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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 spondyloarthritid (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 spondyloarthritides are a group of common inflammatory rheumatic diseases characterized by axial and peripheral arthritis, extraskelatal manifestations including uveitis, and an extremely strong association with HLA-B27 (B27) (1, 2). Ankylosing spondylitis (AS) is the most common of the spondyloarthritides, and 94% of patients are B27+ compared with 9.4% of control subjects, with an odds ratio of 171 (1). Reactive arthritis (ReA) is a related form of spondyloarthritis (SpA), typically following gastrointestinal or genitourinary infection with certain Gram-negative bacteria including Salmonella and Chlamydia species. The pathogenic role of B27 remains unknown (reviewed in Ref. 2). Like all classical class 1 proteins, HLA-B27 presents peptide Ags to CD8 T cells, but it also shows the unusual propensity to form H chain homodimers (“B272”). This is mediated by an unpaired cysteine at position 67, and we have shown that B27 dimers, unlike classical HLA-B27, interact with the killer-cell Ig-like receptor (KIR) KIR3DL2.

Recent genomewide association studies have implicated additional genes whose products are involved in innate and/or adaptive immune function, including the IL-23R (3). IL-23 is expressed by a subset of proinflammatory CD4 T cells known as Th17 cells (4), whose development is stimulated by cytokines including IL-23. The signature cytokine produced by these cells is IL-17A (henceforth termed IL-17). Increased levels of IL-17 have been described in serum (5, 6) and synovial fluid (7, 8). Two recent studies have found increased frequencies of Th17 cells in AS (9, 10). Th17 cells have been implicated in the pathogenesis of other autoimmune diseases including psoriasis, rheumatoid arthritis (RA), multiple sclerosis, and inflammatory bowel disease (11, 12). Th17 cells have been identified both in blood and at sites of inflammation including psoriatic skin (13) and rheumatoid joints (14), and have potent proinflammatory actions, including stimulation of human macrophages to produce TNF and IL-1β (15), and neutrophil recruitment (16, 17). Th17 cells also play an important role in protective immune responses to certain microbial pathogens such as Klebsiella pneumoniae (16) and Bordetella pertussis (18).

KIR are a family of MHC class I-binding receptors expressed by NK and minor subsets of T cells. KIR3DL2 is a three-Ig domain KIR whose cognate ligands include HLA-A3 and A11 (19). Our studies have shown that KIR3DL2 does not recognize HLA-B27/β2-microglobulin/peptide heterotrimeric complexes but binds to B272, which are expressed both on leukocytes of AS patients and on transfected cell lines (20, 21).

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Abbreviations used in this article: AS, ankylosing spondylitis; KIR, killer-cell Ig-like receptor; PB, Pacific Blue; ReA, reactive arthritis; SEB, staphylococcal enterotoxin B; SFMC, synovial fluid mononuclear cell; SpA, spondyloarthritis; UC, ulcerative colitis.

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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. Supplemental 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-B*27+ 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), staphylococcal enterotoxin B (SEB), 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 mM] 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 PE (Beckman Coulter), anti–IL-23R (FITC, FAB14001F; 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 CD158a, CD158b, and Z27 mAbs (BD Biosciences and Beckman Coulter). Dead cells, monocytes, and B cells were excluded by staining with PE-labeled antibodies (Invitrogen, anti-CD14, and anti-CD19 (BioLegend). Intracellular cytokine staining was performed using standard protocols (BD Biosciences) and anti–IL-17A (allophycocyanin/PE; ebioscience), anti–IFN-γ, and anti–TNF-α (FITC; BD Biosciences). Analysis was done with FlowJo software (version 8.8.6). A total of 0.5 × 10⁶ events were acquired, except cell viability and proliferation experiments, when all cells were acquired. IL-17+ KIR3DL2+ and IL-17+ KIR3DL2+ CD4 T cell numbers/ million cells were calculated after exclusion of doublets. The KIR3DL2+ CD4 T cell gate was set at 10× IgG2a background staining. ELISAs for IL-17 and IFN-γ were performed following the manufacturer’s instructions (eBioscience).

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.

Generation of T cell lines

FACS-sorted PBMC and SFMC KIR3DL2+ CD4 T lines were maintained as described previously (21). Short-term 5- to 10-d FACS-sorted CD4 T cells or negatively selected CD4 T cell lines (Miltenyi Biotec) were maintained by stimulation with anti-CD3 and anti-CD28 beads in DMEM with 10% FCS, supplements, and 40 IU/ml rIL-2 (D10/rIL-2) with or without rIL-23 and rIL-1 (10 ng/ml of each cytokine; R&D Systems).

Coculture of T cell lines with HLA-B27+–expressing APCs

LBL721.220 parental B lymphocyte-derived cell lines (220) transfected with B7, B27, B27 C67S, and B27 with human tapasin have been described previously (21, 24). CD4 T cells were labeled with CFSE following the manufacturer’s instructions (Invitrogen). A total of 100,000/500,000,000,000 irradiated 220 APCs were incubated with T cells (at a 1:1 ratio) and 100 ng/ml SEB (Sigma, Poole, U.K.) in 100 µl D10/rIL-2. Day 3 supernatants were taken for IL-17 and IFN-γ ELISA. On day 6, cells were stimulated with PMA and ionomycin for 5 h before FACS staining.

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 (B272+). We have shown previously that the LBL721.220 cells (hereafter abbreviated to 220), transfected with B27, protect KIR3DL2+ NK cells from apoptosis (22). This cell line lacks functional tapasin and expresses high levels of B272, 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. IA). 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 transfected with 220 or control 220 transfectants lacking B272 (220B27C67S), expressing reduced levels of B272 (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. IB). KIR3DL2–ve CD4 T cells and CD4 T cells expressing “other KIR” did not show the same stimulation of proliferation with 220B27 cells (Fig. IA 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 B272, 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 B272 (25).

Fig. 2A shows that bulk CD4 T cells from AS patients, stimulated with SEB presented by 220B27, produced more IL-17 than cells stimulated with control APCs with reduced or no B272.
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− 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+ control CD4 T cells also expressed more KIR3DL2+ than B27− control subjects (mean values ± SD; B27+ control subjects: 11.2 ± 2.3% compared with 7.9 ± 3.2% on B27− 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).

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-FACS-sorted KIR3DL2+ CD4 T cells from AS patients secreted cytokines by this subset between patients and control subjects (data not shown). Fig. 5C shows that sy-novial fluid KIR3DL2+ CD4 T cells 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).

**FIGURE 4.** KIR3DL2+ Th17 cells from SpA patients express high levels of IL-23R and CCR6, and respond to IL-23 and IL-1. A, FACS staining of PBMC from an AS patient for CCR6 and IL-23R expression. B, A total of 32.2 ± 25.2% of peripheral blood KIR3DL2+ CD4 T cells and 3.1 ± 3.4% of KIR3DL2+ (n = 19; 16 B27+ AS and 3 B27+ ReA patients) from SpA patients, and 15.1 ± 8.6% of KIR3DL2+ and 2.5 ± 2.9% of KIR3DL2+ CD4 T cells from B27+ healthy control subjects (HC) express IL-23R (n = 17). All data are presented as mean ± SD. C, A total of 45 ± 25.9% of peripheral blood KIR3DL2+ CD4 T cells and 17.1 ± 11.4% of KIR3DL2+ (n = 20; 17 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 (KIR+) (left panel) and KIR3DL2+CD4 T (KIR+) from healthy B27+ HC (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+ 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 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 also produced TNF-α, and 60% produced IFN-γ.

Fig. 6B shows data from paired peripheral blood and knee synovial fluid samples from the above and two further AS patients, together with three 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. 4D, 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).

**FIGURE 5.** SpA KIR3DL2+ Th17 produce TNF-α and IFN-γ in addition to IL-17. A, Representative ex vivo FACS stain of TNF-α, IFN-γ, and IL-17 production by peripheral blood KIR3DL2+ and KIR3DL2+ CD4 T cells from an AS patient activated with PMA and ionomycin. B, Proportions of FACS stained peripheral blood KIR3DL2+ and KIR3DL2+ CD4 T cells from SpA patients and B27+ healthy control subjects producing IL-17 and IFN-γ (%IL-17%IFN-γ). Data are presented as mean ± SD with ANOVA. C, TNF-α, IFN-γ, and IL-17 production by a KIR3DL2+ CD4 T cell line from a ReA patient activated with PMA and ionomycin. Representative of results from four different cell lines with two B27+ AS and two B27+ ReA patients.
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 subjects (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 proinflammatory cytokines.

**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).
flammatory 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 (B27α) (31). Unlike classical HLA-B27, B27α bind the KIR KIR3DL2 (20, 22, 32). In this article, we show that B27α 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/ B27α interaction, because the effect is reduced by reducing B27α 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 B27α on the survival of NK cells and of CD4 T cells that do not make IL-17 (22 and unpublished observations), so it is likely that B27α-KIR interactions primarily exert their effect by promoting leukocyte survival rather than other effects such as effects on T cell polarization.

In this study, we also show that some of these effects are also found in healthy B27 individuals. Thus, we found a modest expansion of total KIR3DL2+ and of KIR3DL2+ Th17 numbers in healthy HLA-B27+ individuals, without clear evidence of increased cytokine production. To our knowledge, these data show for the first time evidence of an immunological phenotype in healthy HLA-B27+ individuals, and further support a direct pathogenic role for HLA-B27. We propose that this increase in KIR3DL2+CD4 T cells, potentially skewed toward IL-17 production, may predispose to SpA.

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 B27α-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 has recently been shown to be effective in the treatment of a number of inflammatory diseases including psoriatic arthritis (33). Our study provides scientific rationale for treatment trials of anti-Th17 therapy in AS.

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Disclosures
The authors have no financial conflicts of interest.

References

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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

KIR3DL2 APC

CD3 PerCP

58%

HC

KIR3DL2 APC

CD3 PerCP

5.2%

**B**

P=0.005 N=6

IL17 pg/ml/500,000 cells

AS

Control

KIR3DL2+

KIR3DL2-
Figure 3S

A. Ex vivo KIR3DL2+ CD4 T Cell Lines

- Control
- +LPS
- +s. typhimurium
- +SEB

B. KIR3DL2+ CD4 T Cell Lines

- resting
- +αCD3

- CD3 PerCP
- CD3 PerCP
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<th><strong>Spondyloarthritis</strong> (n=26)†</th>
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†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.