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Comparison of blood and synovial fluid Th17 and novel PI16 Treg cell subsets in juvenile idiopathic arthritis

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Abstract

Objective: Early recognition and treatment of JIA can prevent joint damage and minimise side effects of medication. The balance between pro- and anti-inflammatory mechanisms is known to be important in JIA, and we therefore investigated T cell subsets including T helper cells (Th), auto-aggressive 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 this study. Frequency, phenotype and function of T lymphocytes in PB and SF were characterised 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, IL23R 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 PI16+ Treg in active joints compared with peripheral blood. Coexpression of CCR4 and CCR6 was reduced on PI16+Treg in PB and SF of JIA subjects compared with healthy children, however the ability of these cells to migrate towards their ligands was unaffected.

Conclusion: This is the first comprehensive characterisation of novel PI16+ Treg and Th17 cells in matched blood and synovial fluid samples of JIA patients. 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.

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List of abbreviations

ANA= anti-nuclear antibodies; CBA = cytometric bead array; ERA = enthesitis-related arthritis; FCS = foetal calf serum; FLSs = fibroblast-like synoviocytes; IFN = interferon; IL = interleukin; JIA = juvenile idiopathic arthritis; JPsA= juvenile psoriatic arthritis; MTX= methotrexate; NSAID= non steroid anti-inflammatory drugs; NK = natural killer; OA = oligoarthritis; PA = polyarthritis; PB = peripheral blood; PBMC = peripheral blood mononuclear cells; RA = rheumatoid arthritis; SF = synovial fluid; SFMC = synovial fluid mononuclear cells; SoJIA=systemic onset JIA; TGF = transforming growth factor; Th = T helper cells; TNF = tumour necrosis factor; Treg = regulatory T cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DK and CB were responsible for the design of the study, acquisition of data, analysis and interpretation of the data, and manuscript preparation. RHG and DM were responsible for acquisition of data and manuscript preparation. ICN and HZ were responsible for the design of the study, interpretation of the data, and manuscript preparation. SB provided critical review of the manuscript.

Running Title: Th17 & PI16+Treg in JIA

Introduction

Juvenile idiopathic arthritis (JIA) is the most common rheumatologic disease of childhood, occurring in up to 1:500 children [1]. The impact upon 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 has been demonstrated previously, it is important to recognise 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, interleukin-17-producing cells (Th17) cells are believed to be the major inducers of severe autoimmune tissue inflammation and destruction [4-6]. 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 which attract neutrophils and other immune cells [9]. In the synovial fluid 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]. Interleukin-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 within the joint and peripheral blood in JIA patients. 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 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 T helper cells [13]. We found that PI16 is expressed by a T cell subset that expresses high levels of FOXP3. These cells have a memory (CD45RO+) phenotype and express high levels of the chemokines CCR4 and CCR6. *In vitro* assays show that PI16+ Treg cells are able to suppress helper T cell function and also migrate to ligands CCL17 and CCL20 [50].

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

Materials and methods

Patients

When possible, paired peripheral blood (PB) and synovial fluid (SF) samples were obtained from children with JIA. The study cohort of 50 patients is summarised 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 JIA patients 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 JADAS 100 [14]. The 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. Peripheral blood samples from healthy children used in this 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.

Cell isolation and culture

A small aliquot of centrifuged synovial fluid was stored at -80 C for cytokine and chemokine analysis using the Cytometric Bead Array System (BD Biosciences, San Diego, CA). Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated by standard density gradient centrifugation (Lymphoprep; Fresenius Kabi, Bad Homburg, Germany). Up to $5x10^6$ PBMC and SFMC were stained with antibodies against surface molecules and analysed by flow cytometry. For detection of intracellular cytokines, PBMC and SFMC samples were cultured in RPMI 1640 (HyClone Laboratories, South Logan, Utah) supplemented with 2mM L-glutamine (SAFCBiosciences, Lenexa, Kansas), penicillin/streptomycin (Sigma Aldrich, Steinheim, Germany), and 10% FCS (SAFCBiosciences). Cells were stimulated in 96 U-well culture plates ($0.5-1x10^6$/well) with *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich; 1µg/ml) for 18h and GolgiPlug (BD Biosciences, San Jose, CA) was added after 2h of stimulation. For detection of secreted cytokines, cells were stimulated as described above but without the addition of GolgiPlug. Supernatants were collected and kept at –80C until use.

Flow cytometry

For cell surface immunostaining, stimulated and unstimulated PBMC and SFMC ($0.5-1x10^6$/50µl) were stained with monoclonal antibodies against CD4, CD8, CD25, CD27, CD45RA, CD62L, CD127, CD161, CCR2, CCR4, CCR5, CCR6, CXCR3 (BD Biosciences, San Jose, CA), FOXP3 (eBioscience,San Diego, CA, USA), IL23R and TGFβR (R&D Systems, Minneapolis, MN) and PI16 (monoclonal antibody CRCBT-02-001 kindly provided by the CRC for Biomarker Translation, Australia). For intracellular staining, stimulated cells were first stained with monoclonal antibodies against surface antigens and then fixed and

permeabilised using Fix/Perm solution (BD Biosciences, San Jose, CA) in Eppendorf tubes. Cells were then washed with Perm/Wash buffer (BD Biosciences) and stained with monoclonal antibodies against intracellular antigens: IL-17A (eBioscience, San Diego, CA), IL-10,IFNγ, FoxP3 (BD Biosciences), IL-22 and IL-27 (R&D Systems). Cell data were acquired on a FACSAria II flow cytometer and analysed 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, TNFα, IFNγ and IL-17A were measured in synovial fluid and supernatants from stimulated PBMC and SFMC using the Human Th1/Th2/Th17 Cytometric Bead Array (CBA) Kit and CBA Flex Kits according to the manufacturer's instructions (BD Biosciences) and analysed by flow cytometry. The chemokines CCL2, CCL3, CCL4 and CCL5 were measured in synovial fluid 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 synovial fluid 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, 100ng/ml CCL17, 100ng/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 $1x10^6$ cells per well and the cells were allowed to undergo chemotaxis for 2h at 37C. 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* (2008). The number of CD4+/CD25^{hi}/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 analysed using two-tailed Student's *t* test for paired and unpaired samples. P values less than 0.05 were considered significant. Pearson correlations were used to measure the statistical relationship between samples.

Results

Enrichment of memory T cells in synovial fluid compared with matching blood samples

To evaluate whether cells in the peripheral blood are indicative of those within active joints of JIA patients, we measured the distribution of different T cell subtypes in synovial fluid (SF) compared with the peripheral blood (PB) in the same patients using flow cytometry. Although we saw no significant difference in the percentage of total CD4+ T helper (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 Th-1 like cells (CD45RA-/CD62L-) within SF (*p<0.0001*). In contrast, the proportion of CD27+/CD45RA+ naïve Th cells was significantly increased in PB compared with SF (*p<0.0001*) (Figure 1A).

Increased expression of chemokine and cytokine receptors on synovial fluid cells

To understand the recruitment of Th cells into the joints we analysed Th cells within SF and PB of JIA patients 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+/CCR4- cells within the SF compared with PB (Figure 1B). Expression of CXCR3 on CCR4-negative cells indicated a Th1-like immune response. To analyse polarisation of T helper cells in SF compared with PB, we stimulated T cells overnight with SEB and analysed intracellular cytokine production. We found a significant bias towards Th1 immune responses in SF, with T helper cells producing more IFNγ (19±6.8% (mean±SD)) 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 (55.6ng/ml (mean)) and IFNγ (31ng/ml) but in lower concentration than SF T cells (241.2ng/ml and 90.3ng/ml respectively). IL-4 secretion was virtually undetected in the SF and PB. Proinflammatory cytokines IL-6 and TNFα were secreted by both SF and PB Th cells.

Increased frequency of IL-17-producing cells in synovial fluid compared with matching blood samples

Analysis of stimulated CD4+ T cells from paired peripheral blood and synovial fluid 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 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 peripheral blood and synovial fluid

In light of the hypothesis that IL-17-producing cells are responsible for tissue damage within active joints of JIA patients, we characterised these cells in more detail (Figure 2). The analysis of IL-17 producing T helper 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 % (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 IFNγ (42 ±4.9%) and 31.9±5.5% produced IL-22 [15]. 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 JIA patients, we analysed 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 within the peripheral had increased expression of CD62L (32.2±7.2%) and CXCR3 (17.5±6.7%), but comparable levels of CD161 (54.9±3.1%) (Figure2C). Circulating IL-17 cells had reduced expression of CCR5 (14.1±4.5%), IFNγ (4.1±3.1%), IL-22 (7.7±7.5%) and IL-10 (0.3±0.1%), compared to the synovial fluid cells (Figure 2C).

Treg and PI16

Figure 3 shows an increase in the proportion of Th17+ and CD4+/CD25^{hi}/CD127^{low}/FOXP3+ Treg within the SF compared with the PB of JIA patients (Figure 3A and B, respectively). By analysing the ratio between Th17 and Treg in SF and PB we showed 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+/CD25^{hi}/CD127^{low}/FOXP3+ regulatory T cells in SF (10.6±0.8%) of JIA patients compared with matching PB (2.7±0.2%) ($p<0.0001$).

As shown in Figures 4A, B and C, there is a high variation of PI16 expression on CD4+ T cells. Total PI16+ expression was reduced ($p=0.003$) in Oligoarthritis (OA) patients (Figure 4B), but there was no correlation between concentration of PI16 in plasma and synovial fluid. We found a significant decrease ($p=0.02$) in the percentage of CD4+/CD25^{hi}/CD127^{low}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 [50]. PI16+ Treg were reduced in PB ($p=0.02$) and SF ($p=0.05$) of oligoarticular JIA patients compared with the PB of aged matched healthy children (Figure 4F). In contrast, patients with JPsA, ERA and SoJIA had variable levels of the PI16+ Treg cells within PB, but elevated levels within SF (Figure 4F). However, these patient numbers are too low to draw any conclusion, and will require a larger cohort to evaluate. CD4+/CD25^{hi}/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 oligoarticular onset JIA patients, respectively. We saw no correlation between the percentage of circulating or synovial CD4+/CD25^{hi}/PI16+ Treg and disease duration, therapy or 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 synovial fluid of JIA patients (Figure 4C).

Recent studies [16-18] have suggested that Th17 cells express chemokine receptors CCR2, CCR4, CCR6 but not CXCR3 and CCR5. We have confirmed that PI16+ Treg also co-express chemokine receptors CCR4 and CCR6. As shown in Figure 5, the co-expression of

these homing chemokines on PI16+ Treg was significantly lower in PB and SF of JIA patients when compared with healthy children (*p=0.02*). CCR4+/CCR6+ co-expression 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 synovial fluid of JIA patients respectively (Figure 5).

Migration

The ability of CD4+/CD25^{hi}/PI16+ Treg cells from PB and SF to migrate in response to the CCR4 and CCR6 ligands CCL17 and CCL20, was tested using 5μm pore Transwells™. There was no significant difference in cell viability before migration of SFMC (8.3%) when compared to PBMC (range 3.0-16.8%) as determined by propidium iodide staining. CD4+/CD25^{hi}/PI16+ Treg cells migrated towards CCL17, CCL20 and both CCL17 + CCL20 ligands (Figure 6). After 2h chemotaxis, the chemotactic index of PI16+ Treg cells from healthy children was11.4±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 oligoarthritis JIA patients 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 ongoing leukocyte recruitment and retention within synovial tissue and fluid [19]. The key immunological mechanisms causing joint inflammation are still largely uncharacterised. The identification of factors involved in inducing and regulating tissue damage in JIA may provide a tool to individualise treatment in the future. To investigate whether blood or synovial fluid reflect those mechanisms we analysed T cells, mainly Th17 and novel PI16 Treg cells in peripheral blood and synovial fluid of patients with JIA.

Our data show a significant enrichment of memory T cells in the synovial fluid compared with peripheral blood samples. Since CD27 is considered a reliable marker of T cell effector status [20, 21], co-staining of CD27 and CD45RA was used to determine activation and memory status of CD4+ T cells [22]. Our results are consistent with previous studies in patients with autoimmune disease [23, 24] which have shown that memory T cells are recruited to the site of inflammation where they become re-activated. The subsequent enrichment of effector memory T cells within JIA SF is reflected in the decrease of CD27 expression on these cells. We found a balance between memory and naïve CD4+ T cells in the blood of JIA patients, similar to recently published data on RA and healthy controls [24]. In contrast, synovial fluid contained more 'Th1-like' memory T cells (CD62L-/CD45RA-) [25] than the matched blood samples, suggesting an enrichment of Th1 cells within SF. Although some published studies have shown a Th1 bias in the synovial fluid of patients with RA and JIA [26-28], other studies have refuted these findings [29, 30].

Th17 cells have been described as the master mediators of tissue damage in a variety of autoimmune diseases with IFNγ inhibiting Th17 cells and protecting tissues [26]. On the other hand, recent data demonstrates that Th1 and Th17 cells are independently capable of inducing disease in two established models of autoimmunity [29]. The enrichment of Th1 and

Th17 cells within the inflamed joints of children with JIA as presented in this manuscript indicate that both cell types may contribute to joint pathology.

Highly proinflammatory IL-17-secreting CD4+ T cells (Th17) cells have been shown to be the major mediators in the prolongation of inflammation and the induction and persistence of joint damage in adult rheumatoid arthritis (RA) patients [4-6] and more recently in JIA [8, 31, 32]. However, there is evidence that RA and JIA are different diseases [33]. In the present study 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 towards sites of inflammation. Th17 cells are also enriched in the peripheral blood in RA [34]. We found that blood IL-17 positive cells showed a similar phenotype to IL-17 producing cells in JIA synovial fluid. However, we did not see an increased proportion of IL-17 producing cells in peripheral blood, supporting one of the few studies on Th17 cells in JIA that suggest a direct impact of IL-17 producing cells at sites of inflammation [8]. Since the CCR6 ligand CCL20 has been shown to be upregulated in human Th17 cells and in the SF of RA and JIA patients it remains to be determined if differences in the autocrine production of CCL20 contribute to differences in Th17 recruitment between RA and JIA 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 JIA patients. A majority of IL-17 producing CD4+ cells expressed the C-type lectin-like receptor CD161, previously found on NK and CD8 T cells [35]. It has been shown that human IL-17 producing cells originate from a CD161+/CD4+ precursor cell [36]. Notably, CD161 is expressed on resting Th17 cells that can be activated by IL-23 and mediate destructive tissue inflammation [37]. Therefore, the increased expression of CD161

and IL23R on IL-17 producing cells in JIA SF might reflect the role of CD161 in supporting activation-induced T cell expansion and tissue destruction through additional co-stimulatory pathways.

In the present 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 [38, 39], but also on memory T cells and Th17+ cells in healthy adults [40]. Interestingly, we found low levels of MIPα (CCL3) but high levels of RANTES (CCL5) in the synovial fluid of our JIA patients, both ligands that bind CCR5. This result contrasts with data from RA patients in which high levels of both MIPα and RANTES have been reported [41-44] supporting the hypothesis that JIA and RA are clinically and mechanistically distinct arthritides.

Treg, like Th17 cells, are found in high numbers with in the inflamed synovium of RA [45] and JIA [46-48] patients compared with the circulating blood. Our data confirm this enrichment with increased CD4+/CD25^{hi}/CD127^{low}/FoxP3+Treg cells within the synovial fluid of JIA patients. Peptidase inhibitor 16 (PI16) has recently been described as a novel marker for memory regulatory T cells [13, 50]. PI16-positive Treg have suppressing activity in suppressor assays and show enhanced migration towards 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 novel distinct subset of functional memory Treg with the highest expression of the Treg transcription factor FoxP3, and which can migrate to sites of inflammation and regulate the pro-inflammatory response at those sites.

Although there is an enrichment of Treg in inflamed joints of JIA patients, 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 in order to keep the balance.

Oligoarticular JIA subjects had a reduced number of PI16+ Treg cells within the blood and synovial fluid when compared with healthy children and JIA patients with ERA, PA and SoJIA. However, the trend of an increase in the number of PI16+ Treg cells in ERA, JPsA and SoJIA subjects has not been statistically analysed due to the lack of sufficient patient numbers for each subtype. Furthermore, 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 towards joints. This hypothesis is not supported by the data, with similar levels of soluble PI16 observed within the plasma of healthy children, JIA patients and also the SF of JIA patients. 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. Peripheral blood Treg from JIA patients migrated towards inflammatory ligands CCL17 and CCL20, unlike those from synovial fluid of the same patients. We have shown that the failure of SFMC migration towards CCL17 and CCL20 is not due to poor viability but may be part of a generalised chemotactic defect. Nonetheless, we hypothesise 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.

In conclusion, our data show that IL-17 producing cells are enriched in the synovial fluid of JIA patients compared with peripheral blood, 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 peripheral blood does not reflect processes in JIA SF and therefore, is less likely to be useful for disease diagnosis or prognosis. We hypothesise that in individuals who are able to bring inflammation under control there is a balance between PI16-positive Treg and Th17 cells homing to same sites of inflammation, with the Treg controlling the Th17 immune response. A reduced number of PI16-positive Treg within active joints of JIA patients may contribute to their inability to regulate disease activity and consequently, result in tissue damage. Therapeutic intervention 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 JIA patients.

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Table

Table 1

Subtype	n 50	ANA +ve	NSAID alone	NSAID +MTX	Other +MTX	No treatment
Oligoarticular	36	21	29	0	0	7
Polyarticular	8	4	1	6	0	1
Enteitis-related arthritis	2	0	0	2	0	0
Systemic	2	0	0	0	2	0
Juvenile psoriatic arthritis	2	0	0	1	1	0

Figures

Figure 1

Figure 2

Figure 3

A

IL-17+ cells
***

% of CD4 T cells

PBMC   SFMC

B

Treg cells

(CD4+ CD25^{hi} CD127^{low} FOXP3+)

***

% CD4 T cells

PBMC   SFMC)

C

IL-17: Treg ratio

□ CD25^{hi} CD127^{low}
■ IL-17

1:4

1:8

% of CD4 T cells

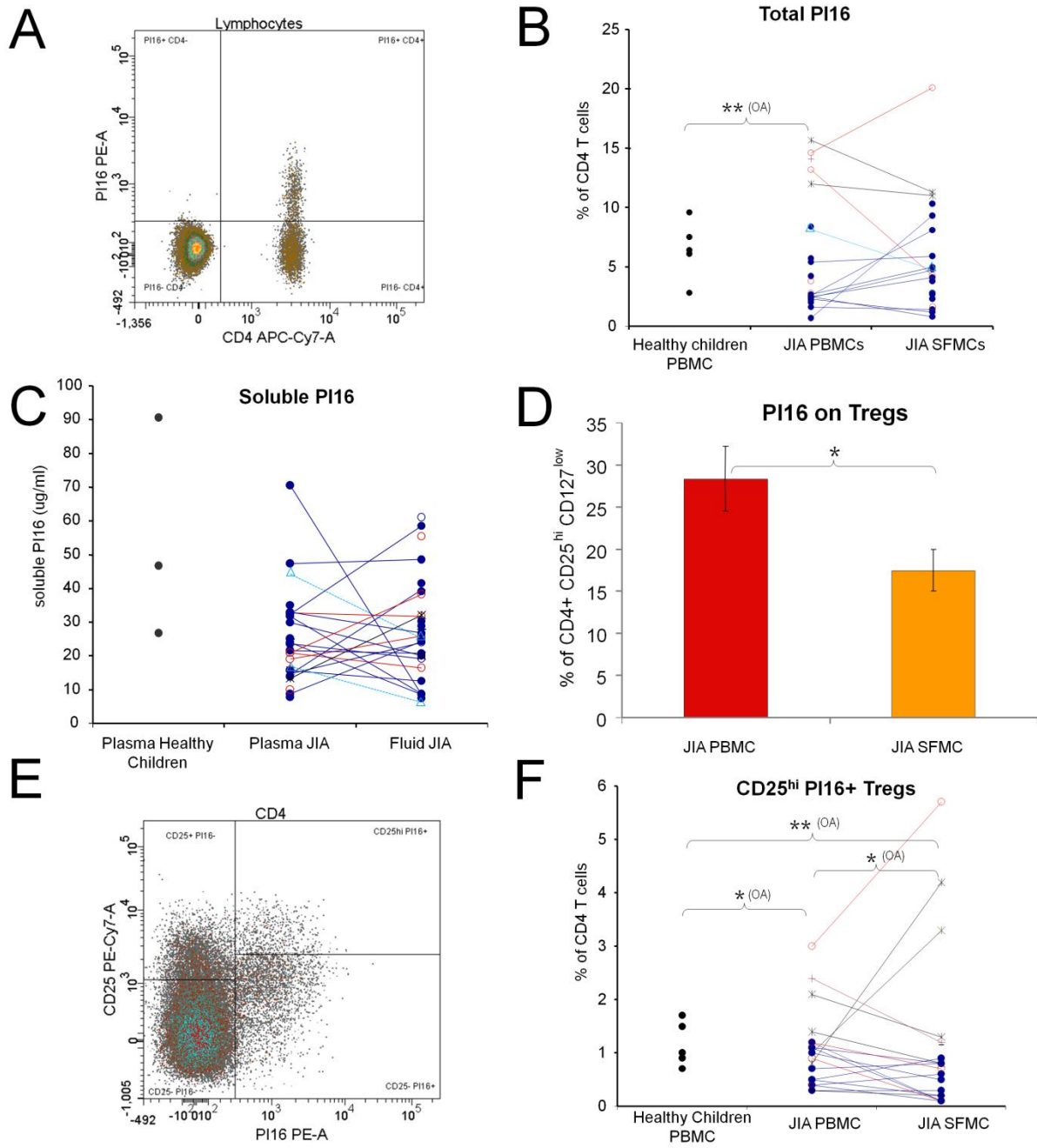PBMC   SFMC
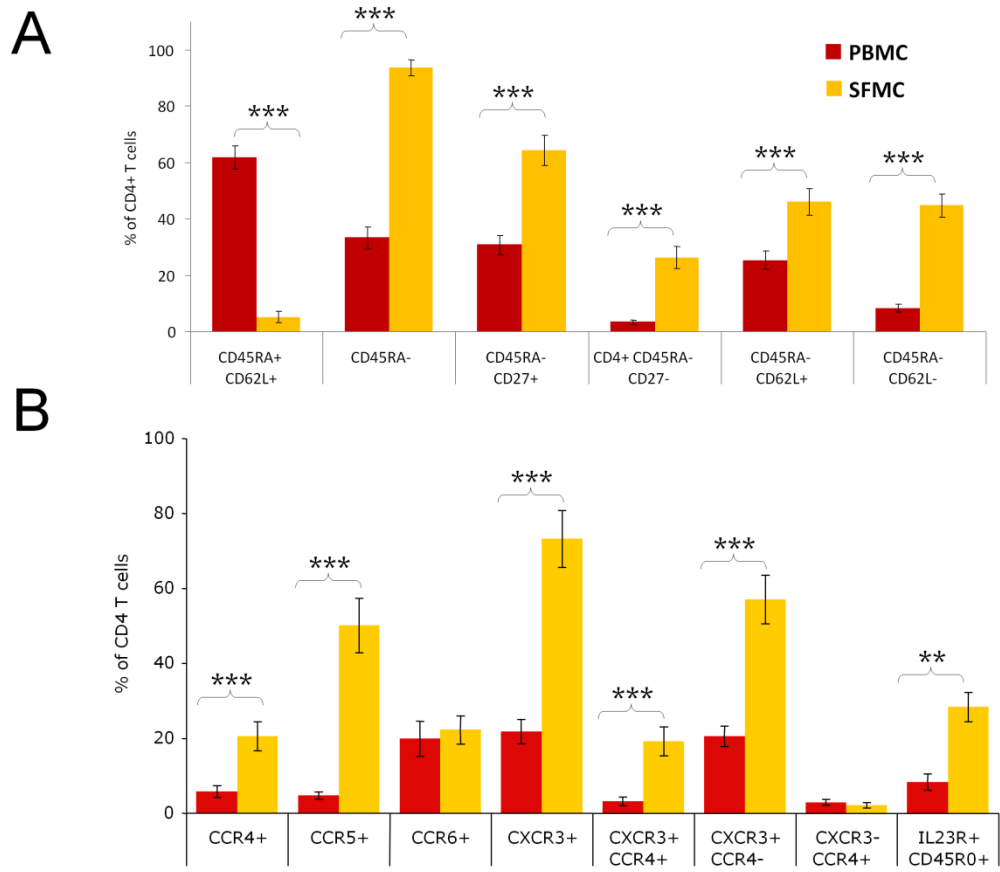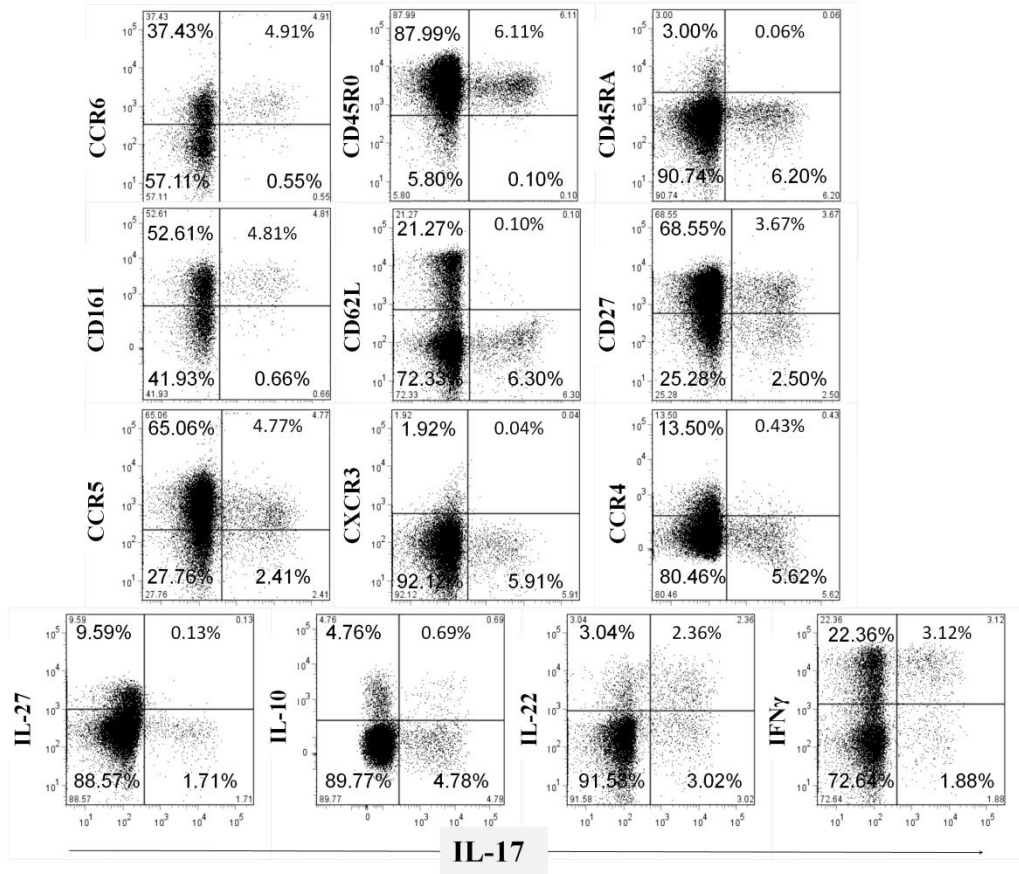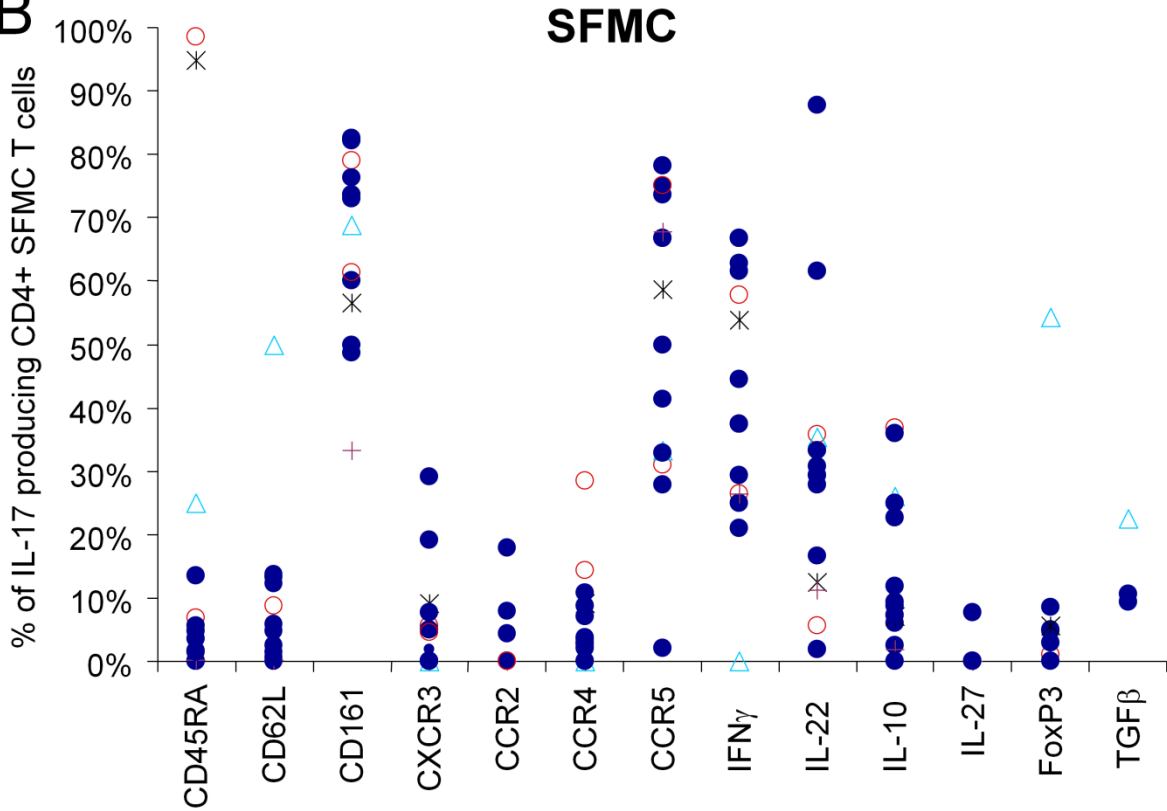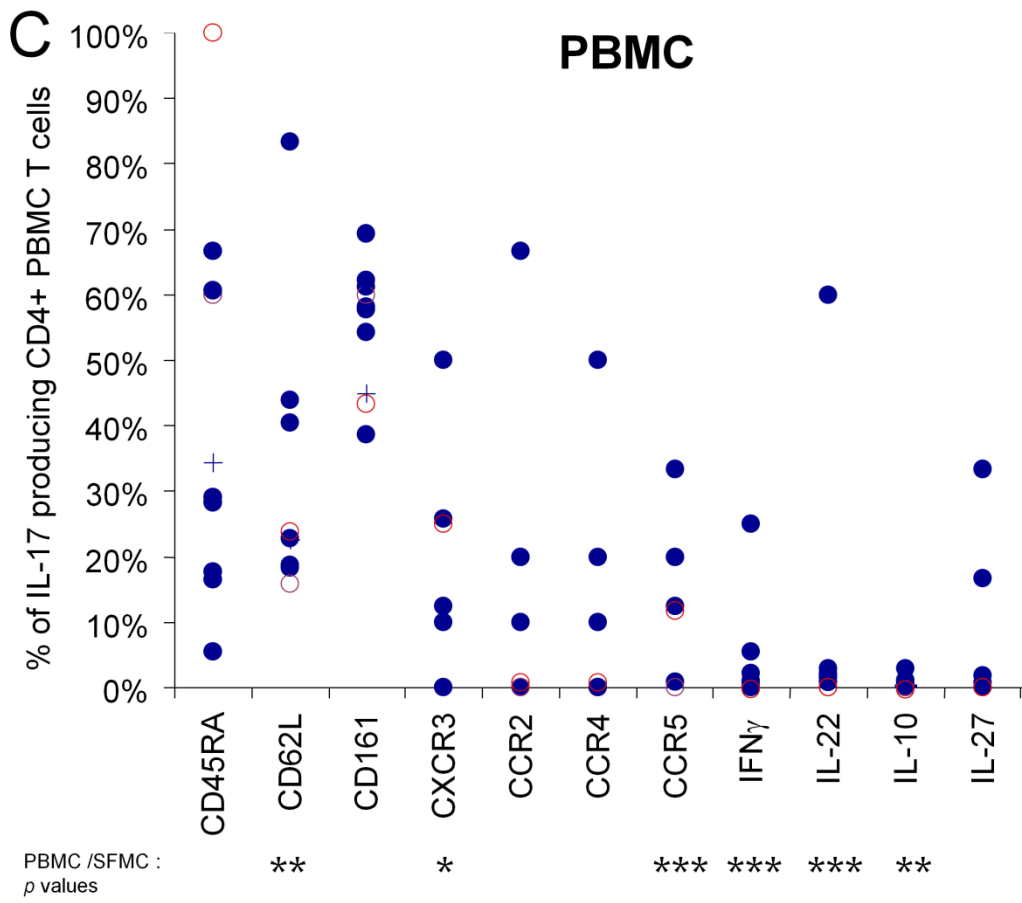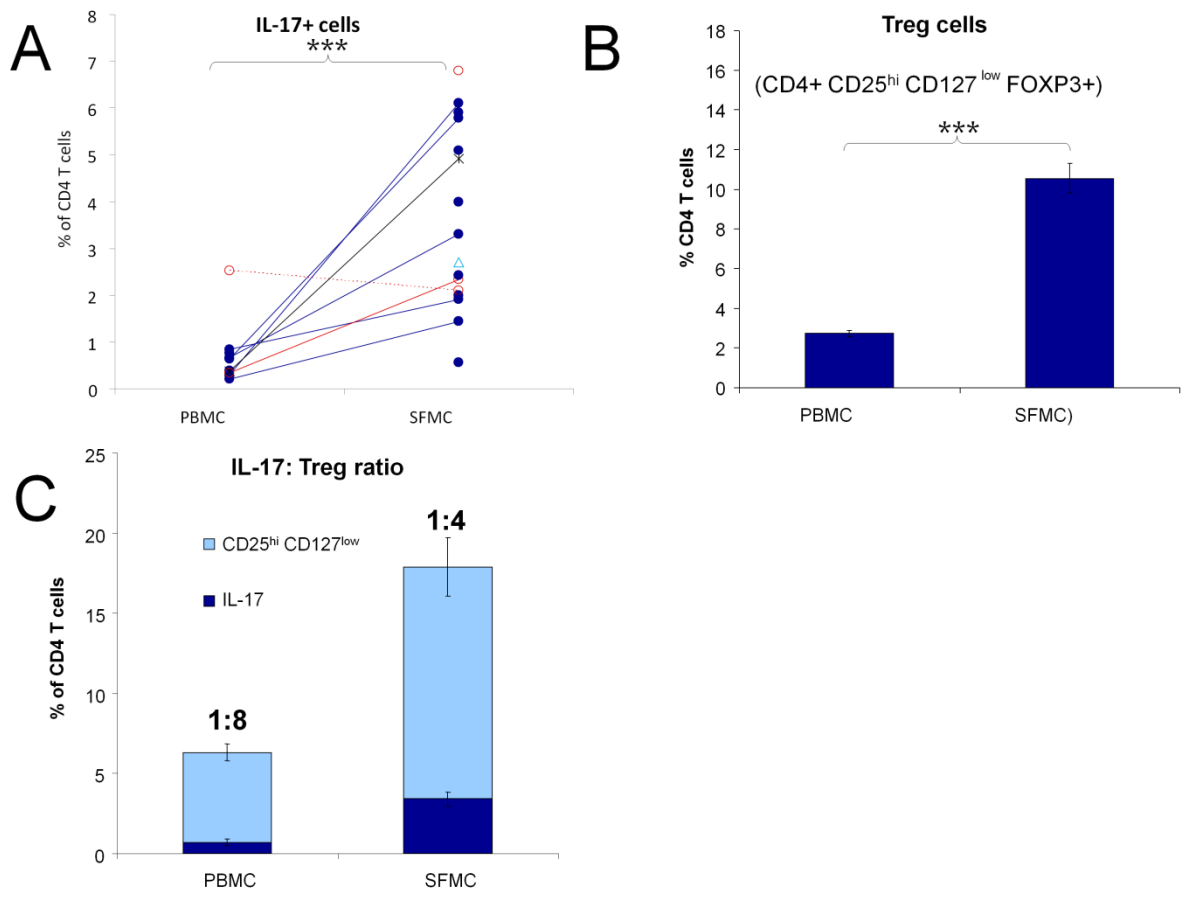
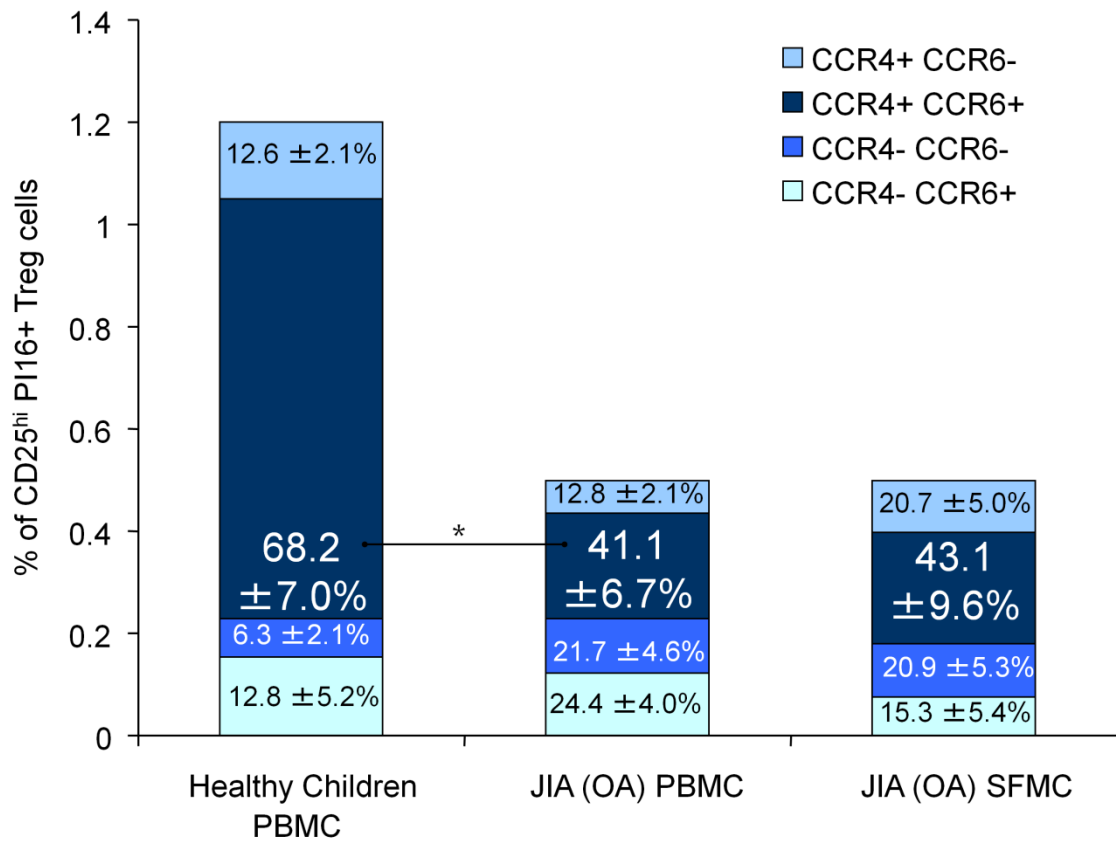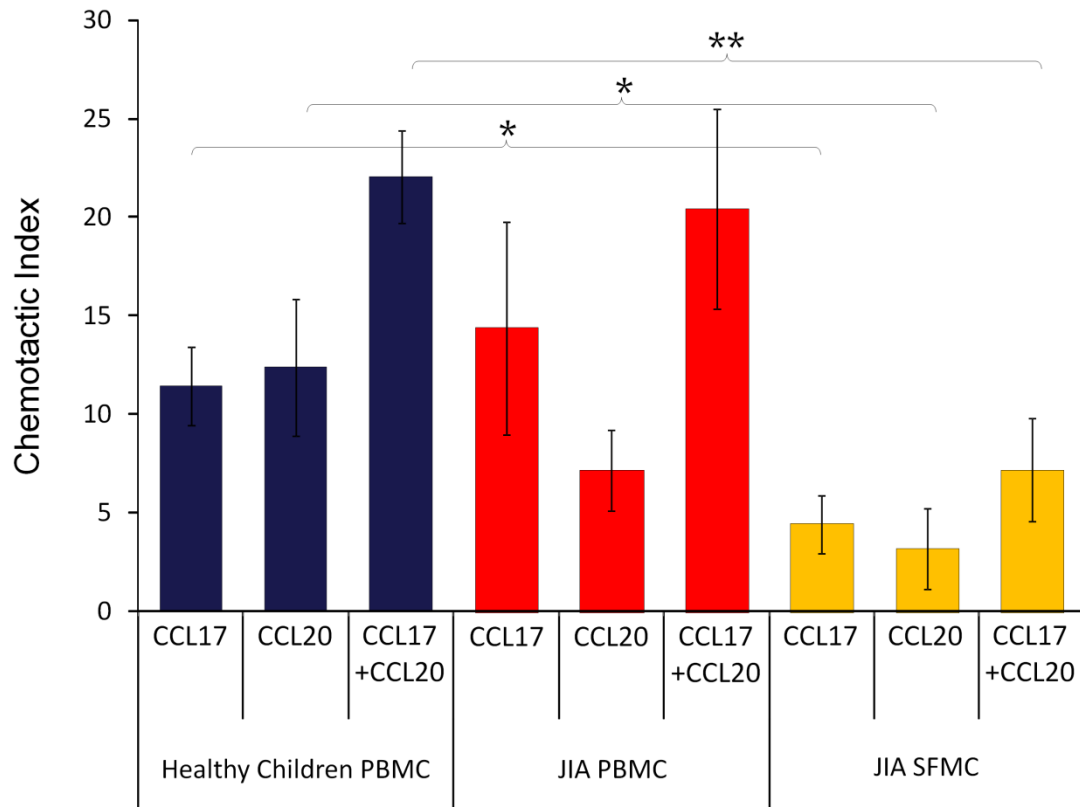**Figure 4**

Figure 5

Figure 6

Table Legend

Table 1: Summary of JIA subtype, ANA status and treatment.

Figure Legends

Figure 1: Distribution of T helper subtypes, chemokine receptors and cytokine receptor IL-23R within peripheral blood (PB) and synovial fluid (SF) of JIA patients. The matched samples were analysed *ex-vivo* by flow cytometry, gated on live lymphocytes and CD4+ cells. (A) Percentages of CD45RA- (memory T cells), CD27+/CD45RA+ (naïve T helper 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 IL23R+/CD45RO+ . The data are expressed as the mean±SEM (n=15-23). ✳✳✳ *p<0.0001*, ✳✳ *p<0.01*, ✳ *p<0.05*.

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 dot plots of IL-17 producing CD4+ T 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-27, IFNγ, FoxP3 and TGFβ.Analysis of the above staining for JIA patient sample comparing (B) PBMC and (C) SFMC. Cells were analysed gated on live lymphocytes and CD4+/IL-17+ cells. The data were shown as: Oligoarthritis ●; Extended Oligoarthritis △; ERA ✳; Polyarthritis ○; Systemic Arthritis ＋.

Figure 3: Analysis of (A) Th17 (CD4+/IL-17+: PBMC (n=10) and SFMC (n=18)), (B) Treg (CD4+/CD25^{hi}/CD127^{low}/FOXP3+: PBMC (n=20) and SFMC (n=19)) and (C) ratio of IL-17 (PBMC (n=10) and SFMC (n=18)): Treg cells (PBMC (n=30) and SFMC (n=32)) in peripheral blood and synovial fluid. The data were shown as: Oligoarthritis ●; Extended Oligoarthritis △; ERA ✳; Polyarthritis ○ (dashed line juvenile psoriatic arthritis polyarthritis onset); Systemic Arthritis ＋.

Figure 4: Analysis of PI16 and Treg in JIA patients. PBMC and 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 (OA; *p=0.003*) and SFMC from JIA patients. (C) Soluble PI16 in plasma (n=27) and synovial fluid (n=27). (D) Percentage of PI16+ cells in the Treg gate (CD4+/CD25^{hi}/CD127^{low}) for PBMC (n=10) and SFMC (n=10) (*p=0.02*) (E) Representative dot plot gated on CD4+ T cells and showing CD25^{hi} PI16+ Treg cell population (F) Comparison of CD25^{hi} PI16+ Treg cells normal healthy children compared with matched PBMC (OA; *p=0.03*) and SFMC (OA; *p=0.001*) from JIA patients. The data were shown as: Oligoarthritis (OA) ●; Extended Oligoarthritis △; ERA ✳; Polyarthritis ○; Systemic Arthritis ＋.

Figure 5. Analysis of PI16+ Treg and co-expression of CCR4 and CCR6 in healthy children and oligoarthritis JIA patients. PBMC from healthy children, PBMC and SFMC from JIA patients with oligoarthritis were stained with monoclonal antibodies against CD4, CD25, PI16, CCR4 and CCR6. Lymphocytes were gated on CD4+/CD25^{hi}/PI16+ and the co-expression of CCR4+/- and CCR6+/- analysed.

Figure 6: Migration of CD4+ CD25^{hi} PI16+ Treg cells to CCR4 ligand CCL17, CCR6 ligand CCL20 and both CCL17 and CCL20 from healthy children PBMC (n=5) compared with PBMC (n=10) and SFMC (n=6) of JIA patients. Cells were analysed by flow cytometry, gated on the CD4+ CD25^{hi}CD127^{low} 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.