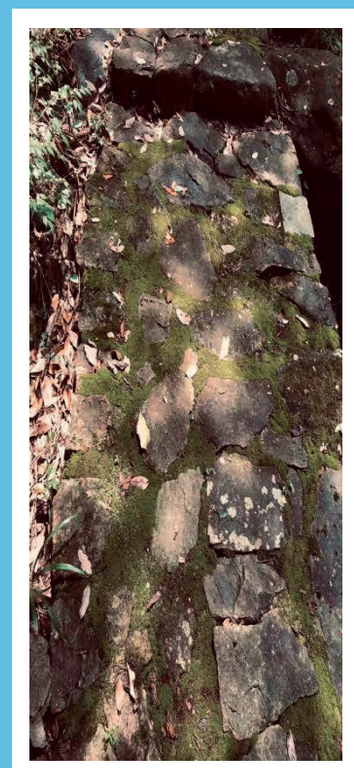
The IL-17A/IL-17R Pathway in Immune-Mediated Inflammatory Diseases

Xiaofei XU



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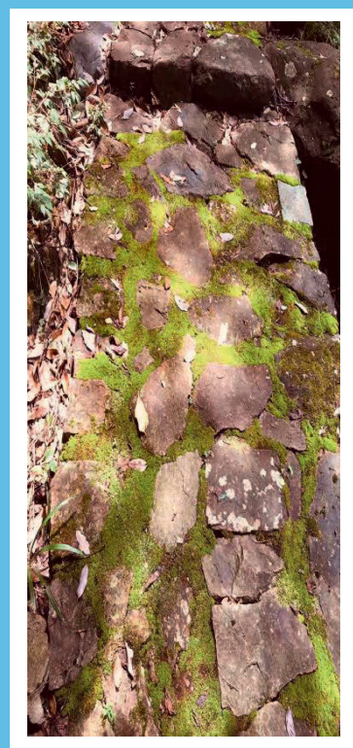

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ABSTRACT

Objective: Interleukin-17A (IL-17A) and TNFα contribute to the pathogenesis of psoriatic arthritis (PsA). However, their functional relationship in PsA synovitis has not been fully elucidated. In addition, although CD8+ T-cells in PsA have been recognized as a source of IL-17A using flow cytometry, it is not clear whether CD8+ T-cells secrete IL-17A under more physiological conditions. Clarifying these issues are the objectives of this study.

Methods: IL-17A+ T-cells were identified in PsA synovial fluid (SF, n=20), treatment-naïve PsA blood and healthy blood (n=22 each) by flow cytometry. IL-17A+ T-cells were sorted from PsA SF (n=12) and stimulated using anti-CD3/anti-CD28 or phorbol myristate acetate and ionomycin (PMA/ion) *ex vivo* alone (n=3), with autologous monocytes (n=3) or with allogeneic PsA fibroblast-like synoviocytes (PsA-FLS, n=5-6). To evaluate the differential effects of neutralizing IL-17A and TNFα, SF CD4+ T-cell and PsA-FLS co-cultures were used (n=5-6).

Results: PsA-SF CD4+ (0.71 (0.35-1.50)%, median (IQR)) and CD8+ T-cells (0.44 (0.17-1.86)%) are IL-17A+ using flow cytometry. However, only CD4+ T-cells secreted IL-17A after anti-CD3/anti-CD28 activation ($p<0.05$). Similar results were observed in co-cultures with PsA monocytes or PsA-FLS ($p<0.05$). Remarkably, CD8+ T-cells only secreted IL-17A after 4- or 72-hours stimulation with PMA/ion. Anti-IL-17A and anti-TNF treatments both inhibited PsA synovitis *ex vivo*. Neutralizing IL-17A strongly inhibited IL-6 ($p<0.05$) and IL-1β ($p<0.01$), while anti-TNF was more potent in reducing MMP-3 ($p<0.05$) and MMP-13.

Conclusion: PsA-SF CD8+ T-cells, in contrast to CD4+ T-cells, did not secrete IL-17A after TCR activation. Overlapping but also distinct effects at the level of inflammatory cytokines and MMPs were found after neutralizing IL-17A or TNFα in a human *ex vivo* PsA synovitis model.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis that develops in up to 30% of patients with a history of or active psoriasis (1). Activated T cells have long been reported to contribute to arthropathies including PsA pathogenesis (2), and therapies depleting lymphocytes were tested in PsA patients with limited clinical response (3). The lack of efficacy during depletion therapy was attributed to a modest synovial lymphopenia despite of significant reduction in peripheral blood (3). This pinpoints to the pathogenic role of local T cells in PsA joints. Moreover, enhanced local clonal expansions of CD4+ and CD8+ T cells were identified in PsA synovial fluid compared to PsA peripheral blood (4), further suggesting that intra-articular T cell activation drives PsA joint inflammation.

Activated T cells excrete a wide range of proinflammatory cytokines including IL-17A and TNFα, both of which have been shown to be elevated in PsA synovial fluid or synovium (5-7). Evidence from studies of PsA patients and other arthropathies points to the involvement of IL-17A in arthritis pathogenesis (8,9). It has been suggested that CD4+ (10,11), CD8+ T cells (12-14) and group 3 innate lymphoid cells (ILC3) (15) can be potential sources of IL-17A in PsA synovial fluid or synovium. However, which of the above cell types is the main producer of IL-17A in local PsA joints is still not clear. Recently, ILC3 reportedly fail to express IL-17A upon *in vitro* stimulation in spondyloarthritis joints (16). Nevertheless, direct *ex vivo* comparison of IL-17A production upon T cell receptor (TCR) activation by CD4+ and CD8+ T cells remains unknown in PsA synovial fluid.

TNFα is a proinflammatory cytokine present at high levels in PsA (5,6). Neutralization of TNFα proved to be effective in reducing local inflammation while slowing or halting joint destruction in patients with PsA (17). However, with success rates of around 60-70% in active PsA patients, standard anti-TNF therapies fail to achieve satisfactory results in remaining non-responding patients (18,19). Even among responders, initial efficacy with TNFα inhibition fails to sustain in subgroups of patients (18). Interestingly, in psoriasis patients not responsive to the TNF blocker, etanercept, persistent levels of serum IL-17A were observed (20). Recent research showed that antibodies targeting IL-

17A, including secukinumab and ixekizumab, were effective in treating PsA patients (21,22) and both were approved for treatment in active PsA. Similar to anti-TNF biologics, IL-17A blockades also successfully suppress joint inflammation and prevent radiographic progression (21,22). Patients intolerant or irresponsive to TNFα inhibition still showed disease improvement with anti-IL-17A therapy (23,24). Further understanding of potential overlapping and distinct roles between anti-IL-17A and anti-TNF treatments is still relevant. It will help to guide future clinical practice and potentially achieve more sustainable therapeutic effects for patients with PsA.

MATERIAL & METHODS

Study design

IL-17A+ CD4+ and CD8+ T cells were first identified using flow cytometry in: 1) synovial fluid (SF) of active PsA patients; 2) peripheral blood from treatment naïve early PsA patients; and 3) peripheral blood from age and sex matched healthy volunteers as controls. SF CD4+ and CD8+ T cells were then sorted to compare ex vivo IL-17A secretion with or without stimulation (anti-CD3/anti-CD28 or PMA/ion). Sorted CD4+ and CD8+ T cells were further co-cultured with autologous PsA monocytes or allogeneic PsA fibroblasts to check ex vivo IL-17A secretion. With TCR activation, sorted SF CD4+ T cells were co-cultured with PsA fibroblasts to evaluate the effects of IL-17A neutralization in comparison to TNF blockade.

Patients

Synovial fluid (SF) of active PsA patients (n=20) were collected. Peripheral blood of treatment naïve early PsA patients and age-/sex-matched healthy volunteers (n=22 each) were also included. All PsA diagnoses were performed by rheumatologists according to classification for psoriatic arthritis (CASPAR) criteria. This study is part of the Dutch South-West Psoriatic Arthritis Register (DEPAR) study and is approved by medical ethics review board of Erasmus Medical Center Rotterdam. Details of PsA patients and healthy volunteers are summarized in supplementary table S1.

Flow cytometry and cell sorting

Cell pellets from PsA-SF were stained for surface markers following standard practice with or without density gradient centrifugation. Peripheral blood of early PsA patients and healthy volunteers were additionally lysed for red blood cells and stained for surface markers as stated above. For intracellular staining, parts of the cell pellets were stimulated for 4 hours (hrs) with 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich), 500 ng/ml ionomycin and Golgistop (BD Biosciences). Afterwards cells were stained for surface markers and Fixable Viability Dye eF506 (eBioscience 65-0866-14) following manufacturer's instructions. Cells were then fixed with 2% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 0.5% saponin buffer (0.5% BSA, 0.05% NaN3 in PBS).

Sorted CD4+ and CD8+ T cells were *ex vivo* cultured or co-cultured for 72 hours and restimulated to examine intracellular staining as described above. LSR II flow cytometer (BD Biosciences) was used to analyze samples and FlowJo software (TreeStar) was used to process results. Cells were sorted with FACSAria cell sorter (BD Biosciences) and purity of obtained cell populations was ≥ 98%.

Flow cytometry antibodies

Following antibodies were used during staining. From BD Biosciences: CD8-PE-CF594 (clone RPA-T8), TCRγδ-PE Cy7 (clone 11F2), CD19-PE Cy7 (clone SJ25C1), CD45-PE-CF594 (clone HI30), CD14-APC H7 (clone MϕP9), CD45RO-PerCP Cy5.5 (clone UCHL1). From BioLegend: CD56-AF700 (clone 5.1H11), CD15-AF700 (clone W6D3), CD16-BV785 (clone 3G8), IFN-γ-AF488 (clone 4S.B3), TNFα-BV421 (clone MAb11), CD25-PE Cy7 (clone BC96). From eBioscience: IL-17A-PE (clone eBio64DEC17), IL-22-eF660 (clone 22URTI). From Sony Biotechnology: CD45-PerCP Cy5.5 (clone HI30), CD3-BV785 (clone OKT3), CD4-BV711 (clone OKT4), CD4-FITC (clone RPA-T4).

Cell culture

Sorted T cells were seeded 2.5×10^4 per well in 96-well round bottom culture plates and stimulated with 0.3 µg/ml soluble anti-CD3 and 0.4 µg/ml soluble anti-CD28 (both from Sanquin, Amsterdam, The Netherlands) for 4 or 72 hrs in IMDM (Lonza) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin/streptomycin, 2 mM L-Glutamine (both from Lonza) and 50 µM β-

mercapto-ethanol (Merck). PsA-FLS was isolated and cultured as described previously (25). After reaching 90% confluence, allogeneic PsA-FLS was seeded 1.0×10^4 per well in 96-well flat bottom culture plates and after overnight incubation, 2.5×10^4 T cells per well were added with soluble anti-CD3/anti-CD28 as above for 72 hrs. In co-cultures with autologous monocytes, 1.0×10^4 per well CD14+ monocytes were seeded with 2.5×10^4 per well T cells in 96-well round bottom culture plates and incubated with soluble anti-CD3/anti-CD28 for 72 hrs.

During neutralization experiments, 100 µg/ml anti-IL-17 (secukinumab, Novartis) and 1µg/ml anti-TNF (adalimumab, AbbVie) were used. An isotype IgG1κ (Sigma-Aldrich) was included as control and soluble anti-CD3/anti-CD28 activation was used as stimulation.

Enzyme-linked immunosorbent assay (ELISA)

IFN-γ, IL-6 and IL-8 in culture supernatants were measured with ELISA (Invitrogen) following manufacturer's instructions. IL-17A, TNFα, MMP-1 and MMP-3 were measured with ELISA Duoset (R&D systems) following manufacturer's instructions.

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

RNA was isolated with Total RNA Miniprep Kit (Sigma Aldrich). cDNA was synthesized with 10U/µl Superscript II after treatment with 0.1 U/µl DNAse (both from Invitrogen). RT-PCR was performed with ViiA7 sequence detection system (Life Technologies). Probes were chosen from the universal probe library (Roche Applied Science) and primers were designed using ProbeFinder software. Gene expression data was normalized to house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primer sequences are available upon request.

Statistical analysis

Flow cytometry results were shown as median (interquartile range (IQR)) and *ex vivo* stimulation data are mean ± standard error (SEM). Statistical differences were determined with paired or unpaired student's *t* test. All data analyses were performed with GraphPad Prism V5 and P-values <0.05 were considered as significant.

RESULTS

Subsets of lymphoid and myeloid cells, except B cells and NK cells, are comparable in PsA synovial fluid and peripheral blood

Peripheral blood from early PsA and matched healthy volunteers (n=22 each) as well as synovial fluid (SF) from active PsA patients (n=20), were stained for surface markers to identify immune cells. Gating strategies for cell populations were shown in supplementary figure S1. As shown in figure 1A, percentages of CD3+ T cells were 44.8 (8.6-55.4)% (median (IQR)) in PsA-SF, 43.3 (25.6-58.2)% in PsA blood and 47.5 (26.7-60.3)% in blood of healthy volunteers. For myeloid cells, percentages of CD14+

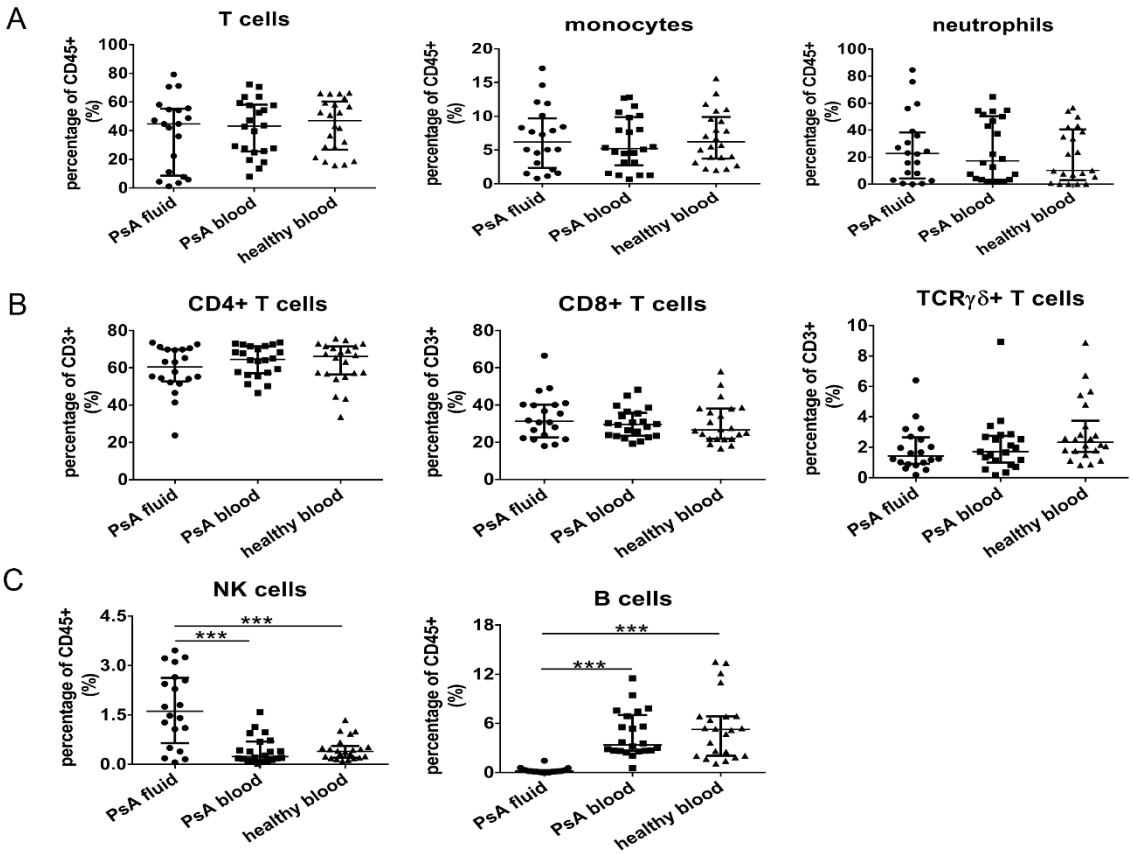

Figure 1. **Differences in percentages of B cells and NK cells in PsA synovial fluid compared to PsA and healthy blood.** SF (n=20) of active PsA patients, peripheral blood of early PsA patients (n=22) and age/sex- matched healthy volunteers (n=22) were stained for surface markers. Gating as shown in supplementary figure S1. **A**. Frequencies of T cells (CD3+), myeloid cells (CD14+), and neutrophils (CD15+CD16+) among CD45+ immune cells. **B.** Among T cells, percentages of CD4+, CD8+ and γδT subsets were shown. **C.** Percentages of B cells (CD3-CD19+) and NK cells (CD3-CD56bright) among CD45+ immune cells. Data are median values and interquartile ranges, and asterisks indicate significance: ***p<0.001.

monocytes in PsA-SF, PsA blood and healthy blood, were 6.2 (2.4-9.7)%, 5.2 (2.7- 9.9)%, and 6.2 (3.7-9.9)%, respectively, while percentages of CD15+CD16+ neutrophils were 22.8 (4.3-38.3)%, 17.3 (3.3-50.4)%, and 10.2 (3.1-40.6)% (figure 1A).

Among CD3+ T cell subsets, CD4+ T cells on average accounted for over 60% of the T cell population with 60.5 (52.7-69.9)% in PsA-SF, 64.6 (57.1-71.7)% in PsA blood, and 66.2 (56.5-71.6)% in healthy blood (figure 1B). About 30% of total T cells were CD8+ with 31.3 (22.6-40.3)% in PsA-SF, 29.7 (23.5-35.8)% in PsA blood, and 26.7 (21.9-38.2)% in healthy blood (figure 1B). TCRγδ+ T cell percentages were around 1.5-2.5% among groups with 1.4 (0.92-2.7)% in PsA-SF, 1.7 (0.99-2.8)% in PsA blood, and 2.3 (1.7-3.7)% in healthy blood (figure 1B).

As shown in figure 1C, significant enhanced accumulation of CD56+(bright) NK cells in PsA-SF was observed comparing to blood of either treatment naïve early PsA patients or healthy volunteers (1.62 (0.64-2.62)% PsA-SF versus 0.24 (0.13-0.69)% PsA blood and 0.39 (0.20-0.56)% healthy blood). On the contrary, CD19+ B cell percentage in PsA-SF was significantly lower in comparison to either blood samples with 0.17 (0.08-0.30)% in PsA-SF, 3.38 (2.65-7.71)% in PsA blood, and 5.26 (2.05-6.87)% in healthy blood, respectively (figure 1C).

Enriched percentage of IL-17A+ CD8+ T cells in PsA synovial fluid compared to peripheral blood

Cell pellets from active PsA-SF (n=20), blood from treatment naïve early PsA and healthy volunteers (n=22 each) were intracellularly stained for IL-17A, IFN-γ, TNFα and IL-22. Surface marker CD4 but not CD8 was down-regulated by PMA and ionomycin (PMA/iono) stimulation during intracellular staining (supplementary figure S2). Therefore, the CD45+CD3+CD8- population was considered CD4+ T cells. Figure 2A showed representative staining results of IL-17A, IFN-γ, TNFα and IL-22 among CD4+ and CD8+ T cells in PsA-SF, PsA blood and blood of healthy volunteers.

In PsA-SF, 0.71 (0.35-1.50)% (median (IQR)) of CD4+ T cells was IL-17A+ and not significantly different comparable to 0.44 (0.17-1.86)% IL-17A+ CD8+ T cells (figure 2B). In PsA blood, however, the percentage of IL-17A+ CD8+ T

cells (0.13 (0.09-0.22)%) was significantly lower than IL-17A+ CD4+ T cells (0.95 (0.77-1.36)%) (figure 2B). Similar result was found in healthy blood (1.15 (0.73-1.78)% CD4 vs 0.13 (0.08-0.21)% CD8) (figure 2B). For IFN-γ staining, 44.4 (24.7-64.8)% CD8+ T cells were positive in PsA-SF, which is significantly higher than 28.8 (13.5-41.2)% of IFN-γ+

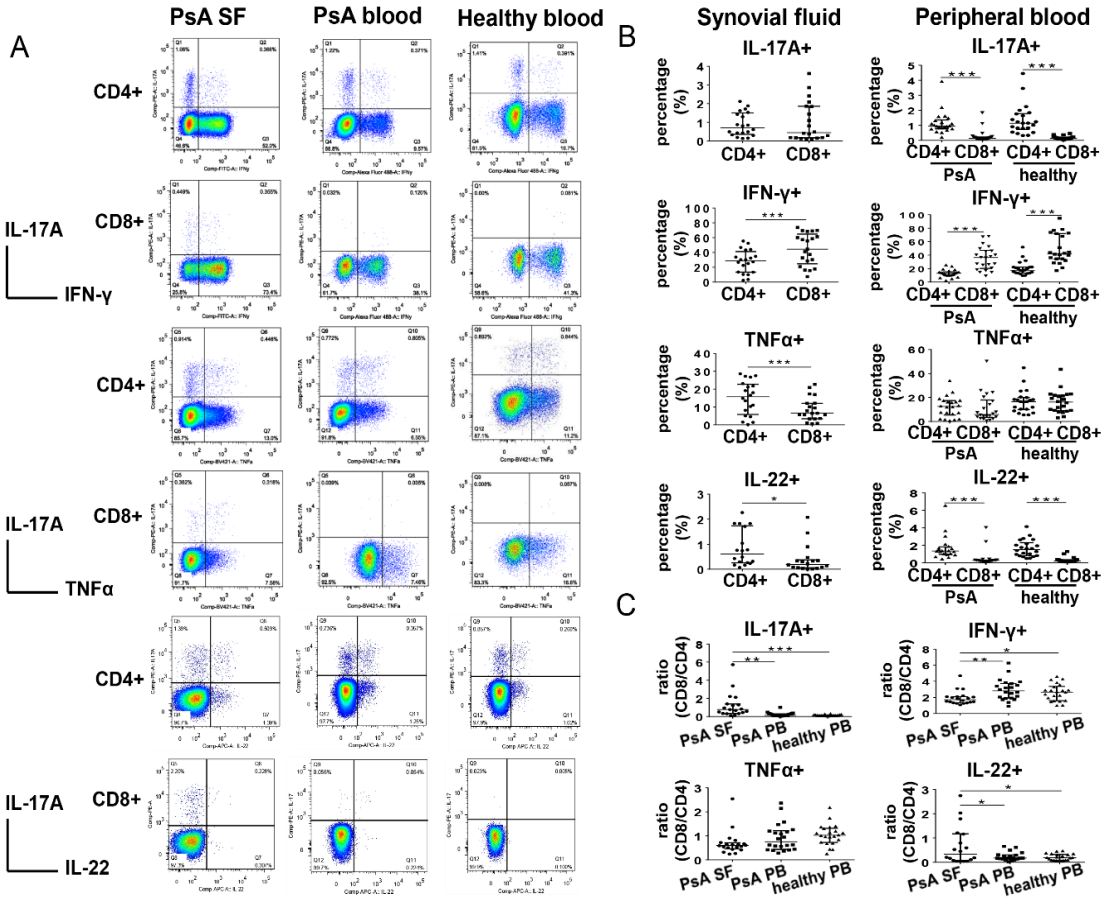

Figure 2. Enriched percentage of IL-17A+ CD8+ T cells in PsA-SF compared to peripheral blood. A. Representative intracellular staining results of IL-17A, IFN-γ TNFα and IL-22 among PsA-SF (n=20), PsA blood (n=22) and healthy blood (n=22) after 4hrs' PMA/ion stimulation. **B.** Summary of IL-17A+, IFN-γ TNFα and IL-22 staining results in CD4+ and CD8+ T cells in PsA-SF (left) and peripheral blood (right). **C.** Percentage ratio between CD8+ and CD4+ T cells of each individual sample was calculated and summarized for each cytokine staining. Data are median values and interquartile ranges, and asterisks indicate significance: *p<0.05 **p<0.01 ***p<0.001.

CD4+ T cells. Similar differences were observed in PsA blood (13.1 (10.0-15.2)% CD4 vs 36.4 (20.3-47.2)% CD8) and healthy blood (17.6 (13.3-22.2)% CD4 vs 42.1 (34.0-72.0)%) (figure 2B). The percentage of TNFα+ CD4+ T cells was significantly higher than TNFα+ CD8+ T cells in PsA-SF (15.7 (5.8-

22.7)% CD4 vs 6.5 (3.4-12.1)% CD8) (figure 2B). However, similar results were observed between CD4+ and CD8+ T cells in PsA blood (12.5 (5.3-17.4)% CD4 vs 5.9 (3.5-17.9)% CD8) and healthy blood (16.5 (9.9-20.1)% CD4 vs 16.2 (9.1-22.0)% CD8) (figure 2B). IL-22+ cells were higher in CD4+ T cells than in CD8+ T cells in PsA-SF (0.61 (0.26-1.74)% CD4 vs 0.20 (0.06-0.38)% CD8) (figure 2B). Similar results were observed in PsA blood (1.35 (0.96-1.82)% CD4 vs 0.27 (0.16-0.32)% CD8) and healthy blood (1.51 (0.81-2.30)% CD4 vs 0.20 (0.15-0.34)% CD8).

To compare directly percentage differences between CD4+ and CD8+ T cells between PsA-SF, PsA blood and healthy blood, percentage ratios (PR) were calculated. The percentage of IL-17A+ CD8+ T cells was divided by the percentage of IL-17A+ CD4+ T cells. Similar calculations were performed for IFN-γ, TNFα and IL-22. With PR close to 1 in PsA-SF (0.77 (0.33-1.39), median (IQR)), it indicated that nearly equal percentages of CD8+ and CD4+ T cells were IL-17A+ (figure 2C). In contrast, PR in PsA and healthy blood were 0.12 (0.09-0.30) and 0.11 (0.08-0.18), both significantly lower than PsA-SF (figure 2C). This suggested that a specific enrichment of IL-17A+ CD8+ T cells over CD4+ T cells in PsA-SF compared to peripheral blood. For IFN-γ+ cells, PR equals to 1.72 (1.34-2.10) in PsA-SF, 2.81 (1.90-3.75) in PsA blood, and 2.67 (1.59-3.36) in healthy blood (figure 2C), indicating 2-3 times more IFN-γ+ CD8+ T cells versus IFN-γ+ CD4+ T cells in all groups. TNFα+ PRs were comparable in PsA-SF (0.60 (0.46-0.71)), PsA blood (0.75 (0.44-1.21)), and healthy blood (1.02 (0.72-1.31)) (figure 2C). This suggested that almost equal percentages of CD8+ and CD4+ T cells were TNFα+ in PsA-SF and peripheral blood. Similar to IL-17A, IL-22+ PR were significantly higher in PsA-SF (0.33 (0.07-1.17)) compared to in PsA blood (0.17 (0.14-0.31)) and healthy blood (0.19 (0.09-0.32)), indicating enhanced accumulation of IL-22+ CD8+ T cells over CD4+ T cells in PsA-SF compared to peripheral blood.

CD4+ but not CD8+ T cells in PsA synovial fluid secrete IL-17A upon TCR activation

To compare IL-17A production and secretion between CD4+ and CD8+ T cells, PsA-SF CD4+ and CD8+ T cells were sorted from synovial fluid mononuclear cells (SFMC). These sorted cells were anti-CD3/anti-CD28 stimulated and *ex*

vivo cultured for 72 hours. Sorted CD4+ T cells did not include CD4+CD14+ monocytes and CD4+CD25high T regulatory cells. After 72 hours, the concentration of IL-17A was measured in culture supernatants using ELISA. In addition, cells were used for intracellular staining of IL-17A. For this, cells were stimulated with PMA/ion for another 4 hours. As shown in figure 3A, intracellular staining of IL-17A was positive for both CD4+ and CD8+ T cells. However, in contrast to the intracellular IL-17A staining, IL-17A was only secreted and measured in supernatants of CD4+ but not CD8+ T cells. Similar results were observed for IL-17F as shown in supplementary figure S3.

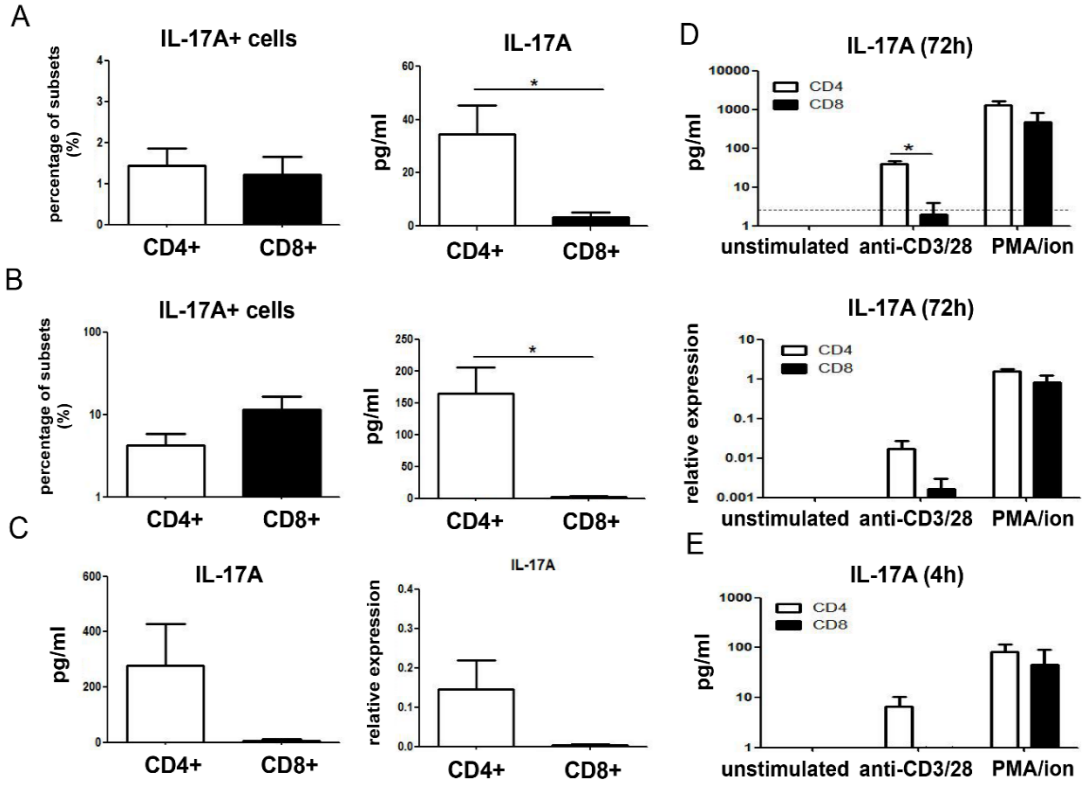

Figure 3. PsA-SF CD4+ but not CD8+ T cells produce IL-17A upon anti-CD3/anti-CD28 activation. A. IL-17A staining in cells and protein level in culture supernatants for sorted SF CD4+ and CD8+ T cells after 72 hrs' *ex vivo* anti-CD3/anti-CD28 activation (n=5 or 6 from two independent experiments). **B.** IL-17A staining in cells and protein level in culture supernatants for SF CD4+ and CD8+ T cells co-cultured with allogeneic PsA-FLS and activated with anti-CD3/anti-CD28 after 72 hrs (n=5 or 6 from two independent experiments). **C.** IL-17A in culture supernatants and mRNA expression in cells for SF CD4+ and CD8+ T cells after 72 hrs' *ex vivo* co-culture with autologous CD14+ monocytes and anti-CD3/anti-CD28 activation (different patients from A/B, n=3). **D.** IL-17A in culture supernatants and mRNA expression in cells for

sorted SF CD4+ and CD8+ T cells after *ex vivo* stimulation with anti-CD3/anti-CD28, PMA/ion, or without stimulation for 72 hrs (different patients from A/B, n=3) (dash line indicates IL-17A detection limit for IL-17A) and (**E**) IL-17A in culture supernatants after similar stimulation for 4 hrs (n=3). Data are mean ± SEM, and asterisks indicate significance: *p<0.05.

To further confirm that anti-CD3/anti-CD28 activation didn't result in secretion of IL-17A from CD8+ T cells, CD4+ and CD8+ T cells were sorted from PsA SFMC and co-cultured with allogeneic PsA fibroblast-like synoviocytes (FLS) or autologous CD14+ PsA monocytes. In line with the findings described above, flow cytometry revealed that CD8+ T cells stained positive for IL-17A after 3 days of co-culture with PsA-FLS (figure 3B). However, excreted IL-17A levels were not detectable in supernatants of anti-CD3/anti-CD28 activated CD8+ T cells co-cultures with PSA-FLS (figure 3B). Similarly, in co-cultures of 72 hours with autologous monocytes, anti-CD3/anti-CD28 activation resulted IL-17A secretion in co-culture supernatants and mRNA IL-17A expression only in CD4+ but not CD8+ T cells (figure 3C). To further explain this discrepancy, PsA-SF CD4+ and CD8+ T cells were cultured with anti-CD3/anti-CD28, PMA/ion, or without stimulation for 4 and 72 hrs. As shown in figure 3D, at 72 hrs, only CD4+ T cells produced IL-17A after anti-CD3/anti-CD28 activation at both protein and mRNA levels, while PMA/ion stimulated both CD4+ and CD8+ T cells to produce IL-17A. Similar results were confirmed with 72 hrs co-cultures of T cells and autologous monocytes (supplementary figure S4). More importantly, as shown in figure 3E, even after 4 hrs of PMA/ion stimulation, CD8+ T cells produced as much IL-17A as CD4+ T cells, but no IL-17A secretion was detectable by CD8+ T cells after 4 hrs of anti-CD3/anti-CD28 activation. Time-course analysis including 24, 48 and 72 hrs with another three PsA patients confirmed above findings (data not shown).

IL-17A induces overlapping and differential effects in PsA fibroblast-like synoviocytes compared to TNFα

As established above, PsA-SF CD4+ T cells are the main IL-17A producers with anti-CD3/anti-CD28 activation. To evaluate overlapping and differential therapeutic effects of TNFα and IL-17A blockers, CD4+ T cells were isolated from PsA SFMCs, stimulated with anti-CD3/anti-CD28 and co-cultured with allogeneic PsA fibroblast-like synoviocytes (FLS). Expression of IL-17RA and

IL-17RC was confirmed on both PsA-FLS and CD14+ PsA monocytes (supplementary figure S5). Pre-titrations of antibodies were performed as shown in supplementary figure S6, and concentrations were chosen to comparably achieve 60-70% of inhibition for each cytokine.

Anti-IL-17A and anti-TNF antibody treatment specifically and significantly reduced the production of either IL-17A or TNFα, respectively in the co-culture system (figure 4A). IFN-γ was not affected compared to an isotype antibody control (figure 4A). Both anti-IL-17A and anti-TNF treatments significantly reduced IL-8 levels compared with the isotype antibody group (figure 4B). Combining two antibodies showed a significant additive effect compared to anti-IL-17A alone (figure 4B). Interestingly, anti-TNF did not significantly influence IL-6 production in these co-cultures compared to isotype control, while anti-IL-17A significantly suppressed IL-6 level compared to both isotype control and anti-TNF (figure 4B). Similar results were observed during antibody titrations (supplementary figure S6). Although anti-TNF significantly reduced mRNA expression of IL-1β, it was significantly less effective compared to anti-IL-17A (figure 4B). Levels of MMP-1 were significantly down-regulated by the combination treatment of anti-IL-17A and anti-TNF compared to isotype control or anti-IL-17A alone (figure 4C). MMP-3 levels were significantly suppressed after neutralizing TNFα compared to both anti-IL-17A and isotype control (figure 4C). Similar to MMP-1, expression of MMP-9 was significantly reduced only by the combination of anti-IL-17A and anti-TNF compared to isotype control (figure 4C). Similar to MMP-3, anti-TNF significantly down-regulated the expression of MMP-13 compared to isotype control, and combination with anti-IL-17A achieved a significant further reduction of MMP-13 compared to either antibody alone (figure 4C).

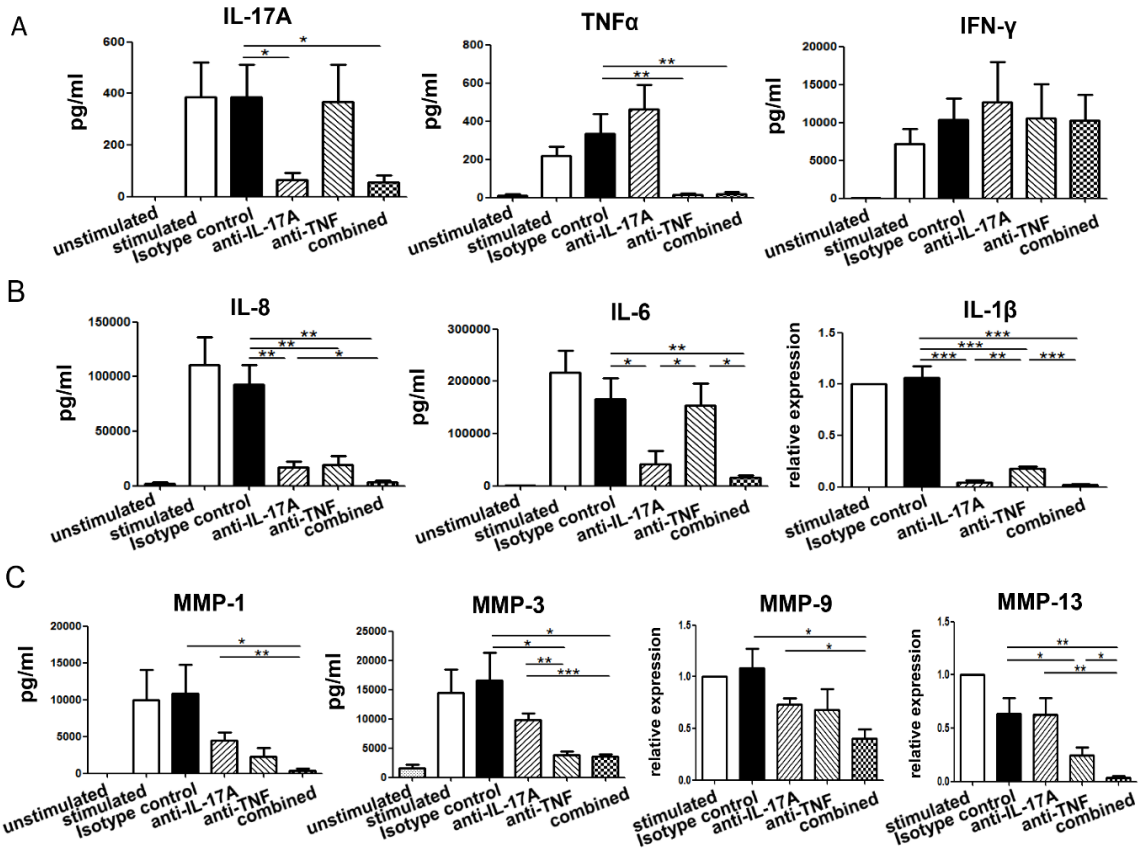

Figure 4. IL-17A induces differential effects compared to TNFα in PsA FLS. CD4+ T cells were sorted from PsA-SF and co-cultured with allogeneic PsA-FLS for 72 hrs with or without anti-CD3/anti-CD28 activation. **A.** Anti-IL-17A and anti-TNF specifically reduced IL-17A and TNFα, respectively, in culture supernatants compared to isotype control antibody, but not IFN-γ, in co-culture system. **B.** IL-6 and IL-8 levels in culture supernatants and IL-1β expression level in co-cultured cells after 72 hrs' co-culture. **C.** MMP-1 and MMP-3 levels in culture supernatants and MMP-9 and MMP-13 expression levels in co-culture cells after 72 hrs. Data (n=5 or 6) are pooled from two independent experiments and plotted as mean ± SEM. Asterisks indicate significance: *p<0.05 **p<0.01 ***p<0.001.

DISCUSSION

The role of IL-17A in PsA pathogenesis is currently widely accepted following the clinical success of biologicals targeting the IL-17 pathway (21,22). Various cell types have been suggested to be a potential source of IL-17A in PsA. However, a detailed *ex vivo* comparison of IL-17A producing cells with various techniques is still relevant. Here we present evidence that CD4+ but not CD8+ T cells from synovial fluid of PsA patients excrete IL-17A upon *ex vivo* activation with anti-CD3/anti-CD28. This indicates that upon TCR activation, CD4+ T cells will be the main producer of IL-17A in local PsA joints despite that both are IL-17A-producing with PMA and ionomycin stimulation. To better reflect in-tissue situations, synovial fluid T cells were co-cultured with fibroblast-like synoviocytes. With this *ex vivo* PsA co-culture assay, we showed that anti-IL-17A and anti-TNF both have therapeutic effects in inhibiting the proinflammatory activation loop between T cells and stromal cells. Interestingly, differential effects between anti-IL-17A and anti-TNF were also observed. Anti-IL-17A exhibits stronger inhibition of inflammatory cytokines such as IL-6 and IL-1β, while anti-TNF is more potent in reducing the production of MMPs.

Earlier reports suggested that CD8+ T cells are predominant in PsA (26). However, our findings show that CD4+ T cells are the major T cell subset in PsA-SF similar to PsA blood. Possible explanations for the discrepancy may lie in the methods of handling samples. Firstly, during our experiments, surface staining was performed directly after cell pelleting without density gradient separation to keep all subsets as genuine as *in vivo.* Secondly, percentages of cell subsets were analyzed without *ex vivo* stimulation and separated from intracellular staining. PMA and ionomycin stimulation conventionally used during intracellular staining can strongly downregulate CD4 but not CD8 surface expression as shown in supplementary figure S1. Therefore, research conditions should represent the infiltrated immune cell characteristics in PsA synovial fluid of patients with PsA as closely as possible.

IL-17A+ CD8+ T cells have been reported in both psoriasis and PsA (12-14,27,28), and particularly in PsA, it was found that percentages of IL-17A+ CD8+ T cells correlate with disease activity and progression of joint damage (12). Our results confirmed the enrichment of IL-17A+ CD8+ T cells in PsA

synovial fluid. However, most of the previous published IL-17A+ results were obtained via intracellular staining with PMA and ionomycin stimulation (11-14,27,28). Such strong stimuli don't resemble the physiological state of T cell activation. On the contrary, anti-CD3/anti-CD28 simulation used in our study activates T cells *in vitro* by providing both primary TCR signal and secondary costimulatory signal. Our findings proved that CD8+ T cells *ex vivo* activated with anti-CD3/anti-CD28 did not produce measurable amounts of IL-17A in contrast to CD4+ T cells. Of note, both CD4+ and CD8+ T cells were IL-17A positive during intracellular staining. Furthermore, in a separate study by Raychaudhuri SP, et al (10), anti-CD3/anti-CD28 activation for three days was used during intracellular staining instead of PMA and ionomycin, and they found that percentages of IL-17A+ CD8+ T cells were negligible in PsA synovial fluid compared to distinct IL-17A+ CD4+ staining (10). However, in their study, mixed T cell subsets were activated simultaneously and chances of IL-17A endocytosis can't be ruled out. We avoided this caveat by sorting CD4+ and CD8+ T cells from PsA synovial fluid and clearly showed that CD4+ but not CD8+ T cells secrete IL-17A upon *ex vivo* anti-CD3/anti-CD28 activation. This indicated that in PsA joints, TCR activation promotes CD4+ T cells but not CD8+ T cells to produce IL-17A. Similar results were observed for IL-17F (supplementary figure S3), and it implies that CD4+ T cells may be the dominant source of both IL-17A and IL-17F in PsA synovial fluid upon TCR activation. Recently, a dual specific antibody targeting both IL-17A and IL-17F was tested in PsA and supported that neutralizing both could achieve rapid and sustained therapeutic effects (29). Group 3 ILCs were another cell type that has been reported as a potential source of IL-17A in PsA synovial fluid (15). However, a recent study reported that though these ILC3s are expanded in inflamed arthritis joints, they failed to express IL-17A upon *in vitro* stimulation (16). These data resemble what we have found for CD8+ T cells. Therefore, the contribution of CD8+ T cells and ILC3 to IL-17 production in PsA joints may be limited under physiological conditions in contrast to CD4+ T cells.

Biologics targeting IL-17A or TNFα prove effective in treating PsA patients, but clinical response rates vary considerably. Failure with one biologic will need to switch to an alternative, which can be a different biological from the same class or a biological with a different mode of action. We used an *ex vivo* model of

human PsA synovitis to explain differential effects and showed that although both biologics efficiently inhibited the proinflammatory loop in the co-culture system, distinct strengths existed between anti-IL-17A and anti-TNF. We provided evidence that neutralizing IL-17A reduced more strongly inflammatory cytokines such as IL-6 and IL-1β, while anti-TNF was more potent in reducing MMPs like MMP-3 and MMP-13. Therefore, to achieve better and sustainable treatment results, it is rational to target both IL-17A and TNFα. Recently, a bispecific antibody, ABT-122, has been developed, and first phase II study in PsA showed that dual inhibition had efficacy and safety similar to adalimumab, a TNF blocker (30, 31). Synergistic effects of anti-IL-17A and anti-TNF were also observed in our experiments and by others (32). Combinations or sequential therapy using both neutralizing agents may be another choice. However, adverse effects such as infection should be closely monitored during all dual neutralizations.

The percentage of IFNγ positive CD4+ and CD8+ T cells are relatively high in blood of patients with PsA compared to IL-17A. In addition, the levels of IFNγ were much higher compared to IL-17A and TNF in the human ex vivo PsA synovitis model. However, neutralization of IL-17A or TNF did not influence the level of IFNγ in these co culture experiments but significantly decreased the inflammation in our synovitis model of PsA, indicating that IFNγ is not a major player in the T cell – PsA synovial fibroblast activation.

We found lower percentages of CD19+ B cells but higher percentages of CD56+(bright) NK cells in PsA synovial fluid which is in line with data from other groups (33-35). B cells tend to function in a protective way in psoriasis or PsA, and can be a source anti-inflammatory cytokines, such as IL-10 (34, 36). Interestingly, increased percentage of CD56+(bright) NK subset was reported to trigger differentiation of monocytes into dendritic cells in PsA synovial fluid (37). Monocytes and dendritic cells are major sources of IL-23 and the IL-23-IL-17 axis plays a pivotal role in inflammatory arthritis (38, 39). Biologicals targeting IL-23 subunits, p40 and p19, were successfully tested in PsA clinical trials (40,41). Whether synovial fluid CD56+(bright) NK cells modulate IL-23 expression by monocytes/dendritic cells and thereby influence IL-23-IL-17 axis is still not known and warranted further research.

In summary, our study showed that the contribution of CD8+ T cells to IL-17A local production in PsA synovial fluid needs further examination as TCR activation mimicked by anti-CD3/anti-CD28 didn't induce IL-17A release from CD8+ in contrast to CD4+ T cells. IL-17A and TNF blockades showed differential effects in our human *ex vivo* PsA synovial inflammation model with the former more potent in dampening inflammatory cytokines while the latter more potent in lowering MMPs. We acknowledged the limitations of our *ex vivo* study systems, and further validation of a combination or sequential therapy in patients with IL-17A and TNFα neutralizing agents is needed. This study indicates that targeting both IL-17A and TNFα may complement each other and may possess additive benefits in coping with destructive synovitis in patients with PsA.

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Supplementary Figures

Parameters	PsA fluid	PsA blood	healthy blood
Age (mean ± SD, years)	47.7 ± 13.7	45.4 ± 12.4	45.3 ± 12.3
Sex (female/male)	7/13	16/6	16/6
DAS28 (mean ± SD)	3.16 ± 1.39	n.a.	0
DMARD therapy, n(%)	18(90)	0(0)	0(0)
oral predisone therapy, n(%)	0(0)	0(0)	0(0)
Biologic therapy, n(%)	4(20)	0(0)	0(0)

Table S1. Clinical characteristics of PsA patients and healthy volunteers
Synovial fluid (SF) of established PsA patients (n=20), peripheral blood of treatment naïve early PsA patients and age-/sex-matched healthy volunteers (n=22 each) were collected. (n.a. indicates not available.)

Supplementary
Figure S1

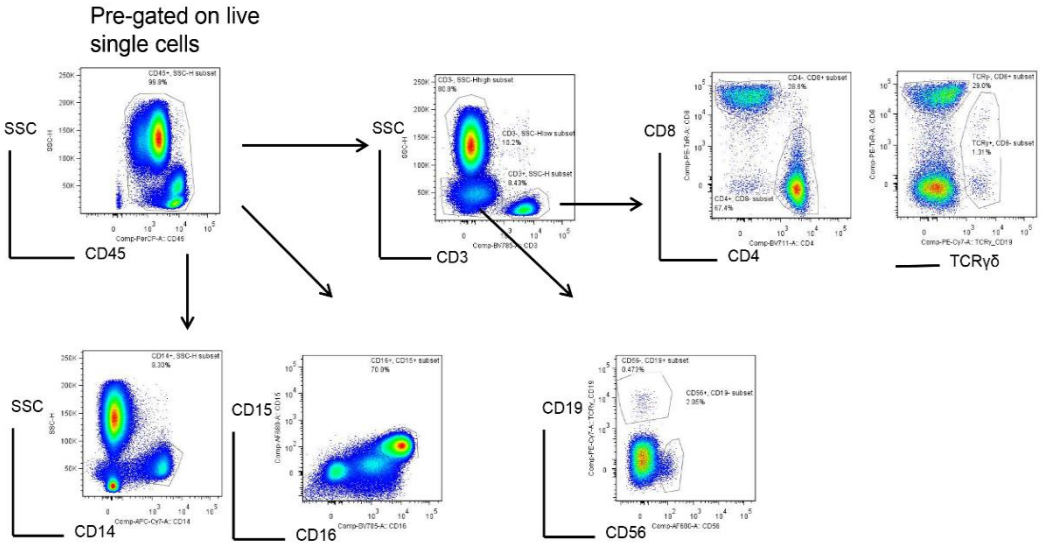


Figure S1. Gating strategy for cell subsets in surface staining results. Cell pellets directly from PsA-SF, PsA blood and healthy blood were stained and analyzed for frequencies in samples. T cells (CD3+), B cells (CD19+), NK cells (CD56+), monocytes (CD14+), and neutrophils (CD15+CD16+) were identified among CD45+ immune cells. Among T cells, CD4+, CD8+, and TCRγδ T cells were further identified.

Supplementary Figure S2

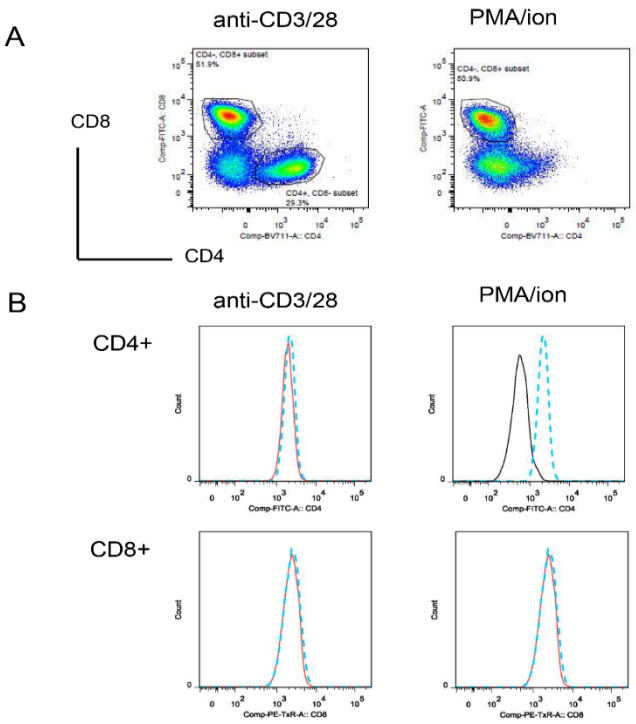


Figure S2. PMA and ionomycin stimulation down-regulated CD4 surface marker. A. Healthy peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3/anti-CD28 or PMA/ion for 4 hrs and percentages of CD4+ and CD8+ T cells were examined after stimulation. **B.** CD4+ and CD8+ T cells were sorted from healthy PBMCs, and *ex vivo* stimulated for 4 hrs. Histogram of CD4 and CD8 surface expression were plotted after stimulation with anti-CD3/anti-CD28 (solid red) or PMA/ion (solid dark) and compared with unstimulated (dash blue) conditions (n=3).

Supplementary Figure S3

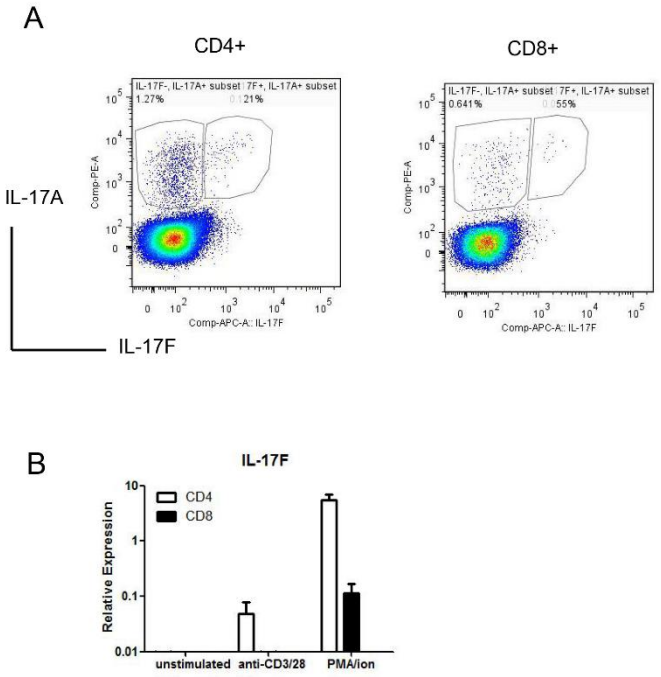


Figure S3. Expression and induction of IL-17F in CD4+ and CD8+ T cells from PsA-SF. A. Representative flow cytometry staining of IL-17A and IL-17F in CD4+ and CD8+ T cells of PsA-SF. **B.** mRNA expression of IL-17F in CD4+ and CD8+ T cells sorted from PsA-SF and stimulated with anti-CD3/anti-CD28, PMA/ion, or without stimulation for 72 hrs (n=3).

Supplementary Figure S4

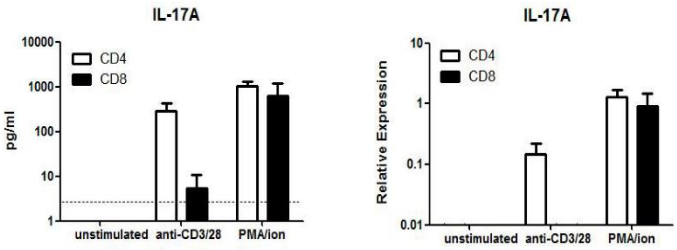


Figure S4. PMA/ion, but not anti-CD3/anti-CD28, induced IL-17A from PsA-SF CD8+ T cells and monocytes co-cultures. IL-17A levels in co-culture supernatants and mRNA expression in cells after SF CD4+ and CD8+ T cells were *ex vivo* co-cultured with autologous CD14+ monocytes and activated with anti-CD3/anti-CD28, PMA/ion, or without stimulation for 72 hrs (n=3) (dash line indicates ELISA detection limit for IL-17A).

Supplementary Figure S5

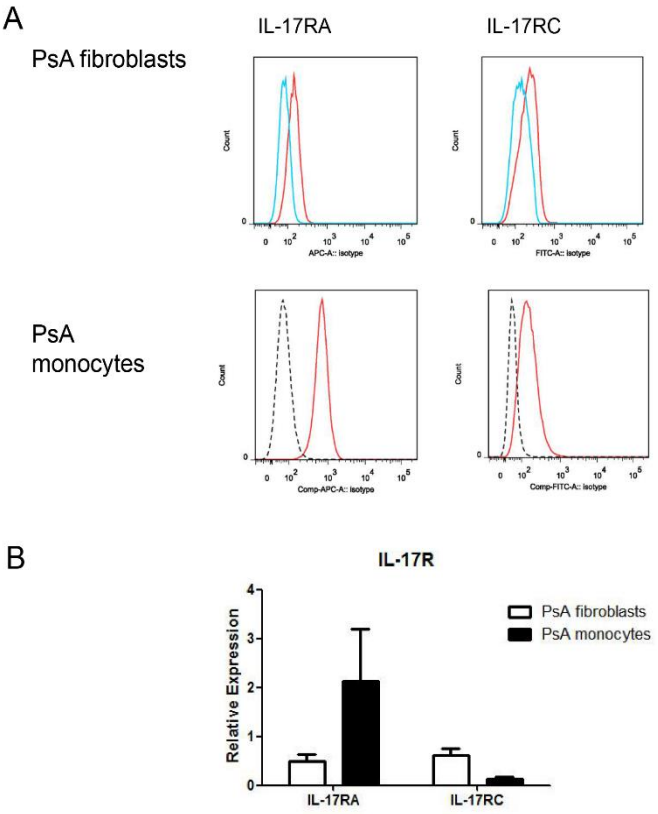
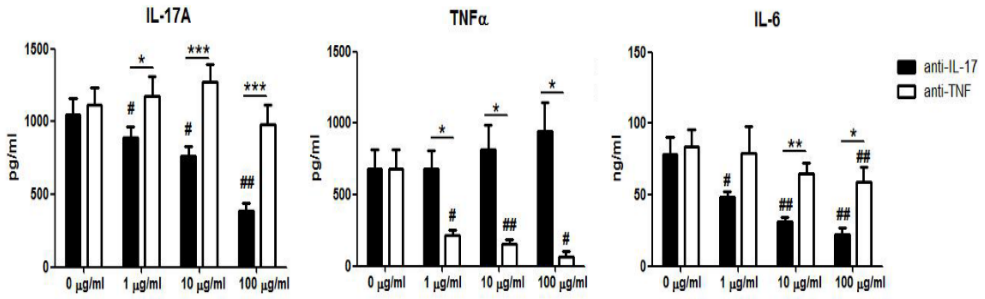


Figure S5. PsA-FLS and monocytes expressed IL-17 receptor. A. Representative flow cytometry staining of IL-17RA and IL-17RC on PsA-FLS and monocytes (solid red) compared to isotype antibody (solid blue or dash dark) controls (n=2). **B.** mRNA expression of IL-17RA and IL-17RC in PsA-FLS and monocytes (n=2).

Supplementary
Figure S6

A



B

IL-17A		
anti-IL-17A	Average concentration (pg/ml)	Percentage reduction (%)
0 µg/ml	1045	0
1 µg/ml	889	15
10 µg/ml	765	27
100 µg/ml	387	**63**

TNF		
anti-TNF	Average concentration (pg/ml)	Percentage reduction (%)
0 µg/ml	683	0
1 µg/ml	214	**69**
10 µg/ml	154	77
100 µg/ml	67	90

Figure S6. Pre-titration of anti-IL-17A and anti-TNF in co-cultures of PsA-FLS and CD4+ T cells from PBMCs of healthy donors. A. IL-17A, TNFα, and IL-6 ELISA results in co-culture supernatants after 72 hrs' activation with anti-CD3/anti-CD28 with 0, 1, 10, 100 µg/ml of either antibody added. Data (n=6) are pooled from two independent experiments. (* indicates significant differences between two antibody groups and # shows significant differences compared to respective null antibody group.) **B.** Average concentrations of IL-17A and TNFα in culture supernatants of each group were summarized and percentage of reduction was calculated compared to null antibody group.

3

Interleukin-17A drives IL-19 and IL-24 expression in skin stromal cells regulating keratinocyte proliferation

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Abstract

IL-17A has been shown to be up-regulated in psoriasis lesions and is central to psoriasis pathogenesis. IL-19, along with other IL-20 subfamily cytokines such as IL-20 and IL-24, is induced by IL-17A and contributes especially to epidermal hyperplasia in psoriasis. However, the regulation, cellular sources of IL-19 and whether targeting of IL-17A by biologics influence IL-19 expression is not completely understood. To investigate the regulation of IL-19 by IL-17A in psoriasis, the imiquimod-induced psoriasis mouse (IMQ) model was used. Enhanced expression of IL-17A in the IMQ model was achieved by anti-IL-10 antibody treatment. Assessments of skin inflammation macroscopically, by histology and flow cytometry, all confirmed increased psoriatic symptoms. Interestingly, depletion of IL-10 markedly upregulated IL-23/IL-17 pathway related cytokines followed by a significant increase in IL-19 and IL-24. The up-regulation of IL-19 and IL-24, but not IL-17A, coincided with increased keratinocyte proliferation. To investigate the cellular source and effects of biologics on IL-19, human skin fibroblasts from healthy controls and psoriasis patients were cultured alone or co-cultured with activated memory CD4+ T cells. Besides IL-1β, IL-17A induced direct expression of IL-19 and IL-24 in skin fibroblasts and keratinocytes. Importantly, intrinsic higher expression of IL-19 in psoriatic skin fibroblasts was observed in comparison to healthy skin fibroblasts. Neutralization of IL-17A in the human skin fibroblast-T cell co-culture system significantly suppressed IL-19 and IL-24 expression. Together, our data show that IL-17A-induced IL-19 and IL-24 expression in skin stromal cells contribute to keratinocyte proliferation.

INTRODUCTION

Psoriasis is a chronic autoimmune skin disease affecting around 2 to 3% of the Western population (1,2). Psoriasis vulgaris, the plaque-forming phenotype, is the most common type and accounts for 85-90% of all patients with psoriasis (1,3). Aberrant regulation of pro-inflammatory and anti-inflammatory cytokines is considered important in the pathogenesis of psoriasis (1-4). Increased levels of IL-17A and elevated percentages of IL-17A-producing lymphocytes are found in psoriatic plaques (5-9). IL-17A alone, and in combination with other inflammatory cytokines such as TNFα, stimulate keratinocyte activation, proliferation, amplifies the immune response and perpetuates cell infiltration (10,11). Biologics targeting IL-17A alone or in combination with IL-17F or the IL-17 receptor A (IL-17RA) are efficacious in the treatment of plaque psoriasis, highlighting the central role of the IL-17A pathway in psoriasis pathogenesis (12). Interleukin-19, a member of the IL-20 subfamily together with IL-20 and IL-24, is up-regulated in psoriatic lesions and contributes to keratinocytes hyperplasia in psoriasis (13). IL-19 is reportedly down-stream of the IL-23/IL-17 cascade and autocrine production of IL-19 by keratinocytes is shown to elicit keratinocyte proliferation (14). Recent data show that serum IL-19 levels reflect clinical improvement induced by anti-IL-17 biologic treatment (15). However, whether other resident cells, such as skin fibroblasts, are a source of IL-19 and whether in situ IL-19 expression is normalized in patients with psoriasis treated with anti-IL-17 therapy is not clear.

Previously, our group established a psoriasis-like skin inflammation model in mice using topical application of imiquimod (IMQ) (16). This model successfully re-captures most critical features of acute plaque formation in psoriasis such as keratinocyte hyper proliferation, acanthosis and parakeratosis (16). Like in human psoriasis, enhanced activity of the IL-23/IL-17 pathway was also involved in the IMQ-induced psoriasis mouse model (16). However, in contrast to the chronic natural course in human psoriasis, this mouse model does not develop into a chronic state of psoriasis, because of stabilization and even improvement of skin inflammation after 5 to 6 days. Interestingly, a clinical study in psoriasis patients showed that, non-lesional skin treated with IMQ initially developed typical features of psoriasis such as acanthosis and parakeratosis

(17). Nevertheless, both clinical and histological features subsided thereafter and in this human model of IMQ-induced psoriasis, the induced lesions showed spontaneous improvement after 5 to 6 days. This improvement was accompanied by significantly lower expression of IL-17A and with a higher expression of IL-10 (17). This suggests that upregulation of IL-10 is involved in the spontaneous improvement of psoriasis symptoms after 5 to 6 days in murine IMQ model and probably explains the spontaneous improvement observed in the IMQ mouse model. Therefore, we used an anti-IL-10 antibody to investigate whether we could achieve enhanced expression of IL-17 in the IMQ-induced psoriasis mouse model and the accompanying visible psoriatic symptoms beyond day 5. *In vitro* assays with human skin fibroblasts from patients with psoriasis and healthy skin were performed to evaluate the direct induction of IL-19 by IL-17. In addition, an ex vivo human psoriasis skin co-culture system was used to examine the effects of biologics targeting IL-17A on IL-19 expression.

MATERIAL AND METHODS

IMQ-induced psoriasis mouse model

BALB/C mice (8-11 week-old) received daily topical application of 62.5mg 5% Aldara (3M Pharmaceuticals) on their shaved back skin. Control mice (n=6, pooled from two independent experiments) were treated with a thin layer of petrolatum (Fagron). Daily evaluation of the local psoriasis area and severity index (PASI) has been described previously (16). Every other day, 20 mg/kg body weight of anti-IL-10 or isotype control antibody (n=10 each, pooled from two independent experiments) was intraperitoneally (i.p.) injected, or 5 mg/kg body weight of dexamethasone (n=7, pooled from two independent experiments) was subcutaneously (s.c.) injected as an anti-inflammatory gold standard. Five and ten days after IMQ induction, mice were sacrificed for analysis. Food and water were provided ad libitum, and mice were kept under specific pathogen-free conditions. All experiments were approved by the Erasmus MC Dutch Animal Ethics Committee (DEC).

Histology and Immunohistochemistry

After sacrifice, skin biopsies were taken and snap-frozen in TissueTek (Bayer). Sections were cut with a Leica cryostat. Gr-1 antibody (clone RB6-8C5) and Ki-67 antibody (Dako, A0047) were used for IHC staining. Subsequent steps were performed as described earlier (15).

Images were analyzed with LAX V4.12 program (Leica microsystems) or NDP view2 (Hamamatsu photonics). To measure epidermal thickness, the average of four measurements was used as the representative thickness per sample. To reduce variance between different experiments, thickness ratios were calculated. Specifically, each skin thickness was divided by the mean skin thickness of the isotype group from that experiment, and thereby mean values of thickness for isotype groups were always set at one.

Flow cytometry

Back skin (ca. 1 cm2) was digested in 50 µg/mL Liberase (Roche) at 4^0C overnight and then at 37^0C for 1 hour to create a single cell suspension and cells were stained with the following antibodies: CD45-BV785 (Biolegend, clone 104), CD11b-eF450 (eBioscience, clone M1/70), Ly6C-APC-Cy7 (BD pharmingen, clone AL-21) and Ly6G-PE-CF594 (BD horizon, clone 1A8). Samples were analyzed with LSR II flow cytometer (BD Biosciences) and results processed with FlowJo software (TreeStar).

Healthy peripheral blood mononuclear cells (PBMC) were obtained from buffycoats (Sanquin, Amsterdam, the Netherlands) and harvested with Ficoll density gradient centrifugation. For the co-culture experiments, memory T cells (CD4+CD45RO+CD14-CD25low/int) were sorted using the FACSAria cell sorter (BD Biosciences) and co-cultured with skin fibroblasts. Cells were stained with the following antibodies: CD14-APC-H7 (clone MϕP9), CD45RO-PerCP-Cy5.5 (clone UCHL1), both from BD Biosciences, and CD4-FITC (clone RPA-T4), CD25-PE Cy7 (clone BC96), both from Sony Biotechnology. The obtained cell purity was $\geqslant$ 98%.

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

RNA was isolated with TRIzol (Thermo Fisher) or Total RNA Miniprep Kit (Sigma-Aldrich). cDNA was synthesized with Superscript II after DNase treatment (both from Invitrogen). RT-PCR was performed with ViiA7 sequence

detection system (Life Technologies). Gene expression of IL-17A, IL-17F, IL-19, IL-22, IL-23p19, IL-24 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in mouse samples and hypoxanthine-guanine phosphoribosyltransferase (HPRT) in human samples. Primer sequences were summarized in supplementary Table S1.

Human primary skin fibroblast cultures and co-cultures with T cells

Fibroblasts (n=6) from lesional skin of psoriasis patients were obtained from our biobank collection. Healthy skin samples (n=6) were obtained from healthy individuals who underwent cosmetic surgeries in the Sint Franciscus Hospital (Rotterdam, The Netherlands). Signed consents were provided by all healthy participants. Human primary skin fibroblasts were cultured from above skin samples as described previously (18). Passages 3-8 fibroblasts were seeded 1.0×10^4 per well in 96-well culture plates and stimulated with IL-1β (0.01 ng/mL, 201-LB), TNFα (5 ng/mL, 210-TA), IL-17A (50 ng/mL, 317-IL), and IL-17F (500 ng/mL, 1335-IL) (all from R&D systems) for 24 hours (hrs).

In co-culture experiments, skin fibroblasts ($1.0x10^4$) were co-cultured with CD4+CD45RO+CD14-CD25low/int T memory cells (2.5×10^4) sorted from buffy coats (n=6). Soluble anti-CD3 and anti-CD28 (both from Sanquin, Amsterdam, The Netherlands) were added for 72 hrs. In addition, 100 µg/ml anti-IL-17Aantibody (secukinumab, Novartis), 1µg/ml anti-TNF antibody (adalimumab, AbbVie), and an isotype IgG1κ antibody (Sigma-Aldrich) were used.

Enzyme-linked immunosorbent assay (ELISA)

Human IL-8 and IL-19 in culture supernatants was measured with ELISA (Invitrogen) and ELISA Duoset (R&D systems) following manufacturers' instructions.

Data set analysis

Public microarray data (GSE13355) were analyzed to compare mRNA expression of psoriatic lesional (n=64), non-lesional skin (n=58) and skin from healthy controls (n=58). Based on GSE13355, values of 216876_s_at (IL-17A), 220745_at (IL-19), 206569_at (IL-24) and 212021_s_at (MKI67) from gene expression profile GDS4602 were plotted. Dataset GSE53552 was analyzed for mRNA expression of psoriatic skin lesions following treatment with

brodalumab (AstraZeneca). Psoriatic non-lesional (n=23), lesional (n=25), and day 8 (n=4), day 15 (n=19), day 43 (n=16) after treatments were included. 220745_at (IL-19), 206569_at (IL-24), 212022_s_at (MKI67) values from GDS5420 were plotted based on GSE53552.

Statistics

Statistical differences were determined with paired or unpaired student's *t* test. All data analyses were performed with GraphPad Prism V5 and P-values <0.05 were considered as significant.

RESULTS

IL-10 neutralization enhances skin thickness and scaling in the IMQ-induced psoriasis mouse model

The design of the IL-10 neutralization experiments is summarized in **Supplementary Figure S1A,B**. Local psoriasis area and severity index (PASI) score was used to evaluate psoriasis symptoms including skin scaling, thickness and redness. As shown in **Figure 1A**, ten days after IMQ treatment, macroscopic scores of skin scaling and thickness were significantly higher in the anti-IL-10 treated group (anti-IL-10) compared to the isotype antibody control group (isotype). This resulted in a significant higher PASI score after neutralizing IL-10 compared to the isotype control (**Figure 1A**). Dexamethasone treatment significantly improved both symptoms compared to either the anti-IL-10 or isotype group. No significant difference for skin redness was observed among groups. Details of kinetic data of the macroscopic scores were summarized in **Supplementary Figure S2A**. These data indicate that IL-10 neutralization enhances skin thickness and scaling in the IMQ-induced psoriasis mouse model beyond day 5.

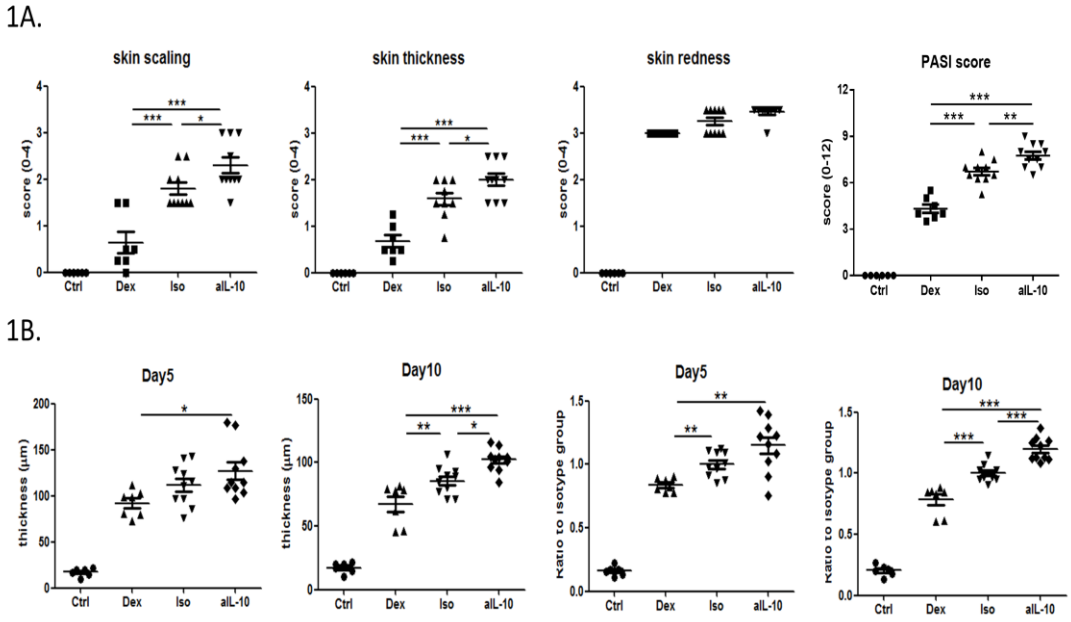

Figure 1. IL-10 neutralization worsens psoriatic symptoms and epidermal thickness in the IMQ-induced psoriasis mouse model. (A) At day 10, scores for skin scaling, thickness, redness and PASI in various groups following IMQ treatment. **(B)** Measured average epidermal

thickness in psoriasis-like skin at days 5 and 10, and thickness ratios compared to isotype antibody controls at days 5 and 10. Data are shown as means ± SEMs. * P<0.05, ** P<0.01, and *** P<0.001.

IL-10 neutralization increases epidermal thickness and keratinocyte proliferation in the IMQ-induced psoriasis mouse model

To confirm the increased skin thickness observed macroscopically after anti-IL-10 treatment, H&E staining was performed and epidermal thickness was measured microscopically (**Figure 2A**). At day 5, epidermal thickness was significantly higher in the anti-IL10 compared to dexamethasone treatment (**Figure 1B**). At day 10, anti-IL-10 significantly increased epidermal thickness compared to the isotype group, whereas dexamethasone significantly reduced this compared to both groups (**Figure 1B**). This is in line with our macroscopic findings of increased skin thickness with the PASI score in the anti-IL-10 treated group (**Figure 1A**).

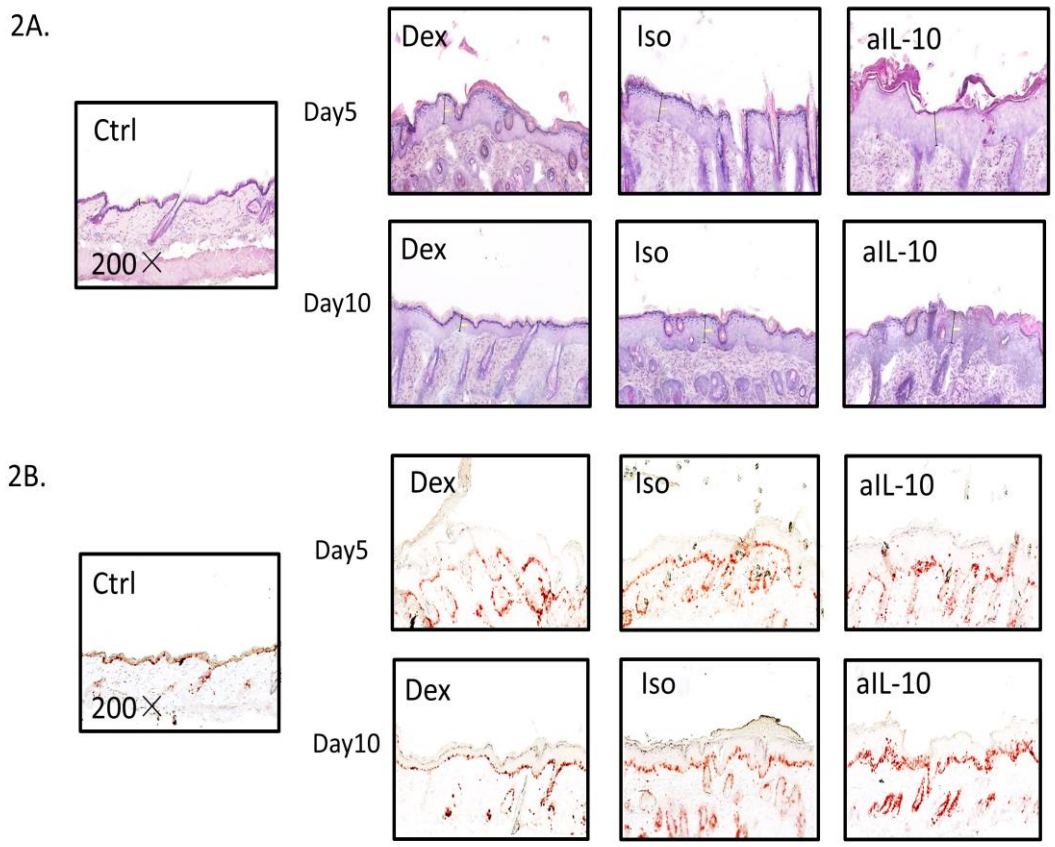

Figure 2. IL-10 neutralization increases keratinocyte proliferation and neutrophil accumulation in the IMQ-induced psoriasis mouse model.

(A) Representative H&E staining results of skin sections in IMQ-induced psoriasis mouse model at days 5 and 10. **(B)** Representative IHC staining of Ki-67+ proliferating keratinocytes in skin sections of IMQ-induced psoriasis mouse model at days 5 and10. All images were taken with 200× magnification.

As indicated before, compared to isotype group, ratios of epidermal thickness in the anti-IL-10 treated group were slightly increased at day 5, but significantly increased at day 10 (**Figure 1B**). Both epidermal thickness and thickness ratios in the isotype group were comparable to saline-treated IMQ groups (**Supplementary Figure S2B**). Ki-67 staining was performed to further identify proliferating cells. As shown in **Figure 2B**, 1-2 layers of keratinocytes in the epidermal stratum basale were Ki-67+ in dexamethasone group, 2-3 layers were Ki-67+ in isotype group, while 4-5 layers were Ki-67+ in anti-IL-10 group (at days 5 and 10). This is in line with the increased thickness of the epidermis in the anti-IL-10 treated group at day 10 (**Figure 1B**). Taken together, our data indicate that in the IMQ-induced psoriasis mouse model, IL-10 neutralization enhances skin thickness through facilitating keratinocyte proliferation.

IL-10 neutralization increases the recruitment of neutrophils and monocytes into the skin

Flow cytometry showed representative staining results of infiltrating neutrophils (CD11b+Ly6CintLy6G+) in lesional skin at day 10 (**Figure 3A**). Both neutrophils numbers and percentages were increased after neutralization of IL-10 compared to the isotype group (**Figure 3B**). Treatment with dexamethasone significantly reduced both parameters compared to the other groups (**Figure 3B**). At days 5 and 10, IHC staining confirmed the enhanced recruitment of Gr-1+ neutrophils in IMQ lesional skin in the anti-IL-10 treated group compared to the isotype group (**Figure 3C**). Neutrophil chemokine CXCL2, but not CXCL1, was upregulated after neutralizing IL-10 and correlated with enhanced neutrophil recruitment (**Figure 3B**). Similar results were found for CD11c+Ly6Cint monocytes-derived dendritic cells (mono/DCs) (**Figure 3D,E**). The isotype group showed similar cell numbers and percentages of neutrophils and mono/DCs compared to the saline-treated IMQ group (**Supplementary Figure S2C,D**), indicating that no specific immune effects were induced by the isotype antibody injections. These data indicate that IL-10 neutralization in the

IMQ-induced psoriasis mouse model results in enhanced persistent inflammation, as evidenced by enhanced influx of neutrophils and mono/DCs into lesional skin.

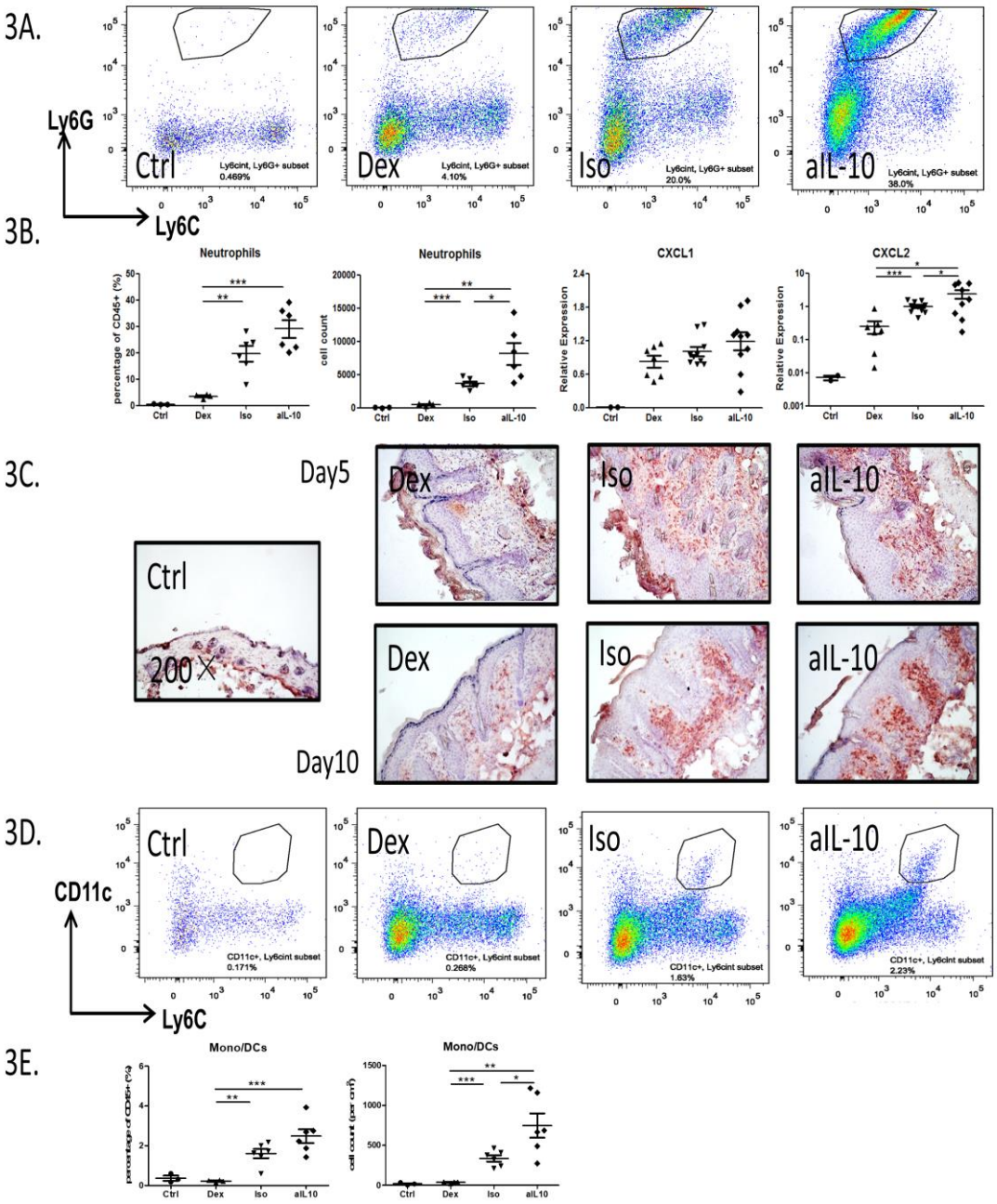

Figure 3. IL-10 neutralization increases neutrophil and monocyte/DC infiltration in the IMQ-induced psoriasis mouse model.

(A) Flow cytometry staining of Ly6G+Ly6Cint neutrophils among pregated CD45+CD11b+ cells in lesional psoriasis-like skin at day10. **(B)** Percentages and cell numbers of neutrophils among

total CD45+ immune cells at day10 in flow cytometry analysis and levels of the neutrophil chemokines, CXCL1 and CXCL2, in lesional psoriasis-like skin. **(C)** Representative IHC staining of Gr-1+ neutrophils in skin sections of the IMQ-induced psoriasis mouse model at days 5 and 10. **(D)** Flow cytometry staining of CD11c+Ly6Cint monocyte-derived dendritic cells in lesional psoriasis-like skin at day10. **(E)** Percentages and cell numbers of monocyte-derived dendritic cells (mono/DCs) among total CD45+ immune cells at day10 using flow cytometry. Data are shown as means ± SEMs. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

IL-10 neutralization in the IMQ-induced psoriasis mouse model results in an early upregulation of the IL-23/IL-17 immune pathway related cytokines followed by a subsequent later increase of IL-19 and IL-24 in the skin

In psoriasis, IL-17-producing T helper cells (Th17) are central in the pathogenesis and Th17-related cytokines such as IL-17A, IL-17F and IL-22, together with TNFα, drive epidermal hyperplasia (9,16,19). Therefore, we investigated the effects of IL-10 neutralization on T cells and T cell cytokine expression in the IL-23/IL-17-dependent IMQ-induced psoriasis mouse model (16). Neutralizing IL-10 did not change the number of CD3+ T cells, including CD4+ and γδ T cells at day 10 (**Supplementary Figure S3A-C**). However, at day 5, IL-23p19, IL-22, IL-17A and IL-17F were significantly increased in the anti-IL-10 treated group compared to the isotype control group (**Figure 4A**). Interestingly, no difference in expression of these cytokines was found between these two groups at day 10 (**Figure 4A**). In contrast, the IL-20 subfamily cytokines, IL-19 and IL-24, were significantly upregulated only at day 10 but not at day 5 (**Figure 4B**) which correlated with the significant increase of epidermal thickness at day 10. This indicates that IL-19 and IL-24, rather than IL-22, IL-17A or IL-17F, were responsible for the late stage (day 10) keratinocyte hyper-proliferation and acanthosis in the IMQ-induced psoriasis mouse model during anti-IL-10 treatment.

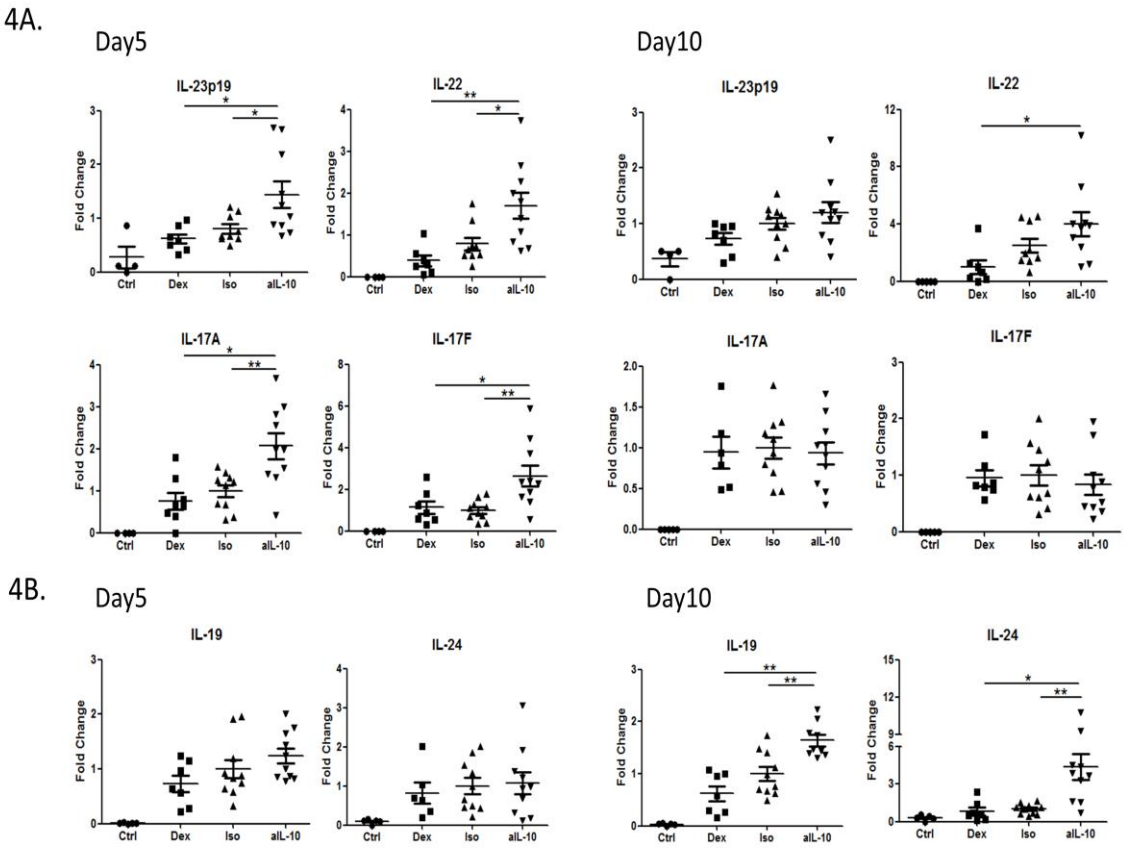

Figure 4. Early and late up-regulation of IL-23/IL-17 cytokines and IL-20 subfamily cytokines in the skin during anti-IL-10 treatment in IMQ-induced psoriasis mouse model. **(A)** At days 5 and 10, mRNA expression of IL-23, IL-22, IL-17A and IL-17F in various groups following IMQ treatment. **(B)** At days 5 and 10, mRNA expression of IL-19 and IL-24 in various groups following IMQ treatment. Data are shown as means ± SEMs. * *P*<0.05, ** *P*<0.01.

IL-17 induces human skin fibroblasts to produce IL-19 and IL-24

As the increase in IL-17 family cytokines preceded the upregulation of IL-19 and IL-24, we assumed that the IL-17 family cytokines induced IL-19 and IL-24. Therefore, the expression of IL-19 and IL-24 in skin fibroblasts was examined after stimulation with IL-1β, TNF, IL-17A, or IL-17F for 24 hours. The expression of the IL-17 receptor, IL-17RA and IL-17RC, was confirmed on skin fibroblasts from psoriasis patients or healthy volunteers (**Supplementary Figure S4A**). As shown in **Figure 5A**, both IL-17A and IL-1β significantly increased IL-19 and IL-24 mRNA expression compared to the unstimulated group. TNF, on the other hand, was only a significant inducer of IL-24 (**Figure 5A**). Additionally, we observed an intrinsic higher expression of IL-19 and IL-24 in psoriatic skin fibroblasts compared to skin fibroblasts from healthy controls (**Figure 5A**).

To further explore the induction of IL-19 and IL-24 by IL-17A, healthy and psoriasis skin fibroblasts were co-cultured with CD4+CD45RO+CD14-CD25low/int T memory cells for 72 hours with or without stimulation. Neutralizing antibodies for IL-17A, TNF or the combination were added to these co-cultures. **Figure 5B** showed that anti-IL-17A treatment significantly reduced mRNA expression of IL-19 and IL-24 compared to either the isotype antibody or anti-TNF. In contrast, anti-TNF treatment only significantly reduced expression of IL-19 but not of IL-24 (**Figure 5B**). No additive effect was shown when both IL-17A and TNF were neutralized compared to anti-IL-17 treatment alone. In line with the mRNA expression data, ELISA data showed that both anti-IL-17A and anti-TNF significantly reduced the protein levels of IL-19 in the co-culture supernatants with anti-IL-17 more potent than anti-TNF, but without additive effect when treatment was combined (**Figure 5C**). However, anti-IL17A and anti-TNF both significantly reduced the levels of IL-8, with additive effect when combined (**Figure 5C**). Also in these skin fibroblast-T cell co-cultures, IL-19 and IL-24 mRNA expression as well as protein levels of IL-19 and IL-8 were all lower when skin fibroblast were derived from healthy controls compared to patients with psoriasis (**Figure 5B,C**). This indicates an intrinsic higher expression of both cytokines in psoriasis lesions.

IL-17A-induced expression of IL-19 was also checked in healthy human skin primary keratinocytes. Expression of the IL-17 receptor, IL-17RA and IL-17RC, was confirmed in these keratinocytes (**Supplementary Figure S4A**). IL-17A stimulation of human keratinocytes resulted in increased IL-19 mRNA expression. On the other hand, inhibition of IL-17A in keratinocyte-T cell co-cultures reduced IL-19 mRNA expression (**Supplementary Figure S4B**). Overall, these data indicate that IL-17A induces IL-19 and IL-24 expression in human skin resident cells such as fibroblasts and keratinocytes, and IL-17A blockade reduces their expression.

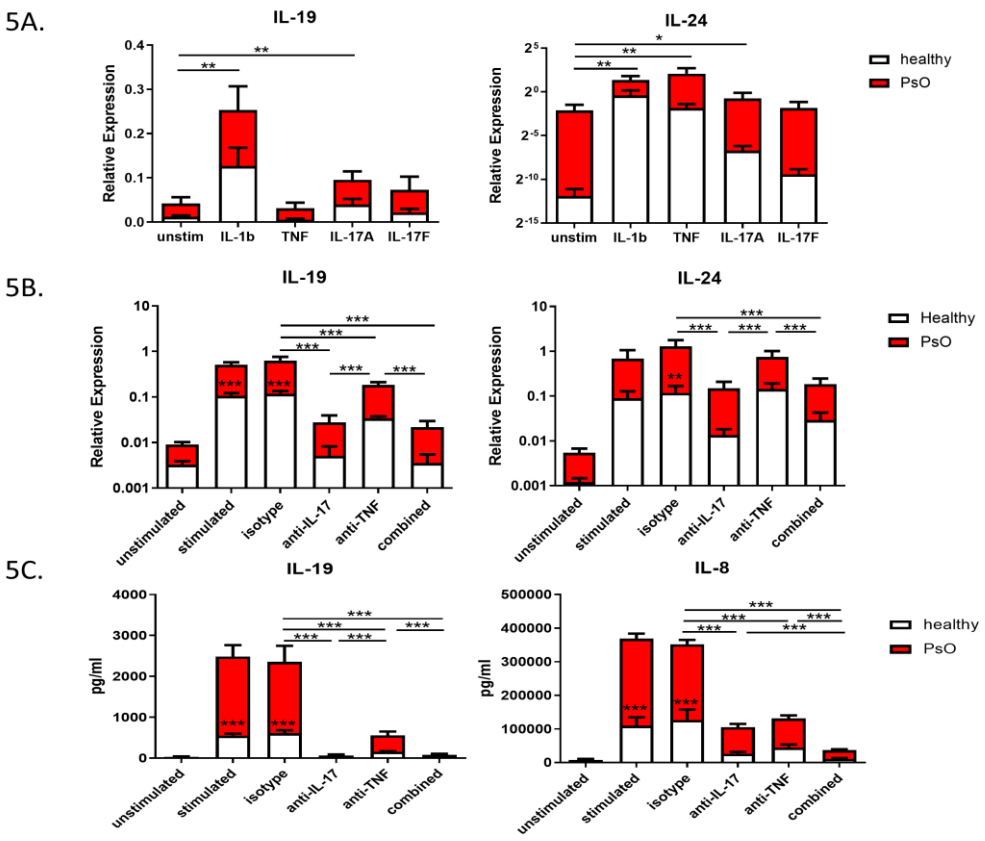

Figure 5. IL-17A induces IL-19 and IL-24 expression in human skin resident cells such as fibroblasts and keratinocytes, and IL-17A blockade reduces their expression. (A) IL-19 and IL-24 mRNA expression in fibroblasts of healthy donors (n=6) and psoriasis patients (n=4) without stimulation or stimulated with IL-1β, TNFα, IL-17A and IL-17F for 24 hours. **(B)** Healthy control skin fibroblasts and psoriatic fibroblasts (n=6 each) were co-cultured with unstimulated or anti-CD3/anti-CD28 stimulated memory CD4+ T cells from healthy controls for 72 hours. IL-19 and IL-24 mRNA expression in these co-cultures without treatment or treated with an anti-IL-17A antibody, anti-TNF antibody, their combination or an isotype control antibody. **(C)** Levels of human IL-19 and IL-8 protein in the supernatant of the above- mentioned co-cultures without treatment or treated with an anti-IL-17A antibody, anti-TNF antibody, their combination or an isotype control antibody. Data are shown as means ± SEMs. * P<0.05, ** P<0.01, and *** P<0.001.

IL-17A neutralization reduces IL-19 and cell proliferation in psoriatic skin lesions

Gene expression data from psoriatic lesional skin, psoriatic non-lesional skin, and healthy skin, obtained in clinical trials with anti-IL-17 biologics in psoriasis, were analyzed using the Genomic Spatial Event (GSE) database. As shown in **Figure 6A**, IL-17A, IL-19, and IL-24 were all significantly higher in psoriatic

lesional skin compared to either psoriatic non-lesional skin or healthy skin. This corroborated our findings of a higher expression of IL-19 and IL-24 in psoriatic skin fibroblasts compared to healthy fibroblasts (**Figure 5**). Furthermore, Ki-67, was significantly increased in psoriatic lesional keratinocytes (**Figure 6A**). Significant upregulation of IL-17A, IL-19, IL-24, and Ki-67 in psoriasis was confirmed in another independent dataset (**Supplementary Figure S5A**).

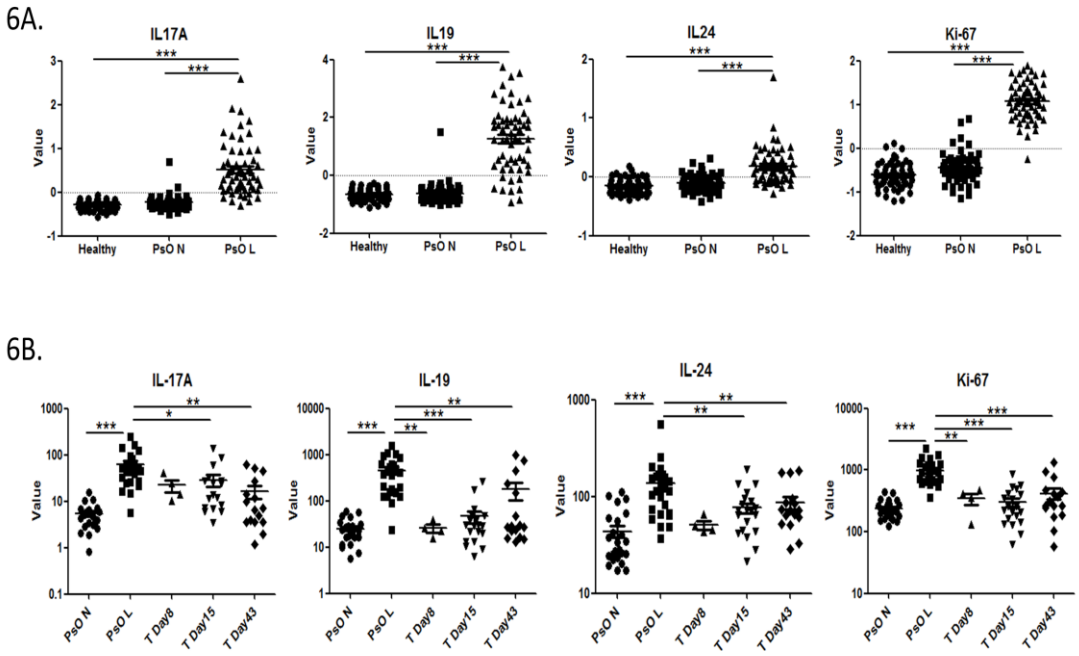

Figure 6. Expression of human IL-17A, IL-19, IL-24 and Ki-67 in psoriatic lesions and the effects of anti-IL-17RA therapy. (A) Expression of IL-17A, IL-19, IL-24 and Ki-67 in lesional and non-lesional skin biopsies from 58 psoriasis patients and in healthy skin biopsies from 64 normal controls in data set GSE13355. **(B)** Expression of IL-17A, IL-19, IL-24 and Ki-67 in lesional and non-lesional skin biopsies from 25 psoriasis patients before anti-IL-17RA treatment, 8, 15 and 43 days after treatments in data set GSE53552. Data are shown as means ± SEMs. * P<0.05, ** P<0.01, and *** P<0.001.

In a separate dataset, in which an IL-17 receptor A (IL-17RA) antibody was used in the treatment of psoriasis, a significant reduction of the expression of IL-19, IL-24, and Ki-67 in psoriatic lesional skin was found, reducing their expression levels close to those of non-lesional skin after treatment (**Figure 6B**). Similar significant down-regulation of IL-19 and Ki-67 was confirmed in another study using anti-IL-17A treatment (**Supplementary Figure S5B**). To summarize, multiple gene expression data of psoriatic patients before and after

targeting the IL-17 pathway support our data that IL-17A regulates IL-19 expression in fibroblasts and keratinocytes.

DISCUSSION

In the present study, we showed that IL-10 neutralization enhanced skin inflammation, thickness and scaling in the IMQ-induced psoriasis mouse model beyond day 5, via upregulation of the IL-17/IL-19 axis. IL-17A induced IL-19 and IL-24 expression in human dermal fibroblasts and epidermal keratinocytes, and IL-17A neutralization reduced the expression of both cytokines. Gene array expression data also show high expression of IL-17A, IL-19, IL-24 and proliferation marker Ki-67 in psoriatic skin lesions, and that anti-IL-17 therapy reduced their expression. In addition to keratinocytes, dermal fibroblasts, through interaction with immune cells and cytokines such as IL-17A and TNF in psoriasis, can be a major source of IL-19 and IL-24 that contribute to perpetuation of psoriatic symptoms such as keratinocyte proliferation and acanthosis.

In psoriasis, recombinant human IL-10 treatment has been demonstrated to improve psoriatic symptoms in clinical trials (20-23). Similarly, in the IMQ-induced psoriasis mouse model, a subset of IL-10-producing B cells was identified, and adoptive transfer of these IL-10-producing B cells reduced disease severity (24). In contrast, as shown by our and other groups, IL-10 neutralization or IL-10 deficiency induced persistent psoriasis-like inflammation after IMQ application (25). In psoriatic skin, macrophages and DC both can be producers as well as direct target cells of IL-10 (28). Frequency of Th17 cells can also be directly controlled by IL-10 and vice versa (29,30).

IL-10 belongs to the cytokine family of IL-19, IL-20, IL-22 and IL-24 and are located in the same cluster on chromosome 1 (31,32). Although IL-10 is the only anti-inflammatory cytokine in this family, simultaneous induction of IL-10 and the IL-20 subfamily has been observed in monocytes by stimulants (33). IL-10 production during inflammation acts as a natural counter-balance to limit the side effects of inflammation. Without this Yin-Yang dynamic equilibrium, inflammation will be skewed towards uncontrolled harmful diseases. For instance, in psoriasis, low levels of IL-10 have been reported in comparison to other inflammatory skin conditions, while contrarily, enhanced expression of IL-

23/IL-17 pathway cytokines has been widely confirmed in psoriatic lesions (9,34,35).

Overexpression of IL-19 and IL-24 has also been observed in psoriatic skin and both induce keratinocyte hyper-proliferation in a reconstituted human skin model (36-38), suggesting a pathogenic role in psoriasis. Myeloid cells are producers of both cytokines, and keratinocytes are also potential producers (31,32,39). Recently, IL-19 was suggested as an important mediator of the IL-23/IL-17 cascade in psoriasis, and IL-17A-induced expression of IL-19 in keratinocytes amplifies keratinocyte responses via auto-paracrine regulation (14). Here we confirmed the induction of IL-19 by IL-17A in human keratinocytes and extended to show that IL-17A neutralization reduced IL-19 in human keratinocyte-T cell co-cultures (**Supplementary Figure S4B**). In addition, our study provided evidence that dermal fibroblasts also produced IL-19 and IL-24 in response to IL-1β and lL-17A, and, when co-cultured with activated memory T cells, fibroblasts produced significant protein levels of IL-19 (**Figure 5A-C**). Synergistic induction of IL-20 subfamily cytokines by IL-1β and IL-17A has also been observed in recent publications (40). Interestingly, in psoriatic fibroblast-T cell co-cultures, higher levels of IL-19 was observed in comparison to healthy fibroblast co-cultures, further supporting the contribution of psoriatic fibroblasts to local IL-19 production. Compared to epidermal keratinocytes, dermal fibroblasts are positioned to encounter more frequently with inflammatory cells including T cells as most infiltrating T cells accumulate in the dermis (**Supplementary Figure S3A**). Therefore, in psoriasis, dermal fibroblasts could be an important local source of IL-20 subfamily cytokines and contribute to keratinocyte hyper-proliferation through a paracrine mechanism.

Discordant regulation of IL-20 in contrast to IL-19 and IL-24 has been found in our study. This could be due to different immune and resident cell types as potential cellular sources for these cytokines (32). Like skin fibroblasts and keratinocytes, other tissue cells such as endothelial cells and fibroblast-like synovial cells can also produce IL-20 subfamily cytokines (32,41). Nevertheless, whether blocking the IL-20 subfamily cytokines will be viable options in psoriasis treatments still warrants further research.

In summary, we show that IL-10 regulates the expression of cytokines related to the IL-23/IL-17 axis via IL-19 and IL-24 influencing a.o. skin thickness and scaling. These data give further insight into the cytokine network in the stromal milieu of psoriasis plaques. Strategies to upregulate local or systemic production of IL-10 in patients with psoriasis could help increasing the effectiveness of current therapies.

DATA AVAILABILTIY STATEMENT

All datasets generated in this study are provided in the article/supplementary material.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

XX contributed to the study design, performed experiments and wrote the manuscript. EF, PA, and AM performed experiments and revised the manuscript. PL and LB supported experiments and revised the manuscript. EP contributed to the study design and revised the manuscript. EL designed the study and revised the manuscript.

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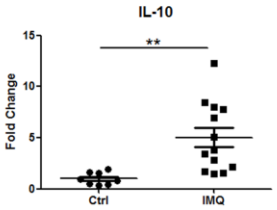
Supplementary tables

Table S1. Primer sequences used in this study.

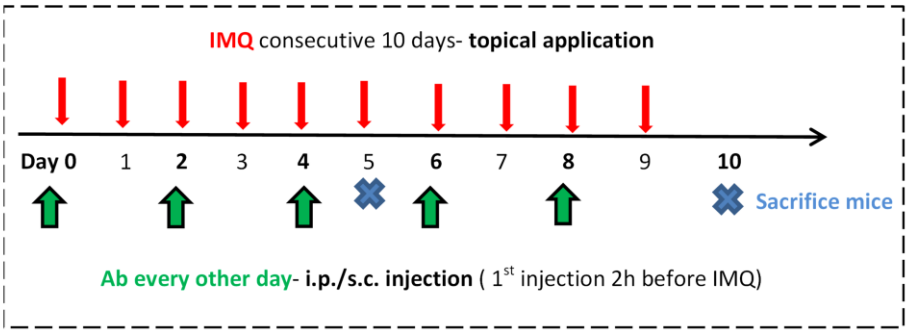
Name	Species	Sequence
mIL-23p19 F	Mouse	CACCTCCCTACTAGGACTCAGC
mIL-23p19 R	Mouse	TGGGCATCTGTTGGGTCT
mIL-22 F	Mouse	TTTCCTGACCAAACTCAGCA
mIL-22 R	Mouse	CTGGATGTTCTGGTCGTCAC
mIL-17A F	Mouse	TTTTCAGCAAGGAATGTGGA
mIL-17A R	Mouse	TTCATTGTGGAGGGCAGAC
mIL17F F	Mouse	CAAGAAATCCTGGTCCTTCG
mIL17F R	Mouse	GAGCATCTTCTCCAACCTGAA
mIL-19 F	Mouse	TGGAGAACCTCAGGAGCATT
mIL-19 R	Mouse	GAATGTCAGCAGGTTGTTGG
mIL-24 F	Mouse	AGAACCAGCCACCTTCACAC
mIL-24 R	Mouse	GTGTTGAAGAAAGGGCCAGT
mCXCL2 F	Mouse	AAAATCATCCAAAAGATACTGAACAA
mCXCL2 R	Mouse	CTTTGGTTCTTCCGTTGAGG
mKC (CXCL1) F	Mouse	GACTCCAGCCACACTCCAAC
mKC (CXCL1) R	Mouse	TGACAGCGCAGCTCATTG
mGAPDH F	Mouse	AGCTTGTCATCAACGGGAAG
mGAPDH R	Mouse	TTTGATGTTAGTGGGGTCTCG
IL-19 F	Human	GGAGACTCTGCAGATCATTAAGC
IL-19 R	Human	GATCCTTGAACACCCTGTCC
IL-24 F	Human	GAAGAATTGAGGCTGCTTGG
IL-24 R	Human	GAGGGCAGAAGGGTCTGG
HPRT F	Human	TGACCTTGATTTATTTTGCATACC
HPRT R	Human	CGAGCAAGACGTTCAGTCCT

Supplementary Figures

S1A



S1B



Groups:	***Abbreviation***
Group1- Control	Ctrl
Group2- IMQ + Saline (s.c.)	IMQ
Group3- IMQ +Dexa (s.c.)	Dex
Group4- IMQ + Isotype (i.p.)	Iso
Group5- IMQ + IL-10 Ab (i.p.)	aIL-10

Figure S1. Experimental design of anti-IL-10 therapy in the IMQ-induced psoriasis mouse model. (A) Expression of IL-10 in lesional skin after topical Aldara application in the IMQ-induced mouse model at day 5. (B) Experimental design and groups of the anti-IL-10 treatment experiments in IMQ-induced psoriasis mouse model sacrificing mice at days 5 and 10. Data are shown as means ± SEMs. ** *P*<0.01.

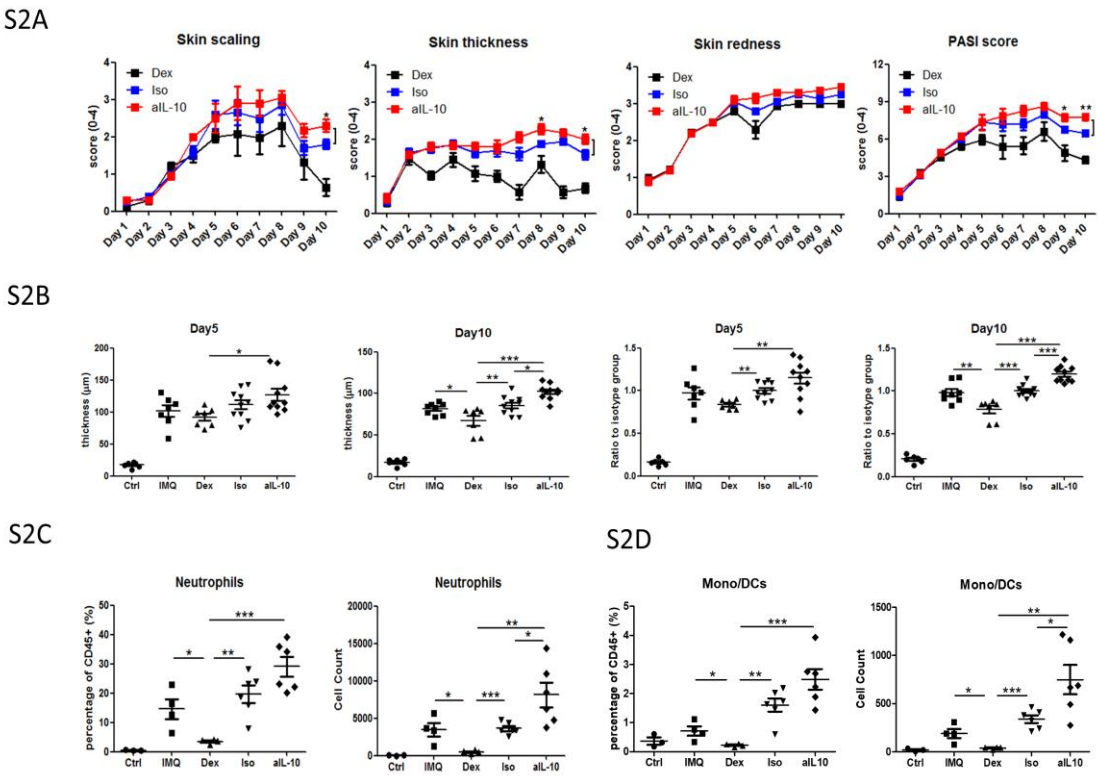


Figure S2. Kinetic information of macroscopic and histologic scores and infiltration of myeloid cells after anti-IL-10 treatment in the IMQ-induced mouse model. (A) Time-course follow-up of local PASI scores, including skin scaling, thickness and redness, in anti-IL-10, isotype control antibody and dexamethasone-treated groups during 10 days of Aldara application. (B) Summary of days 5 and 10 epidermal thickness and thickness ratios in all groups including saline-treated IMQ group. (c&d) Summary of percentages and cell numbers of neutrophils (C) and monocyte-derived dendritic cells (D) in about 1 cm^2lesional digested skin from all groups including saline-treated IMQ group at day10. Data are shown as means ± SEMs. * P<0.05, ** P<0.01, and *** P<0.001.

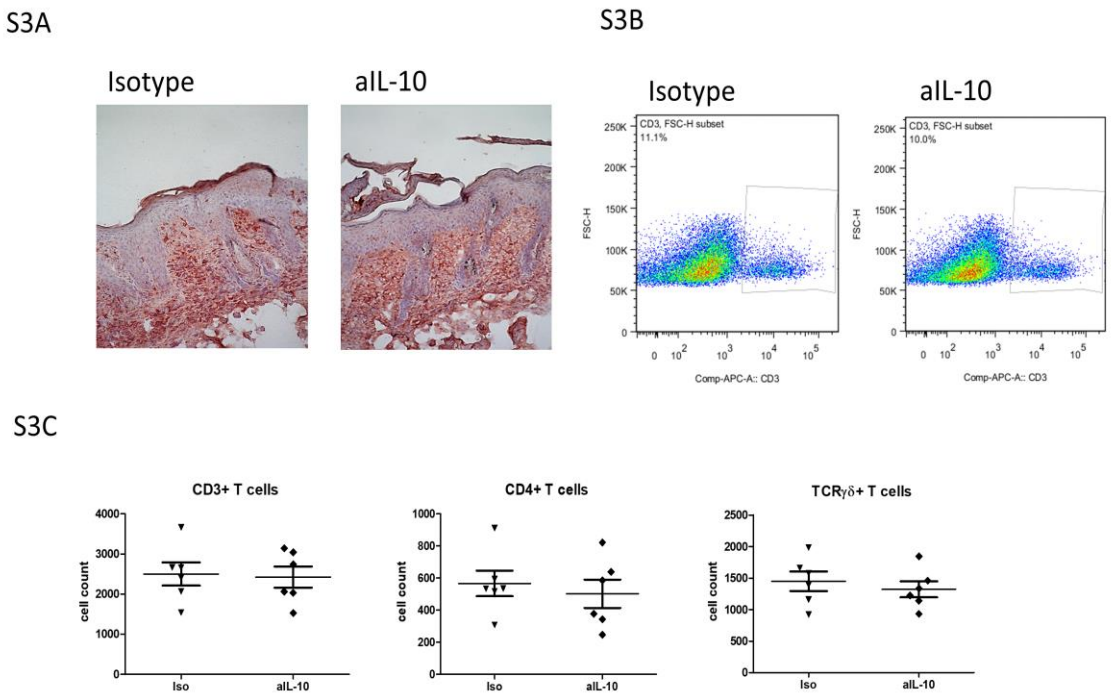


Figure S3. T cell subsets infiltration in IMQ-applied skin after IL-10 neutralization compared to isotype antibody group. (A) Representative CD3+ IHC staining in anti-IL-10 and isotype antibody groups 10 days after IMQ application. (B) Representative flow cytometry staining of CD3+ T cells among CD45+ immune cells in anti-IL-10 and isotype antibody groups 10 days after IMQ application. (C) Cell number summary of infiltrating CD3+ T cells, CD3+CD4+ T cells, and CD3+TCRγδ+ T cells in anti-IL-10 and isotype antibody groups (n=6 each) 10 days after IMQ application.

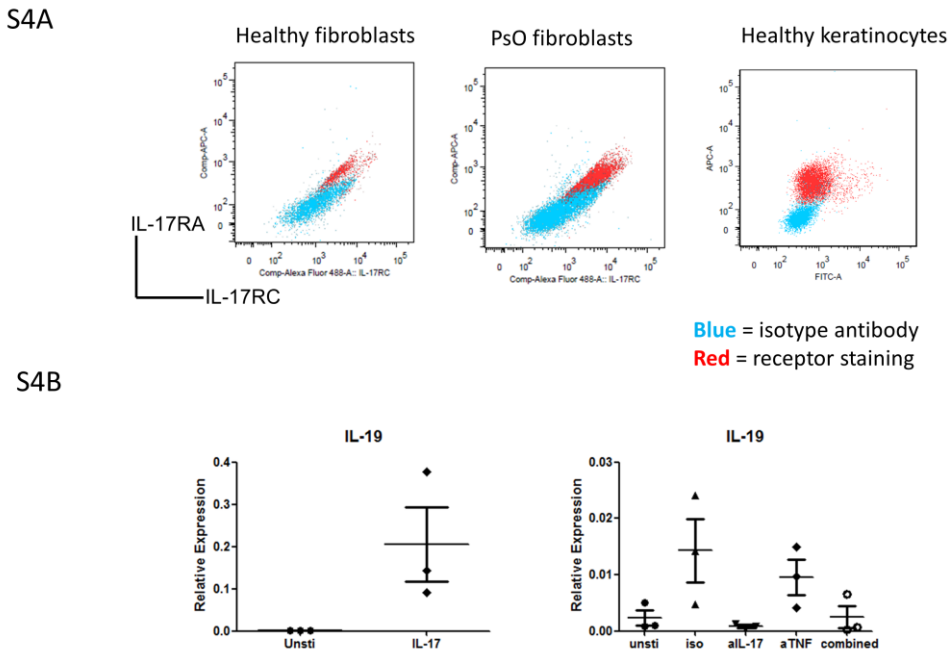


Figure S4. Fibroblasts and keratinocytes express IL-17RA and IL-17RC and respond to IL-17A stimulation. (A) Representative staining of IL-17RA and IL-17RC compared to isotype antibody controls on healthy and psoriatic skin fibroblasts, as well as healthy skin keratinocytes. (B) mRNA expression of IL-19 in keratinocytes stimulated with IL-17A for 24 hrs (left panel) and in anti-CD3/anti-CD28 stimulated co-cultures of keratinocytes and sorted healthy CD4+ T memory cells in which neutralizing antibodies against IL-17A, TNF, the combination of these antibodies or an isotype control antibody was added (right panel).

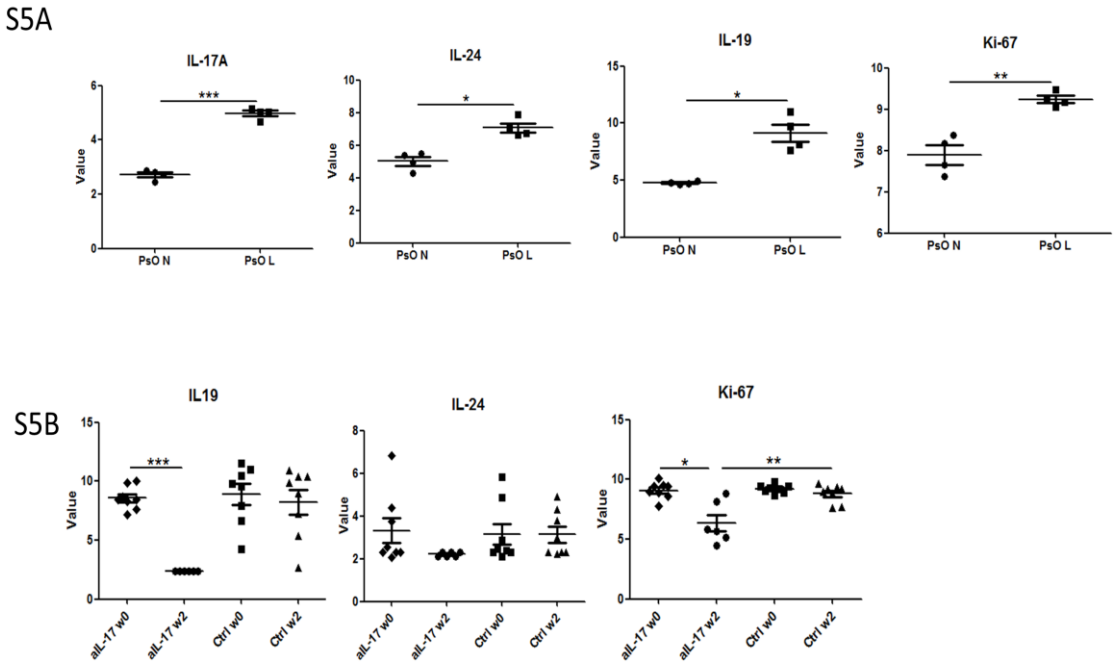


Figure S5. Expression of IL-19, IL-24 and Ki-67 in psoriatic lesions and the effect of anti-IL-17A antibody treatment. (A) Expression of IL-17A, IL-19, IL-24 and Ki-67 in lesional and non-lesional skin biopsies from 4 psoriasis patients in dataset GSE50790. (B) Normalization of IL-19, IL-24 and Ki-67 in lesional biopsies from psoriasis patients before (n=8) and after (n=6) 2-week anti-IL-17A treatment with placebo (n=8 each) treatment controls in dataset GSE31652. Data are shown as means ± SEMs. * *P*<0.05, ** *P*<0.01, and *** *P*<0.001.