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Memory regulatory T cells reside in human skin

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Regulatory T cells (Tregs), which are characterized by expression of the transcription factor Foxp3, are a dynamic and heterogeneous population of cells that control immune responses and prevent autoimmunity. We recently identified a subset of Tregs in murine skin with properties typical of memory cells and defined this population as memory Tregs (mTregs). Due to the importance of these cells in regulating tissue inflammation in mice, we analyzed this cell population in humans and found that almost all Tregs in normal skin had an activated memory phenotype. Compared with mTregs in peripheral blood, cutaneous mTregs had unique cell surface marker expression and cytokine production. In normal human skin, mTregs preferentially localized to hair follicles and were more abundant in skin with high hair density. Sequence comparison of TCRs from conventional memory T helper cells and mTregs isolated from skin revealed little homology between the two cell populations, suggesting that they recognize different antigens. Under steady-state conditions, mTregs were nonmigratory and relatively unresponsive; however, in inflamed skin from psoriasis patients, mTregs expanded, were highly proliferative, and produced low levels of IL-17. Taken together, these results identify a subset of Tregs that stably resides in human skin and suggest that these cells are qualitatively defective in inflammatory skin disease.

Introduction

Foxp3-expressing regulatory T cells (Tregs) play an indispensable role in establishing and maintaining immune homeostasis. It was originally thought that Tregs are a relatively homogenous population generated exclusively in the thymus. However, subsequent studies revealed that an additional subset was derived from cells induced to become Tregs outside of the thymus (1), adding to the complexity of the ontogeny of this cell population. Emerging data suggest the existence of even more complexity, as multiple Treg subsets are being defined with specialized functions and unique cell fates. Perhaps the most distinct subsets of Tregs are those that reside in peripheral tissues. In the gastrointestinal tract, a population of Tregs is induced by microbial flora and is specialized to secrete IL-10 (2). In visceral adipose tissue (VAT), a population of Tregs preferentially expresses the peroxisome proliferator–activated receptor γ (PPARγ), which confers highly specialized functions, including the expression of genes involved in lipid metabolism (3).

We recently characterized a distinct population of Tregs in murine skin (4, 5). Using an inducible model of cutaneous self-antigen expression, we found that upon induction of antigen, thymus-derived Tregs are activated and accumulate in skin. A subset of these cells is maintained in the tissue for relatively long periods in the absence of antigen and has an enhanced capacity to suppress cutaneous autoimmunity when antigen is reexpressed. These cells fit stringent criteria for effector memory cells and were named memory Tregs or mTregs. Consistent with an effector memory phenotype, mTregs require IL-7 for their maintenance in skin (4).

Although studies characterizing specialized Treg subsets in murine tissues are beginning to emerge, very little is known about Tregs in human tissues. Given the limited accessibility of fresh human tissue, functional characterization of Tregs in humans has largely been limited to peripheral blood. Comprehensive analysis of Tregs in blood reveals a heterogeneous population composed of resting Tregs with a "naive" phenotype, "activated" Tregs with characteristics of memory cells, and Foxp3-expressing cells that secrete effector cytokines and lack suppressive capacity (6).

It is important to elucidate the fundamental biology of Tregs in human peripheral tissues in order to define abnormalities in these cells in inflammatory diseases and to exploit Tregs for treating such disorders. Tregs are thought to mediate the majority of their functions in the tissues in which they reside (7), and optimal therapeutic approaches directed at either augmenting or inhibiting Tregs will most likely require strategies that target specific subsets, in an attempt to efficiently treat disease and limit systemic side effects. In this report, we phenotypically and functionally characterize Tregs in human skin. Similar to the mTreg population

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identified in mice, almost all Tregs in human skin have an effector memory phenotype. We show that mTregs in human skin have distinct cell surface marker expression, cytokine production, in situ localization, TCR expression, and functional capacity in both normal and diseased skin. These results reveal a unique subset of Tregs resident in human skin and suggest that these cells may be qualitatively defective in inflammatory skin disease.

Results

Tregs in normal human skin. A comprehensive analysis of immune cell populations in human skin has been hampered by technical challenges in obtaining adequate numbers of cells from relatively harsh digestion protocols. Consequently, the majority of studies published to date rely on either immunohistochemical analyses or assays in which skin cells are cultured for several days, sometimes in the presence of growth factors (8, 9). In order to objectively study Tregs in human skin, we optimized a chemical digestion method for isolating leukocytes from this tissue. Surgically discarded samples of clinically normal-appearing skin were gently digested overnight (in the absence of exogenous growth factors), and single-cell suspensions were immediately analyzed by 12-color flow cytometry. In order to control for potential artifacts in the isolation process, human PBMCs were processed in a manner identical to that used for skin. Adult human skin contains readily detectable Foxp3-expressing $CD4^+$ T cells (Figure 1A). Whereas approximately 5% of $CD4^+$ T cells consistently express Foxp3 in adult peripheral blood, approximately 20% of $CD4^+$ T cells in adult skin express Foxp3, with considerably more variability when compared with that found in blood. Interestingly, greater than 95% of $CD4^+Foxp3^+$ cells in the skin express CD45RO, whereas 75%–80% of skin $CD4^+Foxp3^-$ cells express this marker (Figure 1B). These results indicate that almost all of the $CD4^+Foxp3^+$ cells in adult skin have previously seen antigen (outside of the thymus), consistent with a memory T cell phenotype. Interestingly, the percentage of $CD4^+Foxp3^+$ cells in human fetal skin is markedly reduced when compared with that in adult skin, with a significantly lower percentage of these cells expressing CD45RO (Figure 1C). As expected, CD45RO expression is also reduced on $CD4^+Foxp3^-$ cells in fetal skin when compared with its expression in adult skin (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI72932DS1). Taken together, these data support the notion that Tregs encounter skin-associated antigens over time and gradually accumulate in this tissue.

Tregs in human skin are activated memory cells. mTregs in murine skin express markers of prior activation as well as markers typical of effector memory T cells (4, 5). The fact that greater than 95% of Tregs in normal human skin express CD45RO suggests that this population is a bona fide memory T cell population. To explore this further, we examined these cells for both activation and effector memory T cell markers (Figure 2A). Compared with $CD45RO^+CD4^+Foxp3^+$ cells in adult peripheral blood (hereafter referred to as mTregs), the equivalent cell population in skin expressed significantly higher levels of the Treg activation markers CTLA-4 (extracellular), CD25, and ICOS (Figure 2A). Cutaneous mTregs also expressed higher levels of Foxp3 when compared with mTregs in peripheral blood (Supplemental Figure 2). With respect to memory markers, mTregs in the skin expressed high levels of CD27 and BCL-2 (Figure 2A). Interestingly, mTregs in human skin did not express high levels of IL-7Rα (CD127). This is in contrast to mTregs in murine skin, which have increased expression of CD127 (relative to Tregs in skin-draining lymph nodes) and require IL-7 for their maintenance in skin in the absence of antigen (4).

Approximately 24 hours after initiation of TCR stimulation in vitro, human $CD4^+$ T cells begin to show increased Ki67 expression (10). In the presence of persistent TCR stimulation, the percentage of Ki67-expressing cells peaks at 72 hours and steadily declines to approximate baseline levels 5 days later. In addition, the majority of naive human $CD45RO^-$ Tregs convert to $CD45RO^+$ cells within 4 days of TCR stimulation (11). If similar kinetics apply in vivo, expression of Ki67 and CD45RO can be used to approximate the window of time that a T cell has encountered antigen in humans. It follows that the majority of cells that express both Ki67 and CD45RO are likely to have seen antigen 5 days or less prior to harvesting the tissue, whereas the majority of cells that express only CD45RO (i.e., $Ki67^-$ cells) are likely to have seen antigen greater than 5 days before harvest. Consistent with previous reports (11, 12), approximately 10%–20% of $CD45RO^+$ Tregs in peripheral blood are actively cycling, as evidenced by intracellular Ki67 expression (Figure 2A). In contrast, a significantly lower percentage of mTregs in the skin are cycling. This suggests that in the steady state, a larger percentage of mTregs in peripheral blood have recently seen antigen compared with mTregs in skin, despite the fact that cutaneous mTregs express higher levels of Treg activation markers.

Lack of cytokine expression and demethylation of genomic DNA in intron 1 of the *FOXP3* gene (TSDR locus) strongly correlate with

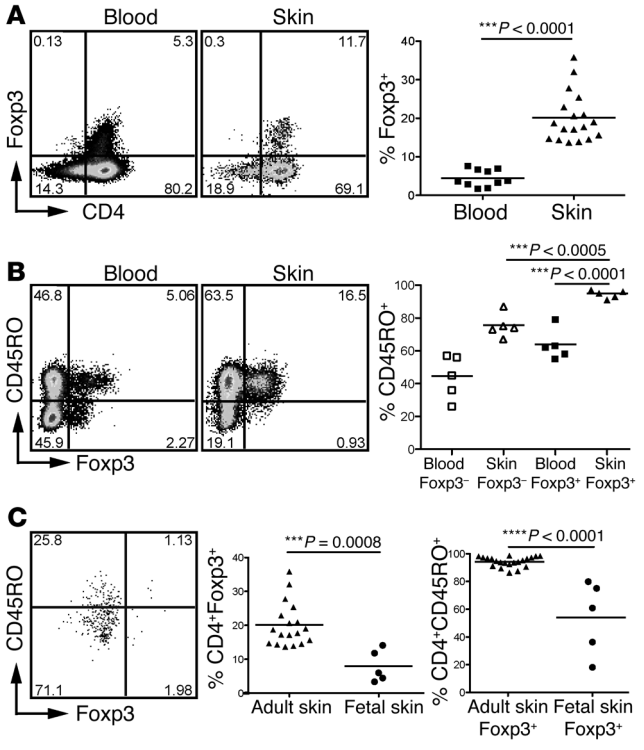


Figure 1

Foxp3 expression in human adult and fetal skin. (**A**) Expression of Foxp3 on $CD3^+$ T cells in PBMCs (Blood) and skin isolated from healthy adults. Scatter plot is gated on $CD3^+CD4^+$ cells. (**B** and **C**) Expression of Foxp3 and CD45RO on $CD3^+CD4^+$ T cells in adult PBMCs and in adult skin as well as human fetal skin. Results are combined data from five or more independent experiments. *P* values were determined using a 2-tailed unpaired Student's *t* test.

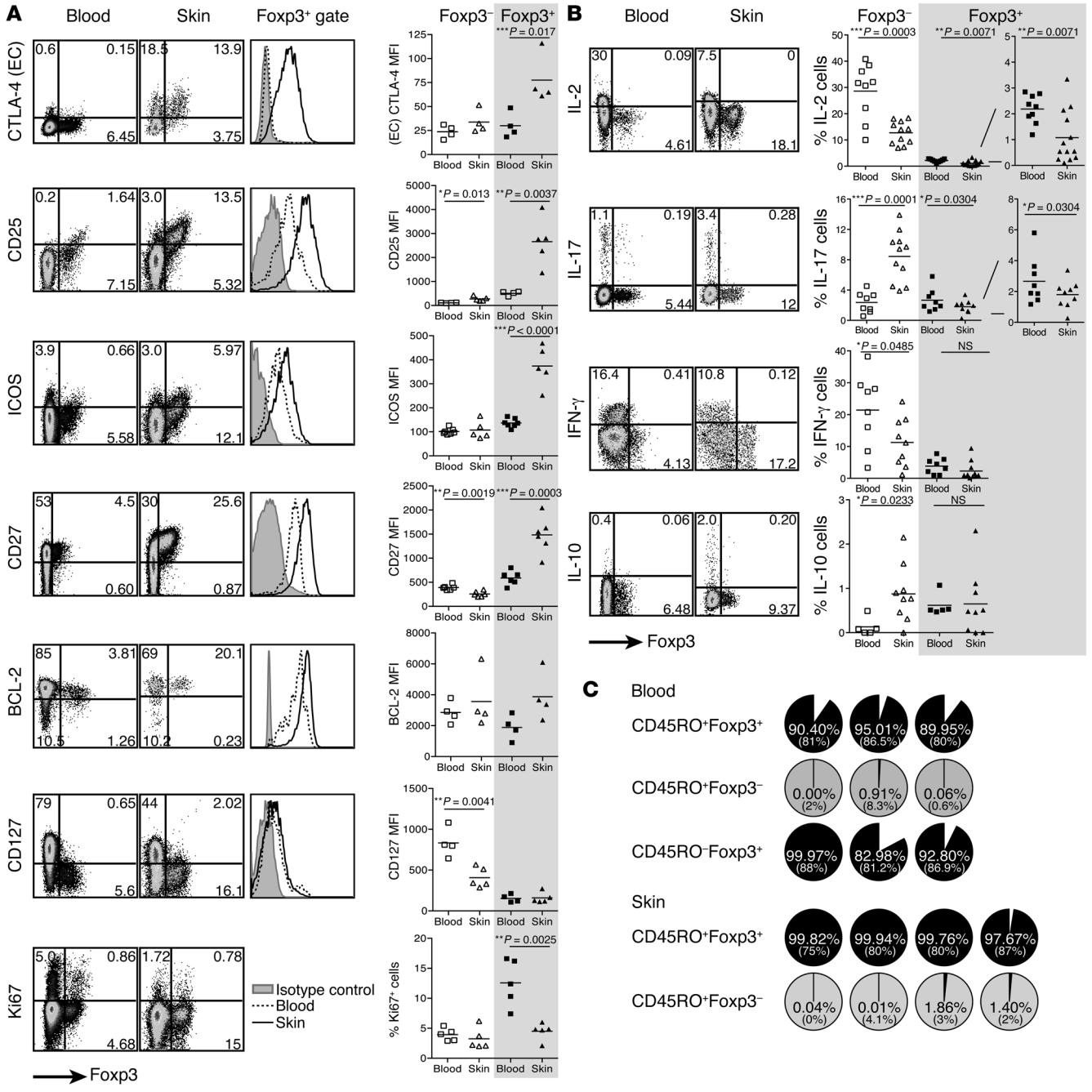


Figure 2

Tregs in human skin have an activated effector memory phenotype. (**A** and **B**) Expression of activation markers, memory markers, and cytokines from viable CD3+CD4+CD45RO+ T cells in PBMCs and skin isolated from healthy adults. Skin used for cytokine analysis in **B** was harvested from face or scalp. Scatter plots in **B** represent the percentage of cytokine-producing cells within Foxp3+ and Foxp3− gates. All gates are based on isotype control staining or unstimulated controls (for cytokine production in **B**). EC, extracellular. (**C**) Percentage of demethylation of genomic DNA in intron 1 of the *FOXP3* gene (TSDR locus) of mTregs and mTconvs in PBMCs and skin isolated from healthy adults. Each pie chart represents a sorted population purified from a different donor, and numeric values within pie charts represent the percentage of demethylation at the TSDR locus. Numeric values in parentheses below each chart represent the percentage of Foxp3-expressing cells within each purified cell population. Cell sorting strategy is shown in Supplemental Figure 4. Results in **A** and **B** are representative data from more than fifteen independent experiments. Results in **C** are from three or more replicate experiments. *P* values were determined using a 2-tailed unpaired Student's *t* test.

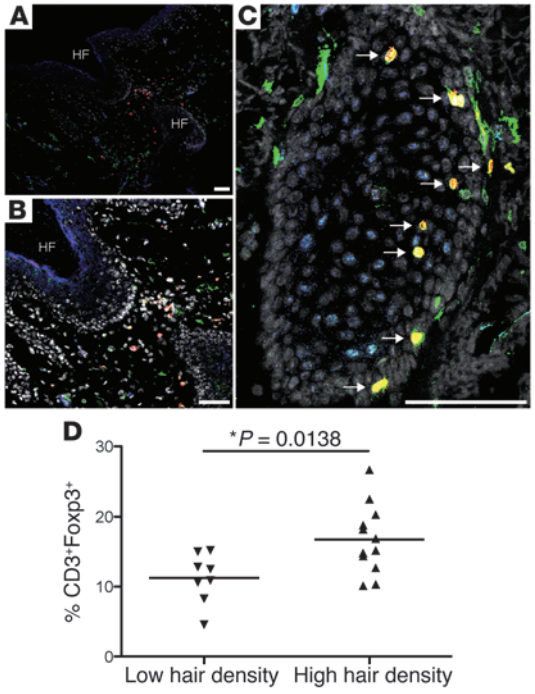


Figure 3
mTregs localize to HFs in human skin. (**A**–**C**) Confocal microscopy of normal human skin. Sections were stained for CD3 (green), Foxp3 (red), CD1a (blue), and DAPI (gray). Scale bars: 100 μm. HFs autofluoresce in blue. Arrows denote CD3+Foxp3+ cells (yellow). (**D**) Quantification of Foxp3+ cells (within the CD3+ gate) in human adult skin by flow cytometry. Low hair density represents skin harvested from anatomical sites with relatively lower HF density (trunk and upper proximal extremities), whereas high hair density represents skin harvested from anatomical locations with relatively higher hair density (scalp and face). Results are representative of more than five replicate experiments. *P* values were determined using a 2-tailed unpaired Student's *t* test.

the suppressive function of mTregs isolated from human blood (6). When compared with mTreg cells in peripheral blood, mTregs isolated from skin expressed significantly less IL-2 and IL-17, with no difference in IFN-γ or IL-10 expression (Figure 2B). Consistent with a phenotype of stably differentiated Tregs, cutaneous mTregs are fully demethylated at the TSDR region of the *FOXP3* gene (Figure 2C). Taken together, these results suggest that Tregs in normal adult skin are a highly activated, stable effector memory Treg population.

mTregs in human skin preferentially localize to hair follicles. In order to define the anatomic localization of mTregs, we performed Treg-specific immunofluorescence microscopy on normal human skin. Consistent with findings in mice (4, 13), we observed that Tregs in human skin preferentially localized to hair follicles (HFs) (Figure 3, A–C). We observed very few CD4+Foxp3+ cells in the interfollicular dermis, as most cells were localized in close proximity to the follicular epithelium. In contrast, CD4+ Foxp3− conventional T cells (Tconvs) displayed a more diverse distribution with little predilection for HFs. Consistent with these findings, flow cytometric quantification showed that skin with high hair density (i.e., scalp and face) had significantly higher percentages of Tregs when compared with skin with low hair density (Figure 3D). We found no significant difference in Treg percentages when data were stratified by gender and age (data not shown); however, only adult skin (>19 years of age) was available for analysis.

mTregs in human skin express unique TCRs. Discovering the nature of the antigens that T cells recognize in tissues is of fundamental importance in elucidating the biology of these cells. This is especially true for human tissue, in which antigen-specific T cell–directed therapies are being implemented to treat autoimmune diseases and cancer (14). An essential question that remains to be answered is whether Tconvs and Tregs found in the same tissue recognize the same or different antigens. To begin to answer this question, we performed deep sequencing of the TCR β chain (TCRβ) from mTregs and memory CD4+ Tconvs (mTconvs) purified from normal human skin. TCR sequence diversity in the CDR3 region is an indirect measure of TCR specificity (15, 16). Thus, we compared TCRβ sequences between mTconvs and mTregs, with the rationale that sequences shared between these two cell populations suggest the possibility that they recognize the same antigens, whereas nonoverlapping sequences suggest that they recognize different antigens. We chose sequencing of TCRβ, because this chain of the TCR contains the most sequence diversity (15). Surprisingly, we found that very few TCRβ sequences were shared between mTconvs and mTregs (Figure 4 and Table 1), suggesting that these two T cell populations predominantly recognize different antigens. Although sequencing depth was limited by the number of cells we could extract from skin for these analyses, we used a series of Monte Carlo statistical simulations (see Methods) in four separate experimental replicates to determine that the mTreg population is qualitatively different from the mTconv population. Taken together, these results indi-

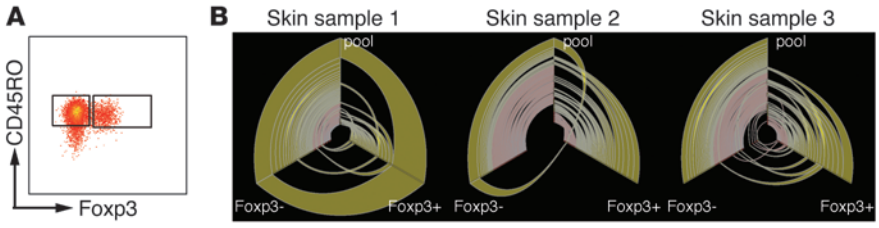


Figure 4
TCRβ sequencing of mTregs and memory Tconvs in human skin. (**A**) Gating strategy for sorting of mTconvs and mTregs from human skin harvested from healthy adults. Cells are pregated on viable CD3+CD4+ cells. (**B**) Hive plots showing relative abundance of unique TCRβ sequence clones and relative overlap of unique sequences between the Foxp3+ and Foxp3− pools. Each line represents one unique clone. The width of the lines denotes the relative abundance of each clone in the Foxp3+ and Foxp3− fractions or the pool of both populations. The most abundant clones (i.e., the widest lines) are positioned distally from the center of the hive, whereas the least abundant clones (thinnest lines) are located centrally. Lines that connect the Foxp3+ and Foxp3− fractions represent unique sequences shared between these cell populations. For example, in skin sample 2, the most abundant sequence in the Foxp3− fraction is present in relatively low frequency in the Foxp3+ fraction and represents the most abundant sequence in the entire pool. In this sample, this sequence is the only one shared between the Foxp3− and Foxp3+ cell populations. *P* values were determined using Monte Carlo simulation for the probability that Foxp3+ cells were not a random sampling of the pooled population (see Methods).

Table 1
TCRβ sequencing of mTregs and mTconvs in normal human skin

Skin sample	Unique Foxp3$^+$ clones	Unique Foxp3$^-$ clones	Shared sequences Foxp3$^{+/-}$	*P* value
1	447	317	5	$P < 0.0001$
2	1,508	8,717	1	$P < 0.0001$
3	773	4,342	9	$P < 0.0001$
4	466	1,832	46	$P < 0.0001$

Quantification of unique TCRβ sequence clones after deep sequencing of genomic DNA from mTregs and mTconvs purified as described. Data shown are results from skin samples obtained from four different healthy donors.

cate that mTregs in the skin possess a distinct TCR repertoire, suggesting that they recognize different antigens than those recognized by cutaneous mTconvs.

mTregs in human skin are nonmigratory. Based on chemokine receptor expression patterns, it has been postulated that the majority of T cells in human skin are resident in this tissue and are thus nonmigratory (9). To a large extent, this conclusion is based on the lack of CCR7 expression seen on T cells isolated from skin. Given that CCR7 mediates the migration of memory T cells to secondary lymphoid organs (17), we analyzed the expression of this chemokine receptor on cutaneous mTregs and mTconvs. Consistent with previous reports (9), the majority of T cells isolated from human skin lacked CCR7 expression (Figure 5A). However, we observed a marked dichotomy within the small fraction of T cells that did express CCR7. This chemokine receptor was almost exclusively expressed on mTconvs (Figure 5A). These results suggested that mTregs are a more skin-resident (i.e., nonmigratory) cell population when compared with mTconvs. To test this, we used an in vivo model of human T cell migration. When human skin is grafted onto immunodeficient mice, a functional human microcirculation develops, allowing for the migration of human and murine cells to and from the grafted tissue (18). To determine the relative migratory potential of cutaneous mTregs and mTconvs in vivo, we grafted normal human skin onto immunodeficient NSG mice and assayed for these cell populations in the blood and spleen 3 and 7 weeks after grafting. We performed the analyses 3 or more weeks after grafting to allow inflammation and wound healing to resolve and to allow human dermal microvessels to anastomose with murine blood vessels (18). Consistent with the relative differences in CCR7 expression, we readily detected mTconvs in blood and spleen at all time points analyzed, whereas mTregs were undetectable in the blood and spleen and were found only in skin grafts (Figure 5B). Interestingly, we found that mTconvs proliferated in grafted skin, as evidenced by intracellular Ki67 expression; in contrast, mTregs maintained the basal proliferation levels (~5% Ki67-expressing cells) we observed in nongrafted normal skin (Figure 2A). Taken together, these results suggest that mTregs in human skin are nonmigratory and relatively anergic when compared with cutaneous mTconvs.

mTregs proliferate and produce IL-17 in psoriatic skin. The studies outlined above phenotypically and functionally characterize mTregs in normal human skin. Because it is important to know how these cells function in both homeostatic and inflammatory contexts, we set out to functionally define these cells in the skin of patients with psoriasis. Due to inherent difficulties in obtaining adequate numbers of cells for functional analyses (from relatively small skin biopsy specimens), previous studies examining Tregs in psoriasis patients have relied on analyzing peripheral blood and/or performing semiquantitative microscopic analyses on tissue sections (19, 20). It has become increasingly clear from both mouse models and studies of human disease that the secondary lymphoid organs and peripheral blood may not accurately represent the pathologic processes in tissues. We optimized our skin digestion protocol to functionally characterize mTregs from 4-mm skin punch biopsies obtained from patients with clinically active psoriasis. In the majority of these studies, we compared lesional skin with nonlesional skin (defined as >10 cm away from a psoriatic plaque harvested from the same anatomical site) obtained from the same patient in an attempt to

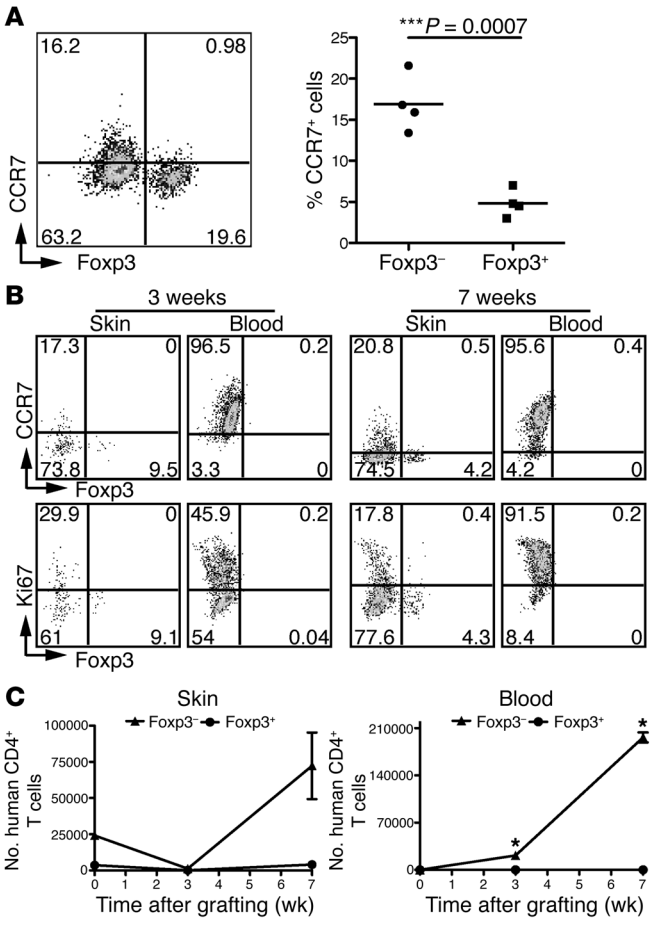


Figure 5
mTregs in human skin are nonmigratory. (**A**) CCR7 expression on mTregs and mTconvs in human skin harvested from healthy adults. Cells were pregated on viable CD3$^+$CD4$^+$CD45RO$^+$ cells. (**B**) Human skin was grafted onto immunodeficient NSG mice, and at specific times thereafter, grafted skin, blood, and spleen (not shown) were harvested and human T cells analyzed by flow cytometry. Cells were pregated on viable CD3$^+$CD4$^+$hCD45$^+$ cells. (**C**) Absolute numbers of CD3$^+$CD4$^+$ hCD45$^+$Foxp3$^+$ and CD3$^+$CD4$^+$hCD45$^+$Foxp3$^-$ cells in grafted skin and blood 3 and 7 weeks after grafting. Results are pooled data from two of five representative experiments with two or more mice per group. *P* values were determined using a 2-tailed unpaired Student's *t* test.

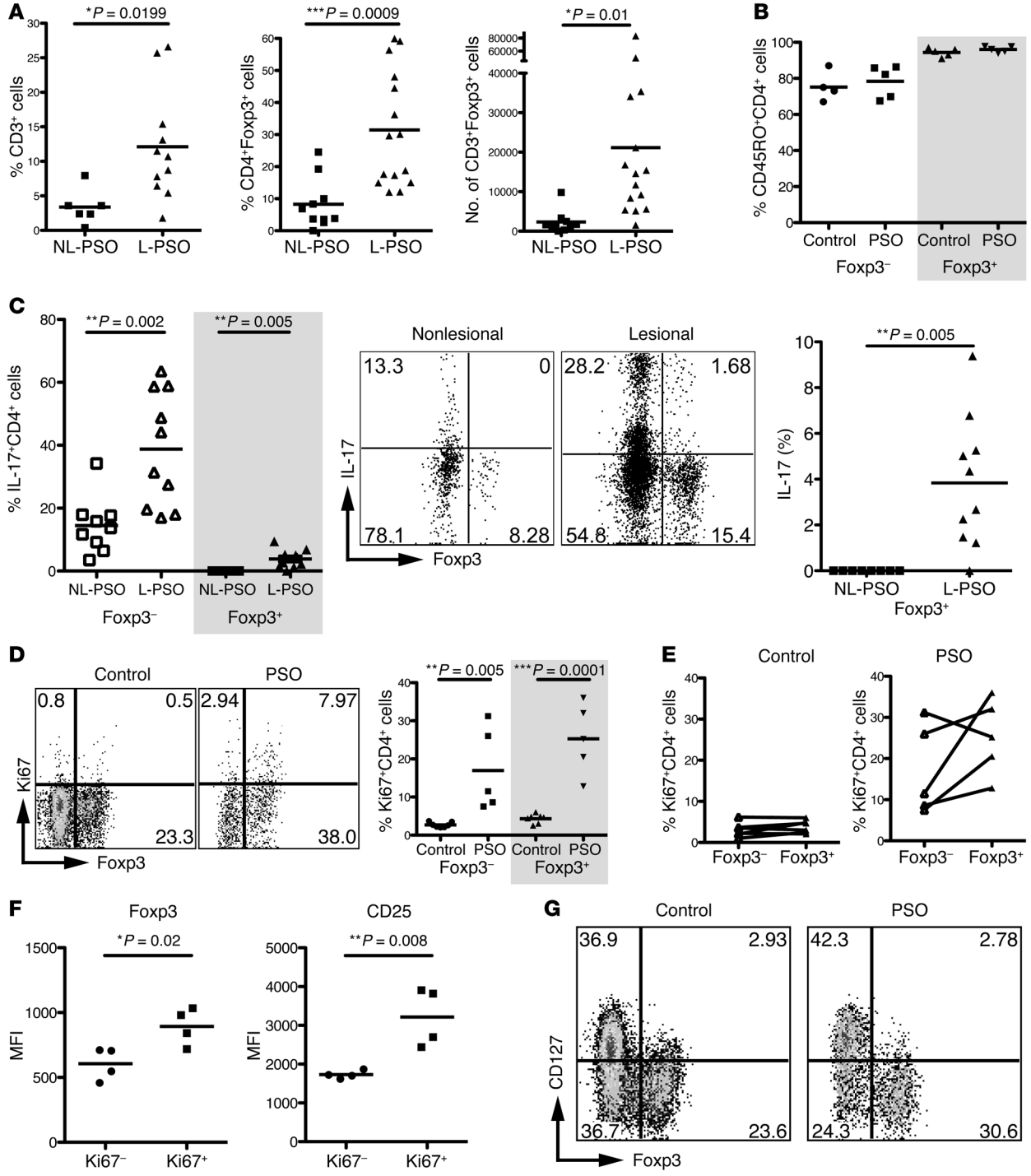


Figure 6

Functional analysis of cutaneous mTregs in patients with psoriasis. (**A**) Percentage and absolute number of T cells and Tregs in nonlesional psoriatic (NL-PSO) and lesional psoriatic (L-PSO) skin from patients (middle and right panels gated on $CD4^+$ and $CD3^+$ cells, respectively). (**B**) Percentage of mTregs and mTconvs in L-PSO skin or normal healthy adult skin (Control), pregated on viable $CD3^+$ cells. (**C**) Intracellular IL-17 production from mTregs and mTconvs in NL-PSO and L-PSO skin, gated on viable $CD3^+CD4^+CD45RO^+$ cells. Middle panel is a representative flow cytometric plot of IL-17 production, and the scatter plot shows percentages of IL-17–producing cells within the Foxp3 gate. (**D**) Expression of Ki67 in mTregs and mTconvs in L-PSO skin or site-matched control skin, gated on viable $CD3^+CD4^+CD45RO^+$ cells. (**E**) Expression of Ki67 in mTregs and mTconvs in L-PSO or control skin, gated on viable $CD3^+CD4^+CD45RO^+$ cells. Lines represent paired data from a single patient. Mean deltas between $Foxp3^-$ and $Foxp3^+$ cells when comparing PSO versus control skin are 8.340 ± 5.05 versus 0.735 ± 0.50, respectively ($P = 0.132$). (**F**) MFI of Foxp3 and CD25 expression on $Ki67^+$ and $Ki67^-$ mTregs in L-PSO skin, gated on viable $CD3^+CD4^+CD45RO^+Foxp3^+$ cells. (**G**) Foxp3 and CD127 expression on mTregs and mTconvs in L-PSO or site-matched control skin, gated on viable $CD3^+CD4^+CD45RO^+$cells. Results are combined data from five or more replicate experiments.

internally control for variability between patients and between anatomic locations. However, in situations where this was not possible, we compared cells obtained from psoriatic skin with site-matched skin from normal healthy controls. As expected, when compared with nonlesional skin, psoriatic skin had an increased percentage of T cells (Figure 6A). We found that increased percentages and absolute numbers of Foxp3-expressing T cells were present in psoriatic skin (Figure 6A). The percentage of $Foxp3^+$ cells that coexpressed CD45RO was similar between lesional and nonlesional skin (Figure 6B), suggesting that the increased Foxp3-expressing cells we observed in lesional skin were not secondary to an influx of naive cells. When compared with nonlesional skin, $CD45RO^+CD4^+Foxp3^+$ cells in lesional skin produced more IL-17 (Figure 6C). We have observed that the frequency of IL-17–producing T cells in normal skin varies with anatomic location. Normal facial/scalp skin contains a higher percentage of IL-17–producing T cells when compared with normal trunk skin (Supplemental Figure 3). Interestingly, lesional skin harvested from the trunk of psoriasis patients contained a higher percentage of IL-17–producing mTregs when compared with either nonlesional trunk skin (harvested from the same patient) or facial/scalp skin harvested from normal healthy controls (Supplemental Figure 3). There was also a trend toward increased IFN-γ production from $CD45RO^+CD4^+Foxp3^+$ cells isolated from lesional psoriatic skin compared with that seen in nonlesional skin; however, this was not statistically significant (Supplemental Figure 4). Interestingly, in contrast to both nonlesional skin and normal skin grafted onto immunodeficient mice (described above), $CD45RO^+CD4^+Foxp3^+$ cells in psoriasis lesions were actively proliferating, with approximately 15% to 35% of cells expressing Ki67 compared with baseline levels of 5% in the skin of healthy controls (Figure 6D). A higher percentage of $Foxp3^+$ cells were cycling compared with $Foxp3^-$ cells in 4 of 5 patients (Figure 6E). It is known that Foxp3 is transiently expressed in recently activated human $CD4^+$ Tconvs (21, 22). Thus, it is possible that increased Foxp3-expressing cells in psoriatic skin represent a recently activated, proliferating Tconv population. However, human Tconvs that transiently express Foxp3 express this protein at lower levels compared with activated Tregs (23). In addition, Foxp3-expressing Tconvs express lower levels of CD25 and do not repress CD127 expression or IL-2 production (23). In psoriatic skin, $CD45RO^+CD4^+Foxp3^+$ cells have a phenotype that is completely different from that of Foxp3-expressing Tconvs. Proliferating $Foxp3^+$ cells in psoriatic skin express high levels of Foxp3 and CD25 (Figure 6F), maintain low levels of CD127 expression (Figure 6G), and do not produce IL-2 (Supplemental Figure 4). Thus, our data suggest that $Foxp3^+$ cells proliferating in psoriatic skin are bona fide mTregs and are not transiently activated Tconvs. These results raise the intriguing possibility that excessive proliferation, perhaps associated with some production of effector cytokines, is a property of mTregs in inflammatory diseases such as psoriasis. Whether these putative Treg abnormalities are the cause or the consequence of inflammation is, of course, a question of fundamental importance for understanding the role of these cells in the disease process.

Discussion

In the studies described herein, we phenotypically and functionally characterize Tregs in human skin. We define this population as being bona fide effector memory cells with distinct cell surface marker expression, cytokine production, in situ localization, TCR expression, and functional capacity in two different biological contexts. Because of these attributes, we propose that this subset of Tregs is a unique cell population resident in human skin.

Our data are in agreement with previous reports showing that Tregs comprise approximately 10% of all T cells in normal human skin (8). We define this population as being highly activated memory cells similar to the "activated Treg" or "aTreg" population previously described in human blood (6). However, compared with aTregs in blood, we observed that mTregs in skin expressed higher levels of Treg activation markers, contained a lower percentage of cycling cells, and produced less IL-2 and IL-17 (Figure 2). These findings suggest that mTregs may be further along the Treg differentiation pathway than aTregs and perhaps represent the final stage in Treg differentiation, a stage present only in peripheral tissues.

Sakaguchi and colleagues' characterization of Tregs in human blood and our studies in transgenic mice support a model in which the life cycle of a Treg involves activation of naive cells by antigen, followed by proliferation and differentiation to become more potent suppressor cells (5, 6). We have previously shown that murine Tregs activated by self-antigen expressed in skin progressively accumulate in this tissue (5). Here, we show that human fetal skin has significantly less Tregs than adult skin, with fewer CD45RO-expressing cells (Figure 1). This directly supports a model in which throughout the life of an individual, Tregs are continually exposed to cutaneous antigens and progressively accumulate in skin. The nature of the antigens recognized by mTregs (i.e., self-proteins expressed in the postnatal environment, foreign pathogens, and/or commensal microbes) remains to be determined.

It is intriguing that in both mice and humans, mTregs preferentially localize to the epithelium of HFs (Figure 3 and refs. 4, 13). This may be a consequence of the antigens that mTregs recognize and/or a unique niche provided by these highly specialized and dynamic organelles. HFs are constantly cycling, with waves of new protein expression at each stage of the cycle that change over time (24). In addition, HFs are colonized by a multitude of microbes (25). Thus, it is likely that many HF-associated antigens are not expressed in the thymus, making peripheral tolerance mechanisms essential to prevent autoimmune attack. In addition, epidermal stem cells reside in a specific HF niche, providing another important reason to prevent autoimmunity directed at these structures (26). It is possible that HFs recruit the services of Tregs for this purpose. Consistent with the idea that Tregs play an important role in regulating HF-associated autoimmunity are recent studies of alopecia areata (AA). AA is one of the most common autoimmune diseases in humans and results from a T cell–mediated autoimmune attack on HFs (27). Interestingly, the largest genome-wide association study in patients with AA shows a strong correlation with polymorphisms in *CTLA4*, *CD25*, and *EOS* (28), all of which play essential roles in Treg function (29, 30). A subsequent study directly correlates polymorphisms in the *FOXP3* promoter with AA (31).

Our comparison of TCRβ sequences between mTconvs and mTregs in the skin shows very little overlap, suggesting that these two populations recognize different antigens (Figure 4 and Table 1). Previous studies examining TCR repertoires between Tconvs and Tregs in human blood and murine lymphoid organs have revealed various degrees of overlap (32–35). However, few studies have examined these T cell populations in tissues. Perhaps the best-characterized tissue-specific Tregs to date are those that reside in VAT (3, 36). Consistent with our data, TCR repertoires between Tregs and Tconvs in murine VAT show very little overlap (36).

Thus, it seems that tissues are enriched for T cells that have a narrow TCR repertoire when compared with blood and secondary lymphoid organs and that TCR specificities in tissues differ between Tregs and other $CD4^+$ T cell populations. It is intriguing to speculate that in normal healthy tissue, Tregs recognize self-antigens and/or commensal microbes, whereas Tconvs recognize foreign pathogens. To the best of our knowledge, this is the first report of TCR sequencing of cells purified from peripheral tissue in humans. Determining the antigens that Tregs and Tconvs recognize in tissues may have a profound impact on our ability to specifically manipulate these cell populations for therapeutic benefit.

Examination of mTregs in human skin grafted onto immunodeficient mice revealed this population to be nonmigratory and relatively anergic compared with cutaneous Tconvs (Figure 5). This is consistent with recent studies performed in murine skin, where Tregs were relatively nonmigratory in the steady state when compared with other T cell populations in skin (13). In our humanized mouse model, it is unknown whether Tconvs cycle in response to lymphopenia or to recognition of xenoantigen and whether entry into the cell cycle contributes to their migratory potential. Furthermore, although differences in CCR7 expression could predict differences in migratory capacity, the extent to which this chemokine receptor mediates Tconv migration out of grafted skin in this model is not known. Nevertheless, grafting human skin onto immunodeficient mice represents one of the best in vivo models for studying human cutaneous T cell migration. Our assay clearly reveals mTregs to have markedly less migratory potential compared with that of Tconvs. It is intriguing to speculate that mTregs in skin have evolved to become tissue-resident cells as a result of a variety of essential functions they play within the tissue, making their continued presence essential for their tissue-specific function. Indeed, VAT Tregs in mice (and not VAT Tconvs) have been shown to play a role in both glucose and lipid metabolism that may be independent of their ability to suppress inflammation (3).

Although mTregs minimally proliferate in normal skin, we found that these cells actively cycle in inflamed lesions of psoriasis (Figure 6). Despite increasing in percentage relative to Tconvs, mTregs are unable to resolve disease. Thus, we observed that mTregs in psoriatic skin appear to be qualitatively defective in controlling inflammation. This is supported by previous work showing that local production of IL-6 impairs Treg function in patients with psoriasis (20). In this study, DCs, ECs, and T cells in psoriatic skin expressed high levels of IL-6, and Tregs in lesional skin expressed IL-6R. Peripheral blood–derived DCs from psoriatic patients inhibited Treg-mediated suppression in vitro in an IL-6–dependent fashion, with no effect on Treg proliferation. The inability of mTregs to resolve disease may also in part be a result of aberrant IL-17 production by this cell population. A small but significantly increased percentage of mTregs in psoriatic skin produced IL-17 compared with mTregs in nonlesional skin (Figure 6C), and expression of this cytokine was normally suppressed in mTregs when compared with aTregs in peripheral blood (Figure 2B). Consistent with this, a recent report showed that peripheral blood–derived Tregs from patients with psoriasis more readily differentiate into IL-17A–producing cells upon stimulation ex vivo (19).

The magnitude and nature of Treg defects in autoimmune diseases remain fundamental and largely unresolved issues. In order to address this, it is essential to first define the properties of these cells in healthy individuals. Because of the emerging idea that immune regulation occurs largely in tissues, we chose to address these questions in an accessible human tissue, the skin. The studies described herein are initial experiments attempting to define long-lived Tregs in healthy human skin. Our preliminary studies of psoriasis patients have already raised the possibility of unexpected Treg defects associated with this disease. Further elucidating the fundamental biology of Tregs in tissues and the factors that influence their maintenance and functions in health and disease is an essential next step in our attempt to understand how immune responses are regulated in humans.

Methods

Human specimens. Normal human skin was obtained from patients at UCSF undergoing elective surgery, in which healthy skin was discarded as a routine procedure. Skin was obtained from psoriasis patients (female and male; age range 20–76 years) who had been off all systemic therapy for greater than 3 weeks prior to surgery. All patients had greater than 10% body surface area involvement, with psoriasis severity index scores (PASI) ranging from 10 to 40. Four-millimeter punch biopsies were obtained from psoriatic plaques (lesional) and from clinically normal-appearing skin (nonlesional) at least 10 cm away from the lesional skin biopsies (i.e., in the same anatomic location).

Mice. All animal studies were performed in compliance with the US Department of Health and Human Services guidelines for the care and use of laboratory animals and were approved by the Laboratory Animal Resource Center of UCSF. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from The Jackson Laboratory.

Skin digestion and flow cytometry. Skin samples were stored at 4°C in a sterile container with PBS and gauze until the time of digestion. Subcutaneous fat and hair were removed, and skin was minced finely with dissection scissors and mixed in a 6-well plate with 3 ml of digestion buffer consisting of 0.8 mg/ml Collagenase Type 4 (4188; Worthington), 0.02 mg/ml DNAse (DN25-1G; Sigma-Aldrich), 10% FBS, 1% HEPES, and 1% penicillin/streptavidin in RPMI medium. Samples were incubated overnight in 5% CO_2 and harvested with wash buffer (2% FBS, 1% penicillin/streptavidin in RPMI medium), then double filtered through a 100-μm filter, centrifuged, and counted. Human PBMCs were prepared by Ficoll-Hypaque gradient centrifugation. Fresh, thawed PBMCs were incubated overnight with digestion buffer, similarly to the skin samples. For detection of intracellular cytokine production, skin cells and PBMCs were stimulated with 70 ng/ml PMA and 700 ng/ml ionomycin in the presence of brefeldin A (Sigma-Aldrich) for 4 to 5 hours. The following antibodies were used for flow cytometry: anti–hBCL-2-PE (BD Biosciences); anti–hCCR7-PE (BioLegend); anti–hCD3 (-Alexa700, -APC, -APC-eFluor780, -eFluor605, -FITC, or -PE from eBioscience or -PE-Cy7 from BioLegend); anti–hCD4 (-PE-Cy7 from BD or -PerCP from BioLegend); anti–hCD25-PE-Cy7 (BD); anti–hCD27-APC-eFluor780 (eBioscience); anti–hCD45RO-FITC (eBioscience); anti–hCD127-Brilliant Violet 650 (BioLegend); anti–hCTLA-4-PE (eBioscience); anti–hFoxp3 (-eFluor 450 or -APC from eBioscience); anti-hICOS-APC (eBioscience); anti–hIFN-γ (-APC from eBioscience or -Alexa700 from BioLegend); anti–hIL-2-Alexa700 (BioLegend); anti–hIL-10-APC (BD); anti–hIL17A -PE (eBioscience); anti–Ki67 (-Alexa647 or -PE-Cy7 from BD); anti–hTNF-α-PE-Cy7 (eBioscience); and LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies). Data were acquired by an LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.).

FOXP3 gene DNA methylation. Multiplex quantitative PCR was used to analyze the methylation status of specific CpG dinucleotides in the TSDR region of the human *FOXP3* gene as described previously (37). PCR primers and probes used in these assays were: GGTTTGTATTTGGGTTTTGTTGTTATAGT (forward); CTATAAAATAAAATATCTACCCTCTTCTCTTCCT

(reverse); VIC-TGGTGGTTGGATGTGTTG-MGBNFQ (unmethylated probe); and 6FAM-CGGTCGGATGCGTC-MGBNFQ (methylated probe). The sorting strategy for mTreg and mTconv purification is outlined in Supplemental Figure 5. Briefly, viable $CD3^+CD4^+CD45RO^+CD25^{hi}CD27^{hi}$ cells and $CD3^+CD4^+CD45RO^+CD25^-CD27^-$ cells were FACS purified from discarded skin harvested from normal healthy males. In addition, viable $CD3^+CD4^+CD45RO^+CD25^{hi}CD27^{hi}$ cells, $CD3^+CD4^+CD45RO^-CD25^{hi}CD27^{hi}$ cells, and $CD3^+CD4^+CD45RO^+CD25^-CD27^-$ cells were purified from healthy male human PBMC samples. Foxp3 staining on sorted cell populations verified that $CD25^{hi}CD27^{hi}$ cells were greater than or equal to 75% $Foxp3^+$ and that $CD25^-CD27^-$ cells were less than or equal to 8.3% $Foxp3^+$.

Immunofluorescence microscopy. Skin specimens were immediately placed in Tissue-Tek OCT Compound (Fisher Scientific), frozen on dry ice, and stored at –80°C. Cryosections (6-mm) were fixed in 100% acetone and stained for Foxp3 (rat IgG2a, clone PCH101, dilution 1:50; eBioscience), CD3 (rabbit IgG, polyclonal, dilution 1:500; Abcam), and CD1a (mouse IgG1, clone 7A7, dilution 1:100; Abcam). Secondary antibodies recognizing rat (donkey IgG, Alexa Fluor 488), rabbit (donkey, Alexa Fluor 555) and mouse (goat, Alexa Fluor 647) were used at 1:1,000 and obtained from Life Technologies, as was the DAPI used for nuclear staining. Confocal images were acquired using a Nikon C1si spectral confocal microscope.

TCR sequencing. Genomic DNA was isolated from FACS-purified $CD3^+CD4^+CD45RO^+Foxp3^+$ and $CD3^+CD4^+CD45RO^+Foxp3^-$ subsets. TCRβ repertoire sequencing was performed using GigaMune Rep-Seq molecular kits and ClonoByte repertoire analysis software (GigaGen). Briefly, genomic DNA was amplified by PCR with a set of 45 primers targeting the TCRβ V genes paired with 13 primers targeting the TCRβ J genes. This set of 58 primers amplifies the CDR3 region of TCRβ and also introduces universal priming sites to the amplicons. A second round of PCR was performed on the resulting amplicons using universal primers. Each sample was indexed with a unique 6-nucleotide tag, allowing demultiplexing of samples after sequencing. Samples were then sequenced on a MiSeq sequencer (Illumina) to a length of 150 bp. The sequencing reads for each sample were analyzed using ClonoByte TCRβ repertoire analysis software (GigaGen). ClonoByte aligns each sequence to the set of TCRβ V and TCRβ J genes and identifies the conserved cysteine and phenylalanine that form the boundaries of the CDR3. For quality purposes, reads that did not have a uniquely identifiable V gene, were out of frame, as defined by the conserved cysteine and phenylalanine, or contained a stop codon or a sequencing error in the form of an uncalled base, were discarded. All other nucleotide sequences were translated into their amino acid equivalent. To avoid distorting the diversity of the TCRβ CDR3 repertoire by the tail of low-abundance clones caused by PCR and sequencing errors, we ran a no-template negative control (NTC) alongside each batch of samples for every stage in the process, including sequencing. In the rare cases in which the NTC showed a strong spurious CDR3 signal, we removed those clones from all corresponding samples. Additionally, we removed all reads mapping to clones whose frequency was less than 0.1% of the most abundant clone's frequency and then normalized the remaining frequencies to the total number of remaining reads. This conservative criterion minimizes the chance of unique clones likely to have arisen due to PCR and sequencing errors being treated as real. A Monte Carlo simulation was conducted for each skin sample, wherein a simulated pool of $Foxp3^+$ and $Foxp3^-$ cells was created, followed by a random subsampling of clones equal to the number of clones in the Foxp3 population for that sample. After 10,000 random subsamplings, we calculated the Bhattacharyya coefficient (BC) for each subsample and pool pair, which quantifies the similarity between each subsample and the pool pair and is calculated as:

$$\sum_{j=1}^{n}\sqrt{f_{j,1}\times f_{j,2}} \qquad \text{(Equation 1)}$$

where $f_{j,1}$ and $f_{j,2}$ are the frequencies of clonotype j in samples 1 and 2, respectively, and n is the number of unique clonotypes present across samples 1 and 2. The distribution of BC scores in the Monte Carlo simulation were then used to calculate a P value for the probability that the $FoxP3^+$ cells were not a random sampling of the full skin TCRβ cell population.

Skin grafting. Normal human skin (~1 cm × 1 cm) was grafted onto the backs of NSG mice as previously described (38). Briefly, healthy skin was dermatomed to a thickness of 0.4 mm and transplanted using an absorbable tissue seal (Vetbond; 3M). Bandages were maintained for 1 week after grafting. Transplanted human skin, peripheral blood, and spleens were harvested, processed, and analyzed by flow cytometry 3 and 7 weeks after grafting.

Statistics. Analysis of the TCR sequencing data is described above. Other group-versus-group data comparisons were performed using a 2-tailed unpaired Student's t test. P values of less than 0.05 were considered significant.

Study approval. Studies using human tissue were approved by the UCSF Committee on Human Research (study number 10-02830) and by the IRB of UCSF. All patients provided written informed consent prior to biopsies. Fetal skin samples were obtained from the UCSF Medical Center and San Francisco General Hospital (San Francisco, California, USA). Samples were from second trimester skin that would have otherwise been discarded at the time of the procedure. Blood samples were obtained from healthy adult volunteers (study number 12-09489). Animal experiments were approved by the IACUC of the UCSF.

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