T CELLS

Regulatory T cells in skin are uniquely poised to suppress profibrotic immune responses

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At the center of fibrosing diseases is the aberrant activation of tissue fibroblasts. The cellular and molecular mechanisms of how the immune system augments fibroblast activation have been described; however, little is known about how the immune system controls fibroblast function in tissues. Here, we identify regulatory T cells (Tregs) as important regulators of fibroblast activation in skin. Bulk cell and single-cell analysis of Tregs in murine skin and lungs revealed that Tregs in skin are transcriptionally distinct and skewed toward T helper 2 (Th2) differentiation. When compared with Tregs in lung, skin Tregs preferentially expressed high levels of GATA3, the master Th2 transcription factor. Genes regulated by GATA3 were highly enriched in skin “Th2 Treg” subsets. In functional experiments, Treg depletion resulted in a preferential increase in Th2 cytokine production in skin. Both acute depletion and chronic reduction of Tregs resulted in spontaneous skin fibroblast activation, profibrotic gene expression, and dermal fibrosis, all of which were exacerbated in a bleomycin-induced murine model of skin sclerosis. Lineage-specific deletion of Gata3 in Tregs resulted in an exacerbation of Th2 cytokine secretion that was preferential to skin, resulting in enhanced fibroblast activation and dermal fibrosis. Together, we demonstrate that Tregs play a critical role in regulating fibroblast activation in skin and do so by expressing a unique tissue-restricted transcriptional program that is mediated, at least in part, by GATA3.

INTRODUCTION

Fibroblast activation is a key event in tissue remodeling after injury. However, excessive deposition of extracellular matrix (ECM) components by activated fibroblasts leads to tissue fibrosis, a hallmark of several life-threatening diseases, including systemic sclerosis, idiopathic pulmonary fibrosis, and end-stage liver disease (1). It is becoming increasingly evident that the immune system plays a major role in the activation of quiescent fibroblasts to ECM-producing myofibroblasts (2). Effector T cell (Teff) production of the T helper 2 (Th2) cytokines, interleukin-4 (IL-4) and IL-13, drives fibroblast activation via signaling through their cognate receptors expressed on fibroblasts and/or on tissue myeloid cells, resulting in increased ECM deposition (3–5). Expression of transforming growth factor-β (TGF-β) by multiple immune and nonimmune cell types directly induces myofibroblast differentiation and drives the progression of tissue fibrosis (6).

A major function of the immune system is to regulate inflammation in tissues. Defined populations of regulatory cells reside in nonlymphoid organs and act to mitigate tissue damage, both in the steady state and after injury. Regulatory T cells (Tregs) are the best characterized regulatory cell subset and are defined by the expression of the X-linked transcription factor FoxP3 (7, 8). Tregs, have a predilection to stably reside in barrier organs such as the gastrointestinal tract, lungs, and skin (7–11). In these locations, they use tissue-specific mechanisms to control inflammatory responses (12). In addition, these Tregs play major roles in mediating tissue-specific functions, such as muscle regeneration (13), lipid and glucose metabolism (14), hair follicle cycling (11), and epidermal repair (15). Although the mechanisms by which the immune system activates fibroblasts have been extensively studied, it is currently unknown whether the regulatory arm of the immune system can control these cells and play a role in attenuating tissue fibrosis. Furthermore, whether these mechanisms differ in specific tissues remains to be elucidated. Tregs have been shown to secrete the profibrotic cytokine TGF-β (6); however, these cells are also capable of suppressing the production of profibrogenic cytokines, such as IL-4 and IL-13 (16). These mechanisms appear to be both tissue and context dependent (17–20). Thus, despite our increasing understanding of how Tregs function, it is currently unclear as to whether the net effect of these cells is to augment or attenuate tissue fibrosis. In addition, it is currently unclear how tissue-specific transcriptomes in Tregs inform fibrosis biology. In the current study, we show that Tregs in skin are uniquely differentiated to attenuate dermal fibroblast activation and skin fibrosis.

RESULTS

Single-cell transcriptional analysis of Tregs in murine skin reveals a predominance of “type 2” Tregs

It is becoming increasingly appreciated that tissue-resident Tregs are composed of multiple subsets that have unique mechanisms of action that largely depend on the tissues in which they reside (12). To begin to better understand the functional heterogeneity of Tregs in skin, we performed bulk cell and single-cell RNA sequencing (scRNAseq) of Tregs isolated from murine skin during the telogen phase of the hair follicle cycle (11). For bulk cell analysis, we performed whole transcriptome RNAseq on live CD45+CD3+CD4+CD25+FoxP3-GFP+ cells that were sort-purified from skin and skin-draining lymph nodes (SDLNs) of FoxP3-GFP reporter mice (21). Differential
gene expression analysis of Treg in skin versus Treg in SDLNs revealed that Treg in skin express relatively high levels of TGFβ-associated genes (Fig. 1A). Genes associated with TGFβ2 differentiation that were highly expressed in skin Treg include the master TGFβ2 transcription factor Gata3 (22) and the TGFβ2-associated transcription factor Irf4 (Fig. 1A) (23). Ingenuity Pathway Analysis revealed that Treg in skin express significantly higher levels of genes associated with TGFβ2 differentiation when compared with Treg in SDLNs (ratio = 40:150; 0.267), with a z score of 1.616 (indicating activation of the pathway) and a Benjamini-Hochberg false discovery rate (FDR)–corrected P value of 5.74 × 10^{-14} (Fig. 1B). Gene set enrichment analysis (GSEA) (24, 25) revealed that a significant number of genes differentially expressed in skin Treg versus SDLNs Treg are regulated by GATA3 (26), with a P value of <0.0001 and FDR of <0.0001 (Fig. 1C and table S1). In an attempt to elucidate whether TGFβ2-associated genes were expressed by all Treg in skin or restricted to a specific subset, we sort-purified Treg from murine skin (using the same strategy as outlined above) and performed scRNAseq using the 10X Genomics platform (27). Data displayed as a dimensionally reduced t-SNE (t-distributed stochastic neighbor embedding) plot revealed skin Treg clustering in seven transcriptionally distinct subsets (Fig. 1D). A heatmap of the top five differentially expressed genes defining each cluster is shown in Fig. 1E. High levels of Gata3 expression were observed across all subsets (Fig. 1F). In contrast, transcription factors that define other Treg subsets, such as Rorc [Tbet; (28)] and Tbx21 [Tbet; (29)], were not expressed at appreciable levels in any of the skin Treg clusters (Fig. 1F). The TGFβ2-associated transcription factor Irf4 that was observed to be differentially expressed between Treg in skin and Treg in SDLNs at the bulk cell “population” level (Fig. 1A) showed a similar expression pattern to Gata3 at the single-cell level. However, Irf4 expression was detected in fewer cells when compared with Gata3 at the single-cell level (Fig. 1F). Genes known to be regulated by GATA3 (i.e., Fos, Il1r1, Ero1l, Maf, and Nfkbi2) (26) showed similar levels of expression and a similar expression pattern across skin Treg clusters when compared with Gata3 (Fig. 1, F and G). Areg (amphiregulin), a gene shown to be regulated by GATA3 and involved in imparting Treg with the capacity for tissue repair (27), was enriched in cluster 3. To directly compare Treg from lung to elucidate whether TH2-associated genes were expressed by all of these cells may be more poised to regulate Tgfβ2 immune responses relative to Treg in lung.  

### Treg depletion results in increased TGFβ2 cytokine production in skin

Treg express transcription factors and chemokine receptors that overlap with those of the effector cells that are most poised to suppress, without expressing appreciable levels of the respective effector cytokines (20, 31–33). Transcription factor analysis of T effector cells (CD4+, Foxp3+) revealed that TGFβ2 cells were enriched in skin compared with lung (fig. S3). Because Treg in skin are predominantly TGFβ2 skewed, we asked whether these cells were most poised to suppress type 2 immune responses in this tissue. Diphtheria toxin (DT) was administered to Foxp3DTR mice (34) to deplete Treg and 5 days later, cytokine production from skin and lung CD4+ T cells was quantified by flow cytometry. In these experiments, equivalent levels of Treg depletion were observed in skin and lung (fig. S4A). Treg depletion resulted in a significantly increased accumulation of IL-13– and IL-4–producing TGFβ2 cells in skin with no appreciable increase in interferon-γ (IFN-γ)–producing TGFβ1 cells or IL-17–producing TGFβ17 cells (Fig. 2A). In contrast, Treg depletion resulted in modest increases in TGFβ1, TGFβ2, and TGFβ17 cells in lung during the same 5-day time period (Fig. 1A). In lung, increases in TGFβ1 and TGFβ17 cells were greater than increases in TGFβ2 cells after Treg depletion (Fig. 2A). No statistically significant differences were observed in myeloid cell populations (i.e., neutrophils, eosinophils, monocytes, and inflammatory macrophages) in either skin or lung of Treg-depleted mice in this 5-day time period (fig. S4, B to E). Flow cytometric staining for intracellular cytokines is limited to quantifying cytokine production from T cells. Thus, we wanted to take a more holistic approach to determine how Treg depletion influenced cytokine gene expression at the whole-tissue level. To do so, we treated Foxp3DTR mice or wild-type (WT) control mice with DT for 7 days and harvested RNA from full-thickness dorsal skin and whole lung tissue for Qiagen cytokine quantitative polymerase chain reaction (qPCR) array analysis. Treg depletion resulted in increased expression of RNA for several cytokines; however, there was a notable preferential increase in IL13 expression (>20 log2-fold change) in skin when compared with all other cytokines (Fig. 2B). Heightened IL13 expression was not observed in lung tissue (Fig. 2B). Thus, both at the protein level in T cells and at the RNA level in whole tissue, our results suggest that Treg preferentially regulate TGFβ2 immune responses (particularly IL-13) in skin in the steady state.  

### Acute depletion of Treg results in dermal fibroblast activation and probiotic gene expression in skin

An emerging body of literature suggests that qualitative and/or quantitative defects in Treg are associated with chronic tissue fibrosing diseases such as hepatic fibrosis (35) and systemic sclerosis (36–39)

Fig. 1. Bulk cell and single-cell analysis of T\(_{reg}\) in murine skin reveals a skewing toward T\(_{H2}\) differentiation. (A to C) FoxP3-GFP\(^{+}\)CD25\(^{+}\)T\(_{reg}\) were sorted from SDLNs and skin of FoxP3\(_{GFP}\) reporter mice for whole transcriptome RNAseq. (A) Volcano plot comparing expression profiles of skin versus SDLN T\(_{reg}\). (B) Ingenuity Pathway Analysis of differentially expressed genes between skin and SDLN T\(_{reg}\). (C) GSEA of Gata3-regulated genes in skin and SDLN T\(_{reg}\). (D to G) FoxP3-GFP\(^{+}\)CD25\(^{+}\)T\(_{reg}\) were sort-purified from skin of FoxP3\(_{GFP}\) reporter mice for scRNAseq using the 10X Genomics platform. (D) t-SNE plot of skin T\(_{reg}\). (E) Heatmap with genes enriched in each skin T\(_{reg}\) cluster. (F) Feature plot of specific transcription factors and (G) genes regulated by Gata3. (H) Combined clustering t-SNE plot of skin and lung T\(_{reg}\). (I) Feature plot of specific transcription factors and (J) genes regulated by Gata3 in combined t-SNE plot of skin and lung T\(_{reg}\). (K) Representative contour plots and flow cytometric quantification of GATA3 expression in T\(_{reg}\) isolated from SDLNs, skin, and lungs of healthy WT mice. Cells are pregated on live CD45\(^{+}\)CD3\(^{+}\)CD4\(^{+}\)T cells. MFI, mean fluorescence intensity. Data are representative of three independent experiments, \(n = 3\) to 5 mice per group, per experiment. Data are mean ± SEM. One-way ANOVA, *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\); ****\(P < 0.0001\); ns, not significant.
SMA-RFP reporter mice to activation. To test this, we crossed highly active in profibrogenic myofibroblasts (42, 43). The resultant αSMA-RFP/FoxP3ΔTR mice have red fluorescent protein (RFP) expressed under the control of the α smooth muscle actin (αSMA) promoter, a promoter that is highly active in profibrogenic myofibroblasts (42, 43). Tregs were depleted in these mice, and αSMA-expressing dermal myofibroblasts were quantified by histology and flow cytometry (Fig. 3A). Consistent with our hypothesis, we observed an increase in αSMA-expressing dermal myofibroblasts with time after Treg depletion by immunohistochemistry (Fig. 3B). Flow cytometric quantification of αSMA-expressing myofibroblasts in skin corroborated these results with about fivefold increase in accumulation of these cells 5 days after depleting Tregs (Fig. 3C and fig. S5A). To comprehensively elucidate the role of Tregs in regulating fibroblast activation in the steady state, we performed 96-gene quantitative reverse transcription PCR (qRT-PCR) “fibrosis pathway” arrays on whole skin tissue harvested from FoxP3ΔTR mice and control mice 5 days after beginning DT treatment. Compared with WT mice, Treg-depleted mice had a marked increase in profibrotic genes with minimal changes in antifibrotic genes (Fig. 3D). In addition, Treg depletion resulted in heightened expression of many genes in the TGF-β pathway (Fig. 3D). Together, these results suggest that Tregs attenuate fibroblast activation and fibrosis-associated gene expression in skin in the steady state.

Chronic reduction of Tregs results in skin fibrosis

In the Treg depletion experiments described above (Fig. 3), Tregs were completely ablated in an attempt to definitively establish their role in regulating fibroblast activation in skin. Because mice completely devoid of Tregs succumb to multiorgan autoimmunity ~15 to 20 days after Treg depletion (34), they do not live long enough to develop tissue fibrosis. Thus, we set out to determine whether chronic reduction of Tregs results in persistent fibroblast activation and skin fibrosis. To do so, we took advantage of the fact that FoxP3 is expressed on the X chromosome (44). Because FoxP3ΔTR mice were generated by “knocking-in” the DTR allele into the endogenous FoxP3 locus (34), female mice that are heterozygous for the DTR allele (i.e., FoxP3-DTR+/−) have one copy of this transgene and one normal endogenous copy of FoxP3. Because of random X-chromosome inactivation, about 50% of Tregs in female FoxP3-DTR+/− mice are susceptible to DT-mediated depletion, whereas the remaining 50% are impervious to this treatment. To determine whether Tregs could be chronically reduced in this model, we treated female FoxP3-DTR+/− mice or WT mice with DT three times per week for 4 weeks and quantified the percentages and absolute numbers of Tregs in skin and SDLNs. Female FoxP3-DTR+/− mice treated with DT showed a ~50% reduction in Tregs in skin (fig. S6A). To determine whether chronic reduction of Tregs results in fibroblast activation and skin fibrosis, we compared the dorsal skin of female FoxP3-DTR+/− mice with female αSMA-RFP/FoxP3-DTR+/− mice treated with female αSMA-RFP/FoxP3-DTR+/− mice control mice 28 days after initiating DT treatment (Fig. 4A). Consistent with our results after acute depletion of Tregs, chronic reduction of Tregs in αSMA-RFP/FoxP3-DTR+/− mice resulted in a significant accumulation of αSMA-expressing dermal myofibroblasts, as quantified by flow cytometry (Fig. 4B) and immunohistochemistry (Fig. 4C). In addition, chronic Treg reduction resulted in increased dermal collagen density (Fig. 4D) and significantly increased dermal thickening, with a significant reduction in subcutaneous dermal adipose tissue (Fig. 4E). qRT-PCR fibrosis pathway analysis showed a very similar pattern to that observed in experiments where Tregs were completely depleted in the acute setting, with increased expression of profibrotic genes and a reduction in antifibrotic genes (Fig. 4F). In addition, chronic Treg reduction resulted in increased expression of several genes in the

![Fig. 2] Tregs preferentially regulate Th2 cytokines in skin. C57/B6 (WT) or FoxP3ΔTR+/− (FoxP3ΔTR) mice (homozygous for the FoxP3ΔTR transgene) were treated with DT every 2 days, and skin and lung tissues were harvested on day 5. Cells from skin and lung from WT (black circles) and FoxP3ΔTR mice (red circles) were stimulated with PMA/ionomycin, and intracellular cytokine production was quantified by flow cytometry. (A) Representative flow cytometric plot for each cytokine tested and quantification shown below. (B) qRT-qPCR for cytokine genes using Qiagen RT2 Profiler Arrays on mRNA isolated from whole skin or lung of WT or FoxP3 DTR mice 7 days after beginning treatment with DT with three replicates each. Log2-fold change in expression levels of cytokine genes with a statistical significance of P < 0.05 is shown.

TGF-β pathway as well as IL-13 and IL-5 (Fig. 4F). Together, these results further support a major role for T<sub>reg</sub> in regulating dermal fibroblast activation and skin fibrosis in the steady state.

**T<sub>reg</sub> attenuate bleomycin-induced skin fibrosis**

The experiments described above suggest a major role for T<sub>reg</sub> in regulating fibroblast activation in skin in the absence of tissue injury or inciting stimuli. We hypothesized that T<sub>reg</sub> mediate these effects indirectly by preferentially regulating T<sub>H</sub>2 cytokine production in skin. T<sub>H</sub>2 immune responses have been shown to play critical roles in driving bleomycin-induced skin fibrosis in mice (45–48). Thus, we set out to determine whether T<sub>reg</sub> play a role in attenuating fibrosis in this model. To do so, we used the T<sub>reg</sub> reduction approach in female αSMA-RFP/FoxP3-DTR<sup>−/+</sup> mice as described above (Fig. 4). Female αSMA-RFP/FoxP3-DTR<sup>−/+</sup> mice were treated with daily subcutaneous injections of bleomycin and given DT every 3 days for 14 days to reduce T<sub>reg</sub> numbers but not completely deplete these cells (see above and fig. S3A). As controls, female αSMA-RFP/FoxP3-DTR<sup>+/−</sup> mice were treated with bleomycin but not T<sub>reg</sub>-depleted (i.e., not treated with DT), and female αSMA-RFP mice were treated with DT but not given bleomycin. Compared with DT-treated αSMA-RFP mice, bleomycin-treated αSMA-RFP/FoxP3-DTR<sup>−/+</sup> mice had significantly higher intensity of αSMA staining (Fig. 5A), increased collagen density (Fig. 5B), and increased dermal thickening with reduced dermal fat (Fig. 5C), with enhanced accumulation of αSMA-expressing myofibroblasts (Fig. 5D). These results indicate that skin fibrosis is readily apparent after 14 days of bleomycin treatment and that DT treatment alone does not induce fibroblast activation or fibrosis. Consistent with results when T<sub>reg</sub> were reduced in the steady state, T<sub>reg</sub> reduction in bleomycin-treated αSMA-RFP/FoxP3-DTR<sup>−/+</sup> mice resulted in significantly augmented fibrosis, with increased αSMA expression, collagen density, dermal thickness, and myofibroblast accumulation (Fig. 5, A to D). Together, these results indicate that T<sub>reg</sub> play a role in attenuating bleomycin-induced skin fibrosis.

**T<sub>reg</sub> expression of GATA3 plays a role in suppressing fibroblast activation and dermal fibrosis**

GATA3 has been coined the master regulator of T<sub>H</sub>2 differentiation (21). Skin T<sub>reg</sub> preferentially express high levels of GATA3, and these T<sub>reg</sub> are significantly enriched in genes regulated by this transcription factor (Fig. 1). Thus, we hypothesized that expression of GATA3 is responsible for mediating the antifibrotic effects of T<sub>reg</sub> in skin. To test this, we crossed FoxP3<sup>CreERT2</sup> mice (17) with Gata3<sup>fl/fl</sup> (49) mice to generate FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> animals that have inducible and selective deletion of Gata3 in T<sub>reg</sub> after administration of tamoxifen. FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> mice were treated with tamoxifen every 3 days starting on the same day as the initiation of bleomycin treatment. As controls, FoxP3<sup>CreERT2</sup> mice were treated with bleomycin and tamoxifen. In addition, in some experiments, FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> were left untreated (i.e., were not given tamoxifen or bleomycin) to control for induction of fibrosis. Administration of tamoxifen and bleomycin in FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> mice resulted in a significant reduction of GATA3 in skin T<sub>reg</sub> relative to controls (Fig. 6A). This relatively short-term deletion of Gata3 in T<sub>reg</sub> did not result in reduced T<sub>reg</sub> numbers in skin, SDLNs, or lung (Fig. 6B). In addition, deletion of Gata3 in T<sub>reg</sub> did not affect their activation and stability, as measured by the mean fluorescence intensity of FoxP3, CD25, and CTLA4 over the 14-day treatment period (fig. S7, A to C). Deletion of Gata3 in T<sub>reg</sub> resulted in a significant increase in the percentage of IL-13− and IL-4− producing T<sub>H</sub>2 cells in skin, with no change in...
the percentage of IFN-γ– and IL-17–producing T effs (Fig. 6C). There was also a trend toward increased IL-13 production from ILC2s (type 2 innate lymphoid cells) in skin upon Gata3 deletion in T regs (fig. S7, D and E). Consistent with the differential expression of GATA3 between skin-resident and lung-resident Tregs (Fig. 1, I and K), deletion of Gata3 in T regs resulted in approximately 80% increase in the percentage of T H 2 cells (as defined by T H 2 cytokine production) in skin, with no increase in T H 2 cells observed in lung (Fig. 6D).

There was no difference in skin-infiltrating proinflammatory macrophages or neutrophils in mice deficient in Gata3-expressing Tregs compared with controls (Fig. 6E and fig. S8A). Consistent with an increase in T H 2 cells, FoxP3CreERT2Gata3fl/fl mice treated with both tamoxifen and bleomycin had increased dermal fibroblast activation, as evidenced by significantly augmented αSMA-expressing myofibroblasts when compared with tamoxifen- and bleomycin-treated FoxP3CreERT2 control mice (Fig. 6F). Collagen density and dermal thickness were also increased in these animals with a concomitant reduction in dermal adipose tissue (Fig. 6, G and H). Histologic examination of lung tissue harvested from these animals revealed no significant differences between any of the groups (fig. S9). Together, these results suggest that GATA3 mediates a transcriptional program in Tregs that confers these cells with the ability to preferentially suppress...
Fig. 5. Chronic T<sub>reg</sub> reduction exacerbates bleomycin-induced skin fibrosis. αSMA-RFP: FoxP3<sup>DTR<sup>−/−</sup> mice (heterozygous for the FoxP3<sup>DTR</sup> transgene) were treated with either bleomycin only or with bleomycin and DT for 14 days. αSMA-RFP mice treated with DT were used as baseline controls. (A) Representative αSMA staining of dorsal skin on day 14 and histologic quantification of αSMA staining intensity from control (αSMA-RFP + DT, black circles), αSMA-RFP: FoxP3<sup>DTR<sup>−/−</sup> treated with bleomycin (B) alone (gray circles), and αSMA-RFP: FoxP3<sup>DTR<sup>−/−</sup> treated with bleomycin and DT (red circles). (B) Representative Masson's trichrome staining of dorsal skin on day 14 and histologic quantification of collagen density. (C) Representative H&E stain on day 14 and quantification of dermal thickness. (D) Representative histograms and flow cytometric quantification of αSMA expression on dermal fibroblasts after T<sub>reg</sub> reduction. Data are representative of three independent experiments, n = 3 to 5 per group, per experiment. Data are mean ± SEM. One-way ANOVA, **P < 0.01, ***P < 0.001, ****P < 0.0001. Scale bars, 100 μm.
Fig. 6. T<sub>reg</sub> expression of Gata3 plays a major role in controlling dermal fibrosis. FoxP3<sup>CreERT2</sup> or FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> mice were treated with bleomycin (B) and tamoxifen (T) for 14 days. FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> treated with PBS (no bleomycin or tamoxifen) were used as baseline controls. (A) Representative contour plots and flow cytometric quantification of GATA3 expression in skin CD4<sup>+</sup> T cells on day 14. (B) Flow cytometric quantification of T<sub>reg</sub> (live CD45<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>) cells isolated from skin, SDLNs, and lungs on day 14. (C) Flow cytometric quantification of percent of cytokine (IL-17, IL-13, IL-4, and IFN-γ)–producing T<sub>eff</sub> (live CD45<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>−</sup>) after PMA/ionomycin stimulation for 4 hours from skin. (D) Fold change in the percentage of CD4<sup>+</sup> TH subsets from skin and lung of bleomycin- and tamoxifen-treated FoxP3<sup>CreERT2</sup>/Gata3<sup>fl/fl</sup> mice versus FoxP3<sup>CreERT2</sup> mice. (E) Percentage of neutrophils (CD45<sup>+</sup>MHCII<sup>−</sup>Ly6G<sup>+</sup>) and inflammatory macrophages (CD45<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup>). (F) Representative αSMA tissue staining of dorsal skin and histologic quantification of αSMA staining intensity on day 14. (G) Representative Masson’s trichrome staining and histologic quantification of collagen density. (H) Representative H&E staining and quantification of dermal thickness. Data are representative of two independent experiments, n = 4 to 7 per group, per experiment. Data are mean ± SEM. One-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Scale bars, 100 μm.
T Helper 2 immune responses and, in doing so, enables optimal regulation of dermal fibroblast activation and skin fibrosis.

We set out to determine whether suppression of T Helper 2 cytokines by skin regulatory T cells (Tregs) plays a role in their ability to regulate fibroblast activation and suppress skin fibrosis. In these experiments, we used FoxP3CreERT2Gata3fl/fl mice to acutely delete Gata3 in Tregs in our bleomycin model and determined whether blocking of T Helper 2 cytokines was capable of reversing the enhanced skin fibrosis observed in these animals. To block T Helper 2 cytokines, we used an anti–IL-4Rα (anti-CD124) antibody, which blocks both IL-4 and IL-13 signaling (50, 51). Isotype or anti-CD124 antibody was given intraperitoneally every 2 days along with tamoxifen to delete Gata3 in Tregs. Bleomycin was injected every other day intradermally for the duration of the study (14 days). In these experiments, we observed a partial but significant reduction in collagen density and dermal thickness in skin of mice receiving anti-CD124 antibody compared with isotype-treated control animals (fig. S10). We did not observe a reduction in αSMA staining. A possible explanation for the lack of a complete rescue in collagen density and dermal thickness with no change in αSMA staining could be from incomplete neutralization of IL-4 and IL-13 signaling using this pharmacologic approach. In addition, factors other than IL-4 and IL-13 (i.e., IL-5, IL-9, and/or IL-10) may contribute to enhanced fibrosis when Gata3 is deleted in Tregs. Nevertheless, these results indicate that suppression of T Helper 2 cytokines plays a role, at least in part, to the mechanisms by which GATA3-expressing Tregs regulate skin fibrosis.

**DISCUSSION**

In recent years, Tregs have emerged as a highly dynamic immune cell subset influencing the function of nonimmune cells in tissues (12). Our data suggest that Tregs are important mediators of myofibroblast accumulation in skin and chronic reduction of Treg predisposes to clinically evident fibrosis. Tregs control fibroblast activation in skin by adapting a unique transcriptional program, which enables them to suppress profibrotic immune responses. Co-opting T helper 2 transcriptional pathways to suppress specific immune responses is consistent with the unique functions of Tregs in tissues (19, 20).

Multiple lines of evidence suggest that tissue Tregs not only prevent autoimmunity but also are actively involved in tissue maintenance and repair (11, 12, 15). It has been shown previously that Treg, in skin and adipose tissue adopt a "T Helper 2 phenotype," which is identified by the expression of Il11r1 (ST2; IL-33 receptor) and Areg (30). Our data support these findings and expand on them, suggesting that although most Treg in skin express Gata3, Areg and Il11r1 are preferentially expressed on specific subsets (Fig. 1). This expression pattern is quite different than that observed in lung-resident Treg. Overall, our results suggest that Tregs in murine skin as an entire population are poised to regulate T Helper 2 immunity, and perhaps a subset of these cells are more differentiated toward having tissue reparative capacity.

The role of Treg in fibrosis has been controversial due to several conflicting reports about their relative accumulation in fibrotic tissues and their ability to produce the profibrotic cytokine TGF-β (39, 52). It has been suggested that unstable Treg accumulate in fibrotic tissues where they transdifferentiate into pathogenic T Helper 1/T Helper 17 cells (37). However, these studies did not go beyond correlating Treg frequencies with inflammatory disease. The ontogeny and functional capacity of these cells remain to be elucidated. Although Treg have been shown to make TGF-β in specific contexts, they produce less of this cytokine in skin based on our RNAseq analysis. However, skin Treg do express relatively high levels of several TGF-β receptors (10). Thus, we speculate that skin-resident Treg could function as "TGF-β sinks," acting to sequester this cytokine. In doing so, they would both prevent free TGF-β from activating fibroblasts and may use this cytokine to maintain and/or enhance their regulatory capacity.

In Treg, Gata3 partners with FoxP3 to form a positive feedback loop to increase transcription of Treg-related genes. Gata3 expression is important for maintaining the stability of Treg under inflammatory conditions by reinforcing FoxP3 expression in dividing Treg (53). Treg that lack Gata3 expression have been shown to be unstable and have the capacity to make IL-17A (54). We did not observe increased IL-17A production from Gata3-deficient Treg in our fibrosis studies, nor did we observe any change in Treg numbers. We also did not observe differences in expression levels of CD25, CTLA-4, or FoxP3 between control and Gata3-deficient Treg (fig. S5, A to C). This suggests that deletion of Gata3 in Treg did not result in "unstable" cells in our experiments. We speculate that this is most likely secondary to the inducible Cre-lox system used in our study, which deleted Gata3 in Treg for a relatively short period of time (~14 days). This time frame may be too short to observe the Treg instability seen in models where constitutive Gata3 deletion occurs in Treg throughout development and the entire lifespan of the animal. Nevertheless, our data support a model whereby Gata3 mediates a transcriptional program in Treg that acts to attenuate type 2 immune responses in skin independent of the role it plays in Treg stability.

Our findings suggest that quantitative or qualitative defects in Treg may predispose to initiation of the fibrotic cascade. We show that these cells use a distinct transcriptional program to regulate fibrosis in skin. However, this is most likely only one of many mechanisms used by Treg, including acting as TGF-β sinks, secretion of IL-10, and/or possibly direct interactions with fibroblasts to attenuate their activity. Further studies are required to discern the potential and relative roles of these and other pathways that Treg use to control fibroblast activation. Given the differences in Treg, resident in healthy skin and lung shown here, it is possible that these cells use different mechanisms to regulate fibroblasts depending on the tissue. When compared with lung-resident Treg, Treg in skin preferentially express Gata3 (Fig. 1). Thus, we speculate that Treg control of profibrotic T Helper 2 responses is relatively skin specific or Treg in lungs use a different mechanism to regulate T Helper 2 immunity. Recently, reduced Treg in the liver have been implicated in the pathogenesis of T Helper 2–mediated fibrosis of this organ (35), underscoring the idea that these cells most likely have multiple mechanisms to attenuate fibrosis in a tissue-dependent manner.

In conclusion, our study underscores the role of the regulatory arm of the immune system in the control of fibroblast activation and tissue fibrosis. Our results suggest that treatment modalities focused on augmenting the function of Treg in skin may have beneficial effects in treating fibrosis in this organ. In addition, in contrast to targeting a single antifibrotic or profibrotic pathway, we speculate that augmenting cell types that naturally suppress fibrosis will capitalize on the multiple mechanisms used by these cells to mediate their antifibrotic effects.

**MATERIALS AND METHODS**

**Mice**

WT C57BL/6, FoxP3GFP, FoxP3DTIR, αSMA-RFP, FoxP3YFPcre, and FoxP3CreERT2 mice were purchased from The Jackson Laboratory.
FoxP3\textsuperscript{DTR} (34) mice were crossed to αSMA-RFP mice (41) in house to generate FoxP3\textsuperscript{DTR}/αSMA-RFP mice. Gata3\textsuperscript{fl/fl} (49) mice were a gift from Z. Werb (University of California, San Francisco). We crossed FoxP3\textsuperscript{CreERT2} (17) to Gata3\textsuperscript{fl/fl} mice to generate FoxP3\textsuperscript{CreERT2}/Gata3\textsuperscript{fl/fl} mice in our mouse facility. Animal experiments were performed on 7- to 12-week-old mice. Mice were maintained through routine breeding at the University of California San Francisco (UCSF) School of Medicine in a specific pathogen--free facility. All animal experiments were performed in accordance with guidelines established by Laboratory Animal Resource Center at UCSF. All experimental plans and protocols were approved by Institutional Animal Care and Use Committee beforehand.

Administration of DT, bleomycin, anti-CD124 antibody, and tamoxifen

Tregs were depleted from FoxP3\textsuperscript{DTR} mice by intraperitoneal injection of DT (30) (50 mg/kg body weight; Sigma-Aldrich) every other day for 5 days (three injections). Tregs were reduced from FoxP3\textsuperscript{CreERT2}/Gata3\textsuperscript{fl/fl} (50 mg/kg body weight; Sigma-Aldrich) every other day for 5 days (three injections). Tregs were reduced from FoxP3\textsuperscript{CreERT2} mice by intraperitoneal injection of DT (30 mg/kg body weight; Sigma-Aldrich) three times per week for 4 weeks (12 injections). Tissues were harvested on the indicated days as described in Results. Mice were compared with age- and gender-matched DT-treated WT mice or non--DT-treated littersmates. In experiments using FoxP3\textsuperscript{CreERT2}/Gata3\textsuperscript{fl/fl} mice, Cre recombinase was activated with tamoxifen (Sigma-Aldrich; 2.5 mg intraperitoneally dissolved in corn oil) every 2 days for 14 days starting with concomitant injections of bleomycin (day 0). Mice were treated with bleomycin (Teva, NDC# 00703-3154-01) dissolved in phosphate-buffered saline (PBS; 1 U/ml) every day for 14 days subcutaneously starting on day 0. In certain experiments, mice were treated with 100 μg of anti-CD124 (anti–IL-4Rα) antibody (BD Pharmingen, clone mIL4R-M1) or isotype (IgG2a, BD Pharmingen, clone R-35-95) every 2 days for 14 days starting on day 0.

Cell preparation from tissues and stimulation for intracellular cytokine staining

Single-cell suspensions of SDLNs were mechanically dissociated through a 100-μm filter and 2.5-cm\textsuperscript{2} dorsal skin that was processed as previously described (55). Single cells were washed in tissue culture media and filtered. Cells were counted using an automated cell counter (NucleoCounter NC-200; ChemoMetec) to determine the absolute number of specific cell populations in skin by flow cytometry. Single cells (3 × 10\textsuperscript{6} to 4 × 10\textsuperscript{6}) were stained for flow cytometry or cultured for intracellular cytokine staining using a phorbol 12-myristate 13-acetate (PMA) and ionomycin cell stimulation cocktail (Tonbo Biosciences).

Flow cytometry

Single-cell suspensions prepared above were pelleted and incubated with anti-CD16/anti-CD32 (BD Biosciences; 2.4G2) in PBS. Cells were washed and stained with Ghost Viability dye (Tonbo Biosciences) in PBS. After a wash in PBS, cells were stained for surface markers in PBS. For intracellular staining, cells were fixed and permeabilized with a FoxP3 buffer set (eBioscience). Samples were run on a Fortessa analyzer (BD Biosciences) in the UCSF Flow Cytometry Core and collected using FACs Diva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (TreeStar). Fluorophore-conjugated antibodies specific for mouse surface and intracellular antigens were purchased from eBioscience, BD Biosciences, and BioLegend. The following antibodies and clones were used: anti-Ly6G (1A8), anti-CD11b (M1/70), anti-CD11c (HL3), anti-MHC (major histocompatibility complex) class II, (M5/114.15.2), anti-Ly6C (HK1.4), anti–IL-17 (TCA11-18H10.1), anti–IL-4 (11B11), anti–IL-13 (eBio13a), anti–IFN-γ (XMG1.2), anti-CD3 (145-2C11), anti-CD4 (RM4–5), anti–CD8 (SK1), anti–TCRγδ (T cell receptor γδ) (GL3), anti-Gata3 (TWAJ), IL-10 (JES3), anti–CD45 (30–F11), and anti–FoxP3 (FJK-16s).

RNAseq analysis and qRT-PCR array

For the analysis of fibrosis-related genes and chemokines, a 3-mm skin biopsy from mouse back skin was homogenized in tissue lyser (gentleMACS; Miltenyi Biotec). RNA was isolated with the RNeasy fibrous tissue kit (Qiagen) and used to synthesize complementary DNA (cDNA) with a first-strand synthesis kit (Qiagen). A mouse chemokine and cytokine array (Qiagen, RT\textsuperscript{2} Profiler PCR Array PAMM150-Z) was used to detect cytokine genes from back skin of mice. A mouse fibrosis array (Qiagen; RT\textsuperscript{2} Profiler PCR Array PAMM120-Z) was used in experiments to detect expression of fibrosis-related genes in skin. For RNAseq, Tregs were isolated by gating on live CD45\textsuperscript{+} CD3\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{+} CD25\textsuperscript{+} CD27\textsuperscript{hi} cells, which contained greater than 90% FoxP3-expressing Tregs from SDLNs and skin. Sorted cell populations were flash-frozen in liquid nitrogen and sent overnight on dry ice to Expression Analysis, Quintiles (Morrisville, NC). RNA samples were converted into cDNA libraries using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina). RNA was isolated for expression analysis using Qiagen RNaseq Spin Column and was quantified with a NanoDrop ND-8000 spectrophotometer. RNA quality was checked by Agilent Bioanalyzer Pico Chip. cDNA was created from 220 pg of input RNA with the SMARTer Ultra Low Input Kit and sequenced to a 25-million read depth with Illumina RNASeq. Reads were aligned to Ensembl mouse GRCm38.p4 reference genome with TopHat software (v. 2.0.12). SAM files were generated with SAMtools from alignment results. Read counts were obtained with htseq-count (0.6.1p1) with the union option. Differential expression was determined using the R/Bioconductor package DESeq2 (Ref 2). Differentially expressed genes between skin Tregs and SDLN Tregs were analyzed by Ingenuity Pathway Analysis. To assess GATA3-regulated genes in Tregs, a curated gene list was generated from published dataset (26) and GSEA was performed (25).

Single-cell RNA sequencing

Skin Tregs were prepared for scRNAseq by harvesting back skin from four C57BL/6 FoxP3\textsuperscript{GFP} female adult mice. Back skin was harvested, digested, and processed as detailed above. Flow cytometry was performed on the post-digested single-cell suspension, and Ghost dye ‘CD45+CD3+CD4+GFP’ cells were sorted to high purity.

Droplet-based scRNAseq

Immediately after sorting, Ghost dye ‘CD45+CD3+CD4+GFP’ skin cells were run on the 10X Chromium [10X Genomics (27)] platform, and library preparation was performed by the Institute for Human Genetics at UCSF following the recommended protocol for the Chromium Single Cell 3’ Reagent Kit (v2 Chemistry). Libraries were run on the HiSeq4000 for Illumina sequencing. Post-processing and quality control were performed by the Genomics Core Facility in the Institute for Human Genetics at UCSF using the 10X Cell Ranger package (v1.2.0, 10X Genomics). Reads were aligned to mm10 reference assembly (v1.2.0, 10X Genomics). Primary assessment using
this software showed 2058 median unique molecular identifiers (UMIs; transcripts) per cell, and 1035 median genes per cell were sequenced to 91.6% sequencing saturation with 88,229 mean reads per cell.

**Unsupervised clustering of scRNAseq data**

The Seurat R package (version 2.2) (56) was used for graph-based clustering and visualizations. All functions mentioned are from the Seurat R package (version 2.2) or the standard R version 3.4.2 package and were used with default parameters unless otherwise noted. Cells (unique barcodes) that passed quality control processing and expressed at least 200 genes and only genes that were expressed in three or more cells were analyzed. Cells with greater than 10% mitochondrial genes and greater than 30% ribosomal protein genes were removed from downstream analysis.

Library size normalization was applied to each cell with the NormalizeData function. Normalized expression for gene i in cell j was calculated by taking the natural log of the UMI counts for gene i in cell j divided by the total UMI counts in cell j multiplied by 10,000 and added to 1. To reduce the influence of variability in the number of UMIs, mitochondrial gene expression, and ribosomal gene expression between cells on the clustering, we used the Scale-Data function to linearly regress out these sources of variation before scaling and centering the data for dimensionality reduction. Principal component analysis was run using the RunPCA function on the variable genes calculated with the FindVariableGenes function. On the basis of the PCElbowPlot function result, we decided to use 19 principal components for clustering. We ran the FindClusters function to apply shared nearest neighbor (SNN) graph-based clustering (0.6 resolution) and used the FindAllMarkers function (Wilcoxon rank sum test, min.pct = 0.25, only.pois = True, thresh.use = 0.25) to identify small clusters of macrophages (Cd68) and fibroblasts (Col1a1) to be removed. The non-\( T_{reg} \) may have been collected because of doublets and/or sorting impurity. The remaining cells in each sample were then normalized and scaled as above. Log-normalized gene expression data were used for visualizations with \( t \)-SNE plots (FeaturePlot).

**Gene set enrichment analysis**

Data were used for visualizations with doublets and/or sorting impurity. The remaining cells in each sample were

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**Histology and immunofluorescence microscopy**

For histopathology, skin tissue was fixed in 10% formalin and paraffin-embedded. Tissue was stained with hematoxylin and eosin (H&E), Masson’s trichrome, and anti-αSMA by the UCSF Mouse Pathology Core. H&E quantifications of epidermal hyperplasia, collagen density, and αSMA intensity were performed using ImageJ64 software [National Institutes of Health (NIH), USA]. For quantification, three to five measurements per slide/sample for each mouse per group (\( n = 3 \) to 7) were taken, with every measurement plotted and the average of all measurements shown.

**Statistical analyses**

Statistical analyses were performed with Prism software package version 7.0 (GraphPad). \( P \) values were calculated using two-tailed unpaired Student’s \( t \)-test or one-way analysis of variance (ANOVA). Pilot experiments were used to determine sample size for animal experiments. No animals were excluded from analysis, unless due to technical errors. Mice were age- and gender-matched and randomly assigned into experimental groups. Appropriate statistical analyses were applied, assuming a normal sample distribution. All in vivo mouse experiments were conducted with at least two to three independent animal cohorts. RNAseq experiments were conducted using three to four biological samples. Data are reported as mean ± SEM.

\( P \) values correlate with symbols as follows: ns, not significant, \( P > 0.05 \), \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \), \( **** P < 0.0001 \).

**SUPPLEMENTARY MATERIALS**

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Fig. S1. scRNAseq of lung \( T_{reg} \) and comparison with skin \( T_{reg} \).

Fig S2. TH2-associated chemokine and cytokine receptors are preferentially enriched in skin compared with lung \( T_{reg} \).

Fig. S3. TH2 cells are enriched in skin.

Fig. S10. Blockade of TH2 cytokines partially rescues fibrosis observed in mice with inducible deletion in Tregs.

Fig. S9. No evidence of lung fibrosis after subcutaneous bleomycin injection and Gata3 deletion in \( T_{reg} \).

Table S1. Raw data Excel file.

**REFERENCES AND NOTES**


Acknowledgments: We thank the UCSF Parnassus Flow Cytometry Core, which is supported by Diabetes Research Center (DRC) grant NIH P30 DK063720, and the UCSF Mouse Pathology Core, which is supported by NIH SP30CA082103-15. Funding: This work was primarily funded by M.D.R.'s Scleroderma Research Foundation (SRF) grant. Additional grant support was provided by M.D.R.'s NIH K08-AR062064, NIH DP2-AR068130, Pfizer ASPIRE Grant W1229090, and NIH RO1-AR071944, and L.A.K.'s Frontiers in Medical Research Fellowship from California Foundation of Molecular Biology and QB3. A.H. was supported by a Dermatology Foundation Career Development Award. Author contributions: M.D.R. conceived the project and directed research. M.D.R. and L.A.K. designed the study and wrote the manuscript. L.A.K. performed the experiments and collected and analyzed the data. J.N.C., N.P., P.M.S., A.N.M., J.M.M., M.M.L., and A.N. assisted with experiments and data generation. P.J.W., A.H., and F.B. provided research input. Competing interests: M.D.R. is the founder of TRex Bio, a cofounder of Sitryx, and a consultant with Celgene. The other authors declare that they have no competing interests. Data and materials availability: RNAseq data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus and are available under accession number GSE136160. All other data needed to evaluate the conclusions of the paper are present in the paper and/or the Supplementary Materials.

Submitted 11 December 2018
Accepted 9 August 2019
Published 6 September 2019
10.1126/sciimmunol.aaw2910

Regulatory T cells in skin are uniquely poised to suppress profibrotic immune responses
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Skin Tregs rely on Gata3 to curb fibrosis
Hyperactive fibroblasts that overproduce extracellular matrix components are a root cause of tissue fibrosis, a shared feature of many chronic inflammatory diseases. Kalekar et al. found that depletion of regulatory T cells (Tregs) in mice resulted in increased skin fibrosis, prompting them to investigate the mechanisms by which skin-resident Tregs normally prevent excess fibroblast activation. RNA sequencing analysis of skin Tregs revealed preferential expression of transcription factors typically associated with Th2 differentiation, including GATA3 and IRF4. Genetic deletion of Gata3 in Tregs resulted in increases in Th2 cytokine production and excessive dermal fibrosis. These findings reveal that GATA3 expression by skin-resident Tregs is an adaptation to the cutaneous microenvironment that enables these specialized Tregs to maintain homeostatic control of fibroblast activity. See the related Focus by Bal and Stadhouders.

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