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Dendritic Cell-Specific Disruption of TGF-β Receptor II Leads to Altered Regulatory T Cell Phenotype and Spontaneous Multiorgan Autoimmunity

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In vitro data and transgenic mouse models suggest a role for TGF-β signaling in dendritic cells (DCs) to prevent autoimmunity primarily through maintenance of DCs in their immature and tolerogenic state characterized by low expression of MHC class II (MHCIId) and costimulatory molecules and increased expression of IDO, among others. To test whether a complete lack of TGF-β signaling in DCs predisposes mice to spontaneous autoimmunity and to verify the mechanisms implicated previously in vitro, we generated conditional knockout (KO) mice with Cre-mediated DC-specific deletion of Tgfbr2 (DC-Tgfbr2 KO). DC-Tgfbr2 KO mice die before 15 wk of age with multiorgan autoimmune inflammation and spontaneous activation of T and B cells. Interestingly, there were no significant differences in the expression of MHCIId, costimulatory molecules, or IDO in secondary lymphoid organ DCs, although Tgfbr2-deficient DCs were more proinflammatory in vitro and in vivo. DC-Tgfbr2 KO showed attenuated Foxp3 expression in regulatory T cells (Tregs) and abnormal expansion of CD25 Foxp3+ Tregs in vivo. Tgfbr2-deficient DCs secreted elevated levels of IFN-γ and were not capable of directing Ag-specific Treg conversion unless in the presence of anti–IFN-γ blocking Ab. Adoptive transfer of induced Tregs into DC-Tgfbr2 KO mice partially rescued the phenotype. Therefore, in vivo, TGF-β signaling in DCs is critical in the control of autoimmunity through both Treg-dependent and -independent mechanisms, but it does not affect MHCIId and costimulatory molecule expression.

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ransforming growth factor-β belongs to a family of evolutionarily conserved molecules with pleiotropic roles in development, carcinogenesis, fibrosis, wound healing, and immune responses (1). In the immune system, TGF-β is critical for the maintenance of peripheral tolerance, an effect believed to be primarily mediated through TGF-β signaling in T cells (1). However, regulation of the innate immune cells, specifically the dendritic cells (DCs), by TGF-β still remains to be characterized in detail. DC maturation induced by TLR ligand/cytokines has been reported to lead to insensitivity to the immunosuppressive effects of TGF-β (2), thus suggesting that loss of TGF-β signaling in DC triggered by inflammation may contribute to the pathogenesis of autoimmune diseases.

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Abbreviations used in this article: B6, C57BL/6J; BM, bone marrow; BMDC, bone marrow-derived dendritic cell; CM, complete medium; Ct, threshold cycle; DC, dendritic cell; Flt3L, Flt3 ligand; HMDC, human monocyte-derived dendritic cell; iTreg, induced regulatory T cell; KO, knockout; LN, lymph node; MHCIId, MHC class II; MLN, mesenteric lymph node; pDC, plasmacytoid dendritic cell; qPCR, quantitative PCR; SF, single-positive; TBP, TATA box binding protein; Treg, regulatory T cell.

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the models used to date have not been able to conclusively and definitively address the role of TGF-β signaling in DCs in vivo.

An important aspect of DC-mediated tolerance requires their functional interaction with regulatory T cells (Tregs), immunosuppressive cells that play a dominant role in maintaining tolerance to self-Ags. One of the mechanisms by which Tregs exert their immunosuppressive function relies on their ability to modulate DC function. Tregs not only downmodulate the expression of co-stimulatory molecules such as CD80/CD86 on DCs but also induce a tolerogenic phenotype (17). DCs are also involved in Treg homeostasis, and a feedback loop between the numbers of DCs and Tregs in vivo has been postulated as crucial for the balance between immunity and tolerance (18). In addition, DCs also actively induce Foxp3+ Tregs from naive T cell precursors in the presence of TGF-β (19). However, although the direct effect of TGF-β on T cells in this process has been well documented, the role of TGF-β signaling in DCs to maintain Treg homeostasis and differentiation has not been examined in detail.

To assess the in vivo significance of TGF-β signaling in DCs in a more comprehensive fashion, we developed a conditional knockout (KO) mouse model (DC-Tgfbr2 KO) by crossing DC-specific Cre deleter mouse strain (20) with mice having exon 2 of Tgfbr2 gene flanked by loxP sites (21). CD11c-Cre mice are BAC transgenics in which Cre recombinase replaced CD11c exon I in the entire Itgax (CD11c) gene, which lacks the 5' end of the adjacent Itgam (CD11b) gene, thus preventing the overexpression of the latter (20). DC-Tgfbr2 KO mice die by 14 wk of age with multiorgan autoimmune inflammation. Despite no difference in MHCIi and costimulatory molecule expression, Tgfbr2-deficient DCs were more proinflammatory and less immunosuppressive as evidenced in the adoptive DC and T cell cotransfer studies. We observed decreased number of CD25+Foxp3+ peripheral Tregs with concomitant expansion of CD25+Foxp3+ cells, as well as increased numbers of activated effector T cells in DC-Tgfbr2 KO mice. The DCs from the KO mice were unable to direct Ag-specific induced Treg (iTreg) differentiation because of elevated IFN-γ production. These findings reveal the importance of TGF-β signaling in DCs in preserving both DC and Treg function, independently of Ag presentation or costimulation.

Materials and Methods

Mice

B6.129S6-Tgfbr2tm1Hlm mice, carrying homozygous loxP site insertion flanking exon 2 of Tgfbr2 gene (21) were obtained from National Cancer Institute (Frederick, MD) mouse repository (strain 01XN5). CD11c-Cre transgenic mice (B6.Cg-Tg(Itgax-cre)-1Reiz/J) (20), OT-II transgenic mice (B6.Cg-Tg(TcraTcrb)425Cbn/J), Rag1−/− (B6.129S7-Rag1tm1Mom/J) (22), and wild-type C57BL/6J (B6) were obtained from The Jackson Laboratory. All mice were maintained in a conventional animal facility at the University of Arizona. A separate colony of Cre− and DC-Tgfbr2 KO was established and maintained in an ultraclean (Helicobacter sp.-free) facility through embryo transfer. All animal experiments were approved by the University of Arizona Institutional Animal Care and Use committee.

Polymerase chain reaction

Cre-mediated recombination in T cells and BMDCs was tested using PCR with primers specifically designed to span exon 2 of Tgfbr2 gene. DNA was

![Figure 1](http://www.jimmunol.org/)
extracted from cells using the DNA isolation kit from Qiagen (Valencia, CA) and subjected to PCR amplification. Each PCR mixture contained 50–100 ng DNA, 5 μl 10× AccuPrime Reaction mix (Life Technologies, Grand Island, NY), 0.5 μl 10 μM gene-specific forward and reverse primers, 0.4 μl AccuPrime DNA polymerase (Life Technologies), and water to 50 μl. Primers used for exon 2 were 5'-GAGAGGGTATAACTCTCCATC-3' (forward) and 5'-GTTGGATGGATGGTCTATTAC-3' (reverse) and for exon 5 were 5'-TAGCCACACAGCCATCTCTCA-3' (forward) and 5'-TTGGATGGATGCCATCTTTCTGGG-3' (reverse).

**Generation of BMDCs**

BMDCs were prepared as described previously (23). Briefly, BM cells were resuspended in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone, Thermo Scientific, Rockford, IL), 50 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 mM glutamine (complete medium [CM]). For GM-CSF/IL-4–DC culture, BM cells were resuspended at 1.5 × 10⁶/ml in CM containing 10 ng/ml GM-CSF and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) and seeded at 3 ml/well in 6-well tissue culture plates. At days 3 and 5, half the medium was removed and fresh medium with cytokines was added to the cells. For Flt3 ligand (Flt3L)-DC culture, BM cells were resuspended at 1 × 10⁶ cells/ml in CM containing 100 ng/ml human recombinant Flt3L (Cell Signaling Technology, Danvers, MA) and seeded at 3 ml/well in 6-well tissue culture plates. At day 6 for GM-CSF/IL-4–DC or day 8 for Flt3L DC, loosely adherent cells were collected and CD11c⁺ cells were purified by magnetic selection using CD11c⁺ microbeads (Miltenyi Biotec, Auburn, CA). Cells were then replated at 1 × 10⁶ cells/ml in CM. Maturation of the DCs was induced by adding LPS (Calbiochem, EMD Millipore, Billerica, MA) at 100 ng/ml. All cells were incubated at 37˚C with 10% CO₂.

**Flow cytometry**

Single-cell suspensions were prepared from the thymus, spleen, and mesenteric lymph nodes (MLNs) and subjected to red cell lysis. After blocking for 15 min with anti CD16/CD32 Ab, the cells were labeled with fluorescent conjugated Abs and incubated for 30 min at 4˚C. Samples were analyzed using FACS-Calibur, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). Anti-mouse CD11c-allophycocyanin/PE, CD3-Percp, MHCII-FITC, CD80-allophycocyanin, CD86-allophycocyanin, CD40-FTC/PE, CCR7-Pe, CD11b-PE, CD86-FITC/PE-Cy7, Qa-2-FTC, CD26-PE, CD44-allophycocyanin/PE-Cy7, CD25-allophycocyanin, and Foxp3-PE were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). PDCA-1-PE and B220-FTC were purchased from Miltenyi Biotec. Intracellular staining for Foxp3 was carried out using the Treg staining kit from eBioscience.

**Adaptive transfer of BMDCs in an induced model of colitis**

BMDCs were generated using GM-CSF and IL-4, and 3 × 10⁶ CD11c⁺ cells were i.p. injected into B6 Rag-1⁻/⁻ mice that had received naive CD4⁺CD45RB⁺ T cells 2 wk earlier. Ten days posttransfer, mice were sacrificed, and colons were collected for histological analysis and explants cultures. Cytokine expression was determined by quantitative RT-PCR.

**Microarray analysis**

Splenic CD11c⁺ DCs were magnetically (CD11c isolation kit; Miltenyi Biotec) sorted from at least three control or DC-Tgfb2 KO mice. For isolation of MLN DCs, single-cell suspensions of the LNs were stained with a mixture of Abs consisting of CD11c, CD11b, Qa-2-FITC, CD45-PerCP, MHCII-FITC, CD80-allophycocyanin, CD86-allophycocyanin, and CD103-PE, and PDCA-1-PE and CD11c-allophycocyanin/PE, CD3-Percp, MHCII-FITC, CD80-allophycocyanin, CD86-allophycocyanin, CD40-FITC/PE, CCR7-PE, OR). Anti-mouse CD11c-allophycocyanin/PE, CD3-Percp, MHCII-FITC, CD80-allophycocyanin, CD86-allophycocyanin, CD40-FITC/PE, CCR7-PE, and CD103-PE (all from eBioscience). Samples were sorted in a BD FACSaria at the Cytometry Core facility at the Arizona Cancer Center. Cells were gated on CD45⁺MHCII⁺ and further gated on the combined population of CD11c⁺ and CD11c⁺CD11b⁻ cells. These cells were then sorted based on CD103 expression. Samples were pooled from four different mice, and RNA was isolated using a RNAqueous-Micro kit (Ambion, Life Technologies) to yield three samples per genotype. RNA integrity was evaluated with Agilent 2100 BioAnalyzer microfluidics-based platform (Agilent Technologies, Foster City, CA). RNA samples were subsequently processed to yield biotinylated cRNA for hybridization to Affymetrix Mouse Exon 1.0 ST arrays. The expression data were obtained in the form of .CEL files and imported into GeneSpring GX 10.0.2 (Agilent Technologies).

**FIGURE 2.** TGFBR2 expression in T cells. (A) Tgfb2 mRNA expression in CD4⁺ T cells from the spleen of Cre− and DC-Tgfb2 KO mice (top panel). Each sample was normalized to TBP expression. Error bars represent means + SEM of five individual mice; TGFBR2 expression in CD4⁺CD62L⁻ T cells from the spleen of Cre− and DC-Tgfb2 KO mice (middle panel). GAPDH was used as a loading control. Data are representative of at least three different experiments; percentage of CD4⁺CD62L⁻ T cells generated after stimulation of naive CD4⁺CD62L⁻ T cells with anti-CD3/CD28 beads and indicated concentrations of TGF-β for 3 d (bottom panel). Error bars represent means + SEM (n = 3). Student t test. (B) PCR detection of Cre-mediated excision of exon 2 of Tgfb2 in genomic DNA extracted from CD4⁺CD62L⁻ T cells from control or DC-Tgfb2 KO mice. CD11c⁺ BMDCs from either control or DC-Tgfb2 KO mice were used as positive controls. Amplicon from exon 5 is shown as an input control.
software package for data quality control and statistical analysis of the microarray data. Stringent empirical and statistical analyses were used to compare gene expression profiles between Cre and DC-Tgfbr2 KO mice with cross-gene error model based on replicates (data deposited to Gene Expression Omnibus database [http://www.ncbi.nlm.nih.gov/geo/]; accession code GSE39651).

Western blot analysis

Whole-cell lysates were collected in radioimmunoprecipitation assay buffer, and protein concentration in lysates was determined using bicinchoninic acid reagent (Thermo Fisher Scientific, Rockford, IL). Twenty micrograms of protein was subjected to SDS-PAGE electrophoresis, and separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), blocked at least for 1 h in 5% BSA or milk in 1 × TBS buffer containing 0.1% Tween 20 (TBST), and probed with pSmad2 (Cell Signaling Technology, Danvers, MA) or TGFBR2 (Santa Cruz Biotechnology, Santa Cruz, CA) Ab overnight at 4˚C. The membranes were washed three times with TBST buffer, followed by incubation with appropriate HRP-coupled secondary Ab. Super Signal West Pico detection kit (Thermo Fisher Scientific, Rockford, IL) was used for chemiluminescent detection. Blots were probed for total Smad2 or GAPDH to confirm equal loading. Autoantibodies were detected according to a previously described protocol (24).

Real-time PCR

Total RNA was isolated from tissues or cells using TRIzol reagent (Life Technologies), and its integrity was confirmed by denaturing agarose gel electrophoresis and calculated densitometric 28S/18S ratio. Total RNA (250 ng) was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories). Subsequently, 20 μl PCRs were set up in 96-well plates containing 10 μl 2× IQ Supermix (Bio-Rad Laboratories), 1 μl TaqMan respective primer/probe set (Applied Biosystems, Foster City, CA), 2 μl of the cDNA synthesis reaction (10% of reverse transcription reaction), and 7 μl nuclease-free water. Reactions were run and analyzed on a Bio-Rad CFX96 iCycler real-time PCR detection system. Cycling parameters were determined, and resulting data were analyzed by using the comparative threshold cycle (Ct) method as means of relative quantification, normalized to an endogenous reference (TATA box binding protein [TBP]) and relative to a calibrator (normalized Ct value obtained from control mice) and

![Figure 3](http://www.jimmunol.org/Downloadedfrom)
expressed as $2^{-\Delta\Delta Ct}$ (Applied Biosystems User Bulletin number 2: Rev B “Relative Quantification of Gene Expression”).

**Histology**

Tissues were fixed in 10% formalin and embedded in paraffin, and 5-μm sections were stained with H&E.

**Immunohistochemistry**

Sections of the liver, stomach, and pancreas were harvested, fixed in Tissue-Tek (Sakura Finetek, Torrance, CA), and snap frozen in liquid nitrogen. Sections (5 μm) were cut, mounted on slides, and fixed for 10 min in cold methanol. After washing in 1× TBS, residual endogenous peroxidase activity was quenched by incubation in 3% H2O2 in water for 10 min. Slides were then incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h in TBST. Next, sections were incubated with a primary Ab against CD4 differentiation Ag (1/50; BD Biosciences) and CD11c+ cell types including B cells, NK cells, and macrophages from DC-Tgfbr2 KO mice (Fig. 1D) were used as control mice throughout the study. Efficient reduction of Tgfbr2 mRNA was confirmed by quantitative PCR (qPCR) in CD11c+ BMDCs from DC-Tgfbr2 KO mice (Fig. 1A). Phosphorylation of Smad2 induced by exogenous TGF-β was also significantly reduced in BMDCs (Fig. 1B) from DC-Tgfbr2 KO mice compared with BMDCs from control Cre− littermates. In splenic DCs from DC-Tgfbr2 KO mice, TGFBR2 protein expression was significantly reduced compared with those from Cre− mice (Fig. 1C). However, TGFBR2 protein expression was not affected in other CD11c+ cell types including B cells, NK cells, and macrophages from DC-Tgfbr2 KO mice (Fig. 1D-F). Tgfbr2 mRNA was decreased by

**Treg conversion assay**

CD11c+ Flt3L BMDCs were pretreated with indicated concentrations of OVA (Sigma-Aldrich, St. Louis, MO) for 18 h, washed three times with CM, and cocultured with CD4+CD62L+ T cells from the spleens of OT-II mice in the presence or absence of 5 ng/ml TGF-β. Cells were incubated for 90 h at 37°C. CD11c+ MLN DCs were cocultured with naïve OT-II T cells in the presence of 1 mg/ml OVA and 5 ng/ml TGF-β for 90 h at 37°C. Foxp3 staining was performed as described earlier. In some conditions, an anti–IFN-γ neutralizing Ab (2 μg/ml, clone XMG 1.2; eBioscience) or an isotype control Ab (rat IgG1) was used.

**ELISA and multiplex assays**

Cytokines in cell culture supernatants or colonic explant cultures were detected using appropriate ELISA kits from eBioscience. Igs in serum were detected using Mouse Ig isotyping kit (EMD Millipore, Billerica, MA) on a Luminex-100 workstation (Liquichip; Qiagen) and analyzed using MasterPlex 2010 software (Hitachi Solutions America, MiraiBio Group, South San Francisco, CA).

**Relative Quantification of Gene Expression**

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA) or an isotype control Ab (rat IgG1). Statistical tests, whenever appropriate.

**CD4+CD62L+ T cells from wild-type mice were stimulated with anti-CD3/anti-CD28 beads (Life Technologies), TGF-β (5 ng/ml), IL-2 (20 ng/ml; PeproTech), and 0.5 μM retinoic acid (Sigma-Aldrich) for 4 d. More than 85% of T cells expressed Foxp3 on day 4 as determined by flow cytometry (data not shown).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA) using one-way ANOVA, followed by Newman–Keuls or Bonferroni post hoc test or by a Student t test, whenever appropriate.

**Results**

**Specificity of Tgfbr2 deletion in DC-Tgfbr2 KO mice**

Initial comparison of wild-type and Cre−/Tgfbr2−/− (B6.129S6-Tgfbr2tm1Hlm) mice revealed no effect of loxP sites on TGFBR2 protein or mRNA expression (data not shown). Therefore, Cre−/Tgfbr2−/− mice (referred to as Cre−) were used as control mice throughout the study. Efficient reduction of Tgfbr2 mRNA was confirmed by quantitative PCR (qPCR) in CD11c+ BMDCs from DC-Tgfbr2 KO mice (Fig. 1A). Phosphorylation of Smad2 induced by exogenous TGF-β was also significantly reduced in BMDCs (Fig. 1B) from DC-Tgfbr2 KO mice compared with BMDCs from control Cre− littermates. In splenic DCs from DC-Tgfbr2 KO mice, TGFBR2 protein expression was significantly reduced compared with those from Cre− mice (Fig. 1C). However, TGFBR2 protein expression was not affected in other CD11c+ cell types including B cells, NK cells, and macrophages from DC-Tgfbr2 KO mice (Fig. 1D-F). Tgfbr2 mRNA was decreased by

**FIGURE 4.** Elevated cytokine expression in DC-Tgfbr2 KO mice. (A and B) mRNA expression of indicated cytokines in the forestomach (A) and proximal colon (B) of Cre− and DC-Tgfbr2 KO mice. Samples were normalized to TBP. Error bars represent means + SEM of samples from five mice. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student t test). (C) TNF and IFN-γ expression in the colonic explant cultures of Cre− and DC-Tgfbr2 KO mice. Results are expressed as cytokine release per 50 mg of tissue. Error bars represent means + SEM of at least six individual mice. All p values were obtained using a Student t test.
FIGURE 5. Tgfbr2 KO DCs are more proinflammatory. (A) Staining of indicated DC subsets in the CD19- and DX5-depleted splenocytes from 8-wk-old Cre− (top panel) and DC-Tgfbr2 KO (bottom panel) mice. Dot plots are gated on CD3− cells (data representative of at least four different experiments); (B) MHCII, CD80, CD86, and CD40 on CD11chi splenic classical DCs (cDCs) (top panel) and CD11c+PDCA-1+ pDCs (bottom panel) of Cre− and DC-Tgfbr2 KO mice. Cells were prepared and gated as described in (A). Data representative of at least four experiments; (C) CCR7 expression in CD11c+ splenic DCs from Cre− and DC-Tgfbr2 KO mice. Bar graph represents the frequency of CD11c+CCR7+ DCs in the spleen of Cre− and DC-Tgfbr2 KO mice are indicated on the right. Error bars represent means ± SEM of ≥6 mice. All p values obtained using a Student t test. (D) Cytokine mRNA expression in CD11c+ BMDCs differentiated with GM-CSF and IL-4 ± LPS for 18 h. Samples were normalized to TBP. Error bars represent (Figure legend continues)
28% in total splenic CD4⁺ T cells isolated from DC-Tgfbr2 KO mice, although without reaching statistical significance (p > 0.16) (Fig. 2A, top panel). There was no difference in TGFBR2 protein expression in naïve splenic CD4⁺CD62L⁺ T cells (Fig. 2A, middle panel). Moreover, we demonstrated the same rates of iTreg (CD25⁺Foxp3⁺) conversion from naïve CD4⁺CD62L⁺ T cells isolated from the spleen of Cre⁻ or DC-Tgfbr2 KO mice in the presence of TGF-β and anti-CD3/anti-CD28 beads, thus confirming intact TGF-β signaling in naïve T cells (Fig. 2A, bottom panel). We have also adoptively transferred naïve CD4⁺CD45RB⁺ T cells from Cre⁻ or DC-Tgfbr2 KO mice into Rag1⁻/⁻ recipients and observed no difference in the pathogenic (colitis) effects of the T cells from the two donor strains (data not shown). To address potential Cre-mediated recombination in activated CD4⁺ CD11c⁺ T cells, we isolated genomic DNA from splenic CD4⁺ CD62L⁻ T cells from healthy Cre⁻ and symptomatic DC-Tgfbr2 KO mice and performed PCR with primers specific to exon 2 (and exon 5 as input control) of Tgfbr2 gene. Efficient recombination of exon 2 could only be demonstrated in CD11c⁺ BMDCs but not in activated T cells from DC-Tgfbr2 KO mice (Fig. 2B). Collectively, these data demonstrate efficient deletion of Tgfbr2 and abrogation of TGF-β signaling specifically in DCs. Absence of TGF-β signaling in DCs leads to multiorgan autoimmune inflammation DC-Tgfbr2 KO mice were phenotypically normal until ~3–4 wk of age; they became symptomatic and moribund by 4–14 wk of age (Fig. 3A, 3B) and gradually developed wasting disease with body weight reduced by ~30–40% in surviving mice at 12 wk of age (Fig. 3C). Histopathology of DC-Tgfbr2 KO mice revealed mild-to-moderate subacute multifocal hepatitis with focal fibrosis (Fig. 3D); mild-to-severe subacute multifocal pancreatitis with loss of exocrine cells (Fig. 3D); mild-to-moderate subacute multifocal colitis with loss of goblet cells; and mild-to-moderate crypt hyperplasia and predominantly lymphocytic or mixed lymphocytic and neutrophilic infiltrates (Fig. 3D) and severe subacute diffuse gastritis with mucosal hyperplasia (Fig. 3D). Premature involution of the thymus with complete loss of the thymic cortex was also observed in DC-Tgfbr2 KO mice. DC-Tgfbr2 KO mice also developed hydrocephalus, which was confirmed histologically to be because of the complete blockage of Aqueduct of Sylvius and dilation of lateral ventricles (Fig. 3D). DC-Tgfbr2 KO mice had elevated proinflammatory cytokine expression both in the stomach and in the colon (Fig. 4A, 4B). In addition, TNF and IFN-γ secretion was significantly elevated in colonic explant cultures from DC-Tgfbr2 KO mice (Fig. 4C). These findings reveal that loss of TGF-β signaling in DCs leads to widespread autoimmune inflammation in multiple organs of the digestive tract. Similar mortality and pathology has been observed in DC- Tgfbr2 KO rederived via embryo transfer into an ultraclean body weight reduced by ∼30% starting at 8 wk of age (Fig. 4A, 4B). In addition, TNF and IFN-γ secretion was significantly elevated in colonic explant cultures from DC-Tgfbr2 KO mice (Fig. 4C). These findings reveal that loss of TGF-β signaling in DCs leads to widespread autoimmune inflammation in multiple organs of the digestive tract. Similar mortality and pathology has been observed in DC-Tgfbr2 KO mice. Tgfbr2 deficiency does not affect differentiation of major DC subtypes or MHCII and costimulatory molecule expression in vivo To determine whether lack of TGF-β signaling in DCs affects their differentiation, we looked at the major DC subsets in the spleen and LNs of DC-Tgfbr2 KO mice using flow cytometry. Splenocytes depleted of B and NK cells or MLN cells were stained with a mixture of Abs to identify the myeloid (CD11c⁺CD11b⁺), lymphoid (CD11c⁺CD8⁺), and plasmacytoid (CD11c⁺PDCA-1⁺) population of DCs, based on the gating strategy described in Supplemental Fig. 1. We found no significant difference in the frequency of different subsets between Cre⁻ and Cre⁺ mice both in the spleen and MLN (Fig. 5A; data not shown). Contrary to in vitro observations (3), there was no difference in the expression of MHCII, CD80, CD86, or CD40 in either CD11c⁺ classical DCs or plasmacytoid DCs (pDCs) in the spleen and MLN of DC-Tgfbr2 KO at 10 wk of age (Fig. 5B; data not shown). However, we observed a significant increase in the frequency of CD11c⁺ CCR7⁺ DCs in the spleen and MLN of DC-Tgfbr2 KO mice (Fig. 5C; data not shown) indicating increased presence of migratory DCs. Therefore, on the basis of MHCII and costimulatory molecule expression, loss of TGF-β signaling in DCs is unlikely to affect their Ag-presenting capacity in vivo. DC from DC-Tgfbr2 KO mice are more proinflammatory TGF-β prevents the development of a subset of inflammatory DCs that express E-cadherin (6). Consistently, we observed a similar increase in the frequency of E-cadherin⁺CD11c⁺ DCs in the MLN of DC-Tgfbr2 KO mice (Supplemental Fig. 2), thus suggesting that Tgfbr2-deficient DCs are indeed more proinflammatory. To test this hypothesis, we analyzed major proinflammatory gene expression by qPCR in CD11c⁺ BMDCs generated from 4- to 6-wk-old asymptomatic mice. DCs from DC-Tgfbr2 KO mice had significantly elevated expression of TNF, even at the basal level as compared with control mice. LPS treatment did not further increase TNF expression, but IL-6 and IL-12 were significantly upregulated compared with Cre⁻ DCs (Fig. 5D). To functionally test the proinflammatory potential of Tgfbr2 KO DCs, we examined their ability to affect the early stages of T cell-mediated colitis. Rag⁻/⁻ mice received CD4⁺CD45RB⁺ T cells 2 wk prior to adoptive transfer of CD11c⁺ BMDCs and were monitored for another 10 d, after which time, the degree of colonic inflammation was assessed based on the proinflammatory gene expression profile. Administration of Tgfbr2 KO DCs but not Cre⁻ DCs led to a significant increase in the colonic gene expression of TNF, IL-1β, IL-6, and IFN-γ (Fig. 5E, left panel). Although with the exception of IFN-γ, cytokine secretion from colonic explants did not reach significance (likely because of the early stage of colitis selected because of short lifespan of transferred DCs), they were markedly elevated compared with mice injected with Cre⁻ DCs (Fig. 5E, right panel). These results demonstrate that Tgfbr2 KO DCs are more proinflammatory and can exacerbate T cell-mediated pathology. We also performed a complete gene expression profiling in total splenic CD11c⁺ DC population and MLN CD11c⁺CD103⁺ DCs isolated from 8-wk-old Cre⁻ or asymptomatic DC-Tgfbr2 KO mice using mouse Exon ST1.0 arrays (Affymetrix). Two hundred seventy-eight and 208 genes were differentially regulated in splenic DCs and MLN DCs, respectively, from DC-Tgfbr2 KO mice (up- or down-regulated; p < 0.05) with a fold change of ≥1.3 (Fig. 6A, 6D). These genes were categorized based on their biological function and sorted according to the Expression Analysis Systematic Explorer score (Fig. 6B, 6E). Among the genes involved in Th1 type inflammatory responses, we found upregulation of TNF (1.7-fold) in splenic DCs and IFNγ (≈2-fold) in MLN DCs. In addition, we found increased

mean ± SEM of triplicate samples; (E) relative mRNA (left panel) and protein (right panel) expression of indicated cytokines in the colon of Rag⁻/⁻ mice transferred with or without CD4⁺CD45RB⁺ naïve T cells (0.5 × 10⁶ cells), followed by either Cre⁻ or DC-Tgfbr2 KO BMDCs (3 × 10⁶ cells). Samples were normalized to TBP. Error bars represent SEM of at least five to six mice per group. Protein expression in colonic explant cultures was determined by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (one-way ANOVA with a Bonferroni post hoc test).
FIGURE 6. Microarray analysis of *Tgfbr2* KO DCs. (A) Histogram depicting the number of genes/probe sets whose expression was increased or reduced at *p* < 0.05 in CD11c⁺ splenic DCs from DC-*Tgfbr2* KO mice relative to their wild-type littermates. Increasing stringency of analysis (1.2- to 2-fold change on x-axis) demonstrates the magnitude of change in splenic DC gene expression profile in DC-*Tgfbr2* KO mice; (B) gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery functional annotation tool (http://david.abcc.ncifcrf.gov/) of the 535 gene/probe sets, which indicated >1.4-fold change at *p* < 0.05 (Student *t* test with Benjamini and Hochberg false-discovery rate as multiple testing correction). Genes categorized based on biological process were grouped and ranked (threshold of 5; *p* < 0.05). Categories were sorted according to the Expression Analysis Systematic Explorer score, a modified Fisher exact *p* value; (C) table of genes up- or downregulated in DC-*Tgfbr2* KO mice as compared with Cre⁻ mice; (D, E) same as described in (A) and (B) but depicts the gene expression changes in MLN CD11c⁺CD103⁺ DCs from DC-*Tgfbr2* KO mice as compared with Cre⁻ mice; (F) selected genes upregulated in DC-*Tgfbr2* KO mice as compared with Cre⁻ mice. *n* = 3/genotype with RNA pooled from at least four mice per sample.
mRNA expression of the T cell chemoattractants CXCL9, CXCL10, CXCL11, CCL6, and CCL9 (Fig. 6C, 6F). Expression of CCR9 chemokine receptor, which may be used to discriminate between immature and mature DCs (25), was downregulated (Fig. 6C), thus suggesting a more mature phenotype of Tgfbr2 KO DCs. The results of the microarray analysis confirm that Tgfbr2-deficient DCs are more proinflammatory and the observed pathology in DC-Tgfbr2 KO mice may be at least in part because of increased production of proinflammatory cytokines like TNF and IFN-γ, which augment Th1 inflammatory responses.

TGF-β has been shown to induce IDO expression in DC through autocrine signaling (9). However, our microarray results did not reveal lower expression of IDO both in splenic as well as MLN DCs from DC-Tgfbr2 KO mice. To confirm these results, we determined IDO expression in splenic CD11c+ DCs by Western blot analysis and real-time PCR, respectively. As shown in Fig. 7A and 7C, we did not find any significant difference in the baseline IDO expression at the protein or transcript in splenic DCs between Cre− and Cre+ mice. Similarly, we observed the same pattern of IDO mRNA expression in MLN CD11c+ DC (Fig. 7B). In addition, we also treated both splenic and BM-derived CD11c+ DCs with IFN-γ or TGF-β1 and analyzed IDO expression. Consistently, we detected no difference in the basal expression of IDO between Cre− and Cre+ mice and no significant effect of TGF-β1–stimulated IDO expression in DCs from Cre+ mice. The response to IFN-γ was largely preserved in both strains, albeit somewhat dampened by TGFBR2 deficiency (Fig. 7C, 7D).

**Impaired TGF-β signaling in DCs leads to T and B cell activation**

As previously described, DC-Tgfbr2 KO mice display premature thymic involution and thymocytes from 10-wk-old DC-Tgfbr2 KO mice showed a 2-fold increase in the frequency of CD4 single-positive (SP) cells, although the total numbers were not significantly different from Cre− mice (Fig. 8A). However, there was a significant increase in both the frequency and number of Qa2+ CD24hi cells among the CD4 SP population in DC-Tgfbr2 KO mice compared with Cre− mice, suggesting that the increased frequency of CD4+ SP cells was a result of recirculation of peripheral CD4 T cells into the thymus, a classic phenomenon in autoimmunity (Fig. 8B) (26). We found a marked increase in the frequency of CD62L+CD44hi cells among both CD4+ and CD8+ T cells in the periphery (spleen and MLN) of DC-Tgfbr2 KO mice (Fig. 8C, Supplemental 3A, 3B). Consistent with these findings, we also demonstrated CD4+ T cell infiltration by immunohistochemistry in the stomach, pancreas, and liver of DC-Tgfbr2 KO mice but not in Cre− mice (Supplemental Fig. 3C).

DC-Tgfbr2 KO mice had significantly increased levels of serum IgG1 and IgM (Fig. 9A). To test for the presence of autoantibodies, we probed tissue lysates from Rag−/− mice with serum from either Cre− or DC-Tgfbr2 KO mice. Several distinct bands were observed only in tissues probed with serum from DC-Tgfbr2 KO mice (Fig. 9B). Overall, these results suggest that abrogation of TGF-β signaling in DCs result in spontaneous activation of self-reactive T and B cells.

**Alteration of Treg phenotype in DC-Tgfbr2 KO mice**

Loss of CD4+CD25+Foxp3+ Tregs leads to a fatal multiorgan autoimmune syndrome (27). To determine whether a similar loss of Tregs in DC-Tgfbr2 KO mice was responsible for the autoimmune pathology, we looked at Treg frequency and numbers in the lymphoid organs of DC-Tgfbr2 KO mice. In 4-wk-old mice with intact thymus, we found no significant difference in the percentage of thymic CD4+Foxp3+ T cells between Cre− and DC-Tgfbr2 KO mice. However, in 8-wk-old mice, we observed a significant decrease in the frequency and number of thymic CD4+Foxp3+ T cells in the thymus of DC-Tgfbr2 KO mice but not in Cre− mice (Supplemental Fig. 3C).

**FIGURE 7.** Lack of TGF-β signaling in DCs does not affect IDO expression. (A) Western blot analysis for IDO expression in splenic CD11c+ DCs from control and DC-Tgfbr2 KO mice. Corresponding densitometric analysis of the Western blot relative to GAPDH is shown on the right. Error bars represent means + SEM of three individual mice. (B) IDO mRNA expression in MLN CD11c+ DCs. Each sample was normalized to TBP expression. Error bars represent means + SEM of three individual mice. (C and D) mRNA expression of IDO in splenic (C) or BM-derived CD11c+ DCs (D) treated with or without IFN-γ (200 U/ml) or TGF-β (20 ng/ml) for 18 h. Each sample was normalized to TBP expression. Error bars represent means + SEM of three repetitions. The p values were obtained using one-way ANOVA, followed by a Newman–Keuls post hoc test. *p < 0.05 in Cre− versus DC-Tgfbr2 KO within respective treatment, *p < 0.05 control versus IFN-γ or control versus TGF-β within respective genotype.
mice (Fig. 10A, left panel). With involution of the thymus at 10 wk, the frequency of CD4⁺Foxp3⁺ T cells increased in DC-Tgfbr2 KO mice, but the total numbers were not significantly different (Fig. 10A, middle and right panels). Interestingly, the proportion (both percentage and number) of CD4⁺Foxp3⁺ T cells in the spleen and MLN was increased in DC-Tgfbr2 KO mice compared with Cre⁻⁻ mice (Fig. 10B), but the intensity of Foxp3 expression was significantly reduced in CD4⁺ T cells from DC-Tgfbr2 KO mice (Fig. 10C). Although in Cre⁻⁻ mice almost all Foxp3⁺ cells were CD25⁺ (Fig. 11), in DC-Tgfbr2 KO mice, there was a considerable decrease in the proportion of CD25⁺Foxp3⁺ cells and an expansion of CD25⁻ Foxp3⁺ cell population (Fig. 11A–C). These results suggest that the phenotype of peripheral Tregs in DC-Tgfbr2 KO mice is altered, which in turn may affect the function of these cells (see Discussion), leading to or contributing to the development of autoimmunity.

Increased IFN-γ production by Tgfbr2 KO DCs inhibits Ag-specific Treg differentiation

In the presence of TGF-β, activation of naive T cells by DCs leads to the induction of Foxp3 transcription factor and Treg differentiation (19, 28). To determine whether DC-Tgfbr2 KO DCs are capable of driving Ag-specific Treg differentiation, we cocultured OVA-pretreated Flt3L-BMDCs from Cre⁻⁻ or DC-Tgfbr2 KO mice with CD4⁺CD62L⁺ naive T cells from OT-II mice in the presence of recombinant TGF-β. Despite the presence of TGF-β, DCs from DC-Tgfbr2 KO mice were significantly less efficient in inducing CD4⁺CD25⁺Foxp3⁺ Treg differentiation than Cre⁻⁻ DCs (Fig.
12A). We saw a similar effect at two different concentrations of OVA (data not shown). Analysis of cell coculture supernatants by ELISA revealed substantial increase in IFN-γ but not IL-6 levels in DCs from DC-Tgfbr2 KO mice (Fig. 12B). qPCR analysis revealed ~40-fold increase in IFN-γ expression by Flt3L-BMDCs from DC-Tgfbr2 KO mice (Fig. 12C). To test whether IFN-γ

FIGURE 9. Deficient TGF-β signaling in DCs results in B cell activation. (A) Ig isotype concentrations in the serum of Cre− and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of 10–12 mice/genotype; (B) Western blot analysis of indicated Rag1−/− tissue lysates showing the staining pattern of autoantibodies present in the serum of control (n = 3–4) and DC-Tgfbr2 KO mice (n = 2–4). All p values were obtained using a Student t test.

FIGURE 10. Altered Treg phenotype in DC-Tgfbr2 KO mice. (A) Left panel, Percentage of CD4+Foxp3+ T cells in the thymus of 4-wk-old DC-Tgfbr2 KO mice. Error bars represent means ± SEM from at least three individual mice. Percentage (middle panel) and numbers (right panel) of CD4+Foxp3+ Tregs in the thymus of 10-wk-old control and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of six mice; not significant (ns), Student t test; (B) percentage (left panel) and numbers (right panel) of CD4+Foxp3+ Tregs in the spleen and MLN of 10-wk-old control and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of six mice; (C) Foxp3 expression in CD4+ T cells from the spleen and MLN of Cre− and DC-Tgfbr2 KO mice (representative of ≥6 mice per group). Bar graph represents the mean fluorescence intensity of Foxp3 among CD4+ T cells in spleen and MLN of Cre− and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of ≥6 mice.
overexpression inhibited Treg conversion, we performed an analogous Treg conversion assay with anti–IFN-γ neutralizing Ab or an isotype control Ab. As shown in Fig. 12D, isotype control Ab did not affect Treg conversion. However, neutralization of IFN-γ restored the percentage of CD4+CD25+Foxp3+ cells to that observed with Cre− DCs (Fig. 12D). Because microarray analysis also indicated overexpression of IFN-γ in MLN CD103+ DCs, we also examined the potential of MLN DCs to induce Treg differentiation. Similar to our results with BMDCs, we found that MLN DCs from DC-Tgfr2−/− KO mice were ineffective in converting naive T cells into Tregs (Fig. 12E), and elevated levels of IFN-γ were observed in the supernatants from Tgfbr2−/− KO DC coculture (Fig. 12F). Addition of neutralizing Ab to IFN-γ rescued the conversion rate to levels close to that observed with control DCs (Fig. 12G).

In vitro-generated iTregs partially protect from autoimmunity in DC-Tgfr2−/− KO mice

Transfer of Tregs into young mice has been shown to prevent the development of autoimmune disease in mice (27). To determine whether adoptive transfer of CD4+CD25+Foxp3+ iTregs into DC-Tgfr2−/− KO mice can alleviate the autoimmune phenotype, polyclonal iTregs were generated in vitro by stimulating naive CD4+ T cells with anti-CD3/CD28 in the presence of TGF-β, retinoic acid, and IL-2 for 4 d. These iTregs were then adoptively transferred i.v. into 2- to 3-wk-old Cre− or DC-Tgfr2−/− KO mice. Control groups received PBS. The mice were monitored for 6 wk. Thirty-three percent of DC-Tgfr2−/− KO mice injected with PBS died during the course of the study, while no mortality was observed in DC-Tgfr2−/− KO mice transferred with iTregs (Fig. 13A). DC-Tgfr2−/− KO mice transferred with iTregs showed a significant decrease in the percentage of activated CD62L-CD44hi CD4+ and CD8+ T cells in the spleen, compared with DC-Tgfr2−/− KO mice injected with PBS (Fig. 13B). Adoptive transfer of iTregs into DC-Tgfr2−/− KO mice significantly lowered TNF and IFN-γ expression in the proximal and distal colon, as compared with DC-Tgfr2−/− KO mice injected with PBS (Fig. 13C). Interestingly, iTreg transfer did not influence the development of gastritis, with no significant difference in TNF or IFN-γ expression in DC-Tgfr2−/− KO mice injected with iTregs or PBS (Fig. 13D). Similar results were obtained with other tissues including pancreas and liver (data not shown), thus suggesting that the Treg-mediated suppression of autoimmune inflammation may be tissue-specific or may require Ag-specific Tregs and is not sufficient to completely rescue the phenotype of DC-Tgfr2−/− KO mice.

Discussion

DCs maintain a fine balance between immune activation to foreign Ags and tolerance to self-Ags. Under steady-state conditions, DCs mediate both clonal deletion of self-reactive T cells in the thymus and control of T cells specific responses to self- or harmless Ags in the periphery (29). These DCs are termed as tolerogenic DCs, but the signals that drive the tolerogenic pathways in these cells are just beginning to be understood (30). In this article, we provide conclusive in vivo evidence that TGF-β provides a signal that is essential to maintain the tolerogenic function of DCs, although in a manner not consistent with the reported in vitro studies. Loss of TGF-β signaling in DCs makes them more proinflammatory and less immunosuppressive, which in turn leads to the development of autoimmunity in mice.

The pathology of DC-Tgfr2−/− KO mice resembles that of Tgfb−/− null KO mice (Tgfb−/−) (31), although with a delayed onset. Therefore, it is conceivable that apart from the impaired T cell homeostasis and enhanced T cell activation (32, 33), impaired DC function may also greatly contribute to disease severity in Tgfb−/− mice. Although spontaneous upregulation of MHC class I and II were attributed to the observed autoimmune phenotype in Tgfb−/− mice, we did not see any difference in the expression of MHCIi or the costimulatory molecules CD80, CD86, and CD40 in DC-Tgfr2−/− KO mice, suggesting that loss of TGF-β signaling does not alter the Ag-presenting function of DCs as a direct effect. However, Tgfr2−/−-deficient DCs were more proinflammatory in agreement with previous in vitro studies using BMDCs (3, 6). We observed increased TNF expression by splenic DCs and increased IFN-γ expression by MLN DCs. The difference in the expression of inflammatory cytokines by these two populations of DCs may be due to the differences in the DC lineage in these lymphoid organs. It is known that during autoimmune disease a subset of inflammatory DCs (TNF-inducible NO synthase-producing DCs) that produce TNF and iNOS populate the spleen. These DCs arise from monocyte precursors and can be cultured in vitro from BM precursors using GM-CSF and IL-4 (34). Indeed, we found in-

FIGURE 11. Expansion of CD25−Foxp3+ T cells in DC-Tgfr2−/− KO mice. (A) Contour plots showing the frequency of CD25−Foxp3+ (upper right quadrant) and CD25+ Foxp3+ T cells (lower right quadrant) in the spleen (top panel) and MLN (bottom panel) of 8-wk-old asymptomatic control (left panel) and DC-Tgfr2−/− KO mice (right panel). Plots are gated on CD4+ cells; (B) percentage of CD4+CD25−Foxp3+ (top panel) and CD4+CD25+Foxp3+ (bottom panel) T cells in the spleen and MLN of control and DC-Tgfr2−/− KO mice. Error bars represent means ± SEM of six mice. The p values were obtained using a Student t test; (C) bar graphs representing the number of CD25− (top panel) and CD25+ (bottom panel) Foxp3+ T cells in the spleen and MLN of Cre− and DC-Tgfr2−/− KO mice. Error bars represent means ± SEM of six individual mice. All p values were obtained using Student t test.
FIGURE 12. Increased IFN-γ production by Tgfbr2 KO DCs inhibits Treg differentiation. (A) Treg differentiation assay using Cre− or DC-Tgfbr2 KO CD11c+ Flt3L BMDCs. OVA-pretreated DCs (500 μg/ml) were cocultured with CD4+CD62L+ naive OT-II T cells (1:10) in the presence/absence of TGF-β (5 ng/ml) for 90 h. Percentage of CD4+CD25+Foxp3+ Tregs was determined by flow cytometry (representative of at least two experiments); (B) IFN-γ (top panel) and IL-6 (bottom panel) concentrations in the supernatants of Treg conversion assay as described in (A). Error bars represent means ± SEM of triplicate samples. *p < 0.05 (one-way ANOVA with a Newman–Keuls post hoc test; data representative of at least two independent experiments); (C) Ifng mRNA in Flt3L CD11c+ BMDCs from Cre− or DC-Tgfbr2 KO mice. Samples were normalized to TBP. Error bars represent SEM of triplicates. The p-values were obtained using Student t-test; (D) Treg conversion assay was performed using Cre− (top panel) and DC-Tgfbr2 KO DCs (bottom panel) as described in (A) either alone (left panel) or with an isotype control Ab (middle panel) or anti–IFN-γ Ab (2 μg/ml) (right panel); (E) CD25+Foxp3+ Tregs (left panel) obtained using CD11c+ MLN DCs from Cre− or DC-Tgfbr2 KO mice. Protocol similar to that described in (A). (Figure legend continues)
creased TNF production in BMDCs differentiated using GM-CSF and IL-4, and these DCs were able to exacerbate T cell-mediated colitis. MLN CD103+ DCs (and CD103− DC; data not shown), as well as Flt3L-differentiated BMDC from DC-Tgfbr2 KO, showed elevated IFN-γ expression without additional stimulation. Interestingly, microarray analysis of splenic DCs from DC-Tgfbr2 KO mice showed a clear IFN-γ signature response, although expression of IFN-γ itself was not elevated. Similarly, splenic DCs from CD11c+CD103− mice did not produce IFN-γ even after stimulation with IL-12 and IL-18 (15). These two observations suggest differences in IFN-γ induction in the splenic and mucosal/LN DC lineages and/or that DCs migrating to the spleen are primed by IFN-γ at the mucosal/parenchymal sites. This is also consistent with the fact that CD103+ DCs produce higher levels of active TGF-β (35), which may act in an autocrine or paracrine manner to suppress IFN-γ expression, both in CD103+ and CD103− DCs.

Although TGF-β has been shown to induce expression of IDO in DCs (9, 36), we did not find any significant differences in the expression of IDO in either splenic DCs or MLN CD103+ DCs. Expression and activity of IDO protein in Tgfbr2 KO mucosal except that DCs were cocultured with T cells (1:2) in the presence of 1 mg/ml OVA; (F) IFN-γ expression (ELISA) in the supernatants of Treg conversion assay with MLN DCs. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA with Newman–Keuls post hoc test). (G) Treg conversion assay was performed using Cre+ (top panel) and DC-Tgfbr2 KO DCs (bottom panel) as described in (E) (DC/T cell ratio, 1:10) either alone (left panel), with an isotype control (middle panel), or anti–IFN-γ neutralizing Ab (2 μg/ml) (right panel). Representative data from one of three repetitions is shown.

FIGURE 13. Adoptive transfer of Tregs partially rescues the autoimmune phenotype of DC-Tgfbr2 KO mice. (A) Survival curve of Cre− or DC-Tgfbr2 KO mice injected with PBS or adoptively transferred at 2–3 wk of age with Foxp3+ iTregs (2 × 106 cells/mouse) generated as described in Materials and Methods; (B) percentage of CD4+ (left panel) and CD8+ (right panel) CD62LloCD44hi T cells in the spleen of recipient mice at the end of the experiment described in (A); (C) cytokine mRNA expression in the proximal and distal colon of recipient mice as described in (A). Samples were normalized to TBP expression. Error bars represent means ± SEM of six to seven individual mice; (D) cytokine mRNA expression in the forestomach and glandular stomach of recipient mice as described in (A). Error bars represent means ± SEM of six to seven individual mice. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA with a Newman–Keuls post hoc test).
DCs remain to be investigated. Lack of detectable changes in IDO expression in MLN or splenic DCs may represent the net effect of the absence or reduction of stimulatory TGF-β signaling and compensatory autocrine effects of elevated IFN-γ, which is known to induce IDO expression (37). In pDCs, TGF-β also controls nonenzymatic cell signaling functions of IDO without affecting its expression levels (10). It is therefore plausible that this pathway may also be affected, thus leading to the loss of regulatory phenotype in pDCs, a response skewed toward the canonical NF-κB pathway, more inflammatory state, and ultimately loss of tolerance in DC-Tgfb2 KO mice.

Immunosuppressive Tregs control T cell activation and prevent the development of autoimmune disease. Expression of Foxp3 in secondary lymphoid CD4+ T cells was significantly decreased, along with a decrease in the frequency of CD4+CD25+Foxp3+ Tregs in DC-Tgfb2 KO mice. Moreover, among the CD4+Foxp3+ T cells, we observed a dramatic increase in the proportion of CD4+CD25+ Foxp3+ T cells, a population also reported recently in patients with systemic lupus erythematosus (38, 39) and relapsing patients with multiple sclerosis (40). The immunosuppressive potential of CD4+CD25+Foxp3+ Tregs is not entirely clear. Functional analyses of this population from SLE patients revealed a partial loss of function (39, 41). Zelenay et al. (42) showed that CD4+CD25+Foxp3+ T cells constitute a reservoir of committed Tregs that regain CD25 expression upon homeostatic proliferation in a lymphopenic host. However, the same group showed that Tregs identified as the CD45RBloCD25− population from SLE patients failed to show suppressive function in vitro when freshly isolated from mice (42). It has also been shown that Tregs with attenuated Foxp3 expression have lower levels of CD25 and that these cells have a tendency to convert to Th2 type cells (43). On the basis of these studies, it is highly likely that the CD4+CD25+Foxp3+ T cells in DC-Tgfb2 KO mice are less immunosuppressive thereby contributing to the autoimmune pathology. Such alterations in Treg homeostasis and spontaneous autoimmunity were not observed in CD11cΔdKO mice, suggesting that residual TGF-β signaling in DCs may have been sufficient to maintain self-tolerance under basal conditions and that the consequences of impaired TGF-β signaling in DCs may represent a continuum depending on the degree of suppression. Future studies with DC-Tgfb2 KO crossed with Foxp3-RFP knockin mice should further clarify the nature and function of the CD4+CD25+Foxp3− T cells.

We have also demonstrated that in vitro, DC-mediated Ag-specific Treg conversion is impaired because of elevated production of IFN-γ by Tgfb2 KO DCs. However, we did not observe the expansion of the CD4+CD25+Foxp3+ population as we did in vivo. Therefore, the mechanisms leading to the potential loss of function of Tregs in DC-Tgfb2 KO mice remain unclear. Bidirectional DC-Treg interactions are required to maintain immunological tolerance (44). DCs induce Treg differentiation and proliferation through Ag-dependent and -independent interactions but in a cell–cell contact- and IL-2–dependent mechanism (45). However, the suppressive function of the expanded Tregs may still require additional signals that remain unidentified. Adoptive transfer of in vitro-generated polyclonal Foxp3+ iTregs into young DC-Tgfb2 KO mice prevented early mortality and partially rescued the autoimmune phenotype with significant reduction in the proportion of activated T cells and protection from colitis. It did not, however, protect from gastritis, pancreatitis, or hepatitis. Although the optimal timing of transfer and lifespan of the injected Tregs may be debatable, loss of their immunosuppressive function in vivo in DC-Tgfb2 KO mice cannot be ruled out. In contrast, iTregs are generally thought to suppress immune responses to environmental, food allergens, and commensal microbiota, whereas natural Tregs prevent autoimmunity by raising the threshold for activation of immune response to self-Ags (46). Consistent with our observations, non–Ag-specific iTregs have been shown to be protective in mouse models of colitis (46). In contrast, natural Tregs are selected in the thymus through MHCII-dependent TCR interactions and may mediate suppression in an Ag-specific manner. Ag-specific Tregs were required to prevent autoimmune gastritis in mice (47), which may explain persistent gastritis in our Treg rescue model.

This novel mouse model with DC-specific Tgfb2 deletion highlights the critical importance of TGF-β signaling in DCs in the maintenance of immune homeostasis and in the prevention of autoimmunity. However, these functions may be independent of the previously ascribed TGF-β control of Ag presentation and costimulation. Although a mechanistic relationship between TGF-β signaling in DCs and Foxp3+ Treg responses still remains to be elucidated in detail, the phenotype of our novel mouse model can be exploited to advance our understanding of the pathogenesis of complex autoimmune disorders, including the in vivo expansion and function of CD25+Foxp3+ Tregs.

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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1: Gating strategy for analysis of dendritic cells from the spleen

Splenocytes depleted of CD19+ B cells and DX5+ NK cells were incubated with different antibodies to identify dendritic cell subsets. Live cells (gate A) were gated based on forward and side scatter. CD3+ T cells were excluded from further analysis. The CD3- (gate B) cells were selected for further analysis and examined for the expression of CD11c and PDCA-1. The CD11c^hi (gate C) population includes both myeloid (CD11b+, gate E) and lymphoid populations (CD8+, gate F) and these represent classical DCs. CD11c^loPDCA-1^+ (gate D) population represents pDCs and were further analyzed for B220 expression.

Figure S2: Increased E-cadherin expression in CD11c+ DCs from MLN of DC-Tgfbr2 KO mice

(A) Histogram of E-cadherin expression among CD11c^+ MLN cells from Cre^- and DC-Tgfbr2 KO mice by flow cytometry. Cells were gated on CD3^- cells; Numbers indicate percentage of CD11c^-E-cadherin^+ cells (B) Bar graph representing the percentage of E-cadherin^-CD11c^- DCs in the MLN of Cre^- and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of at least 3 individual mice. p values obtained using Student’s t test.

Figure S3: Increased frequency of activated T cells in the MLN of DC-Tgfbr2 KO mice and infiltration of peripheral organs by CD4+ T cells

(A) and (B) Frequency of CD62L^lo (left) or CD44^hi (right) cells among the CD4 (A) and CD8 (B) T cell population in the MLN of Cre^- and DC-Tgfbr2 KO mice. Error bars represent means ± SEM of at least 4-6 individual mice. p values were obtained using Student’s t test; (C)
Immunohistochemistry for CD4\(^+\) T cells in the indicated organs of Cre\(^-\) and DC-\textit{Tgfbr2}\(^{\text{KO}}\) mice. Data are representative of at least 6 individual mice.
Figure S1
Figure S3