

Fli1 deficiency suppresses RALDH1 activity of dermal dendritic cells and related induction of regulatory T cells: a possible role in scleroderma

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Abstract

Background

Aldehyde dehydrogenase 1 (RALDH1)-producing dermal dendritic cells (DCs), a conventional DC subset regulating skin fibrosis, are decreased in the involved skin of patients with systemic sclerosis (SSc). In this study, we investigated the contribution of Fli1 deficiency, a potential predisposing factor of SSc, to the phenotypical alteration of RALDH1-producing dermal DCs by using SSc model mice and SSc skin samples.

Methods

Bleomycin (BLM)-induced skin fibrosis was generated with Fli1^{+/-} and wild-type mice. The proportions of DC and CD4⁺ T cell subsets were determined by flow cytometry in the dermis of BLM-treated mice. Fli1 expression in dermal DCs was evaluated by immunofluorescence with skin samples of SSc and healthy control subjects.

Results

RALDH activity of dermal DCs was significantly decreased in BLM-treated Fli1^{+/-} mice compared with BLM-treated wild-type mice, whereas the proportion of CD103-CD11b- dermal DCs, a major DC subset producing RALDH1 in response to BLM injection, was comparable between groups. Relevant to this finding, the proportion of regulatory T cells (Tregs) in the dermis was decreased in BLM-treated Fli1^{+/-} mice relative to BLM-treated wild-type mice, while the proportions of Th1, Th2 and Th17 cells were unaltered. In the involved skin of SSc patients, Fli1 was downregulated in CD11c⁺ cells, including dermal DCs.

Conclusions

Fli1 deficiency inhibits RALDH1 activity of CD103-CD11b- dermal DCs and related induction of Tregs in BLM-treated mice. Considering Fli1 reduction in SSc dermal DCs, Fli1 deficiency may impair the dermal DC-Treg system, contributing to the development of skin fibrosis in SSc.

Background

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vasculopathy and fibrosis of the skin and certain internal organs [1–3]. Although its etiology remains enigmatic, skin fibrosis is believed to be caused by the complex, but orchestrated interaction of interstitial fibroblasts with immune cells, vascular cells, keratinocytes and adipocytes [4–6]. Among immune cells, previous research attention has focused on B cells, T cells and macrophages in SSc [7]. Regarding dendritic cells (DCs), recent studies have revealed the contribution of plasmacytoid DCs (pDCs) to the development of SSc [8], but the role of other DC subsets, such as conventional DCs (cDCs) and monocyte-derived DCs, had remained unknown. However, our recent study identified aldehyde dehydrogenase 1 (RALDH1)-producing

CD103⁻CD11b⁻ cDCs as a critical DC subset regulating the development of inducible Foxp3⁺ regulatory T cells (Tregs) in BLM-treated mice and demonstrated decreased numbers of RALDH1-producing dermal DCs in SSc involved skin [9]. Since dermal DCs serve as a key mediator of skin immunity [10], further characterization of RALDH1-producing dermal DCs would enable us to better understand the pathogenesis of SSc.

In the skin, DCs and macrophages are the principal antigen presenting cells. Langerhans cells in the epidermis and cDCs in the dermis uptake internal and external antigens, migrate to lymph nodes, and present them to naïve T cells, thus serving as a bridge between innate and adaptive immunity. Under physiological conditions a fraction of cDCs undergoes homeostatic maturation, then transport cutaneous self-antigen to the T cell zones of draining lymph nodes and activate an abortive program of autoreactive T cells. In contrast, when encountering external antigen, cDCs experience terminal differentiation, migrate to draining lymph nodes and promote clonal expansion of naïve antigen-specific T cells and the acquisition of T cell effector functions. During this highly organized process, cDCs regulate Treg development from conventional naïve CD4⁺ T cells by producing retinoic acid [11]. Retinoic acid is generated from vitamin A through a sequential step tightly regulated by three enzymes, namely, alcohol dehydrogenase, retinol dehydrogenase and aldehyde dehydrogenase (RALDH). Although the former two enzymes are ubiquitously produced in various cell types, RALDH is exclusively expressed in certain cell types, including cDCs. Thus, RALDH-producing cDCs promote retinoic acid-dependent Treg development together with a submitogenic dose of antigen, low costimulation and high levels of transforming growth factor (TGF)- β [12–15]. In bleomycin (BLM)-induced skin fibrosis, CD11b⁻CD103⁻ dermal cDCs express RALDH1 and promote Treg development [9]. Importantly, the number of RALDH1-producing dermal cDCs is decreased in the involved skin of SSc patients and tends to correlate with the severity of skin fibrosis [9], suggesting that retinoic acid-dependent induction of Tregs by cDCs is involved in the development of SSc-associated skin fibrosis.

Friend leukemia virus integration 1 (Fli1), a member of Ets transcription factor family, is broadly downregulated in various types of cells in the skin of SSc patients [16]. Fli1 deficiency seems to be a potent predisposing factor of SSc due to the following reasons; (i) Fli1 deficiency induces SSc-like phenotypes in dermal fibroblasts, endothelial cells, macrophages and keratinocytes [17–27], (ii) Fli1 heterozygous deficiency enhances SSc-like phenotypes in dermal fibroblasts, endothelial cells, and macrophages in mice treated with BLM [28], (iii) endothelial cell-specific Fli1 knockout mice resemble vascular structural and functional abnormalities characteristic of SSc vasculopathy [29], (iv) bosentan, a dual endothelin receptor antagonist that prevents the development of new digital ulcers, increases endothelial Fli1 expression in SSc patients [30], (v) epithelial cell-specific Fli1 knockout mice develop dermal and esophageal fibrosis characterized by the activation of stratified squamous epithelia and interstitial lung disease due to the downregulation of the autoimmune regulator (AIRE) in the thymus, recapitulating selective organ fibrosis of SSc [31]. Thus, Fli1 deficiency-related studies have provided new insights into the pathogenesis of SSc.

Based on these backgrounds, we investigated the contribution of Fli1 deficiency to the phenotypical alteration of dermal DCs in the context of SSc and related conditions by using BLM-treated Fli1^{+/-} mice and SSc skin samples.

Methods

A BLM-induced murine SSc model

BLM (200 µg, Nippon Kayaku, Tokyo, Japan) dissolved in phosphate buffered saline (PBS) or control PBS was injected subcutaneously into a single location on the back of 8-week-old female wild-type (WT) mice (C57BL/6) and Fli1^{+/-} mice daily. The injection was conducted consecutively for 1 week.

Flow Cytometry

The day following the completion of 1-week BLM injection, lymphocytes from draining lymph nodes and the dermis of the lower back skin were obtained. In the surface staining, cells were stained with antibodies against CD11b (M1/70), CD11c (N418), and CD103 (2E7; all from BioLegend, San Diego, CA, USA). In intracellular cytokine staining, cells were stimulated with 10 ng/ml phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO) and 1 µg/ml ionomycin (Sigma-Aldrich) in the presence of 1 µg/ml brefeldin A (GolgiStop; BD PharMingen) for 4 hours. Cells were washed, stained for the surface markers CD4 (RM4-5) and CD25 (PC61; all from BioLegend), treated with fixation/permeabilization working solution (BD PharMingen), and then stained with anti-interleukin (IL)-4 (11B11), anti-IL-17A (TC11.18H10), and anti-interferon (IFN)-γ (XMG1.2; all from BioLegend) antibodies. To analyze transcription factors, antibody against Foxp3 (FJK-16 s; eBioscience, San Diego, CA, USA) were used. Cells were analyzed on a FACSVerser flow cytometer (BD Biosciences, San Diego, CA, USA). The populations of positive and negative cells were determined using nonreactive isotype-matched antibodies as controls. Gating strategies are shown in supplementary Fig. S1.

Analysis Of RALDH Activity At The Single-cell Level

The presence of cells displaying RALDH activity was determined using an ALDEFLUOR staining kit (StemCell Technologies, Vancouver, BC, Canada). The following modifications were introduced into the manufacturer's protocol. Briefly, cells (1×10^6 cells/mL) were incubated in the dark for 45 minutes at 37 °C in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate, with or without the RALDH inhibitor diethylaminobenzaldehyde (DEAB). Cells were subsequently stained using the specified antibodies in ice-cold ALDEFLUOR assay buffer. Cells were subsequently washed in ALDEFLUOR assay buffer, resuspended in ALDEFLUOR assay buffer, and kept on ice before analysis on a FACSVerser flow cytometer (BD Biosciences).

Immunofluorescence

Mouse anti-CD11c antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-Fli1 antibody (Santa Cruz Biotechnology) were used as primary antibodies, and Alexa Fluor 555-conjugated donkey anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG antibody (Santa Cruz Biotechnology) were used as secondary antibodies for human skin sections. Coverslips were mounted by Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA), and staining was examined with Biozero BZ-8000 (Keyence, Osaka, Japan) at 495 nm (green), 565 nm (red), and 400 nm (blue). Ten random grids were evaluated under high magnification by 2 independent researchers in a blinded manner.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.30 (GraphPad Software, San Diego, CA, USA). The Mann-Whitney U-test was used for two-group comparison. The Kruskal-Wallis test followed by the Dunn's multiple comparison test was used for three-group multiple comparison. Statistical significance was defined as a P value of < 0.05.

Results

The number of dermal DCs with RALDH activity is decreased in BLM-treated Fli1^{+/-} mice

As an initial experiment, we focused on the RALDH1 activity in dermal DCs of Fli1^{+/-} and WT mice because RALDH1 is a key enzyme regulating the induction of Tregs in BLM-treated mice [9]. To this end, we employed an ALDEFUOR staining kit in which we can evaluate the summation of RALDH1, -2, and -3 activities. As shown in Fig. 1a and 1b, the number of dermal DCs exhibiting RALDH activity was decreased in BLM-treated Fli1^{+/-} mice compared with BLM-treated WT mice, and the majority of dermal DCs with RALDH activity were CD11b⁻CD103⁻ DCs in both strains. Furthermore, mean fluorescence intensity of RALDH activity was significantly lower in dermal DCs of BLM-treated Fli1^{+/-} mice than in those of BLM-treated WT mice (Fig. 1c). Given that BLM selectively induces RALDH1 activity as demonstrated in our previous study [9], these results indicate that Fli1 heterozygous deficiency suppresses the activity of RALDH1 in CD11b⁻CD103⁻ dermal DCs after the injection of BLM.

The impact of Fli1 deficiency on the proportion of dermal DC subsets

Generally, dermal DCs are classified into CD103⁺ DCs, CD103⁻CD11b⁻ DCs and CD103⁻CD11b⁺ DCs in mice; the former two subsets are cDCs, but CD103⁻CD11b⁺ DCs comprise cDCs, monocyte-derived DCs

and macrophages [10]. Therefore, we compared the proportions of these dermal DC subsets in the dermis of BLM-treated Fli1^{+/-} and WT mice. In the dermis, the proportion of CD103⁺ DCs was significantly increased, while the proportions of CD103⁻CD11b⁺ DCs and CD103⁻CD11b⁻ DCs were unaltered in BLM-treated Fli1^{+/-} mice compared with BLM-treated WT mice (Fig. 2). Taken together with the results in Fig. 1, these results indicate that the decreased RALDH1 activity is not due to the decreased proportion of CD103⁻CD11b⁻ DCs. Therefore, RALDH1-producing CD103⁻CD11b⁻ dermal cDC-associated biological events are likely inhibited in the dermis of BLM-treated Fli1^{+/-} mice.

The proportion of Tregs is decreased in the dermis of BLM-treated Fli1^{+/-} mice

To determine if Treg induction is disturbed in BLM-treated Fli1^{+/-} mice, we assessed the proportions of CD4⁺ T cell subsets, such as Th1, Th2, Th17 and Tregs, in the dermis of BLM-treated Fli1^{+/-} and WT mice. As shown in Fig. 3, the proportion of Tregs was significantly decreased in BLM-treated Fli1^{+/-} mice compared with BLM-treated WT mice, while the proportions of Th1, Th2 and Th17 cells revealed similar results in the two groups. Taken together with the results in Fig. 2, these results suggest that RALDH1-producing CD103⁻CD11b⁻ dermal cDC-dependent induction of Tregs is inhibited in the dermis of BLM-treated Fli1^{+/-} mice.

Fli1 Is Downregulated In Dermal DCs Of SSc Patients

Finally, we explored the expression of Fli1 in dermal DCs in SSc patients. To this end, we carried out double immunofluorescence for CD11c and Fli1. When anti-Fli1 antibody was used at a 50-fold dilution, the number of double positive cells was comparable among diffuse cutaneous SSc patients, limited cutaneous SSc patients and healthy controls (Fig. 4a). Importantly, when anti-Fli1 antibody was diluted 200-fold, the number of double positive cells was significantly decreased in diffuse cutaneous SSc patients compared with healthy controls (Fig. 4b). These results indicate that Fli1 expression is decreased in dermal CD11c⁺ cells, including dermal DCs, in SSc lesional skin.

Discussion

Previous clinical and experimental studies on the role of DCs in SSc development have been predominantly focused on pDCs that contribute to sterile inflammation related to autoimmune rheumatic disorders through type I interferon (IFN-I) production in response to self-nucleic acids [8]. Indeed, the IFN signature has been observed in the peripheral blood, skin and lungs of SSc patients [32, 33], and circulating SSc pDCs secrete elevated levels of IFN-I due to the aberrantly expressed Toll-like receptor (TLR) 8 as well as TLR7 and TLR9. Importantly, pDC depletion not only prevents the development of skin fibrosis, but also ameliorates established skin fibrosis in BLM-treated mice [34]. Thus, it is currently

accepted that pDCs play a critical role in SSc pathogenesis. On the other hand, although cDCs act as a critical regulator of skin immunity [10], their roles in immunopathology of SSc have remained poorly understood.

In this study, we employed Fli1^{+/-} mice to investigate the phenotypical alteration of cDCs with relevance to SSc-associated disease pathology because BLM-treated Fli1^{+/-} mice exhibit SSc-like features in various cell types [28, 35]. Since BLM injection induces RALDH1 production in CD103⁻CD11b⁻ dermal cDCs and subsequently promotes Treg development in WT mice [9], the enhancement of BLM-dependent dermal fibrosis in Fli1^{+/-} mice suggests that Fli1 deficiency impairs the regulatory function of CD103⁻CD11b⁻ dermal cDCs. As expected, RALDH activity was decreased in CD103⁻CD11b⁻ dermal cDCs of BLM-treated Fli1^{+/-} mice. Furthermore, the Treg proportion was decreased in the dermis of BLM-treated Fli1^{+/-} mice. Although we could not exclude the possibility that Fli1 deficiency directly affects the differentiation of naïve CD4⁺ T cells to Tregs, these current results indicate that decreased RALDH1 activity of CD103⁻CD11b⁻ DCs at least partially contributes to the dysregulated Treg induction in the skin of BLM-treated Fli1^{+/-} mice. This notion is supported by our previous finding that Raldh1 siRNA suppresses Treg induction in the skin of BLM-treated WT mice [9]. Considering decreased expression of Fli1 and RALDH1 in CD11c⁺ cells as shown in our previous and current studies and exclusive expression of RALDH in cDCs [9, 11], we can plausibly conclude that Fli1 deficiency induces SSc-like phenotypes in dermal DCs, in addition to previously shown fibroblasts, endothelial cells, macrophages and epithelial cells [20, 28, 29, 31, 36, 37].

The alteration of T cell subsets has been well studied in the field of SSc research. Generally, the balances of Th1/Th2 and Th17/Treg immune responses skew toward Th2 and Th17 predominance [38–41], and Treg function is impaired in the active stage of SSc [42]. In addition, the proportion of Th2-like Tregs is increased in the involved skin of SSc patients [43]. Importantly, these characteristic alterations of CD4⁺ T cell balance are reproduced in BLM-treated Fli1^{+/-} mice [28], and some of the potential underlying mechanisms have been shown. For instance, Fli1 deficiency induces the expression of IL-33 and galectin-9 in dermal fibroblasts, resulting in the promotion of Th2-like Treg induction and the suppression of Th1 development, respectively [28, 35]. Also, Fli1 deficiency facilitates IL-6 production in dermal fibroblasts, promoting Th17 differentiation together with active TGF-β and IL-1β that are elevated in the skin of BLM-treated Fli1^{+/-} mice [35]. The current study added a new insight into the contribution of Fli1 deficiency to the induction of SSc-like CD4⁺ T cell balance; namely, the decrease in Treg proportion due to the reduced RALDH1 expression in CD103⁻CD11b⁻ dermal cDCs. Thus, Fli1 deficiency may serve as a key regulator of skin inflammation, as well as skin fibrosis and vasculopathy, in SSc.

The current study suggests that administration of retinoic acid is a potential therapeutic strategy to increase the proportion of Tregs in SSc [44]. However, it has been long recognized that overexposure to retinoic acid causes widespread teratogenesis in humans [45]. An alternative therapeutic strategy is to restore Fli1 expression in cDCs, possibly resulting in increased RALDH1 activity of cDCs and subsequent induction of Tregs in the involved skin of SSc patients. From this perspective, it is quite important to

identify the mechanism by which Fli1 is downregulated in SSc cDCs. In dermal fibroblasts and dermal microvascular endothelial cells, endothelin-1 stimulation suppresses Fli1 expression and induces SSc-like phenotypes [36, 37, 46]. Importantly, endothelin-1 (ET-1) directly induces the phenotypic maturation of bone marrow-derived DCs that preferentially prime T cells to produce Th17 cytokines [47]. Given that RALDH1-producing cDCs skew Th17/Treg balance toward Treg predominance [9, 44], ET-1 may be a possible factor suppressing RALDH1 production in cDCs, in which ET-1-dependent Fli1 suppression may be involved. Since bosentan, a dual antagonist of endothelin receptors, reverses SSc-like phenotypes of dermal microvascular endothelial cells by increasing Fli1 expression and improves SSc vascular features at the molecular and morphological levels [30, 36, 37, 48–50], this reagent may modulate skin immunity of SSc patients. Further studies are required to clarify this point.

Conclusion

This is the first report demonstrating the critical role of Fli1 deficiency in CD103[−]CD11b[−] dermal cDC-dependent induction of Tregs in BLM-treated mice, an established SSc animal model. The current data indicate that dermal DCs are yet another cell type in which Fli1 deficiency induces an SSc-like phenotype. Although further studies are required to clarify the role of Fli1 deficiency in human cDCs, this study strengthens the notion that Fli1 deficiency serves as a potential predisposing factor of SSc.

List Of Abbreviations

SSc, systemic sclerosis; DCs, dendritic cells; pDCs, plasmacytoid DCs; cDCs, conventional DCs; RALDH1, aldehyde dehydrogenase 1; Tregs, regulatory T cells; RALDH, aldehyde dehydrogenase, TGF, transforming growth factor; Fli1, Friend leukemia virus integration 1; BLM, bleomycin,; AIRE, autoimmune regulator; PBS, phosphate buffered saline; WT, wild-type; DEAB, diethylaminobenzaldehyde; IFN-I, type I interferon; TLR, Toll-like receptor; ET-1, endothelin-1.

Declarations

Ethics approval and consent to participate

This study was approved by the ethical committee and the committee on animal experimentation of The University of Tokyo Graduate School of Medicine and performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design, SM and YA; acquisition of data, SM, RS, TY, KN, MH, TM and AY; Analysis and interpretation of data, SM, RS, TY, KN, MH, TM, AY, MT, SS and YA; Obtaining funding, SS and YA; Supervision, SS and YA.

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Figures

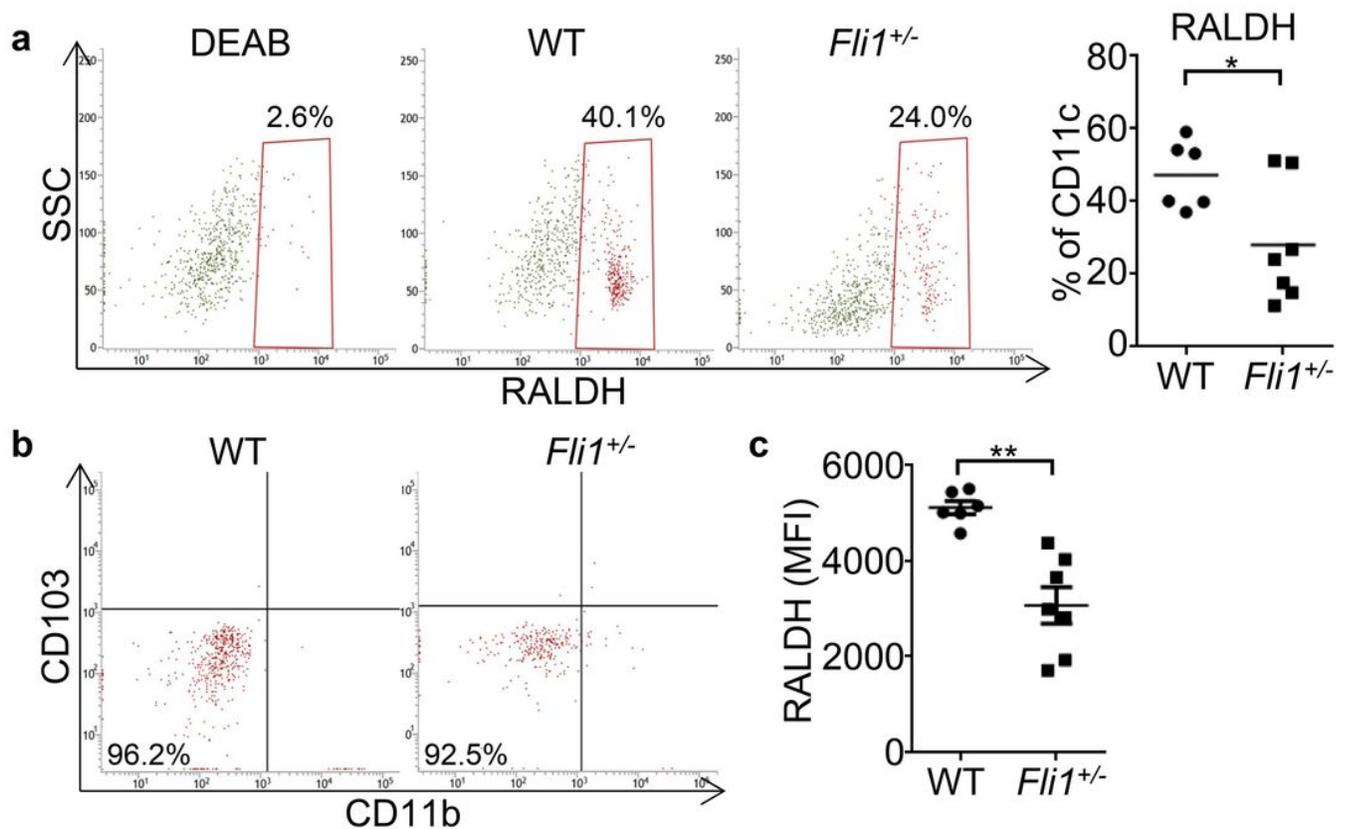


Figure 1

The number of dermal DCs with RALDH activity is decreased in BLM-treated *Fli1*^{+/-} mice. Wild-type (WT) and *Fli1*^{+/-} mice were treated with bleomycin (BLM) for a week. a. Representative flow cytometry plots of RALDH staining in CD11c⁺ cells from the dermis of BLM-treated WT and *Fli1*^{+/-} mice are shown (left panels). The result in the presence of the RALDH inhibitor diethylaminobenzaldehyde (DEAB) is shown as control. The proportions of CD11c⁺ cells positive for RALDH are summarized in a right graph (n = 6-7 per group). b. Representative flow cytometry plots of CD11b and CD103 staining in RALDH-producing CD11c⁺ cells from the dermis of BLM-treated WT and *Fli1*^{+/-} mice are shown (n = 6-7 per group). c. Mean fluorescence intensity (MFI) of RALDH was examined in CD11b-CD103⁻ DCs isolated from the dermis. Each graph indicates mean ± SEM of the indicated parameters. * P < 0.05; ** P < 0.01.

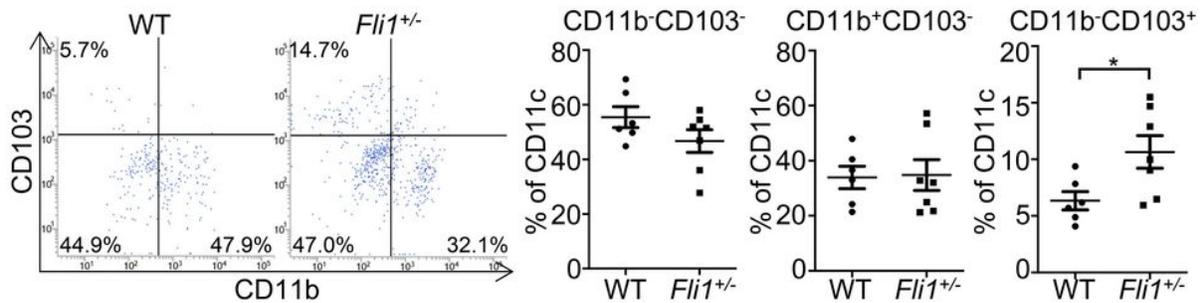


Figure 2

The impact of *Fli1* deficiency on the proportion of dermal DC subsets. Wild-type (WT) and *Fli1*^{+/-} mice were treated with bleomycin (BLM) for a week. Representative flow cytometry plots of CD11b and CD103 staining in CD11c⁺ cells from the dermis are shown. The percentages of CD11b⁻CD103⁻, CD11b⁺CD103⁻, and CD11b⁻CD103⁺ cells in CD11c⁺ cells in WT and/or *Fli1*^{+/-} mice are summarized in graphs. Flow cytometry profiles are representative of 6-7 individual animals from at least 2 separate experiments. Bars show the mean ± SEM. * P < 0.05.

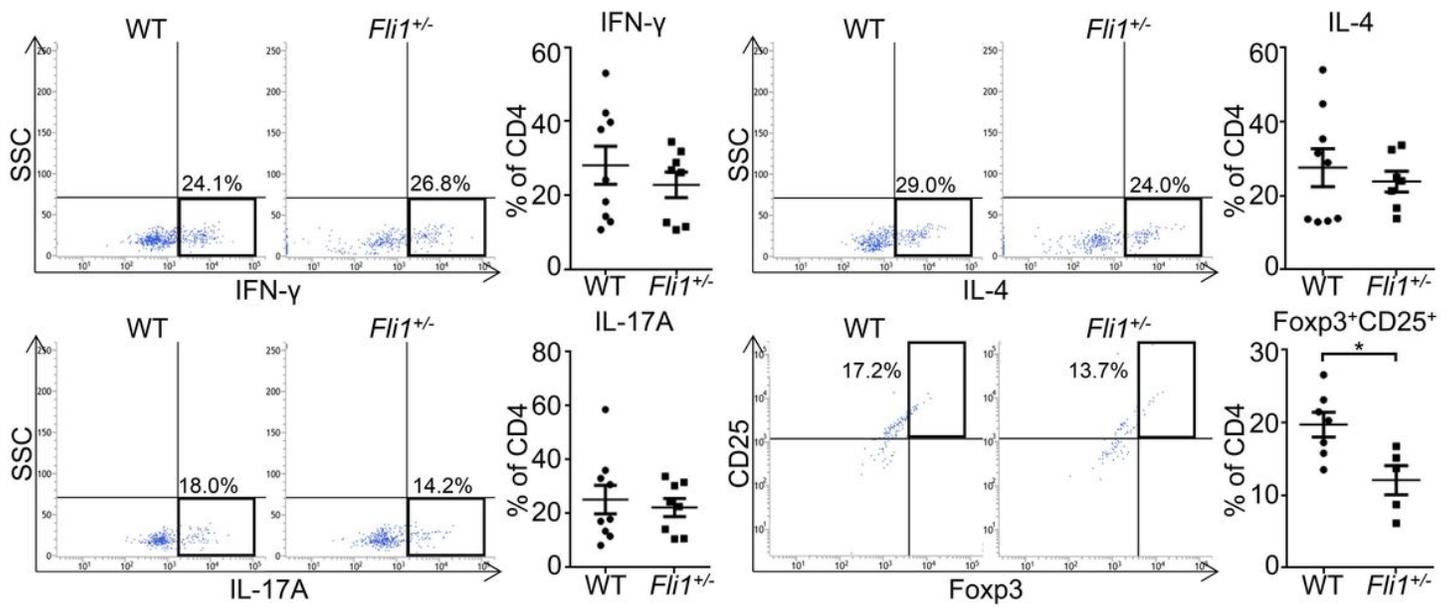


Figure 3

The proportion of Tregs is decreased in the dermis of BLM-treated *Fli1*^{+/-} mice. Wild-type (WT) and *Fli1*^{+/-} mice were treated with bleomycin (BLM) for a week. The percentages of IFN- γ , IL-4- and IL-17A-producing CD4⁺ T cells and CD4⁺CD25⁺Fopx3⁺ Tregs in the dermis were determined by flow cytometry. Flow cytometry profiles are representative of 5-9 individual animals from at least 2 separate experiments. Each graph indicates mean \pm SEM of the indicated parameters. * P < 0.05.

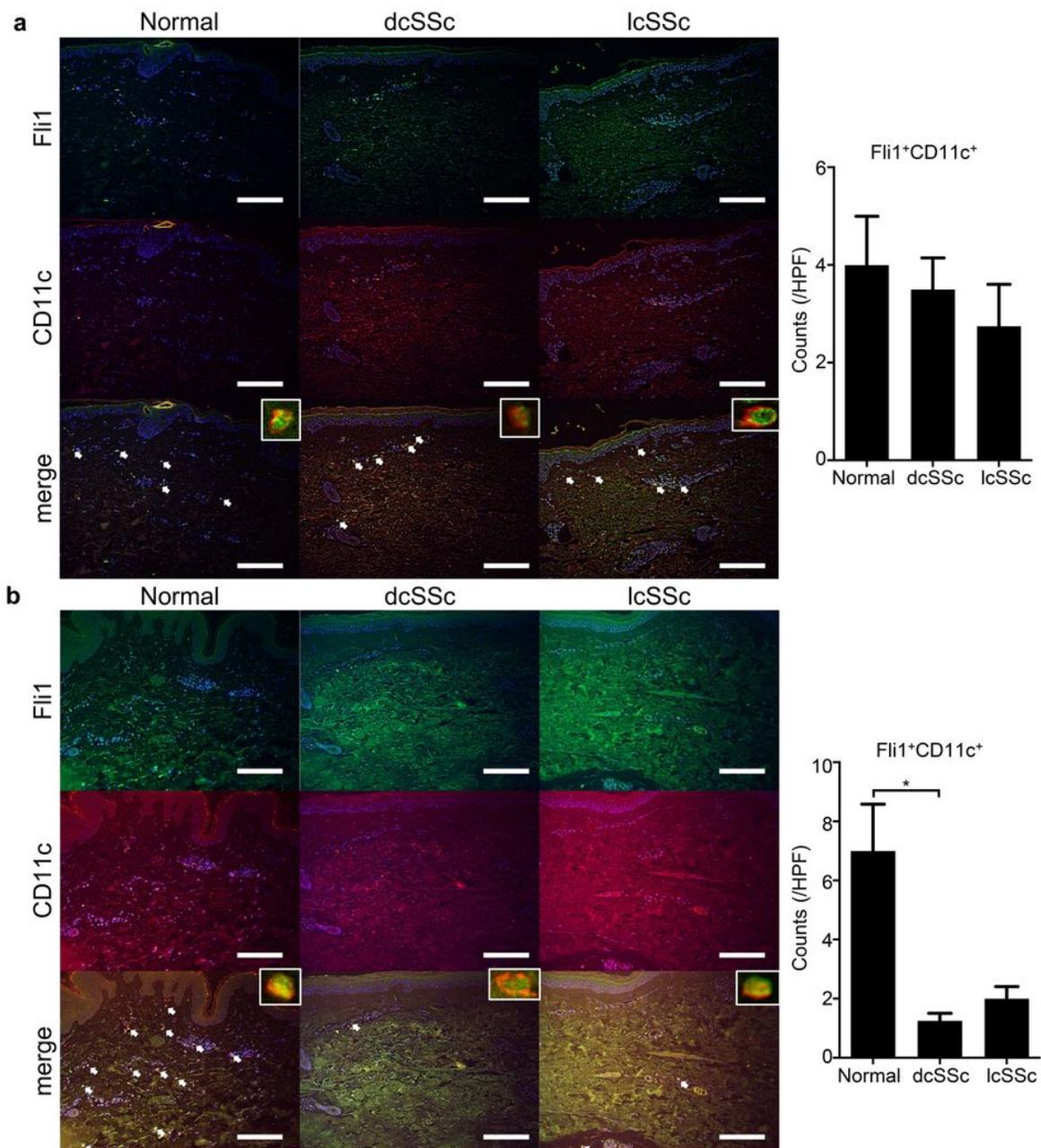


Figure 4

Fli1 is downregulated in dermal DCs of SSc patients. a, b. Immunofluorescence with antibodies against CD11c and Fli1 was conducted with skin samples from systemic sclerosis (SSc) and healthy control subjects. Anti-Fli1 antibody was diluted 50-fold (a) and 200-fold (b) in reaction buffer. Representative immunofluorescence images are shown (left panels). Double positive cells were counted in a $\times 100$ high-power field (HPF; right graph; $n = 4$, each). dcSSc, diffuse cutaneous systemic sclerosis. lcSSc, limited cutaneous systemic sclerosis. Graph indicates mean \pm SEM. Arrows indicate positive cells. Scale bars are 100 μm . * $P < 0.05$.

Supplementary Files

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