Foxp3 + Regulatory T Cells of Psoriasis Patients Easily Differentiate into IL-17A-Producing Cells and Are Found in Lesional Skin

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Psoriasis is an autoimmune-related chronic inflammatory skin disease that is strongly associated with IL-23 and T helper-17 (Th17) effector cytokines. In addition, CD4 + CD25^{high} regulatory T-cell (Treg) function appeared to be impaired in psoriasis. CD4 + CD25^{high}Foxp3 + Tregs are typically considered inhibitors of autoimmune responses. However, under proinflammatory conditions, Tregs can differentiate into inflammation-associated Th17 cells—a paradigm shift, with as yet largely unknown consequences for human disease initiation or progression. Th17 cells are highly proinflammatory T cells that are characterized by IL-17A and IL-22 production and expression of the transcription factor retinoic acid-related orphan receptor γt (RORγt). We here show that Tregs of patients with severe psoriasis, as compared with those of healthy controls, have an enhanced propensity to differentiate into IL-17A-producing cells on *ex vivo* stimulation. This enhanced Treg differentiation was linked to unexpectedly high RORγt levels and enhanced loss of Foxp3. Notably, IL-23 boosted this Treg differentiation process particularly in patients with psoriasis but less so in controls. IL-23 further reduced Foxp3 expression while leaving the high RORγt levels unaffected. The histone/protein deacetylase inhibitor, Trichostatin-A, prevented Th17 differentiation of Tregs in psoriasis patients. Importantly, IL-17A + /Foxp3 + / CD4 + triple-positive cells were present in skin lesions of patients with severe psoriasis. These data stress the clinical relevance of Treg differentiation for the perpetuation of chronic inflammatory disease and may pave novel ways for immunotherapy.

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INTRODUCTION

Several human autoimmune disorders are associated with pathogenic T helper-17 (Th17) cells (Tesmer *et al.*, 2008) as well as with dysfunctional regulatory T cells (Tregs; Brusko *et al.*, 2008). A recent paradigm shift with regard to T-cell lineage development (Koenen *et al.*, 2008; Yang *et al.*, 2008b; Ayyoub *et al.*, 2009; Beriou *et al.*, 2009; Voo *et al.*, 2009; Zhou *et al.*, 2009) may point to a new role for Tregs in the perpetuation of inflammatory processes, rather than in the suppression thereof.

Psoriasis is an autoimmune-related chronic inflammatory skin disease (Nestle *et al.*, 2009). The disease is strongly associated with the Th17-driving cytokine IL-23 (Nair *et al.*, 2009) and Th17 cells, which express the transcription factor retinoic acid-related orphan receptor γt (RORγt; Teunissen *et al.*, 1998; Wilson *et al.*, 2007; Lowes *et al.*, 2008). Th17 cells produce among others the effector cytokines IL-17A and IL-22, which may have a prominent pathogenic role in skin inflammation and the development of psoriatic plaques (Teunissen *et al.*, 1998; Wilson *et al.*, 2007; Lowes *et al.*, 2008). Increased numbers of IL-17A- and IL-22-producing T cells were present in the peripheral blood of psoriasis patients (Kagami *et al.*, 2010). Psoriasis has also been associated with impaired suppressive capacity of Tregs (Sugiyama *et al.*, 2005). Tregs are essential for immune homeostasis by virtue of their capacity to suppress the function of other lymphocytes, thereby suppressing immune responses, inflammation, and tissue destruction. Stable expression of the Treg master transcription factor, Foxp3, is crucial for Treg function (Zhou *et al.*, 2009).

Human Th17 cells were shown *in vitro* to differentiate from naive or memory CD4 + T cells, dependent on the presence of the cytokines transforming growth factor-β, IL-1β, IL-6, IL-21, and IL-23 (Wilson *et al.*, 2007; costa-Rodriguez *et al.*, 2007; Manel *et al.*, 2008; Volpe *et al.*, 2008; Yang *et al.*, 2008a). Interestingly, we and others have recently

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Abbreviations: Th17, T helper-17; Treg, regulatory T cell

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demonstrated that CD4 + CD25^{high} Foxp3 + Tregs obtained from healthy individuals can convert into inflammation-associated Th17 cells (Koenen *et al.*, 2008; Ayyoub *et al.*, 2009; Beriou *et al.*, 2009; Voo *et al.*, 2009). This Treg differentiation was further promoted by the proinflammatory cytokines, such as IL-1β, IL-21, and IL-23 (Koenen *et al.*, 2008). In mice, the adoptive transfer of differentiated Tregs led to autoimmunity (Zhou *et al.*, 2009). This brought us to propose that next to dysfunction of Tregs, differentiation of Tregs into an inflammatory cell type might very well contribute to the perpetuation of chronic autoimmunity, such as psoriasis, in humans.

Here we present data that reveal that differentiated IL-17A-producing Tregs might have a role in human chronic inflammatory processes. This is substantiated by the finding that peripheral-blood-derived Tregs of severe psoriasis patients easily differentiate into a Th17-associated phenotype, in patients this differentiation process appeared to be very adequately promoted by IL-23. Most importantly, Foxp3 + IL-17A-producing cells can be found in the lesional skin of patients with severe psoriasis, and likely contribute to the perpetuation of immune pathology. This to our knowledge, previously unreported concept may well influence the choice and the development of future treatment modalities.

RESULTS AND DISCUSSION

Ex vivo-stimulated CD4 + CD25^{high} Tregs from patients with severe psoriasis show enhanced IL-17A production

To examine whether differentiation of Tregs to Th17 can have a role in human chronic inflammatory disease processes, we first set out to analyze peripheral blood Tregs from psoriasis patients. Previously, it was shown that in psoriasis patients, the suppressor capacity of peripheral blood Tregs was reduced, whereas their frequency and anergic phenotype appeared unchanged (Sugiyama *et al.*, 2005). Indeed, we here show that this holds true for Tregs from patients with mild (Psoriasis Area and Severity Index 5.1) and severe (Psoriasis Area and Severity Index 15.8) plaque psoriasis (Supplementary Figure 1a,b/2a,b online). Next, we studied the potential of these Tregs to produce the inflammatory cytokines, such as IL-17A, IL-22, and IFN-γ, known to be involved in the pathogenesis of psoriasis (Teunissen *et al.*, 1998; Lowes *et al.*, 2008; Nestle *et al.*, 2009). Importantly, all three cytokines were produced, but in stimulated Tregs from severe psoriasis patients, we found significantly ($P<0.05$) increased amounts of IL-17A in the culture supernatants as compared with Tregs from healthy controls (Figure 1a). No significant difference was observed for IFNγ or IL-22. In contrast to Tregs, stimulated CD4 + CD25− conventional T cells of psoriasis patients revealed similar levels of IL-17A, IL-22, and IFNγ as those found for healthy controls (Figure 1a). These findings suggest that Tregs of severe psoriasis patients are particularly prone to differentiate into IL-17A-producing cells. Thus, we sought to investigate in more depth.

Increased IL-17A production by Tregs from severe psoriasis patients is the result of enhanced numbers of IL-17A-producing cells and increased IL-17A expression levels per cell

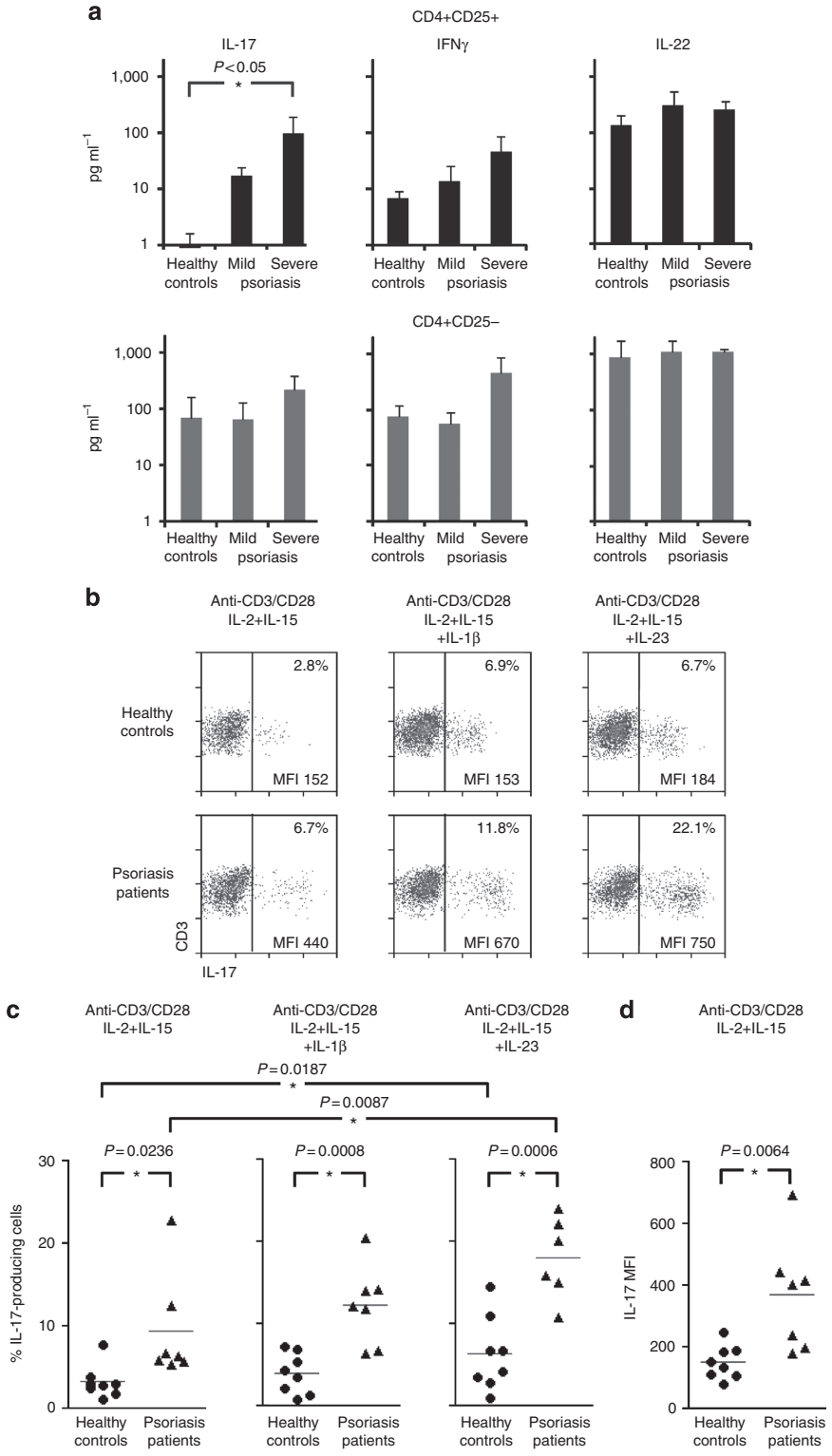
We evaluated the increased IL-17A-producing capacity of Tregs from psoriasis patients (Figure 1b) at the single-cell level using flowcytometry. To this end, CD4 + CD45RA-CD25^{high} Tregs were isolated by high-purity flowcytometric cell sorting, resulting in >98% Foxp3 + cells that lack CD127 expression (data not shown). Following *ex vivo* stimulation of Tregs from severe psoriasis patients, we found a significant increase in the percentage of intracellular IL-17A-producing cells as compared with healthy controls (mean ± SEM; 9.3 ± 2.4%, $n=7$; 3.2 ± 0.7%, $n=8$, respectively; $P=0.0236$; Figure 1b and c). In addition, these cells produced higher levels of IL-17A on a per cell basis (fluorescence intensity; 366 ± 68, $n=7$ vs. 150 ± 19, $n=8$, respectively; $P=0.064$; Figure 1b and d). The majority of the cells were IL-17A + /IFNγ-producing Th17 cells (Supplementary Figure 3 online).

These findings indicate that highly purified Tregs from severe psoriasis patient, as compared with healthy controls, have an increased potential to produce IL-17A following *ex vivo* stimulation. Thus, we interpret as an enhanced propensity of Tregs to differentiate into IL-17A-producing cells. The Treg population is heterogeneous with respect to the expression of cell surface markers and the transcription factor Foxp3, although both Foxp3^{low}- and Foxp3^{high}-expressing Tregs can produce IL-17A, it appeared that within the Foxp3^{low} population more cells were able to produce IL-17A (Miyara *et al.*, 2009). Further investigation is needed to reveal whether the Foxp3^{low} or Foxp3^{high} Tregs are differentiating into IL-17A-producing cells in psoriasis patients. Importantly, directly *ex vivo* isolated Foxp3 + Tregs did not produce IL-17A (Figure 2).

This supports our finding that the increased IL-17A production found in psoriasis patients is truly due to Treg

Figure 1. *Ex vivo*-stimulated CD4 + CD25^{high} T cells from patients with severe psoriasis show increased IL-17A production. (**a**) Cytokine production measured by a Luminex cytokine assay (IL-17A, IFNγ) and ELISA (IL-22) in cell culture supernatants of isolated CD4 + CD25 + (upper panel) and CD4 + CD25− T cells (lower panel) after 4 days of *ex vivo* stimulation with anti-CD3/CD28 mAb-coated beads. Cells were derived from healthy controls and mild and severe psoriasis patients. Data show SEM and mean of $n=4$–5 controls or patients. (**b**) Flowcytometry of the intracellular IL-17A production in high-purity cell sorted CD4 + CD45RA-CD25^{high} from healthy controls (upper panel) and severe psoriasis patients (lower panel) on *ex vivo* stimulation as indicated at the top. Intracellular IL-17A measurement was performed at day 8 of the cultures after stimulation with phorbol 12-myristate 13-acetate plus ionomycin in the presence of brefeldin-A. Dot plots show the percentage and mean fluorescence intensity (MFI) of CD3 + IL-17A-producing cells. Representative experiments from $n=6$–7 controls or patients are shown. (**c**) Summarized data showing the percentages and (**d**) mean fluorescence intensity (MFI) of IL-17A-producing cells in sorted and *ex vivo* stimulated CD4 + CD25^{high} from healthy controls and severe psoriasis patients as shown in **b**. Each data point represents one separate experiment conducted with cells obtained from different healthy blood donors or severe psoriasis patients.

differentiation. Moreover, we excluded the possibility that the IL-17A-producing cells in our Treg cultures arose from contaminating CD4+CD25+ effector, memory cells or CD4+CD25− T –cells, or CD161+ Th17 cell precursors (Koenen *et al.*, 2008; Ayyoub *et al.*, 2009; Beriou *et al.*, 2009; Voo *et al.*, 2009; and data not shown). The observation that Tregs reveal plasticity and have the ability to differentiate into IL-17-producing cells has more recently been confirmed by others (Koenen *et al.*, 2008; Ayyoub *et al.*, 2009; Beriou *et al.*, 2009; Voo *et al.*, 2009).



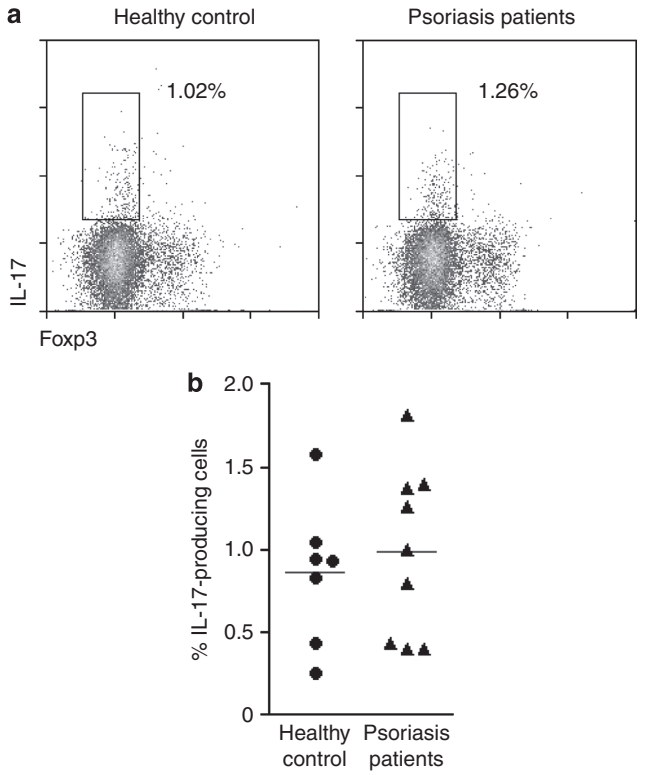


Figure 2. Lack of intracellular IL-17A production by freshly isolated Foxp3+ CD4+ cells. (**a**) Flowcytometry of the intracellular IL-17A production and Foxp3 expression in isolated CD4+ cells from healthy controls (left panel) and severe psoriasis patients (right panel) directly after isolation and following stimulation of phorbol 12-myristate 13-acetate plus ionomycin in the presence of brefeldin A. Dot plots show Foxp3 (*x*-axis) and IL-17 (*y*-axis) expression. Percentages of IL-17-producing cells are indicated in the dot plots. Representative experiments are shown. (**b**) Summarized data showing the percentage of IL-17-producing cells (*y*-axis). Each data point represents one separate experiment conducted with cells obtained from different healthy blood donors or severe psoriasis patients.

Increased Treg differentiation in patients with severe psoriasis is associated with high expression levels of RORγt and enhanced loss of Foxp3

According to expectation, using flowcytometry, we observed that IL-17A-producing Tregs derived from severe psoriasis patients and controls expressed the Th17-associated transcription factor RORγt (Koenen *et al.*, 2008; Ayyoub *et al.*, 2009; Voo *et al.*, 2009; Figure 3). However, interestingly, we found that on stimulation, patient-derived IL-17A-producing Tregs revealed far higher levels of RORγt as compared with healthy controls. This may explain, at least in part, the increased Treg differentiation rate of patient-derived Tregs.

Foxp3 can directly bind to RORγt and antagonize Th17 differentiation of T cells (Zhou *et al.*, 2008; Yang *et al.*, 2008b). The balance between Foxp3 and RORγt may determine whether a Tregs or Th17 differentiation program will be induced (Yang *et al.*, 2008b). *In vivo* in mice, inflammatory conditions were shown to induce loss of Foxp3 expression by Tregs (Yang *et al.*, 2008b; Zhou *et al.*, 2009) and result in proinflammatory effector cytokine production (IL-17A, IFNγ) by these cells (Zhou *et al.*, 2009). In our previous work, we showed that human Foxp3+ Tregs that convert into IL-17A-producing cells gradually lost expression of Foxp3 (Koenen *et al.*, 2008): early after stimulation the Tregs co-expressed Foxp3 and IL-17A, whereas later in time Foxp3 expression was progressively lost. This reveals that, Tregs that differentiate into an IL-17A-producing phenotype pass through a Foxp3/IL-17A-double-positive stage. Having established that on *ex vivo* stimulation, Tregs from severe psoriasis patients showed both increased RORγt and IL-17A levels (Figures 1 and 3), we wondered whether this feature was indeed also associated with progressive loss of Foxp3 expression. Following stimulation of Foxp3+ Tregs from severe psoriasis patients, we found that significantly more cells lost expression of Foxp3 as compared with healthy controls (% Foxp3-positive cells: $47.8 \pm 2.2\%$, $n=7$ vs. $62.6 \pm 1.9\%$, $n=8$, respectively; $P=0.002$; Figure 4a and b). In line with our previous observations (Koenen *et al.*, 2008), we found cells that were positive for both Foxp3 and IL-17A, as well as cells that had already lost Foxp3 expression (Figure 4c).

IL-23 strongly boosts Treg differentiation in psoriasis patients, which is associated with enhanced loss of Foxp3

Interestingly, supplementation of IL-23, a cytokine that is strongly associated with psoriasis and present in psoriatic plaques (Nair *et al.*, 2009), greatly promoted the percentage of IL-17A-producing Tregs, particularly in psoriasis patients ($17.0 \pm 2.6\%$, $n=6$ patients vs. $6.3 \pm 1.6\%$, $n=8$ healthy controls, respectively; $P=0.0006$; Figures 1b and c and 4c). In psoriasis patients, this was accompanied by the enhanced loss of Foxp3 expression (Figure 4a, b and c), whereas the increased RORγt levels were maintained (Figure 3).

Genome-wide association studies have indicated the IL-23 receptor (*IL-23R*) locus as a susceptibility gene in psoriasis (Nair *et al.*, 2009). We reasoned that increased expression of the IL-23R might be an alternative possibility of why Tregs from psoriasis patients could be more prone to differentiate into IL-17A-producing cells. However, we did not observe an increased expression levels of the IL-23R or increased numbers of IL-23R-expressing $CD25^{high}$/Foxp3 cells, as these were similar for both patients and healthy controls. Therefore, the increased response of the patient Tregs toward IL-23 was not due to the increased expression of the IL-23R.

Although IL-1β supplementation increased IL-17A production in Tregs from both psoriasis patients and healthy controls (Figures 1b and c and 4c), this, in contrast to IL-23, was not accompanied by a similar drop in Foxp3 levels (Figure 4a, b and c).

Histone/protein deacetylases regulate chromatin remodeling, gene expression and the functions of many transcription factors, and non-histone proteins. Acetylation of histones leads to an open chromatin structure, which is permissive for the initiation of gene transcription and expression. Histone/protein deacetylase inhibition by Trichostatin-A (TSA) increased histone acetylation resulting in increased Foxp3 protein expression in mouse Tregs (Tao *et al.*, 2007). In human Tregs, TSA prevented the production of IL-17A and sustained Foxp3 expression (Koenen *et al.*, 2008).

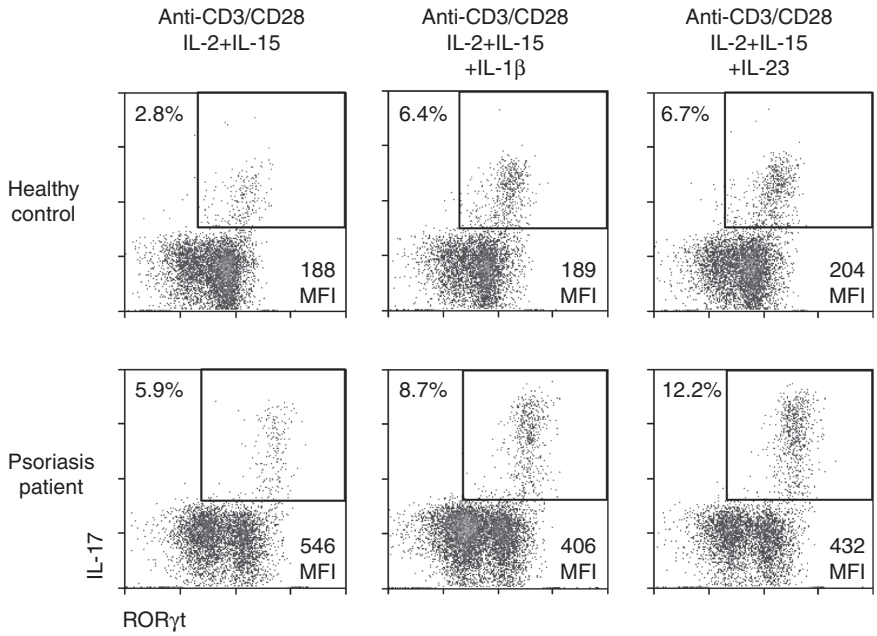


Figure 3. *Ex vivo*-stimulated CD4+ CD25^{high} T cells from patients with severe psoriasis show increased retinoic acid-related orphan receptor γt (RORγt) expression levels. Flowcytometry of intracellular IL-17A and RORγt expression in high-purity cell sorted CD4+CD45RA-CD25^{high} from healthy controls (top panel) and severe psoriasis patients (lower panel) on stimulation as described under Figure 1. Dot plots show the percentage of IL-17A-producing RORγt+ double-positive cells and RORγt expression levels (mean fluorescence intensity (MFI)). Data are representative of three separate experiments conducted with cells obtained from different healthy blood donors or severe psoriasis patients.

Importantly, in psoriasis patients, the enhanced differentiation of Tregs into a Th17-like phenotype could be inhibited by TSA (Figure 4d), which might be of interest for the development of new therapeutic modalities.

IL-17A-producing Foxp3+ CD4+ T cells are present in the dermis of the lesional skin of severe psoriatic patients

To date, we demonstrated that Tregs isolated from the peripheral blood of severe psoriasis patients have an enhanced capacity to differentiate into IL-17A-producing cells. A role for these cells in the perpetuation of the local inflammatory process would be likely if we could detect such cells directly at the site of active inflammation. Thus, we assessed the presence of Foxp3/IL-17A-double-positive cells in the lesional skin of patients with severe psoriasis using immunohistochemistry. Notably, CD4+ cells positive for both Foxp3 and IL-17A were clearly present in the psoriatic dermis (Figure 5a and b), but absent in either lesional epidermis or non-lesional skin (data not shown). We also found CD4+ IL-17A-producing cells that lacked Foxp3 expression (Figure 5a and b).

Collectively, our current work indicates that Foxp3+ Tregs in severe psoriatic patients have an enhanced propensity to lose Foxp3 expression, while maintaining a high level of RORγt expression, all in favor of a high RORγt:Foxp3 ratio to promote the induction of a proinflammatory IL-17A transcription program. The cytokine IL-23 strongly drives this process. Clinical trials with monoclonal antibodies that target IL-12p40 subunit, which is shared by both IL-23 and IL-12, have shown high efficacy in psoriasis (Griffiths *et al.*, 2010). Limiting the IL-23-driven differentiation program of Tregs may explain at least part of this success.

Importantly, the presence of Foxp3+ IL-17A-producing cells in the proinflammatory environment of the psoriatic dermis suggests that the conversion of Tregs into IL-17A-producing cells might take place *in vivo* in humans. This, in turn, might inadvertently contribute to the perpetuation of the inflammatory condition. These findings, together with the recent observations in mouse models, which show that the differentiation of Tregs into pathogenic-cytokine-secreting cells is able to induce autoimmunity (Zhou *et al.*, 2009), and this finding might provide to our knowledge, previously unreported mechanistic understanding of the process that drives chronic autoimmune inflammation.

MATERIALS AND METHODS

Psoriasis patients and healthy volunteers

Patients with mild-to-moderate psoriasis (Psoriasis Area and Severity Index ~5), severe psoriasis (Psoriasis Area and Severity Index ⩾15), and healthy volunteers were recruited according to the Declaration of Helsinki and following approval of medical ethics committee of the Radboud University Medical Centre. After given informed consent, 100 ml of peripheral blood was collected. Punch biopsies (4 mm) were taken from the center of a psoriatic lesion and from uninvolved skin on local anesthesia. Healthy controls did not have a positive (family) history of psoriasis or other autoimmune or skin diseases. Patients did not use any topical treatment for at least 1 month and were naive to photo(chemo)therapy or systemic treatment for psoriasis. Furthermore, they were otherwise healthy and did not use any other systemic medication.

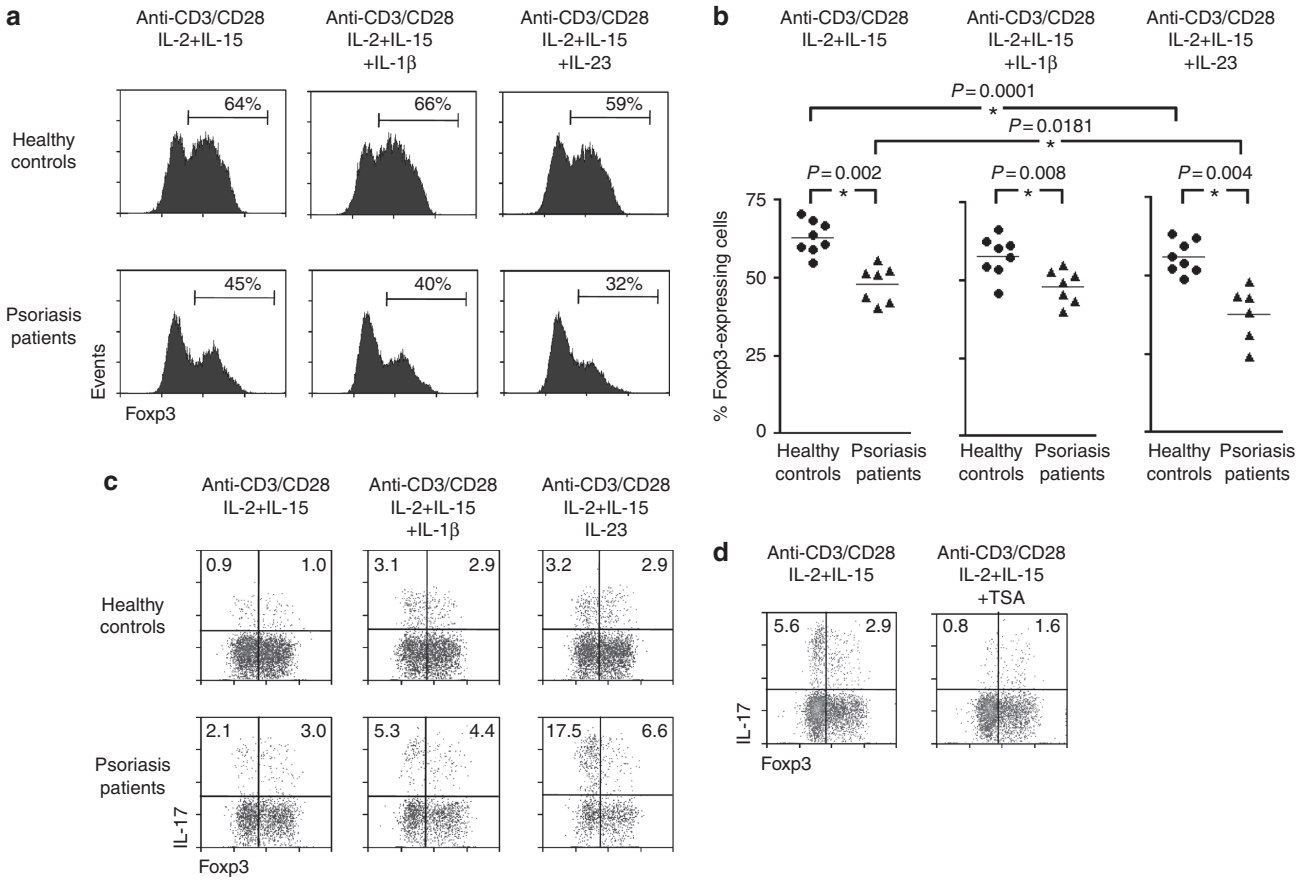


Figure 4. *Ex vivo*-stimulated CD4 + CD25^{high} T cells from patients with severe psoriasis show a reduced number of Foxp3 expressing cells. (**a**) Flowcytometry of intracellular Foxp3 expression in high-purity cell sorted CD4 + CD45RA-CD25^{high} from healthy controls (upper panel) and severe psoriasis patients (lower panel) on stimulation as indicated at the top. Measurements were taken at day 8. Histograms show Foxp3 expression (*x*-axis) and number of events (*y*-axis). Percentages of Foxp3-expressing cells are indicated in the histograms. Representative experiments from $n = 6$–7 controls or patients are shown. (**b**) Summarized data showing the percentages of Foxp3 expressing (*y*-axis) in sorted and *ex vivo*-stimulated CD4 + CD45RA-CD25^{high} from healthy controls and severe psoriasis patients as indicated in **a**. Each data point represents one separate experiment conducted with cells obtained from different healthy blood donors or severe psoriasis patients. (**c**) Flowcytometry of intracellular Foxp3 and IL-17A expression in high-purity cell sorted CD4 + CD45RA-CD25^{high} cells from healthy controls (upper panels) and severe psoriasis patients (lower panels) on *ex vivo* stimulation as mentioned under **a**. (**d**) Stimulation of psoriasis patient-derived Tregs in the absence or of presence Trichostatin-A (TSA). Dot plots show percentage of IL-17A-producing cells that are Foxp3− or Foxp3 +. Representative experiments from $n = 6$–7 controls or patients are shown.

Cell isolation and culture of cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway). CD4 + T cells were purified from peripheral blood mononuclear cells by negative selection as described previously (Koenen *et al.*, 2008) or by positive selection using the magnetic-activated cell sorting CD4-beads (Miltenyi-Biotec, Bergisch-Gladbach, Germany) as indicated by the manufacturer. To isolate CD4 + CD45RA-CD25^{high} Tregs, purified CD4 + T cells were labeled with CD4(MT310)FITC (Dako, Glostrup, Denmark), CD25(MA251)PE (BD-Biosciences, Erembodegem, Belgium), CD45RA(2H4)ECD (Beckman-Coulter, Mijdrecht, the Netherlands), thereafter CD4 + CD25^{high}CD45RA− cells (Tregs) were isolated by high-purity flowcytometric cell sorting using an Altra cell sorter (Beckman-Coulter). A re-run was performed to analyze the cell purity of the sorted cells, sorted cells were always >98% pure. In some experiments, CD4 + CD25 + T cells were isolated using negative CD4 selection combined with CD25 + magnetic-activated cell sorting isolation (Miltenyi-Biotec) using half the amount of beads as recommended by the manufacturer. Cells were cultured as described previously (Koenen *et al.*, 2008).

Reagents

Recombinant human cytokines IL-2 (12.5 U ml^{-1}, Proleukine, Amsterdam, the Netherlands), IL-15 (10 ng ml^{-1}), IL-1β (50 ng ml^{-1}; Biosource, Etten-Leur, the Netherlands), and IL-23 (50 ng ml^{-1}; R&D-Systems, Abingdon, UK). TSA (Sigma, Zwijndrecht, the Netherlands; 50 ng ml^{-1}).

Flowcytometry and antibodies

Cells were analyzed by five to six color flowcytometry as described previously (Koenen *et al.*, 2008) using an FC500 or CyAn flowcytometer (Beckman-Coulter). The following conjugated antibodies were used: CD3(UCHT1), CD4(MT310), CD45RA(4KB5) (Dako), CD25(M-A251), CD127(M21) (BD biosciences), CD4(T4) (Beckman-Coulter). Intracellular analysis of Foxp3(259D/C7)AF647

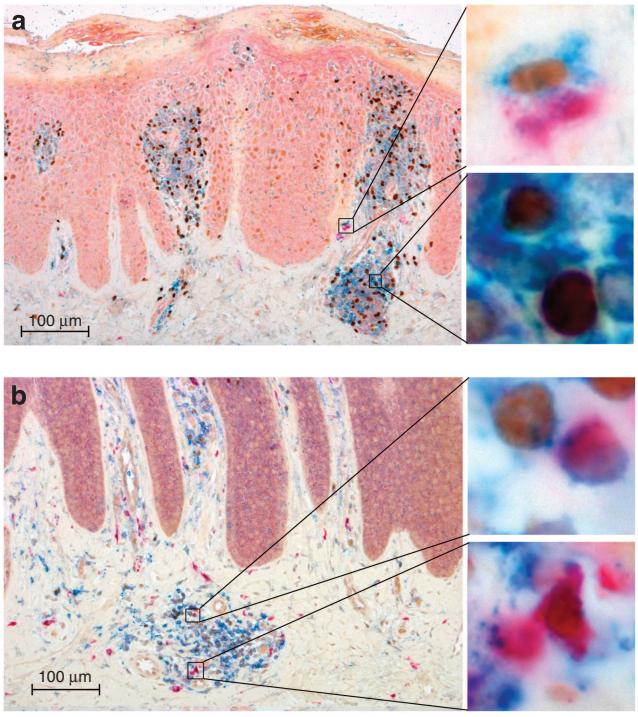


Figure 5. IL-17A-producing Foxp3+ CD4+ T cells are present in the epidermis of the affected skin of severe psoriatic patients. Immunohistochemistry, showing triple staining of Foxp3 (brown), IL-17A (red), and CD4 (blue) in the lesional skin of two different patients with psoriasis (**a**, **b**). In one patient, the infiltrate was dense and mainly present in the upper part of the dermis (**a**), whereas in the other patient the infiltrate was more restricted to the middle part of the dermis (**b**). At the right side, higher magnifications of the indicated cells are shown. Representative immunostainings of sections from biopsies of severe psoriasis patients ($n=3$) are shown.

(BD biosciences), Foxp3(PCH101)FITC, PE or pacific blue, RORγ t(AFKJS-9)PE or APC and IL-17A(6CAP17)PE of AF647 (eBioscience, SanDiego, CA) was performed as described previously (Koenen *et al.*, 2008). Isotype-matched antibodies were used to define marker settings.

T-cell proliferation and co-culture suppression assays

The proliferative capacity of isolated CD4+CD25+ or CD4+CD25− T cells (5×10^4) was analyzed by ^{3}H-thymidine incorporation, as described previously (Koenen *et al.*, 2008), after stimulation with anti-CD3/CD28-coated beads (Invitrogen, Breda, the Netherlands) with or without exogenously added recombinant human IL-2 (12.5 U ml^{-1}). The suppressor capacity of T cells was studied in co-culture assays (Koenen *et al.*, 2008). In brief, CD4+CD25− (5×10^4) T cells were stimulated with anti-CD3/CD28-coated beads in the absence and presence of decreasing numbers of CD4+CD25+ or CD4+CD25− T cells. Cell proliferation was analyzed at day 4 of the cultures.

Cytokine measurement in cell culture supernatants

Cytokines were determined in the supernatant of the T-cell cultures by Luminex cytokine assays (IL-17A, IFNγ; Biorad, Veenendaal, the Netherlands) and ELISA (IL-22; R&D-systems) according to the manufacturer's instructions.

Immunohistochemistry of skin biopsies

Tissue sections of 4–6 μm were generated after embedding the skin biopsies in paraffin. Triple immunostainings were conducted using anti-human primary antibodies: CD4 (BC/1F6; SantaCruz Biotechnology, SantaCruz, CA), FoxP3(PCH101), and polyclonal goat IL-17A (R&D-Systems). For the first immunostaining, antigens were retrieved by boiling the sections in Tris/EDTA buffer (50 mM Tris, 2 mM EDTA, pH 9.0) for 3 minutes. After washing, sections were incubated overnight with anti-FoxP3 antibody (1:100). To detect Foxp3+ cells, the section were sequentially washed and incubated (30 minutes) with biotinylated anti-rat antibody (1:200; Vector Laboratories, Burlingame, CA), avidin–biotin complex, horseradish peroxidase solution (1:50; Vectastain ABC-elite kit; Vector Laboratories), and visualized using 3,3′ diaminobenzidine. For the second immunostaining, the sections were washed, pre-incubated for 15 minutes with 20% normal swine serum and incubated overnight with anti-IL-17A antibody (1:500). To detect IL-17A-producing cells, the section were stained using the Labeled Streptavidin Biotin method (Universal LSAB+ Kit/AP; Dako) and visualized using Permanent Red (Dako). Finally, for the third immunostaining, the sections were preincubated as described above and incubated overnight with anti-CD4 antibody (1:100). CD4 expressing cells were detected by the LSAB method and visualized using Vector Blue (Vector Laboratories). Sections were photographed at an objective magnification of ×20 or ×40 using a microscope (Axioskop2 MOT; Zeiss, Sliedrecht, the Netherlands), digital camera (Axiocam MRc5; Zeiss) and AxioVision software (Zeiss).

Statistics

Results are presented as mean ± SEM if not otherwise stated. Analyses of variance and two-tailed *t*-tests were performed were appropriate. Differences were considered significant at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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