Comparison of Blood and Synovial Fluid Th17 and Novel Peptidase Inhibitor 16 Treg Cell Subsets in Juvenile Idiopathic Arthritis

RANDALL H. GROSE, DEBORAH J. MILLARD, CHRIS MAVRANGELOS, SIMON C. BARRY, HEDDY ZOLA, IAN C. NICHOLSON, WENG TARNG CHAM, CHRISTINA A. BOROS, and DOREEN KRUMBIEGEL

ABSTRACT. ***Objective.*** Early recognition and treatment of juvenile idiopathic arthritis (JIA) can prevent joint damage and minimize side effects of medication. The balance between proinflammatory and anti-inflammatory mechanisms is known to be important in JIA, and we therefore investigated T cell subsets including Th cells, autoaggressive Th17 cells, and regulatory T cells (Treg), including a novel Treg subset in peripheral blood (PB) and synovial fluid (SF) of patients with JIA.

Methods. Fifty children with JIA were enrolled in our study. Frequency, phenotype, and function of T lymphocytes in PB and SF were characterized using flow cytometry. Migration capabilities of PB and SF cells were compared.

Results. Synovial T cells showed different phenotype and function compared with PB T cells, with an increased proportion of memory T cells, expression of CCR4, CCR5, CXCR3, interleukin 23R, and an increased ratio of Th17 to Treg. Although Treg were increased in SF compared with the PB, we found a significant decrease in the numbers of peptidase inhibitor 16 (PI16)+ Treg in active joints compared with peripheral blood. Coexpression of CCR4 and CCR6 was reduced on PI16+ Treg in PB and SF of patients with JIA compared with healthy children, however the ability of these cells to migrate toward their ligands was unaffected.

Conclusion. This is a comprehensive characterization of novel PI16+ Treg and Th17 cells in matched blood and synovial fluid samples of patients with JIA. Despite an increased number of Treg within the inflamed joint, lower numbers of PI16+ Treg but high numbers of Th17 cells might contribute to the inability to control disease. (J Rheumatol First Release August 15 2012; doi:10.3899/jrheum.111421)

Key Indexing Terms:
JUVENILE IDIOPATHIC ARTHRITIS PEPTIDASE INHIBITOR 16 Th CELLS

Juvenile idiopathic arthritis (JIA) is the most common rheumatologic disease of childhood, occurring in up to 1:500 children[1]. The effect on children with JIA can be significant, with pain, disability, deformity, growth failure, and osteoporosis[2]. The disease can affect not only the child's education, social, and psychological functioning, but also his or her family[3]. As demonstrated, it is important to recognize and treat JIA early to prevent soft tissue deformities, irreversible joint damage, and medication side effects[4].

T cells have been demonstrated to play key roles in the pathogenesis of JIA. Specifically, cells that produce interleukin 17 (IL-17; Th17) are believed to be the major inducers of severe autoimmune tissue inflammation and destruction[4,5,6]. By binding the chemokine CCL20 produced by activated monocytes, chemokine receptor CCR6-expressing Th17 cells migrate along a CCL20 gradient into sites of inflammation[7,8], where they induce the production of proinflammatory cytokines and chemokines that attract neutrophils and other immune cells[9]. In the synovial fluid (SF) of patients with rheumatoid arthritis (RA), it is known that IL-17 is responsible for osteoclastogenesis[10], and that it promotes cartilage and bone destruction and resorption[11]. IL-17 also promotes the generation, attraction, and expansion of further Th17 cells, which sustain the inflammatory response within the joint. However, little is known about T cells with-

From the Women's and Children's Health Research Institute (WCHRI), North Adelaide, and the Discipline of Paediatrics, University of Adelaide, Adelaide, Australia.

Supported by the Co-operative Research Centre (CRC) for Biomarker Translation, Australia.

R.H. Grose, PhD; D.J. Millard, Dip Biomed; C. Mavrangelos, BSc, WCHRI, Leukocyte Biology Laboratory, CRC for Biomarker Translation; S.C. Barry, PhD, WCHRI, Leukocyte Biology Laboratory, Molecular Immunology Laboratory, and Department of Gastroenterology, Children, Youth and Women's Health Service, Discipline of Paediatrics, University of Adelaide, and CRC for Biomarker Translation; H. Zola, PhD, WCHRI, Leukocyte Biology Laboratory, Discipline of Paediatrics, University of Adelaide, and CRC for Biomarker Translation; I.C. Nicholson, PhD, WCHRI, Leukocyte Biology Laboratory, Discipline of Paediatrics, University of Adelaide, Flinders University, CRC for Biomarker Translation; W.T. Cham, MBBS, MRCPCH; C.A. Boros, MBBS, PhD, FRACP, Department of Rheumatology, Women's and Children's Hospital, Discipline of Paediatrics, University of Adelaide; D. Krumbiegel, PhD, WCHRI, Leukocyte Biology Laboratory, Discipline of Paediatrics, University of Adelaide, CRC for Biomarker Translation.

Dr. Boros and Dr. Krumbiegel contributed equally to the study.

Address correspondence to Dr. D. Krumbiegel, Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, SA 5006, Australia. E-mail: doreen.krumbiegel@adelaide.edu.au

Accepted for publication June 4, 2012.

in the joint and peripheral blood (PB) in patients with JIA. Previous studies have shown that IL-17-producing cells are enriched in the synovial fluid of children with extended oligoarthritis compared with patients with persistent oligoarthritis in a reciprocal relationship with regulatory T cells (Treg)[8]. An inverse relationship between the increased synovial expression of the Th17 transcription factor RORC2 and the Treg transcription factor FOXP3 has also been demonstrated in the joints of children with JIA[12].

As part of a search for surface surrogates of FOXP3 on human Treg, we have recently identified a novel surface molecule peptidase inhibitor 16 (PI16) as being overexpressed on expanded Treg compared with Th cells[13]. We found that PI16 is expressed by a T cell subset that expresses high levels of FOXP3. These cells have a memory phenotype (CD45RO+) and express high levels of the chemokines CCR4 and CCR6. *In vitro* assays show that PI16+ Treg cells are able to suppress Th cell function and also migrate to ligands CCL17 and CCL20[14].

A detailed understanding of the mechanism(s) of inflammation in JIA may be crucial in helping to predict which children will develop severe or persistent disease. We have characterized T cell subsets including Th17 and Treg cells in PB and SF from patients to further understand the mechanisms involved, ultimately providing a target for the therapeutic intervention of JIA.

MATERIALS AND METHODS

Patients. When possible, paired PB and SF samples were obtained from children with JIA. The study cohort of 50 patients is summarized in Table 1. Thirty-one females and nineteen males, aged 6 months to 18 years, with mean age of onset of 9.0 ± 5.0 years, and mean disease duration of 3.4 ± 3.4 years, were included in the study. All patients with JIA fulfilled the revised International League of Associations for Rheumatology classification criteria for JIA[1]. Disease activity at the time of joint injection was calculated using Juvenile Arthritis Disease Activity Scores 100[15]. Our study was approved by the Women's and Children's Hospital Research Ethics Committee (REC2101/9/11) and written informed consent was obtained for all participants. Paired PB and SF samples were obtained at the time of therapeutic steroid joint injection and processed immediately. PB samples from healthy children used in our study were obtained from a parallel study which was approved by the Women's and Children's Hospital Research Ethics Committee (REC2264/3/13) and written informed consent was obtained for all participants.

Table 1. Summary of juvenile idiopathic arthritis subtype, antinuclear antibody (ANA) status, and treatment.

Subtype	n = 50	ANA+	NSAID Alone	NSAID + MTX	Other + MTX	No Treatment
Oligoarticular	36	21	29	0	0	7
Polyarticular	8	4	1	6	0	1
Enthesitis-related arthritis	2	0	0	2	0	0
Systemic	2	0	0	0	2	0
Juvenile psoriatic arthritis	2	0	0	1	1	0

NSAID: nonsteroidal antiinflammatory drugs; MTX: methotrexate.

Cell isolation and culture. A small aliquot of centrifuged SF was stored at –80˚C for cytokine and chemokine analysis using the Cytometric Bead Array System (BD Biosciences, San Diego, CA, USA). PB mononuclear cells (PBMC) and SF mononuclear cells (SFMC) were isolated by standard density gradient centrifugation (Lymphoprep; Fresenius Kabi, Bad Homburg, Germany). Up to 5 × 10^6 PBMC and SFMC were stained with antibodies against surface molecules and analyzed by flow cytometry. For detection of intracellular cytokines, PBMC and SFMC samples were cultured in RPMI 1640 (HyClone Laboratories, South Logan, UT, USA) supplemented with 2 mM L-glutamine (SAFCBiosciences, Lenexa, KS, USA), penicillin/streptomycin (Sigma Aldrich, Steinheim, Germany), and 10% fetal calf serum (SAFCBiosciences). Cells were stimulated in 96 U-well culture plates (0.5-1 × 10^6/well) with *Staphylococcus enterotoxin* B (SEB; Sigma-Aldrich; 1 μg/ml) for 18 h and GolgiPlug (BD Biosciences, San Jose, CA, USA) was added after 2 h of stimulation. For detection of secreted cytokines, cells were stimulated as described but without the addition of GolgiPlug. Supernatants were collected and kept at –80˚C until use.

Flow cytometry. For cell surface immunostaining, stimulated and unstimulated PBMC and SFMC (0.5-1 × 10^6/50 μl) were stained with monoclonal antibodies against CD4, CD8, CD25, CD27, CD45RA, CD62L, CD127, CD161, CCR2, CCR4, CCR5, CCR6, CXCR3 (BD Biosciences), FOXP3 (eBioscience, San Diego, CA, USA), IL-23R and transforming growth factor-βR (R&D Systems, Minneapolis, MN, USA), and PI16 (monoclonal antibody CRCBT-02-001 kindly provided by the Co-operative Research Centre for Biomarker Translation, Australia). For intracellular staining, stimulated cells were first stained with monoclonal antibodies against surface antigens and then fixed and permeabilized using Fix/Perm solution (BD Biosciences) in Eppendorf tubes. Cells were then washed with Perm/Wash buffer (BD Biosciences) and stained with monoclonal antibodies against intracellular antigens: IL-17A (eBioscience), IL-10, interferon-γ (IFN-γ), FOXP3, IL-22, and IL-27 (R&D Systems). Cell data were acquired on a FACSAria II flow cytometer and analyzed using FACSDiva software v 6.1.3 (BD Biosciences). For data analysis, 100,000 lymphocytes were acquired for each sample.

The cytokines IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFN-γ, and IL-17A were measured in SF and supernatants from stimulated PBMC and SFMC using the Human Th1/Th2/Th17 Cytometric Bead Array (CBA) Kit (provided by Homero Sepulveda, BD Biosciences, San Diego, CA, USA) and CBA Flex Kits according to the manufacturer's instructions (BD Biosciences) and analyzed by flow cytometry. The chemokines CCL2, CCL3, CCL4, and CCL5 were measured in SF using the Human Chemokine Bead Array Kit according to the manufacturer's instructions (BD Biosciences).

PI16 ELISA. Soluble PI16 was measured in blood plasma and SF using a PI16 ELISA kit (USCN Life Sciences Inc., Wuhan, China) according to the manufacturer's instructions.

Chemotaxis assay. Recombinant human thymus and activation regulated chemokine (CCL17) and recombinant human macrophage inflammatory protein-3 alpha (CCL20) were purchased from Raybiotech (Norcross, GA, USA). Propidium iodide (Molecular Probes Inc.) was used to determine PBMC and SFMC cell viability. Chemotaxis assays were performed using Transwell plates with 5-μm pores (Corning, NY, USA). Bottom wells contained media alone, 100 ng/ml CCL17, 100 ng/ml CCL20, or 100ng/ml of both CCL17 and CCL20. PBMC and SFMC were isolated by standard-density gradient centrifugation. Upper wells were loaded with 1 × 10^6 cells per well and the cells were allowed to undergo chemotaxis for 2 h at 37°C. After chemotaxis, cells in the top (no migration) and bottom (migrated) wells were collected and stained with monoclonal antibodies against CD4, CD25, PI16, CCR4, and CCR6. The chemotactic index was calculated as previously shown by Nistala, *et al*[8]. The number of CD4+/CD25hi/PI16+ Treg cells that migrated in response to chemokine ligands was divided by the number of cells that migrated spontaneously to control medium.

Statistical analysis. The results are expressed as either mean ± SD or mean ± SEM. The data were analyzed using 2-tailed Student's t test for paired and unpaired samples. P values < 0.05 were considered significant. Pearson correlations were used to measure the statistical relationship between samples.

RESULTS

Enrichment of memory T cells in SF compared with matching blood samples. To evaluate whether cells in the peripheral blood are indicative of those within active joints of patients with JIA, we measured the distribution of different T cell subtypes in SF compared with the PB in the same patients using flow cytometry. Although we saw no significant difference in the percentage of total CD4+ Th cells, when the CD4+ Th cells were further divided into subtypes (Figure 1A), we detected a clear enrichment of CD45RA- memory Th cells within SF ($p < 0.0001$). This included both CD27+ (mixed memory; $p < 0.0001$) and CD27– (effector memory, $p < 0.0001$) T cells. Equally, we observed an increase of memory Th1-like cells (CD45RA-/CD62L-) within SF ($p < 0.0001$). In contrast, the proportion of CD27+/CD45RA+-naive Th cells was significantly increased in PB compared with SF ($p < 0.0001$; Figure 1A).

Increased expression of chemokine and cytokine receptors on SF cells. To understand the recruitment of Th cells into the joints, we analyzed Th cells within SF and PB of patients with JIA and revealed a significant increased expression of the chemokine receptors CCR4, CCR5, and CXCR3 and the cytokine receptor IL23R within the SF (Figure 1B). We found an increase in the percentage of CXCR3+/

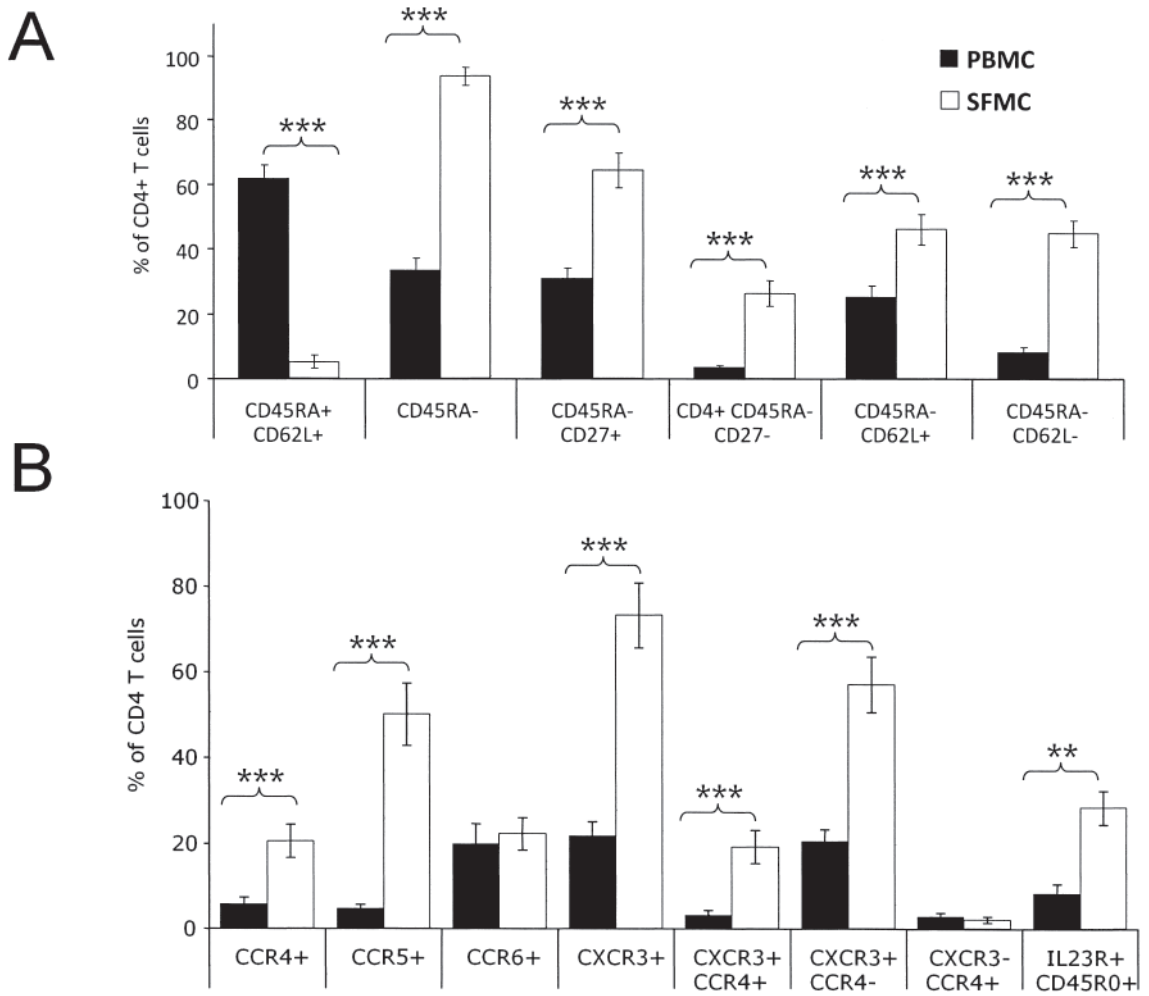

Figure 1. Distribution of Th subtypes, chemokine receptors, and cytokine receptor (IL-23R) within peripheral blood (PB) and synovial fluid (SF) of patients with juvenile idiopathic arthritis. The matched samples were analyzed *ex-vivo* by flow cytometry, gated on live lymphocytes and CD4+ cells. A. Percentages of CD45RA- (memory T cells), CD27+/CD45RA+ (naive Th cells), CD27-/CD45RA- (effector memory cells), and CD27+/CD45RA- (mixed memory cells). B. Percentage of chemokine receptors CCR4, CCR5, CCR6, CXCR3, CXCR3+/CCR4+, CXCR3+/CCR4-, CXCR3-/CCR4+, and IL-23R+/CD45RO+. The data are expressed as the mean ± SEM (n = 15-23). *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$. PBMC: peripheral blood mononuclear cells; SFMC: synovial fluid mononuclear cells.

CCR4- cells within the SF compared with PB (Figure 1B). Expression of CXCR3 on CCR4-negative cells indicated a Th1-like immune response. To analyze polarization of Th cells in SF compared with PB, we stimulated T cells overnight with SEB and analyzed intracellular cytokine production. We found a significant bias toward Th1 immune responses in SF, with Th cells producing more IFN-γ (19 ± 6.8%) and IL-10 (8.7 ± 6.2%) compared with PB (1.8 ± 1.8%, $p < 0.001$; and 0.4 ± 0.3%, $p < 0.05$, respectively). Further analysis of the supernatants of stimulated T cells revealed that PB T cells also secreted Th1 cytokines IL-2 [55.6 ng/ml (mean)] and IFN-γ (31 ng/ml) but in lower concentration than SF T cells (241.2 ng/ml and 90.3 ng/ml, respectively). IL-4 secretion was virtually undetected in the SF and PB. Proinflammatory cytokines IL-6 and TNF-α were secreted by both SF and PB Th cells.

Increased frequency of IL-17-producing cells in SF compared with matching blood samples. Analysis of stimulated CD4+ T cells from paired PB and SF samples revealed that SF contained a higher percentage of IL-17-producing cells [mean 3.4% (range 0.5-6.8)] than PB [mean 0.7% (range 0.2-1.1; $p < 0.001$)]. The concentration of IL-17 in the supernatant of stimulated SFMC was also higher compared with the supernatant of stimulated PBMC, and we found a strong correlation between the proportion of IL-17-producing T cells and concentration of IL-17 in the supernatants of stimulated cells ($R = 0.95$).

Phenotypic analysis of IL-17-producing cells in PB and SF. To test the hypothesis that IL-17-producing cells are responsible for tissue damage within active joints of patients with JIA, we characterized these cells in more detail (Figure 2). The analysis of IL-17-producing Th cells in SF confirmed that the majority of Th17 cells have a memory phenotype (CD45RA-/CD45RO+) and express CCR6 on their surface but do not express CD62L (Figure 2). Because published data describing the expression of CD161, CCR4, and CXCR3 on Th17 cells are inconsistent, we investigated the expression of these receptors and found that 69 ± 3.6%

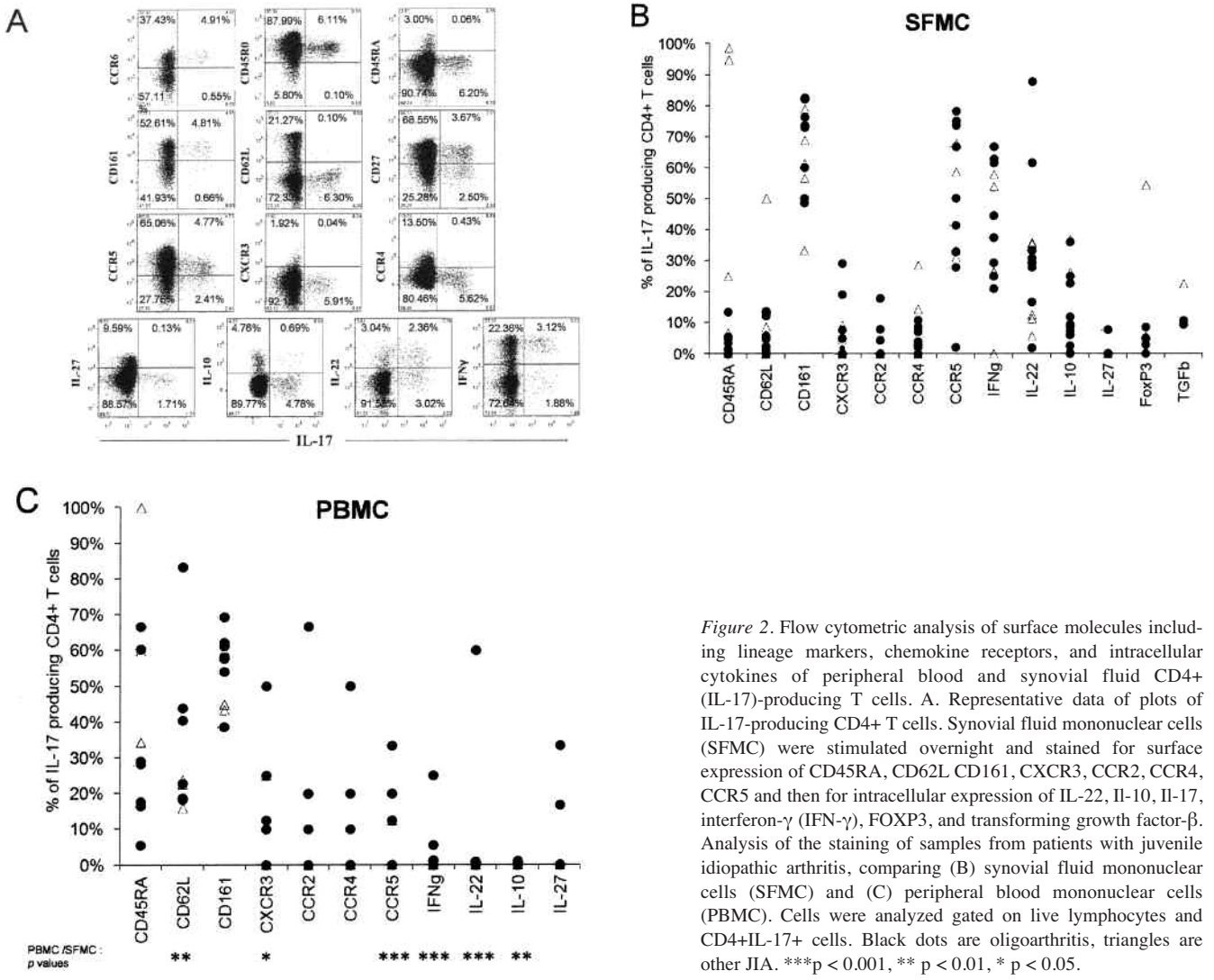

Figure 2. Flow cytometric analysis of surface molecules including lineage markers, chemokine receptors, and intracellular cytokines of peripheral blood and synovial fluid CD4+ (IL-17)-producing T cells. A. Representative data of plots of IL-17-producing CD4+ T cells. Synovial fluid mononuclear cells (SFMC) were stimulated overnight and stained for surface expression of CD45RA, CD62L CD161, CXCR3, CCR2, CCR4, CCR5 and then for intracellular expression of IL-22, Il-10, Il-17, interferon-γ (IFN-γ), FOXP3, and transforming growth factor-β. Analysis of the staining of samples from patients with juvenile idiopathic arthritis, comparing (B) synovial fluid mononuclear cells (SFMC) and (C) peripheral blood mononuclear cells (PBMC). Cells were analyzed gated on live lymphocytes and CD4+IL-17+ cells. Black dots are oligoarthritis, triangles are other JIA. ***$p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

(mean ± SEM) of IL-17-producing cells expressed CD161, whereas only a few expressed CCR4 (5.9 ± 1.9%) and CXCR3 (4.7 ± 1.8%) on their cell surface (Figure 2B). Analysis of intracellular cytokine expression confirmed recently reported data that almost half of IL-17-producing cells also secreted IFN-γ (42 ± 4.9%) and 31.9 ± 5.5% produced IL-22[16]. It has been suggested that Th17 cells are controlled by IL-27 and IL-10. To investigate autocrine feedback mechanisms of IL-17-producing cells in patients with JIA, we analyzed the co-production of IL-17 and IL-10 or IL-27, respectively. Fifteen percent of IL-17-producing cells also produced IL-10 (15 ± 3.0%), whereas only 2.2 ± 1.5% co-produced IL-27 (Figure 2B).

In contrast, IL-17-producing cells in the PB had increased expression of CD62L (32.2% ± 7.2%, $p < 0.01$) and CXCR3 (17.5% ± 6.7%, $p < 0.05$), but comparable levels of CD161 (54.9% ± 3.1%; Figure 2C). Circulating IL-17 cells had reduced expression of CCR5 (14.1% ± 4.5%, $p < 0.001$), IFN-γ (4.1% ± 3.1%, $p < 0.001$), IL-22 (7.7% ± 7.5%, $p < 0.001$) and IL-10 (0.3% ± 0.1%, $p < 0.01$), compared with the SF cells (Figure 2C).

Treg and PI16. Figure 3 shows an increase in the proportion of Th17+ and CD4+/CD25hi/CD127low/FOXP3+ Treg within the SF compared with the PB of patients with JIA (Figure 3A and B, respectively). By analyzing the ratio between Th17 and Treg in SF and PB we demonstrated a difference in the relationship between Th17 and Treg with a ratio of 1:8 in blood and a ratio of 1:4 in active joints (Figure 3C). Figure 3B illustrates the significant increase of CD4+/CD25hi/CD127low/FOXP3+ regulatory T cells in SF (10.6 ± 0.8%) of patients with JIA compared with matched samples of PB (2.7 ± 0.2%; $p < 0.0001$).

As shown in Figures 4A, 4B, and 4C, there is a high variation of PI16 expression on CD4+ T cells. Total PI16+ expression was reduced ($p = 0.003$) in patients with oligoarthritis (Figure 4B), but there was no correlation between concentration of PI16 in plasma and SF. We found a significant decrease ($p = 0.02$) in the percentage of CD4+/CD25hi/CD127low Treg expressing PI16 in SF (15.8 ± 1.8%) compared with matched JIA blood samples (24.7 ± 3.1%; Figure 4D). We have recently shown that PI16+ Treg express high levels of FOXP3, CD45RO, and Th17-like chemokine receptors CCR4 and CCR6[14]. PI16+ Treg were reduced in PB ($p = 0.02$) and SF ($p = 0.05$) of oligoarticular patients with JIA compared with the PB of age-matched healthy children (Figure 4F). In contrast, patients with juvenile psoriatic arthritis (JPsA), enthesitis-related arthritis (ERA), and systemic-onset JIA (SoJIA; other JIA) had variable levels of the PI16+ Treg cells within PB, but elevated levels within SF (Figure 4F). However, patient numbers in these cohorts are too low to draw any firm conclusions, and will require larger numbers to evaluate these findings fur-

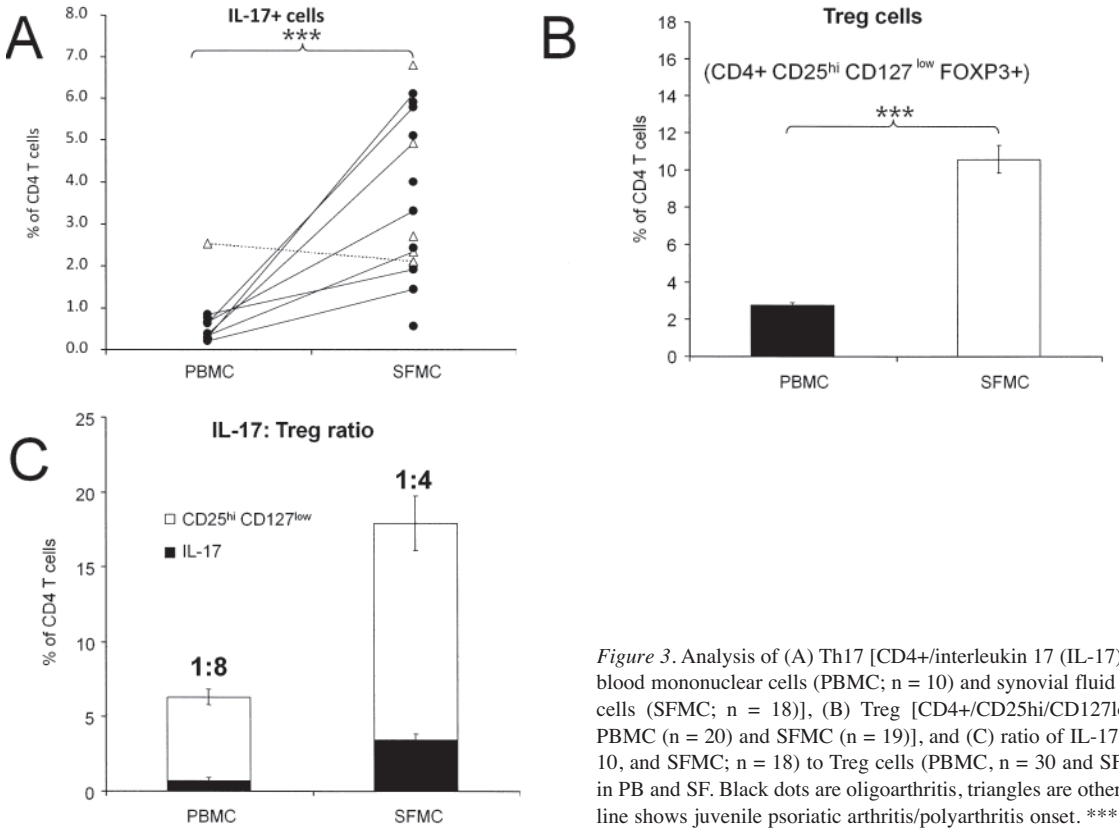

Figure 3. Analysis of (A) Th17 [CD4+/interleukin 17 (IL-17)+: peripheral blood mononuclear cells (PBMC; n = 10) and synovial fluid mononuclear cells (SFMC; n = 18)], (B) Treg [CD4+/CD25hi/CD127low/FOXP3+: PBMC (n = 20) and SFMC (n = 19)], and (C) ratio of IL-17 (PBMC; n = 10, and SFMC; n = 18) to Treg cells (PBMC, n = 30 and SFMC, n = 32) in PB and SF. Black dots are oligoarthritis, triangles are other JIA. Dashed line shows juvenile psoriatic arthritis/polyarthritis onset. *** $p < 0.001$.

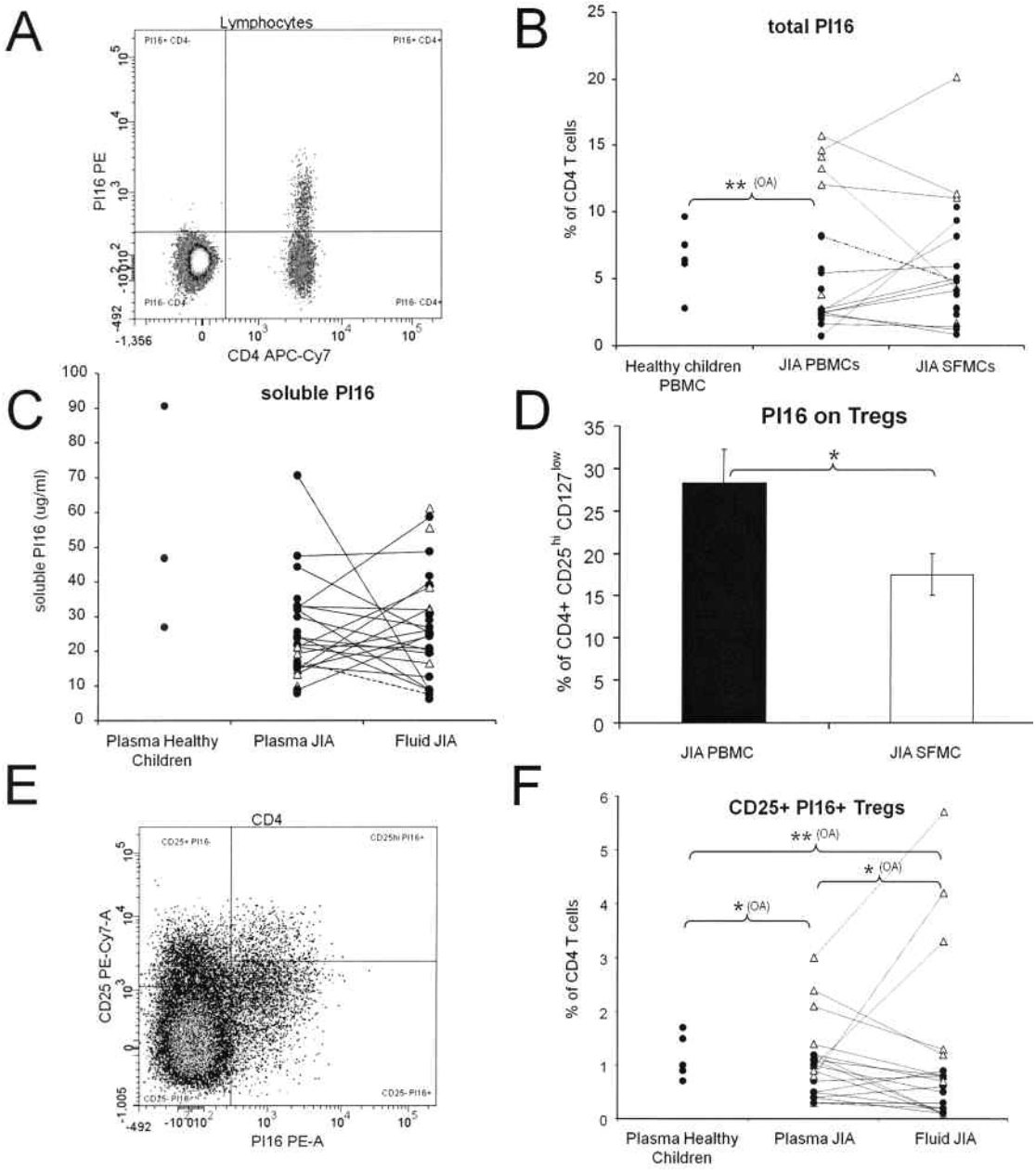

Figure 4. Analysis of peptidase inhibitor 16 (PI16) and Treg in patients with juvenile idiopathic arthritis (JIA). Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were stained with the Treg cocktail (CD4, CD25, CD127), FOXP3, and PI16. A. Representative dot plot of total CD4+ PI16+ cells, gated on total lymphocytes and the (B) comparison of total PI16+/CD4+ cells from healthy children, PBMC [oligoarthritis (OA); $p = 0.003$], and SFMC from patients with JIA. C. Soluble PI16 in plasma (n = 27) and SF (n = 27). D. Percentage of PI16+ cells in the Treg gate (CD4+/CD25hi/CD127low) for PBMC (n = 10) and SFMC (n = 10; $p = 0.02$). E. Representative plot gated on CD4+ T cells and showing CD25hi PI16+ Treg cell population. F. Comparison of CD25hi PI16+ Treg cells in healthy children compared with matched PBMC (OA; $p = 0.03$) and SFMC (OA; $p = 0.001$) from patients with JIA. Black dots are oligoarthritis, triangles are other JIA. ** $p < 0.01$, * $p < 0.05$.

ther. CD4+/CD25hi/PI16+ Treg represented 1.2 ± 0.2% (mean ± SEM) of total CD4+ cells for healthy children compared with 0.48 ± 0.05% and 0.5 ± 0.11% in PB and SF in oligoarthritis patients with JIA, respectively. We saw no correlation between the percentage of circulating or synovial CD4+/CD25hi/PI16+ Treg and disease duration, arthritis medications, or antinuclear antibodies (ANA) status. Our original hypothesis was that PI16 may be shed at the site of inflammation; however, we saw no difference in the amount of soluble PI16 in plasma compared with that in SF of patients with JIA (Figure 4C).

Studies have suggested that Th17 cells express

chemokine receptors CCR2, CCR4, and CCR6 but not CXCR3 and CCR5[17,18,19]. We have confirmed that PI16+ Treg also coexpress chemokine receptors CCR4 and CCR6. As shown in Figure 5, the coexpression of these homing chemokines on PI16+ Treg was significantly lower in PB and SF of patients with JIA compared with healthy children ($p = 0.02$). CCR4+/CCR6+ coexpression was present on 68 ± 7.0% (mean ± SEM) PI16+ Treg in healthy children compared with 41.1 ± 6.7% and 43.1 ± 9.7% in blood and SF of patients with JIA, respectively (Figure 5).

Migration. The ability of CD4+/CD25hi/PI16+ Treg cells from PB and SF to migrate in response to the CCR4 and CCR6 ligands CCL17 and CCL20 was tested using 5 μm-pore Transwell plates. There was no significant difference in cell viability before migration of SFMC (92%) compared with PBMC (range 83–97%) as determined by propidium iodide staining. CD4+/CD25hi/PI16+ Treg cells migrated toward CCL17, CCL20, and CCL17 + CCL20 ligands (Figure 6). After 2 h chemotaxis, the chemotactic index of PI16+ Treg cells from healthy children was 11.4 ± 2.0, 12.4 ± 3.5 and 22.1 ± 2.3, in response to ligands CCL17, CCL20, and CCL17 + CCL20, respectively (Figure 6). A similar trend was observed in JIA patients with oligoarthritis with the chemotactic index of 14.4 ± 5.4, 7.2 ± 2.0, and 20.4 ± 5.1 in response to the chemokine ligands. In contrast, these PI16+ Treg cells from the SF did not migrate in response to ligands CCL17, CCL20, and/or CCL17 + CCL20, having a chemotactic index of only 4.4 ± 1.5 ($p = 0.02$), 3.2 ± 2.0 ($p = 0.04$), and 7.2 ± 2.6 ($p = 0.002$), respectively (Figure 6).

DISCUSSION

Inflammation in JIA persists as a consequence of continuing leukocyte recruitment and retention within synovial tissue and fluid[20]. The key immunological mechanisms causing joint inflammation are still largely uncharacterized. The identification of factors involved in inducing and regulating tissue damage in JIA may provide a tool to individualize treatment in the future. To investigate whether blood or SF reflect those mechanisms, we analyzed T cells, mainly Th17 and novel PI16 Treg cells in PB and SF of patients with JIA.

Our data show a significant enrichment of memory T cells in the SF compared with PB samples. Because CD27 is considered a reliable marker of T cell effector status[21,22], costaining of CD27 and CD45RA was used to determine activation and memory status of CD4+ T cells[23]. Our results are consistent with previous studies in patients with autoimmune disease, which have shown that memory T cells are recruited to the site of inflammation, where they become reactivated[24,25]. The subsequent enrichment of effector

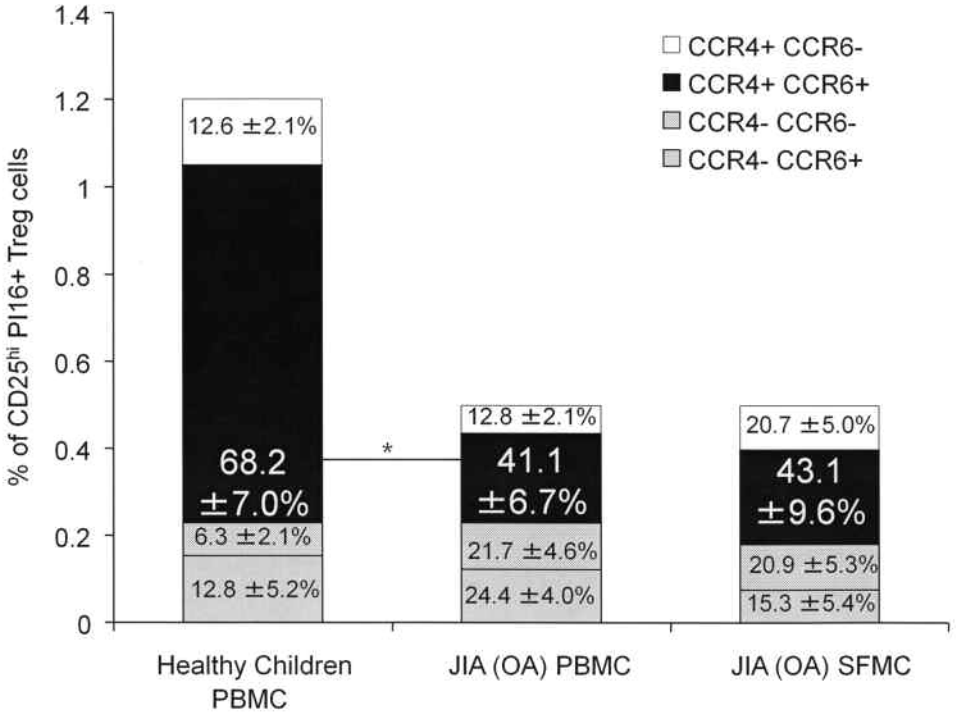

Figure 5. Analysis of peptidase inhibitor 16 (PI16)+ Treg and coexpression of CCR4 and CCR6 in healthy children and in patients with juvenile idiopathic arthritis (JIA) and oligoarthritis (OA). Peripheral blood mononuclear cells (PBMC) from healthy children and PBMC and synovial fluid mononuclear cells (SFMC) from JIA patients with oligoarthritis were stained with monoclonal antibodies against CD4, CD25, PI16, CCR4, and CCR6. Lymphocytes were gated on CD4+/CD25hi/PI16+ and the coexpression of CCR4± and CCR6± was analyzed. * $p < 0.05$.

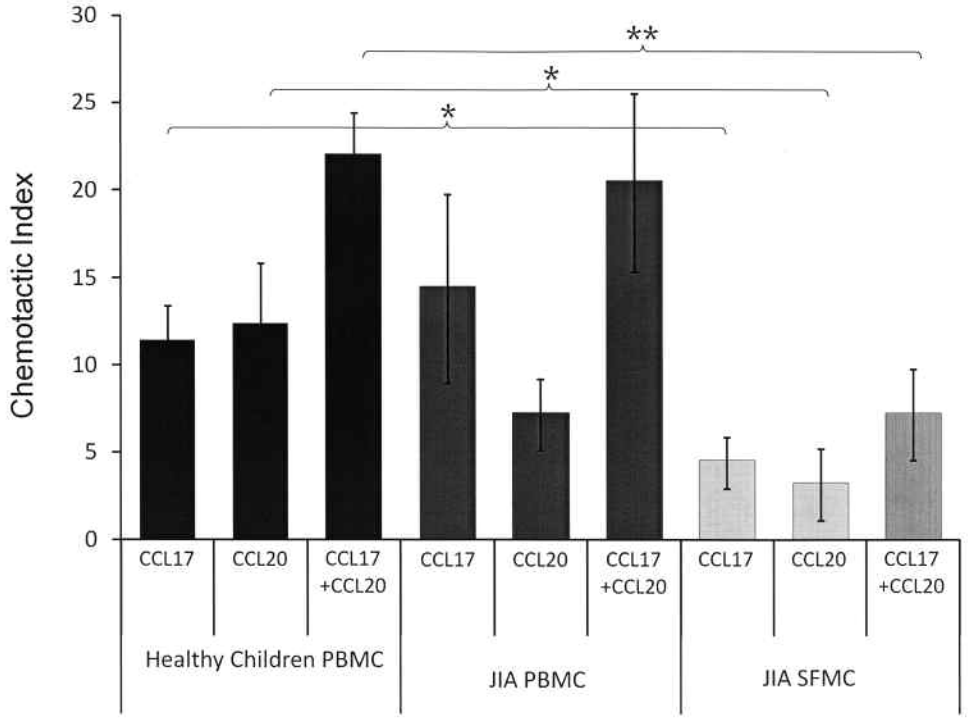

Figure 6. Migration of CD4+ CD25hi peptidase inhibitor 16 (PI16)+ Treg cells to CCR4 ligand CCL17, CCR6 ligand CCL20, and both CCL17 and CCL20 from peripheral blood mononuclear cells (PBMC) of healthy children (n = 5) compared with PBMC (n = 10) and synovial fluid mononuclear cells (SFMC; n = 6) of patients with juvenile idiopathic arthritis (JIA). Cells were analyzed by flow cytometry, gated on the CD4+ CD25hiCD127low PI16+ Treg cell population. Data are shown as the chemotactic index (ratio of the cells migrating to the chemokine ligand divided by the cells migrating to the control medium). The data are shown as mean ± SEM. ** $p < 0.01$, * $p < 0.05$.

memory T cells within JIA SF is reflected in the decrease of CD27 expression on these cells. We found a balance between memory and naive CD4+ T cells in the blood of patients with JIA, similar to recently published data on RA and healthy controls[25]. In contrast, SF contained more Th1-like memory T cells (CD62L–/CD45RA–)[26] than the matched blood samples, suggesting an enrichment of Th1 cells within SF. Although some studies have shown a Th1 bias in the SF of patients with RA and JIA[27,28,29], other studies have refuted these findings[30,31].

Th17 cells have been described as the master mediators of tissue damage in a variety of autoimmune diseases, with IFN-γ inhibiting Th17 cells and protecting tissues[31]. On the other hand, recent data demonstrate that Th1 and Th17 cells are independently capable of inducing disease in 2 established models of autoimmunity[30]. The enrichment of Th1 and Th17 cells within the inflamed joints of children with JIA as presented here indicates that both cell types may contribute to joint pathology.

Highly proinflammatory IL-17-secreting CD4+ T cells (Th17) have been shown to be the major mediators in the prolongation of inflammation and the induction and persistence of joint damage in adult patients with RA[4,5,6] and more recently in JIA[8,32,33]. However, there is evidence that RA and JIA are different diseases[34]. We examined 50 children with JIA and showed that IL-17-producing cells were highly enriched within SF compared with the matched PB samples. The IL-17-producing cells were uniformly contained within the T helper memory subset (CD4+/CD45RA-) and expressed the chemokine receptor CCR6, which enables Th17 cells to migrate toward sites of inflammation. Th17 cells are also enriched in the PB in RA[35]. We found that blood IL-17-positive cells showed a phenotype similar to IL-17-producing cells in JIA SF. However, we did not see an increased proportion of IL-17-producing cells in PB, supporting one of the few studies on Th17 cells in JIA that suggests a direct effect of IL-17-producing cells at sites of inflammation[8]. Because the CCR6 ligand CCL20 has been shown to be upregulated in human Th17 cells and in the SF of patients with RA or JIA, it remains to be determined whether differences in the autocrine production of CCL20 contribute to differences in Th17 recruitment among these patients.

Conflicting data have been reported regarding the phenotype of IL-17-producing cells and we therefore investigated the expression of surface molecules and intracellular cytokines in cells from the synovial fluid of patients with JIA. A majority of IL-17-producing CD4+ cells expressed

the C-type lectin-like receptor CD161, previously found on natural killer and CD8 T cells[36]. It has been shown that human IL-17-producing cells originate from a CD161+/CD4+ precursor cell[37]. Notably, CD161 is expressed on resting Th17 cells that can be activated by IL-23 and mediate destructive tissue inflammation[38]. Therefore, the increased expression of CD161 and IL-23R on IL-17-producing cells in the SF of patients with JIA may reflect the role of CD161 in supporting activation-induced T cell expansion and tissue destruction through additional costimulatory pathways.

In our study, the majority of IL-17-producing cells also expressed the chemokine receptor CCR5. It has been reported that CCR5 is expressed on activated T cells that show Th1 characteristics[39,40], but also on memory T cells and Th17+ cells in healthy adults[41]. Interestingly, we found low levels of macrophage inflammatory protein (CCL3) but high levels of RANTES (CCL5) in the SF of our patients with JIA. Both are ligands that bind CCR5. This result contrasts with data from patients with RA in which high levels of both CCL3 and RANTES have been reported[42,43,44,45], supporting the hypothesis that JIA and RA are clinically and mechanistically distinct arthritides.

Treg, like Th17 cells, are found in high numbers in the inflamed synovium of patients with RA[46] and JIA[47,48,49] compared with the circulating blood. Our data confirm this enrichment with increased CD4+/CD25hi/CD127low/FOXP3+ Treg cells within the SF of patients with JIA. PI16 has been described as a novel marker for regulatory T cells[13,14]. PI16-positive Treg cells have suppressing activity in suppressor assays and show enhanced migration toward the inflammatory chemokines CCL17 and CCL20 compared with PI16-negative Treg in healthy adults. The role of PI16 on Treg is yet to be demonstrated, but our data suggest that PI16 identifies a distinct subset of functional memory Treg with the highest expression of the Treg transcription factor FOXP3, which can migrate to sites of inflammation and regulate the proinflammatory response at those sites[14].

Although there is an enrichment of Treg in inflamed joints of patients with JIA, fewer of these Treg express PI16 compared with the Treg in the periphery, and the ratio between Treg and Th17 is reduced by half in the joint compared with the blood. That means that in contrast to blood, in the inflamed joint there are few Treg and even fewer PI16+ Treg to control the increased numbers of Th17 cells to keep the balance.

Oligoarticular JIA subjects had a reduced number of PI16+ Treg cells within the blood and SF when compared with healthy children and JIA patients with ERA, PsA, and SoJIA. However, the trend of an increase in the number of PI16+ Treg cells in patients with ERA, JPsA, and SoJIA has not been statistically analyzed because of the lack of sufficient patient numbers for each subtype. Further, we did not observe a correlation between the percentage of circulating or synovial PI16+ Treg cells and disease duration, medication, or ANA status in children with oligoarthritis.

Our original hypothesis was that PI16 might be shed within active joints or during the process of migrating toward joints. This hypothesis is not supported by the data, with similar levels of soluble PI16 observed within the plasma of healthy children and that of patients with JIA, as well as in the SF of patients with JIA. However, the degradation or loss of soluble PI16 from inflamed tissue has yet to be investigated.

We have shown that PI16+ Treg cells express the same chemokine receptors as Th17 cells (CCR4/CCR6), indicating similar homing characteristics. PB Treg from patients with JIA migrated toward inflammatory ligands CCL17 and CCL20, unlike those from SF of the same patients. We have shown that the failure of SFMC migration toward CCL17 and CCL20 is not due to poor viability but may be part of a generalized chemotactic defect. Nonetheless, we hypothesize that circulating PI16+ Treg and Th17 cells are capable of migrating to the same sites of inflammation, with the Treg controlling the Th17 immune response. A defect or reduced number of PI16+ Treg, on the other hand, might contribute to autoimmunity and consequently to tissue damage.

Our data show that IL-17-producing cells are enriched in the SF of patients with JIA compared with PB, supporting the hypothesis that IL-17-producing cells contribute to the tissue damage within active joints. In contrast to adult RA, our results show that PB does not reflect processes in JIA SF and therefore is less likely to be useful for disease diagnosis or prognosis. We hypothesize that in individuals who are able to bring inflammation under control, there is a balance between PI16-positive Treg and Th17 cells homing to the same sites of inflammation, with the Treg controlling the Th17 immune response. A reduced number of PI16-positive Treg within active joints of patients with JIA may contribute to their inability to regulate disease activity and consequently result in tissue damage. Therapeutic interventions to downregulate Th17 cells in the joint, or to increase the recruitment of functional (PI16+) Treg to the joint may provide a significant therapeutic benefit for patients with JIA.

REFERENCES

1. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol 2004;31:390-2.
2. Pain CE, LJ McCann. Challenges in the management of juvenile idiopathic arthritis with etanercept. Biologics 2009;3:127-39.
3. Foster HE, Marshall N, Myers A, Dunkley P, Griffiths ID. Outcome in adults with juvenile idiopathic arthritis: a quality of life study. Arthritis Rheum 2003;48:767-75.
4. Pene J, Chevalier S, Preisser L, Venereau E, Guilleux MH, Ghannam S, et al. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. J Immunol 2008;180:7423-30.
5. Singh R, Aggarwal A, Misra R. Th1/Th17 cytokine profiles in patients with reactive arthritis/undifferentiated spondyloarthropathy.

J Rheumatol 2007;34:2285-90.
6. Shahrara S, Huang Q, Mandelin AM 2nd, Pope RM. TH-17 cells in rheumatoid arthritis. Arthritis Res Ther 2008;10:R93.
7. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 2008;180:214-21.
8. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. Arthritis Rheum 2008;58:875-87.
9. Shahrara S, Pickens SR, Dorfleutner A, Pope RM. IL-17 induces monocyte migration in rheumatoid arthritis. J Immunol 2009;182:3884-91.
10. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 1999;103:1345-52.
11. Van Bezooijen RL, Van Der Wee-Pals L, Papapoulos SE, Lowik CW. Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction in vitro. Ann Rheum Dis 2002; 61:870-6.
12. Olivito B, Simonini G, Ciullini S, Moriondo M, Betti L, Gambineri E, et al. Th17 transcription factor RORC2 is inversely correlated with FOXP3 expression in the joints of children with juvenile idiopathic arthritis. J Rheumatol 2009;36:2017-24.
13. Sadlon TJ, Wilkinson BG, Pederson S, Brown CY, Bresatz S, Gargett T, et al. Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. J Immunol 2010;185:1071-81.
14. Nicholson IC, Mavrangelos C, Bird DR, Bresatz-Atkins S, Eastaff-Leung NG, Grose RH, et al. PI16 is expressed by a subset of human memory Treg with enhanced migration to CCL17 and CCL20. Cell Immunol 2012;275:12-8.
15. Consolaro A, Ruperto N, Bazso A, Pistorio A, Magni-Manzoni S, Filocamo G, et al. Development and validation of a composite disease activity score for juvenile idiopathic arthritis. Arthritis Rheum 2009;61:658-66.
16. Eid RE, Rao DA, Zhou J, Lo SF, Ranjbaran H, Gallo A, et al. Interleukin-17 and interferon-gamma are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. Circulation 2009;119:1424-32.
17. Sato W, Aranami T, Yamamura T. Cutting edge: Human Th17 cells are identified as bearing CCR2+CCR5- phenotype. J Immunol 2007;178:7525-9.
18. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 2007;8:639-46.
19. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204:1849-61.
20. Ravelli A, Martini A. Juvenile idiopathic arthritis. Lancet 2007;369:767-78.
21. Tortorella C, Schulze-Koops H, Thomas R, Splawski JB, Davis LS, Picker LJ, et al. Expression of CD45RB and CD27 identifies subsets of CD4+ memory T cells with different capacities to induce B cell differentiation. J Immunol 1995;155:149-62.
22. Lens SM, Tesselaar K, van Oers MH, van Lier RA. Control of lymphocyte function through CD27-CD70 interactions. Semin Immunol 1998;10:491-9.
23. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997;186:1407-18.
24. Kohem CL, Brezinschek RI, Wisbey H, Tortorella C, Lipsky PE, Oppenheimer-Marks N. Enrichment of differentiated CD45RBdim,CD27- memory T cells in the peripheral blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 1996;39:844-54.
25. Dejaco C, Duftner C, Klauser A, Schirmer M. Altered T-cell subtypes in spondyloarthritis, rheumatoid arthritis and polymyalgia rheumatica. Rheumatol Int 2010;30:297-303.
26. Kanegane H, Kasahara Y, Niida Y, Yachie A, Sughii S, Takatsu K, et al. Expression of L-selectin (CD62L) discriminates Th1- and Th2-like cytokine-producing memory CD4+ T cells. Immunology 1996;87:186-90.
27. Canete JD, Martinez SE, Farres J, Sanmarti R, Blay M, Gomez A, et al. Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies. Ann Rheum Dis 2000;59:263-8.
28. Scola MP, Thompson SD, Brunner HI, Tsoras MK, Witte D, Van Dijk MA, et al. Interferon-gamma:interleukin 4 ratios and associated type 1 cytokine expression in juvenile rheumatoid arthritis synovial tissue. J Rheumatol 2002;29:369-78.
29. Wedderburn LR, Robinson N, Patel A, Varsani H, Woo P. Selective recruitment of polarized T cells expressing CCR5 and CXCR3 to the inflamed joints of children with juvenile idiopathic arthritis. Arthritis Rheum 2000;43:765-74.
30. Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, et al. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. J Exp Med 2008;205:799-810.
31. Amadi-Obi A, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, et al. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat Med 2007;13:711-8.
32. Cosmi L, Cimaz R, Maggi L, Santarlasci V, Capone M, Borriello F, et al. Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. Arthritis Rheum 2011;63:2504-15.
33. Agarwal S, Misra R, Aggarwal A. Interleukin 17 levels are increased in juvenile idiopathic arthritis synovial fluid and induce synovial fibroblasts to produce proinflammatory cytokines and matrix metalloproteinases. J Rheumatol 2008;35:515-9.
34. De Benedetti F, Pignatti P, Gerloni V, Massa M, Sartirana P, Caporali R, et al. Differences in synovial fluid cytokine levels between juvenile and adult rheumatoid arthritis. J Rheumatol 1997;24:1403-9.
35. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum 2009;60:1647-56.
36. Lanier LL, Chang C, Phillips JH. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. J Immunol 1994;153:2417-28.
37. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, et al. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. J Exp Med 2008;205:1903-16.
38. Kleinschek MA, Boniface K, Sadekova S, Grein J, Murphy EE, Turner SP, et al. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. J Exp Med 2009;206:525-34.
39. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A 1997;94:1925-30.
40. Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, et al. CCR5 is characteristic of Th1 lymphocytes. Nature 1998;391:344-5.

41. Lim HW, Lee J, Hillsamer P, Kim CH. Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. J Immunol 2008;180:122-9.
42. Suzuki N, Nakajima A, Yoshino S, Matsushima K, Yagita H, Okumura K. Selective accumulation of CCR5+ T lymphocytes into inflamed joints of rheumatoid arthritis. Int Immunol 1999;11:553-9.
43. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis 2007;66:589-98.
44. Mahendra A, Misra R, Aggarwal A. Th1 and Th17 predominance in the enthesitis-related arthritis form of juvenile idiopathic arthritis. J Rheumatol 2009;36:1730-6.
45. Tanida S, Yoshitomi H, Nishitani K, Ishikawa M, Kitaori T, Ito H, et al. CCL20 produced in the cytokine network of rheumatoid arthritis recruits CCR6+ mononuclear cells and enhances the production of IL-6. Cytokine 2009;47:112-8.
46. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS. CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum 2004;50:2775-85.
47. Jiao Z, Wang W, Jia R, Li J, You H, Chen L, et al. Accumulation of FoxP3-expressing CD4+CD25+ T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis. Scand J Rheumatol 2007;36:428-33.
48. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol 2004;172:6435-43.
49. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. Blood 2011;118:3538-48.