Title:
Regulatory T cells in skin facilitate epithelial stem cell differentiation

Abstract:
The maintenance of tissue homeostasis is critically dependent on the self-renewal and differentiation capacity of epithelial stem cells (SCs)\(^1\). How extrinsic signals, including tissue resident immune cells, govern SC behavior is largely unknown. We have previously observed that regulatory T cells (T\(_\text{regs}\)) in skin preferentially localize to hair follicles (HFs)\(^3,4\), which house a major subset of skin SCs (HFSCs). HF regeneration is critically dependent on HFSCs\(^5\). Here, we mechanistically dissect the role of T\(_\text{regs}\) in HFSC biology. We found that T\(_\text{reg}\) abundance and activation state in skin precisely correlate with the synchronous HF cycle. Lineage-specific cell depletion revealed that T\(_\text{regs}\) are required for HF regeneration. 

T\(_\text{regs}\) preferentially localize to the bulge region of HFs and facilitate HF cycling by directly augmenting HFSC proliferation and differentiation. Transcriptional and phenotypic profiling of T\(_\text{regs}\) and HFSCs revealed that T\(_\text{regs}\) in skin preferentially express high levels of the Notch ligand family member, Jag1, and the Notch signaling pathway was significantly enhanced in HFSCs when T\(_\text{regs}\) were present.

In functional experiments, expression of Jag1 in T\(_\text{regs}\) was required for HFSC function and efficient HF regeneration. Taken together, our work functionally dissects the role of T\(_\text{regs}\) in HF biology and establishes a mechanistic link between tissue resident immune cells and epithelial SCs. These findings have clinical implications in human diseases of HFs, such as Alopecia Areata (AA), where T\(_\text{regs}\) are thought to play a role in disease pathogenesis\(^6-8\).
Hair follicles in mammalian skin undergo bouts of regeneration, cycling between highly synchronized phases of quiescence (telogen) and growth (anagen). Both epithelial intrinsic and extrinsic environmental signals regulate the coordinated activation of telogen HF to enter anagen. Two findings have raised the possibility that skin-resident immune cells, specifically Tregs, are involved in this process. First, Tregs are concentrated in the vicinity of follicular epithelium, where HFSCs take residence. Second, genome wide association studies have linked single nucleotide polymorphisms in Treg-associated genes to AA, a disorder of immune-mediated disruption of HF regeneration. In addition, augmenting Tregs clinically has shown efficacy in treating patients with this disease. However, the contribution of Tregs to HFSC biology, or the mechanisms underlying such a pathway of SC regulation, is currently unknown. To test the hypothesis that Tregs play a functional role in HF biology, we began by performing comprehensive immune profiling of Tregs in murine skin at specific stages of the synchronous HF cycle. The proportion of CD4+Foxp3+ Treg cells in skin draining lymph nodes (SDLNs) of adult C57BL/6 mice showed little variability. In contrast, Treg percentages and abundance in dorsal skin fluctuated significantly at different stages of the HF cycle. Tregs were significantly more abundant in the telogen phase of the HF cycle when compared to anagen. The proliferative index and activation state of skin Tregs (as evidenced by expression of Ki67, CD25, ICOS, GITR, and CTLA-4) also correlated with stage of the HF cycle, as Tregs displayed a more activated phenotype in telogen skin.

Given that both Treg abundance and activation correlated with HF stage, we sought to determine whether these cells play an active role in the process of HF cycling. To do so, we employed a well-characterized model of depilation-induced HF regeneration. In this model, mice with dorsal skin HFs in telogen are depilated to remove hair shafts. This treatment rapidly initiates the telogen-to-anagen transition.
homogenously across the entire depilated dorsum (anagen induction), and thus begins the process of hair regeneration. To determine if $T_{\text{regs}}$ play a role in this process, we utilized mice transgenic for the diphtheria toxin receptor under the control of the Foxp3 promoter ($\text{Foxp3}^{\text{DTR}}$)\textsuperscript{12}. These mice allow for robust depletion of $T_{\text{regs}}$ following administration of Diphtheria toxin (DT). Importantly, $T_{\text{regs}}$ begin to repopulate lymph nodes and peripheral tissues soon after the last DT treatment, permitting the study of transient $T_{\text{reg}}$ loss at specific times during HF regeneration\textsuperscript{12}. Ablation of $T_{\text{regs}}$ markedly reduced hair regrowth when compared to wild type (WT) control mice treated with or without DT (Fig. 1c, d). Whereas control mice had complete hair regrowth by 14 days post-depilation, mice depleted of $T_{\text{regs}}$ showed less than 20% regrowth at this time (Fig. 1d). In these initial experiments, $T_{\text{regs}}$ were continually depleted (i.e., DT administered every 2 days for 14 days). Because a major function of $T_{\text{regs}}$ is to suppress inflammation, mice deficient in these cells for prolonged periods of time succumb to multi-organ autoimmunity\textsuperscript{12}. Thus, we set out to determine whether attenuation of HF cycling in the absence of $T_{\text{regs}}$ was simply a result of systemic inflammation. In addition, we wanted to precisely define a potential ‘window’ of time for $T_{\text{reg}}$ requirement in HF cycling. To do so, we treated WT or $\text{Foxp3}^{\text{DTR}}$ mice with DT ‘early’ after depilation (up to 4 days post-depilation), ‘late’ after depilation (starting at 7 days post-depilation), or throughout the entire 14-day period of hair regeneration. Depletion of $T_{\text{regs}}$ early after depilation completely recapitulated the attenuation of HF regeneration observed with constant $T_{\text{reg}}$ depletion (Fig. 1e and Extended Data Fig. 3). In contrast, late $T_{\text{reg}}$ depletion showed normal hair regrowth (Fig. 1e and Extended Data Fig. 3). Histologic examination of skin showed a marked diminution of anagen HFs in early $T_{\text{reg}}$-depleted mice. Whereas HFs in control mice displayed an elongated phenotype extending deep into the subcutaneous fat (indicative of anagen induction)\textsuperscript{11}, HFs from $T_{\text{reg}}$-depleted mice were significantly shorter in length and failed to extend beyond the superficial dermis (Fig. 1f, g and
Extended data Fig. 3). Taken together, these results indicate that activated T\textsubscript{regs} are more abundant in telogen skin and that these cells play an essential role in the telogen-to-anagen transition of the HF cycle. Consistent with these findings, mice that lack all T cells (Rag2\textsuperscript{−/−}) have a significant delay in depilation-induced hair regeneration and anagen onset during the synchronous HF cycle (Extended Data Fig. 4).

Given that telogen HFs from T\textsubscript{reg}-depleted mice failed to enter anagen after depilation, we sought to elucidate the cellular mechanisms responsible for this phenomenon. The HF bulge region is the best characterized niche for adult skin epithelial SC residence\textsuperscript{10}. Activation of bulge HFSCs is required anagen onset\textsuperscript{13,14}. Upon anagen induction, HFSCs are activated, begin to proliferate and eventually differentiate to form all cell lineages of the newly generated HF\textsuperscript{15-17}. Because T\textsubscript{regs} function early to facilitate anagen induction, we set out to determine their influence on HFSC activation during the telogen-to-anagen transition. To do so, we utilized a previously established flow cytometric approach to delineate HFSCs\textsuperscript{18}. Epidermal cell suspensions were prepared from dorsal skin and stained for CD45, Sca-1, EpCAM, CD34 and integrin α6 (ITGA6). Bulge HFSCs were defined as CD45\textsuperscript{neg}Sca-1\textsuperscript{neg}EpCAM\textsuperscript{low}CD34\textsuperscript{high} cells (Fig. 2a). High levels of ITGA6 expression on this population distinguishes basal bulge residing HFSCs from those in the suprabasal layer\textsuperscript{17} (Fig. 2a). Analysis of mid-telogen WT skin confirmed the presence of quiescent bulge-resident HFSCs\textsuperscript{10}, as evidenced by lack of expression of the proliferative marker Ki67 (Fig. 2b, c). Consistent with previous reports\textsuperscript{19}, HFSC activation closely followed depilation-induced anagen, with an increased percentage of HFSCs expressing Ki67 4-days after depilation (Fig. 2b, c). Induction of HFSC proliferation was significantly reduced in mice depleted of T\textsubscript{regs} in the early window of time after depilation (Fig. 2b, c). This proliferative defect appeared to be selective for the bulge HFSC compartment, as no difference in Ki67 expression was observed in non-bulge keratinocytes between T\textsubscript{reg}-sufficient and T\textsubscript{reg}-depleted mice (Fig. 2d). To
determine if T\textsubscript{regs} play a similar role in the natural telogen-to-anagen transition of the HF cycle, we utilized Foxp3-Scurfy mice (Foxp3\textsuperscript{Sf}) that fail to develop functional Foxp3-expressing T\textsubscript{reg} cells\textsuperscript{20}. While WT littermate controls have entered 1\textsuperscript{st} anagen on post-natal day 28, Foxp3\textsuperscript{Sf} mice fail to do so, as evidenced by diminished proliferative potential of HFSCs, lack of dorsal skin pigmentation, and significantly reduced HF length (Extended Data Figure 5). These data suggest that T\textsubscript{regs} are required to promote HFSC function during the normal cycle of hair follicle regeneration.

To elucidate how T\textsubscript{regs} influence HFSC activation and differentiation, we performed whole transcriptome RNA sequencing (RNAseq) of bulge HFSCs. Lineage tracing studies in the SC niche have identified a role for basal cell progenitors and their progeny in adopting a HF fate\textsuperscript{13,16,21–23}. We therefore sequenced iTGA6\textsuperscript{high} basal bulge HFSCs purified from T\textsubscript{reg}-sufficient and T\textsubscript{reg}-depleted mice 4 days post-depilation. Consistent with our Ki67 flow cytometric data, there was a marked proliferative defect in HFSCs isolated from T\textsubscript{reg}-depleted mice, as evidenced by a significant reduction in genes associated with cell proliferation (Supplementary Table 1). In addition, HFSCs isolated from T\textsubscript{reg}-depleted mice showed a significant reduction in genes associated with differentiation to HF keratinocyte lineages\textsuperscript{24} (Fig. 2e). Collectively, these results suggest that a major mechanism by which T\textsubscript{regs} mediate anagen induction is through preferentially facilitating the activation and differentiation of bulge HFSCs.

T\textsubscript{regs} are well known to play a major role in suppressing inflammation\textsuperscript{25}. However, these cells have recently been show to have alternative functions in tissues, independent of their role in immunosuppression. In visceral adipose tissue, T\textsubscript{regs} express the peroxisome proliferator-activated receptor-\textgamma, enabling them to function in lipid and glucose metabolism\textsuperscript{26}. In addition, T\textsubscript{regs} in muscle and lung express high levels of the epidermal growth factor ligand, amphiregulin, giving them the ability to directly facilitate tissue repair\textsuperscript{27,28}. Alternative functions of T\textsubscript{regs} in skin are currently unknown. It is
therefore possible that T<sub>regs</sub> produce factors that directly promote the activation and differentiation of HFSCs. Alternatively, T<sub>regs</sub> may indirectly facilitate anagen entry by suppressing inflammatory cells in and around the HFSC niche<sup>29</sup>. To determine if suppression of inflammation was the dominant mechanism by which T<sub>regs</sub> mediate HFSC activation, we comprehensively assessed the inflammatory response in skin of T<sub>reg</sub> depleted mice early after anagen induction. Hallmarks of skin inflammation, such as epidermal hyperplasia and immune cell infiltrate, were not significantly different between control and T<sub>reg</sub> depleted mice (Fig. 3a-b). In addition, absolute numbers of dermal γδ T cells, dendritic epidermal T cells, cytotoxic CD8<sup>+</sup> T cells, CD4<sup>+</sup>Foxp3<sup>−</sup> T effector cells, CD3<sup>−</sup>γδ<sup>−</sup> lineage negative cells, dendritic cells, neutrophils, and macrophages remained unchanged between T<sub>reg</sub> depleted and T<sub>reg</sub> sufficient mice during this acute anagen induction period (Fig. 3c and Extended Data Figure 6). Similarly, skin effector T cell production of the cytokines interleukin (IL)-22, interferon-γ (IFNγ), IL-17 and tumor necrosis factor-α were comparable between control and T<sub>reg</sub> depleted mice (Fig. 3c-e and Extended Data Figure 6b-d), suggesting that the absence of T<sub>regs</sub> during this defined window of anagen induction does not result in overt skin inflammation. To functionally test whether T<sub>regs</sub> indirectly facilitate anagen onset by suppressing inflammatory cells, the major immune cell populations in skin were co-depleted with T<sub>regs</sub> and the proliferative capacity of HFSCs in response to anagen induction was assayed. If T<sub>regs</sub> facilitate HFSC activation by suppressing inflammatory cells, we would predict that co-depletion of these cells with T<sub>regs</sub> would rescue depilation-induced HFSC proliferation. However, we observed that suppression of HFSC proliferation following T<sub>reg</sub> depletion was not rescued by co-depletion of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, Gr-1 expressing myeloid cells, or CD11c-expressing myeloid cells (Fig. 3f). In addition, both antibody neutralization and genetic deletion of the interferon-γ pathway, a major effector cytokine suppressed by T<sub>regs</sub><sup>30</sup>, did not rescue HFSC activation (Fig. 3f). These findings were
consistent with the lack of skin inflammation observed in our early transient T\textsubscript{reg} ablation model (Fig. 3a-e). Taken together, these data suggest that suppression of local immune cells is not the dominant mechanism by which T\textsubscript{regs} facilitate HFSC differentiation.

To determine if T\textsubscript{regs} directly interact with HFSCs, we first set out to establish the extent of co-localization of these two cell populations. Immunofluorescence microscopy revealed that T\textsubscript{regs} reside proximal to the lower portions of telogen HFs, clustering in close association with the bulge region (Fig. 3g). Co-staining of Foxp3 with Keratin-15 and ITGA6, markers of bulge resident SCs\textsuperscript{16}, revealed a sub-population of bulge-associated T\textsubscript{regs} in direct interaction with HFSCs (Fig. 3h and Extended Data Figure 7). Cells that are in constant contact with other cells typically have characteristic dynamic behavior. Specifically, cell-to-cell contacts alter the shape of cells such that they are no longer spherical\textsuperscript{31}. Thus, we hypothesized that bulge-associated T\textsubscript{regs} would have fundamentally different cell shape and cell dynamics when compared to T\textsubscript{regs} found outside this region. To test this, we developed a non-invasive vacuum suction approach for live intravital 2-photon imaging of T\textsubscript{regs} in dorsal skin of Foxp3\textsuperscript{GFP} reporter mice\textsuperscript{32}. Utilizing this system, we observed that bulge-associated T\textsubscript{regs} differ markedly with respect to shape and behavior when compared to T\textsubscript{regs} found >20 µm away from follicular epithelium. Bulge-associated T\textsubscript{regs} displayed a more amoeboid cell morphology with increased protrusive activity (Extended Data Figure 7 and Supplementary Video 1 and Video 2). This differential cell shape was quantified in individual skin T\textsubscript{regs} by applying a measure of relative sphericity\textsuperscript{33,34}. When compared to non-bulge T\textsubscript{regs}, the sphericity of bulge-associated T\textsubscript{regs} was significantly lower (Extended Data Figure 7), indicating the presence of a more dynamically active cell population in constant interaction with the HF milieu. Taken together with images obtained by static immunofluorescence microscopy, these observations suggest that T\textsubscript{regs} directly interact with HFSCs in the bulge region of the HF.
We next set out to elucidate potential mechanisms by which $T_{\text{regs}}$ may directly influence HFSC function. We hypothesized that pathways involved in this process would be preferentially expressed in skin $T_{\text{regs}}$ when compared to $T_{\text{regs}}$ found in SDLNs. Thus, we performed whole transcriptome RNA sequencing on $T_{\text{regs}}$ purified from telogen skin and compared expression profiles to $T_{\text{regs}}$ isolated from SDLNs (Supplementary Table 2). Consistent with heightened activation of $T_{\text{regs}}$ in tissues\textsuperscript{4,28,35}, differential expression analyses revealed increased expression of genes associated with $T_{\text{reg}}$ function in skin $T_{\text{regs}}$ compared to SDLN $T_{\text{regs}}$ (Fig. 4a). Most notably, Jag1, a ligand of the Notch signaling pathway, was among the highest differentially expressed genes between skin and SDLN $T_{\text{regs}}$ (Fig. 4a). $T_{\text{regs}}$ in skin expressed approximately 150-fold more Jag1 transcript than SDLN $T_{\text{regs}}$ (p-value=3.4x10\textsuperscript{-32}; Fig. 4b). Assessment of Jag1 protein expression by flow cytometry confirmed preferential expression on skin $T_{\text{regs}}$ relative to other adaptive immune cell populations in skin (Extended Data Figure 8). Aside from preferential expression on skin $T_{\text{regs}}$, this molecule was an especially interesting candidate given previous mechanistic studies identifying Jag1 and the canonical Notch signaling pathway as playing a major role in anagen induction and basal SC commitment to HF regeneration\textsuperscript{15,36–38}. Having identified skin $T_{\text{reg}}$ expression of Jag1, a known HF cycle inducer, we next sought to determine if Notch target gene transcripts\textsuperscript{39} were differentially expressed in HFSCs in the presence of $T_{\text{regs}}$. Genome wide RNAseq analysis of HFSCs isolated 4 days after anagen induction in the presence or absence of $T_{\text{regs}}$ revealed a differential signature of Notch target genes (Fig 4d, e). Of the 1174 differentially expressed genes between the two groups, 84 genes (~7%) were known transcriptional targets of the Notch signaling pathway (p-value of the overlap = 5.21x10\textsuperscript{-27}). Collectively, these results indicate that $T_{\text{regs}}$ in skin express high levels of the Notch ligand, Jag1, and that in the absence of $T_{\text{regs}}$, Notch signaling is attenuated in HFSCs. To functionally determine if Notch signaling plays a role in $T_{\text{reg}}$-mediated enhancement of
HFSC activation, we attempted to rescue the proliferative defect observed in HFSCs in T\textsubscript{reg}-depleted mice by the exogenous addition of Jag1\textsuperscript{40}. Microbeads coated with Jag1-Fc or control Fc were subcutaneously administered to DT-treated Foxp3\textsuperscript{DTR} mice and activation of HFSCs was quantified by flow cytometry 4 days post-depilation. In these experiments, exogenous Jag1 was able to partially rescue HFSC activation and induction of anagen (as measured by HF length) imparted by the absence of T\textsubscript{regs} (Fig. 4f, g).

To definitively test whether Jag1 expression on T\textsubscript{regs} is required for anagen induction, mice expressing a Jag1 conditional allele\textsuperscript{41} (Jag1\textsuperscript{fl/fl}) were crossed to Foxp3\textsuperscript{-cre} mice\textsuperscript{42} to specifically ablate Jag1 expression in T\textsubscript{regs}. Early after anagen induction, Foxp3\textsuperscript{Cre/Cre}Jag1\textsuperscript{fl/fl} mice had significantly attenuated proliferative capacity of integrin-\(\alpha_6\)high CD34\(^+\) bulge HFSCs when compared to age- and gender-matched littermate controls (Fig. 4h-i). In addition, we assessed the epidermal expression of key differentiation associated genes identified in our whole genome RNA-sequencing analysis. Relative to control mice, deletion of Jag1 in T\textsubscript{regs} resulted in a significant reduction in the expression of Bgn, Ccnd1, Gdf10, Sox4, Sox7 and Timp3 (Fig. 4j).

Given the early requirement for T\textsubscript{regs} in HFSC activation, we then monitored these mice for the earliest clinical sign of anagen entry - dorsal skin pigmentation\textsuperscript{11}. To determine the anagen induction capacity in the absence and presence of Jag1 on T\textsubscript{regs}, Foxp3\textsuperscript{Cre/Cre}Jag1\textsuperscript{fl/fl} and control mice were depilated and dorsal skin pigmentation was quantified. Consistent with the diminished proliferative and differentiation capacity of HFSCs, the pigmentation intensity of Foxp3\textsuperscript{Cre/Cre}Jag1\textsuperscript{fl/fl} dorsal skin was significantly decreased relative to control dorsal skin (Fig. 4k, l), revealing an essential function for Jag1 expression on T\textsubscript{regs} for anagen induction. Collectively, these findings suggest that T\textsubscript{regs} in skin utilize the Notch pathway, at least in part, to directly augment HFSC proliferation and differentiation.
In recent years, there has been a growing appreciation that tissue resident immune cells contribute to homeostatic and wound-induced regeneration\(^2\). The non-immunologic functions of cutaneous T\(_{\text{regs}}\), a major skin-resident immune cell population, have yet to be defined. Here, we identify a functional requirement of skin T\(_{\text{regs}}\) for the regeneration of HFs through enhancing bulge HFSC activation and differentiation. T\(_{\text{reg}}\)-mediated induction of SC activation provides a novel pathway by which an immune cell population influences SC behavior in tissues, with a marked impact on regeneration, and ultimately tissue function. These results also suggest that T\(_{\text{reg}}\) impairment may contribute to dysregulated HF cycling observed in patients with AA, establishing a foundation for new therapeutic strategies targeting this disease and potentially other tissue regenerative disorders.

Bibliography


**Figure Legends**

**Figure 1.** *T*<sub>reg</sub> *s* are required for hair regeneration. **a,** *T*<sub>reg</sub> cell abundance in skin draining lymph nodes (SDLNs) and skin of adult 4-14 week old WT mice as measured by flow cytometry. Pre-gated on live CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> cells. **b,** Flow cytometric quantification of skin *T*<sub>reg</sub> from dorsal skin of C57BL/6 mice at specific stages of the synchronous HF cycle (*n* = 5-12 mice per time point). Foxp3<sup>DTR</sup> mice or control WT mice were treated with DT on days -2, -1, and depilated on day 0 to induce anagen. DT administration was continued from Day 1 and then every other day until the termination of the experiment at day 14. **c,** Representative photos and **(d)** kinetics of hair regrowth in WT and *T*<sub>reg</sub> depleted (Foxp3<sup>DTR</sup>) mice (*n* = 3-4 mice per group). *T*<sub>reg</sub> were depleted either up to day 4 (early), from day 7 onwards (late) or constitutively (con) throughout the experimental period. **e,** Hair regrowth at day 14 in WT and Foxp3<sup>DTR</sup> mice with DT treatments as indicated. **f,** Representative H&E staining of skin from WT and Foxp3<sup>DTR</sup> mice on day 14. Arrows indicate anagen HF extension into subcutaneous fat. Scale Bars, 100 µm. **g,** Quantification of HF length on day 14 by line measurement tool from image acquisition software, Zen (Zeiss). Data are mean ± s.e.m. **** *P*<0.0001, Student’s *t*-test.

**Figure 2.** *T*<sub>reg</sub> *s* are required for HFSC proliferation and differentiation. **a,** Flow cytometric gating strategy to identify CD34<sup>+</sup> integrin α6<sup>high</sup> (ITGA6) bulge HFSCs. Foxp3<sup>DTR</sup> mice or control mice were treated with DT on days -2, -1, depilated on day 0 to induce anagen and DT administered again on days 1 and 3 (*i.e.*, early regimen). **b,** Representative flow cytometric plots of Ki67 expression in bulge HFSCs between WT and Foxp3<sup>DTR</sup> mice 4 days after depilation. **c,** Flow cytometric quantification of Ki67<sup>+</sup> bulge HFSCs and **(d)** non-bulge keratinocytes 4 days after depilation. RNA sequencing was
performed on FACS purified bulge HFSCs at day 4 post-depilation from control (WT) or T\textsubscript{reg} depleted (Foxp\textsuperscript{3DTR}) mice. e, Fold change in HFSC differentiation genes in WT and Foxp\textsuperscript{DTR} mice, expressed as fold change relative to WT (where a value of 1 = no change). Genes to the left of the solid line represent control genes. Significance values are calculated based on transcript expression level. Data are mean ± s.e.m. **** P<0.0001, ns = no significant difference, Student’s t-test.

Figure 3. T\textsubscript{regs} facilitate HFSC activation independently of suppressing skin inflammation. Control wild-type (WT) or Foxp3\textsuperscript{DTR} mice were depilated and treated with DT according to the ‘early’ depletion protocol. a, total dermal infiltrate and (b) epidermal hyperplasia in DT treated control (WT + DT) and T\textsubscript{reg} depleted (Foxp3\textsuperscript{DTR} + DT) Dorsal skin on day 4 as measured by routine histology. c, The absolute cell number of innate and adaptive immune cell subsets in skin as measured by flow cytometry. d, The proportion of cytokine producing CD4\textsuperscript{+} T\textsubscript{eff} Cells, and (e) CD8\textsuperscript{+} T cells in dorsal skin. f, Flow cytometric quantification of Ki67\textsuperscript{+} bulge HFSCs at day 4 post-depilation in immune cell depleted and interferon-\textgamma neutralized (or interferon-\textgamma signaling deficient) mice. g, Representative immunofluorescent image of Foxp3\textsuperscript{+} T\textsubscript{regs} in telogen skin of Foxp3\textsuperscript{GFP} reporter mice. Dashed line indicates outline of HF. ‘B’ indicates bulge region. h, T\textsubscript{reg} cell co-staining with Keratin-15. Scale Bars, 50 µm. One representative experiment of two, with n = 3-4 mice per group (a-e). Combined data from two experiments (f). Data are mean ± s.e.m. ns = no significant difference, Student’s t-test.

Figure 4. T\textsubscript{reg} expression of Jagged-1 (Jag1) is required for efficient HFSC activation and anagen induction. RNA sequencing was performed on telogen skin
T regs and T regs isolated from SDLNs. a, Volcano plot comparing expression profile of skin versus SDLN T regs. b, Raw gene counts of Jag1 transcripts as quantified by RNA sequencing (n = 4 for skin T regs and n = 3 for SDLN T regs). RNA sequencing was performed on FACS purified bulge HFSCs at day 4 post-depilation from control (WT) or T reg depleted (Foxp3 DTR) mice. c, Hierarchial clustering of differential expression of Notch target genes in HFSCs sequenced from control (WT) and T reg depleted (DTR) mice, depicted as a heat map d, Venn diagram depicting the overlap between the total differentially expressed (DE) genes and known Notch target genes. P-value represents the significance of the overlap as determined by a chi-squared test. Jag1-Fc coated or control Fc coated beads were administered subcutaneously in T reg depleted mice on days -2, -1, 1 and 3. All mice were depilated on day 0. e, Representative flow cytometric plots 4 days post-depilation gated on HFSCs from WT and Foxp3 DTR mice treated with control or Jag1-Fc coated beads. f, Quantification of Ki67+ bulge HFSCs and (g) HF length 4 days post-depilation. Control (i.e., Foxp3 Cre/CreJag1 wt/wt or Foxp3 wt/wtJag1 fl/fl) or Foxp3 Cre/CreJag1 fl/fl mice were depilated to induce anagen. h, Representative flow cytometric plots and i, quantification of Ki67+ bulge HFSCs j, Fold change in HFSC differentiation genes as measured by qRT-PCR. k, Representative photos and (l) quantification of skin pigmentation. RNA-Seq experiments were conducted using 2-4 biological samples (a-d). Data are combined from three independent experiments (e-g). One representative experiment of two (h-l). Data are mean ± s.e.m. *P<0.05, **P<0.01 ***P<0.001, ****P<0.001, Student’s t-test.
Figure 1

- **a**
  - Graph showing Treg percentages in Adult SDLN and Adult Skin.

- **b**
  - Graph depicting Age (days) vs. Treg percentages for 1st and 2nd cycles.

- **c**
  - Images of WT, WT + DT, and Foxp3<sup>DTR</sup> + DT hair under depilation.

- **d**
  - Graph showing Hair Regrowth (%) over Days Post-depilation for WT, WT + DT, and Foxp3<sup>DTR</sup> + DT (early).

- **e**
  - Graph illustrating Hair Regrowth (%) with Mouse: WT, WT + DT, Foxp3<sup>DTR</sup> + DT, and DTR + DT (early).

- **f**
  - Images of Hair Follicle Length (μm) for WT, WT + DT, and Foxp3<sup>DTR</sup> + DT (early).

- **g**
  - Graph depicting Hair Follicle Length (μm) with WT, WT + DTR + DT (early).
Figure 2

(a) Flow cytometry analysis of CD45, Sca-1, CD34, and EpCAM.

(b) Ki67 expression in WT and WT + Depilation and Foxp3^{DTR} + Depilation.

(c) Box plot showing % Ki67 (Bulge CD34) and % Ki67 (Non-bulge KCs).

(d) Fold change in gene expression for WT and Foxp3^{DTR} in different conditions.

(e) Graph showing fold change in gene expression with WT and Foxp3^{DTR}.
Rosenblum 2015-12-16763B
Figure 3
Rosenblum 2015-12-16763B

Figure 4

a. Graph showing Log10 (padj) vs. Log2 Fold Change with data points indicating higher expression for Jag1, Il10, and Icos.

b. Bar chart showing Jag1 gene counts across different conditions.

c. Heatmap illustrating gene expression patterns with color intensity indicating expression levels.

d. Venn diagram showing the overlap of DE Genes and Notch Target Genes with 1174 overlapping genes.

e. Comparison of Foxp3^DTR^WT vs. Foxp3^DTR^DT + Jag1 conditions for Ki67 expression.

f. Graph showing percent Ki67+ (Bulge CD34+) in different conditions.

g. Graph depicting Hair Follicle Length (μm) across different groups.

h. Comparison of Pigmentation Index across different conditions.

i. Fold change in hair follicle length (μm) with error bars indicating variance.

j. Graph showing % K67+ (Bulge CD34+) with fold change (Rel. to Control) for different conditions.

k. Photographs demonstrating Pigmentation index differences.

l. Graph comparing Pigmentation index with error bars.