Functional aspects of the adaptive immune system in arthritis

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CHAPTER 5

IL-17-PRODUCING CD4+ T CELLS ARE INCREASED IN EARLY, ACTIVE AXIAL SPONDYLOARTHRITIS INCLUDING PATIENTS WITHOUT IMAGING ABNORMALITIES

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ABSTRACT

Introduction Increased numbers of IL-17-producing CD4+ T cells have been observed in ankylosing spondylitis. However, it is not known if these CD4+ T cells are already present in early disease or if this is a late disease phenomenon only. Therefore we aimed to investigate whether IL-17-producing CD4+ T cells are involved in early active axial spondyloarthritis including patients without imaging abnormalities, by determining the frequency and phenotype of IL-17-producing CD4+ T cells in these patients.

Methods Flow cytometry was used to analyse the cytokine production and surface marker expression of peripheral blood mononuclear cells from 31 patients suffering from early active HLA-B27-positive axial spondyloarthritis fulfilling the Assessment of SpondyloArthritis International Society (ASAS) criteria with or without MRI abnormalities and 21 healthy controls.

Results Patients with early active axial spondyloarthritis showed an increased percentage of IL-17-producing CD4+ T cells compared to the healthy controls (mean 1.1% *vs* 0.4% respectively, p=0.013). The percentage of IL-17-producing CD4+ T cells was equally increased in patients with and without MRI abnormalities (1.2% *vs* 1.1% respectively, p=0.81). These IL-17-producing CD4+ T cells expressed the αβ T-cell receptor but not the γδ T-cell receptor, exhibited a memory phenotype and expressed CD161, but only sporadically expressed killer cell immunoglobulin-like receptor 3DL2 (KIR3DL2).

Conclusion IL-17-producing CD4+ T cells are increased in patients with early active axial spondyloarthritis both with and without MRI abnormalities. This finding shows that the frequency of IL-17-producing CD4+ T cells is enhanced in the early stages of disease.

INTRODUCTION

Axial spondyloarthritis (SpA) is a common chronic disease involving the sacroiliac (SI) joints and the axial skeleton. The most well-known form of axial SpA is ankylosing spondylitis (AS) which is characterized by sacroiliitis on conventional radiographs. However, radiographs become positive for sacroiliitis at a rather late stage, as they detect only structural damage as a consequence of inflammation and not the inflammation itself. The term nonradiographic axial spondyloarthritis (nr-axSpA) has recently been introduced to identify patients with axial SpA without structural changes in the sacroiliac joints. The new Assessment of SpondyloArthritis International Society (ASAS) criteria were subsequently developed as classification criteria for axial SpA covering both radiographic axial SpA (AS) and nr-axSpA[1]. Patients with nr-axSpA can either show inflammation on MRI of the SI joints with one SpA feature (imaging arm) or be HLA-B27-positive with two additional SpA features (clinical arm).

SpA is an HLA-B27-associated inflammatory disease and several lines of evidence suggest that the pro-inflammatory cytokine interleukin 17-A (IL-17) is involved in the pathogenesis of AS. In animal models it has been described that IL-17-producing CD4+ T cells are expanded in SpA-prone HLA-B27 transgenic rats[2] and that IL-17-producing CD4+ T cells are increased in regional lymph nodes of male BXSB x NZB F1 mice that spontaneously develop seronegative ankylosing enthesitis in the ankle or tarsal joints[3]. In humans, an increase in the number of IL-17-producing CD4+ T cells in the blood of patients with AS compared with healthy controls has been reported[4-6]. However, other studies have not been able to reproduce these findings[7-9]. In addition, AS is associated with genetic polymorphisms of the IL-23 receptor with the susceptibility-conferring R381Q allele variant characterized by enhanced Th17 responses[10,11]. Finally, an anti-IL17A monoclonal antibody has shown promising results in the treatment of AS patients[12]. Irrespective of all the evidence for the involvement of IL-17 in the pathogenesis of AS, the underlying mechanism connecting HLA-B27 and IL-17 production is incompletely understood. Bowness *et al.* showed that HLA-B27 is capable of forming homodimers and that these homodimers are able to bind the killer cell immunoglobulin-like receptor 3DL2 (KIR3DL2)[13]. Furthermore, they showed that AS patients have increased levels of IL-17-producing CD4+ T cells that express KIR3DL2 and that binding of KIR3DL2 to HLA-B27 induces proliferation and production of IL-17 by these CD4+ T cells, potentially linking HLA-B27 to IL-17 production[14].

It is not known whether IL-17-producing CD4+ T cells play a role in disease initiation or maintenance. Furthermore, the expression of KIR3DL2 on IL-17-producing CD4+ T cells early in disease has not been examined. To investigate whether IL-17-producing CD4+ T cells are already involved at the onset of disease, we set out to determine the frequency and phenotype of IL-17-producing CD4+ T cells in early active axial SpA (axial SpA).

METHODS

Patients

Peripheral blood was obtained from patients visiting the outpatient clinic of the Leiden University Medical Center, Leiden, the Netherlands, classified with axial spondyloarthritis according to the ASAS criteria[1]. Fourteen patients had a negative MRI of the SI joints and 17 patients had a positive MRI with bone marrow oedema highly suggestive of sacroiliitis according to the ASAS definition. None of the MRI-negative patients had sacroiliitis on radiographs compared with 6 out of 17 patients with a positive MRI. All patients were HLA-B27-positive and on average had a high Ankylosing Spondylitis Disease Activity Score (ASDAS; Table 1). Median back pain duration was 16 months. The median number of SpA features in the MRI-negative patients was four (range three to six) and also four in MRI-positive patients (range two to six). Five patients used DMARDs because of an extra-axial manifestation; sulfasalazine was used in two patients for IBD and in two patients for peripheral arthritis. One patient used methotrexate for arthritis. Two patients used a TNF blocker (both adalimumab), one for IBD and one for psoriasis.

The average number of days between the MRI being performed and peripheral blood mononuclear cell (PBMC) isolation was 2.4 days (range 0-21). Written informed consent was obtained from all participating patients. The study has been reviewed and approved by the medical ethical committee of the Leiden University Medical Center.

Controls consisted of 21 healthy blood donors of which buffy coats were obtained from the blood bank (Sanquin, the Netherlands). Of these healthy controls, 12 individuals were HLA-B27 positive and 9 individuals were HLA-B27 negative to investigate the influence of HLA-B27 on the frequency of IL17-producing CD4+ T cells. Detailed patient and control characteristics are provided in Table 1.

Intracellular cytokine and surface staining

PBMCs were isolated from peripheral blood of buffy coats using Ficoll Paque gradient centrifugation (Leiden University Medical Center Pharmacy). Cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, St Louis, MO, USA) and 1 μg/ml ionomycin (Sigma) for 5 hours, in the presence of 5 μg/ml Brefeldin A (Sigma) during the final 4 hours of the incubation. After stimulation surface staining was performed using the following antibodies; CD3 PE-Cy7 (SK7), CD4 APC-Cy7 (RPA-T4), CD8 AlexaFluor 700 (RPA-T8), CD14 Pacific Blue (M5E2), CD28 FITC (CD28.2), CD45RO PE-CF594 (UCHL1), T cell receptor-αβ (TCRαβ) AlexaFluor 488 (WT31) and TCRγδ PE (B1) all purchased from BD Biosciences (San Jose, CA, USA), CD56 Brilliant Violet 605 (HCD56) and CD161 Brilliant Violet 421 (HP-3G10) purchased from Biolegend (San Diego, CA, USA) and KIR3DL2 PE (DX31) from the University of California San Francisco (San Francisco, CA, USA). Intracellular staining was performed using the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit from BD Biosciences and IL-17A AlexaFluor 647 (eBio64CAP17; eBioscience, San Diego, CA, USA) and IFNγ PE (4S.B3; BD Biosciences). All samples were measured on a BD LSRFortessa cell analyser (BD Biosciences)

and analysed using BD FACSDIVA software (BD Biosciences) and FlowJo version 7.6.5 (FlowJo, Ashland, OR, USA).

Statistical analysis

All statistical analysis were performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) including regression analysis. The different patient and control groups were compared using a Mann-Whitney test. *p*-values <0.05 were considered to be significant.

Table 1. Characteristics of patients with axial spondyloarthritis with and without imaging abnormalities and healthy controls

	MRI negative (*n*= 14)	MRI positive (*n*= 17)	Controls (*n*= 21)
Age median (range), years	29 (19-47)	30 (23-50)	47 (29-60)
Males, *n* (%)	5 (36)	14 (82)	12 (57)
Duration of back pain, median (range), months	17 (4-45)	16 (6-36)	n/a
Family history of SpA, *n* (%)	3 (21)	7 (41)	n/a
Inflammatory backpain, *n* (%)[a]	13 (93)	12 (71)	n/a
Heel pain, *n* (%)	3 (21)	3 (18)	n/a
Anterior uveitis, *n* (%)[b]	6 (43)	1 (6)	n/a
Arthritis, *n* (%)[b]	4 (29)	5 (29)	n/a
Dactylitis, *n* (%)[b]	1 (7)	2 (12)	n/a
Psoriasis, *n* (%)[b]	1 (7)	0 (0)	n/a
Inflammatory bowel disease, *n* (%)	2 (14)	0 (0)	n/a
HLA-B27 positive, *n* (%)	14 (100)	17 (100)	12 (57)
Elevated CRP or ESR, *n* (%)	3 (21)	9 (59)	n/a
Sacroiliitis on MRI, *n* (%)	0 (0)	17 (100)	n/a
Sacroiliitis on X-ray, *n* (%)	0 (0)	6 (36)	n/a
Current NSAID use, *n* (%)	9 (64)	14 (82)	n/a
Current DMARD use, *n* (%)	3 (21)	2 (12)	n/a
Current TNF-blocker use, *n* (%)	1 (7)	1 (6)	n/a
ASDAS-CRP, median (range)	3.1 (1.5-5.3)	3.0 (2.0-5.0)	n/a

[a]According to ASAS definition. [b]Physician observed. ASAS: Ankylosing SpondyloArthritis International Society; ASDAS-CRP: Ankylosing Spondylitis Disease Activity Score with CRP; n/a: not applicable.

RESULTS

Increased frequency of IL-17-producing CD4+ T cells in patients with axial SpA irrespective of imaging abnormalities

Flow cytometry was used to determine the intracellular expression of IL-17 by PBMCs from 31 patients with axial SpA and from 21 healthy controls after stimulation with PMA and ionomycin (patient and control characteristics are provided in Table 1). Patients with axial SpA exhibited a higher percentage of IL-17-producing CD4+ T cells compared with healthy controls (Figure 1A and 1B; mean 1.1% *vs* 0.4% in controls; *p*= 0.013). The percentage of IL-17-producing CD4+ T cells was equally increased in axial SpA patients with (*n*= 14) and without (*n*= 17) MRI abnormalities (mean 1.2% *vs* 1.1%, *p*= 0.81). Moreover, this increase was disease related and not HLA-B27 specific as the healthy controls positive (*n*= 12) and negative (*n*= 9) for HLA-B27 showed comparable percentages of IL-17-producing CD4+ T cells (mean 0.4% *vs* 0.3%, *p*= 0.45; Figure 1B). IL-17 production by CD8+ T cells was determined as well, however,

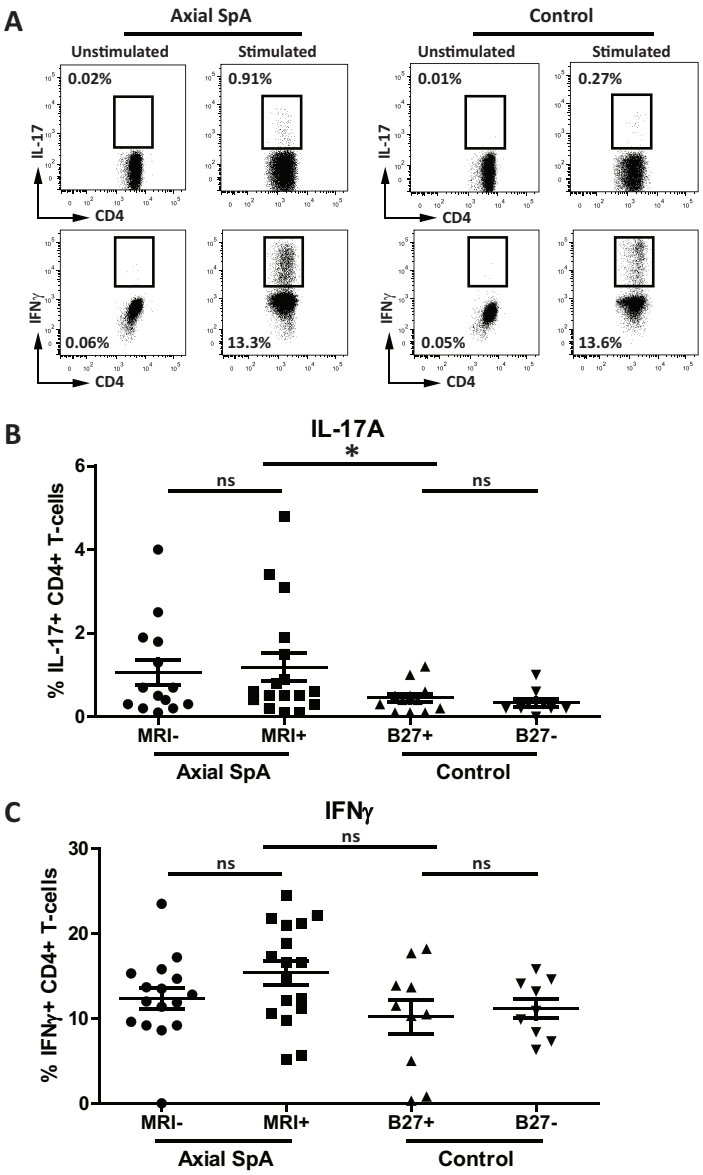

Figure 1. Increased percentage of IL-17-producing CD4+ T cells in patients with axial SpA irrespective of imaging abnormalities. PBMCs of 31 axial SpA patients and 21 healthy controls were stimulated with PMA and ionomycin for 5 hours and subsequently surface stained with CD3, CD4, CD8, CD14 and CD56 and intracellularly stained with IL-17A or IFNγ. The monocytes and natural killer cells were excluded by gating on the CD14- and CD56-negative cells and subsequently the T cells were selected by gating on CD3 and CD4 double-positive cells. Then the production of IL-17A and IFNγ by these CD4+ T cells was analysed. (A) Dot plots of a representative patient and control. (B) Overview of the IL-17-producing CD4+ T cells in axial SpA patients either with (MRI+; *n*= 17) or without (MRI-; *n*= 14) MRI abnormalities and healthy controls either positive (HLA-B27+; *n*= 12) or negative (HLA-B27-; *n*= 9) for HLA-B27. (C) Overview of the IFNγ-producing CD4+ T cells in axial SpA patients either with (MRI+) or without (MRI-) MRI abnormalities and healthy controls either positive (HLA-B27+) or negative (HLA-B27-) for HLA-B27. *$p < 0.05$. ns = not significant. PBMCs: peripheral blood mononuclear cells; PMA: phorbol 12-myristate 13-acetate.

IL-17+ CD8+ T cells were not detected in patients irrespective of imaging abnormalities nor in the healthy controls irrespective of their HLA-B27 status (data not shown). In addition to the intracellular expression of IL-17, the intracellular expression of IFNγ by CD4+ T cells was determined as well. The frequency of IFNγ-producing CD4+ T cells was higher in axial SpA patients compared with healthy controls, although no statistical significance was reached (mean 13.9% *vs* to 10.7%, p= 0.07; Figure 1C). Together, these data indicate an expansion of IL-17- and possibly IFNγ-producing CD4+ T cells in the PBMC fraction of axial SpA patients already early in the disease.

As controls were on average older than patients, we analysed whether the increased percentage of IL-17-producing CD4+ T cells in patients was correlated with age. In both patients and controls there was no correlation between age and the number of IL-17-producing CD4+ T cells as linear regression analysis correlating age versus percentage of IL-17-producing CD4+ T cells showed that the slope of the fitted line was not significantly different from zero (p= 0.64 for patients and p= 0.68 for controls; data not shown). Moreover, using the median age to dichotomize groups showed that the mean percentage of IL-17-producing CD4+ T cells was not significantly different between young or old patients compared with controls (mean 0.7% *vs* 1.5%, p= 0.15 and mean 0.3% *vs* 0.4%, p= 0.65, respectively; data not shown).

In addition, we investigated whether the presence of extra-axial manifestations or the use of DMARDs and biologicals in patients explained the finding. Both DMARD/biological use and a history of extra-axial manifestations was associated with a higher percentage of IL-17-producing CD4+ T cells compared with patients without (mean 1.9% *vs* 1.0% and mean 1.4 *vs* 1.0%, respectively), however, neither was significant (p= 0.17 and p= 0.98, respectively; data not shown). Of note, when the blood sampling for this study was performed, according to the clinical assessment of the treating physician, none of the patients with extra-axial manifestation had signs of activity of that manifestation except the one patient with psoriasis.

IL-17-producing CD4+ T cells in patients with axial SpA express TCRαβ and CD161 and exhibit a memory phenotype, but sporadically express KIR3DL2

IL-17 production by TCRαβ- and TCRγδ-positive CD4+ T cells in AS has been described[7,8]. Therefore the expression of TCRαβ and TCRγδ by the IL-17-producing CD4+ T cells of 11 axial SpA patients was evaluated using flow cytometry after stimulation with PMA and ionomycin. The IL-17-producing CD4+ T cells in patients with axial SpA did not express TCRγδ, but they did express TCRαβ (Figure 2A). To further characterize the IL-17-producing CD4+ T cells in axial SpA patients, the expression of CD161 was evaluated. CD161 is a marker of IL-17-producing T cells induced by RORC. Indeed, CD161 expression was observed on a large proportion of IL-17-producing CD4+ T cells. On average 50.8% of the IL-17-producing CD4+ T cells expressed CD161 in patients (Figure 2B). Since IL-17-producing CD4+ T cells are thought to reside within the memory pool, the memory status of the IL-17-producing CD4+ T cells was evaluated using the expression of CD28 and CD45RO. As expected, IL-17-producing CD4+ T

cells showed an expression pattern compatible with early memory (CD28+ CD45RO+) and memory phenotype (CD28- CD45RO+). On average 83.0% of the IL-17-producing CD4+ T cells were positive for CD28 and CD45RO and 10.8% of the IL-17-producing CD4+ T cells were CD28-CD45RO+ (Figure 2C).

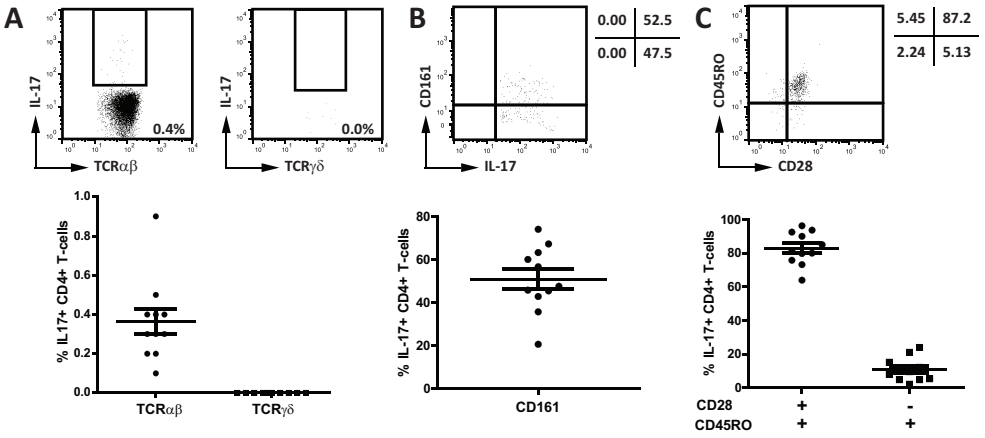

Figure 2. IL-17 is produced by TCRαβ-positive CD4+ T cells that exhibit a memory phenotype and show a heterogeneous expression of CD161. TCR expression of IL-17-producing CD4+ T cells of axial SpA patients was evaluated by determining the expression of TCRαβ and TCRγδ and subsequently the IL-17 production by these cells using flow cytometry. (A) Dot plots of a representative patient (top). Summary of 11 patients with each dot representing one patient (bottom). The expression of CD161 by IL-17-producing CD4+ T cells was also determined by flow cytometry. (B) Dot plot of a representative donor (top). Cells were gated on CD3+CD4+ IL17+ T cells and their IL-17 production (*x*-axis) and CD161 expression (*y*-axis) is depicted. A summary of 11 patients is depicted in the bottom graph. (C) Memory phenotype of the IL-17-producing CD4+ T cells. The cells were gated on CD3+CD4+ T cells positive for IL-17 and subsequently the memory phenotype was determined by different combinations of expression of CD28 and CD45RO. CD28+CD45RO- cells were considered to be naïve T cells, CD28+CD45RO+ cells were considered to be early memory T cells, CD28-CD45RO- cells were considered to be memory T cells and CD28-CD45RO- cells were considered to be late memory cells. Representative dot plot of CD45RO and CD28 expression by IL-17-producing CD4+ T cells (top). Summary of 11 patients is depicted in the bottom graph.

HLA-B27 has been described to bind to KIR3DL2 expressed by CD4+ T cells in AS patients, thereby stimulating IL-17 production[14]. Therefore, the expression of KIR3DL2 on the IL-17-producing CD4+ T cells of patients with axial SpA was determined by flow cytometry. Expression of KIR3DL2 was detected on a small percentage of CD4+ T cells, on average 0.8% of the CD4+ T cells expressed KIR3DL2 (Figure 3A and 3B). Of these KIR3DL2+ CD4+ T cells, on average 2% produced IL-17 after stimulation. Thus, although a relative increase in IL-17-producing cells was observed within the KIR3DL2+ CD4+ T cells versus the total CD4+ T cell population (i.e. 2% *vs* 1.2%), only 0.02% of CD4+ T cells were double positive for IL-17 and KIR3DL2 (Figure 3C) as a result of the relative infrequency of KIR3DL2+ CD4+ T cells in peripheral blood. The expression of other KIRs was also evaluated using an antibody that recognizes KIR2DL2, KIR2DL3 and KIR2DS2 (KIR2DL2/3). Expression of KIR2DL2/3 was

detected on average on 0.1% of the CD4+ T cells (Figure 3D and 3E). Unexpectedly, a relative large fraction of these cells produced IL-17 as, on average 11.9% of the KIR2DL2/3+ CD4+ T cells stained positive for IL-17. Nonetheless, KIR2DL2/3 is expressed by a minority of CD4+ T cells, as only 0.01% of the total CD4+ T cells were IL-17 and KIR2DL2/3 positive (Figure 3F).

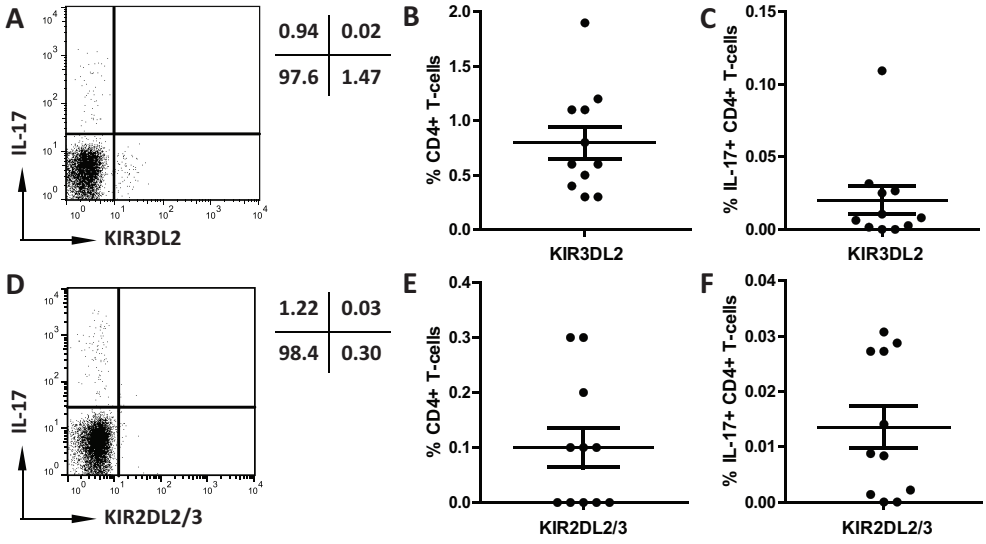

Figure 3. A small minority of IL-17-producing CD4+ T cells express KIRs. Killer cell immunoglobulin-like receptor (KIR) expression of IL-17-producing CD4+ T cells of patients with axial SpA was analysed using FACS. Cells were gated on CD3+CD4+ T cells and subsequently KIR and IL-17 expression was determined. Representative dot plots of IL-17 and KIR expression are depicted: (A) KIR3DL2 and (D) KIR2DL2/3. Summary of expression of (B) KIR3DL2 and (E) KIR2DL2/3 on CD4+ T cells of 11 patients. (C) and (F) represent the percentage of double-positive CD4+ T cells; either (C) IL-17 and KIR3DL2 or (F) IL-17 and KIR2DL2/3 double-positive cells.

DISCUSSION

Increased levels of IL-17-producing CD4+ T cells have been described in patients with AS with long disease duration and in animal models for SpA[2,4-6,15]. Whether this T cell population is already present in early disease is not known. Therefore, patients with axial SpA with short disease duration were tested for IL-17-producing CD4+ T cells and compared with healthy controls either positive or negative for HLA-B27. Patients with axial SpA showed an increased percentage of IL-17-producing CD4+ T cells compared with healthy controls. The percentage of IL-17-producing CD4+ T cells was similar in patients with and without MRI abnormalities. This increased percentage of IL-17-producing CD4+ T cells was disease related and not HLA-B27 specific as the healthy controls positive and negative for HLA-B27 exhibited comparable percentages of IL-17 producing CD4+ T cells. The IL-17-producing CD4+ T cells expressed TCRαβ and CD161 and exhibited a memory phenotype consistent with previous reports[4,5,8].

In contrast to our results, Appel *et al.*[9] reported no increase of IL-17-producing CD4+ T cells

in peripheral blood in SpA patients. Furthermore, a recent published study performed in Spain reported a decreased percentage of Th17 cells in patients with non-radiographic axial SpA compared with healthy controls[8]. Selection of patients could explain these differences. Compared with our patients, the Spanish patients had on average low disease activity based on the BASDAI, only a few patients had increased CRP and the number of SpA features was low[8]. In addition, despite a relatively short disease duration with a median back pain duration of only 16 months, already 6 out of 17 patients (36%) with a positive MRI showed signs of sacroiliitis on radiographs in our study. There are also technical differences, as the Spanish study used different isolation and stimulation of the cells and in particular a much longer stimulation of cells with PMA and ionomycin for 16 hours. Collectively, these data suggest that IL-17-producing CD4+ T cells are elevated only in active disease, which would limit the potential diagnostic use of measuring IL-17-producing CD4+ T cells in the peripheral blood of axial SpA patients. Moreover, increased levels of IL-17-producing CD4+ T cells have also been reported in rheumatoid arthritis[4,16-21] and in most reports the levels correlated with disease activity, demonstrating that this is not a disease-specific finding, but a general inflammation-related phenomenon. On the other hand, IL-17-producing CD4+ T cells were elevated irrespective of the presence of MRI abnormalities, making our results relevant to patients fulfilling both the imaging and the clinical arm of the ASAS classification criteria.

Our results were obtained from the peripheral blood compartment. However, the site of interest is the primary site of inflammation and the frequency of IL-17-producing cells in this compartment could be different from the peripheral blood compartment[9,22]. Biopsies of the affected target tissue in the spine are difficult to obtain in patients suffering from SpA which represents a clear limitation of the current study.

SpA is strongly associated with HLA-B27 and several hypotheses have been proposed to explain the role of HLA-B27 in the disease pathology[23]. One of these hypotheses proposes that HLA-B27 heavy chains can form homodimers that are able to bind KIR3DL2[13,24]. KIRs are typically expressed by natural killer (NK) cells, however, a subset of CD4+ T cells has been found to express KIR3DL2[25] consistent with our findings. CD4+ KIR3DL2+ T cells have been reported to be increased in AS patients and reportedly proliferate and produce IL-17 upon binding to HLA-B27 homodimers[14]. We found expression of KIR3DL2 by IL-17-producing CD4+ T cells in only a small minority of cells. This indicates that an interaction between KIR3DL2 and HLA-B27 is not required for induction and expansion of IL-17-producing CD4+ T cells in early disease. In addition, the higher frequency of KIR3DL2+ CD4+ T cells reported in later stages of disease suggests that these KIR3DL2+ CD4+ T cells expand as the disease becomes more chronic and since our patients suffered from active disease argues against a role in disease initiation. Nonetheless, given the relatively low frequency of KIR3DL2+ CD4+ T cells in peripheral blood, it would be interesting to investigate the absolute number of IL-17-producing KIR3DL2+ CD4+ T cells in peripheral blood in patients with established disease. Likewise, it would be interesting to study whether IL-17-production is confined to the

KIR3DL2+ CD4+ T cell pool or whether other KIR-expressing T cells, as surrogates for T cells with an activated/memory phenotype, are also more often IL-17 positive, as suggested by the data presented in the current article.

In summary, we report an increased frequency of IL-17-producing CD4+ T cells in axial SpA patients compared with healthy controls irrespective of MRI abnormalities. These results demonstrate that the frequency of IL-17-producing CD4+ T cells is enhanced in the early stages of disease.

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