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Transcription Factor 7 Limits Regulatory T Cell Generation in the Thymus

Melanie M. Barra,* David M. Richards,* Jenny Hansson,† Ann-Cathrin Hofer,* Michael Delacher,* Jan Hettinger,* Jeroen Krijgsveeld,† and Markus Feuerer*

Regulatory T cells (Tregs) differentiate in the thymus, but the mechanisms that control this process are not fully understood. We generated a comprehensive quantitative and differential proteome of murine Tregs and conventional T cells. We identified 5225 proteins, 164 of which were differentially expressed in Tregs. Together with the comparative analysis of proteome and gene expression data, we identified TCF7 as a promising candidate. Genetic elimination of transcription factor 7 (TCF7) led to increased fractions of Tregs in the thymus. Reduced levels of TCF7, found in the heterozygote, resulted in a greater potential for Treg precursors to differentiate into the Treg lineage. In contrast, activation of TCF7 through β-catenin had the opposite effect. TCF7 levels influenced the required TCR signaling strength of Treg precursors, and TCF7 deficiency broadened the repertoire and allowed lower TCR affinities to be recruited into the Treg lineage. FOXP3 was able to repress TCF7 protein expression. In summary, we propose a regulatory role for TCF7 in limiting access to the Treg lineage. The Journal of Immunology, 2015, 195: 000–000.

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gulatory T cells (Tregs) are indispensable in the regulation of immune responses throughout life. They ensure immune homeostasis and bring inflammation to a proper resolution. They are characterized by the expression of FOXP3, which was identified as the major transcription factor and is crucial for Treg function (1–3). Dysfunction or impaired development of Tregs leads to severe autoimmune diseases (4). The thymus has a dual role in T cell tolerance: by the negative selection of autoreactive T cells and by the generation of the thymus-derived Treg (tTreg) pool (5).

T cell maturation in the thymus is a multistep process that involves different molecular and cellular mechanisms. T cell precursors sequentially differentiate from the immature CD4 and CD8 double-negative (DN) stage to the CD4 and CD8 double-positive (DP) stage. Finally, they develop into CD4 or CD8 single-positive (SP) thymocytes (6). During this transition between positive and negative selection, some precursors get committed to the Treg lineage in a process that still needs to be resolved in detail. Analysis of mice with limited TCR diversity demonstrated only little overlap in the TCR CDR3 sequences between Tregs and conventional CD4+ T cells (Tconvs), indicating that different TCR specificities could account for the alternative fate (7–9). TCR signaling strength seems to have an important role in the selection into the Treg lineage, and several studies suggested that Tregs have a higher level of self-reactivity than Tconvs (5). It is believed that Treg development in the thymus occurs in a window during which the TCR avidity for self-antigens lies close to or even overlaps with the avidity range, leading to negative selection (5). This is supported by N4aI(Nur77)-GFP reporter mice, in which the TCR signal strength, as measured by reporter activity, required for Treg differentiation is substantially higher than for Tconvs (10). In addition to TCR signaling, other factors contribute to the Treg lineage decision. Intraclonal competition, likely caused by limited access to Ag (11), and costimulation via CD28 are important variables (12). The two common γ-chain cytokines IL-2 and IL-15 are additional factors that are essential for Treg differentiation (13, 14). In fact, it was proposed that Treg development occurs in two consecutive steps. The first step is TCR dependent, whereas the second step is TCR independent but requires common γ-chain signaling by IL-2 or IL-15 (13). Each of these steps has corresponding precursor populations in the thymus: the “early Treg precursor” is defined as CD4SP CD69+CD25+FOXP3+, and the “late Treg precursor” is characterized as CD4SP CD69+CD25−FOXP3− (13, 14).

Transcription factor 7 (TCF7; also known as TCF1) is important for T cell development in the thymus. Tcf7-deficient (Tcf7−/−) mice have greatly reduced numbers of thymocytes (15), and it was shown that TCF7 is involved in thymocyte differentiation at several important checkpoints (16–18). The early T cell precursors require TCF7 for efficient T cell lineage specification and differentiation (19, 20). TCRβ selection and the maturation of DN thymocytes to the DP stage are compromised (15, 17). In DN and preselected DP thymocytes, TCF7 restrains expression of lymphoid enhancer–binding factor 1 (LEF1) and components of the Notch signaling pathway to prevent malignancy (21). TCF7 was also shown to promote commitment into the CD4+ T cell lineage (22). Lastly, negative selection and cell survival at the DP stage also were shown to depend on TCF7 (23, 24). Because this is the...
stage when TCR-mediated positive and negative selection of thymocytes occurs, an obvious question is whether TCF7 also influences Treg generation in the thymus.

Gene expression profiles of Tregs from different tissues and subphenotypes, as well as under various activation conditions, have been studied (25–28). Those studies revealed important information about Treg molecular signatures on the mRNA level; however, a precise description of the Treg proteome and its correlation with mRNA expression are lacking.

In this study, we analyzed the whole proteome of Tregs compared with Tconvs and identified TCF7 as an interesting candidate. Based on its known function during thymocyte development, we investigated the role of TCF7 in Treg differentiation and demonstrated that its presence restricts the development of Tregs. TCF7 deficiency broadened the repertoire and allowed lower TCR affinities to be recruited into the Treg lineage.

Materials and Methods

Mice

Wild-type C57BL/6 (B6) mice were obtained from the Charles River Breeding Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). Tcf7+/− mice (Tcf7tm1'T2a, ΔVII) were a gift from Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands) (15). Tcf7+/- mice were crossed to Foxp3-YFP mice [B6.129(Cg)-Foxp3tm14CAG藐c/BoyJ; Jackson catalog no. 016959] (29) or Nr4a1-GFP mice [Tg(Nr4a1-EGFP/cre)820Khog/J; Jackson catalog no. 016617] (10) in the German Cancer Research Center animal facility. We used littersmates as control animals for all experiments. Foxp3-GFP mice [B6.129(Cg)-Foxp3tm14CAG藐c/BoyJ; Jackson catalog no. 016958] (30) were crossed to a CD45.1 (B6.SL-Ppcre'Pepc'/BoyJ; Jackson catalog no. 002014) or CD90.1 (B6.PL-Thyl1/CyJ; Jackson catalog no. 000406) background. All mice were maintained under specific pathogen–free conditions in the animal facility of the German Cancer Research Center. All animal experiments were approved by the governmental committee for animal experimentation in Karlsruhe, Germany.

Tissue isolation and sample preparation

For tissue isolation, spleen, thymus, and lymph nodes (LNs; axillary, brachial, and inguinal) were removed, and organs were processed by mashing and filtering to prepare single-cell suspensions. Ammonium chloride–potassium bichromate lysis buffer was used to lyse erythrocytes in spleen samples. Samples that were purified by FACS for Treg protein isolation (mass spectrometry [MS] experiments) were first enriched for CD25. Samples that were purified by FACS for Treg precursors and quantitative real-time PCR experiments were first depleted of CD8+ cells. For enrichment and depletion, cells were labeled with biotin-conjugated Abs and anti-biotin MicroBeads (Miltenyi Biotec). Magnetic cell separation was performed using an AutoMACS Pro Separator (Miltenyi Biotec).

Flow cytometry

Cells were labeled with Abs/streptavidin: CD3ε (145-2C11), CD4 (RM4-5 or GK1.5), CD8a (53-7.3), CD8ε (53-6.7), CD11b (M170), CD11c (N418), CD19 (5D5), CD25 (PC61), CD44 (IM7), CD61 (2C9.G2 [HMb3-1]), CD69 (H1.2F3), CD86 (GL-1), CD122 (5H4), CD304 (N43-7), CD357 (DTA-1), Foxp3 (FJK-16s) (from Cell Signaling); LEF1 (C12A5) and TCF1 (C63D9) (from Cell Signaling); CD19 (6D5), CD25 (PC61), CD44 (IM7), CD61 (2C9.G2 [HMb3-1]), and CD357 (DTA-1), Foxp3 (FJK-16s) (all from BD Pharmingen, BioLegend or eBioscience); LEF1 (C12A5) and TCF1 (C63D9) (from Cell Signaling); CD19 (6D5), CD25 (PC61), CD44 (IM7), CD61 (2C9.G2 [HMb3-1]), and CD357 (DTA-1), Foxp3 (FJK-16s) (all from BD Pharmingen, BioLegend or eBioscience); LEF1 (C12A5) and TCF1 (C63D9) (from Cell Signaling); CD19 (6D5), CD25 (PC61), CD44 (IM7), CD61 (2C9.G2 [HMb3-1]), and CD357 (DTA-1), Foxp3 (FJK-16s) (all from BD Pharmingen, BioLegend or eBioscience); LEF1 (C12A5) and TCF1 (C63D9) (from Cell Signaling).

For counting of total thymus subpopulations using beads, AccuCheck Counting beads (Invitrogen) were added to the sample prior to acquisition. A total of 2 × 10^6 beads was added to 20% of the thymus sample. The number of cells in the total thymus was calculated according to the manufacturer’s instructions.

In vitro Treg-differentiation assay

FACS-purified early (CD4SP CD69−CD25−Foxp3−) or late (CD4SP CD69+CD25+Foxp3+) Treg precursors were seeded at 1 × 10^6 cells/well in 96-well bottom-plate and cultured for 1 or 3 d before analysis. Cells were cultured with different combinations of human IL-2 (100 U/ml, Proleukin; S. Novartis), murine IL-15 (100 ng/ml, PeproTech), and anti-CD3/CD28 Dynabeads (Invitrogen) at a cell/bead ratio of 1:1. For Wnt-activation assays, 1 × 10^5 sorted Treg precursors were cultured under the conditions described above. In addition, DMSO-dissolved Wnt-activator 6-bromodirindirubin-3'-oxide (BIO) or 6-[2-[(4,2,4- Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl][amino] ethyl][amino]-3-pyridinecarboxonitile (CHIR99021) (both from BiOMOL) was added to the cultures at 1 or 2 μM. Control wells were cultured with DMSO concentrations corresponding to the highest DMSO content in the wells containing the Wnt activators.

For the in vitro Treg-differentiation assay with titration of anti-CD3 stimulus, 96-well flat-bottom plates were coated overnight with different concentrations (0.1, 0.25, 0.5, or 2 μg/ml) of purified anti-CD3 in PBS. Plates were washed with PBS prior to use. Purified anti-CD3 (10 μg/ml) was added together with 1 × 10^5 Treg precursors, IL-2, and IL-15 to the culture media.

Flow cytometric analysis of all in vitro experiments, Fixable Viability Dye eFluor 506 (eBioscience) was used to exclude dead cells.

Immunofluorescence imaging

Sections (5 μm) from cryopreserved thymi from Tcf7+/− and Tcf7+/+ mice were prepared by step sectioning every 25 μm. Sections were fixed in ice-cold acetone, blocked in PBST with 5% (v/v) FCS and 1% (w/v) BSA, and stained with Ab against K14 (Covance). Next, sections were stained with Anti-Rabbit IgG-Alexa Fluor 488 (Jackson ImmunoResearch) and DAPI (Sigma). Imaging was performed on an Axiosmager.Z1 microscope (Zeiss). Images were analyzed in AxioVision (Zeiss) and Photoshop (Adobe).

Quantitative real-time PCR

Cells were FACs purified into TRizol reagent (Life Technologies), and mRNA was extracted according to the manufacturer’s protocol. For cDNA synthesis, SuperScript reverse transcriptase (Invitrogen) and oligo(dt) primers were used. cDNA samples were analyzed by quantitative real-time PCR using Power SYBR Green Master Mix and the ViiA 7 system (both from Life Technologies). Appropriate primers for Hprt and Tcf7 were used. The following primer sequences were used: HprFor: 5'-CCTGTCGACCTGTGATGT-3', and HprtRev: 5'-ATAGTCCCCGGTTGACGTGAT-3', and Tcf7For: 5'-CGAGAAGACGAGCCAACTGA-3', and Tcf7Rev: 5'-CCTGGTGGTGATCTTCTGAT-3'. Tcf7 mRNA (gene X) abundance was calculated as percentage relative to the expression of Hprt by the change-in-threshold method (100*(relative expression = 2^(-CT (gene X) – CT (Hprt)))).

Retroviral transduction of FOXP3

Phoenix-ECO cells were transduced with calcium phosphate using a murine stem cell virus plasmid (IRES-C90.1, empty or encoding wild-type human FOXP3) (28). Supernatants containing murine stem cell virus retroviral particles were harvested at day 3 after transfection. Wells were prepared for T cell culture by coating with goat-anti-hamster IgG (MP Biomedicals), followed by purified anti-CD3 and anti-CD28. LN and spleen cells were depleted of CD8, CD19, CD11b, CD11c, CD25, and CD49b using an AutoMACS Pro Separator (Miltenyi Biotec) to enrich for Tconvs. T cells were seeded at 2.5 × 10^6 per well in a precoated 24-well plate. IL-2 (20 U/ml) was added to the T cell culture. T cells were spin-transduced with fresh viral supernatants containing Polybrene (Sigma-Aldrich) ~40 h after the start of T cell stimulation. Retrovirally transduced T cells were identified by C90.1 expression and analyzed 3 d after transduction.

Protein digestion, labeling of peptides with stable isotopes, and fractionation

FACS-purified cell pellets were snap-frozen in liquid nitrogen. After lysis of cells in 0.1% RapiGest (Waters)/50 mM ammonium bicarbonate, extracted proteins were reduced/alkylated with 5 mM DTT and 10 mM iodoacetamide and digested overnight with sequencing-grade modified trypsin (Promega). Resulting peptides were dimethyl labeled on a column, as previously described (31). Briefly, peptides were labeled on Sep-Pak C18 cartridges (Waters) with the labeling reagent ("light" or "intermediate" using CH3O (Fisher) + NaBH4[CN] [Fisher] or C0 [Biotec] + NaBH4-CN, respectively). In the second, third, and fourth biological replicate experiments, cell population reagents were swapped. The "light"- and "intermediate"-labeled samples were mixed at a 1:1 ratio based on cell number. Sample complexity
was reduced by fractionating the peptides with pH 3-10 IPG strips and a 3100 OFFGEL Fractionator (Agilent) into 12 fractions.

**Liquid chromatography–electrospray ionization–tandem MS analysis**

Peptides were separated using the nanoACQUITY UPLC system (Waters) fitted with a trapping column (nanoACQUITY Symmetry C18, 5 μm particle size, 180 μm inner diameter × 20 mm length) and an analytical column (nanoACQUITY BEH C18, 1.7 μm particle size, 75 μm inner diameter × 200 mm length). Peptides were separated on a 120-min gradient and analyzed by electrospray ionization–tandem MS on an LTQ Orbitrap Velos (Thermo Fisher Scientific). Full-scan spectra from m/z 300 to 1700 at resolution 30,000 FWHM (profile mode) were acquired in the Orbitrap MS. From each full-scan spectra, the 15 ions with the highest relative intensity were selected for fragmentation in the ion trap. A lock mass correction using a background ion (m/z 445.12003) was applied.

**Protein identification and quantification**

MS raw data files were processed with MaxQuant (version 1.2.0.18) (32). Cysteine carboxamidomethylation and methionine oxidation were selected as fixed and variable modifications, respectively. The derived peak list was searched using the built-in Andromeda search engine (version 1.2.0.18) in MaxQuant against the Uniprot mouse database (2011.06.21). Initial maximal allowed mass tolerance was set to 20 ppm for peptide masses, followed by 6 ppm in the main search, and 0.5 Da for fragment ion masses. A 1% false discovery rate was required at the protein level and the peptide level. In addition to the false discovery rate threshold, proteins were considered identified if they had at least one unique peptide. The protein identification was reported as an indistinguishable “protein group” if no unique peptide sequence to a single database entry was identified.

**Bioinformatic and statistical analysis**

Statistical analysis was performed for the proteins quantified in at least two replicates using the Limma package in R/Bioconductor (33, 34). Proteins with an adjusted p value < 0.05 were considered differentially expressed between Tregs and Tconvs. Network analysis was done using STRING (35), and visualization was performed in Cytoscape v2.8 (36). Treg-specific genes (over- and underrepresented) were identified based on mRNA microarray expression data that we published earlier (25, 28). Tregs and Tconvs from LN and thymus were included. Genes that were differentially expressed >1.5-fold between Tregs and Tconvs were considered significantly different (p < 0.05). Student t test, two-tailed. Generated gene lists were cross-referenced with our proteomic data based on gene names and used for further analysis.

**Accession numbers**

The MS proteomics data have been submitted to the ProteomeXchange consortium (http://www.proteomexchange.org) via the PRIDE partner repository (37) under dataset identifier PXD000794.

**Results**

**Differential quantitative MS of the Treg proteome**

We performed MS-based quantitative proteomic analysis of murine Tregs, comparing them with Tconvs. To this end, cells were highly enriched by FACS, and proteins were isolated and differentially labeled with stable isotopes (31) (Fig. 1A). Peptide fractions were analyzed by high-resolution nano liquid chromatography-tandem MS, and relative protein abundance was based on relative MS signal intensities (Fig. 1B). Four replicates identified a total of 5225 unique proteins, with 4859 detected in at least two replicates and 3756 detected in all replicates (Fig. 1C, Supplemental Table I). The identified proteins covered all protein classes and cell compartments, from the nucleus to the cell surface (Fig. 1D). Among the identified proteins, 164 were significantly (p < 0.05) differentially expressed between Tregs and Tconvs (Supplemental Table I). Of these, 51 proteins were underrepresented and 113 proteins were overrepresented in Tregs. The quantified proteins are plotted as fold change Treg versus Tconv against p value to visualize differences (Fig. 2A).

**Expression of the transcription factor TCF7 in Tregs**

For the accurate function and differentiation of cells, it is often essential to repress the expression of specific genes. Therefore, we were particularly interested in underrepresented proteins in Tregs. To screen for interesting targets, we compared LN and thymus mRNA expression data sets (25, 28) with the set of 51 proteins underrepresented in Tregs. We were able to map 43 protein candidates to gene expression data and, thereby, identified a cluster of genes that was prominently underrepresented in Tregs (Fig. 2A, highlighted in red). This cluster included three genes that were previously described in the context of Treg development and function (Fig. 4A): Pde3b, Sall1, and Ikh (38–40). This cluster also contained Tcf7, which was one of the most strongly underrepresented proteins in Tregs (Supplemental Table I). TCF7 was established to be important during thymocyte differentiation (15), but its function in the context of tTreg generation has not been analyzed.

Recently, a Tcf7-Lef1 coregulated cluster of genes was described by the Immunological Genome Consortium (41). We compared this Tcf7-Lef1 coregulated cluster with our proteomics data and identified 19 members in our data set (Fig. 4B). Those included, in addition to TCF7 and LEF1, THEMIS, ITK, and multiple CD3 family members. Interestingly, the whole cluster was significantly (p = 0.00015) underrepresented in Tregs in our proteome data set (Fig. 4B, 4C).

The data obtained from our quantitative MS analysis identified TCF7 as significantly underrepresented in Tregs, and it was differentially expressed on the gene level. Hence, we performed intracellular flow cytometric staining for TCF7 and LEF1 and confirmed that both were underrepresented in Tregs, as detected by our MS analysis (Fig. 4D, 4E). Because our comparative proteomics data set identified the transcription factor TCF7 as a promising target differentially expressed in thymic Tregs, we wondered about its role in Treg generation.

**Dosage effect: reduction in TCF7 results in greater Treg-generation capacity**

To study the role of TCF7 in thymic Treg differentiation, we analyzed a Tcf7-deficient mouse model (15). Flow cytometric analysis
and cell counting showed that the total numbers of DN, DP, CD4 and CD8 SP thymocytes were reduced in Tcf7Δ/Δ mice compared with the heterozygous (Tcf7Δ/+Δ) and wild-type (Tcf7Δ+/Δ) littermate controls (Supplemental Fig. 1A). Immunohistological staining of Tcf7Δ/Δ and Tcf7Δ+/Δ thymi with K14, a marker for thymic medullary epithelial cells, showed an overall reduced thymus size but relatively normal organization into medullary and cortical regions (Supplemental Fig. 1B, 1C).
When we analyzed the fraction of Tregs among the CD4 SP population, we found that Tcf7\(^{-/-}\) mice harbored approximately three times more thymic Tregs compared with Tcf7\(^{+/+}\) mice (5 versus 15\%) (Fig. 5A). However, the total number of Tregs was not elevated, because there is also a reduction in the total number of CD4 SP cells in the Tcf7\(^{-/-}\) thymus (Supplemental Fig. 1A).
Because FOXP3+ cells are detectable as early as the DP stage (42), we looked at DP cells and found that Tcf7 deficiency resulted in a significantly increased fraction of FOXP3+ cells (Fig. 5B). These observations indicate that the presence of TCF7 influences the frequency of Tregs in the thymus.

Because of the greatly reduced thymocyte number in Tcf72/2 mice, it was not clear whether this increased Treg frequency in Tcf72/2 mice was caused by influencing Treg development or by the decreased number of cells in the Tconv compartment. Therefore, we investigated Treg differentiation in heterozygous Tcf7+/- mice, which had normal thymocyte numbers. Examination of TCF7 expression levels in thymus, LN, and spleen cells from Tcf7+/+, Tcf7+/-, and Tcf7-/- mice showed a significant decrease in TCF7 expression in Tregs and Tconvs in Tcf7+/- mice compared with the Tcf7+/+ littermate mice (Fig. 5C, Supplemental Fig. 2A). In contrast, no difference was observed for the expression level of LEF1 in the thymus, LN, and spleen between Tcf7+/+ and Tcf7+/- mice (Supplemental Fig. 2B).

These results showed that Tcf7+/+ mice are an ideal model to study the dosage effect of Tcf7 expression in the absence of significant changes in total thymocyte numbers. Because we were interested in Treg differentiation, we analyzed Treg precursors, including the TCR-dependent early Treg precursor (CD4SP CD69+CD252 FOXP32), as well as the TCR-independent, but common γ-chain signaling-dependent, late Treg precursor (CD4SP CD69+CD25+FOXP32) (13, 14). Tcf7-specific real-time PCR revealed that gene expression levels of Tcf7 in both Treg precursor populations in Tcf7+/- mice were about half those in Tcf7+/+ mice (Fig. 5D).

Next, we analyzed the thymic Tregs and Treg precursors more closely in Tcf7+/- mice. In line with the previous observation in Tcf7+/- mice, the percentages of CD4SP FOXP3+ Tregs and late...
FIGURE 4. Identification of TCF7 and confirmation of TCF7 and LEF1 expression. (A) Heat map of gene expression of Tregs and Tconvs isolated from LN or thymus with the greatest (red) and least (blue) gene expression. Genes were selected from the 43 significantly underrepresented proteins (Tregs compared with Tconvs) identified by differential MS. Hierarchical clustering using Pearson’s correlation (row normalized). Red line marks cluster. (B) Heat map of the TCF7/LEF1-coregulated cluster–related proteins. (C) Force-directed STRING network of interacting proteins from the TCF7/LEF1 cluster. Flow cytometric analysis of intracellular TCF7 (D) and LEF1 (E) expression in Tregs, Tconvs, and B cells from thymus, LN, and spleen of Tcf7+/+ mice (n = 4). B cells served as a negative control. Symbols represent individual samples, and the horizontal lines indicate the mean value for each group. **p < 0.01, unpaired t test.
FIGURE 5. Reduction of TCF7 results in higher Treg-generation capacity. FOXP3 and CD25 expression in CD4SP cells (n = 5) (A) and DP cells (n = 5) (B) from Tcf7+/+ and Tcf7−/− mice. (C) TCF7 expression in Tregs, Tconv, B cells, and DN cells from thymus and LN from Tcf7+/+, Tcf7+/−, and Tcf7−/− mice (n = 4). Tcf7+/+ data are also plotted in Fig. 4D. (D) Tcf7 mRNA expression in FACS-purified thymic Tconv and Treg precursors from Tcf7+/+, Tcf7+/−, and Tcf7−/− mice via quantitative PCR. Data are presented as percentage relative to Hprt expression. (E) FOXP3 and CD25 expression in CD4SP cells from Tcf7+/+ and Tcf7−/− mice (n = 5). (F) Late Treg precursor frequency in Tcf7+/+ and Tcf7+/− mice (n = 7–12). Representative plots are shown in Supplemental Fig. 2C. (G and H) FOXP3 and CD25 expression in Treg precursors that were FACS purified from Foxp3-YFP+ Tcf7+/+ and Tcf7+/− mice and cultured in vitro. (G) The late Treg precursor was cultured for 1 d with IL-2 and IL-15 (n = 3–4). (H) The early Treg precursor was cultured for 3 d with IL-2 and anti-CD3/CD28 beads (n = 6). Cell count was determined by flow cytometry. Representative plots and quantification shown as described. Symbols represent individual samples, and numbers indicate the percentage of cells within the defined region. The horizontal lines indicate the mean value for each group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, unpaired t test.
Treg precursors were significantly increased in Tcf7+/− mice compared with Tcf7+/+ mice (Fig. 5E, 5F; Supplemental Fig. 2C). To confirm whether the identified precursor was the TCR signaling–independent late Treg precursor, we examined it in vitro. FACS-purified Foxp3+ precursors (see gating scheme, Supplemental Fig. 2D, R4) were incubated with the cytokines IL-2 and IL-15 without TCR stimulus, and the expression of FOXP3 and CD25 was measured after 1 d in culture. Tcf7+/− and Tcf7+/+ precursors differentiated into Foxp3-expressing Tregs, revealing that they were Treg-committed late precursors (Fig. 5G).

Initially, we tried to establish bone marrow (BM) chimera assays to analyze Treg differentiation. Unfortunately, because the development of TCF7-deficient progenitors into T cells is reduced, wild-type BM strongly outcompeted TCF7-deficient BM at all ratios tested [as previously described (19)]. Therefore, we decided to study purified precursors.

To study the early Treg precursor, a TCR-dependent and APC-free differentiation assay was established to avoid any influence from APCs (Supplemental Fig. 2E). The combination of IL-2, IL-15, and anti-CD3/CD28 bead-based stimulus generated the highest output of Tregs in terms of total number and frequency after 3 d of in vitro culture. Sixty to eighty percent of the cells differentiated into Tregs under these conditions (Supplemental Fig. 2E). As a control, we analyzed thymic Tconv (CD4SP Foxp3−) that no longer expressed CD69 (CD695); they had generally lost the ability to differentiate into Tregs under the same conditions, although they were strongly activated, induced CD25 expression, and proliferated (Supplemental Fig. 2E). Side-by-side comparison of FACS-purified early Treg precursors from Tcf7+/− and Tcf7+/+ mice incubated with either the full stimulus (IL-2, IL-15, and anti-CD3/CD28 beads) or a weaker stimulus (without IL-15) showed that significantly more Tregs were generated from the Tcf7+/− Treg precursor (Fig. 5H, Supplemental Fig. 2F).

The results obtained from the ex vivo analysis and in vitro culture suggest that decreased expression of TCF7 leads to enhanced Treg differentiation capacity.

**TCF7 deficiency allows lower TCR affinities to be recruited into the Treg lineage**

TCR signaling strength is a key factor that determines tTreg selection. The current model suggests that the TCR avidity that drives commitment into the Treg lineage is rather high and even overlaps with the affinity range of negative selection. CD5 expression was described to correlate with the overall avidity of the MHC–TCR interaction during positive selection (43). Tregs show higher levels of CD5 with the affinity range of negative selection. CD5 expression was measured after 1 d in culture. Tcf7+/− and Tcf7+/+ precursors differentiated into Foxp3-expressing Tregs, revealing that they were Treg-committed late precursors (Fig. 5G).

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To study the early Treg precursor, a TCR-dependent and APC-free differentiation assay was established to avoid any influence from APCs (Supplemental Fig. 2E). The combination of IL-2, IL-15, and anti-CD3/CD28 bead-based stimulus generated the highest output of Tregs in terms of total number and frequency after 3 d of in vitro culture. Sixty to eighty percent of the cells differentiated into Tregs under these conditions (Supplemental Fig. 2E). As a control, we analyzed thymic Tconv (CD4SP Foxp3−) that no longer expressed CD69 (CD695); they had generally lost the ability to differentiate into Tregs under the same conditions, although they were strongly activated, induced CD25 expression, and proliferated (Supplemental Fig. 2E). Side-by-side comparison of FACS-purified early Treg precursors from Tcf7+/− and Tcf7+/+ mice incubated with either the full stimulus (IL-2, IL-15, and anti-CD3/CD28 beads) or a weaker stimulus (without IL-15) showed that significantly more Tregs were generated from the Tcf7+/− Treg precursor (Fig. 5H, Supplemental Fig. 2F).

The results obtained from the ex vivo analysis and in vitro culture suggest that decreased expression of TCF7 leads to enhanced Treg differentiation capacity.

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expression level of TCF7 is high in both Treg precursors (Fig. 7C). The TCF7 decrease coincided with the first expression of FOXP3. Therefore, we analyzed whether FOXP3 can influence TCF7 levels. To this end, we retrovirally overexpressed FOXP3 in Tconvs in vitro. Transduced T cells showed that expression of FOXP3 induces repression of TCF7 at the protein level (Fig. 7D), which could account for the reduced expression of TCF7 in differentiated tTregs.

In conclusion, the gain-of-function and loss-of-function data suggest that TCF7 interferes with the ability of Treg precursors to differentiate into mature tTregs, and FOXP3 can finally regulate the expression of regulator TCF7.

Discussion
Although generation of tTregs has been studied for years, this process is still not fully understood, and important regulators of this process are still being identified. Based on proteomics and gene expression data comparing Tregs and Tconvs, we identified TCF7 as a promising candidate. The proteomics data set with its 5225 identified proteins by itself is a valuable repository for further Treg research. Although proteomics has been used to study Tregs, previous studies focused either on proteins identified by gel blotting (44) or selective differences in the expression of specific surface molecules (45) or protein kinases (46). One recent report used proteomics to study the FOXP3 complex with all of its interaction partners (47). We now provide a comprehensive quantitative and differential proteomics data set of murine Tregs and Tconvs. Together with our comparative analysis of proteome and gene expression data, we generated a data set that will assist in better understanding the uniqueness of Tregs.

Previous studies of the function of TCF7 in thymocyte development did not distinguish between Tconv and Treg subsets (15, 17, 19, 22, 24). We observed that TCF7 deficiency increased the fraction of Tregs among the CD4 SP and DP cell populations. This, by itself, does not prove that TCF7 is involved in processes that interfere with Treg selection, because the severe reduction in the total number of thymocytes could also influence the Tconv compartment. Therefore, we continued to work with Tcf7-heterozygous mice. We demonstrated that Treg precursors from heterozygous mice expressed about half of the Tcf7 message, and this decreased TCF7 level led to the enhanced Treg-differentiation capacity of Treg precursors. We carefully...
FACS purified TCR-rearranged (i.e., postpositive selection) Treg precursors and studied them in vitro, in the absence of other thymic influences, to determine their ability to differentiate into Tregs. We purposely used an APC-free Treg-differentiation system to control for the number of signals received by the Treg precursors. An APC-free thymic Treg precursor–differentiation assay was introduced previously (14). In this study, the investigators demonstrated that, on day 3, ∼10% of cells had differentiated into FOXP3+ Tregs (14). We refined this assay by introducing an anti-CD3/CD28 bead-based stimulus and different cytokine conditions and were able to improve the efficiency of Treg lineage commitment to up to 80% after 3 d. Importantly, thymic Tconvs (CD4PS CD692) had generally lost the ability to differentiate into Tregs under the same conditions, indicating that Treg commitment is restricted to a narrow developmental window. These data showed that three signals were sufficient for Treg differentiation: TCR, costimulation, and common γ-chain signaling. TCF7 has to interfere with one of these three signals, because the endogenous TCF7 levels in wild-type Treg precursors restrict Treg development under these conditions. Indeed, it was shown that β-catenin and TCF7 signaling were active downstream of TCR signaling (24). When we titrated the TCR stimulus by using different concentrations of anti-CD3, we observed that wild-type levels of TCF7 in Treg precursors limited Treg output over a broad range of stimuli. We calculated that heterozygote precursors with reduced TCF7 levels needed about three times less TCR stimulus compared with Treg precursors from Tcf7 wild-type mice. This indicated that TCF7 increases the requirements of the TCR stimulus for a Treg precursor to differentiate into the Treg lineage and, thereby, helps to move the critical window of Treg commitment to higher-affinity TCR interactions. This idea was supported by our findings in the Nr4a1-GFP mouse. Previous studies described the Nr4a1-GFP reporter mouse as a tool to investigate the TCR avidities in developing T cells and Tregs, because the GFP expression correlates with TCR signaling strength (10, 48). In Tcf7-deficient precursors and thymic Tregs, we detected cells with lower expression of Nr4a1 indicating less TCR affinity, which supports the concept that TCF7 fosters the selection of clones with higher TCR affinities. This observation was also supported by our finding that tTregs from Tcf7-deficient mice harbor many more CD5low Tregs. In contrast, although thymic Tconvs from Tcf7-deficient mice also had lower expression of Nr4a1, there was no difference in the breadth of the TCR affinities compared with Tcf7-sufficient mice. Of note, this analysis also showed that the distribution of affinities in late Treg precursors, as well as thymic Tregs, was significantly lower than that in thymic Tconvs, which supports the concept that Tregs are normally selected in a more narrow TCR affinity range compared with Tconvs.
Other factors have been implicated with such a modulatory effect on the TCR signal.Themis was proposed to control the signaling threshold for positive and negative selection, although Treg development was not analyzed in that study (49). Another recent report suggested that TNFR family members, such as GITR and OX40, could couple TCR signal strength to Treg differentiation (48). Unlike these TNFR family members that support Treg differentiation by lowering the required TCR threshold, TCFT could act antagonistically to restrict generation, exclude low TCR affinities, and, thereby, help to shape the Treg TCR repertoire toward higher-affinity clones. Interestingly, a recent study demonstrated that Tregs from Tcf7-deficient mice are perfectly functional and, if anything, were even more potent in suppression assays (50).

It was observed recently that activation of β-catenin results in significantly fewer Tregs in the thymus (51). Using CD4-specific expression of constitutively active β-catenin and BM chimeras, it was shown that this effect was T cell intrinsic. We took this analysis one step further by demonstrating that Treg precursors are sensitive to TCFT/β-catenin signaling. In the thymus, TCF7 signaling can be triggered through different pathways. For example, in early T cell precursors, Notch signaling was also shown to induce TCFT (19, 20). It is unclear which signaling events are more critical for TCFT induction and, thereby, interference with Treg differentiation. Intriguingly, once FOXP3 is expressed it can facilitate a reduced expression level of TCF7 and, thus, may foster a survival advantage in committed Tregs.

The molecular basis of how a biased TCR repertoire is established in developing iTregs is still not fully understood. Our results suggest that TCFT is part of this program and helps to restrict and shape the TCR repertoire toward higher-affinity clones that access the Treg lineage. By increasing the TCR signaling strength hurdle, TCFT may contribute to the enrichment of self-reactive TCRs within the Treg pool.

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Disclosures
The authors have no financial conflicts of interest.  

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