Th17 Cells Expressing KIR3DL2⁺ and Responsive to HLA-B27 Homodimers Are Increased in Ankylosing Spondylitis

Paul Bowness, Anna Ridley, Jacqueline Shaw, Antoni T. Chan, Isabel Wong-Baeza, Myles Fleming, Fraser Cummings, Andrew McMichael and Simon Kollnberger

*J Immunol* 2011; 186:2672-2680; Prepublished online 19 January 2011;
doi: 10.4049/jimmunol.1002653
http://www.jimmunol.org/content/186/4/2672

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/01/19/jimmunol.1002653.DC1

**References**
This article cites 33 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/186/4/2672.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Th17 Cells Expressing KIR3DL2+ and Responsive to HLA-B27 Homodimers Are Increased in Ankylosing Spondylitis

Paul Bowness,* Anna Ridley,* Jacqueline Shaw,* Antoni T. Chan,* Isabel Wong-Baeza,* Myles Fleming,† Fraser Cummings,‡ Andrew McMichael,* and Simon Kollnberger*

CD4 Th cells producing the proinflammatory cytokine IL-17 (Th17) have been implicated in a number of inflammatory arthritides including the spondyloarthritides. Th17 development is promoted by IL-23. Ankylosing spondylitis, the most common spondyloarthritis (SpA), is genetically associated with both HLA-B27 (B27) and IL-23R polymorphisms; however, the link remains unexplained. We have previously shown that B27 can form H chain dimers (termed B272), which, unlike classical HLA-B27, bind the killer-cell Ig-like receptor KIR3DL2. In this article, we show that B272-expressing APCs stimulate the survival, proliferation, and IL-17 production of KIR3DL2+ CD4 T cells. KIR3DL2+ CD4 T cells are expanded and enriched for IL-17 production in the blood and synovial fluid of patients with SpA. Despite KIR3DL2+ cells comprising a mean of just 15% of CD4 T in the peripheral blood of SpA patients, this subset accounted for 70% of the observed increase in Th17 numbers in SpA patients compared with control subjects. TCR-stimulated peripheral blood KIR3DL2+ CD4 T cell lines from SpA patients secreted 4-fold more IL-17 than KIR3DL2+ lines from controls or KIR3DL2− CD4 T cells. Strikingly, KIR3DL2+ CD4 T cells account for the majority of peripheral blood CD4 T cell IL-23R expression and produce more IL-17 in the presence of IL-23. Our findings link HLA-B27 with IL-17 production and suggest new therapeutic strategies in ankylosing spondylitis/SpA. The Journal of Immunology, 2011, 186: 2672–2680.

The Journal of Immunology
We have previously shown an increase in KIR3DL2-expressing CD4 T cells in the peripheral blood of B27+ SpA patients (22), but the role of these cells in promoting inflammation in SpA and their interaction with B27+ has not been defined. KIR ligation by HLA class I can inhibit activation-induced cell death of NK and T cells (22, 23). Thus, we hypothesized that interaction of B27+ with KIR3DL2 expressed on CD4 T cells might have a net proinflammatory effect by promoting T cell survival and subsequent cytokine production. We asked whether KIR3DL2 interaction with B27+ affected the proliferation, survival, and cytokine production of Ag-stimulated CD4 T cells. We also investigated to what degree KIR3DL2+-expressing CD4 T cells contributed to production of IL-17 in AS.

In this article, we show that B27+-expressing APCs stimulate the survival and proliferation of superantigen-activated KIR3DL2+-expressing CD4 T cells, which can produce IL-17. KIR3DL2 CD4 T cells are expanded, enriched for IL-17 production, and account for the majority of CD4 T cell IL-23R expression in the blood of patients with AS/SpA. These cells are further enriched in SpA synovial fluid and constitute a therapeutic target in SpA.

Materials and Methods

Patients

Fifteen milliliters heparinized venous blood was obtained from 25 patients with AS fulfilling the modified New York criteria. Supplementary Table I shows patient demographics and medication. None had received anti-TNF therapy. Paired knee synovial fluid samples were obtained from three AS and three acute Rea patients. Of 28 SpA patients, 22 were male and 27 were HLA-B*2705+ve by molecular typing. Seventeen healthy HLA-B27+ and six HLA-B*2705+ healthy control subjects were studied. As inflammatory disease control patients, we studied 10 patients with RA and 10 patients with ulcerative colitis (UC) without SpA. Appropriate ethical permission (Centre of Research Ethical Campaign 06/Q1606/139 and Oxfordshire Research Ethics Committee B 07/Q1605/35) and individual informed consent were obtained.

FACS and ELISA analysis

PBMC and synovial fluid mononuclear cells (SFMC) were separated by density gradient centrifugation. Cells were stimulated with PMA and ionomycin for 6 h or overnight with anti-CD3 and anti-CD28 beads (Miltenyi Biotec), phytohaemagglutinin torbertainox (BEB), or LPS (100 ng/ml) with brefeldin A (1 μg/ml, all Sigma). Cells were infected with Salmonella typhimurium araA− (multiplicity of infection, 10 bacteria/cell) for 1 h in antibiotic-free medium. Before culture overnight at 37°C, 5% CO2 in RPMI 1640 containing 10% FCS, antibiotics (penicillin, streptomycin [200 μM] and gentamicin sulfate [50 μg/ml]) and l-glutamine (2.5 mM). Cells were stained for surface markers (anti-IgG2a, anti-DX31, anti-CD3 PerCP; BD Biosciences), anti-CD4 Pacific Blue (PB)/FITC (BioLegend), anti-CD14 (BioLegend), anti-CD161 PE (Beckman Coulter), anti–IL-23R (FITC, FAB14010F; R&D Systems), and anti–CCR6 PE (BD Biosciences). KIR3DL2 mAb DX31 was a kind gift of Jo Phillips (DNAX). CD4 T cells were stained for expression of “other KIR” using a mix of PE-conjugated mAbs. KIR3DL2 mAb DX31 was a kind gift of Jo Phillips (DNAX). CD4 T cells were stained for expression of “other KIR” using a mix of PE-conjugated mAbs. KIR3DL2 mAb DX31 was a kind gift of Jo Phillips (DNAX). CD4 T cells were stained for expression of “other KIR” using a mix of PE-conjugated mAbs.

Statistical analysis

We used GraphPad Prism version 4 software for statistical analysis. Based on the work of Shen et al. (10), a sample size of 26 patients and 18 B27+ control subjects was chosen to achieve an α value of 5% in comparisons of IL-17–producing KIR3DL2 CD4 T cells. One-tailed unpaired t tests with Welch’s correction and ANOVAs with a Bonferroni posttest were performed where shown.

Results

Enhanced proliferation, survival, and IL-17 production of KIR3DL2 CD4 T cells stimulated with APCs expressing HLA-B27 homodimers

We first asked whether superantigen-stimulated KIR3DL2+ Th17 T cells could be preferentially expanded in the presence of cells expressing HLA-B27+ H chain homodimers (B27+). We have shown previously that the LBL721.220 cells (hereafter abbreviated to 220), transfected with B27, protect KIR3DL2 NC patients from apoptosis (22). This cell line lacks functional tapasin and expresses high levels of B27+, the levels being abrogated by mutation of the unpaired cysteine at position 67 to serine and reduced 2-fold if tapasin is overexpressed by cotransfection (21). CFSE-labeled CD4 T cells from AS patients and control subjects were cultured with SEB and equal numbers of 220 cells transfected with B27 or control HLA for 5 d. We compared the proliferation of viable CD4 T cells expressing KIR3DL2 with KIR3DL2–ve CD4 T cells and with CD4 T cells expressing “other” KIR (stained for expression with a mix of mAbs recognizing KIR3DL1/DS1, KIR2DL1/DS1, and KIR2DL2/3/2DS2; Fig. 1A). The greatest proliferation was observed in the KIR3DL2 population stimulated with SEB and B27+, expressing 220B7. Less proliferation of KIR3DL2+ cells was seen when CD4 T cells were stimulated with 220 or control 220 transfectants lacking B27 (220B27C67S), expressing reduced levels of B27+ (220B27 transfected with human tapasin) or expressing other HLA class I (220B7). The percentage of viable CD4 T cells expressing KIR3DL2 was consistently greater when CD4 T cells from patients with AS were stimulated with SEB and 220B27 (Fig. 1B). KIR3DL2 CD4 T cells and CD4 T cells expressing “other KIR” did not show the same stimulation of proliferation with 220B27 cells (Fig. 1A and data not shown). The same effect was observed with FACS-purified KIR3DL2 CD4 T cell lines and was inhibited with the KIR3DL2-specific mAb DX31, showing direct involvement of KIR3DL2 (Fig. 1C). HC10, an Ab to HLA class I H chains that also recognizes B27, also inhibited cell survival (data not shown). LBL721.221 cells expressing tapasin, transfected with HLA-B27, also stimulated proliferation of KIR3DL2 CD4 T cells more than LBL721.221 transfected with control HLA (data not shown). LBL721.221 HLA-B27 transfectants also express cell surface B27 (25).

Fig. 2A shows that bulk CD4 T cells from AS patients, stimulated with SEB presented by 220B7, produced more IL-17 than cells stimulated with control APCs with reduced or no B27
FIGURE 1. Enhanced proliferation and survival of KIR3DL2+ CD4 T cells stimulated with SEB and APC expressing HLA-B27 homodimers (B27+). A. Live CD4+ CFSE+ from a healthy B27+ control subject were gated on KIR3DL2+ KIR3DL2+, or “other” KIR+ (left panels). Proliferation of KIR3DL2+, KIR3DL2-, and CD4 T cells expressing “other” KIR, with SEB presented by 220B27 or control 220B7, 220B27C67S, and 220 cells (right panels). Representative of 12 experiments with 6 patients and 3 control subjects. B. Percentage viable KIR3DL2+ CD4 T cells after stimulation with SEB and 220B27 or control APC compared with stimulation with 220 APC. Data from six independent experiments with six patients. 220B27 HuTPN, 220 transfected with HLA-B27 and human tapasin express reduced levels of B27*. *p < 0.0001, ANOVA for T + 220B27 + SEB compared with other stimuli. C. Percentage viable CFSE+ T cells after stimulation of FACS-purified KIR3DL2+ CD4 T cells with SEB and control 220B27 HuTPN (3.2 ± 2.7%), 220B27 (67 ± 2.2%), or 220B27 with KIR3DL2-specific DX31 mAb (44.2 ± 4%) or IgG2a (62.3 ± 2.4%). Four independent experiments with a KIR3DL2 CD4 T cell line from an AS patient; results in B and C expressed as mean percentage viable cells ± SEM. Representative of seven independent experiments with T cell lines from three AS patients.

Peripheral blood KIR3DL2+ CD4 T cells from AS patients are enriched for IL-17 production

We have previously described increased numbers of KIR3DL2+-expressing CD4 T cells in the peripheral blood of B27+ SpA patients, compared with healthy control subjects and RA patients (22). We asked whether these cells were capable of producing IL-17 ex vivo. We first studied 26 SpA patients (24 with AS, 2 with ReA, 25 B27+), together with 6 B27+ and 18 B27- healthy control subjects, and 10 RA patients and 10 UC patients without SpA as inflammatory disease control patients. The patient demographics are shown in Supplemental Table I. Fig. 3A shows enumeration by intracellular cytokine staining and FACS of IL-17–producing CD4 T (Th17) cells from the peripheral blood of a representative AS patient and a B27- healthy control subject. As compared with only 1.54% of KIR3DL2+ CD4 T cells, 3.31% of AS KIR3DL2+ CD4 T cells produced IL-17 after stimulation with PMA and ionomycin ex vivo. For the healthy control subject, 1.099% of KIR3DL2+ and 0.45% of KIR3DL2- CD4 T cells produced IL-17. Because 16.5% of patient CD4 T cells expressed KIR3DL2, this corresponds to 30% of all the Th17 cells in this particular AS patient. By contrast, for this particular healthy control subject, only 6.4% of IL-17–producing CD4 T cells expressed KIR3DL2.

Fig. 3B shows that KIR3DL2+ CD4 T cells contain a significantly greater proportion of IL-17–producing cells for both SpA patients and, to a lesser extent, healthy control subjects. We observed no difference in IFN-γ production by KIR3DL2+ CD4 T cells between SpA patients and healthy control subjects (Fig. 3B). By contrast, with IL-17, KIR3DL2+ CD4 T cells from SpA patients were not enriched for TNF-α production compared with KIR3DL2- CD4 T cells (data not shown).

Fig. 3C shows that the absolute number of peripheral blood KIR3DL2+ Th17 cells was also increased in SpA patients compared with healthy B27- control subjects, and RA and UC inflammatory disease control patients. We also studied six B27+ healthy control subjects. Both the absolute numbers (Fig. 3C) and percentages of KIR3DL2+ Th17 were increased compared with healthy B27- individuals, although they were lower than the SpA patients. Interestingly, healthy B27+ve control CD4 T cells also expressed more KIR3DL2 than B27-ve control subjects (mean values ± SD; B27+ve control subjects: 11.2 ± 2.3% compared with 7.9 ± 3.2% on B27-ve control subjects; p = 0.01; SpA patients: 15.12 ± 10%; p = 0.0018 compared with B27- control subjects). Fig. 3D shows that the proportion of Th17 cells expressing KIR3DL2 was increased both in SpA patients and healthy B27+ control subjects compared with healthy B27-, RA, and UC disease controls. Thus, a mean of 36% of all Th17 cells in SpA patients expressed KIR3DL2 compared with 12.7, 16, and 17.4% in healthy B27-, RA, and UC controls, respectively. Strikingly, although KIR3DL2+ cells comprised the minority of CD4 T cells, the observed expansion within the KIR3DL2 population...
accounted for 70% of the total difference in Th17 numbers between SpA patients and control subjects. Consistent with two previous studies (9, 10), the absolute number of Th17 cells in the bulk CD4 T cell population was also increased in SpA patients compared with healthy B27− control subjects, but to a lesser extent than the absolute number of Th17 cells in the KIR3DL2+ CD4 T cell population (Supplemental Fig. 1).

We next determined whether IL-17–producing KIR3DL2+ CD4 T cells expressed phenotypic markers consistent with the Th17 phenotype. Fig. 4A shows a representative FACS stain from an AS patient of IL-23R and CCR6 expression by KIR3DL2+ and KIR3DL2− CD4 T cells. In this patient, 72% of KIR3DL2+ CD4 T cells coexpressed IL-23R and CCR6 compared with 5% of KIR3DL2− CD4 T. Overall, a mean of 32% of KIR3DL2+ CD4 T cells expressed IL-23R, compared with only 3.1% of KIR3DL2− CD4 T cells in the SpA patients studied (Fig. 4B). Although KIR3DL2+ CD4 T cells comprised a minority of peripheral blood CD4 T cells, they contained a mean of 57% of the total number of IL-23R+ CD4 T cells in SpA patients. By contrast, a lower proportion of KIR3DL2+ CD4 T cells from B27− control subjects expressed IL-23R (15%; Fig. 4B). There was no difference in the proportion of KIR3DL2+ CD4 T cells expressing IL-23R between SpA patients and healthy controls (Fig. 4B). In addition to IL-23R, KIR3DL2+ CD4 T cells were enriched for expression of CCR6 and CD161 Th17 phenotypic markers (Fig. 4C and data not shown). By contrast with IL-23R and CCR6, we observed no difference in the proportion of KIR3DL2+ CD4 T cells expressing CD161 between SpA patients and healthy controls (data not shown).

We sought to investigate whether KIR3DL2+ cells produced more IL-17 than KIR3DL2− CD4 T cells in response to TCR

**FIGURE 2.** KIR3DL2+ CD4 T cells show enhanced IL-17 production on activation with B27−-expressing 220B27 cells. A, IL-17 ELISA showing CD4 T cells stimulated with SEB and B27−-expressing (220B27) APC produce more IL-17 than cells with SEB and control APC. Data from five independent experiments with five AS patients; results are expressed as mean ± SEM. p < 0.05, Student t test for all samples. (No stimulus: 5.9 ± 1.5; 220B27: 5.9 ± 3.8; 220 + SEB: 134 ± 29; 220B27 + SEB: 298 ± 57; 220B27 transfected with human tapasin [220B27 HuTPN]: 113 ± 15; 220B7 + SEB: 164 ± 42 pg/ml). B, Total numbers of viable IL-17+ KIR3DL2+ CD4 T cells from an AS patient after 5 d of stimulation of CD4 T cells with SEB and B27−-expressing (220B27) APC compared with stimulation with SEB and control APC (220, 220B7, and 220B27 HuTPN). Representative of data from three patients and two control subjects. C, Intracellular cytokine staining for IL-17 of a purified KIR3DL2+ CD4 T cell line after 5 d of stimulation with SEB and APC, in the presence of KIR3DL2-specific (DX31) or isotype IgG2a mAb. Representative data from three independent experiments with this T cell line.
ligation and whether KIR3DL2+ production of IL-17 was promoted by the Th17 cytokines IL-23 and IL-1. We first studied IL-17 secretion by equivalent numbers of FACS-sorted KIR3DL2+ and KIR3DL2− CD4 T cells stimulated with Abs against CD3 and CD28 for 5 d by ELISA. Fig. 4D shows that, with this stimulus, KIR3DL2+ CD4 T cells from SpA patients produce 4-fold more IL-17 than KIR3DL2− CD4 T cells or KIR3DL2+ CD4 T cells from healthy B27− control subjects (mean ± SD; SpA KIR3DL2+...
25.9% of peripheral blood KIR3DL2+ CD4 T cells and 17.1% of blood KIR3DL2+ (KIR+) CD4 T cells from AS patients activated with anti-CD3 and anti-CD28 secrete more IL-17 than equivalent numbers of purified KIR3DL2+ CD4 T cells from patients with active AS (Fig. 5A). A total of 50 ± 25.9% of peripheral blood KIR3DL2+ CD4 T cells and 17.1 ± 11.4% of KIR3DL2+ CD4 T cells from B27+ healthy control subjects (HC) express IL-23R (n = 17). All data are presented as mean ± SD. A total of 45 ± 25.9% of peripheral blood KIR3DL2+ CD4 T cells and 17.1 ± 11.4% of KIR3DL2+ CD4 T cells from B27+ B27+ AS and 3 B27+ ReA patients) from SpA patients, and 29.5 ± 12.9% of KIR3DL2+ and 15.1 ± 5.7% of KIR3DL2+ CD4 T cells from B27+ HC express CCR6 (n = 17). D. Purified peripheral blood KIR3DL2+ (KIR+) CD4 T cells from AS patients activated with anti-CD3 and anti-CD28 secrete more IL-17 than equivalent numbers of purified KIR3DL2+ CD4 T cells from AS patients (KIR+) (left panel) and KIR3DL2+CD4 T (KIR+) from healthy B27+ (right panel). IL-17 secretion by KIR3DL2+ CD4 T is enhanced by coculture with IL-23 and IL-1, and this effect was more pronounced (>5-fold increase) for KIR3DL2+ CD4 T cells from SpA patients (Fig. 4A).

**KIR3DL2+ Th17 cells are polyfunctional, frequently producing TNF-α and IFN-γ.**

We next asked whether KIR3DL2+ IL-17–producing CD4 T cells were capable of producing additional cytokines. Fig. 5A shows that ex vivo-stimulated KIR3DL2+ Th17 from the peripheral blood of an AS patient also produce TNF-α and IFN-γ, in addition to IL-17. The majority of IL-17–producing KIR3DL2+ CD4 T cells also produced TNF-α. KIR3DL2+ Th17 were enriched for production of IFN-γ compared with KIR3DL2+ Th17 (Fig. 5A, 5B). Increased proportions of KIR3DL2+ CD4 T cells staining for both IL-17 and IFN-γ production were found in SpA patients compared with healthy B27+ and disease controls (Fig. 5B and data not shown). Fig. 5C shows a representative FACS stain for IL-17, TNF-α, and IFN-γ production by a KIR3DL2+CD4 T cell line from a B27+ ReA patient. Most IL-17–producing cells from FACS-sorted KIR3DL2+CD4 T cells from this and other SpA patients also produced TNF-α, with some also producing IFN-γ. KIR3DL2+ CD4 T cells also produced IL-17 after stimuli known to induce SpA, such as infection with *Salmonella typhimurium* or stimulation with anti-CD3 or superantigen (Supplemental Fig. 3).

**KIR3DL2+ Th17 cells are highly enriched in synovial fluid in SpA patients with active joint disease.**

Because the hallmark of SpA is inflammation of entheses and synovial joints, we next asked whether the joints of SpA patients were enriched for KIR3DL2+ Th17 cells. Fig. 6A shows that synovial fluid KIR3DL2+ CD4 T cells from the inflamed knee of a patient with AS produce IL-17. Ninety-five percent of IL-17–producing KIR3DL2+ CD4 T cells from AS patients and B27+ patients with acute ReA after *Salmonella* sp. or *Chlamydia* sp. infection. A total of 15.5 ± 4.4% of synovial CD4 T: 344 ± 221 versus 80.63 ± 81.7 pg/ml/50,000 cells for KIR3DL2–; KIR3DL2–CD4 T cells from healthy control subjects: 86.4 ± 32.5 pg/ml/50,000 cells, n = 7). By contrast with the KIR3DL2+ population, there was no difference in production of IL-17 between KIR3DL2+ CD4 T cells from AS patients and healthy control subjects. We also observed no difference in production of TNF-α and IFN-γ by this subset between patients and control subjects (data not shown). Thus, although KIR3DL2− cells constituted a minority of CD4 T cells, IL-17 production by this subset accounted for the majority of the increase in production of this cytokine in SpA patients (Fig. 4A, Supplemental Fig. 2A, 2B). FACS-sorted KIR3DL2+ CD4 T cells from AS patients secreted up to 58% of all CD4 T cell IL-17 (Supplemental Fig. 2A). IL-17 production was further enhanced by addition of the Th17-promoting cytokines IL-23 and IL-1, and this effect was more pronounced (>5-fold increase) for KIR3DL2+ cells from SpA patients (Fig. 4D).

SpA KIR3DL2+ Th17 T cells are polyfunctional, frequently producing TNF-α and IFN-γ.

We next asked whether KIR3DL2+ IL-17–producing CD4 T cells were capable of producing additional cytokines. Fig. 5A shows that ex vivo-stimulated KIR3DL2+ Th17 from the peripheral
fluid CD4 T cells expressed KIR3DL2, compared with 8.4 ± 1.4% of peripheral blood CD4 T from matched samples from these patients. In all but one case, SFMC were further enriched for IL-17 production and CD161 expression compared with peripheral blood, and all patients’ SFMC KIR3DL2+ CD4 T cells showed increased CCR6 and IL-23R expression. Synovial fluid KIR3DL2+ CD4 T cells also frequently produced more TNF-α and IFN-γ than matched peripheral blood KIR3DL2+ CD4 T cells (Fig. 6B). Increased proportions of KIR3DL2+ CD4 T cells producing both IL-17 and IFN-γ were found in the synovial fluid of patients compared with peripheral blood. Fig. 6C shows that FACS-sorted KIR3DL2+ CD4 T cells from SFMC produced more IL-17 than KIR3DL2− CD4 T cells, when stimulated with anti-CD3 and anti-CD28 for 5 d.

Discussion

The spondyloarthritides, of which AS is the most common, are a group of inflammatory arthritides strongly associated with possession of HLA-B27. In this article, we show that CD4 T cells expressing the killer cell Ig-like receptor KIR3DL2+ specifically survive, proliferate, and produce IL-17 on stimulation with cells expressing B27 dimers (termed B27γ). We also show that the KIR3DL2+ CD4 T cell subset is expanded and enriched for IL-17 production in the peripheral blood and synovial fluid of patients with SpA. Thus, IL-17–producing KIR3DL2+ CD4 T cells accounted for 70% of the observed increase in Th17 numbers in SpA patients (compared with control subjects), despite this subset comprising a mean of only 15% of CD4 T cells in the peripheral blood of SpA patients. Furthermore, KIR3DL2+ from AS patients secreted more IL-17 than KIR3DL2− lines and KIR3DL2+ CD4 T cells from healthy control subjects ex vivo after TCR stimulation.

An important role for Th17 cells in the pathogenesis of AS has recently been suggested by the strong genetic linkage with IL-23R polymorphisms (3), and by the findings of increased IL-17 levels and Th17 cell numbers in AS patients (7, 9, 10). IL-23R is typically, although not exclusively, expressed by Th17 cells. Strikingly, we show in this article that the KIR3DL2+ CD4 T population accounts for the majority of peripheral blood CD4 T cell IL-23R expression in SpA patients. We also show that IL-23 (together with IL-1) stimulates KIR3DL2+ CD4 T cell IL-17 production. Increased IL-23 expression has been demonstrated in the terminal ileum of AS patients (26), and IL-23 has been shown to be critical for maintenance of pathogenic Th17 cells producing IFN-γ in a murine colitis model (27). IL-17 acts on a variety of cell types to induce secretion of multiple proin-

FIGURE 6. SpA patients with active joint disease have increased numbers of KIR3DL2+ Th17 cells in synovial fluid. A, FACS comparison of IL-17, TNF-α, and IFN-γ production by PMA ionomycin activated KIR3DL2+ and KIR3DL2− CD4 T of synovial fluid leukocytes from an AS patient. B, Plot of comparative data from matched PBMC and SFMC samples from three AS and three ReA HLA-B27+ patients, showing expression of CCR6, CD161, and IL-23R, production of IL-17, TNF-α, and IFN-γ, and proportions of IL-17+IFN-γ+ cells by synovial fluid KIR3DL2+ CD4 T compared with peripheral blood KIR3DL2+ CD4 T cells. Significance was calculated using a Wilcoxon signed rank test. C, Equivalent numbers of FACS-purified synovial fluid KIR3DL2+CD4 T cells secreted more IL-17 than KIR3DL2− CD4 T (1904 ± 401 pg/ml/50,000 cells versus 403 ± 164 pg/ml, respectively; mean ± SD of values for three patients). Resting KIR3DL2− cells produced <50 pg/ml (data not shown).
flamatory cytokines, chemokines, and prostanoids (15–17). Our data support the concept that IL-17–producing T cells contribute to the pathogenesis of AS.

The B27-responsive IL-17–producing CD4 T cells described in this article carry Th17 phenotypic markers including CCR6, IL-23R, and CD161, but also commonly produce TNF-α and/or IFN-γ (furthermore, production of all three cytokines is increased in response to IL-23). There is now mounting evidence not only that such polyfunctional cells are common and increased in SpA, but that cells with an overlapping Th17 and Th1 phenotype may have increased pathogenicity (27, 28). Interestingly, the chemokine CCL20, a potent chemotactic factor for CCR6+ T cells, is increased in SpA (and RA) synovial fluid (29). Forty-five percent of KIR3DL2+ T cells in SpA patients in our study are CCR6+. CCL20 has been shown to be secreted by human articular chondrocytes on dynamic compression (30). This may be of relevance given the predilection of inflammation in SpA for the spine and entheses, areas of high mechanical stress.

We have previously shown that B27 can form H chain dimers (B272) (31). Unlike classical HLA-B27, B272 bind the KIR3DL2 (20, 22, 32). In this article, we show that B272 expressing APCs stimulate the survival, proliferation, and IL-17 production of KIR3DL2+ CD4 T cells. We provide evidence that this effect is mediated directly through the KIR3DL2/B272 interaction, because the effect is reduced by reducing B272 expression and inhibited by a KIR3DL2-specific mAb. The molecular basis requires further elucidation, although we show that cell death by apoptosis is reduced and cell proliferation is increased. We have observed similar antiapoptotic effects of KIR3DL2 ligation by B272 on the survival of NK cells and of CD4 T cells with seronegative spondylarthritides. Arthritis Rheum. 58: 2307–2317.

We show in this study that AS patients have increased KIR3DL2+ Th17 cells, confirming previous findings of increased numbers of circulating KIR3DL2+ CD4 T cells (22), and increased Th17 cells (9, 10), and for the first time, to our knowledge, link both these findings to each other. Expression of HLA-B27 likely explains both findings, because we have shown that KIR3DL2+ IL-17-secreting CD4 T cells selectively proliferate on coculture with B272-expressing cells. Our data are consistent with the hypothesis that Th17 cells play a significant role in AS pathogenesis. The mAb ustekinumab against the p40 subunit of IL-12 and IL-23 promoting APCs stimulate the survival, proliferation, and IL-17 effects on T cell polarization.

Acknowledgments
We thank the Thames Valley Comprehensive Local Research Network and Claire Farrar for patient recruitment, Jo Phillips for KIR3DL2 (DX31) mAb, and Prof. Brigitte Askonas and Sarah Rowland Jones for comments.

Disclosures
The authors have no financial conflicts of interest.

References


Figure 1S. Increased numbers of Th17 cells in the peripheral blood of AS patients compared with healthy B27- and disease controls. A Total numbers of Th17 cells per/million leukocytes are shown. AS 2981/10^6 PBMC +/-2398: B27- control 1751+/-1367 B27+control 2355+/-978; RA 2564+/-1529 UC 1889+/-2055. ANOVA not significant. (All data presented as mean+/-SD; p=0.03 students t test).

Figure 2S. KIR3DL2+ CD4 T cells from AS patients secrete more IL17 and comprise a greater proportion of total Th17 than in healthy B27- controls. A Representative FACS staining of KIR3DL2+ expression by peripheral blood CD4 T cells from an AS patient and healthy control (HC). Representative ELISAs show IL17 secreted by equivalent numbers (50,000 cells) of FACS-sorted KIR3DL2+ and KIR3DL2- CD4 T stimulated with anti-CD3 and anti-CD28 for 5 days. Numbers indicate pg/ml of IL17 secreted by each of the sorted populations. Percentages indicate the proportion of total CD4 T cell IL17 secreted by the KIR3DL2+ fraction. B Extrapolated mean values of IL17 production by KIR3DL2+ CD4 T and KIR3DL2-CD4 T in 500,000 cells in AS patients and healthy controls after calculating the amount of IL17 that would be produced by each population given the proportion of cells that express KIR3DL2 in the original unsorted sample. Significance calculated using an unpaired students t test (n=6).

Figure 3S. SpA KIR3DL2+ Th17 produce TNFα and IFNγ and make IL17 in response to diverse antigenic stimuli. A. Ex vivo ICS FACS stain of SFMC KIR3DL2+ CD4 T from a ReA patient showing production of IL17 and TNFα in response to overnight stimulation with s.typhimurium lipopolysaccharide (1μg/ml), or s.typhimurium infection (MOI 50 bacteria/cell). B. FACS of 2 KIR3DL2+ CD4 T cell lines showing production of IL17 in response to anti-CD3 and SEB stimulation.
Figure 1S

P = 0.03

Total CD4 IL17+ T/10^6 cells

AS, Control, Control B27+, UC, RA
Figure 2S

A

**AS**

58%

**HC**

5.2%

B

P=0.005 N=6
### TABLE I: PATIENTS IN THIS STUDY

<table>
<thead>
<tr>
<th></th>
<th>Spondyloarthritis (n=26)†</th>
<th>Healthy Controls (n=24)</th>
<th>Rheumatoid Arthritis (n=10)</th>
<th>Ulcerative Colitis (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range) years</td>
<td>44.3 (28-69)</td>
<td>40.8 (25-67)</td>
<td>50.8 (36-71)</td>
<td>54.5 (41-67)</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>22/4</td>
<td>15/9</td>
<td>4/6</td>
<td>7/3</td>
</tr>
<tr>
<td>HLA-B27+ no. (%)</td>
<td>25 (96)</td>
<td>6 (25)</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>Corticosteroid treatment, no. (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMARD use no. (%)</td>
<td>3 (11)</td>
<td>0</td>
<td>4/ (40)</td>
<td>9/(90)</td>
</tr>
<tr>
<td>Anti-TNF use no. (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

†Twenty three patients had Ankylosing Spondylitis (AS) and 3 had Reactive Arthritis. AS patients’ mean ± SD scores for the Bath AS Metrology Index, the Bath AS Functional Index, and the Bath AS Disease Activity Index, respectively were 4.1±2.4, 4.6±2.3, and 5.0±2.0. RA patients’ mean DAS ESR scores were 4.4±1.4. UC patients’ Montreal classification disease activity index and the UC disease activity index scores were respectively 0.6±7, and 1.5±2.1.