Nonfunctional Regulatory T Cells and Defective Control of Th2 Cytokine Production in Natural Scurfy Mutant Mice

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Foxp3+ regulatory T cells (Tregs) are crucial for preventing autoimmunity. We have demonstrated that depletion of Foxp3+ Tregs results in the development of a scurfy-like disease, indicating that Foxp3− effector T cells are sufficient to induce autoimmunity. It has been postulated that nonfunctional Tregs carrying potentially self-reactive T cell receptors may contribute to scurfy (sf) pathogenesis due to enhanced recognition of self. Those cells, however, could not be identified in sf mice due to the lack of Foxp3 protein expression. To address this issue, we crossed the natural sf mouse mutant with bacterial artificial chromosome transgenic DEREG (depletion of regulatory T cells) mice. Since DEREG mice express GFP under the control of an additional Foxp3 promoter, those crossings allowed proving the existence of “would-be” Tregs, which are characterized by GFP expression in the absence of functional Foxp3. Sf Tregs lost their in vitro suppressive capacity. This correlated with a substantial reduction of intracellular CAMP levels, whereas surface expression of Treg markers was unaffected. Both GFP+ and GFP− sf cells produced high amounts of Th2-type cytokines, reflected also by enhanced Gata-3 expression, when tested in vitro. Nevertheless, sf Tregs could be induced in vitro, although with lower efficiency than DEREG Tregs. Transfer of GFP+ sf Tregs, in contrast to GFP− sf T cells, into RAG1-deficient animals did not cause the sf phenotype. Taken together, natural and induced Tregs develop in the absence of Foxp3 in sf mice, which lack both suppressive activity and autoreactive potential, but rather display a Th2-biased phenotype. The Journal of Immunology, 2009, 183: 5662–5672.

Central tolerance is mediated by deletion of self-reactive T cells in the thymus, while small numbers of potentially pathogenic T cells, which escape central tolerance, can be controlled in the periphery by regulatory T cells (Tregs)1 (1, 2).

Foxp3 has been shown to be the major transcription factor associated with Treg development and function (3–5) and mutations within the Foxp3 gene locus can lead to IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) disease, a rare autoimmune disease in humans associated with eczema, severe enteropathy, type 1 diabetes, thyroiditis, hemolytic anemia, and thrombocytopenia (6, 7). Severity and outcome of the disease are highly dependent on the site of mutation, and different grades of autoimmunity have been observed for complete loss, truncated forms of Foxp3 protein, or single point mutations (8). It is evident that disease severity can be closely linked to mutations in different functional regions of the protein; however, environmental factors may also contribute to IPEX pathogenesis (8).

In the genetically equivalent mouse-model named scurfy (sf), a natural frame-shift mutation in exon 8 (insertion of two additional adenosines) causes a premature stop and truncation of the forkhead functional regions of the protein; however, environmental factors associated with Treg development and function (3–5) and mutations within the Foxp3 gene locus can lead to IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) disease, a rare autoimmune disease in humans associated with eczema, severe enteropathy, type 1 diabetes, thyroiditis, hemolytic anemia, and thrombocytopenia (6, 7). Severity and outcome of the disease are highly dependent on the site of mutation, and different grades of autoimmunity have been observed for complete loss, truncated forms of Foxp3 protein, or single point mutations (8). It is evident that disease severity can be closely linked to mutations in different functional regions of the protein; however, environmental factors may also contribute to IPEX pathogenesis (8).

In the genetically equivalent mouse-model named scurfy (sf), a natural frame-shift mutation in exon 8 (insertion of two additional adenosines) causes a premature stop and truncation of the forkhead box region of the protein, resulting in a highly unstable and non-detectable Foxp3 protein (9). The sf mutation leads to the development of a severe lymphadenopathy (10) with a similar spectrum of immune-mediated tissue inflammation as observed in IPEX patients (9, 11). The sf phenotype has in part been associated with typical Th2 pathology, for example eosinophilia, hyper-IgE syndrome, and blepharitis also occurring in Th1-prone C57BL/6 mice (12–14) and IPEX patients (10). Massive autoimmunity in sf mice results in death during the first month after birth but can be cured by neonatal adoptive transfer of wild-type (WT) Foxp3+ Tregs (5, 15, 16). The disease has been shown to be mediated by CD4+ T cells (14), although it was not possible to distinguish between activated effector T cells and lineage-committed Tregs due to a lack of specific Treg markers. It has been shown that under certain experimental conditions using a fixed transgenic TCR β-chain, the Treg TCR repertoire is shifted toward self-Ag recognition (17–19), and the presence of nonfunctional self-reactive Tregs has been
discussed as a potential cofactor for the induction of autoimmunity in sf mice (17). In previous studies we and others were able to demonstrate in transgenic mouse models that specific ablation of Tregs in neonates was sufficient to induce fatal autoimmunity with a severity comparable to the naturally occurring sf phenotype (20, 21). We thereby concluded that the mere absence of Tregs was sufficient for the development of the disease. Nevertheless, it could not be ruled out that mutated, Foxp3-deficient sf Tregs also contributed to the fatal phenotype in sf mice.

By crossing DEREG (depletion of regulatory T cells) mice (mice expressing diphtheria toxin receptor-enhanced GFP under the control of the Foxp3 promoter in a bacterial artificial chromosome (BAC)-transgenic approach (20)) with sf mice, we were able to assess GFP Foxp3 “would-be” Tregs in the thymus and periphery of natural Foxp3 mutants. We observed that, although these cells lost their suppressive capacity, they expressed bona fide Treg markers such as CD25, GITR, and CTLA-4. The only observed difference between functional Tregs and nonfunctional sf Tregs was intracellular cAMP, which was highly diminished in sf Tregs when compared with DEREG Tregs. Regardless of sf Tregs could no longer suppress naïve T cell proliferation and were nonanergic in in vitro cultures, they did not elicit any pathogenic consequences when transferred into RAG1−/− mice, whereas mice receiving the effector T cell population from sf donors developed the sf phenotype. Furthermore, we show that sf Tregs and sf CD4+ GFP T cells, here on a C57BL/6 background, display a substantial shift toward a Th2 phenotype as measured by their capacity to produce high amounts of IL-4, IL-10, and IL-13, correlating with high Gata-3 expression.

Materials and Methods

Mice

DEREG and C57BL/6 mice were bred at the animal facility of the Institute for Medical Microbiology, Immunology and Hygiene (Technische Universität Muenchen, Munich, Germany). SF mice (B6.Cg-Foxp3<sup>FLRT</sup>) were purchased from Charles River Laboratories. TCR-HA and pkg-HA mice were bred at the Research Institute for Molecular Pathology (Vienna, Austria). All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines.

Abs, FACS staining, and sorting

The following Abs and secondary reagents were purchased from eBioScience: anti-CD3ε (50A2), anti-CD4 (GK1.5), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD103 (M290), anti-CD127 (A7R34), anti-Foxp3 (FJK-16s), FixPermx, streptavidin, and appropriate isotype controls. Anti-GITR and anti-CTLA-4 were generated in our laboratory. Rabbit anti-GFP and Cy3-conjugated goat anti-rabbit polyclonal Abs were obtained from Jackson ImmunoResearch Laboratories. TCR-HA transgenic T cells were detected by a FITC-conjugated mAb (6.5) generated in the laboratory of L. Klein (22). For in vitro restimulation, anti-CD3ε (145-2C11) and anti-CD25 (35.71) from eBioScience or PMA/ ionomycin (Sigma-Aldrich) were used. For intracellular staining, anti-IL-4 and anti-IFN-γ Abs from eBioScience or anti-CD28 (37.51) from eBioscience or PMA/ionomycin (Sigma-Aldrich) were used. Cytometric analysis and sorting were performed using a CyAn (Dako) flow cytometer, immunofluorescence staining, histology, and paraffin-embedded organs. Foxp3 immunohistochemistry was performed as described (24).

Microscopy, immunofluorescence staining, histology, and immunohistochemistry

Immunofluorescent stainings were performed as described previously (23). Histologic stainings on various organs were performed on formalin-fixed and paraffin-embedded organs. Foxp3 immunohistochemistry was performed as described (24).

CDR3 spectratyping

CD4+ GFP+ and GFP+ CD4+ cells from sf × DEREG and DEREG mice were sorted. RNA was extracted by TRIzol-LS reagent (Invitrogen), and cDNA was prepared using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). PCR amplification and 3′-run off reactions were performed as described (25), with the exception that we used the primer C (26) as reverse primer for the initial V-J reactions. Thus, the analysis included 24 TCR variable regions in all possible combinations with one Cβ and 12 Jβ elements (reviewed in Ref. 27). Fragment lengths were analyzed on an ABI377 DNA sequencer (Applied Biosystems). Polyclonal populations show Gaussian distributions. Clonal expansions appear as distinct peaks.

In vitro suppression assay

CD4+ Tregs and would-be CD4+ GFP Foxp3+ T cells were sorted from DEREG and sf × DEREG mice as described above. CD25+ CD4+ responder T cells were purified from lymph nodes of C57BL/6 mice by CD4-negative isolation followed by depletion of CD25+ cells via anti-CD25-PE staining and anti-PE microbeads (Miltenyi Biotech). Purification of responder T cells was followed by CFSE labeling (5 μM). CD90+ APCs were purified from C57BL/6 mice using anti-CD90 microbeads (Miltenyi Biotech) and were irradiated before culture. Cultures were established in 96-well round-bottom plates using 1 × 10⁶ responder cells and 2 × 10⁵ APCs per well. Tregs were added at a 1:1 ratio. Cells were activated using 1 μg/ml anti-CD3e mAb, and CFSE dilution was assessed by flow cytometry after 4 days.

Real-time RT-PCR

RNA was isolated from CD4+ GFP+ and CD4+ T cells using the RNeasy kit (Qiagen) following cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed on LightCycler 480 (Roche) using Roche’s SYBR Green Master PCR kit and specific primers were optimized to amplify 90- to 250-bp fragments from the different genes analyzed. A threshold line was set in the linear part of the amplification curve to determine the corresponding crossing point for every gene. Relative mRNA levels were determined by using included standard curves for each individual gene and further normalization to Rps9 (ribosomal protein S9). Melting curves established the purity of the amplified band. Primer sequences are: Foxp3 (5′-CTG-GCG-AAC-CTG-GTA-GTC-CT-3′, 5′-CTC-CCA-GAG-CCC-ATG-GCA-GAA-GT-3′), Il10 (5′-ATT-TCT-GGG-CCA-TGC-TTC-TCT-GC-3′, 5′-CTG-GAC-AAC-ATA-CTG-ACA-GAC-TTC-3′), Il4 (5′-AGC-ACC-TGG-GAA-GCC-CTA-CAG-AC-3′, 5′-GGA-CCG-CAT-CGA-CCG-AAG-TA-3′), Igake (integrin alpha E, epithelial-associated) (5′-GCT-GGG-CCC-TCC-TTG-TGC-TCT-3′, 5′-GAA-CTG-CCG-CAT-GGT-GTA-ATA-3′), Cebpa (5′-GCA-ACC-CGG-TAC-CTA-GAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (TNF receptor superfamily, member 18) (5′-CAT-AGG-GCC-CAA-TGG-ATA-ACC-3′, 5′-GGA-CCG-GGC-GAG-CAG-ACA-GA-3′), C4a (5′-CCT-CTT-GTT-GCA-TTA-AC-TTG-TCT-3′, 5′-GCG-CTG-CTG-CAT-ATT-TTC-3′), Lgal1 (lectin, galactoside binding, soluble 1) (5′-TCC-GGC-GTC-ATG-TTG-AT-3′, 5′-AAC-CTG-CCT-TCT-CCT-TCC-3′).

For Gata3 and Rorc, the following method was used: cDNA was prepared using the μMACS One-Step cDNA kit and a thermostable magnetic separator (both from Miltenyi Biotech) according to the manufacturer’s instructions. Validated intron-spanning primer sets were designed employing the Universal Probe Library Assay Design Center (www.roche-applied-science.com). The following primer pairs were used: Gata3 (5′-TCA-AGC-CCA-AGA-GCA-G-3′, 5′-TGG-TGA-TGT-GAT-GAC-GAC-3′), Rorc (5′-GGC-ACC-GAC-GGT-TAT-GAG-GTG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (5′-GGA-CG-AAA-GGC-GAG-CCT-CAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (TNF receptor superfamily, member 18) (5′-CAT-AGG-GCC-CAA-TGG-ATA-ACC-3′, 5′-GGA-CCG-GGC-GAG-CAG-ACA-GA-3′), Gata3 (5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′).

For Gata3 and Rorc, the following method was used: cDNA was prepared using the μMACS One-Step cDNA kit and a thermostable magnetic separator (both from Miltenyi Biotech) according to the manufacturer’s instructions. Validated intron-spanning primer sets were designed employing the Universal Probe Library Assay Design Center (www.roche-applied-science.com). The following primer pairs were used: Gata3 (5′-TCA-AGC-CCA-AGA-GCA-G-3′, 5′-TGG-TGA-TGT-GAT-GAC-GAC-3′), Rorc (5′-GGC-ACC-GAC-GGT-TAT-GAG-GTG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (TNF receptor superfamily, member 18) (5′-CAT-AGG-GCC-CAA-TGG-ATA-ACC-3′, 5′-GGA-CCG-GGC-GAG-CAG-ACA-GA-3′), Gata3 (5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (TNF receptor superfamily, member 18) (5′-CAT-AGG-GCC-CAA-TGG-ATA-ACC-3′, 5′-GGA-CCG-GGC-GAG-CAG-ACA-GA-3′), Gata3 (5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′), Tnfrsf18 (TNF receptor superfamily, member 18) (5′-CAT-AGG-GCC-CAA-TGG-ATA-ACC-3′, 5′-GGA-CCG-GGC-GAG-CAG-ACA-GA-3′), Gata3 (5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′, 5′-ACC-CCT-CCC-AGT-TTC-TCT-CAG-3′).
MasterMix (AB-1219/b; Abgene). After normalization of the data according to the expression of Eef1a1 mRNA, the relative expression level of Pde3b mRNA was calculated.

cAMP ELISA
To assess cytosolic cAMP content, $1 \times 10^6$ T cells sorted as described above were washed three times in ice-cold PBS and lysed in 0.1 N HCl ($1 \times 10^3$/ml) for 10 min. Lysis was verified by trypan blue staining. Upon lysis, the cAMP-specific ELISA (Correlate EIA Direct Cyclic AMP Enzyme Immunoassay kit, 900-066; Assay Design) was performed according to the manufacturer’s instructions.

In vitro restimulation and cytokine production
T cells ($5 \times 10^5$) from peripheral LN plus an equal number of irradiated APCs per 96 round-bottom wells were cultured in RPMI 1640 medium (10% FCS supplemented). Anti-CD3 and anti-CD28 were added in a concentration of 1 $\mu$/ml, and recombinant human IL-2 (Roche) was used in a final concentration of 100 U/ml. Cultures were incubated for 4 days. All ELISAs were purchased from R&D Systems. For intracellular cytokine staining, cells were washed after 4 days of anti-CD3/anti-CD28 stimulation and further incubated for 3 days with complete medium containing 100 U/ml IL-2. On day 7, cells were restimulated with PMA/ionomycin in the presence of brefeldin A, harvested, and stained intracellularly for IL-4 and IFN-γ.

In vitro induction assay
CD4+ GFP− T cells were isolated from LNs of sf × DEREG and DEREG mice by FACS sorting. T cells ($1 \times 10^5$ per well) were seeded in RPMI 1640 medium (10% FCS supplemented) in the presence of 100 U/ml IL-2, 2 ng/ml TGF-β (PeproTech), and 0.01 $\mu$/ml anti-CD3e. Treg induction was assessed by FACS analysis for expression of GFP, CD25, and CD4 after 2 days.

Adoptive cell transfer
To assess the pathogenicity of Foxp3-deficient T cell populations, GFP+ CD4+ and GFP− CD4+ T cells were sorted from sf × DEREG and DEREG mice. Cells ($3 \times 10^5$) were transferred i.v. into RAG1−/− recipients. Mice were monitored closely and sacrificed for analysis after signs of skin disease development (8–12 wk).

Results
Foxp3+ GFP+ would-be Tregs can be detected in sf mice
We set out to establish an experimental system to study T cells from sf natural mutant mice, which were programmed to be Tregs but did not express functional Foxp3 protein due to a frame-shift mutation within exon 8 of the Foxp3 gene (9). Since none of the commercially available Abs detect Foxp3 protein in sf T cells, it was unclear if sf mice suffer from autoimmune disease solely because they lack Tregs or whether, in addition, nonfunctional, self-reactive Tregs may contribute to disease severity. By crossing BAC-transgenic DEREG mice to sf mice, we wanted to investigate if sf Tregs can be identified in sf × DEREG male mice (carrying the mutated copy of the Foxp3 gene) based on Foxp3 promoter-driven GFP expression mediated by the additional BAC-encoded promoter copy.

By using DEREG mice as reporter, we were able to detect Foxp3+ GFP+ would-be Tregs (designated as sf Tregs) in sf × DEREG male mice within different lymphatic organs (Fig. 1, A and B). Foxp3 Ab staining failed in sf × DEREG males as expected; however, we could detect GFP+ cells in spleens from those mice by immunofluorescence (Fig. 1A). To test if relative numbers of sf Tregs were reduced in comparison to DEREG Tregs, FACS analysis of GFP+ cells from different lymphatic organs was performed (Fig. 1B). Analysis of GFP+ cells from the periphery (lymph nodes, spleens) and thymi of sf × DEREG male mice and DEREG control mice revealed similar percentages of GFP+ cells (Fig. 1B). Given the severe lymphadenopathy of sf mice, the absolute number of lymph node GFP+Foxp3+ sf Tregs was significantly increased compared with the numbers of GFP+Foxp3+ Tregs in DEREG controls (data not shown).

Tregs can be generated both in the periphery from Foxp3− progenitors (induced Tregs) and within the thymus (natural Tregs) (reviewed in Ref. 28). The thymic selection process has initially been discussed to be potentially dependent on functional Foxp3 expression, and peripheral conversion may require a self-amplification loop for Foxp3 (29, 30). When we analyzed the development of thymic natural would-be Tregs (Fig. 1B), we observed comparable proportions of GFP+ cells in sf × DEREG male mice vs DEREG control mice, suggesting that potentially self-reactive natural sf Tregs could be selected in the absence of functional Foxp3 protein expression. To test whether differences in the specificity of T cells from sf × DEREG and DEREG mice can be observed, we analyzed in detail the TCR repertoires of LN GFP+ and GFP− T cells and thymic GFP+ cells from both genotypes by CDR3 spectratyping. This technique allows the detection of expanded clones by measuring the length distributions of the CDR3 regions for each possible Vβ-Jβ combination (reviewed in Ref. 27). Expanded clones yield single peaks, whereas polyclonal populations yield Gaussian length distributions. For each sample, we tested all possible Vβ-Jβ rearrangements using 24 V element-specific forward primers, 12 J-specific primers (25), and 1 C-specific reverse primer (26) in all combinations. In none of the samples from LN (Fig. 1C) or thymus (not shown) did we observe any major difference between GFP+Foxp3− sf and GFP+Foxp3+ DEREG Tregs selected in the absence or presence of functional Foxp3 protein. Most V-J combinations yield Gaussian-like distributions. In some cases the distribution was skewed, which indicates that a particular NDN length is slightly preferred, but no pronounced monoclonal expansions were observed (Fig. 1C). Importantly, there was no remarkable difference in the patterns between sf and DEREG Tregs.

We then wanted to address whether the selection of T cells with a defined TCR specificity may be impaired due to the lack of functional Foxp3 expression. Thereto, we made use of a TCR-transgenic system, where coexpression of a TCR specific for influenza hemagglutinin (TCR-HA) and its cognate Ag (pgk-HA) as corresponding neo-self Ag in the thymus leads to the efficient selection of TCR-HA+ cells into the Treg lineage (22). Lack of functional Foxp3 did not impair this process, as shown by the equal percentages and absolute numbers of TCR-HA+CD25+ cells in the thymi of WT and sf × TCR-HA × pgk-HA mice (Fig. 2, A and B). To test whether up-regulation of CD25 expression in sf × TCR-HA × pgk-HA mice was a consequence of Treg development and not merely a result of T cell activation, Foxp3 mRNA expression was assessed by real-time PCR detecting both WT and mutated Foxp3 mRNA. Foxp3 mRNA was expressed both in WT and sf TCR-HA+ cells of TCR-HA × pgk-HA mice (Fig. 2C), indicating that the selection of a defined TCRspecificity into the Treg lineage is not impaired by the absence of functional Foxp3. Although the percentage of TCR-HA+CD25+ cells was comparable, Foxp3 mRNA levels were slightly decreased in sf mice when compared with their WT counterparts, possibly reflecting a Foxp3-mediated positive feedback mechanism and self-stabilizing function of the Treg phenotype (30).

Sf Tregs lack suppressive activity but express most bona fide Treg markers
Since Foxp3 expression was reported to be necessary for the suppressive phenotype of natural Tregs, we wanted to assess the in vitro suppressive capacity of Foxp3-mutated sf Tregs. Functional properties of sf Tregs were analyzed in an in vitro suppression assay. Whereas GFP+Foxp3+ Tregs from DEREG control mice clearly suppressed the proliferation of CD4+CD25+ responder T cells (Fig. 3A), sf Tregs displayed no suppressive capacity.

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These findings indicate that expression of functional Foxp3 is not only sufficient (5), but also necessary for the suppressive capacity of Tregs.

To seek differentially expressed suppression-involved molecules in sf and WT Tregs, we sorted GFP$^+$ cells from sf × DEREG and DEREG control mice and performed RT-PCR and flow cytometry analysis (Fig. 3, B and C). RT-PCR data were normalized to house-keeping genes and further to conventional GFP$^+$ CD4$^+$ T cells from DEREG mice (Fig. 3B). No gross differences in the mRNA expression levels of typical Treg markers also associated with
regulatory function such as Foxp3, GITR, and CTLA-4 could be observed when peripheral CD4\(^+\)\(\text{GFP}^-\) cells from sf\(\times\)DEREG mice were compared. Activation markers of Tregs such as CD103 and galectin-1 were up-regulated in sf Tregs. Galectin-1, a lectin up-regulated in Tregs upon TCR activation, has been recently implied to be a candidate effector molecule since its blockade can abrogate in vitro suppression by Tregs \(31\). However, mRNA levels of this molecule were markedly up-regulated in both Tregs (Fig. 3\(B\)) and effector T cells (not depicted) from sf \(\times\) DEREG mice when compared with the expression level in DEREG Tregs, most likely attesting to the constant immune activation in male sf mice. Another molecule, which has been recently linked to Treg function, is the G protein-coupled receptor 83 \(\left(G_{\text{pr83}}\right)\) \(32\). RT-PCR revealed that this molecule was slightly down-regulated in sf Tregs when compared with DEREG Tregs.

Next, we performed FACS analysis of sf \(\times\) DEREG Tregs and DEREG Tregs to measure expression of several markers on the protein level (Fig. 3\(C\)). Intracellular Foxp3 protein expression was, as expected, clearly absent from the sf Treg population. The activation and memory markers CD69 and CD103 were found to be marginally up-regulated in male sf Tregs compared with DEREG Tregs (Fig. 3\(C\)), again reflecting the autoimmune phenotype in male mice. Notably, CD103 expression was absent on Foxp3\(^-\) Tregs in female individuals (supplemental Fig. 1).\(^4\) No significant differences for CD44 and CD127 could be observed for sf Tregs compared with DEREG Tregs (Fig. 3\(C\)). For the Treg markers GITR and CTLA-4, only a slight reduction of CTLA-4 expression in sf Tregs could be detected, while equal levels could be detected in Foxp3-deficient and -sufficient Tregs from female sf \(\times\) DEREG mice (supplemental Fig. 1). CD25, as a marker of both Treg lineage and also T cell activation, was nevertheless down-regulated in sf Tregs in both genders (Fig. 3\(C\) and supplemental Fig. 1).

cAMP production by Tregs has been discussed to reflect an important mechanism for suppression of responder cells \(33\). To test whether a lack of intracellular cAMP in sf Tregs could be the cause for disrupted regulatory capacity, we assessed cAMP levels in sf Tregs and DEREG Tregs in comparison to GFP\(\text{CD4}^-\) cells from both mouse strains. Indeed, cAMP levels were significantly lower in sf Tregs when compared with DEREG Tregs, whereas both effector T cell populations did not contain measurable amounts of intracellular cAMP (Fig. 3\(D\)). Real-time data further suggested a higher expression of Pde3b, a cAMP-hydrolyzing enzyme, in sf Tregs (Fig. 3\(D\), inset).

Sf Tregs express high amounts of Th2 cytokines, are not anergic, and can be induced in vitro

We and others have previously shown that the mere absence of Foxp3\(^+\) Tregs was sufficient to induce a scurfy-like phenotype \(20, 21\). To assess whether sf Tregs, in addition to GFP\(^+\) T cells, might

\(^4\) The online version of this article contains supplemental material.
further contribute to disease severity in sf mice, we first analyzed the cytokine expression profile of sf Tregs and sf effector T cells after in vitro culture of CD4^+ T cells. A high percentage of both cell types stained positive for IL-4 (Fig. 4A). This could further be confirmed by direct ex vivo isolation of both cell types followed by real-time measurement (shown for Treg subset in Fig. 3B). We could also detect IFN-γ-producing cells, although to a lower percentage. As controls, CD4^+ T cells from DEREG and WT mice were cultured in the presence of diphtheria toxin to exclude Treg-specific effects. Both cultures contained fewer IFN-γ-producing cells and almost no IL-4-producing cells when compared with sf cultures. Upon restimulation, ex vivo isolated sf Tregs as well as sf effector T cells did express higher levels of the Th2 transcription factor GATA-3 compared with DEREG cells as assessed by FACS staining (Fig. 4B). This was confirmed by RT-PCR on cDNA from directly ex vivo sorted sf Tregs and DEREG Tregs (supplemental Fig. 2).

To further dissect Th2 cytokine production from sf Tregs and sf effector T cells without potential co-influence, both populations were sorted and subjected to in vitro stimulation. GFP^+ WT Tregs and GFP^- effector T cells from DEREG mice served as controls. We could measure high amounts of IL-4, IL-10, and IL-13 in cell-culture supernatants from in vitro restimulated purified sf Tregs and sf effector T cells (Fig. 4C). IL-17 and TGF-β production were not elevated. Th17 cells are known to play a major role in the development of autoimmune diseases, and a reciprocal relationship between Tregs and Th17 cells has been suggested (reviewed in Ref. 34). To confirm the absence of high levels of IL-17 in sf T cells, we performed RT-PCR for the main transcription factor for Th17 cells (supplemental Fig. 2). Rorc was markedly down-regulated in both sf Tregs and sf effector T cells when compared with DEREG control cells.

Since sf Tregs showed a Th2-biased phenotype closely resembling Th2 effector cells, we analyzed whether the absence of Foxp3 rendered these cells nonanergic. Upon stimulation of sorted GFP^+ cells with anti-CD3 and anti-CD28, we were able to recover a substantial amount of sf Tregs, whereas Tregs from DEREG control mice died in the absence of exogenous IL-2 (data not shown). To confirm active proliferation of sf Tregs, we stained cultured cells for the nuclear proliferation marker Ki-67 (Fig. 4D). Upon stimulation of the cultures with IL-2 alone, DEREG Tregs survived but did not proliferate in contrast to sf Tregs. When a
TCR stimulus was provided in the absence of exogenous IL-2, DEREG Tregs did not survive the 4-day culture period, whereas sf Tregs concomitantly underwent active proliferation. As control, proliferation of conventional GFP^+/H11002^/CD4^+/H11001^T cells upon stimulation with anti-CD3 and anti-CD28 was also assessed, and no striking differences were observed between cells derived from sf^+/H11003^DEREG and DEREG control mice. Thus, our data suggest that GFP^+/H11001^sf Tregs do not harbor an anergic phenotype in vitro.

GATA-3-driven Th2 responses have been demonstrated to inhibit TGF-β-induced Foxp3 expression and therefore induction of Tregs both in mice and humans (35, 36). To test if the sf mutation may affect the generation of induced Tregs, we cultivated CD4^+/GFP^- T cells from male sf^+/H11003^DEREG mice in vitro with IL-2, TGF-β, and anti-CD3 similar to published protocols (37, 38) (Fig. 5). We could generate GFP^- induced sf Tregs in the absence of Foxp3 expression, albeit with lower efficacy when compared with conventional GFP^- CD4^+ T cells from DEREG control mice.

Sf Tregs infiltrate sites of inflammation but do not cause autoimmune pathology

Previous studies suggested that Tregs show a TCR repertoire shifted toward the recognition of self Ags (19). Since we demonstrated that Foxp3-mutated would-be Tregs from sf mice are able to escape from negative selection within the thymus, display an activated Th2-biased phenotype, and are not anergic in vitro, we next asked whether sf Tregs could be potentially harmful and could induce autoimmune pathology.

First, we tested for the presence of GFP^- sf Tregs in inflamed peripheral organs of sf^+/H11003^DEREG mice. We could detect high numbers of GFP^-sf Tregs in conjunction with GFP^-effector T cells in the inflamed ears of male sf^+/H11003^DEREG mice, while T cell...
infiltrates in healthy tissues from DEREG control mice were negligible (supplemental Fig. 3).

Since GFP\textsuperscript{-}H11001 nonsuppressive, potentially self-reactive sf Tregs could be found in tissues displaying autoimmune pathology, we next performed adoptive transfer experiments into RAG1\textsuperscript{-}/H11002 mice to investigate whether sf Tregs would lead to the onset of the typical autoimmune pathology observed in sf mice. Interestingly, sf Tregs survived in RAG1\textsuperscript{-}/H11002 hosts but did not induce any signs of inflammation, as observed in skin, pancreas, colon, liver, and lung (Fig. 6). In contrast, massive lymphocyte infiltration and tissue destruction occurred in recipients of effector sf T cells. GFP\textsuperscript{-}H11001 CD4\textsuperscript{+} (Treg) (3 \times 10^5) or GFP\textsuperscript{-}H11002 CD4\textsuperscript{+} (effector T, Teff) (3 \times 10^5) cells from either sf \times DEREG or DEREG mice were transferred into RAG1-deficient recipients. Mice were sacrificed when pathologic consequences were visible, occurring between 8 and 12 wk after transfer. Mesenteric LNs were removed and FACS stained to measure survival of transferred cells. Upper panel, FACS plots show CD4 and GFP expression of live-gated cell suspensions. Recipients were treated as indicated. Other organs were collected and embedded for histologic analysis. Representative H&E stainings of skin, pancreas, colon, liver, and lung are displayed. Experiments were performed three times.

Discussion

We and others demonstrated recently that the lack of Foxp3\textsuperscript{+} Tregs is sufficient to cause severe autoimmunity in mice (20, 21). Given that Tregs can express self-reactive TCRs (17–19), it could not be ruled out from those studies that nonfunctional self-reactive Tregs may additionally contribute to induction of autoimmunity in sf mice (17). On the other hand, self-reactivity of Tregs has been questioned (39), and various infection models (summarized in Ref. 40) suggest that cross-reactive or specific induction and expansion of Tregs can also occur in response to foreign Ag. To test the hypothesis that sf disease is exclusively caused by the lack of natural Foxp3\textsuperscript{+} Tregs, we asked whether cells expressing mutated Foxp3, designated here as would-be sf Tregs, exist in sf mice and, if so, could eventually contribute to the autoimmune disease.

In this study, we could demonstrate that GFP\textsuperscript{-}Foxp3\textsuperscript{-} sf Tregs do exist in sf mice. By crossing BAC-transgenic DEREG mice to sf mutant mice, the additional copy of the Foxp3 promoter together with the GFP reporter gene could be used to detect cells that did not express the functional Foxp3 gene (sf allele on the X chromosome) but may express factors activating the additional Foxp3 promoter (GFP expressed as BAC transgene instead of Foxp3 exon 1). Interestingly, sf Tregs could be found as GFP\textsuperscript{-}H11001 Foxp3\textsuperscript{-} cells in similar percentages as GFP\textsuperscript{-}H11001 Foxp3\textsuperscript{-} cells from DEREG control mice, clearly indicating that sf Tregs do exist in sf mice.

Sf Tregs showed a distribution of TCR V\textbeta-chain expression comparable to WT Tregs, indicating that no particular T cell clone proliferated extensively in sf mice (Fig. 1C). Moreover, selection of a defined TCR specificity was not altered by the absence of functional Foxp3, as shown by a TCR transgenic approach. Thus, it appeared that Foxp3 is not required for thymic selection of Tregs.
and that sf mice possess would be Tregs (Figs. 1 and 2). This is consistent with recent data from the laboratories of Rudensky and Chatila. Here, genetic models have been used to detect nonfunctional Tregs in BALB/c mice harboring a truncated version of Foxp3 (29) or in 129Sv/C57BL/6 mice where the Foxp3 gene was completely knocked out and replaced by GFP (30). Both studies also indicated that, in those genetic models, thymic selection of Tregs is independent of functional Foxp3 expression (29). IPEX disease, the human equivalent of sf disease, has an identical etiology, and IPEX pathogenesis can also be explained by mutations affecting the transcription factor Foxp3 (6, 8). However, autoimmune phenotypes of affected patients vary depending on the site of mutation and possibly additional environmental factors (8). Given the different outcomes of IPEX disease in humans depending on different sites and forms of mutations, we found it intriguing to study nonfunctional sf Tregs in sf mice. In the recently published study of Lin et al. (29), exon 11 was knocked out, resulting in expression of a stable Foxp3 protein, lacking the C-terminal forkhead domain. The truncated Foxp3 protein was nonfunctional, since the forkhead domain drives the translocation of the protein to the nucleus and enables DNA binding. Nevertheless, dominant-negative effects of the truncated protein could not completely be ruled out (29), a fact that gained importance after Ono et al. published that an N-terminal region of the Foxp3 protein is needed to bind AML1/Runx1 (41). Without this interaction there is an abrogation of the energetic state of the Tregs and attenuation of their suppressive capacity (34, 41, 42). Additionally, the N-terminal half of the protein has been shown in human cells to be necessary and sufficient for Foxp3-mediated suppression of a NF-AT-inducible luciferase reporter (43). Therefore, it cannot be excluded that the truncated protein could still bind AML1/Runx1 and directly compete with NF-AT binding, of which the latter is thought to be at least one of the mechanisms resulting in inhibition of activation-induced cytokine expression (42). Furthermore, a forkhead box-independent interaction between Foxp3 and the Th17 driving transcription factor RORγT has been published recently. In the presence of RORγT, a truncated version of Foxp3 lacking the forkhead box was detectable in the nucleus (44).

As expected, Foxp3-mutated sf Tregs did not suppress in an in vitro suppression assay (Fig. 3A). Interestingly, we nevertheless detected all bona fide Treg markers tested by RT-PCR (Fig. 3B). We asked whether the lack of suppressive capacity of GFP<sup>+</sup>Foxp3<sup>-</sup> sf Tregs could be explained by changes in the protein expression levels of known suppression-related molecules such as CTLA-4 or GITR. CTLA-4 has been suggested to be an essential molecule for Treg-mediated suppression, and the knock-out of CTLA-4 results in a phenotype comparable to the sf mouse (45–47). GITR interaction with its ligand GITR-L on accessory cells has been linked to induction of regulatory properties and proliferation of plasmacytoid dendritic cells (48). While Foxp3, as expected, was not detectable on the protein level (Figs. 1 and 3C), GITR expression patterns were not altered in sf Tregs compared with WT Tregs and CTLA-4 was down-regulated only in a minor subset of sf Tregs, probably a consequence of high proliferation and T cell activation (49). CTLA-4 expression was not altered in female Foxp3-deficient Tregs (Fig. 3C and supplemental Fig. 1B), indicating that the expression of both molecules is not solely dependent on Foxp3 expression and, furthermore, is not able to provide suppressive activity when Foxp3 is absent. The surface molecule CD103 is a marker for effector/memory-like Tregs (50, 51), and analysis of this adhesion molecule showed a direct dependency of CD103 on Foxp3 expression. Female sf × DEREG mice stained negative for CD103 in all GFP<sup>+</sup>Foxp3<sup>-</sup> sf Tregs, whereas the Foxp3-competent Tregs of the same mice had normal CD103 expression (supplemental Fig. 1). This is in accordance with the recent finding of Marson et al., showing a direct binding of Foxp3 to the CD103 promoter (52). However, loss of suppressive capacity of sf Tregs cannot simply be explained by loss of CD103 expression, since Tregs from CD103<sup>-/-</sup> mice display a comparable suppressive capacity as do their WT counterparts (53). Furthermore, Foxp3-dependent CD103 expression can be overcome by overt inflammation since CD103 expression could be recovered in male sf mice (Fig. 3, B and C).

Recently, intracellular cAMP has been shown to be highly abundant in Tregs and its delivery into responder cells essentially contributes to the suppressive capacity of Tregs (33). As shown in Fig. 3D, intracellular CAMP levels in sf Tregs are significantly reduced when compared with WT Tregs. It will have to be addressed in future studies if intracellular CAMP levels are directly dependent on Foxp3 protein expression, as could be shown recently for Pde3b<sup>+</sup>, one potential CAMP-degrading enzyme (30). As assessed by real-time PCR, Pde3b was highly expressed in sf Tregs but almost absent in DEREG Tregs (Fig. 3D, inset). Hence, the substantial reduction of CAMP levels in sf Tregs might be one reason for the loss of suppressive capacity.

Would-be Tregs in female heterozygous sf mice were less abundant than their Foxp3-competent counterparts (supplemental Fig. 1), indicating that Foxp3 might influence survival or homeostasis of Tregs. Using quantitative RT-PCR and FACs analysis (Figs. 2 and 3C), we demonstrated here that sf Tregs fail to up-regulate the high-affinity IL-2 receptor chain (CD25), consistent with the finding that Foxp3 induces CD25 expression (52). Consequently, the survival disadvantage of would-be Tregs in healthy female sf mice could be due to decreased signaling of IL-2, a cytokine known to be crucial for the homeostasis of Tregs (52, 54–56). This effect might play a negligible role in sf male mice suffering from severe autoimmunity, since it has been shown that the highly activated status in those mice leads to elevated IL-2 expression levels (12).

We next asked the question whether nonsuppressive sf Tregs might have acquired effector functions. The classical sf phenotype has been primarily attributed to the increased production of Th2 cytokines and typical Th2-type pathology (12–14). Regarding the cytokine expression profiles, the most striking differences between GFP<sup>+</sup> sf Tregs and GFP<sup>+</sup> WT Tregs were observed for the production of Th2-type cytokines, such as IL-4, IL-13, and IL-10, which were markedly up-regulated in sf Tregs. In earlier studies, the Th2-type cytokine expression in sf mice has already been linked to CD4<sup>+</sup>CD25<sup>+</sup> T cells, which under inflammatory conditions mainly comprised activated effector T cells (4). Here, using Foxp3-GFP-reporter mice crossed with sf mice, the enhanced cytokine expression could for the first time be detected in both GFP<sup>+</sup> sf Tregs and GFP<sup>+</sup> effector T cells. Dysregulation of Th2-type cytokines in the latter cell type can be best explained by the absence of functional Foxp3<sup>+</sup> Tregs. The absence of IL-4<sup>-</sup> cells in Treg-depleted cultures from DEREG mice clearly shows that elevated IL-4 production is not directly dependent on the absence of Foxp3<sup>+</sup> cells within the cultures. In sf Tregs, however, expression of Th2 cytokines might be affected more directly by the lack of Foxp3, since Foxp3 has been reported to negatively influence IL-10 and IL-4 production by direct binding to their promoter or by binding to the transactivator NF-AT (29, 42, 57–59). Interestingly, as shown by RT-PCR, galec-1 appeared to be expressed independently of Foxp3 since it was highly up-regulated on sf T cells (Fig. 3B). This elevated expression might have a compensatory effect because of the high T cell activation in these mice and could even play a deleterious role. It has been shown that galec-1 can induce apoptosis in Th1 and Th17 cells, which could
result in a bias toward Th2-type immunity (60). Furthermore, we observed both on the mRNA (supplemental Fig. 2) and protein level (Fig. 4C) increased levels of the master regulator of Th2 cells, GATA-3. Interestingly, IL-17, a cytokine augmented in various autoimmune diseases, was not elevated in supernatants of sf T cell cultures. This is in contrast to the model by Rudensky and colleagues, who showed elevated IL-17 levels in would-be Tregs (30). RT-PCR data revealed a reduction of mRNA of the major Th17 transcription factor in both GFP+ and GFP− sf T cells. These findings are in accordance with a previously published report, even though underlying mechanisms are yet unclear (61). It appears that when Foxp3 is mutated, cells that are prone to produce high amounts of cytokines are unleashed, mostly affecting those showing a Th2 phenotype. Importantly, the Th2 environment of male sf mice did affect, but not completely prevent, generation of Foxp3+CD4+ T cells into Th2 effector cells has been shown to be dependent on CD28/B7 costimulation (62, 63). Since CD28−/− sf mice show a substantially prolonged lifespan and significantly less cytokine production when compared with sf mice with same level of lymphoproliferation (64), it is conceivable that the observed Th2 shift in sf CD4+ T cells is an essential factor for disease development. Also, for the human form of the disease, IPEX, high correlations between disease progression and increased IgE and eosinophilia could be observed (8). In vitro proliferation studies using Ki-67 staining, we could observe that exogenous IL-2 was dispensable for a basal proliferation of sf Tregs that were not anergic, and that additional IL-2 promoted proliferation to a much higher extent (Fig. 4D). Accumulation of sf Tregs in affected organs may reflect rather increased proliferation and migration toward the site of inflammation than the cause of autoimmune pathology. This hypothesis is further strengthened by the fact that the adoptive transfer of sf Tregs did not cause sf-like multiorgan inflammatory disease, while sf CD4 effector cells led to massive lymphocyte infiltration and tissue damage of all observed organs within 8 wk (Fig. 6). In summary, we conclude that Foxp3 mutated would-be sf Tregs can be found in sf mice but appear to be neither the initiators nor the major contributors mediating autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary data

Supplementary figure 1:

(A) Heterozygous sf x DEREG females harbour small percentages of Foxp3-mutated Tregs.

LN and spleen cells from sf x DEREG and DEREG mice were harvested and stained for Foxp3. Plots show expression of Foxp3 and GFP among live gated cells. Small populations of Foxp3^−GFP^+ cells could be detected in sf x DEREG females, whereas in DEREG females, all GFP^+ cells stained also positive for Foxp3.

(B) Differential control of bona fide Treg markers.

Sf x DEREG LN cells were gated on Foxp3^+GFP^+ cells (gray filled histograms) or Foxp3^−GFP^+ cells (thick black open histograms) to compare protein expression by sf Tregs and WT Tregs from one donor. Isotype controls are shown by thin black histograms.

Plots are representative for three independent measurements.

Supplementary Figure 2:

Gata-3 and rorc are differentially expressed in sf x DEREG T cells and DEREG T cells.

GFP^+CD4^+ and GFP^−CD4^+ cells were sorted from sf x DEREG and DEREG mice and cDNA was prepared from the purified populations. Bars show expression of the indicated transcription factors normalized to hprt and further to DEREG Tregs. Gata-3 was significantly upregulated in both cell populations from sf x DEREG mice when compared
to DEREG mice. In contrast, rorc expression was absent in sf x DEREG cells. Measurements were performed twice.

Supplementary figure 3:

(A) Foxp3 deficient Tregs infiltrate affected organs in sf mice.

Ears from sf x DEREG and DEREG mice were digested with 1 mg/ml collagenase D for 1 hour. Cell suspensions were stained for CD4 to monitor cell infiltration by effector T cells and Tregs. Plots show live gated cells of one out of two independent experiments.

(B) Ears from sf x DEREG mice were stained histologically with DAPI (blue), αCD3 (red, clone 500A2, secondary antibody is anti-Syrian hamster-Cy3) and αGFP (Alexa488, green) (magnification x 100), confirming high T cell infiltration of affected organs in sf mice, including GFP⁺ Foxp3-mutated cells.