC), anti-FOXP3-APC, anti-CD25 (PE, BV421), anti-CD45.2 (PE/Cy7, PE), anti-CD45.1-Pacific Blue, anti-Thy1.1-AF700, anti-

Thy1.2-APC, anti-CD3-APC/Cy7, anti-CD8 (FITC, PE), anti-IL-2-PE/Cy7, anti-CTLA-4-PE, anti-IL-10-APC/Cy7, anti-CD44-Percp, anti-CD62L-APC/CY7, anti-CXCR3-Percp/Cy5.5, anti-PD1-BV785, anti-ICOS-PE, and anti-GITR (PE, BV605). LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen cat. L34957) was used to remove dead cells from the analysis. Anti-CD3 (clone 17A2; Biolegend cat. 100202) was used to coat stimulation plates, and Ultra LEAF-purified anti-CD28 (clone 37.51; Biolegend cat. 102116) was used as a soluble stimulator. We used a multiplex ELISA kit from Biolegend (LEGENDplex Mouse Th1 cytokine panel Th 2-plex kit, cat. 741044) and a customized mouse T-cell kit to perform serum cytokine assays. In addition, luciferase substrate was acquired from Gold Bio. All ex vivo cells were analyzed on a BD LSR Fortessa cytometer machine for flow analysis (from Becton Dickinson Biosciences, BD). All flow cytometry data were analyzed with FlowJo as described.[34]

2.4 Chimera production

To produce bone marrow chimeras, Thy1.1 female mice aged 8 to 12 wk were lethally irradiated with 800 cGys in a single dose. Bone marrow was isolated from the femur and tibia of WT mice by (B6Ly5, CD45.1) and TCF-1 cKO (CD45.2) mice, filtered through a 70-μm filter, and counted. Bone marrow cells for the WT mixed bone marrow chimera model were mixed at a 1:1 ratio, and WT TCF-1 cKO bone marrow chimeras were mixed at a 1:4 (WT: TCF-1 cKO) ratio to ensure survival of KO cells with a potential proliferation defect.[34] Bone marrow cells were suspended in sterile 1× PBS for transplantation. The mixed bone marrow was injected into the Thy1.1 recipients via the tail vein at 4 h postirradiation. At 9 wk posttransplantation, blood was collected from the Thy1.1 (B6.PL-Thy1a/CyJ) mice and tested via flow cytometry for the presence of both CD45.1 (WT) and CD45.2 (TCF-1 cKO) cells. At 10 wk posttransplantation, recipient mice were euthanized and splenocytes were obtained for flow cytometry as described above.

2.5 Isolation of lymphocytes from liver

To isolate lymphocytes from liver, recipient mice were euthanized, and the livers were perfused with 5 mL cold 1× PBS. The livers were then mashed through a 70-μm filter, washed with MACS buffer, and mixed with 10% Percoll in RPMI/PBS. The samples in Percoll were spun at 2,200 rpm for 22 min at 22 °C, with no brake or acceleration, leading to isolation of lymphocytes in the pellet. These lymphocytes were then treated with RBC Lysis Buffer to remove RBCs and processed for flow cytometry as described above.[13]

2.6 Isolation of lymphocytes from small intestine

To isolate lymphocytes from the small intestine (SI), mice were euthanized, and following liver perfusion as described above, the entire SI was removed and placed in ice-cold media. The SI was cut open lengthwise to expose the lumen and washed with ice-cold media. The tissue was then placed in a 50-mL tube with 20 mL strip buffer (containing 1× PBS, FBS, EDTA 0.5 M, and DTT 1 M) and shaken at 37 °C for 30 min. This removed the epithelium from the SI, so following incubation, the tissues were vortexed, supernatant was discarded, and the tissue was moved to a new tube. The SI was minced into small pieces, and 10 mL digestion buffer was added to each tube (containing collagenase, DNAse, and RPMI). The tissues were incubated for 30 min while shaking at 37 °C and filtered into a clean tube using

a 70-μm filter. Remaining tissue pieces were ground onto the filter to obtain any remaining lymphocytes while leaving fat and structural cells behind. Finally, these cells were spun down in Percoll as described for livers to isolate lymphocytes. RBC Lysis Buffer was not used on these cells.[43,44]

2.7 Cell culture for Treg markers

To test cells for the Treg markers (FOXP3, CD25, CTLA-4, IL-2, IL-10), splenocytes were obtained from WT or TCF-1 cKO naive mice. These cells were split into three groups. Group 1 was simply stained for flow cytometry. Group 2 was cultured in LAK media for 6 h with Brefeldin A (GolgiPlug), in wells previously "coated" with PBS as a control. Finally, group 3 was cultured in LAK media for 6 h with Brefeldin A in wells coated with anti-CD3 in PBS (1 μg/mL), and anti-CD28 (2 μg/mL) was added to the LAK media for culture. After 6 h of culture, all cells were collected from the stimulation plate and stained for flow cytometry. All cells were stained for extracellular markers, then fixed overnight with the Invitrogen Intracellular Fixation and Permeabilization buffer kit (cat. 88-8824-00). The next day, the cells were permeabilized and stained with intracellular markers (FOXP3 for all, IL-2 and IL-10 for cultured cells only). All samples were then run on a BD LSR Fortessa flow cytometer as described above.

2.8 In vivo T-cell proliferation assay

Host BALB/c animals were irradiated lethally with 800 cGy total, divided into 2 doses of 400 cGy. These mice were then transplanted with allogeneic 10×10^6 bone marrow cells from WT C57BL/6 mice with T cells being depleted as above. Animals were also transplanted with allogeneic 1×10^6 WT C57BL/6 *luc*$^+$ CD8$^+$ T cells[13,43] and 0.5×10^6 FACS-sorted canTregs or ncTregs from TCF-1 cKO C57BL/6-FOXP3^{RFP} and from WT C57BL/6- FOXP3^{RFP} mice, which were administered intravenously to detect in vivo conventional T-cell proliferation through bioluminescence imaging (BLI). IVIS-50 was utilized to image recipient BALB/c mice every day for 31 d. BLI was quantified as described.[13,34] One-way ANOVA was performed for statistical analysis followed by Tukey's multiple comparison test. To determine the clinical score of GVHD, recipient mice were examined 2 to 3 times per week as described.[34] When recipient animals lost more than 30% of their original body weight, they were euthanized. Each animal in these experiments was imaged 3 times a week posttransplantation.[34] The IVIS 200 Imaging System (Xenogen) was used to evaluate tumor growth by BLI as described earlier.[34]

3 Histopathologic examination

Animals were transplanted with allogeneic 10×10^6 bone marrow cells from WT C57BL/6 mice and were also transplanted with 1×10^6 CD8$^+$ T cells from WT C57BL/6 mice and 0.5×10^6 FACS-sorted canTregs or ncTregs from TCF-1 cKO C57BL/6-mice or from WT C57BL/6- mice, which were administered intravenously to lethally irradiated mice. On day 14 posttransplantation, liver and small intestines were isolated from host mice and stained with hematoxylin and eosin (H&E). A pathologist graded the H&E slides blindly, and photos were also taken of the sectioned organs.[43,44]

3.1 Statistics

All statistics were performed using 1-way ANOVA or 2 way ANOVA, or Student's *t* test, depending on the data set. ANOVA analyses included Tukey's multiple comparisons test. *P* values are presented as <0.05 being significant. Data graphing and statistical testing were performed with GraphPad Prism v9 (GraphPad Software). Data are presented as means with standard deviation. All experiments were done with at least 3 mice per group, according to power analyses, and repeated multiple times unless otherwise specified.

4 Results

4.1 Loss of TCF-1 in all T cells leads to increased production of noncanonical Tregs

T-cell factor 1 (TCF-1, encoded by *Tcf7* genes) regulates T-cell development, cell fate specification, and maintenance of tissue homeostasis.[45] A recent publication[20] demonstrated that loss of TCF-1 induces aberrant FOXP3 expression due to the release of suppressive control. However, this work was done with TCF-1 global KO mice or CRISPR-mediated deletion in T cells, allowing potential off-target effects or developmental changes in these cells.[20,46] Therefore, we sought to determine whether loss of TCF-1 specifically in mature T cells would alter Treg populations. We obtained TCF-1$^{flox/flox}$ mice and bred these mice with CD4 cre mice both strain on a C57BL/6J background,[27] and the offspring of these were deficient in TCF-1 only on mature T cells following the double positive (DP) stage of development.[31] This allowed us to study mature T cells that developed normally in the thymus and then lost expression of the Tcf7 gene for TCF-1 at the DP phase.[47] We find that this loss of TCF-1 does not change the frequency of canTregs (CD25$^+$, FOXP3$^+$) but increases the frequency of ncTregs (CD25$^-$, FOXP3$^+$) when compared to the WT C57BL/6 mice and CD4 cre$^{+/+}$ C57BL/6 mice (Fig. 1A). To examine whether the loss of TCF-1 impacts absolute numbers of CD4 T cells that are positive and compared to canTregs and ncTregs to CD4 T cells from WT mice. Using flow cytometry utilizing data from several mice, our data confirm there is a difference in both frequency and absolute numbers (Fig. 1B). We observed a decrease in the absolute number of canTregs, possibly due to reductions in the absolute number of CD4 T cells from TCF-1 cKO mice, which is a significant finding and has not been reported before. Next, we want to determine whether the loss of TCF-1 impacts absolute CD4 T-cell numbers. Thus, we gated on the percent CD4$^+$ T cells, multiplied the percent number by the total spleen cell number, and divided that number by 100 to determine the absolute number. Our data uncovered that the loss of TCF-1 significantly decreases the absolute number and frequency of CD4 T cells (Fig. 1C and D).

In contrast, we find no significant differences in the absolute number of FOXP3$^+$ Tregs among CD3$^+$ T cells from TCF-1 cKO compared to control mice (Fig. 1E). These data demonstrated that TCF-1 expression in mature T cells affects CD4$^+$ canTregs and ncTregs. Therefore, we examined whether there are differences in total CD4$^+$ FOXP3-expressing Tregs. Our data demonstrated that the frequencies of CD4$^+$ T cells from TCF-1 cKO mice were significantly higher in FOXP3$^+$ Tregs than CD4 T cells from WT C57BL/6 mice and CD4 cre$^{+/+}$ C57BL/6 mice (Fig. 1F). Our data provide evidence that CD4 T cells from TCF-1 cKO C57BL/6 mice are reduced in number compared to CD4 T cells from WT C57BL/6 mice.

4.1.1 Eomes and T-bet do not impact noncanonical Treg frequency—

TCF-1 controls the T-cell downstream transcription factor Eomes and may affect T-bet by controlling the TFH/Th1 axis.[28] To examine whether changes in Eomes and T-bet during TCF-1 deficiency could play a role in the expansion of ncTregs, we performed the phenotypic analysis comparing Eomes cKO C57BL/6 mice and T-bet cKO C57BL/6 mice to control WT C57BL/6 mice, CD4cre$^{+/+}$ C57BL/6 mice, and T-bet$^{Flox/Flox}$ and Eomes$^{Flox/Flox}$ C57BL/6 mice (Fig. 2A–G). In agreement with previously reported work, the number of canTregs was found to be increased in the T-bet cKO C57BL/6 mice[21,48] (Fig. 2E and F). However, ncTregs were not impacted by the loss of Eomes or T-bet, suggesting that these factors are not critical for expansion or suppression of these cells (Fig. 2A–G). These findings suggest that the impact of TCF-1 on Tregs is not affected by Eomes or T-bet.

4.1.2 Expansion of noncanonical and canonical Tregs due to TCF-1 deficiency is cell intrinsic—

Changes in phenotype may be cell intrinsic (due to loss of the factor in each individual cell) or cell extrinsic (from changes in the microenvironment in the mouse due to loss of the factor). Therefore, we created bone marrow chimeras to test whether the increased frequency of ncTregs in TCF-1 cKO mice is cell intrinsic or extrinsic. Briefly, bone marrow from CD45.1 WT C57BL/6 was mixed with congenic CD45.2 TCF-1 cKO at a 1:4 ratio. The ratio was chosen based on our previous studies that showed bone marrow–derived T cells from IL-2–inducible T-cell kinase knockout mice (Itk$^{-/-}$) do not proliferate well,[34] similar to TCF-1 cKO C57BL/6T cells that do not proliferate well in vitro. The bone marrow mixture was transplanted into irradiated Thy1.1 mice (donor and host both on H2K^{b} background), and blood was collected at 9 wk and analyzed by flow cytometry to ensure reconstitution. At 10 wk, splenocytes were taken from these mice and phenotyped by flow cytometry. We first asked whether mixed bone marrow–derived absolute spleen cells from TCF-1 cKO mice that develop in the same thymus as WT bone marrow–derived spleen increase or decrease. Thus, we gated on WT bone marrow–derived spleen cells by CD45.1 and TCF-1 cKO bone marrow–derived spleen by CD45.2. We multiplied the frequencies of WT bone marrow–derived spleen or TCF-1 cKO bone marrow–derived spleen by the total number of spleen cells and divided by 100 to obtain the absolute number of WT or TCF-1 cKO bone marrow– derived spleen cells. We observed significantly higher absolute numbers and frequencies of TCF-1 cKO bone marrow–derived spleen cells than WT bone marrow–derived spleens (Fig. 3A and B). We also examined the absolute number of CD4 T cells from total spleen cells (Fig. 3C). Next, we wanted to determine the absolute number of CD4 T cells from either WT or TCF-1 cKO bone marrow–derived cells. Our data showed significantly more WT bone marrow–derived CD4 T cells than TCF-1 cKO bone marrow–derived CD4 cells (Fig. 3D). Next, we examined the frequencies of TCF-1 cKO or WT bone marrow–derived CD3^{+} T cells. First, we gated on CD3, then on CD45.1 or CD45.2. Our data showed significantly more WT mice bone marrow–derived CD3^{+} T cells in absolute numbers and frequencies (Fig. 3D and E). We also examined whether there are differences in either WT or TCF-1 cKO mice bone marrow derived CD8 and CD4 T cells from mixed chimera mice. We gated CD3^{+} CD45.1 to examine WT bone marrow–derived CD4 and CD8 and CD3^{+} CD45.2 to assess TCF-1 cKO mice bone marrow–derived CD4 and CD8 T cells. Our data showed a significant decrease in TCF-1 cKO-derived CD4 T cells compared to WT mice bone marrow–derived CD4 T cells. However, we observed an

increase in TCF-1 cKO bone marrow–derived CD8 T cells compared to WT mice bone marrow–derived CD8 T cells (Fig. 3F). We also examined whether the increase in both canonical and ncTregs is the result of an increase in total CD3 or CD4 T cells in mixed bone marrow chimera models. Thus, we looked at the cell numbers for these Tregs in the chimeric mice. We calculated the number of Treg cells per 100,000 $CD3^+$ T cells of each donor type (because WT C57BL/6 mice and TCF-1 cKO C57BL/6 mice donor T cells were mixed in the mouse). In the chimera, we found no difference in the numbers of canonical or ncTregs in these 2 donors types out of CD3 T cells (Fig. 3G). Therefore, we looked at the number of Tregs per 100,000 $CD4^+$ T cells, and we saw a reduced number of both $CD25^+$ and $CD25^-$ Tregs from WT C57BL/6 mice donors compared to TCF-1 cKO C57BL/6 mice donors (Fig. 3H). Next, we wanted to determine whether the percentage of $FOXP3^+$ Tregs might increase due to the loss of TCF-1. Thus, we gated on either WT bone marrow–derived CD45.1 or TCF-1 cKO bone marrow–derived $CD45.2^+$ $CD4^+$ T cells and $FOXP3^+$. Our data demonstrated that TCF-1 cKO bone marrow–derived CD4 T cells express a significantly higher percentage of FOXP3 Tregs (Fig. 3I). However, the number of ncTreg and canTregs derived from WT C57BL/6 mice T cells in the chimera was increased compared to the numbers found in the WT C57BL/6 naive mice (Fig. 3J). The number of WT C57BL/6 mice bone marrow derived CD25 Tregs out of CD4 T cells was increased in the chimera to the average numbers found in the TCF-1 cKO C57BL/6 naive mice. This could suggest that, when in a mixed environment with WT C57BL/6 mice cells, the TCF-1 cKO C57BL/6 mice T cells induce more WT C57BL/6 mice T cells to adopt the ncTreg phenotype, resulting in a higher frequency and number of these Tregs than would occur in naive WT C57BL/6 mice.

4.1.3 Noncanonical Tregs are found at increased frequency in multiple tissues from TCF-1–deficient mice—

The ncTreg population we observed in this model was identified in the spleen. To determine whether these cells existed only in the spleen or could be found in other organs, we phenotyped lymphocytes from the thymus, liver, small intestine, and lymph nodes of WT C57BL/6 mice, $CD4cre^{+/+}$ C57BL/6 mice, and TCF-1 cKO C57BL/6 mice (Fig. 4). We found that ncTregs were present even in WT C57BL/6 mice and $CD4cre^{+/+}$ C57BL/6 mice in all the tested organs (Fig. 4A–H). Significantly higher frequencies of ncTregs were observed in the thymus, liver, SI, and lymph nodes of TCF-1 cKO C57BL/6 mice compared to control mice (Fig. 4B). Therefore, ncTregs were present in multiple tissues in WT C57BL/6 mice and TCF-1 cKO C57BL/6 mice, with higher frequencies appearing when TCF-1 was lost. Additionally, the thymus of TCF-1 cKO C57BL/6 mice showed an increase in canonical Tregs (Fig. 4A–H). Overall, these data show that $CD25^-$ Tregs are found in multiple tissues, with expansion due to loss of TCF-1 occurring in these peripheral tissues as well.

4.1.4 TCF-1 regulates key molecules of the potent noncanonical Treg phenotype—

Recently, we have published that mice lacking IL-2–inducible T-cell kinase (Itk) express more canTregs and ncTregs, and these ncTregs are more suppressive than canTregs from WT mice,[13] with our findings confirmed by several groups.[43,49,50] To uncover whether canTregs or ncTregs from TCF-1 cKO mice express more or less suppressive molecules compared to canTregs and ncTregs from WT mice. Published studies have shown that GITR activation reduces Tregs' suppressive function.[35,36] To examine

whether canTregs or ncTregs from TCF-1 cKO C57BL/6 mice increase or decrease GITR expression, we isolated spleen $CD3^+CD4^+$ T cells from either WT C57BL/6 mice or TCF-1 cKO C57BL/6 mice using MACS purification. Our data showed that canTregs from WT mice have a higher percentage of GITR-expressing cells. However, ncTregs from WT C57BL/6 mice have significantly less GITR expression than canTregs (Fig. 5A and B). The GITR expression is further reduced by ncTregs from TCF-1 cKO C57BL/6 mice. These findings suggest that the loss of TCF-1 can increase the suppressive ability of the Tregs by downregulating GITR.

Published data have shown that reduced PD-1 expression of Tregs is significantly more suppressive than Tregs with higher PD-1 expression.[37] RNA sequences and cyTOF data showed that Tregs expressing higher PD-1 expression become exhausted and exhibit a reduced suppression function.[38] Therefore, we examined whether canTregs and ncTregs from WT C57BL/6 mice and TCF-1 cKO C57BL/6 mice express PD-1. Our data provide evidence that ncTregs from TCF-1 cKO C57BL/6 mice express less PD-1 than canTregs or ncTregs from WT C57BL/6 mice (Fig. 5C and D). We did not observe any differences in PD-1 expression from canTregs from either strain of mice (Fig. 5C and D). Thus, our finding highlights that the role of TCF-1 is critical for PD-1 expression of ncTregs, which may suggest that TCF-1 positively regulates the exhaustion of the ncTregs.

Chemokines play a critical role in trafficking to the site of inflammation. Most Treg cells use CXCR3 receptors to traffic to the site of inflammation, including to GVHD target organs such as the lungs, liver, and small intestine.[51] Published work has shown that adoptive transfer of Tregs with high CXCR3 expression rescues mice from T-cell–mediated GVHD.[52] Published data also show that deletion of CXCR3 on Tregs limits their recruitment to the site of inflammation.[39] Several lines of published work have shown that CXCR3 expression is critical for Treg localization.[53,54] Our data uncovered that canTregs from TCF-1 cKO C57BL/6 mice expressed more CXCR3, suggesting that canTregs from TCF-1 cKO C57BL/6 mice are more effective in migrating to the site of inflammation (Fig. 5E and F). We also did not observe any differences in CXCR3 expression in the ncTregs from WT C57BL/6 mice or TCF-1 cKO C57BL/6 mice. Our findings highlight that TCF-1 might suppress canTreg migration to the site of inflammation, and canTregs from TCF-1cKO C57BL/6 mice can have a superior suppressive function in GVHD.

Published data have shown that several key molecules, such as CTLA4, IL-2, and IL-10, play roles in Treg inhibition.[55] Other molecules like LAG-3,[37,56–58] local competition for growth factors such as consumption of IL-2.[59,60] Since CTLA-4 is an important factor for Treg function and identity,[61] we examined whether canTregs or ncTregs express differences in CTLA-4, as well as whether they express the cytokines IL-2 and IL-10. However, we did not observe any differences in CTLA, IL-2, and IL-10 expression (Supplementary Fig. 1A–F).

The ICOS signaling pathway endows Tregs with increased generation, proliferation, and survival abilities.[10,62] Maintenance and survival of effector (eTreg) cells mostly relies on ICOS.[63] Our data show that canTregs from TCF-1 cKO C57BL/6 mice showed similar ICOS expression as canTregs from WT mice. Surprisingly, ncTregs from WT C57BL/6

mice expressed significantly less ICOS than canTregs from both WT C57BL/6 and TCF-1 cKO C57BL/6 mice. This finding suggests that ncTregs from TCF-1 cKO C57BL/6 mice could have less of an effector memory phenotype. Since there was less ICOS expression in ncTregs from TCF-1 cKO C57BL/6 mice, we examined the memory phenotype of these $CD25^{+}FOXP3^{+}$ and $CD25^{-}FOXP3^{+}$ Tregs from WT C57BL/6 and TCF-1 cKO C57BL/6 mice. Effector-phenotype Tregs have been defined as $CD62L^{-}CD44^{+}$ activated Tregs, which are superior in suppressive function compared to other Treg subsets.[63,64] Our data showed that canTregs from TCF-1 cKO C57BL/6 mice have significantly more effector-phenotype Tregs than canTregs from WT C57BL/6 mice (Fig. 5I and L). This may mean that canTregs from TCF-1 cKO C57BL/6 mice have a more suppressive ability compared to canTregs from WT C57BL/6 mice. Also, as we suspected, $CD25^{-}FOXP3^{+}$ from TCF-1 cKO C57BL/6 mice have fewer effector-phenotype Tregs compared to the same cells from WT C57BL/6 mice (Fig. 5I and L). Since maintenance of the effector-phenotype Treg is predominantly dependent on ICOS expression, this may explain why ncTregs from TCF-1 cKO C57BL/6 mice have less ICOS expression. We also observed that ncTregs lacking TCF-1 have a more naive phenotype that has been shown to play an important role in long-term survival and maintenance[65–67] (Fig. 5I and K). Long-term survival of the Tregs has been shown to be an important factor mediating the suppressive function of the Tregs in autoimmune processes (Fig. 5I–L).

We have previously reported that the loss of ITK resulted in enhancements of both canTregs and ncTregs,[13] and here we provide evidence that TCF-1 regulates key molecules that regulate potent Tregs.[13,43,44,68,69] Delacher et al.[20] recently showed that a global deficiency of TCF-1 led to aberrant expression of FOXP3 in both CD4 and CD8 T cells, resulting in conventional T cells that appeared to be Tregs but were not. To determine whether aberrant FOXP3 expression was the reason for expansion of ncTregs, we first examined FOXP3 expression of CD8 T cells derived from WT C57BL/6 and TCF-1 cKO C57BL/6 mice. We found that there was no significant increase in $FOXP3^{+}CD25^{-}$ cells among CD8 T cells from TCF-1 cKO naive mice (Supplementary Fig. 2). Therefore, aberrant expression of FOXP3 in CD8 T cells does not occur in this model of TCF-1 deletion. We also showed that increased FOXP3 expression of CD4 T cells was associated with increased inhibitory and migratory factors and cannot be aberrant in the mice lacking TCF-1.

4.1.5 $CD25^{-}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$ Tregs from TCF-1 cKO mice suppress donor T-cell proliferation in vivo—

To examine whether canTregs and ncTregs from TCF-1 cKO C57BL/6 mice suppress GVHD caused by conventional $CD8^{+}$ T cells in the allogeneic transplant model, we specifically examined whether canTregs and ncTregs from TCF-1 cKO C57BL/6 mice suppressed donor T-cell proliferation and the manifestation of GVHD compared to canTregs and ncTregs from WT C57BL/6 mice. In order to do so, we used the TCF-1 cKO C57BL/6 $FOXP3^{RFP}$ mice to be able to sort the canTregs and ncTregs. We sorted canTregs and ncTregs by gating on $CD4^{+}CD25^{+}$ and $FOXP3^{+}$ by RFP from WT $FOXP3^{RFP}$ C57BL/6 mice and TCF-1 cKO $FOXP3^{RFP}$ C57BL/6 mice (Fig. 6A and B). Next, we used these sorted canTregs and ncTregs from both WT $FOXP3^{RFP}$ C57BL/6 and TCF-1 cKO $FOXP3^{RFP}$ mice into a functional assay by transplanting these cells in an allogeneic transplant model as described.[13] In these experiments, we transplanted

$CD8^+$ T cells from luciferase-expressing WT C57BL/6 mice into irradiated BALB/c mice as H2kb-expressing donor cells into H2kd-expressing recipients to induce a complete mismatch model.[13,43] Recipient BALB/c animals were examined for donor T-cell proliferation by BLI[13,43,70] (Fig. 6C). By using this model, we were able to assess the suppressive ability of the canTregs and ncTregs from WT C57BL/6 $FOXP3^{RFP}$ and TCF-1 cKO C57BL/6 $FOXP3^{RFP}$ mice in vivo. BALB/c recipient mice in each group were transplanted with allogeneic B- and T-cell–depleted bone marrow 10×10^6 cells $_{BTCD}$BM from non-*luc* WT C57BL/6 mice. In addition, we transplanted the first group of animals with 1×10^6 $CD8^+$ T cells from WT C57BL/6 *luc* mice (group 1). This group of mice did not have any Tregs transplanted and exhibited an increase in donor $CD8^+$ T-cell proliferation (Fig. 6C). The second group of recipient mice were transplanted with 10×10^6 $_{BTCD}$BM from non-*luc* WT mice and 1×10^6 $CD8^+$ T cells from WT C57BL/6 *luc* mice and further treated with 0.5×10^6 $CD25^+FOXP3^+$ Tregs FACS sorted from WT C57BL/6 $FOXP3^{RFP}$ mice by $CD25^+FOXP3^{RFP}$.[34] Our rationale for using 0.5×10^6 cells was that published data have shown that a 1:1 ratio of Tregs and conventional T cells transplanted into irradiated BALB/c mice were able to rescue BALB/c mice from developing GVHD.[34,64,71] Thus, we want to examine whether the reduced number of Tregs from TCF-1 mice can suppress conventional T-cell proliferation.[13] The second group of animals showed that treatment with 0.5×10^6 canTregs from WT C57BL/6 $FOXP3^{RFP}$ mice led to a reduction in conventional $CD8^+$ T-cell proliferation, but recipient mice eventually died of donor T-cell proliferation and GVHD. The third group of recipient mice were transplanted with 10×10^6 $_{BTCD}$BM from non-*luc* WT C57BL/6 and 1×10^6 $CD8^+$ T cells from WT C57BL/6 *luc* mice and treated with 0.5×10^6 ncTregs from WT C57BL/6 $FOXP3^{RFP}$ mice. This group of mice showed improved survival, but they did not suppress conventional T cells enough and died of GVHD. The fourth group of recipient BALB/c mice were transplanted with 10×10^6 $_{BTCD}$BM non-*luc* from WT C57BL/6 mice and 1×10^6 $CD8^+$ T cells from WT C57BL/6 *luc* mice. Additionally, this group of mice were treated with 0.5×10^6 $CD25^+FOXP3^+$ Tregs from TCF-1 cKO C57BL/6 $FOXP3^{RFP}$ mice sorted by $CD25^+FOXP3^{RFP}$. The fourth group of recipient mice treated with 0.5×10^6 $CD25^+FOXP3^+$ Tregs showed a significantly higher reduction in donor $CD8^+$ T-cell proliferation, and all animals survived for over 30 d (Fig. 6A–G). The fifth group of recipient BALB/c mice were transplanted with 10×10^6 $_{BTCD}$BM non-*luc* from WT C57BL/6 mice and 1×10^6 $CD8^+$ T cells from WT C57BL/6 *luc* mice. This group of mice were also transplanted with 0.5×10^6 $CD25^-FOXP3^+$ Tregs from TCF-1 cKO C57BL/6 $FOXP3^{RFP}$ mice. The cells were sorted by $CD25^-$ $FOXP3^{RFP}$. Similarly, this group were able to suppress luc^+ CD8 T cells from WT C57BL/6 mice in allogeneic transplantation and survived longer (Fig. 6C–G).

All transplanted mice were monitored for BLI (representing donor T-cell proliferation) every day for 30 d (Fig. 6D). Day 1 post-transplantation, there were no differences among any of the groups, but on day 7, we observed differences among groups. Total BLI was used to measure the reduction in donor $CD8^+$ T-cell proliferation. We observed that recipient BALB/c mice treated with canTregs or ncTregs from WT C57BL/6 $FOXP3^{RFP}$ mice died within 25 d of posttransplantation, while recipient BALB/c mice treated with either canTregs or ncTregs from TCF-1 cKO C57BL/6 $FOXP3^{RFP}$ mice survived for more than 30 d post-transplantation (Fig. 6E). Recipient animals were weighed 3 times per week for 30 d.

Recipient BALB/c mice treated with either ncTregs or canTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice initially started to lose weight within the first 7 to 10 d, and this weight loss was due to irradiation and transplantation. However, on post day 10 transplantation, mice in groups 4 and 5 all regained the weight.[72] However, recipient BALB/c mice treated with either canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice were unable to gain weight and died within 25 d of transplantation. Recipient BALB/c mice treated with canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice had better clinical scores compared to recipient BALB/c mice treated with either canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice (Fig. 6G). These functional data show that TCF-1 negatively regulates the suppressive function of the Tregs, and Tregs lacking TCF-1 can ameliorate GVHD by suppressing conventional T cells.

4.1.6 CD25^{-}FOXP3^{+} and CD25^{+}FOXP3^{+} Tregs from TCF-1 cKO mice cause less damage to GVHD target organs—

To examine whether recipient BALB/c mice treated with either canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice induce less tissue damage, experiments were conducted where all groups of BALB/c mice were irradiated as described above and transplanted with 10×10^6 $_{BTCD}$BM from WT C57BL/6 mice and $1{\times}10^6$ CD8^{+} T cells from WT C57BL/6 mice. An experimental group of recipient BALB/c mice as described above were treated with canTregs or ncTregs from either WT C57BL/6 FOXP3^{RFP} mice or TCF-1 cKO C57BL/6 FOXP3^{RFP} mice. Recipient mice were euthanized at day 14, and GVHD target organs were isolated. GVHD target organs, including liver, SI, and skin, from recipient BALB/c mice were treated with either canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice, or TCF-1 cKO C57BL/6 FOXP3^{RFP} mice were used for histologic analysis. GVHD target organs were stained for H&E and were analyzed by a pathologist (L.S.). GVHD target organs showed significant donor T-cell infiltration into liver, SI, and skin from animals treated with canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice (Fig. 7). Recipient BALB/c gross images were taken before the mice were euthanized. Recipient BALB/c mice transplanted with canTregs from WT C57BL/6 FOXP3^{RFP} mice were compared to recipient mice transplanted with canTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice. We observed significantly more skin GVHD compared to what was observed in BALB/c mice transplanted with canTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice (Fig. 7A). Next, we took gross images of recipient BALB/c before euthanasia. Recipient mice transplanted with canTregs from WT C57BL/6 FOXP3^{RFP} mice were compared to recipient mice transplanted with canTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice and ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice (Fig. 7B). We observed significant erythematous ruffling, which could be the result of donor T-cell infiltration. To compare BALB/c mice transplanted with either canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice (Fig. 7A and B), we used an established histologic grading system.[34,73,74] Recipient BALB/c mice transplanted with canTregs from WT C57BL/6 FOXP3^{RFP} mice were scored grade 4, based on the established scoring system. Recipient liver histology was significant for changes of acute GVHD showing interlobular bile duct epithelium (black arrow) that was infiltrated and destroyed predominantly by lymphocytes (red circle). We observed extensive necrosis with degeneration (Fig. 7C). The livers of recipient mice transplanted with ncTregs from WT C57BL/6 FOXP3^{RFP} mice were also graded 4 with extensive necrosis with degeneration

(Fig. 7D). However, recipient mice treated with either canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice showed significantly less donor T-cell infiltration and significantly less GVHD damage to target organs (Fig. 7E and F). Microscopic analysis of SI indicated that recipient BALB/c mice transplanted with either canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice showed extensive necrosis with degeneration and were scored 4 (Fig. 7G and H). Recipient mice treated with either canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice were given grades of 1, because we saw isolated epithelial cell apoptosis but did not observe any crypt loss or destruction (Fig. 7I and J). Microscopic analysis of skin showed that recipient BALB/c mice transplanted with either canTregs or ncTregs from WT C57BL/6 FOXP3^{RFP} mice were given scores of 4 as they showed extensive necrosis with degeneration and significant damage (Fig. 7K and L). Recipient BALB/c mice transplanted with either canTregs or ncTregs from TCF-1 cKO C57BL/6 FOXP3^{RFP} mice were scored 1 as we only saw vascular changes but no apoptosis no lymphocyte infiltration (Fig. 7M and N). Altogether, our compelling data demonstrated that TCF-1 negatively regulates both canTreg and ncTreg suppressive in vivo functions. Strategies can be developed to enhance ncTregs to treat T-cell–mediated alloimmunity.

5 Discussion

TCF-1 is a major T-cell transcription factor that controls T-cell development and lineage commitment, and it also has an important role in T-cell responses to infection.[19–29,75] However, the role of TCF-1 in controlling mature T cells following normal development has not been well studied. Our studies sought to determine whether loss of TCF-1 in mature T cells (following development) would impact Treg cell fates in vivo in the mouse, given that TCF-1 is known to repress FOXP3 expression.[20] Loss of TCF-1 in this context—using a Tcf7$^{flox/flox}$ × CD4cre mouse—resulted in the production of a smaller number and frequency of CD4 T cells, as has been reported previously.[31] We have demonstrated that of the CD4 T cells present, there were higher frequencies and absolute number of ncTregs found in the TCF-1 cKO mice compared to WT mice, while no change in frequency was found for canTregs. However, we observed a decrease in the absolute number of canTregs due to a reduction in the absolute number of CD4 T cells from TCF-1 cKO mice. This suggests that the expression of FOXP3 in ncTregs from the TCF-1 cKO mice may not simply have an aberrant elevated expression of FOXP3, because if that were the case, one would expect more canTregs as well. In addition, the expression of IL-2, IL-10, and CTLA-4 at WT levels among CD25^{-} Tregs suggests that these expanded cells from TCF-1 cKO mice are potentially suppressive of Treg cells and not the product of abnormal FOXP3 expression. This is in direct opposition to what has been reported from the use of global TCF-1 KO mice and CRISPR-mediated deletion of TCF-1, suggesting that TCF-1 may have a different role in mature cells than in developing cells.[20]

Of note, our studies showed that the rise in ncTregs was cell intrinsic and not a result of microenvironment changes in the mouse due to loss of TCF-1, as may be more likely in a TCF-1 global KO mouse.[22,31] In addition, TCF-1 controls Eomes and possibly T-bet expression,[28,75] yet Eomes cKO and T-bet cKO mice showed no changes in ncTregs. This suggests that TCF-1 and loss thereof is directly responsible for the expansion of CD25^{-} FOXP3^{+} Tregs. These ncTregs were found in small intestine, spleen, thymus, and liver,

suggesting that they are capable of migrating to potential sites of immune responses where suppression may be needed. If these cells were simply aberrantly exiting the thymus without expression of CD25, we would expect the frequency of these cells to be higher in the thymus than in other organs, but this was not the case. In fact, the frequency of these cells was higher in liver, gut, and spleen than in the thymus, especially in the TCF-1 cKO mice. Therefore, these ncTregs appear to be stable peripheral Tregs that have lost CD25 expression at some point after exiting the thymus.[14]

Our most important observation from this study came from our mixed bone marrow chimera, which involved mixing bone marrow cells from WT and TCF-1 cKO mice and allowing them to develop to maturity in a WT thymus. We then examined the absolute number and frequency of $CD25^+$ and $CD25^-$ Tregs in the spleen. This allowed us to determine that the expansion of $CD25^-$ Tregs is cell intrinsic, because the rise in frequency of these cells for TCF-1 cKO mice compared to WT mice was preserved for this model. Even though the absolute number and frequencies of bone marrow–derived spleen from TCF-1 increased, we observed a significant reduction in TCF-1 cKO bone marrow–derived CD4 T cells in both absolute number and frequencies. In a similar manner, despite the WT environment, TCF-1–deficient T cells are able to induce more production of ncTregs from WT donor cells. This finding and this trait generally could have a major impact on attempts to raise the yield of suppressive cells for therapeutic purposes. It also shows a novel way by which deficiency within a cell could impact nearby cells, most likely by release of factors into the microenvironment, which can alter fate decisions. To our knowledge, the ability of gene- or gene product–deficient cells to alter WT cells in a WT environment in this fashion has not yet been described, so this trait is a major novel finding.

Our data uncovered that both canTregs and ncTregs from TCF-1 cKO mice utilize key molecules such as a lower expression of GITR for their suppressive function. The lower expression of GITR has been shown to be constitutively expressed in all Treg cells and is critical for their development and activity.[40,76] Thus, there is no direct evidence for which transcription factor regulates GITR expression. Our data suggested that TCF-1 might regulate GITR expression. Another key molecule that plays a critical role in Tregs is PD-1. Studies have shown that PD-1–deficient Tregs are more suppressive against the proliferation of T conventional cells.[37,77] Several lines of published data suggested that Tregs expressing a higher number of CXCR3 receptors can suppress Th1 T cells.[56] Our data indicate that TCF-1 negatively regulates CXCR3 expression of canonical Tregs, which means that TCF-1 not only regulates ncTregs and increases their frequency but also can also decrease the migration of the canonical Tregs. It has also been previously shown that CXCR3-expressing Tregs can suppress the Th1 response.[56] This would suggest that they can migrate and maintain their suppressive ability in the GVHD target organs longer than canonical Tregs from WT mice.[52]

We have previously reported that when we transplanted 1 million $CD8^+$ T cells from WT mice and treated with 0.5 million $CD25^+FOXP3^+$ Tregs from WT mice, recipient mice were not protected from developing GVHD.[13,64] In this report, we have shown that transplantation with 1 million WT T cells and 0.5 million canonical Tregs from TCF-1 cKO mice rescued recipient mice from GVHD development and was able to suppress

T conventional (Tcon) cells, confirming that in a 1:2 Treg to Tcon ratio, they are more suppressive than canTregs from WT mice.

Tregs play an important and critical role in the maintenance of tolerance, and the transfer of Treg cells can ameliorate T-cell–mediated alloimmunity by restoring defective tolerance mechanisms.[51] Given their low numbers in the blood, it is difficult to obtain enough Tregs in the clinic for use as a therapy. One of the biggest challenges is the generation of functional Tregs in large numbers that also have the suppressive ability for immunotherapy.[78] Therefore, understanding the underlying mechanism of canTregs and ncTregs could be an attractive approach for patients with autoimmune disorders. This limitation of cell numbers could be mitigated by in vitro expansion of Tregs, but data have shown that in vitro expansion causes loss of FOXP3 expression.[71] Several recent protocols have been developed using chimeric antigen receptor T-cell therapy. This is a very attractive approach, but it also has considerable limitations, such as problems with trafficking, downregulating chemokine receptors, and cryopreservation.[79,80] Future work should address the functional capacity of these Treg cells, although the suppressive ability of $CD25^{-}$ Tregs from normal WT mice has been proven in several experimental models.[11,12,15] The mechanism or factors by which TCF-1–deficient cells evoke fate changes in WT cells in a mixed cell model should also be examined and could have significant impacts on cell culture strategies for therapeutic uses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

canTreg	canonical regulatory T cell
cGys	centiGrays
cKO	conditional knockout
Eomes	Eomesodermin
eTreg	effector regulatory T cell
IPEX	immune dysregulation polyendocrinopathy and X-linked syndrome

KO	knockout
LAK	lymphocyte-activated killer media
MHC (I/II)	major histocompatibility complex^{-} (I/II)
ncTreg	noncanonical regulatory T cell
RBC	red blood cell
SI	small intestine
TBX21	T-bet T-box transcription factor
TCF-1	T-cell factor 1
Treg	regulatory T cell
WT	wild type

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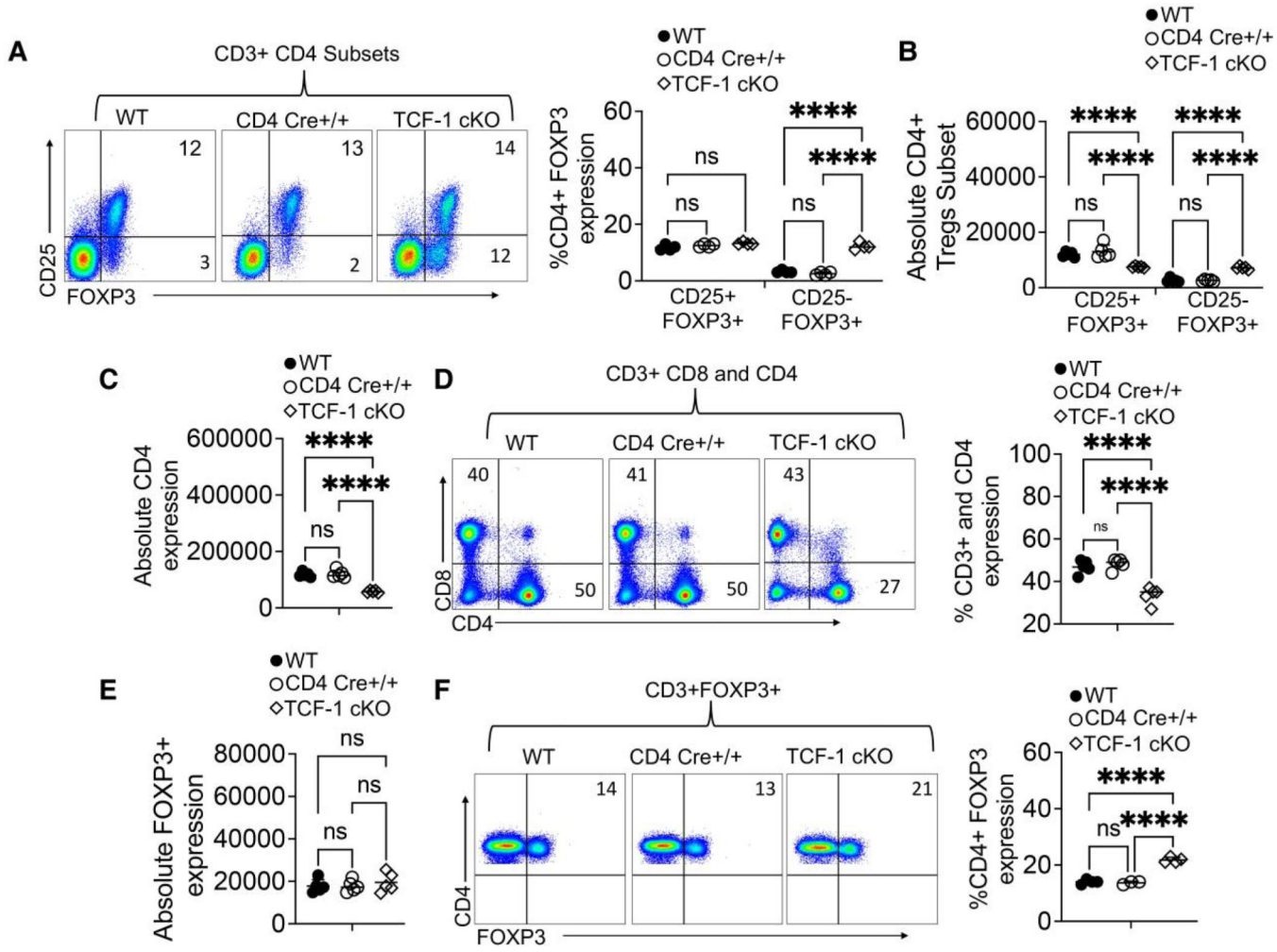

Fig. 1.

Loss of TCF-1 in T cells leads to increased production of noncanonical Tregs. Splenocytes were taken from naive mice and stained for CD4, CD25, and FOXP3 to identify Treg populations. Canonical Tregs are $CD4^+CD25^+FOXP3^+$, and noncanonical Tregs are $CD4^+CD25^-FOXP3^+$. (A) Flow cytometry plots of Treg frequencies in WT, $CD4cre^{+/+}$ homozygous control, and TCF-1 cKO. Cells from naive mice cells were gated on $CD3^+$ and CD4 T cells first and further gated on $CD25^+$ and FOXP3 T cells. One representative plot is shown per group and quantification of several mice. (B) Quantitative analysis of an absolute number of $CD3^+CD4^+$, $CD25^+FOXP3^+$, and $CD25^-FOXP3^+$ Treg subsets from WT, $CD4cre^{+/+}$, and TCF-1 cKO mice. (C) Quantification of the absolute number of CD4 T cells from WT, $CD4cre^{+/+}$, and TCF-1 cKO mice. The frequency of CD4 T cells was multiplied by total spleen cells divided by 100 and presented as quantitative numbers. (D) Spleen cells were gated on $CD3^+$, CD8, or CD4 T cells from WT, $CD4cre^{+/+}$, and TCF-1 cKO mice and quantified for several mice. (E) Quantifying an absolute number of $CD3^+FOXP3^+$ Tregs from WT, $CD4cre^{+/+}$, and TCF-1 cKO mice. (F) Spleen cells were gated on $CD3^+$, $CD4^+$, and $FOXP3^+$ Tregs from WT, $CD4cre^{+/+}$, and TCF-1 cKO mice and quantified for several mice. For all graphs, $n = 4$ to 5 per group, and 1 representative

experiment is shown; the mean and SD are also plotted. *P* values: ****$P \leq 0.0001$, and ns ($P > 0.05$) is not significant. Each experiment was repeated 3 times.

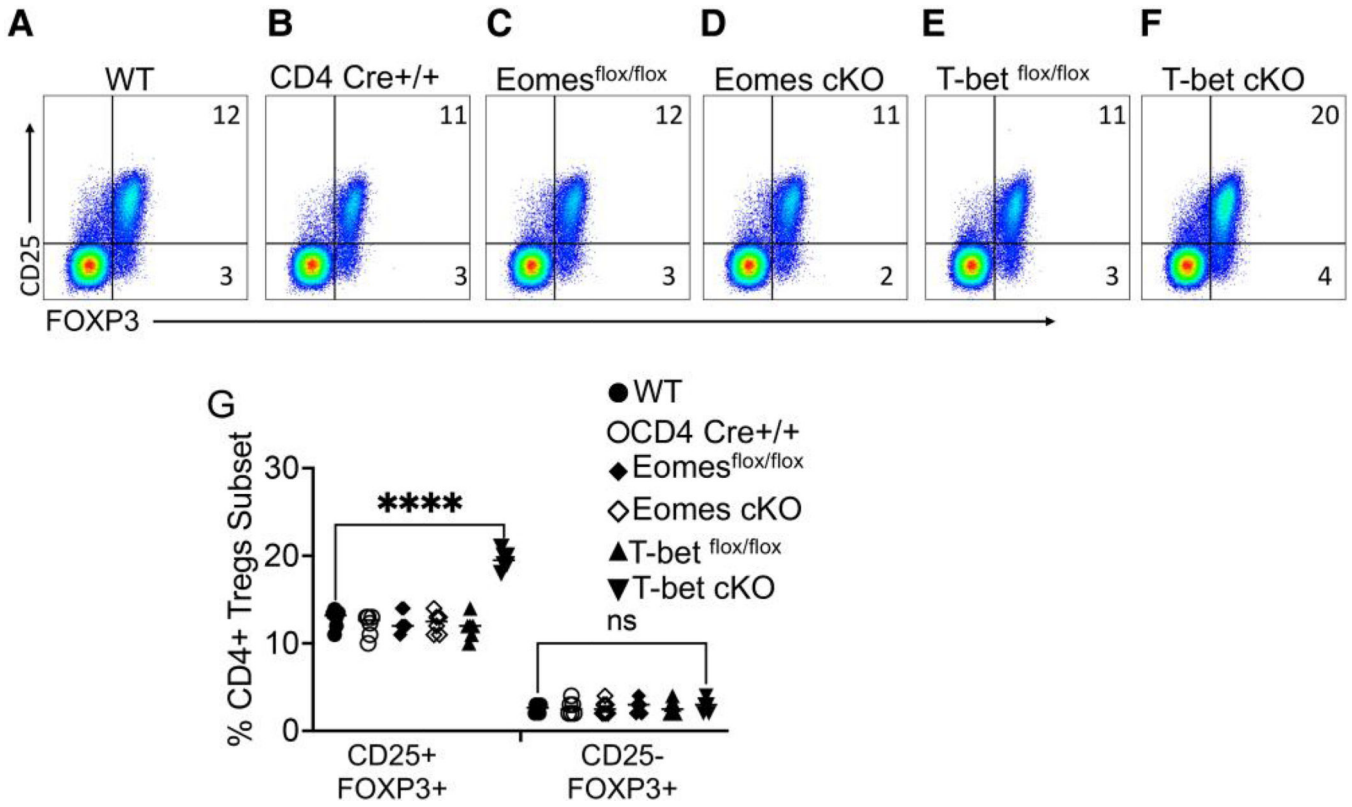

Fig. 2.

Downstream factors Eomes and T-bet do not impact noncanonical Treg frequency. (A–F) Flow cytometry plots of Treg frequencies in WT, CD4cre$^{+/+}$, Eomes flox/Flox, T-bet cKO, or Eomes cKO naive mice. T cells from naive mice were gated on CD3^{+}, CD4^{+} then CD25^{+} and FOXP3^{+} Tregs. One representative plot is shown per group. (G) Quantification of A–F, showing all groups. For all graphs, $n = 4$ to 5 per group, and 1 representative experiment is shown; mean and SD are also plotted. Each experiment was repeated 3 times. P values: **** $P \leq 0.0001$, and ns ($P > 0.05$) is not significant.

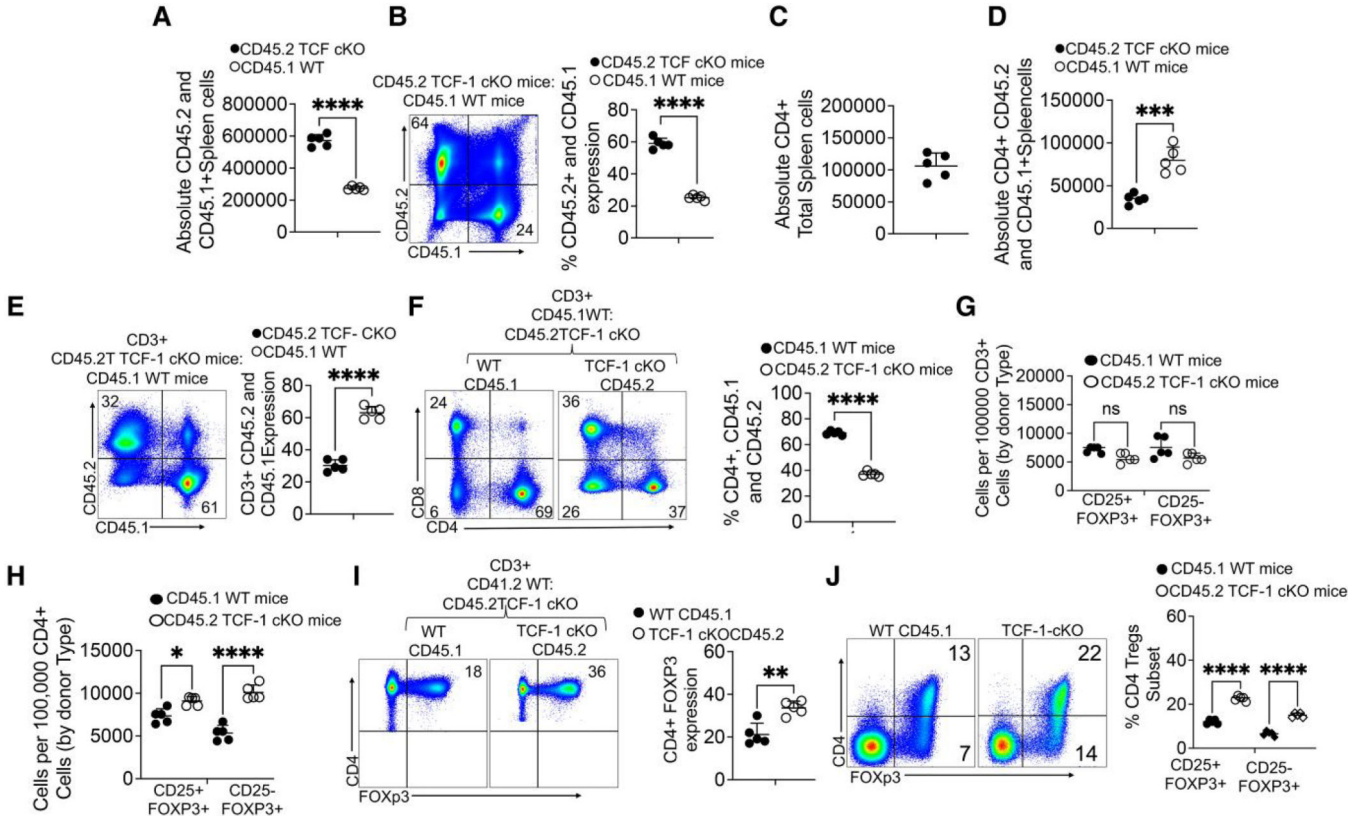

Fig. 3.

Expansion of noncanonical Tregs due to TCF-1 deficiency is cell intrinsic. (A) Bone marrow cells from WT and TCF-1 cKO mice were obtained and mixed at a 4:1 (TCF-1:WT) ratio and injected into lethally irradiated Thy1.1 mice. At 10 wk posttransplantation, flow cytometry was performed to look at Treg markers (CD25, FOXP3). (A) Using flow cytometric analysis, absolute cell numbers of WT bone marrow–derived spleen cells were gated by CD45.1 (WT) mice and TCF-1 cKO bone marrow–derived spleen cells by CD45.2. Quantification of flow cytometry plots of 5 mice is also presented. (B) Flow cytometry plot that shows that mixed bone marrow–derived spleen cells were gated of WT (CD45.1) and TCF-1 cKO (CD45.2) mice. Quantification of flow cytometry plots of 5 mice is also presented. (C) The absolute number of total CD4 T cells from mixed bone marrow from several mice is presented as a graph. (D) Quantification of the absolute number of CD4 T cells from either WT (CD45.1) or TCF-1 cKO (CD45.2) from mixed bone marrow spleen cells is presented. (E) Flow cytometry plots showing the frequency of mixed bone marrow–derived T cells that were gated on $CD3^+$ T cells from either TCF-1 cKO (CD45.2) or WT (CD45.1), and quantification of flow cytometry plots of 5 mice is also presented. (F) Flow cytometric analysis of mixed bone marrow–derived spleen cells included CD4 and CD8 T cells from WT (CD45.1) mice and TCF-1 cKO (CD45.2) mice, with quantification of flow cytometry plots of 5 mice. (G) Number of mixed bone marrow–derived cells gated on $CD25^+$ or $CD25^-$ Tregs was calculated per 100,000 $CD3^+$ WT mice (CD45.1) or TCF-1 cKO mice (CD45.2) within chimeric mice. (H) Number of $CD25^+$ or $CD25^-$ Tregs calculated per 100,000 $CD4^+$ WT mice by CD45.1 or TCF-1

cKO mice by CD45.2 donor-derived T cells within chimeric mice. (I) Flow cytometry plots of mixed bone marrow chimera derived spleen cells gated on $CD3^+$, $CD4^+$, and $FOXP3^+$ of either WT mice (CD45.1) or TCF-1 cKO mice (CD45.2), with quantification of flow cytometry plots of several mice also presented. (J) Flow cytometry plots of Treg frequencies from WT (CD45.1) and donor-derived or TCF-1 cKO (CD45.2) mice donor-derived quantification. For all graphs, $n = 5$ per group, and 1 representative experiment of 2 independent experiments is shown; mean and SD are also plotted. P values: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, and ns ($P > 0.05$) is not significant.

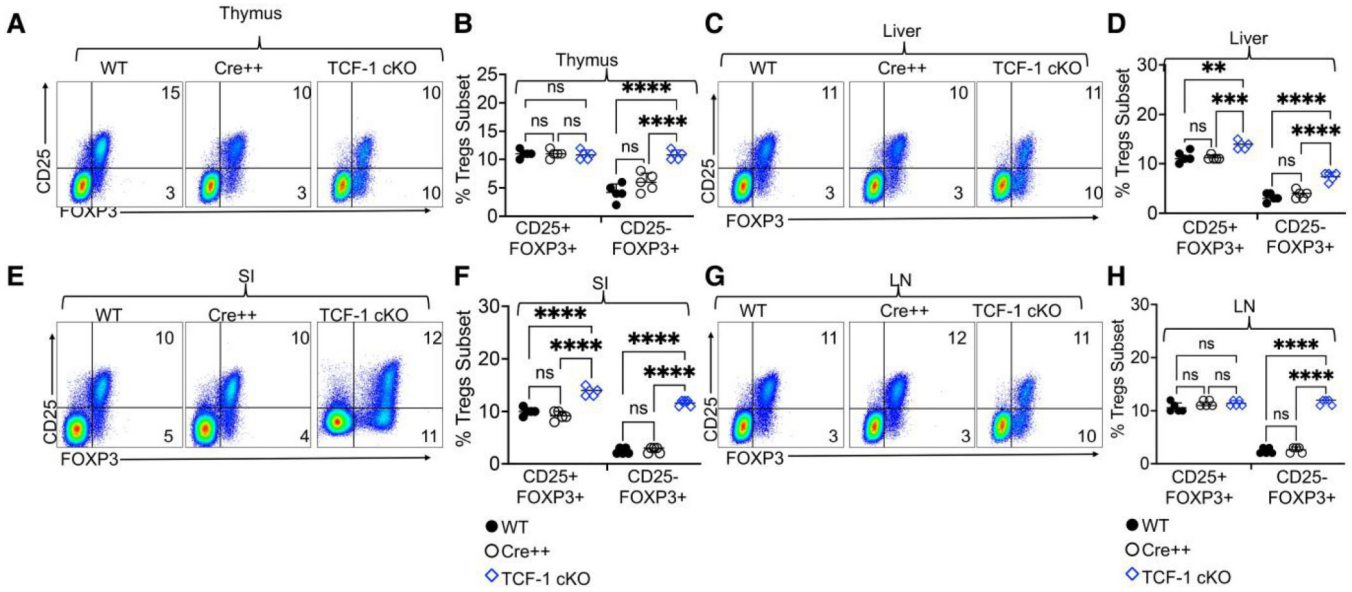

Fig. 4.

Noncanonical Tregs are found at increased frequency in multiple tissues from TCF-1–deficient mice. Flow cytometry plots of Treg frequencies in tissues of WT, CD4cre$^{+/+}$, or TCF-1 cKO mice. One representative plot is shown per group. Lymphocytes were obtained from the thymus (A, B), liver (C, D), small intestine (SI) (E, F), and lymph nodes (G, H), as described in detail in the Methods section. These cells were taken from WT or TCF-1 cKO naive mice and stained for flow cytometry. For all graphs, $n = 5$ per group, and 1 representative experiment of 3 independent experiments is shown; mean and SD are also plotted. P values: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, and ns ($P > 0.05$) is not significant.

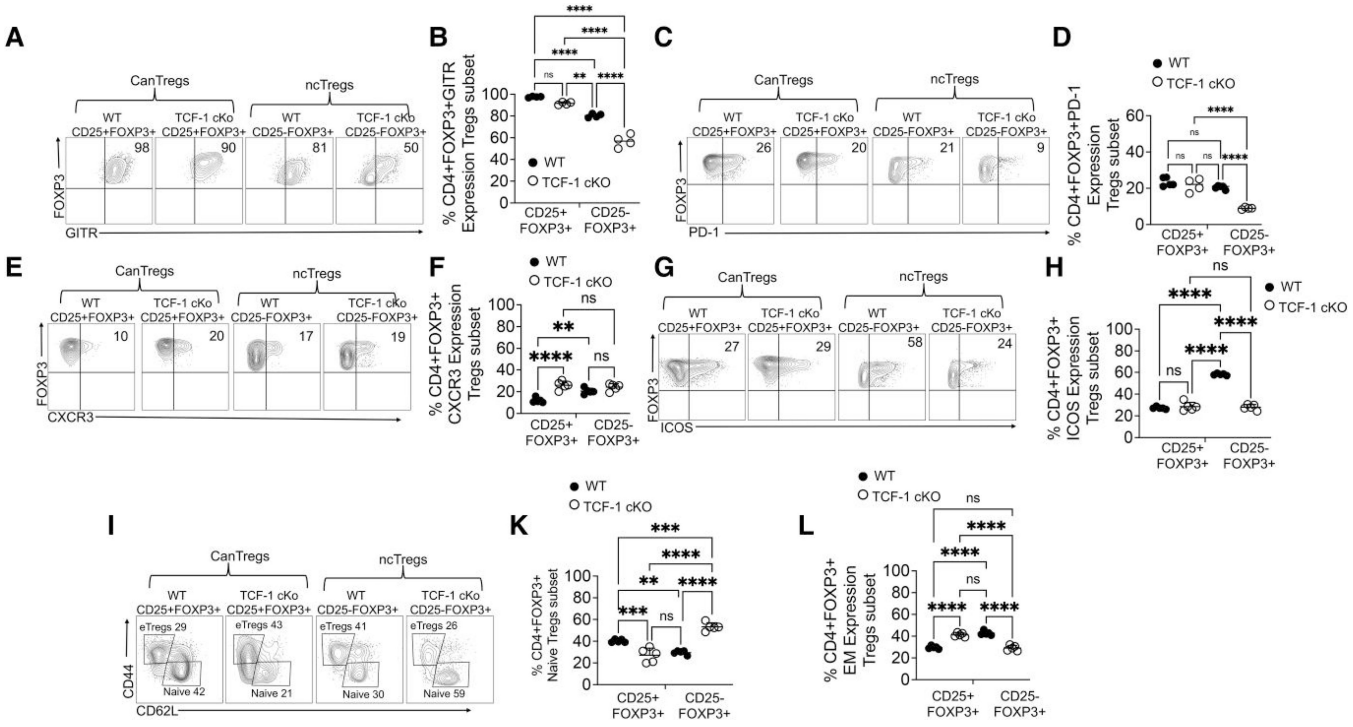

Fig. 5.

TCF-1 regulates key molecules of the potent noncanonical Treg phenotype. (A, B) Spleen cells were stained with $CD3^+CD4^+$ T cells CD25, GITR, PD-1, CXCR3, ICOS with Aqua live and dead markers, and FOXP3 as described in the Methods section. Cells were gated on live and dead markers, $CD3^+CD4^+$, either $CD25^+$ or $CD25^-$, $FOXP3^+$, and GITR positive. (C) These canonical and noncanonical cells were analyzed for PD-1 expression. (D) Quantitative data analysis of PD-1 from several mice is presented. (E) These canonical and noncanonical cells were analyzed for CXCR3 expression. (F) Quantitative data analysis of CXCR3 from several mice is presented. (G) Canonical and noncanonical cells were analyzed for ICOS expression. (H) Quantitative data analysis of ICOS expression from several mice is presented. (I) These canonical and noncanonical cells from WT and TCF-1 cKO mice were analyzed for effector memory by CD44 and CD62L expression. (K, L) Quantitative analysis of naive Tregs and effector memory Tregs from WT and TCF-1 cKO mice. For all graphs, $n = 4$ to 5 per group; 1 experiment is shown as a representative of 2 independent experiments. Statistical analysis was performed using 1-way ANOVA, and P values are presented with each figure. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, and ns ($P > 0.05$) is not significant.

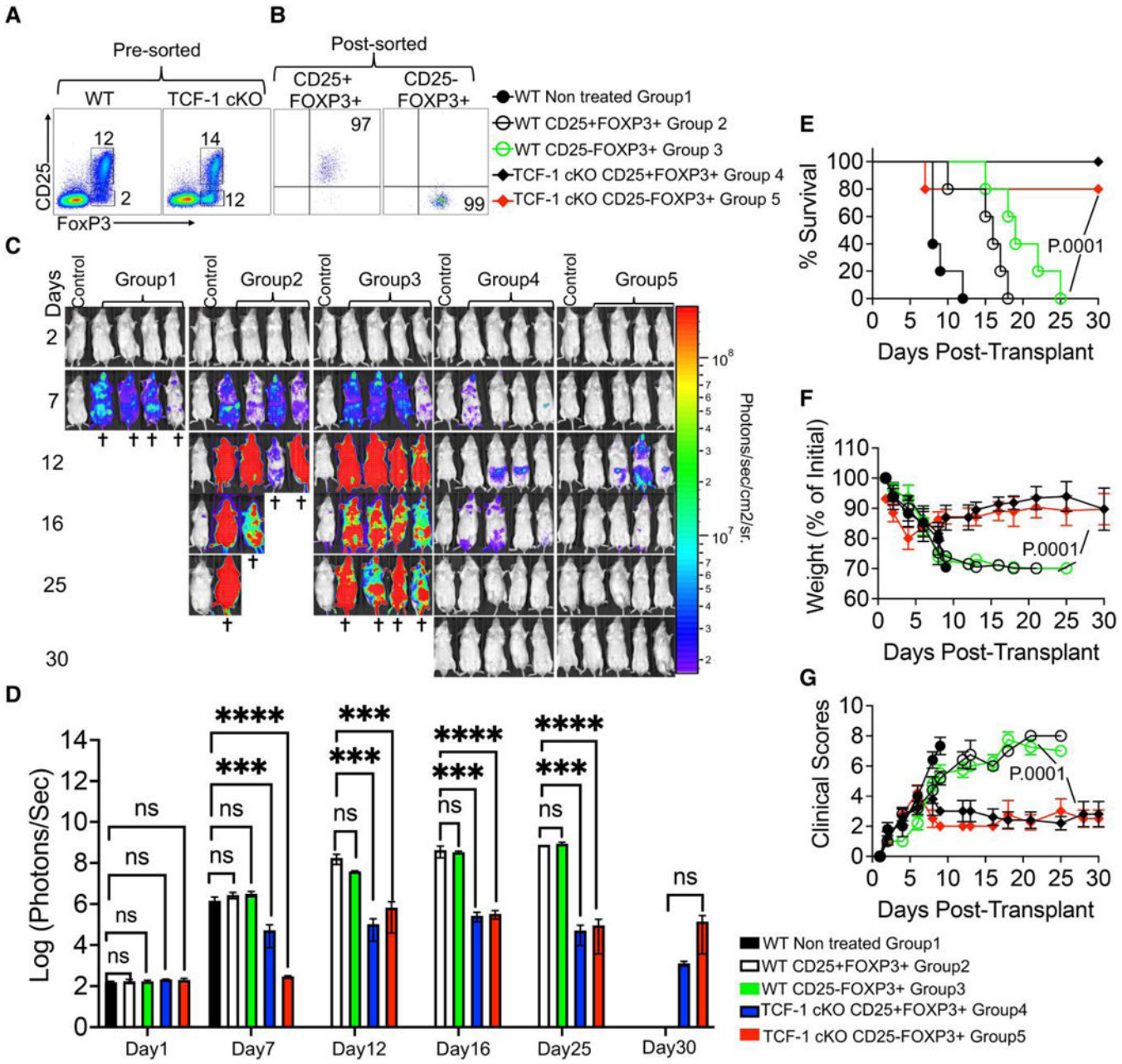

Fig. 6.

$CD25^{-}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$ Tregs from TCF-1 cKO mice better suppress donor T-cell proliferation in vivo. (A) Presorted canonical and noncanonical Tregs from WT and TCF-1 cKO spleens of naive mice were examined for expression of CD25 and FOXP3 by flow cytometry. (B) Next, after FACS, sorted canonical and noncanonical Tregs from WT C57BL/6 and TCF-1 cKO were gated on CD4 and $CD25^{+}$ $FOXP3^{+}$ or CD25-$FOXP3^{+}$ to confirm the presence of canonical and noncanonical Tregs. (C) BALB/c recipient mice for all groups were lethally irradiated and transplanted with 10×10^{6} T-cell–depleted bone marrow cells and 1×10^{6} WT-*luc*$^{+}$ $CD8^{+}$ T cells (donor T cells expressing luciferase). Group 1: recipient mice were not given any additional cells (nontreated).

Group 2: BALB/c recipient mice were treated with FACS-sorted canonical Tregs from WT C57BL/6 mice. Group 3: BALB/c recipient mice were treated with FACS-sorted noncanonical Tregs from WT C57BL/6 mice. Group 4: BALB/c recipient mice were treated with FACS-sorted canonical Tregs from TCF-1 cKO mice. Group 5: BALB/c recipient mice were treated with FACS-sorted noncanonical Tregs from TCF-1 cKO mice. Recipient BALB/c mice were imaged using IVIS 50 for 3 d per week for 30 d posttransplant in order to track the transplanted WT-*luc*$^{+}$ CD8 T-cell proliferation in the different treatment groups. (D) Quantification of luciferase bioluminescence, representing CD8-*luc*$^{+}$ donor T-cell proliferation. Statistical analysis was performed using 1-way ANOVA with Tukey's test; 1 experiment is shown. (E) The mice were monitored for survival. (F) Changes in body weight and (G) clinical score for about 30 d posttransplantation. For all graphs, $n = 5$ per group, and 1 experiment is shown as a representative from 2 independent experiments. Statistical analysis was performed using 1-way ANOVA, and P values are presented with each figure. *** $P \leq 0.001$, **** $P \leq 0.0001$, and ns ($P > 0.05$) is not significant.

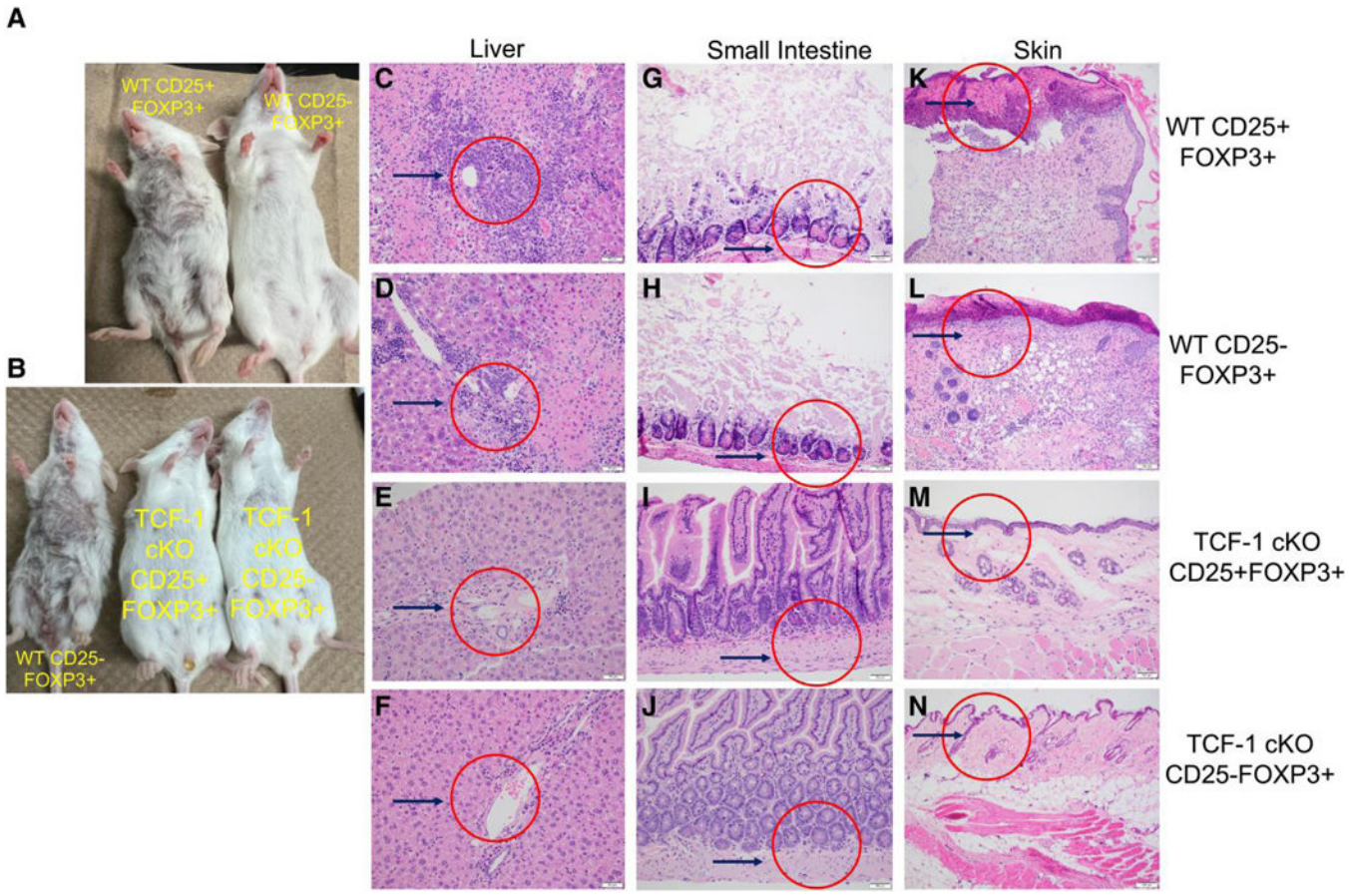

Fig. 7.

$CD25^{-}FOXP3^{+}$ and $CD25^{+}FOXP3^{+}$ Tregs from TCF-1 cKO mice cause less damage to GVHD target organs. BALB/c recipient mice for all groups were lethally irradiated and transplanted with 10×10^{6} T-cell–depleted bone marrow cells and 1×10^{6} WT-*luc*$^{+}$ $CD8^{+}$ T cells (donor T cells expressing luciferase). Group 1: recipient mice were not given any additional cells (nontreated). Group 2: BALB/c recipient mice were treated with FACS-sorted canonical Tregs from WT C57BL/6 mice. Group 3: BALB/c recipient mice were treated with FACS-sorted noncanonical Tregs from WT C57BL/6 mice. Group 4: BALB/c recipient mice were treated with FACS-sorted canTregs from TCF-1 cKO mice. Group 5: BALB/c recipient mice were treated with FACS-sorted ncTregs from TCF-1 cKO mice. (A) A representative gross image of comparing recipient BALB/C mice treated with $CD25^{+}FOXP3^{+}$ T regs from WT mice to recipient BALB/C mice treated with $CD25^{+}FOXP3^{+}$ or $CD25^{-}FOXP3^{+}$ T regs from TCF-1 cKO mice. (B) A representative gross image of comparing recipient BALB/C mice treated with $CD25^{-}FOXP3^{+}$ T regs from WT mice to recipient BALB/C mice treated with $CD25^{+}FOXP3^{+}$ or $CD25^{-}FOXP3^{+}$ T regs from TCF-1 cKO mice. At day 7 posttransplantation, recipient mouse livers, small intestines, and skin were obtained, sectioned, and stained with H&E. Representative photos of recipient organs for each treatment group are shown. One experiment is shown as a representative from 2 independent experiments.