Circulating Th17, Th22, and Th1 Cells Are Increased in Psoriasis

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Th17, Th22, and Th1 cells are detected in psoriatic skin lesions and implicated in psoriasis pathogenesis, but inflammatory T cell numbers in blood, as well as the relative importance of each cell type, is unclear. Using 7-color flow cytometry, circulating Th17, Th22, and Th1 cells were quantified in 21 untreated psoriatics and 17 healthy individuals. CCR6 was the best cell surface marker for IL-17A+ cells when compared with IL-23R or CD161. CCR6+, IL-17A+, IL-22+, CCR6+IL-17A+, CCR6+IL-22+, CCR6+tumor necrosis factor-α+, IL-17A+IFN-γ−, IL-17A+IL-22+IFN-γ−, and IL-17A+IL-22−IFN-γ− cells were increased in psoriatics (all values $P<0.001$), indicating elevations in circulating Th17 cells, using multiple criteria to define these cells. Th22 (IL-17A−IL-22+IFN-γ−, $P<0.05$) and Th1 (IL-17A−IFN-γ+, $P<0.05$) cells were also increased in psoriatics, but to a lesser extent. Inhibition of either NF-κB or STAT3 *in vitro* blocked cytokine production by both Th17 and Th1 cells. Circulating levels of Th17 and Th1 cells decreased in a subset of five psoriasis patients serially evaluated following induction therapy with infliximab. In summary, elevated numbers of circulating inflammatory T cells may contribute to cutaneous inflammation and systemic inflammatory disease that occurs in individuals with psoriasis.

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INTRODUCTION

T-helper (h)17 cells are inflammatory CD4+ T cells that produce IL-17A and not IFN-γ (Miossec *et al.*, 2009). These cells and the cytokines produced by these cells are found in increased levels within skin affected by psoriasis (Chan *et al.*, 2006; Wolk *et al.*, 2006; Wilson *et al.*, 2007; Zaba *et al.*, 2007; Kryczek *et al.*, 2008; Lowes *et al.*, 2008; Harper *et al.*, 2009). Th22 cells are recently described inflammatory CD4+ T cells that produce IL-22, but do not express IL-17A or IFN-γ (Duhen *et al.*, 2009; Liu *et al.*, 2009; Nograles *et al.*, 2009; Trifari *et al.*, 2009). Th22 cells are also increased within psoriasis lesions (Harper *et al.*, 2009; Nograles *et al.*, 2009). Before the discovery of Th17 and Th22 cells, inflammatory CD4+ T cell studies in psoriasis focused on Th1 cells, which produce IFN-γ as their signature cytokine. It has been well established for many years that Th1 cells and cytokines are elevated in psoriatic tissue as well (Uyemura *et al.*, 1993; Szabo *et al.*, 1998; Austin *et al.*, 1999). As the past and current data show that Th17, Th22, and Th1 cells are all present within skin tissue affected by psoriasis, controversy exists as to the relative importance of each T cell type in disease pathogenesis. Thus, in human diseases where Th17, Th22, and Th1 cells are believed to have primary pathogenic roles, such as psoriasis, it is a fundamental aim of researchers in this field to determine (1) the relative contribution of each cell type to disease pathogenesis; (2) whether cell numbers correlate with disease activity and response to therapies; and (3) whether Th17, Th22, and Th1 cells are increased throughout the body as well as within affected tissues or whether these cells are expanded locally only at disease sites.

To help address these critical questions, identification and quantification of human Th17, Th22, and Th1 cells by flow cytometric analyses has been described over the last several years (Acosta-Rodriguez *et al.*, 2007; Annunziato *et al.*, 2007; Wilson *et al.*, 2007; Cosmi *et al.*, 2008; Duhen *et al.*, 2009; Trifari *et al.*, 2009). Th17 cell clones derived through *in vitro* differentiation are CCR6+, and a subset of Th1 clones is also CCR6+ (Acosta-Rodriguez *et al.*, 2007). Coexpression of CCR6 and CCR4 as well as expression of IL-23R helped distinguish Th17 cells from Th1 cells, which were found to be CXCR3+ and IL-23R− (Acosta-Rodriguez *et al.*, 2007). Annunziato *et al.* showed that CCR6 expression was much more commonly expressed by Th17 clones, whereas both this group and Wilson *et al.* confirmed that, as expected, IL-23R was expressed by Th17 clones and not by Th1 clones (Annunziato *et al.*, 2007; Wilson *et al.*, 2007). CD161 was more recently shown to be a cell surface marker for Th17 cells (Cosmi *et al.*, 2008); expression of CD161 has not yet been evaluated in patients with psoriasis. Using mainly cytokine production to label cells, circulating Th17 cells have

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Abbreviation: PASI, Psoriasis Area and Severity Index

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been reported to be elevated in patients with Crohn's disease (Kleinschek *et al.*, 2009), ankylosing spondylitis (Shen *et al.*, 2009), and rheumatoid arthritis (Shen *et al.*, 2009).

In this study, 7-color flow cytometry was used to identify and quantify Th17, Th22, and Th1 cells among circulating primary CD4+ cells isolated from individuals with and without psoriasis. CCR6, IL-23R, and CD161 were used as cell surface markers, and intracellular cytokine expression for IL-17A, IL-22, IFN-γ, and tumor necrosis factor (TNF)-α was assessed. A subset of patients undergoing infliximab therapy was serially assayed over the initial course of their treatment. Th17, Th22, and Th1 cells, as defined by a number of criteria, were clearly increased in blood of psoriatics. Increased numbers of circulating inflammatory T cell subsets may contribute to both cutaneous inflammation and to systemic inflammatory disease as occurs in individuals with psoriasis.

RESULTS

Circulating CCR6+, IL-23R+, and CD161+ CD4+ cells are increased in psoriasis

Table 1 shows the demographic features of individuals analyzed in this study. For all experiments, CD4+ cells were purified from peripheral blood by negative selection (>95% purity, data not shown) and cultured for 1 day with or without phorbol myristate acetate (PMA) and ionomycin for the last 6 hours of culture. In single-color flow analyses, percentages of CD4+ cells that were CCR6+, IL-23R+, or CD161+ were increased in psoriatics compared with healthy individuals (16.6 ± 6.44 vs 8.82 ± 3.12%, $P<0.001$; 19.9 ± 6.36 vs 9.85 ± 5.75%, $P<0.01$; 14.1 ± 4.33 vs 10.9 ± 4.06%, $P<0.05$, respectively) (Figure 1a). It is noted that mitogen activation for 6 hours was found to decrease CCR6 surface expression (13.2 ± 5.76% unstimulated vs 12.0 ± 5.18% stimulated, $P<0.01$, $n=53$), but not IL-23R (15.6 ± 8.98% unstimulated vs 16.1 ± 6.11% stimulated, $n=12$) or CD161 (12.8 ± 4.58% unstimulated vs 13.1 ± 4.69% stimulated, $n=45$) surface expression. Levels of circulating CCR6+, IL-23R+, and CD161+ CD4+ cells did not significantly correlate with skin disease severity (see Supplementary Figure S1a online).

To determine whether CCR6, IL-23R, and CD161 label the same or unique populations of human CD4+ cells, cells were then analyzed for simultaneous expression of all three surface markers. Surprisingly, CCR6, IL-23R, and CD161 did not consistently label the same cells (Figure 1b and c). There were significantly more CCR6+IL-23R+CD161+ cells, CCR6+IL-23R+CD161− cells, CCR6−IL-23R+CD161+ cells, and CCR6−IL-23R+CD161− cells in psoriatics compared with healthy individuals (2.58 ± 1.56 vs 0.90 ± 0.85%, $P<0.01$; 3.62 ± 2.33 vs 1.44 ± 0.86%, $P<0.05$; 1.90 ± 0.91 vs 1.05 ± 0.72%, $P<0.05$; 11.8 ± 3.04 vs 6.44 ± 3.77%, $P<0.01$, respectively) (Figure 1c). By contrast, there were fewer CCR6−IL-23R−CD161− cells in psoriatics (67.5 ± 8.07 vs 79.5 ± 5.59%, $P<0.001$) (Figure 1c).

Table 1. Demographics of healthy individuals and untreated psoriatics evaluated in this study

Age of healthy individuals	34.0 ± 13.0 years
Age of untreated psoriatics	42.3 ± 12.0 years
PASI of untreated psoriatics	22.2 ± 24.9

Abbreviation: PASI, Psoriasis Area and Severity Index.
Data are expressed as mean ± SD.

Circulating Th17 cells are increased in psoriasis, but do not correlate with skin disease severity

As T cells immediately secrete cytokines on production *in vivo*, intracellular detection of cytokines in these cells *ex vivo* normally requires a brief stimulation period in the presence of brefeldin A, which blocks protein secretion. Thus, CD4+ cells were incubated with PMA and ionomycin for 6 hours in the presence of brefeldin A. In single-color analyses, IL-17A+, IL-22+, and TNF-α+ cells were elevated in psoriatics compared with healthy individuals (1.47 ± 0.74 vs 0.73 ± 0.34%, $P<0.001$; 1.71 ± 1.04 vs 1.00 ± 0.31%, $P<0.001$; and 56.4 ± 10.3 vs 41.4 ± 10.2%, $P<0.001$, respectively) (Figure 2a). IFN-γ+ cells were also elevated in psoriatics, but to a lesser extent (10.3 ± 3.87 vs 7.55 ± 2.58%, $P<0.05$) (Figure 2a). Cells were then analyzed for simultaneous expression of IL-17A and IFN-γ (Figure 2b and c). Circulating IL-17A+IFN-γ− cells, a classic definition of Th17 cells, were increased in psoriatics when compared with healthy individuals (1.33 ± 0.71 vs 0.66 ± 0.28%, $P<0.001$) (Figure 2c). IL-17A−IFN-γ+ and IL-17A+IFN-γ+ cells were also increased in psoriatics, indicating elevations in Th1 and Th17/Th1 cells, but to a lesser extent when compared with IL-17A+IFN-γ− cells (8.68 ± 3.28 vs 6.07 ± 1.81%, $P<0.05$ and 0.21 ± 0.14 vs 0.11 ± 0.07%, $P<0.01$, respectively) (Figure 2b and c). Levels of circulating IL-17A+, IL-22+, IFN-γ+, and TNF-α+ CD4+ cells did not significantly correlate with skin disease severity (Supplementary Figure S1b).

Most circulating Th17 cells do not simultaneously express both IL-17A and IL-22

To determine whether IL-17A+, IL-22+, and IFN-γ+ label the same or unique populations of circulating human CD4+ cells, cells were analyzed for simultaneous expression of all three intracellular cytokines. Interestingly, IL-17A and IL-22 production did not consistently occur in the same cells, whereas IL-17A and IFN-γ production did not consistently occur in separate cell populations (Figure 3a and b). For these experiments, Th17 cells were defined as IL-17A+IL-22+IFN-γ− and IL-17A+IL-22−IFN-γ− cells, and by this definition, they were increased in blood of psoriatics compared with healthy individuals (0.19 ± 0.20 vs 0.07 ± 0.04%, $P<0.001$ and 1.02 ± 0.71 vs 0.52 ± 0.25%, $P<0.001$, respectively) (Figure 3b). By contrast, Th1 (IL-17A−IL-22−IFN-γ+), Th17/Th1 (IL-17A+IL-22−IFN-γ+), and Th22 (IL-17A−IL-22+IFN-γ−) cells were increased to lesser degrees in psoriatics compared with healthy individuals (8.84 ± 3.11 vs 5.93 ± 1.86%, $P<0.01$, 0.13 ± 0.11 vs 0.05 ± 0.03%, $P<0.01$, and 1.29 ± 0.78 vs 0.86 ± 0.32%, $P<0.05$, respectively) (Figure 3b). Circulating

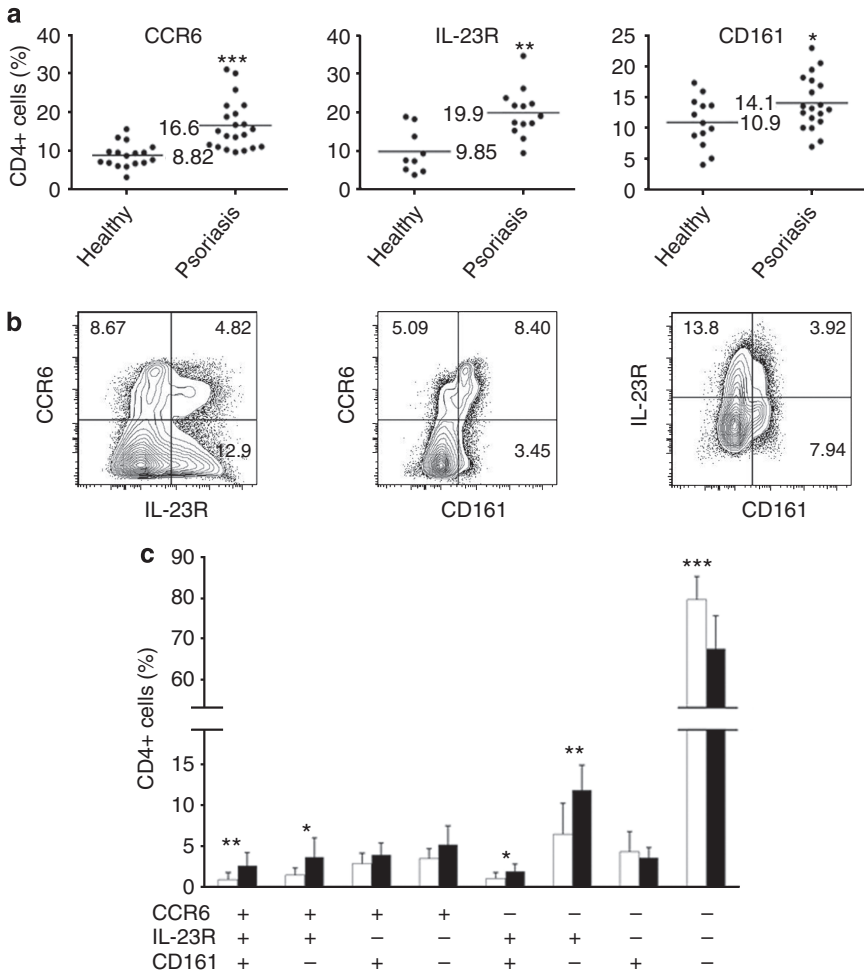

Figure 1. Circulating CCR6+, IL-23R+, and CD161+ cells are increased in psoriasis. (**a**) The percentage of CD4+ cells expressing CCR6, IL-23R, and CD161 among unstimulated cells from 17 healthy individuals and 21 untreated psoriatics (horizontal bars, mean; each circle, single donor). (**b**) Representative dot plot analyses of CCR6, IL-23R, and CD161 expression in circulating unstimulated CD4+ cells from the same individual. (**c**) The percentages of each subset divided by the total number of circulating CCR6, IL-23R, and CD161-positive cells in unstimulated CD4+ cells of healthy individuals (white bars) and psoriatics (black bars). Data expressed as mean ± SD; *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

cells negative for all three of these cytokines were significantly lower in psoriatics (88.3 ± 4.12 vs 92.4 ± 2.15%, $P<0.001$) (Figure 3b), indicating a general trend toward higher levels of inflammatory cytokine-producing CD4+ cells in persons with psoriasis compared with those without this disease.

IL-22 plasma levels are increased in psoriasis and correlate with skin disease severity

Plasma levels of IL-17A, IL-22, IFN-γ, and TNF-α were also measured. Plasma IL-22 and TNF-α levels, but not IL-17A or IFN-γ levels, were significantly higher in psoriatics compared with controls ($P<0.001$ for both) (Supplementary Figure S2a). It is noted that IL-22 plasma levels correlated significantly with PASI ($r>0.45$ and $P<0.05$) (Supplementary Figure S2b). Plasma levels of IL-17A, IFN-γ, and TNF-α did not significantly correlate with skin disease severity (Supplementary Figure S2b).

Based on IL-17A production, CCR6 is a more reliable cell surface marker for circulating Th17 cells when compared with IL-23R or CD161

Most of the scientific literature on the phenotype of human Th17 cells is derived from experiments where naive CD4+ T cells are induced to differentiate *in vitro* into relatively pure polarized populations of Th17 clones, and then examined for cell surface and cytokine expression (Acosta-Rodriguez *et al.*, 2007; Annunziato *et al.*, 2007; Cosmi *et al.*, 2008). In contrast, we studied populations of primary Th17, Th22, and Th1 cells isolated and analyzed directly from human blood. These cells have been identified within the pool of circulating CD4+CD45RO+ (memory) T cells (Miossec *et al.*, 2009). Th17 cells were first defined as CD4+ cells that produced IL-17A and Th1 cells as CD4+ cells that produced IFN-γ. Populations of cells producing IL-17A, IL-22, or IFN-γ were then gated and examined for expression of CCR6, IL-23R, CD161, and

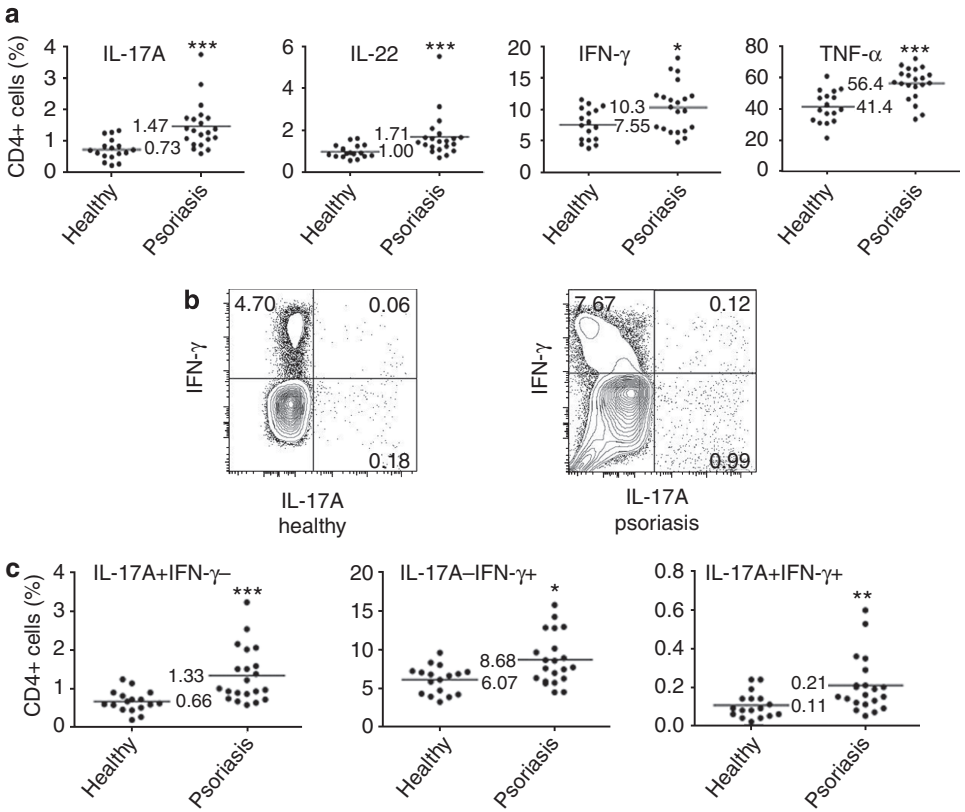

Figure 2. Circulating Th17, Th1, and Th17/Th1 cells are increased in psoriasis. (**a**) In single-color flow analyses, circulating IL-17A+, IL-22+, IFN-g+, and tumor necrosis factor (TNF)-alpha+ cells are increased in psoriasis patients. (**b**) Representative two-color dot plot analyses of IL-17A and IFN-γ expression in stimulated CD4+ cells from a healthy volunteer and a psoriasis patient. (**c**) The percentages of circulating Th17 (IL-17A+IFN-γ−), Th1 (IL-17A−IFN-γ+), and Th17/Th1 (IL-17A+IFN-γ+) cells among stimulated CD4+ cells from healthy individuals and psoriatics. For **a** and **c**, each circle, single donor; horizontal bars, mean; and $*P<0.05$, $**P<0.01$, and $***P<0.001$.

TNF-α. Most IL-17A+ cells and more than half of IL-22+ cells expressed CCR6, whereas only a quarter of IFN-γ+ cells expressed CCR6 (80.9±12.4, 57.7±13.2, and 25.8±9.04%, respectively, $P<0.001$ when comparing CCR6+IL-17A+ or CCR6+IL-22+ cells with CCR6+IFN-γ+ cells) (Figure 4). Surprisingly, only 23% of IL-17A+ cells expressed IL-23R, yet both IL-17A+ and IL-22+ cells were more likely to express IL-23R than IFN-γ+ cells ($P<0.05$ and $P<0.001$, respectively) (Figure 4). CD161 expression was significantly increased by IL-17A+ cells (58.6±15.0% for IL-17A+, 34.7±10.6% for IL-22+, and 25.6±11.2% for IFN-γ+, respectively), but to a lesser extent compared with CCR6 expression (Figure 4). IL-22+ cells were less likely to produce TNF-α simultaneously compared with IL-17A+ or IFN-γ+ cells (83.6±6.88% for IL-17A+, 66.8±13.9% for IL-22+, and 81.6±9.35% for IFN-γ+, respectively) (Figure 4). From these results, we conclude that most primary human circulating Th17 cells express CCR6, CD161, and TNF-α, whereas most primary human circulating Th1 cells express TNF-α, but do not express CCR6 or CD161. These data also suggest that CCR6 is a more reliable cell surface marker when compared with IL-23R or CD161 to help distinguish between circulating human Th17 and Th1 cells.

Circulating CCR6+IL-17A+, CCR6+IL-22+, and CCR6+TNF-α+ cells are increased in psoriatics when compared with healthy individuals

CD4+ cell subsets in individuals with or without psoriasis were then analyzed using combinations of cell surface protein and intracellular cytokine expression. Circulating CCR6+IL-17A+, CCR6+IL-22+, and CCR6+TNF-α+ cells were greatly increased in psoriatics (1.19±0.66 vs 0.55±0.25%, 1.02±0.77 vs 0.42±0.21%, and 14.8±5.94 vs 8.65±3.26%, respectively, $P<0.001$ for all three subsets) (Figure 5). IL-23R+IL-22+ (0.73±0.62 vs 0.17±0.07%) and IL-23R+TNF-α+ cells (8.65±4.32 vs 3.64±2.75%), but not IL-23R+IL-17A+ cells, were increased in psoriatics as well, but to a lesser extent ($P<0.01$ and $P<0.05$, respectively) (Figure 5). Furthermore, CD161+IL-17A+ (0.84±0.41 vs 0.45±0.22%) and CD161+IL-22+ (0.66±0.53 vs 0.28±0.14%), but not CD161+TNF-α+ cells, were significantly increased in psoriatics ($P<0.01$ for both) (Figure 5).

NF-κB and STAT3 inhibition *in vitro* blocks cytokine production by circulating Th17 and Th1 cells

Phorbol myristate acetate and ionomycin, which were used to stimulate cytokine production in this study,

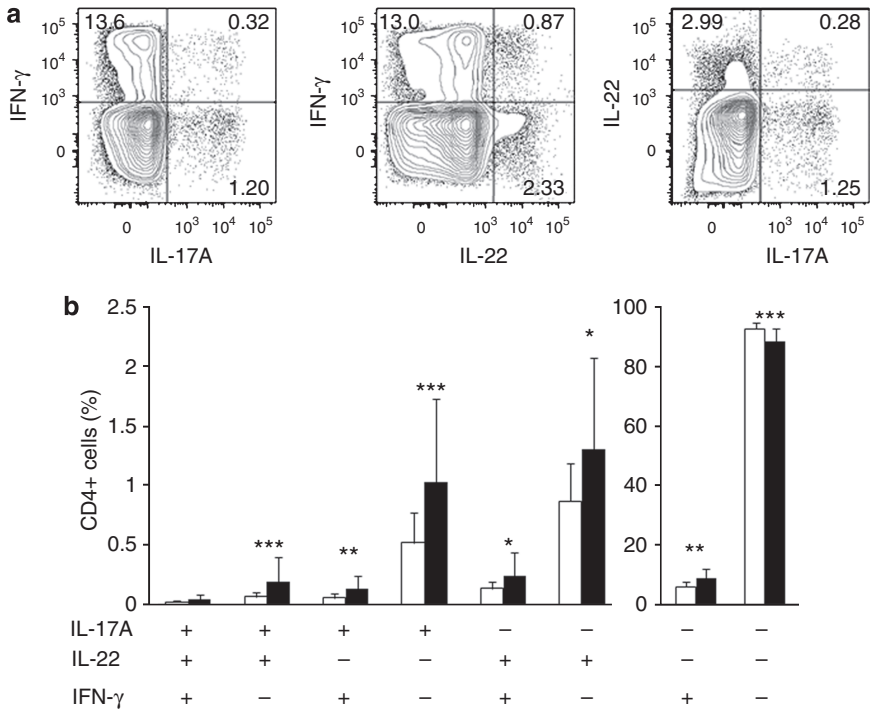

Figure 3. Most Th17 cells do not simultaneously express both IL-17A and IL-22. (**a**) Representative two-color dot plot analyses of IL-17A, IL-22, and IFN-γ expression in stimulated CD4+ cells from a single patient with psoriasis. (**b**) The percentages of circulating CD4+ cells that coexpress IL-17A, IL-22, and IFN-γ in healthy individuals (white bars) and psoriatics (black bars). Data expressed as mean ± SD; $*P<0.05$, $**P<0.01$, and $***P<0.001$.

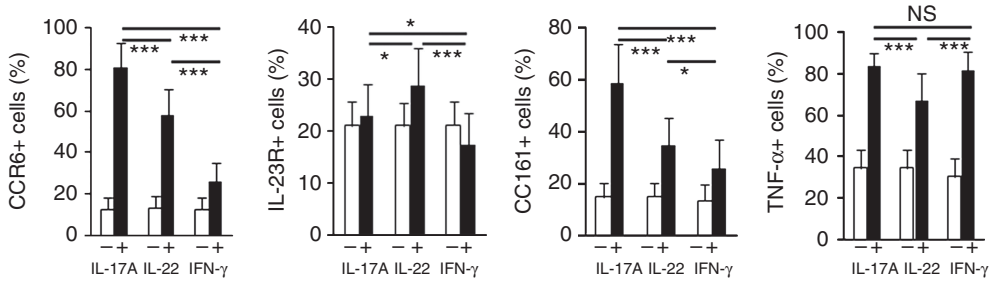

Figure 4. On the basis of IL-17A production, CCR6 is a more reliable cell surface marker for Th17 cells when compared with IL-23R or CD161. IL-17A+ cells, IL-22+ cells, and IFN-γ+ cells from psoriatics were gated and the percentage of cells coexpressing CCR6, IL-23R, CD161, or tumor necrosis factor (TNF)-α was determined (black bars). Similarly, IL-17A− cells, IL-22− cells, and IFN-γ− cells from psoriatics were gated and the percentage of cells coexpressing CCR6, IL-23R, CD161, or TNF-α was determined (white bars). Data expressed as mean ± SD; NS, not significant; $*P<0.05$, $**P<0.01$, and $***P<0.001$.

are known to activate both NF-κB and STAT3 (Caspar-Bauguil *et al.*, 1999; Hartley and Cooper, 2000). Both NF-κB and STAT3 have also have been implicated in mediating IL-17A production in Th17 cells (Cho *et al.*, 2006). Thus, NF-κB or STAT3 pathways were inhibited by adding specific pharmacologic inhibitors of these proteins *in vitro* (parthenolide or stattic, respectively). These agents blocked CD4+ cell production of IL-17A, IL-22, IFN-γ, and TNF-α equally well in both healthy individuals ($n=6$) and psoriatics ($n=4$) (Figure 6). Observed effects were dose dependent and the inhibitors did not decrease cell viability at the indicated concentrations (Figure 6).

Infliximab induces modest decreases in circulating Th17 and Th1 cells over time in a small cohort of psoriatics

Infliximab is a potent TNF-α chimeric mAb that is FDA approved for severe psoriasis. Five of the 21 patients evaluated in our study received infliximab at weeks 0, 2, and 6, and also had serial 7-color flow cytometry performed on circulating CD4+ cells at weeks 0, 2, 6, and 14 (blood drawn just before each infusion). Infliximab therapy over time led to improvement in skin disease scores in all patients (Table 2). Relatively minor decreases in Th17 cells (CCR6+, CCR6+IL-17A+, CCR6+IL-22+, CCR6+TNF-α+) and Th1 cells (IFN-γ+, IL-17A−IFN-γ+, IFN-γ+TNF-α+) were

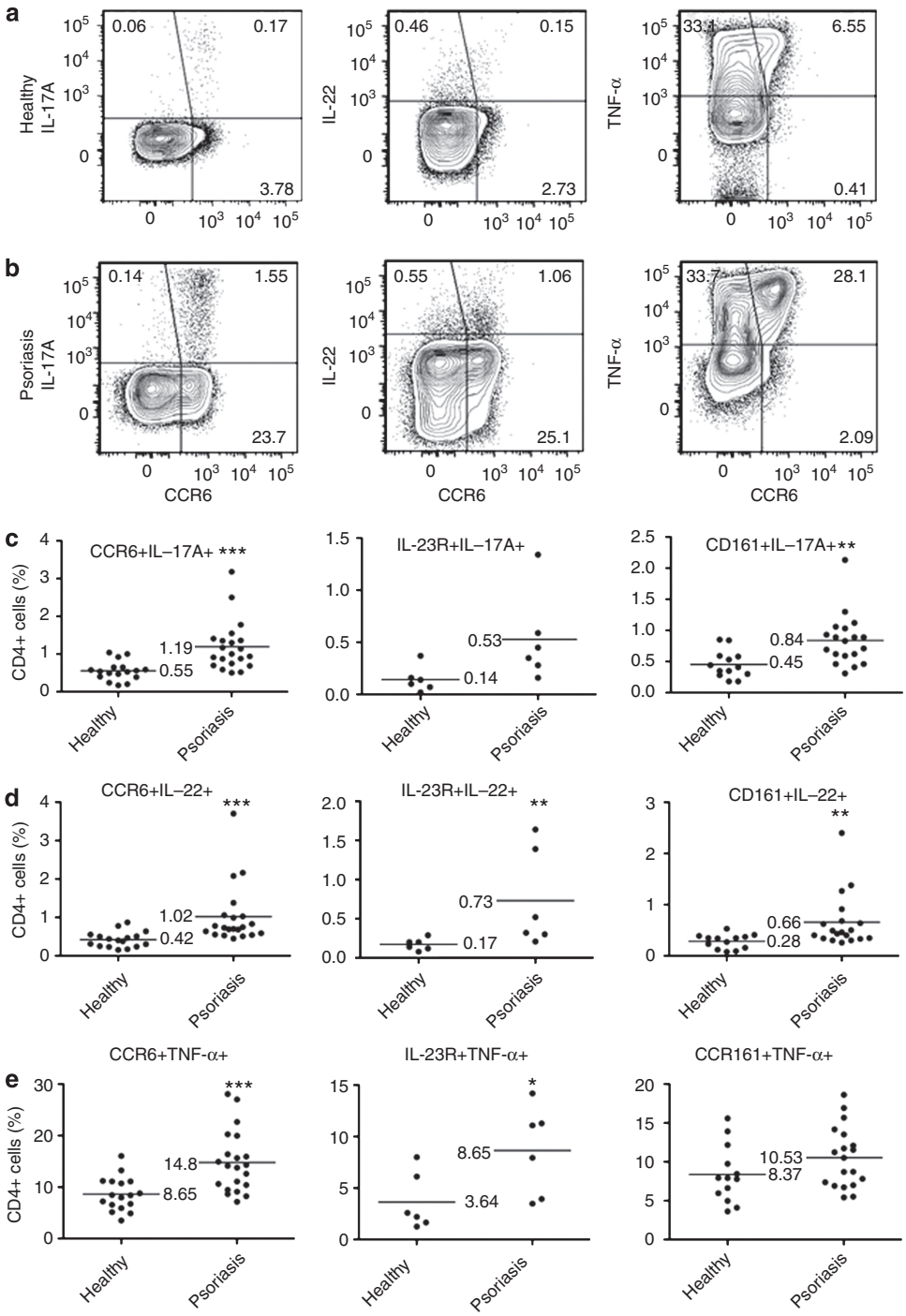

Figure 5. CCR6 + IL-17A +, CCR6 + IL-22 +, and CCR6 + TNF-α + cells are increased in psoriatics compared with healthy individuals. Representative two-color dot plot analyses of CCR6 and IL-17A/IL-22/tumor necrosis factor (TNF)-α expression in stimulated CD4 + cells from a single healthy volunteer (**a**) and a psoriasis patient (**b**). (**c**) The percentages of circulating IL-17A + cells that coexpress CCR6, IL-23R, and CD161 among stimulated CD4 + cells from healthy individuals and psoriatics. (**d**) The percentages of circulating IL-22 + cells that coexpress CCR6, IL-23R, and CD161 among stimulated CD4 + cells from healthy individuals and psoriatics. (**e**) The percentages of circulating TNF-α + cells that coexpress CCR6, IL-23R, and CD161 among stimulated CD4 + cells from healthy individuals and psoriatics. Each circle, single donor; horizontal bars, mean; *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

also seen in all five patients (Table 2), with a suggestion that greater decreases occurred in Th17 cells compared with Th1 cells. Too few patients, however, were evaluated to generate statistically significant differences in these parameters.

DISCUSSION

There are considerable data showing that both Th17 and Th1 cells are increased within involved psoriatic skin (Uyemura *et al.*, 1993; Szabo *et al.*, 1998; Austin *et al.*, 1999; Chan

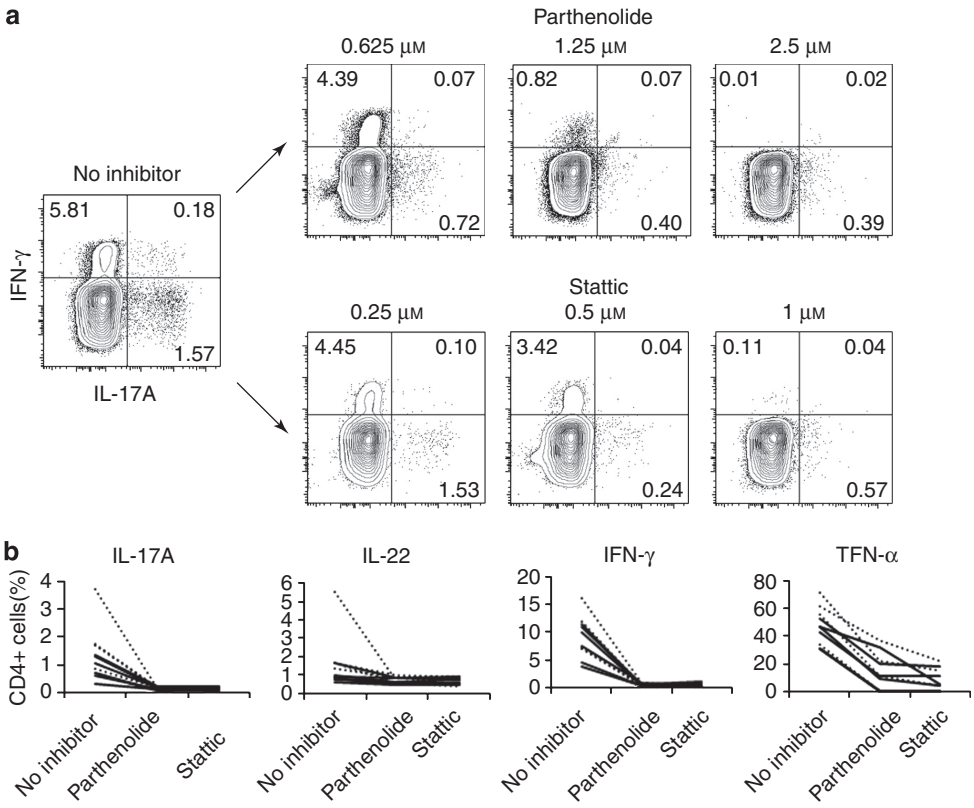

Figure 6. NF-κB and STAT3 inhibition *in vitro* blocks cytokine production by both Th17 and Th1 cells in a dose-dependent manner. (**a**) CD4+ cells were cultured overnight with or without parthenolide or stattic at the indicated concentrations, and then activated with phorbol myristate acetate (PMA)/ionomycin for 6 hours. Representative two-color dot plot analyses of IFN-γ and tumor necrosis factor (TNF)-α expression from a single healthy volunteer are shown. (**b**) The percentage of circulating CD4+ cells producing IL-17A, IL-22, IFN-γ, and TNF-α in healthy individuals (solid lines) and psoriatics (dotted lines) with or without 2.5 μM of parthenolide or 1 μM of stattic. Each line, single donor.

Table 2. Decreases in skin disease severity scores and circulating Th17 and Th1 cell numbers over time in five psoriasis patients after initial treatment with infliximab

	Week 0 (%)	Week 2 (%)	Week 6 (%)	Week 14 (%)
PASI	7.7 ± 6.3[1]	5.6 ± 3.0	4.0 ± 2.6	3.7 ± 2.4
Th17 markers				
CCR6+	16.4 ± 3.40	15.4 ± 2.71	14.2 ± 3.84	10.4 ± 3.17
CCR6+IL-17A+	1.37 ± 0.69	1.28 ± 0.52	1.28 ± 0.72	1.04 ± 0.62
CCR6+IL-22+	0.87 ± 0.34	0.91 ± 0.48	0.68 ± 0.47	0.56 ± 0.23
CCR6+TNF-α+	16.3 ± 2.24	13.5 ± 2.05	13.4 ± 1.84	12.4 ± 3.97
Th1 markers				
IFN-γ+	12.4 ± 4.42	11.5 ± 4.29	11.2 ± 3.44	10.9 ± 3.92
IFN-γ+IL-17A−	9.42 ± 3.83	9.21 ± 3.11	8.00 ± 1.35	7.46 ± 2.29
IFN-γ+TNF-α+	8.42 ± 2.90	7.42 ± 3.36	6.31 ± 1.37	6.58 ± 2.55

Abbreviations: PASI, Psoriasis Area and Severity Index; TNF, tumor necrosis factor.
[1]Data are expressed as mean ± SD.

et al., 2006; Wolk *et al.*, 2006; Annunziato *et al.*, 2007; Wilson *et al.*, 2007; Zaba *et al.*, 2007; Kryczek *et al.*, 2008; Lowes *et al.*, 2008; Harper *et al.*, 2009). Here, we show clear evidence of significant elevations in circulating Th17 cells in psoriasis, using a number of criteria to define these cells. More specifically, we show increases in circulating CCR6+, IL-17A+, IL-22+, IL-17A+IFN-γ−, CCR6+IL-17A+, CCR6+IL-22+, CCR6+TNF-α+, IL-17A+IL-22+IFN-γ−, and IL-17A+IL-22−IFN-γ− CD4+ T cells in untreated psoriatics compared with healthy individuals ($P<0.001$ for all subsets) (Figures 1–3 and 5). Circulating Th1 cells, defined as IFN-γ+, IL-17A−IFN-γ+, and IL-17A−IL-22−IFN-γ+ CD4+ cells, were also elevated ($P<0.05$ for the first two subsets and $P<0.01$ for the third subset) (Figures 2 and 3). It is noted that two groups recently reported normal numbers of circulating Th17 cells in psoriatics (Kryczek *et al.*, 2008; Lowes *et al.*, 2008). There are three possible reasons for this discrepancy: (1) these investigators used intracellular expression of IL-17A and IFN-γ alone to define Th17 cells (we used multiple criteria); (2) CD4+ T cells were not purified before their analyses as we have done; and (3) they studied smaller numbers of patients and controls compared with our current report.

It is unknown how circulating Th17 cells in psoriasis patients may contribute to skin disease and inflammation at

sites other than skin. They may represent cells that are trafficking to and from skin, thus contributing significantly to cutaneous inflammation. However, the xenograft transplantation model of psoriasis, whereby human non-lesional skin from psoriatics is engrafted onto immunodeficient mice, is independent of circulating human T cells (Boyman *et al.*, 2004). Alternatively, and independent of skin disease severity, psoriatics may be genetically predisposed to make greater amounts of IL-23 and to create greater numbers of Th17 cells under the appropriate stimulation conditions, whether the cells are in the blood, skin, or elsewhere. This genetic tendency could be regulated, at least in part, by polymorphisms in *IL-23p19*, *IL-12/23p40*, and *IL-23R* (Nair *et al.*, 2009). Circulating Th17 cells could also have a role in systemic inflammation observed in psoriasis patients, including psoriatic arthritis and atherosclerosis (Gelfand *et al.*, 2006). Recent evidence suggests that Th17 cells and cytokines promote atherosclerosis (Cheng *et al.*, 2008; Eid *et al.*, 2009). Thus, we believe it would be important to study correlations between numbers and functional activity of circulating Th17 cells and the development of atherosclerosis and/or myocardial infarction in individuals with severe psoriasis.

Our data are strengthened by the utilization of 7-color flow cytometry to simultaneously examine both cell surface marker expression and intracellular cytokine expression in various circulating T cell subsets. As there is no universal cell surface marker to delineate Th17 cells from Th1 cells, we used several criteria to help distinguish these two subsets. CCR6 was expressed by 81% of circulating IL-17A+ cells and only 26% of circulating IFN-γ+ cells, making it the best cell surface marker we studied to delineate Th17 cells from Th1 cells (Figure 4). By contrast, CD161 and IL-23R were expressed by fewer circulating Th17 cells and by a minority of circulating Th1 cells, which made them less useful markers in separating these two T cell subsets (Figure 4). It is unclear why so few IL-17A+ cells expressed IL-23R, but this may have been due to downregulation of cell surface receptor expression following mitogenic stimulation of the cells. It is also possible that stimulation of cells by IL-23, and not by PMA/ionomycin, may be a more selective inducer of IL-17A specifically within the population of IL-23R+ Th17 cells. Even though CCR6 was shown to be the best Th17 cell surface marker used here, sensitivity and specificity were too low for CCR6 to be used as a sole marker for Th17 cells. Combining expression of CCR6 with intracellular cytokine production may prove to be a useful tool in future analyses of Th17 and Th1 cells in human disease. Very recently, the skin-homing markers CCR4 and CCR10 have been identified on blood-derived Th22 cells (Duhen *et al.*, 2009; Trifari *et al.*, 2009). It will be interesting to further characterize Th22 and Th17 subsets in blood of psoriatics for their expression of cutaneous lymphocyte antigen, the prototypic skin-homing marker, and these two chemokine receptors.

Little information is known about the function of Th17/Th1 cells and Th22 cells. Th17/Th1 cells were identified in the affected skin of psoriatics (Lowes *et al.*, 2008; Nograles *et al.*, 2009). Zaba *et al.* (2009) recently reported that dermal dendritic cells within psoriasis lesions induce a population of activated T cells that simultaneously produce IL-17A and IFN-γ (i.e., Th17/Th1 cells), which was not observed when using dermal dendritic cells isolated from normal skin. We show here that Th17/Th1 cells, defined as either IL-17A+IFN-γ+ or IL-17A+IL-22−IFN-γ+ cells, are a rare population of circulating cells, representing only 0.05–0.11% of CD4+ cells in healthy blood and 0.13–0.21% of CD4+ cells in psoriatic blood ($P<0.01$ for both subsets) (Figures 2 and 3). Our data suggest that the tendency to develop Th17/Th1 cells may be a general immunologic feature seen in psoriasis patients, and not necessarily a phenomenon restricted to inflamed skin. Another relatively obscure subset of T cells, Th22 cells (defined as IL-17A−IL-22+IFN-γ−), was more commonly detected when compared with Th17/Th1 cells: 1.29% of CD4+ cells in psoriatic versus 0.86% of CD4+ cells in healthy individuals ($P<0.05$) (Figure 3). The clinical relevance of circulating Th22 cells remains to be determined, although Liu *et al.* (2009) recently suggested that these cells are preferentially stimulated by *Candida albicans*, and thus may have a role in defense against fungal infections.

We also measured plasma cytokine levels, which have been notoriously difficult in prior studies of psoriasis, as pro-inflammatory cytokines are usually active and elevated only at sites of tissue inflammation and not within circulation. We showed that plasma IL-22 concentration is higher in psoriatics when compared with healthy individuals and that levels are highly reflective of skin disease activity (Supplementary Figure S2), which is consistent with two previous reports (Wolk *et al.*, 2006; Caproni *et al.*, 2009). We also detected increases in plasma TNF-α (Supplementary Figure S2), similar to several prior reports (Mussi *et al.*, 1997; Arican *et al.*, 2005). In contrast to IL-22 and TNF-α, IL-17A and IFN-γ concentrations are not elevated in plasma of psoriatics (Supplementary Figure S2a). Thus, despite some conflicting data on serum or plasma cytokine data, there appears to be consensus that circulating levels of IL-22 and TNF-α are increased in psoriatics, and that cytokine concentrations, especially for IL-22, correlate with extent of skin disease activity.

NF-κB is important in regulating expression of a wide variety of cytokines. Relevant to psoriasis, NF-κB expression is high in affected skin and normalizes following effective anti-psoriatic therapy with etanercept (Lizzul *et al.*, 2005). NF-κB regulates IL-17A production by inducing phosphorylation of IκB-α (Cho *et al.*, 2006). Here, we showed that specific inhibition of NF-κB by parthenolide led to the complete blockade of IL-17A, IL-22, IFN-γ, and TNF-α production by circulating CD4+ cells *in vitro* (Figure 6). Importantly, IL-17A production by Th17 cells is critically dependent on STAT3 activation through the PI3K pathway (Cho *et al.*, 2006). Furthermore, individuals with genetic mutations in *STAT3* develop Job's syndrome, also known as hyper-IgE syndrome, and are incapable of making Th17 cells (Ma *et al.*, 2008; Milner *et al.*, 2008). Accordingly, we showed that IL-17A, IL-22, IFN-γ, and TNF-α production by CD4+ cells could be completely blocked *in vitro* by stattic, a specific inhibitor of STAT3 (Figure 6). Although systemic

inhibition may be impractical on the basis of feasibility and safety issues, skin-specific inhibition of either NF-κB or STAT3 may be a future therapeutic strategy for patients with localized psoriasis.

Infliximab is a chimeric anti-TNF-α mAb that is highly efficacious in the treatment of individuals with moderate-to-severe plaque psoriasis (Reich *et al.*, 2005). Here, we serially evaluated numbers of circulating Th17 and Th1 cells in five patients receiving induction therapy with infliximab. Infliximab led to decreases in clinical disease scores and circulating levels of both Th17 and Th1 cells, including Th17 and Th1 cells that produced TNF-α (Table 2). Drops in both clinical and immunologic parameters, however, were relatively minor and not statistically significant, although there was a trend toward greater decreases in Th17 cell numbers when compared to Th1 cell numbers. We believe dramatic changes were not observed because the study included too few patients and because of the potential residual clinical and immunologic effects induced by etanercept therapy, which these five patients were receiving 2–4 weeks before their first infliximab infusion. Thus, greater decreases in circulating Th17 and Th1 cells may have been observed if the patients showed more severe skin disease and/or were treatment naive at the beginning of the study. It will be interesting to monitor circulating levels of Th17 and Th1 cells in patients receiving ustekinumab or ABT-874, two new highly effective human mAb therapies for moderate-to-severe psoriasis that target p40 (Kimball *et al.*, 2008; Leonardi *et al.*, 2008; Papp *et al.*, 2008), the subunit shared by IL-23 and IL-12. Drops in circulating levels of both Th17 and Th1 cells are expected, although it is unclear whether this will lead to decreases in systemic immune function.

MATERIALS AND METHODS

Study subjects and sample collection

The Oregon Health and Science University Institutional Review Board approved all aspects of this study according to the Declaration of Helsinki Principles. After giving informed consent, 17 healthy individuals and 21 untreated psoriatics were enrolled. Of these 21 patients, five were also enrolled in a 22-week open-label study to determine the efficacy of infliximab in treating psoriasis in patients who had previously failed etanercept therapy. These individuals were eligible for the study if they had active psoriasis lesions while on etanercept; etanercept therapy for psoriasis was stopped 2–4 weeks before starting infliximab. Each of these patients received intravenous infusions of infliximab 5 mg kg^{-1} at weeks 0, 2, 6, 14, and 22, a dose of infliximab previously shown to be highly effective for psoriasis (Reich *et al.*, 2005). Blood was isolated for T cell analyses just before infusions on weeks 0, 2, 6, and 14. Each time blood was collected from a psoriasis patient, Psoriasis Area and Severity Index (PASI) was determined, which is a standard measure of psoriasis clinical disease activity.

Fifty microliters of blood was collected from each study subject in EDTA-coated blood collection tubes (BD Biosciences, San Jose, CA). Blood samples were diluted 1:2 with phosphate-buffered saline and peripheral blood mononuclear cells were isolated through Ficoll-hypaque (Amersham, Piscataway, NJ) gradient centrifugation. Plasma samples were collected and stored at −20 °C until further use. CD4+ cells were then purified from peripheral blood mononuclear cells by negative selection using the EasySep human CD4+ T cell enrichment kit (Stemcell, Vancouver, Canada).

Antibody reagents

The following mouse anti-human mAbs and corresponding isotype control Abs were used for flow cytometry: phycoerythrin (PE)-conjugated IL-17A (BioLegend, San Diego, CA), Pacific Blue-conjugated IL-17A (BioLegend), PE-conjugated IL-22 (R&D Systems, Minneapolis, MN), PE-conjugated IFN-γ (BD Biosciences), PerCP-Cy5.5-conjugated IFN-γ (BioLegend), PE-conjugated TNF-α (R&D Systems), PE-Cy7-conjugated TNF-α, (BD Biosciences), allophycocyanin-conjugated CCR6 (R&D Systems), FITC-conjugated CD161 (BD Biosciences), allophycocyanin-H7-conjugated CD4 (clone RPA-T4, BD Biosciences). The goat antihuman polyclonal biotinylated IL-23R Ab (R&D Systems), FITC streptavidin (eBioscience, San Diego, CA), and PE streptavidin (eBioscience) were used for IL-23R labeling.

Flow cytometry on circulating CD4+ cells

Blood-derived CD4+ cells were resuspended at 5×10^5 cells ml^{-1} in RPMI 1640 with GlutaMAX-I (Invitrogen, Grand Island, NY) containing 5% human AB serum (Sigma, St Louis, MO), 100 U ml^{-1} of penicillin G sodium, and 100 μg ml^{-1} of streptomycin sulfate (Invitrogen). CD4+ cells were cultured overnight (~14 hours) in six-well plates (Corning, Corning, NY) at 37 °C and 5% CO_2 with or without 2.5 μM of parthenolide (NF-κB inhibitor, EMD Biosciences, La Jolla, CA) or 1 μM of stattic (STAT3 inhibitor, EMD Biosciences). The following morning, CD4+ cells were stimulated for 6 hours using 25 ng ml^{-1} of PMA and 1 μg ml^{-1} of ionomycin in the presence of 0.5 μl ml^{-1} of BD GolgiPlug (BD Biosciences). Cells were then centrifuged, washed, and resuspended in staining buffer (1% fetal bovine serum and 0.09% sodium azide solution in phosphate-buffered saline). Cells were incubated with mAbs for extracellular proteins for 30 minutes at 4 °C, washed twice with staining buffer, and incubated with fluorochrome-conjugated streptavidin for 30 minutes at 4 °C. Cells were then washed twice, fixed, and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Cells were then incubated with antibodies for intracellular cytokines for 30 minutes at 4 °C. Cells were collected using an LSRII flow cytometer equipped with FACSDiva (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR). As CD4+ cells were purified on day 0, percentages of CD4+ cells that express any given cell surface or intracellular protein also reflect absolute increases in cells expressing that protein. It is noted that memory T cell populations were not specifically selected in our experiments, but instead CD4+ T cell populations that contained both naive and memory cells were analyzed.

Intracellular cytokines were not expressed in uncultured cells isolated on day 0 (data not shown). In a subset of initial experiments, CCR6 surface expression following 1 versus 5 days of culture was compared. CCR6 expression decreased considerably to undetectable levels with additional days of culture (data not shown), and thus the 5-day culture experiments were abandoned. In another series of initial experiments, blood samples were obtained from the same healthy volunteers on different days to establish reproducibility of the flow cytometry (data not shown). As the psoriasis patients were

often initiating therapies after having blood samples drawn for this study, data reproducibility on different days could not be established for these individuals.

ELISA

Frozen plasma was allowed to thaw and IL-17A, IL-22, IFN-γ, and TNF-α levels were measured by ELISA (RayBiotech, Norcross, GA for IL-17A and IFN-γ; Quantikine ELISA kit, R&D systems for IL-22, and Quantikine HS ELISA kit, R&D systems for TNF-α), according to the manufacturer's instructions. Optical densities were measured at 450 nm for IL-17A, IL-22, and IFN-γ and 490 nm for TNF-α with a microplate spectrophotometer (Spectramax Plus, Molecular Devices, Sunnyvale, CA). Concentrations were calculated from the standard curves generated by SOFTmax Pro (Molecular Devices).

Statistical analyses

The χ^2 goodness-of-fit test was used to evaluate normality for all parameters. The *F*-test and Bartlett test were used to evaluate equality of variance between two and three groups, respectively. The Student's *t*-test was used when data were normally distributed and variance was equal. Welch's *t*-test was used when data were normally distributed and variance was not equal. Mann–Whitney's *U*-test was used when data were not normally distributed. Regression lines were calculated with the least squares method. Spearman's correlation coefficient by rank test was used to evaluate correlations between two sets of data. For multiple comparisons as shown in Figure 4, the two-factor analysis of variance and Scheffe's *F* tests were used when data were normally distributed and the variance was equal, whereas the Friedman and Scheffe's *F* tests were used when the data were not normally distributed or the variance was not equal. *P*-values of 0.05 or less were considered significant.

CONFLICT OF INTEREST

Dr Blauvelt is a scientific advisor and a clinical study principal investigator for Centocor.

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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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