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Dysfunctional Blood and Target Tissue CD4⁺CD25^{high} Regulatory T Cells in Psoriasis: Mechanism Underlying Unrestrained Pathogenic Effector T Cell Proliferation¹

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The balance between regulatory and effector functions is important for maintaining efficient immune responses, while avoiding autoimmunity. The inflammatory skin disease psoriasis is sustained by the ongoing activation of pathogenic effector T cells. We found that a CD4⁺ T lymphocyte subpopulation in peripheral blood, phenotypically CD25^{high}, CTLA-4⁺, Foxp3^{high} (regulatory T (Treg) cells), is deficient in its suppressor activity in psoriasis. This was associated with accelerated proliferation of CD4⁺ responder T cells in psoriasis, the majority of which expressed CXCR3. Nevertheless, criss-cross experiments isolated the defect to psoriatic Treg cells. To examine Treg cells in a nonlymphoid tissue of a human T cell-mediated disease, Treg cells were also analyzed and isolated from the site of inflammation, psoriatic lesional skin. At the regulatory vs effector T cells ratios calculated to be present in skin, however, the psoriatic Treg cell population demonstrated decreased suppression of effector T cells. Thus, dysfunctional blood and target tissue CD4⁺CD25^{high} Treg cell activity may lead to reduced restraint and consequent hyperproliferation of psoriatic pathogenic T cells in vivo. These findings represent a critical component of human organ-specific autoimmune disease and may have important implications with regard to the possible therapeutic manipulation of Treg cells in vivo. *The Journal of Immunology*, 2005, 174: 164–173.

Recently, several T cell subsets with immunoregulatory functions have been described, and their crucial roles in certain animal autoimmune disease models have been shown (1). One such T cell subset was initially identified in mice, as CD4⁺ T cells that constitutively express CD25, the IL-2R α -chain (regulatory T (Treg)⁴ cells) (2–4). This population is characterized by its ability to inhibit the development of autoimmune gastritis after neonatal thymectomy (2) and constitutes ~10% of peripheral CD4⁺ cells. Murine Treg cells are anergic when stimulated in vitro with anti-CD3 mAbs, but proliferate upon addition of exogenous IL-2 (3, 4) and show remarkable suppressive capacity both in vitro and in vivo (5–9). After TCR-mediated stimulation, CD4⁺CD25⁺ Treg cells suppress the activation and proliferation of other CD4⁺ and CD8⁺ T cells in an Ag-nonspecific manner (3, 10) via a mechanism that requires cell-cell contact and

in most systems is independent of the production of immunosuppressive cytokines (3, 4). Murine CD4⁺CD25⁺ Treg cells constitutively express CTLA-4 (6), which is known as a negative regulator of T cell activation, and expression of this molecule is required for these cells to suppress immune responses in vivo (7, 11).

CD4⁺CD25⁺ Treg cells are also likely to be relevant to immune-mediated diseases in humans. These cells are found in both the thymus and the peripheral blood of humans at similar frequencies as those observed in mice and rats. Functionally these cells are very similar to those identified in mice, in that they fail to proliferate in response to polyclonal stimulation and are able to suppress T cell responses in a cytokine-independent, but contact-dependent, manner (12–15).

Psoriasis, a common disorder affecting 1–2% of individuals in Western societies, bears many features of a T cell-mediated autoimmune disease and shares predisposing genes with the common forms of autoimmunity targeted to peripheral organs, such as multiple sclerosis (16) and Crohn's disease (17). Psoriatic skin lesions are sharply demarcated, erythematous, raised, scaling plaques containing hyperproliferating keratinocytes and endothelial cells as well as a variable infiltrate of granulocytes and a dense mononuclear infiltrate with activated T cells and hyperstimulatory APCs (18–25). Although the pathogenesis of this disease is not well understood, a variety of studies suggest that intralesional activated T cells produce cytokines that trigger primed basal stem cell keratinocytes to proliferate and perpetuate the disease (26–28). Clinical trials demonstrate that targeting of pathogenic activated and memory T cells with fusion proteins or Abs specific for CD2⁺ and CD25⁺ T cells can result in clinical improvement of the disease (29–34). Activated T cells from psoriasis patients, but not healthy controls, induced psoriasis-like changes when injected into human skin samples transplanted onto SCID mice (35). T cell clones established from psoriatic skin lesions had the select capacity of

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⁴ Abbreviations used in this paper: Treg, regulatory T; CLA, cutaneous lymphocyte-associated antigen; Tresp, responder T.

enhancing the proliferation of keratinocytes from uninvolved skin of patients with psoriasis (36, 37), thus translating T cell reactivity in the tissue into organ dysfunction.

How effector T cell activation remains ongoing in patients with psoriasis has yet to be determined. Dysfunction of the CD4⁺CD25⁺ Treg cell population, which is crucial for the prevention of spontaneous autoimmune disease, is a potential explanation for unrestrained pathogenic/effector T cell proliferation in psoriasis. Indeed, Treg cell dysfunction has recently been shown to occur in autoimmune diseases as diverse as multiple sclerosis (38) and autoimmune polyglandular syndrome type II (39). Due to the accessibility of both peripheral blood and target tissue cells involved in the pathogenic process, psoriasis offers a unique opportunity to study the mechanism of Treg cell function in human disease.

Materials and Methods

Patients

Seventeen patients with chronic plaque type psoriasis affecting at least 10% of their total body surface area and 15 healthy volunteers were enrolled in the study. A medication-free period of at least 1 mo for oral drugs was required in both groups, and psoriatic patients refrained from using topical medications or phototherapy for at least 2 wk before the procedures. The protocols involving human subjects were approved by the institutional review boards of University Hospitals of Cleveland, Case Western Reserve University, and the Veterans Affairs Medical Center. Informed consent was obtained from all subjects before performing the studies.

Cell isolation

Human PBMCs were prepared from heparinized venous blood by Hisopaque (Sigma-Aldrich) density gradient centrifugation according to the manufacturer's directions. In several experiments, peripheral blood from psoriatic and normal individuals were prepared simultaneously to ensure that variations between experiments were minimized and that the assays were consistently reproducible. CD4⁺ cells were separated from PBMCs by negative selection on midiMACS columns (CD4⁺ T cell isolation kit; Miltenyi Biotec) according to the manufacturer's instructions. After overnight incubation, CD25⁺ cells were positively selected using anti-CD25 Ab-coated magnetic microbeads (Dynabeads CD25; DYNAL Biotech), according to the manufacturer's instructions. Autologous APCs were isolated by plastic adherence, followed by negative depletion of CD3⁺ cells by CD3 microbeads (Miltenyi Biotec) according to the manufacturer's instructions, and irradiated with 4500 rad.

Epidermal and dermal cell suspensions were prepared from lesional skin keratome biopsies as described previously, with minor modifications (27). Viability was determined by trypan blue exclusion (70–90% range).

In some experiments, dermal cell suspensions were first depleted of plastic-adherent cells and cultured for 48 h to rest, and dermal CD25⁺ cells were then positively selected using anti-CD25 microbeads, as described above for peripheral blood CD4⁺ T cells.

Flow cytometry

Surface markers on blood or skin cells were detected by incubation with the appropriate mAbs, followed by fixation in 4% paraformaldehyde. To detect intracellular Ags, cells were permeabilized using 1% saponin and then stained with the appropriate mAbs. Samples were analyzed using a BD LSR flow cytometer (BD Biosciences). In some experiments, CD25^{high}, CD25^{low}, CD25^{high}cutaneous lymphocyte-associated Ag⁺ (CLA⁺), and CD25^{high}CLA⁻ subsets of CD4⁺ T cells were sorted using an EPICS Elite flow cytometer (Coulter). The following mAbs were used: anti-CD25-FITC, anti-CD45RA-FITC, anti-CD45RO-FITC, anti-CD62L-FITC, anti-CD69-FITC, anti-CLA-FITC, anti-HLA-DR-FITC, anti-CD25-PE, anti-CD69-PE, anti-CD152-PE (CTLA-4), and anti-CD69-PerCP (BD Pharmingen); CXCR3-PE (R&D Systems) and CD4-allophycocyanin from Caltag Laboratories.

Proliferation assays

To analyze the proliferation of CD4⁺CD25^{high} Treg cells in response to polyclonal activation, flat-bottom, 96-well plates (Costar) were coated with 10 µg/ml anti-CD3 mAb (BD Pharmingen). CD4⁺CD25^{high} T cells (2 × 10⁴) were cultured for 4 days in 200 µl of complete medium in the presence or the absence of 1 µg/ml soluble anti-CD28 mAbs (BD Pharmingen) with or without 100 U/ml human rIL-2 (R&D Systems).

To test alloantigen-specific T cell proliferation of CD4⁺CD25⁻ responder T (Tresp) cells, 1–2 × 10⁴ cells were cultured for 6–7 days in round-bottom, 96-well plates (Costar) in the presence or the absence of various numbers of Treg cells and CLA⁺ or CLA⁻ CD4⁺CD25^{high} Treg cells. Mitomycin C (4–5 × 10⁴; Sigma-Aldrich)-treated, allogeneic, plastic-adherent PBMCs were used as APCs. For criss-cross experiments, normal CD4⁺CD25⁻ Tresp cells were mixed at a 1:1 ratio with autologous or psoriatic CD4⁺CD25^{high} Treg cells, and vice versa, in the presence of allogeneic APCs. In separate experiments, psoriatic 1.5 × 10⁴ peripheral blood Tresp cells were cultured for 5 days with 5 × 10⁴ allogeneic APCs in the presence or the absence of various numbers of autologous dermal CD25⁺ cells.

In some experiments, 1 × 10⁴ CD4⁺CD25⁻ Tresp cells were tested for their ability to proliferate in response to soluble 0.01 µg/ml anti-CD3 mAb in the presence of 4 × 10⁴ irradiated autologous APCs. Cells were cultured for 3 days in round-bottom, 96-well plates in the presence or the absence of various numbers of CD4⁺CD25^{high} Treg cells. Proliferation values of anti-CD3-stimulated Treg cells (<9 × 10⁻³ cpm, on the average) were subtracted from the results before calculating inhibition. Cells were pulsed with 1 µCi/well [³H]thymidine for the last 16 h before harvesting on the day described above.

Cell division assay

CD4⁺CD25⁻ Tresp cells were labeled with 5 µM CFSE (Molecular Probes), washed, and recounted, and 8 × 10⁴ cells were cultured with 2 × 10⁵ APCs in the presence or the absence of unlabeled Treg cells at a 1:1 ratio. After 3 days of culture, cells were harvested and stained with anti-CD4-allophycocyanin and anti-CXCR3-PE, and analyzed by flow cytometry.

Real-time quantitative RT-PCR

Total RNA was extracted from CD4⁺CD25^{high} Treg and CD4⁺CD25⁻ Tresp cells of healthy or psoriatic volunteers using an RNeasy mini kit (Qiagen). RNA was reverse transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA was amplified in the presence of specific primers and probes for *Foxp3*, IL-2, IFN-γ, IL-4, IL-10, TGF-β, or 18S rRNA and TaqMan Universal Master Mix in a 96-well microtiter plate format on an ABI PRISM 7700 Sequence Detection System (all from Applied Biosystems). Each PCR was performed in triplicate, using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Copy numbers of samples were calculated against input copy numbers of plasmid standards for both target and 18S rRNA genes and were normalized with the copy numbers of the 18S rRNA gene.

Statistical analysis

Statistical analysis was performed using Student's *t* test. A value of *p* < 0.05 was considered significant.

Results

CD25^{high} Treg cells comprise similar proportions of CD4⁺ T cells in psoriatic and normal blood

To characterize the possible role of Treg cells in the pathogenesis of psoriasis, we first compared Treg proportions in normal and psoriatic blood. Based on their CD25 expression, human CD4⁺ T cells can be divided into three populations: CD25^{high}, CD25^{low}, and CD25⁻ cells (15). In normal volunteers (*n* = 8), we found that CD25^{high} and total CD25⁺ cells comprised an average of 4.0 and 22.0% of CD4⁺ T cells, respectively. In psoriatic patients (*n* = 6), comparable values were obtained; CD25^{high} and CD25⁺ cells represented 4.1 and 27.5% of the CD4⁺ T cell population, respectively. The values of the normal volunteers and the psoriatic patients were not significantly different (Fig. 1, A and B).

We also investigated the expression of several cell surface and intracellular molecules on CD4⁺CD25^{high}, CD4⁺CD25^{low}, and CD4⁺CD25⁻ T cells in both normal volunteers and psoriatic patients (Fig. 1C). In normal PBMC, as previously reported by Baecher-Allan et al. (15), CD4⁺CD25^{high} T cells expressed higher CD45RO (74.1%) and HLA-DR (28.2%) levels than the CD4⁺CD25^{low} (65.4 and 5.7%) or CD4⁺CD25⁻ (14.6 and 3.2%)

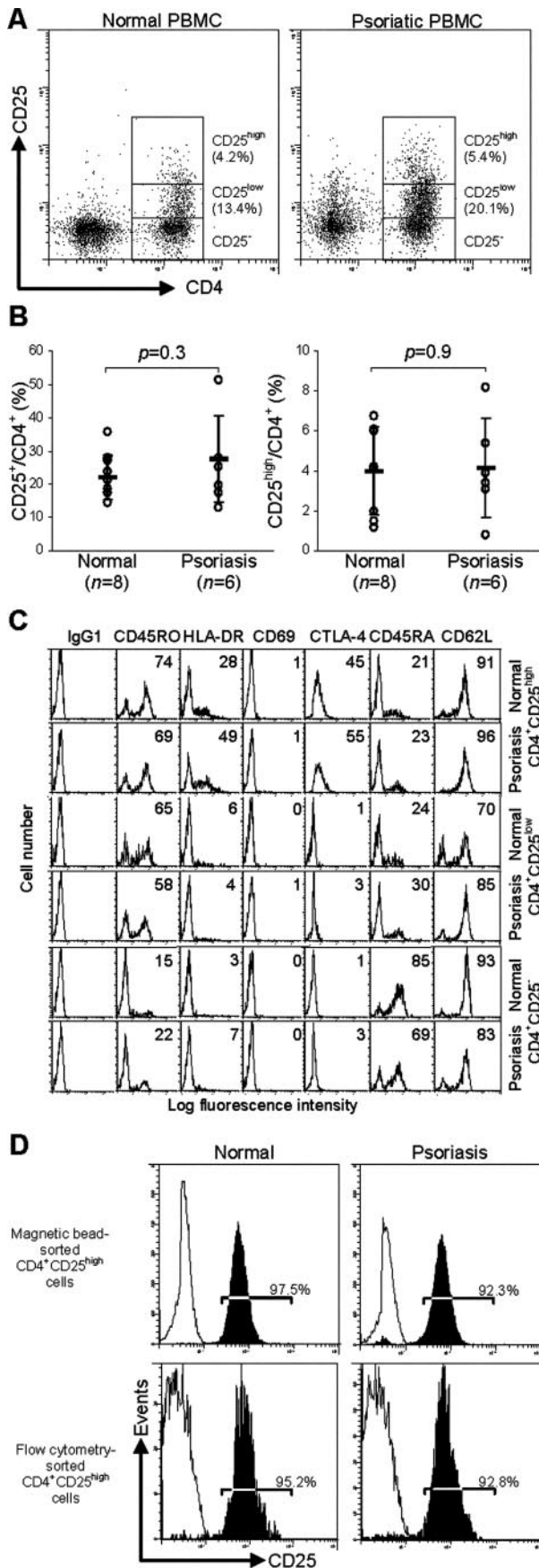


FIGURE 1. Similar proportions of CD4⁺CD25^{high} Treg cells in normal and psoriatic peripheral blood. PBMCs of normal volunteers and psoriatic patients were stained with anti-CD4 and anti-CD25 mAbs and analyzed by flow cytometry. **A**, Representative flow cytometry diagrams showing

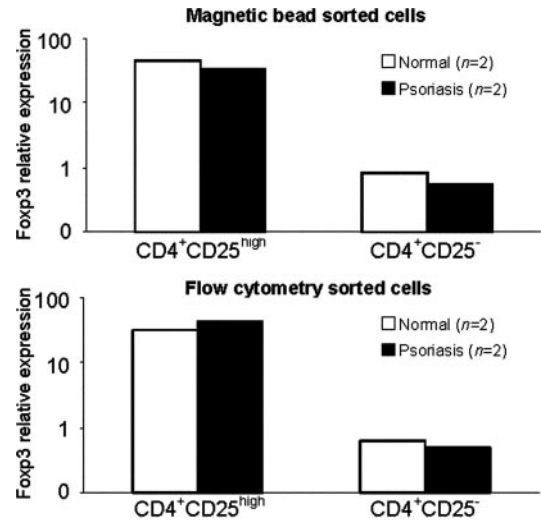


FIGURE 2. CD4⁺CD25^{high} Treg cells showed high copy numbers of *Foxp3* mRNA in psoriatic and normal blood. *Foxp3* mRNA expression was determined by real-time quantitative RT-PCR. Total RNA, extracted from magnetic bead-selected (upper panels) or flow cytometry-sorted (lower panels) CD25^{high}CD4⁺ and CD25^{low}CD4⁺ T cells from normal and psoriatic PBMC, was reverse transcribed and amplified in the presence of specific primers and probes for *Foxp3* or 18S. All assays were performed in triplicate. Results are expressed as relative *Foxp3* copy numbers (mean \pm SEM), where the *Foxp3* expression of one normal individual was designated 1. Data represent the summary of independent experiments of two psoriatic patients and two healthy controls.

T cell population. In contrast, CD45RA expression showed an opposite profile (i.e., 21.5% in the CD4⁺CD25^{high} T cell subset and 84.8% in the CD4⁺CD25^{low} T cell subset).

Similar results were obtained from psoriatic patients (Fig. 1C). Both normal and psoriatic CD4⁺CD25^{high} T cells showed high expression of cytoplasmic CTLA-4 (44.9 and 55.0%, respectively), one of the systematic surface markers associated with Treg cells. CD4⁺CD25^{high} T cells also expressed high levels of CD62L. None of the three T cell populations in either normal or psoriatic subjects showed notable levels of CD69 expression (an early T cell activation marker). Importantly, T cells of normal volunteers and psoriatic patients exhibited similar cell surface and intracellular Ag

CD25^{high}, CD25^{mid}, and CD25^{low} peripheral blood CD4⁺ T cells in a normal volunteer and a psoriatic patient. **B**, Statistical analysis of the CD25^{high}/CD4⁺ and CD25^{low}/CD4⁺ ratios in peripheral blood of normal volunteers and psoriatic patients. Data represent the summary of eight (normal) and six (psoriasis) independent experiments \pm SEM. **C**, Flow cytometric analysis of cell surface and intracellular molecule expression of normal and psoriatic CD4⁺CD25^{high}, CD4⁺CD25^{low}, and CD4⁺CD25^{low} T cells. PBMCs were prepared from peripheral blood of normal volunteers ($n = 2$) and psoriatic patients ($n = 2$). After overnight resting, nonadherent cells were stained with anti-CD4-allophycocyanin, anti-CD25-PE, and anti-CD45RO-FITC; anti-HLA-DR-FITC, anti-CD69-FITC, anti-CD62 ligand (CD62L)-FITC, and anti-CD45RA-FITC; or anti-CD4-APC, anti-CD25-FITC, and anti-CTLA-4-PE. Control samples stained with IgG2a-FITC, IgG2b-FITC, or IgG2a-PE as the third color are not shown, because they were identical with the staining pattern for the IgG1 isotype control. Numbers in the upper right corners represent the percentages of positive cells. A representative result of two independent experiments (for both normal and psoriatic individuals) is shown. **D**, Magnetic bead-selected (upper panels) or flow cytometry-sorted (lower panels) CD25^{high}CD4⁺ T cells from normal and psoriatic PBMC were stained with anti-CD25 mAb (■) or isotype (□) and analyzed by flow cytometry. Representative diagrams show >90% purity of sorted populations.

expression profiles in all three $CD4^+CD25^-$, $CD4^+CD25^{low}$, and $CD4^+CD25^{high}$ populations, defined by the varying levels of CD25 expression.

To isolate $CD4^+CD25^{high}$ T cells for additional functional analysis in subsequent experiments, these cells were sorted by either magnetic bead selection or flow cytometry-based methods. Bead-selected or flow cytometry-sorted Treg cell populations showed identical high expression of CD25 in normal and psoriatic individuals (Fig. 1D).

We also examined the forkhead/winged helix transcription factor gene, *Foxp3*, expression of these cells using real-time quantitative RT-PCR, because this gene is reported to be specifically expressed by Treg cells in mice and to program their development and function (40–42). With either the bead-selected or flow-sorting method, both normal and psoriatic $CD4^+CD25^{high}$ T cells showed comparable and at least 50 times higher numbers of *Foxp3* mRNA copies than $CD4^+CD25^-$ T cells (Fig. 2). Collectively, these findings suggest that bead-selected and flow-sorted Treg cell populations are phenotypically identical.

Psoriatic $CD4^+CD25^{high}$ Treg cells are impaired in their inhibitory functions

We next investigated the functional properties of normal and psoriatic $CD4^+CD25^{high}$ Treg cells in response to alloantigen-specific T cell stimulation. Psoriatic Tresp cells exhibited moderately higher proliferation than normal cells when activated with alloantigens (Fig. 3A, left columns). Neither psoriatic nor normal $CD4^+CD25^{high}$ Treg cells proliferated in response to alloantigens (Fig. 3A, far right columns). Treg cell activity, in contrast, appeared dysfunctional in psoriasis. In contrast to normal $CD4^+CD25^{high}$ Treg cells that, similar to results reported previously (12–15), inhibited $CD4^+CD25^-$ Tresp proliferation by an average of 87.8% at a 1:1 ratio (Fig. 3, A and B), the inhibitory capacity of psoriatic Treg cells was significantly decreased (60.6%; $p = 0.0001$; Fig. 3B), similar to what was recently reported for both multiple sclerosis and autoimmune polyglandular syndrome type II (38, 39).

Next we compared the proliferative responses of normal and psoriatic $CD4^+CD25^-$ Tresp cells at varying Treg:Tresp ratios (Fig. 3D). The Treg:Tresp ratio necessary to achieve similar 50% inhibition efficiency differed markedly between normal and psoriatic cells. Normal $CD4^+CD25^{high}$ Treg cells inhibited Tresp cell proliferation by 50% between ratios of 1:16 and 1:8, whereas an ~8-fold higher number of psoriatic Treg (1:2 to 1:1 ratio) was required to achieve the same inhibition. Moreover, even at Treg cell numbers exceeding T responder numbers (2:1 ratio), psoriatic cells were unable to achieve the almost complete suppression that normal Treg cells provide at a lower (1:1) ratio.

To exclude the possibility that the differences in Treg cell functions were due to diverse alloantigen responses in psoriatic and normal T cells, we next performed polyclonal TCR stimulation assays (Fig. 3C). Normal and psoriatic Tresp cells were cocultured with soluble anti-CD3 for 3 days in the presence or the absence of autologous $CD4^+CD25^{high}$ Treg cells. Normal Treg cells almost completely inhibited autologous Tresp cell proliferation at a 1:1 ratio; in contrast, psoriatic Treg cells showed significantly less inhibitory function. Thus, both alloantigen-specific and polyclonal TCR stimulation elicit impaired suppressor functions in psoriatic Treg cells.

Psoriatic $CD4^+CD25^{high}$ Treg cells fail to suppress normal Tresp cell proliferation

To pinpoint that the defective population in psoriatic patients is indeed that of Treg cells, we performed criss-cross experiments

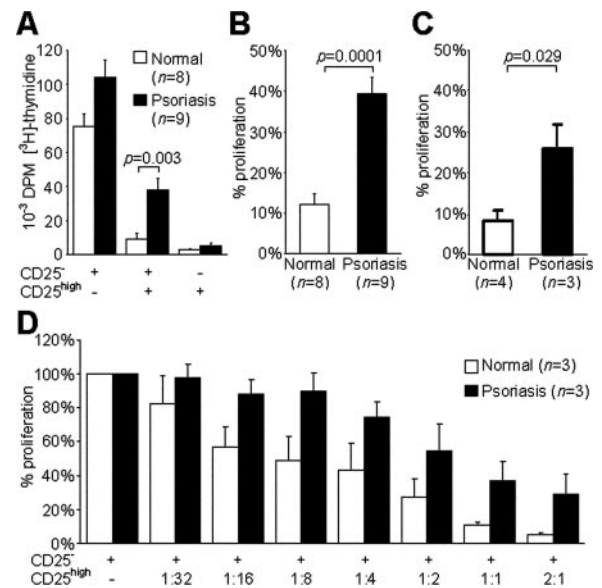


FIGURE 3. Psoriatic peripheral blood Treg cells are less potent inhibitors of T cell proliferation. *A*, Magnetic bead-sorted $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T cells (designated $CD25^-$ and $CD25^{high}$, respectively) purified from peripheral blood of normal volunteers and psoriatic patients were mixed at an equal ratio and cultured with allogeneic APCs. Proliferation was assessed by [³H]thymidine incorporation assay. Data are the summary of eight (normal) and nine (psoriatic) independent experiments. Results are expressed as the mean \pm SEM. *B* and *C*, Comparison of the relative inhibitory functions of alloantigen-stimulated and anti-CD3-stimulated normal and psoriatic Treg cells. The proliferative results are expressed as the percentage response. The proliferation of alloantigen-stimulated (*B*) and anti-CD3-stimulated (*C*) $CD4^+CD25^-$ T cells was normalized to 100%. Data are the summary of eight (normal) and nine (psoriatic) independent experiments (*B*) and four (normal) and three (psoriatic) independent experiments (*C*). Results are expressed as the mean \pm SEM. *D*, $CD25^{high}$ and $CD25^-$ T cells prepared as described in *A* were mixed at the indicated ratios and cultured with allogeneic APCs. The relative proliferative results of normal and psoriatic Treg cells are expressed as the percent proliferation of alloantigen-stimulated $CD4^+CD25^-$ T cells. Data are the summary of three independent experiments for both normal and psoriatic individuals. Results are expressed as the mean \pm SEM.

with psoriatic Treg and normal Tresp cells, and vice versa (Fig. 4). Normal $CD4^+CD25^-$ Tresp cells were incubated with APCs in the presence or the absence of autologous or psoriatic $CD4^+CD25^{high}$ T cells, whereas psoriatic Tresp cells were cocultured with autologous or normal Treg cells. At an equal ratio, normal Treg cells suppressed psoriatic Tresp proliferation by an average of 75.2% (compared with 88.7% inhibition on autologous normal $CD4^+CD25^-$ Tresp cells), whereas psoriatic Treg cells were able to suppress normal Tresp cell proliferation by only 34.4% (52.6% on autologous responder cells). Therefore, psoriatic Treg cells control the activation of responding $CD4^+CD25^-$ Tresp cells isolated from nonpsoriatic individuals far less efficiently than do normal Treg cells. Conversely, normal Treg cells are capable of suppressing the proliferation of hyper-reactive psoriatic Tresp cells more effectively than psoriatic autologous Treg cells. Collectively, psoriatic $CD4^+CD25^{high}$ Treg cells are indeed inherently dysfunctional in their suppressor capacity.

Psoriatic $CD4^+CD25^{high}$ Treg cells are anergic to polyclonal CD3/CD28 TCR stimulation

Because a profound functional difference was observed between normal and psoriatic Treg cells, we next examined how Treg cells responded to TCR stimulation in the presence or the absence of

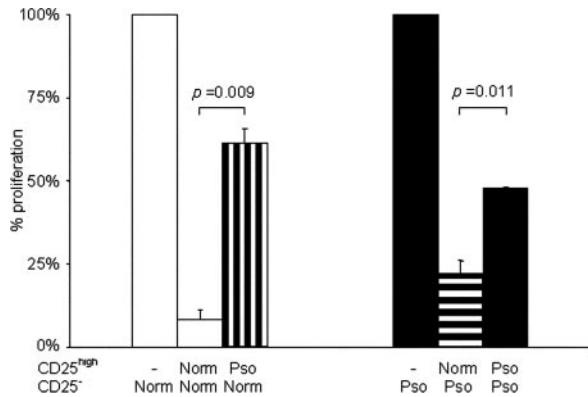


FIGURE 4. Psoriatic Treg cells inadequately control the proliferation of normal Tresp cells. CD4⁺CD25⁻ and CD4⁺CD25^{high} T cells (designated CD25⁻ and CD25^{high}, respectively) were purified by flow cytometry from normal (Norm) and psoriatic (Pso) PBMCs. Normal CD4⁺CD25⁻ T cells were mixed with autologous or psoriatic CD4⁺CD25^{high} T cells at an equal ratio and vice versa and were cultured in the presence of allogeneic APCs. Proliferation was assessed by [³H]thymidine incorporation assay. Results represent the summary of two independent experiments (four individuals) and are expressed as a percentage of the relative proliferation (mean ± SEM). The proliferation of corresponding normal or psoriatic alloantigen-stimulated CD4⁺CD25⁻ T cells was normalized to 100%.

costimulatory signals. Neither normal nor psoriatic Treg cells proliferated when stimulated with plate-bound anti-CD3 Ab alone, a characteristic feature of Treg cells. Combined anti-CD3/anti-CD28 stimulation elicited moderate, but reproducible, proliferative responses in normal Treg cells, as previously described by Levings et al. (13). In contrast, psoriatic CD4⁺CD25^{high} Treg cells remained completely anergic after optimal polyclonal CD3/CD28 TCR stimulation. The difference between the proliferative responses of normal and psoriatic Treg cells was >10-fold and was statistically significant ($p = 0.02$). Addition of exogenous IL-2 to the cultures completely reversed the unresponsive state of psoriatic CD4⁺CD25^{high} Treg cells and yielded almost identical results in psoriatic and normal Treg cells (Fig. 5A).

In some experiments we also examined the endogenous IL-2 mRNA expression of these cells using real-time quantitative RT-PCR. Both normal and psoriatic CD4⁺CD25⁻ Tresp cells expressed comparable and relatively high numbers of IL-2 mRNA copies after CD3/CD28 TCR stimulation. Normal CD4⁺CD25^{high} Treg cells showed ~1 order less, but still significant, amounts of IL-2 mRNA copies after optimal polyclonal TCR stimulation. The same stimulation, however, resulted in a ~5-fold additional decrease in IL-2 mRNA copy number in psoriatic CD4⁺CD25^{high} Treg cells compared with normal Treg cells (Fig. 5B). This suggests that the decreased proliferative response of psoriatic Treg cells may be due to the inability of these cells to produce IL-2 upon optimal polyclonal TCR stimulation, not to their unresponsiveness to IL-2.

CD4⁺CD25^{high} Treg cells from normal and psoriatic peripheral blood express similar copy numbers of Th1- and Th2-type cytokine mRNA

Although Treg cell function occurs in a cytokine-independent fashion, the Th1/Th2 balance as well as production of Th2-specific cytokines have been associated with Treg cell activity. Therefore, we examined the general cytokine profile associated with psoriatic and normal Treg cells. Total RNA from Treg and Tresp cells from normal and psoriatic PBMC were reverse transcribed and amplified in the presence of specific primers and probes for cytokines

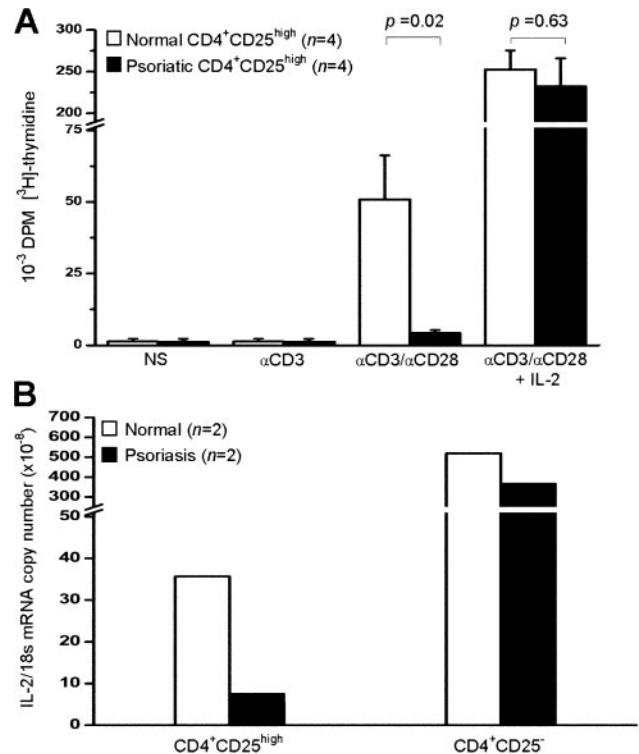


FIGURE 5. Psoriatic Treg cells are completely anergic to CD3/CD28 stimulation. Magnetic bead-sorted CD4⁺CD25^{high} Treg cells from normal volunteers and psoriatic patients were cultured with or without plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence or the absence of rIL-2. A, Proliferation of cells was assessed by [³H]thymidine incorporation assay. Data represent the average results from independent experiments involving four psoriatic patients and four normal individuals. Results are expressed as the mean ± SEM. B, IL-2 mRNA expression by real-time quantitative RT-PCR. Total RNA, extracted from anti-CD3/anti-CD28-stimulated normal and psoriatic CD4⁺CD25^{high} and CD4⁺CD25⁻ T cells, was reverse transcribed and amplified in the presence of specific primers and probes for IL-2 or 18S. All assays were performed in triplicate, and copy numbers were calculated using absolute plasmid standards. Data represent the summary of independent experiments of two psoriatic patients and two healthy controls. Results are expressed as IL-2 copy numbers normalized to 18S rRNA (mean ± SEM).

and 18S ribosomal RNA. We examined the expression of IFN- γ , IL-4, IL-10, and TGF- β (Fig. 6). Globally, Treg cells from either psoriatic or normal individuals expressed lower cytokine levels than autologous Tresp cells. However, among Treg cells, the expressions of these cytokines were comparable in normal and psoriatic individuals.

Psoriatic CD4⁺CD25⁻ Tresp cells exhibit increased early proliferative responses to alloantigens

We examined CD4⁺CD25⁻ Tresp proliferation directly using an alternative approach based on CFSE labeling. After short term (3-day) culture with allogeneic APCs, psoriatic and normal proliferating Tresp cells were observed as discrete CD4⁺CFSE⁺ populations (Fig. 7A). At this time point, dividing cells constituted ~5% of the psoriatic CD4⁺CD25⁻ Tresp population compared with <1% of the normal Tresp cells ($p = 0.003$). Thus, the dividing capacity of freshly obtained psoriatic Tresp cells was slightly greater, or more primed, than that of normal Tresp cells (Fig. 7B). We observed that 80.1% of the cells in the early proliferative T cell population expressed CXCR3 compared with 8.1%

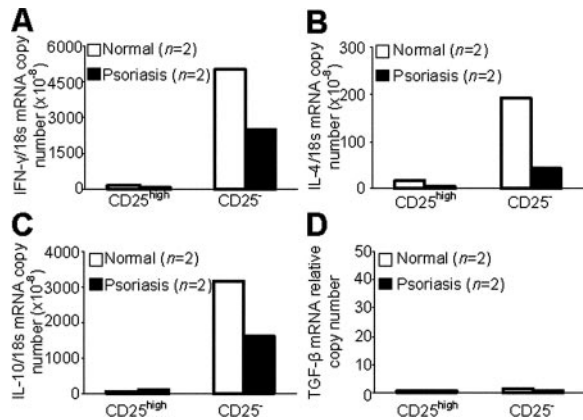


FIGURE 6. CD4⁺CD25^{high} Treg cells from normal and psoriatic peripheral blood express similar copy numbers of Th1- and Th2-type cytokine mRNA. Total RNA, extracted from flow-sorted CD4⁺CD25^{high} and CD4⁺CD25⁻ T cells from normal and psoriatic PBMC, was reverse transcribed and amplified in the presence of specific primers and probes for cytokines or 18S. The ratios of IFN- γ /18S mRNA (A), IL-4/18S mRNA (B), and IL-10/18S mRNA (C) of CD4⁺CD25^{high} and CD4⁺CD25⁻ T cells in normal and psoriatic PBMC were compared. TGF- β results (D) are expressed as the relative TGF- β copy number, where the TGF- β expression of one normal individual's CD4⁺CD25⁻ T cells was designated 1. All assays were performed in triplicate. Data represent the summary of independent experiments with two psoriatic patients and two healthy controls.

of the nonproliferating cells (Table I). This table shows that psoriatic patients had a statistically significant increase in CXCR3 expression among proliferating cells (CFSE⁺) compared with normal cells, although even normal individuals had a much higher percentage of CXCR3⁺ proliferating cells compared with the nonproliferating cell percentages (62 vs 6.2%). Second, there was no statistical difference in normal vs psoriatic CXCR3 (3%) in the nonproliferating cell population.

CD4⁺CD25^{high} Treg cells are enriched in the CLA⁺ skin-homing population

Because skin-homing T cells play a central role in the pathogenesis of skin diseases, including psoriasis (43, 44), we next focused on characterizing the CLA-positive Treg cell populations in normal and psoriatic PBMCs. Total CLA⁺ cells represented an average of 14.6 and 17.2% of the peripheral blood CD4⁺ T cells in normal volunteers ($n = 7$) and psoriatic patients ($n = 6$), respectively (data not shown). Normal CD25^{high} cells comprised an average 11.4% of CLA⁺CD4⁺ T cells, whereas 11.2% of the psoriatic CLA⁺CD4⁺ T cells were CD25^{high}. Interestingly, CD25^{high} cells represented <5% of the total and non-skin-homing CD4⁺ populations in both normal ($n = 8$) and psoriatic ($n = 6$) PBMCs, suggesting that the skin-homing CD4⁺ T cells are enriched for Treg cells (Fig. 8A and B).

To investigate whether CLA⁺ or CLA⁻ psoriatic CD4⁺CD25^{high} Treg cells exhibit differential functional activity, we tested the ability of CLA⁺ Treg cells to suppress the proliferative responses of autologous Tresp cells. CLA⁺ and CLA⁻ Treg cells were sorted by flow cytometry from CD4⁺CD25^{high} cells of psoriatic patients' PBMCs. At a Treg:Tresp ratio of 1:1, both psoriatic CLA⁺ and CLA⁻ Treg cells only partially, and almost equally, inhibited the proliferation of autologous Tresp cells (Fig. 8C). Thus, although Treg cells are enriched within both normal

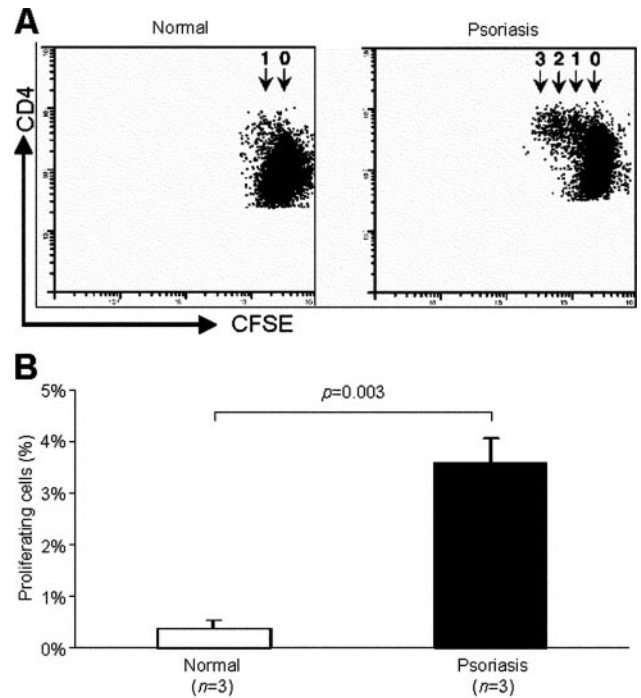


FIGURE 7. Early proliferative response of psoriatic CD4⁺CD25⁻ Tresp cells. A, Magnetic bead-sorted CD4⁺CD25⁻ Tresp cells from normal volunteers and psoriatic patients were labeled with CFSE and cultured with allogeneic APCs. After 3 days, cells were harvested, stained with anti-CD4 mAb, and analyzed by flow cytometry. Up to three divisions were observed in the case of psoriatic CD4⁺CD25⁻ Tresp cells, whereas normal CD4⁺CD25⁻ Tresp cells divided only once. A representative result of three independent experiments (for both normal and psoriatic individuals) is shown. B, The percentage of proliferating populations of total CFSE-labeled CD4⁺CD25⁻ T cells. Data represent the average results from three independent experiments for both normal and psoriatic individuals. Results are expressed as the mean \pm SEM.

and psoriatic CLA⁺CD4⁺ T cells, Treg cells in psoriatic blood are not confined only to the skin-homing population.

Cutaneous Treg cells: high proportions of epidermal and dermal CD4⁺ T cells coexpress CD25 and CTLA-4

Next we determined the possible presence of Treg cells in human psoriatic lesional skin. To avoid the detection of effector T cells transiently expressing CD25 and/or CTLA-4, epidermal and dermal cell suspensions were rested for 48 h before flow cytometric analysis. On CD4⁺CD25⁺ Treg cells, CTLA-4 is expressed up to 7 days after activation (14). However, because CD4⁺CD25⁻ T cells transiently up-regulate both surface and intracellular CTLA-4 expression within 24–48 h after activation, yet quickly down-regulate it thereafter (14), our resting duration was chosen accordingly. After resting, CTLA-4 and CD25 were coexpressed on ~35 and ~14% of psoriatic epidermal and dermal CD4⁺ T cells, respectively (Fig. 9A). Because constitutive CD25 positivity and CTLA-4 expression have been described as highly characteristic features of Treg cells (12–14, 45), these data suggest that regulatory phenotype cells constitute a significant proportion of T cells in psoriatic skin. Based on the above values, we calculated the ratio of CD4⁺CD25⁺ regulatory vs CD4⁺CD25⁻ effector T cells in psoriatic lesional skin to be 1:6 in dermis and 1:2 in epidermis. We also examined CD69 expression as an additional early activation marker and found that 48 h after isolation, <1% of epidermal and dermal CD4⁺ T cells expressed this molecule (data not shown).

Table I. CXCR3⁺ T cells preferentially populate the responder T cells that proliferate and are increased in psoriasis responder T cells escaping Treg cell control

	Proliferating Population			Nonproliferating Population		
	Normal	Psoriatic	<i>p</i>	Normal	Psoriasis	<i>p</i>
With Treg population	51.8 ± 4.6	69.4 ± 11	0.04	6.1 ± 1.5	6 ± 2	0.95
Without Treg population	62.2 ± 6.4	80.1 ± 2	0.04	6.2 ± 2.4	8.1 ± 2.5	0.09

Psoriatic lesional Treg cells exhibit deficient inhibitory effects on Tresp cells

We next attempted to examine the and functionality of Treg cells in lesional psoriatic skin. Psoriatic lesional dermal CD25⁺ cells were selected by anti-CD25 Ab-coated magnetic microbeads, and their ability to suppress the proliferative responses of autologous peripheral blood CD4⁺CD25⁻ Tresp cells to alloantigens was tested in vitro. In rested, nonadherent dermal cells, CD25⁺ cells were almost entirely confined to the CD3⁺ population (~99% of CD25⁺ cells coexpressed CD3), and the vast majority (~92%) of these CD3⁺CD25⁺ cells were also CD4⁺ (data not shown). Thus, magnetic microbead selection yielded a CD4⁺CD25⁺ dermal cell subset with >90% purity.

Psoriatic dermal CD25⁺ cells were anergic to alloantigens (Fig. 9B; Treg alone) and exhibited the capacity to suppress the proliferation of peripheral blood CD4⁺CD25⁻ Tresp cells in a dose-dependent manner (Fig. 9B). However, similar to peripheral blood Treg cells, the level of regulation by psoriatic lesional Treg cells was less than that of normal circulating Treg cells. Moreover, at the Treg:Tresp ratio that presumably reflects the in vivo situation (14 vs 86%, 1:6), dermal Treg cells failed to suppress the proliferation of autologous Tresp cells in vitro, allowing ~88% proliferation, whereas this ratio represents a >50% immunosuppressive dose in the case of normal peripheral blood Treg cells (Fig. 9C). Thus, psoriatic dermal Treg cells are both functionally and numerically insufficient to restrain Tresp cell proliferation.

Discussion

Psoriasis, an inflammatory skin disease demonstrated previously to be driven by T cells (27, 46–49), is a unique source of cutaneous T cells and can therefore provide valuable information about the interaction of regulatory and effector/pathogenic T cells in vivo. We provide evidence that psoriatic CD4⁺CD25^{high} Treg cells are functionally deficient in suppressing effector T cell responses in both alloantigen-specific and polyclonal TCR stimulation assays (Fig. 3). Although this deficiency is not absolute, higher numbers of psoriatic Treg cells are required to provide suppression similar to that of normal regulatory cells (Fig. 3D), and even at the highest concentration tested, psoriatic Treg cells do not fully inhibit effector T cell proliferation. Using a criss-cross experimental setup where psoriatic Treg cells are tested for their ability to inhibit normal Tresp cells and vice versa, we were able to pinpoint that the psoriatic Treg population is indeed dysfunctional. Psoriatic Treg cells are unable to restrain the proliferation of normal Tresp cells, whereas normal Treg are capable of suppressing psoriatic Tresp cells.

Because during the immune response the Treg cell population has to expand to perform its suppressor function (1, 50, 51), it is tempting to speculate that the inferior suppressive capacity of psoriatic Treg cells may originate from a defective proliferation and/or activation pathway. Psoriatic Treg cells are completely unable to proliferate after optimal polyclonal TCR stimulation via CD3/CD28, in contrast with normal regulatory cells that respond with slight, but obvious, expansion (Fig. 5A). In this study we provide

evidence that the decreased proliferative ability of psoriatic Treg cells may be associated with their considerably reduced IL-2 expression after anti-CD3/anti-CD28 stimulation compared with normal Treg cells (Fig. 5B). Proliferation upon polyclonal stimulation

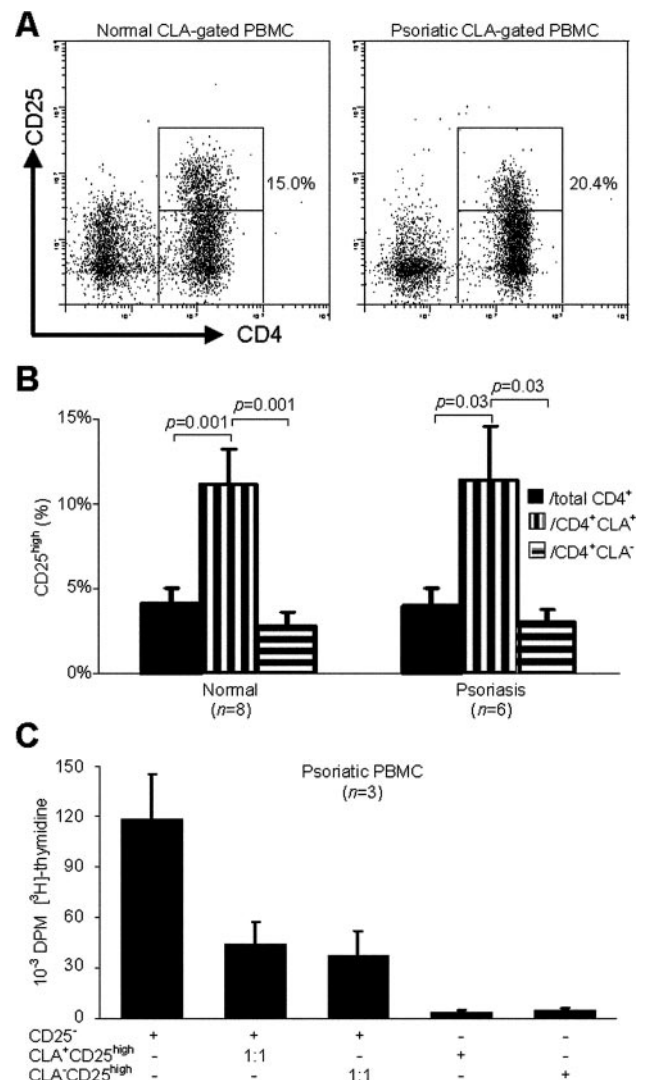


FIGURE 8. The skin-homing CLA⁺CD4⁺ T cell population contains a high proportion of CD4⁺CD25^{high} Treg cells. A, PBMCs of normal volunteers and psoriatic patients were stained with anti-CD4, anti-CD25, and anti-CLA mAbs and analyzed by flow cytometry. One representative figure each of eight (normal) and six (psoriasis) independent experiments is presented; CLA⁺ gated cells are shown. B, Proportion of CD25^{high}CD4⁺ Treg cells in the total CD4⁺, skin-homing (CLA⁺CD4⁺), and non-skin-homing (CLA⁻CD4⁺) T cell populations of normal and psoriatic peripheral blood. C, Skin-homing CLA⁺CD4⁺CD25^{high}, non-skin-homing CLA⁻CD4⁺CD25^{high} psoriatic peripheral blood Treg cells, and autologous CD4⁺CD25⁻ Tresp cells (designated CLA⁺CD25^{high}, CLA⁻CD25^{high}, and CD25⁻, respectively) were cultured with allogeneic APCs. Proliferation was assessed by [³H]thymidine incorporation assay. Data are the summary of three independent experiments. Results are expressed as the mean ± SEM.

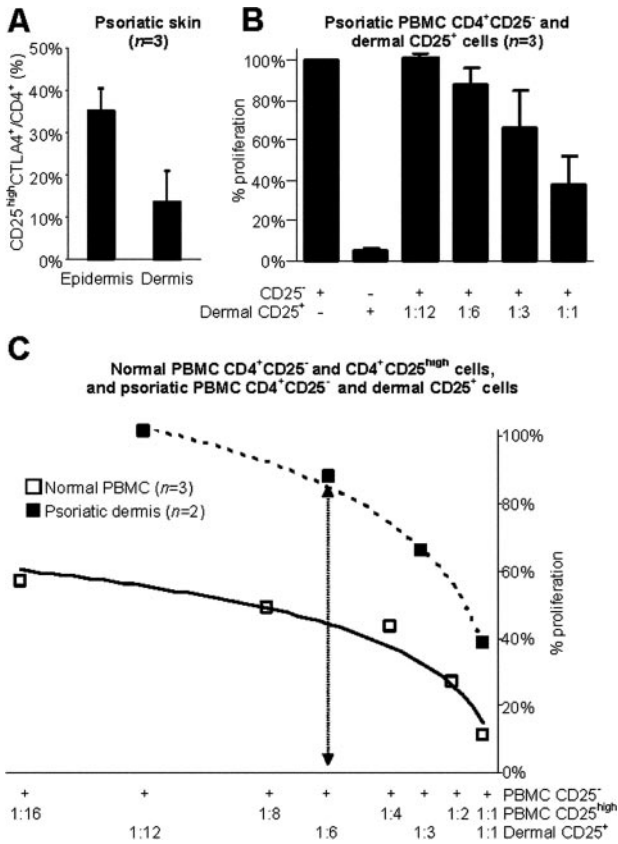


FIGURE 9. Treg cells in psoriatic epidermis and dermis. *A*, The percentage of CD25^{high}CTLA4⁺ Treg cells in psoriatic epidermis and dermis. Data are the summary of three independent experiments. Results are expressed as the mean ± SEM. *B*, Psoriatic peripheral blood CD4⁺CD25⁻ T cells (designated CD25⁻) were cultured with allogeneic APCs in the presence or the absence of lesional skin dermal CD25⁺ cells (designated dermal CD25⁺) at the indicated ratios. Proliferation was assessed by [³H]thymidine incorporation assay. The relative inhibitory function of psoriatic lesional dermal CD25⁺ cells is expressed as the percent response of alloantigen-stimulated CD4⁺CD25⁻ T cells. The proliferation of alloantigen-stimulated CD4⁺CD25⁻ T cells was normalized to 100%. Data are the summary of three (normal) and three (psoriatic) independent experiments. Results are expressed as the mean ± SEM. *C*, Comparison of the relative inhibitory function of psoriatic lesional dermal CD25⁺ cells and normal peripheral blood CD4⁺CD25^{high} T cells (as described in Fig. 3, *B* and *D*, and Fig. 9*B*). The relative inhibitory function of psoriatic lesional dermal CD25⁺ cells and normal peripheral blood CD4⁺CD25^{high} T cells is expressed as the percent response of alloantigen-stimulated psoriatic and normal CD4⁺CD25⁻ T cells at the indicated ratios, respectively. The proliferation of alloantigen-stimulated CD4⁺CD25⁻ T cells was normalized to 100%. Data are the summary of three (normal) and two (psoriatic) independent experiments, each with at least triplicate determinations. Results are expressed as the mean; lines represent best-fit curves (solid, normal; dotted, psoriasis).

is apparently not solely responsible for the homeostatic maintenance of Treg cells, because comparable numbers of Treg cells are detected in normal and psoriatic blood (Fig. 1, *A* and *B*). However, initial failure to obtain a critical number of Treg cells capable of sustaining sufficient peripheral tolerance against autoreactive T cells may allow for the emergence and unrestrained proliferation of psoriatic effector/pathogenic T cells in vivo. Indeed, we provide evidence in this study that a more proliferative subset of CD4⁺CD25⁻ effector T cells is apparent in psoriatic patients (Fig. 7, *A* and *B*).

CD4⁺CD25^{high} T cells may perform their regulatory function by interacting with effector T cells in target tissues (i.e., skin in psoriasis). Both psoriatic epidermis and dermis have extensive Treg populations, demonstrated as CD25⁺CTLA-4⁺ cells within the skin-residing CD4⁺CD69⁻ T cell subset (Fig. 9*A*). These psoriatic cells, however, are deficient in their regulatory functions to an extent similar to that of circulating Treg cells. We also demonstrated an even more profound lack in suppressor function at ratios presumably present in psoriatic lesional dermis (Fig. 9, *B* and *C*). Although the deficiency can be partially overcome using higher numbers of Treg cells, these ratios are not observed, and are not likely to be achieved in vivo, in psoriatic lesional skin.

Another interesting aspect is the peripheral homing of Treg cells, highlighted by the demonstration of CLA expression on a subset of Treg. This finding can be explained by one of two possible scenarios: 1) the extrathymic development of CD4⁺CD25^{high} Treg cells, or 2) the acquisition of peripheral homing properties by thymus-emigrant, naturally occurring Treg cells. Although it seems more plausible that CLA (as an activation/differentiation marker) is acquired in peripheral lymphoid organs during the process of T cell priming, ultimately, additional studies are needed to answer the question of which mechanism leads to the development of peripheral organ-homing Treg cells.

Because a single surface marker has not been identified for Treg cells, it is possible that isolated Treg populations could contain effector T cells. However, this is unlikely, because CD4⁺CD25^{high} cells from both PBMC and lesional dermis displayed all known phenotypic and functional properties of Treg: CD4, CD25, and CTLA-4 coexpression; high expression of *Foxp3* mRNA; low proliferative responses; and inhibition of cocultured Tresp cell proliferation in response to both alloantigen-specific and polyclonal stimulation. Furthermore, both magnetic bead-selected and flow cytometry-sorted normal and psoriatic Treg cells functioned as suppressors in response to both alloantigen-specific and polyclonal stimulation. Additional convincing evidence is provided by the CD3/CD28 TCR stimulation experiments: had our psoriatic Treg populations contained more effector T cells than normal cells, the proliferative response should have been higher and not lower as detected. Thus, the observed impairments in the psoriatic Treg cell functions are very unlikely to be solely attributable to contamination with proliferative effector T cells. One could alternatively argue that the CD4⁺CD25^{high} population in psoriasis contained higher levels of anergic effector T cells. A phenotypic distinction between Treg cells and anergic effector T cells (effector T cells in lesional skin that may have seen their Ag and have become anergic) cannot presently be made. Nonetheless, functionally, the net effect of the presence of anergic effector T cells would be a decrease in Treg cell activity in psoriasis. If the Treg pool were contaminated with these cells, this would ultimately mean that the number of CD25^{high} Treg cells (Fig. 1*B*) is proportionately less in psoriasis than in normal cells. The decrease or absence of the Treg population is associated with the development of autoimmune disease in animal models. Thus, due to a lower number of Treg cells, psoriasis patients would exhibit unrestrained proliferation of effector T cells.

Certain psoriatic therapeutic methods associated with rapid relapse, such as corticosteroids or cyclosporin A, may result in a decrement in both Treg and Tresp populations. Other treatments associated with more durable remissions, such as phototherapy, in addition to eliminating pathogenic infiltrating T cells, may be linked to the induction of suppressive cutaneous T cells (52) as well. One of the ultimate goals of identifying and isolating a Treg population is a therapeutic approach to controlling T cell proliferative diseases. We have shown that Treg cells can be isolated in the

periphery and demonstrate proof of concept that psoriatic Treg cells, at the proper ratio, can function to suppress Tresp cells. Thus, reconstituting the Treg population by adoptive transfer in psoriatic individuals may abort the activation and expansion of a psoriasis-specific T cell population in lymph nodes in the induction phase and ultimately may lead to long term remission. Alternatively, decreasing the size of the memory effector T cell population or increasing its susceptibility to Treg cell suppression could promise similar results. Whether novel remittive therapies, such as Alefacept or Infliximab, increase the ratio of Treg:Tresp or the effectiveness of Treg cells has yet to be addressed.

In this study we show that Treg cells can infiltrate nonlymphoid target tissues, such as psoriatic dermis and epidermis. We hypothesize that the recruitment of Treg cells to the site of inflammation is essential to control, and ultimately to eliminate, inflammatory reactions. However, Treg cells are both functionally and numerically impaired in psoriasis, and as a result they cannot restrain the ongoing chronic inflammation. Once inflammation is initiated in psoriatic skin, an imbalance develops between the pro- and anti-inflammatory processes, which leads to sustained proliferation and activation of pathogenic T cells.

Collectively, our data demonstrate a profound proliferative and functional deficit of Treg cells as well as a hyper-responsiveness of the effector T cell population in the chronic inflammatory skin disease, psoriasis. Although psoriasis has not been directly proven to be an autoimmune disease, it is certainly a favored paradigm among researchers in this field. Our finding that CD4⁺CD25^{high} Treg cells are involved in the pathogenesis of the disease provides important additional evidence to support this hypothesis. Psoriasis is a disease with multiparameter origins, and in combination with other critical factors (e.g., genetic predisposition), Treg cell defects may contribute to overall disease pathogenesis. Compensating for the Treg cell defect or correcting Treg:Tresp ratios to a more favorable Treg balance, as we have demonstrated, should provide benefit for psoriasis patients.

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