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Th17 cells expressing KIR3DL2+ and responsive to HLA-B27 homodimers are increased in Ankylosing Spondylitis

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Abstract

CD4 helper T cells producing the pro-inflammatory cytokine IL17 (Th17) have been implicated in a number of inflammatory arthritides including the Spondyloarthritides. Th17 development is promoted by IL23. Ankylosing Spondylitis (AS), the commonest Spondyloarthritis, is genetically associated with both HLA-B27 (B27) and with IL23 receptor polymorphisms, however the link remains unexplained. We have previously shown that B27 can form heavy chain dimers (termed $B27_2$), which, unlike classical HLA-B27, bind the Killer-cell Immunoglobulin-like Receptor KIR3DL2**.** Here we show that $B27_2$-expressing antigen presenting cells stimulate the survival, proliferation and IL17 production of KIR3DL2+ CD4 T. KIR3DL2+ CD4 T cells are expanded and enriched for IL17 production in the blood and synovial fluid of patients with spondyloarthritis (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 subjects compared to controls. TCR-stimulated peripheral blood KIR3DL2+CD4 T cell lines from SpA patients secreted four fold more IL17 than KIR3DL2+ lines from controls or KIR3DL2- negative CD4 T. Strikingly, KIR3DL2+ CD4 T cells account for the majority of peripheral blood CD4 T cell IL23 receptor expression and produce more IL17 in the presence of IL23. Our findings link HLA-B27 with IL-17 production and suggest new therapeutic strategies in AS/SpA.

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INTRODUCTION

The Spondyloarthritides (SpA) are a group of common inflammatory rheumatic diseases characterised by axial and peripheral arthritis, extra-skeletal manifestations including uveitis, and an extremely strong association with the Human Leucocyte Antigen HLA-B27 (B27) (1, 2). Ankylosing Spondylitis (AS) is the commonest SpA and 94% of patients are B27+ compared to 9.4% controls, with odds ratio 171 (1). Reactive Arthritis is a related form of SpA that follows gastrointestinal or genitourinary infection with certain gram negative bacteria including Salmonella and Chlamydia species. The pathogenic role of B27 remains unknown (reviewed in (2)). Like all classical class 1 proteins, HLA-B27 presents peptide antigens to CD8 T cells, but it also shows the unusual propensity to form heavy chain homodimers ("$B27_2$"). 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 immunoglobulin-like receptor KIR3DL2.

Recent genomewide association studies have implicated additional genes whose products are involved in innate and/or adaptive immune function, including the IL23 receptor (IL23R) (3). IL23R is expressed by a subset of pro-inflammatory CD4 T cells known as Th17 cells (4), whose development is stimulated by cytokines including IL23. The signature cytokine produced by these cells is IL17A (henceforth termed IL17). Increased levels of IL-17 have been described in AS 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 pro-inflammatory actions, including stimulation of human macrophages to produce TNF and IL-1 beta (15), and neutrophil recruitment (16, 17). Th17 cells also play an important role in protective immune responses to certain microbial pathogens such as Klebsiella pnemoniae [16] and Bordetella pertussis (18).

Killer-cell Immunoglobulin-like Receptors (KIR) are a family of MHC class 1-binding receptors expressed by natural killer (NK) and minor subsets of T cells. KIR3DL2 is a three immunoglobulin domain KIR whose cognate ligands include HLA-A3 and A11 (19). Our studies have shown that KIR3DL2 does not recognize HLA-B27/beta 2 microglobulin/peptide heterotrimeric complexes but binds to $B27_2$ which are expressed both on leukocytes of AS patients and on transfected cell lines (20, 21).

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 $B27_2$ has not been defined. KIR ligation by HLA class 1 can inhibit activation induced cell death (AICD) of NK and T cells (22, 23). Thus we hypothesized that interaction of $B27_2$ with KIR3DL2 expressed on CD4 T cells might have a net pro-inflammatory effect by promoting T cell survival and subsequent cytokine production. We asked if KIR3DL2 interaction with $B27_2$ affected the proliferation, survival and cytokine production of antigen-stimulated CD4 T cells. We also investigated to what degree KIR3DL2+ expressing CD4 T cells contributed to production of IL17 in AS.

Here we show that $B27_2$- expressing antigen presenting cells stimulate the survival and proliferation of superantigen-activated KIR3DL2+expressing CD4 T which can produce IL17. KIR3DL2+ CD4 T cells are expanded, enriched for IL17 production and account for the majority of CD4 T cell IL23R 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

15 ml heparinized venous blood were obtained from 25 patients with Ankylosing Spondylitis fulfilling the modified New York criteria. Table S1 shows patient demographics and medication. None had received anti-TNF therapy. Paired knee synovial fluid samples were obtained from 3 AS and 3 acute Reactive Arthritis patients. Of 28 SpA patients, 22 were male and 27 were HLA-B*2705+ve by molecular typing. 17 healthy HLA-B27− and 6 HLA-B*2705+ healthy controls were studied. As inflammatory disease controls we studied 10 patients with Rheumatoid Arthritis and 10 patients with Ulcerative Colitis without spondyloarthritis. Appropriate ethical permission (COREC 06/Q1606/139 and Oxfordshire Research Ethics Committee B – 07/Q1605/35) and individual informed consent was obtained.

FACS and ELISA analysis

Peripheral blood and synovial fluid mononuclear cells were separated by density gradient centrifugation. Cells were stimulated with PMA and ionomycin for 6 hours or overnight with anti-CD3 and -CD28 beads (miltenyi), Staphylococcal enterotoxin B or LPS (100ng/ml) with brefeldin A (1μg/ml; all Sigma). Cells were infected with salmonella typhimurium araA−/− (MOI 10 bacteria/cell) for 1 hour in antibiotic-free medium, before culture overnight at 37°C, 5% CO_2 in RPM1640 containing 10% FCS, antibiotics (penicillin, streptomycin (200mM) and gentamicin sulfate 50μg/ml) and L-glutamine (2.5mM). Cells were stained for surface markers (anti-IgG2a, -DX31, -CD3 PerCP; BD Biosciences), anti-CD4 pacific blue(PB)/ FITC (Biolegend), -CD14 PB, -CD56 or -CD161 PE (Beckman Coulter), -IL-23R (FITC ; R and D systems, FAB14001F) and -CCR6 PE (BD biosciences). KIR3DL2 mAb DX31 was a kind gift of Jo Phillips (DNAX, USA). 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 PB live-dead stain (Invitrogen), anti-CD14 and -CD19 (Biolegend). Intracellular cytokine staining (ICS) was performed using standard protocols (BD Biosciences) and anti-IL-17A (APC/PE; Ebioscience), -IFNγ and -TNFα (FITC; BD biosciences). Analysis was done with FlowJo software (version 8.8.6). 0.5×10^6 events were acquired, except cell viability and proliferation experiments, when all cells were acquired. IL-17+ KIR3DL2+ and IL17+KIR3DL2− CD4 T cell numbers/million cells were calculated after exclusion of doublets. The KIR3DL2+ CD4 T cell gate was set at 10x IgG2a background staining. ELISAs for IL-17 and IFNγ were performed following manufacturers instructions (Ebioscience).

Statistical analysis used graph pad prism version 4 software. Based on the work of Shen *et al* (10), a sample size of 26 patients and 18 B27-controls was chosen in order to achieve an alpha value of 5% in comparisons of IL17 producting KIR3DL2+ CD4 T. One-tailed unpaired T tests with welch’s correction and Anova analysis of variances with a bonferroni post test were performed where shown.

Generation of T cell lines

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

Coculture of T cell lines with HLA-B27-expressing antigen presenting cells (APCs)

LBL.721.220 parental B lymphocyte-derived cell lines (220) transfected with B7, B27, B27 C67S, and B27 with human tapasin have been described (21, 24). CD4 T cells were labelled with CFSE following the manufacturer's instructions (Invitrogen). 100,000/500,0000 γ-irradiated 220 APCs were incubated with T cells (at a 1:1 ratio) and 100ng/ml SEB (Sigma, UK) in 100μl D10/rIL-2. Day 3 supernatants were taken for IL-17 and IFNγ ELISA. On day 6 cells were stimulated with PMA and ionomycin for 5 hours before FACS staining.

RESULTS

Enhanced proliferation, survival and IL17 production of KIR3DL2+ CD4 T cells stimulated with antigen presenting cells expressing HLA-B27 homodimers

We first asked if superantigen-stimulated KIR3DL2+ Th17 T cells could be preferentially expanded in the presence of cells expressing HLA-B27 heavy chain homodimers ($B27_2$). 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 $B27_2$, the levels being abrogated by mutation of the unpaired cysteine at position 67 to serine and reduced 2 fold if tapasin is overexpressed by co-transfection (21). CFSE-labelled CD4 T cells from AS patients and controls were cultured with staphylococcal enterotoxin B (SEB) and equal numbers of 220 cells transfected with B27 or control HLA for five days. 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 recognising KIR3DL1/DS1, KIR2DL1/DS1 and KIR2DL2/3/2DS2, Figure 1A). The greatest proliferation was observed in the KIR3DL2+ population stimulated with SEB and $B27_2$+ expressing 220B27. Less proliferation of KIR3DL2+ cells was seen when CD4 T were stimulated with 220 or control 220 transfectants lacking $B27_2$ (220B27C67S), expressing reduced levels of $B27_2$ (220B27 HuTPN, 220B27 transfected with human tapasin) or expressing other HLA class 1 (220B7). The percentage of viable CD4 T cells expressing KIR3DL2 was consistently higher when CD4 T cells from patients with ankylosing spondylitis (AS) were stimulated with SEB and 220B27 (Figure 1B). KIR3DL2-CD4 T cells and CD4 T cells expressing "other KIR" did not show the same stimulation of proliferation with 220B27 cells (Figure 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 (Figure 1C). HC10, an antibody to HLA-class I heavy chains which also recognises $B27_2$, also inhibited cell survival (data not shown). LBL.721.221 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 $B27_2$ (25)).

Figure 2A shows that bulk CD4 T cells from AS patients, stimulated with SEB presented by 220B27, produced more IL17 than cells stimulated with control APC with reduced or no $B27_2$ expression. Figure 2B shows that KIR3DL2+ CD4 T produce more IL17 under these stimulation conditions. Figure 2C shows that, for a KIR3DL2+ CD4 T cell line, both cell

survival (total number) and numbers of cells producing IL17 upon stimulation can be inhibited by DX31 mAb.

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+ spondyloarthritis (SpA) patients, compared to healthy controls and rheumatoid arthritis (RA) patients (22). We asked if these cells were capable of producing IL-17 *ex vivo*. We first studied 26 SpA patients (24 with Ankylosing Spondylitis (AS), 2 with Reactive Arthritis, 25 B27+), together with 6 B27 + and 18 B27 – healthy controls, and 10 RA and 10 Ulcerative Colitis (UC) patients without SpA as inflammatory disease controls. The patient demographics are shown in Table S1. Figure 3A shows enumeration by Intracellular Cytokine Staining (ICS) and FACS of IL17-producing CD4 T (Th17) cells from the peripheral blood of a representative AS patient and a B27− healthy control. 3.31% of AS KIR3DL2+ CD4 T cells produced IL17 following stimulation with PMA and ionomycin *ex vivo*, as compared with only 1.54% of KIR3DL2− CD4 T cells. For the healthy control, 1.09% of KIR3DL2+ and 0.45% of KIR3DL2− CD4 T cells produced IL17. Since 16.5% of patient CD4 T cells patient expressed KIR3DL2, this corresponds to 30% of all the Th17 cells in this particular AS patient. By contrast for this particular healthy control only 6.4% of IL17 producing CD4 T cells expressed KIR3DL2. Figure 3B shows that KIR3DL2+CD4 T contain a significantly higher proportion of IL17-producing cells for both SpA patients and, to a lesser extent, healthy controls. We observed no difference in IFNγ production by KIR3DL2+ CD4 T between SpA patients and healthy controls (Figure 3B). By contrast with IL17 , KIR3DL2+ CD4 T from SpA patients were not enriched for TNFα production compared to KIR3DL2− CD4 T (data not shown).

Figure 3C shows that the absolute number of peripheral blood KIR3DL2+ Th17 cells was also increased in SpA patients compared to healthy B27− controls, and RA and UC inflammatory disease controls. We also studied six B27+ healthy controls. Both the absolute numbers (Figure 3C) and percentages of KIR3DL2+Th17 were increased compared to 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 controls (B27+ controls; 11.2 %+/−2.3 compared with 7.9%+/− 3.2 on B27− controls p=0.01; SpA patients 15.12 %+/− 10, p=0.0018 compared with B27− controls; mean values+/−SD). Fig. 3D shows that the proportion of Th17 cells expressing KIR3DL2 was increased both in SpA patients and healthy B27+ controls 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, the observed expansion within the KIR3DL2 population accounted for 70% of the total difference in Th17 numbers between SpA patients and controls. 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− controls but to a lesser extent than the absolute number of Th17 cells in the KIR3DL2+ CD4 T cell population (Figure 1S).

We next determined if IL17-producing KIR3DL2+ CD4 T cells expressed phenotypic markers consistent with the Th17 phenotype. Figure 4A shows a representative FACS stain from an AS patient of IL23R and CCR6 expression by KIR3DL2+ and KIR3DL2− CD4 T cells. In this patient 72% of KIR3DL2+ CD4 T cells coexpressed IL23 receptor and CCR6 compared with 5% of KIR3DL2− CD4 T. Overall a mean of 32% of KIR3DL2+ CD4 T cells expressed IL23 receptor, compared to only 3.1% of KIR3DL2−ve CD4 T in the SpA patients studied (Figure 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 IL23R+ CD4 T cells in SpA patients. By contrast, a lower proportion of KIR3DL2+ CD4 T cells

from B27− controls expressed IL23R (15%; Figure 4B). There was no difference in the proportion of KIR3DL2− CD4 T expressing IL23R between SpA patients and healthy controls (Fig 4B). In addition to IL23R, KIR3DL2+ CD4 T cells were enriched for expression of CCR6 and CD161 Th17 phenotypic markers (Fig. 4C and data not shown). By contrast we observed no difference in the proportion of KIR3DL2+ CD4 T expressing CD161 (data not shown).

We wished to investigate if KIR3DL2+ cells produced more IL17 than KIR3DL2− CD4 T cells in response to TCR ligation and whether KIR3DL2+ production of IL17 was promoted by the Th17 cytokines IL23 and IL1. We first studied IL17 secretion by equivalent numbers of FACS-sorted KIR3DL2+ and KIR3DL2− CD4 T stimulated with antibodies against CD3 and CD28 for 5 days by ELISA. Figure 4D shows that, with this stimulus, KIR3DL2+ CD4 T cells from SpA patients produce four fold more IL-17 than KIR3DL2− CD4 T cells or KIR3DL2+ CD4 T cells from healthy B27− controls (SpA KIR3DL2+ CD4 T 344±221 pg/ml/50,000 cells vs 80.63±81.7 pg/ml/50,000 cells for KIR3DL2−; KIR3DL2+CD4 T from healthy controls 86.4±32.5 pg/ml/50,000 cells, n=7; mean ±SD). By contrast with the KIR3DL2+ population, there was no difference in production of IL17 between KIR3DL2− CD4 T cells from AS patients and healthy controls. We also observed no difference in production of TNFα and IFNγ by this subset between patients and controls (data not shown). Thus, although KIR3DL2+ cells constituted a minority of CD4 T, IL17 production by this subset accounted for the majority of the increase in production of this cytokine in SpA patients. FACS-sorted KIR3DL2+ CD4 T cells from AS patients secreted up to 58% of all CD4 T cell IL17 (Figure 2SA). IL17 production was further enhanced by addition of the Th17 promoting cytokines IL23 and IL-1, and this effect was more pronounced (>5 fold increase) for KIR3DL2+ cells from AS patients (Fig. 4D).

SpA KIR3DL2+ Th17 T cells are polyfunctional, frequently producing TNFα and IFNγ

We next asked if KIR3DL2+ IL17-producing CD4 T cells were capable of producing additional cytokines. Figure 5A shows that *ex vivo* stimulated KIR3DL2+ Th17 from the peripheral blood of an AS patient also produce TNFα and IFNγ in addition to IL17. The majority of IL17 producing KIR3DL2+ CD4 T cells also produced TNFα. KIR3DL2+Th17 were enriched for production of IFNγ compared to KIR3DL2− Th17 (Figure 5A and 5B). Increased proportions of KIR3DL2+ CD4 T cells staining for both IL17 and IFNγ production were found in SpA patients compared to healthy B27− and disease controls (Figure. 5B and data not shown). Figure 5C shows a representative FACS stain for IL17, TNFα and IFNγ production by a KIR3DL2+CD4 T cell line from a B27+ ReA patient. Most IL17-producing cells from FACS sorted KIR3DL2+CD4 T cells from this and other SpA patients also produced TNFα with some producing IFNγ in addition.

KIR3DL2+ CD4+ T cells also produced IL17 following stimuli known to induce spondyloarthritis such as infection with *salmonella typhimurium* or stimulation with anti-CD3 or superantigen (Figure 3S).

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

Since the hallmark of SpA is inflammation of entheses and synovial joints, we next asked if the joints of SpA patients were enriched for KIR3DL2+ Th17 cells. Figure 6A shows that synovial fluid KIR3DL2+ CD4 T cells from the inflamed knee of a patient with AS produce IL17. 95% of IL17 producing KIR3DL2+ CD4 T cells also produced TNFα, and 60% produced IFNγ.

Figure 6B shows data from paired peripheral blood and knee synovial fluid samples from the above and 2 further AS patients, together with 3 B27+ patients with acute Reactive Arthritis following Salmonella sp. or Chlamydia sp. infection. 15.5±4.4% of synovial fluid CD4 T cells expressed KIR3DL2, compared to 8.4±1.4% of peripheral blood CD4 T from matched samples from these patients. In all but one case synovial fluid mononuclear cells (SFMC) were further enriched for IL17 production and CD161 expression compared with peripheral blood, and all patients' SFMC KIR3DL2+ CD4 T showed increased CCR6 and IL23R expression. Synovial fluid KIR3DL2+ CD4 T cells also frequently produced more TNFα and IFNγ than matched peripheral blood KIR3DL2+ CD4 T (Figure 6B). Increased proportions of KIR3DL2+ CD4 T producing both IL17 and IFNγ were found in the synovial fluid of patients compared with peripheral blood.

Figure 6C shows that FACS-sorted KIR3DL2+CD4 T from SFMC produced more IL17 than KIR3DL2− CD4 T cells, when stimulated with anti CD3 and anti-CD28 for 5 days.

DISCUSSION

The Spondyloarthritides (SpA), of which Ankylosing Spondylitis (AS) is the commonest, are a group of inflammatory arthritides strongly associated with possession of HLA-B27. Here we show that CD4 T cells expressing the Killer Immunoglobulin Receptor KIR3DL2+ specifically survive proliferate and produce IL17 upon stimulation with cells expressing B27 dimers (termed $B27_2$). We also show that the KIR3DL2+ CD4 T cell subset is expanded and enriched for IL17 production in the peripheral blood and synovial fluid of patients with SpA. Thus IL17-producing KIR3DL2+ CD4 T cells accounted for 70% of the observed increase in Th17 numbers in SpA subjects (compared to controls), despite this subset comprising a mean of only 15% of CD4 T in the peripheral blood of SpA patients. Furthermore KIR3DL2+ from AS patients secreted more IL17 than KIR3DL2− lines and KIR3DL2+ CD4 T from healthy controls *ex vivo* following TCR-stimulation.

An important role for Th17 cells in the pathogenesis of AS has recently been suggested by the strong genetic linkage with IL23R polymorphisms (3), and by the findings of raised IL17 levels and Th17 cell numbers in AS patients (7, 9, 10). IL23R is typically although not exclusively expressed by Th17 cells. Strikingly we show here that the KIR3DL2+ CD4 T population accounts for the majority of peripheral blood CD4 T cell IL23 receptor expression in SpA patients. We have also shown that IL23 (together with IL1) stimulates KIR3DL2+ CD4 T cell IL17 production. Increased IL23 expression has been demonstrated in the terminal ileum of AS patients (26), and IL23 has been shown to be critical for maintenance of pathogenic Th17 cells producing IFNγ in a murine colitis model (27). IL17 acts on a variety of cell types to induce secretion of multiple pro-inflammatory cytokines, chemokines and prostanoids (15-17). Our data support the concept that IL17-producing T cells contribute to the pathogenesis of AS.

The B27-responsive IL17-producing CD4 T cells described here carry Th17 phenotypic markers including CCR6, IL23R and CD161, but also commonly produce TNFα and/or Interferonγ (furthermore production of all three cytokines is increased in response to IL23). There is now mounting evidence not only that such polyfunctional cells are common and increased in SpA, but also 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 elevated in SpA (and RA) synovial fluid (29). 45% of KIR3DL2+T cells in SpA patients in our study are CCR6+. CCL20 has been shown to be secreted by human articular chondrocytes upon 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 heavy chain dimers ($B27_2$) (31). Unlike classical HLA-B27, $B27_2$ bind the Killer-cell Immunoglobulin-like Receptor KIR3DL2 (20, 22, 32). Here we show that $B27_2$-expressing antigen presenting cells stimulate the survival, proliferation and IL17 production of KIR3DL2+ CD4 T. We provide evidence that this effect is mediated directly through the KIR3DL2/ $B27_2$ interaction, since the effect is reduced by reducing $B27_2$ expression and inhibited by a KIR3DL2-specific monoclonal antibody. The molecular basis requires further elucidation, although we show that cell death by apoptosis is reduced and that cell proliferation is increased. We have observed similar anti-apoptotic effects of KIR3DL2 ligation by $B27_2$ on the survival of NK cells and of CD4 T cells that do not make IL17 ((22) and unpublished data), so it is likely that $B27_2$ -KIR interactions primarily exert their effect by promoting leukocyte survival rather than other effects such as effects on T cell polarisation.

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. 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 towards IL17 production, may predispose to SpA.

We show here 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 link both these findings to each other. Expression of HLA-B27 likely explains both findings, since we have shown that KIR3DL2+ IL17-secreting CD4 T cells selectively proliferate upon co-culture with $B27_2$-expressing cells. Our data are consistent with the hypothesis that Th17 cells play a significant role in Ankylosing Spondylitis pathogenesis. The monoclonal antibody ustekinumab against the p40 subunit of IL12 and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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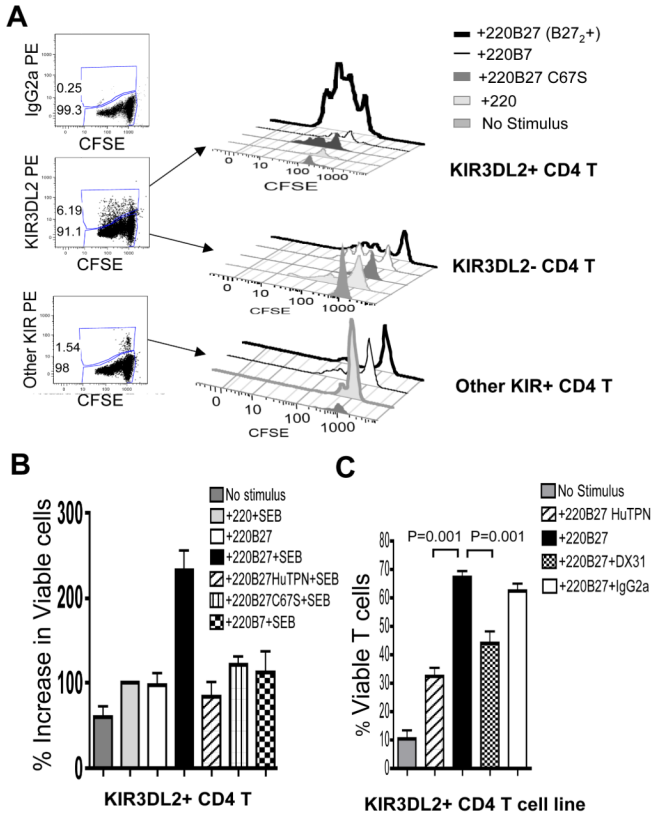

Figure 1. Enhanced proliferation and survival of KIR3DL2+ CD4 T cells stimulated with SEB and antigen presenting cells expressing HLA-B27 homodimers ($B27_2$)

A. Left hand panels. Live CD4+ CFSE+ from a healthy B27− control were gated on KIR3DL2+ , KIR3DL2−, or "other" KIR+. Right hand panels. Proliferation of KIR3DL2+ , KIR3DL2− and CD4 T cells expressing "other" KIR, with SEB presented by 220B27 or control 220B7, 220B27C67S and 220 cells. Representative of 12 experiments with 6 patients and 3 controls. **B.** % Increase in viable KIR3DL2+ CD4 T cells after stimulation with SEB and 220B27 or control APC compared with stimulation with 220 APC. Data from 6 independent experiments with 6 patients. 220B27 HuTPN: 220 transfected with HLA-B27 and human tapasin express reduced levels of $B27_2$. *ANOVA for T +220B27 + SEB compared with other stimuli $p<0.0001$. **C.** %viable CFSE+ T cells after stimulation of FACS-purified KIR3DL2+ CD4 T cells with SEB and control 220B27 HuTPN (32.4±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 % viable cells± s.e.m. Representative of seven independent experiments with T cell lines from 3 AS patients.

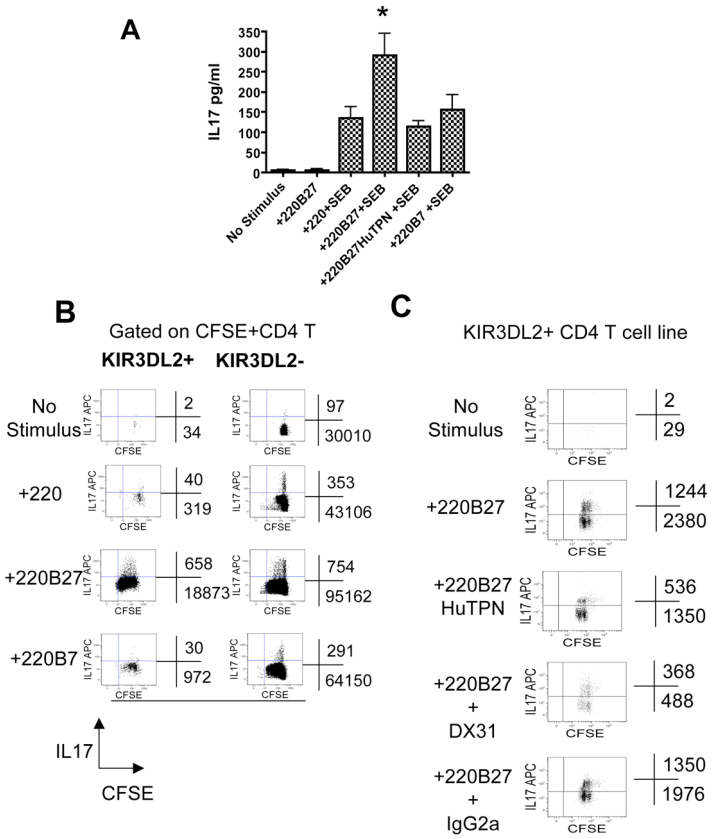

Figure 2. KIR3DL2+ CD4 T cells show enhanced IL17 production upon activation with $B27_2$-expressing 220B27 cells

A. IL17 ELISA showing CD4 T cells stimulated with SEB and $B27_2$-expressing (220B27) APC produce more IL17 than cells with SEB and control APC. Data from 5 independent experiments with 5 AS patients; results expressed as mean ± s.e.m . $p<0.05$ students t test for all samples. (No stimulus 5.9± 1.5; 220B27 5.9± 3.8 ; 220+SEB 134± 29; 220B27+SEB 298± 57; 220B27HuTPN 113± 15; 220B7+SEB 164±42 pg/ml). **B.** Total numbers of viable IL17+ KIR3DL2+ CD4 T cells from an AS patient after 5 day stimulation of CD4 T with SEB and $B27_2$-expressing (220B27) APC compared to stimulation with SEB and control APC (220, 220B7 and 220B27 HuTPN). Representative of data from three patients and two controls. **C.** Intracellular cytokine staining for IL17 of a purified KIR3DL2+ CD4 T cell line after 5 day 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.

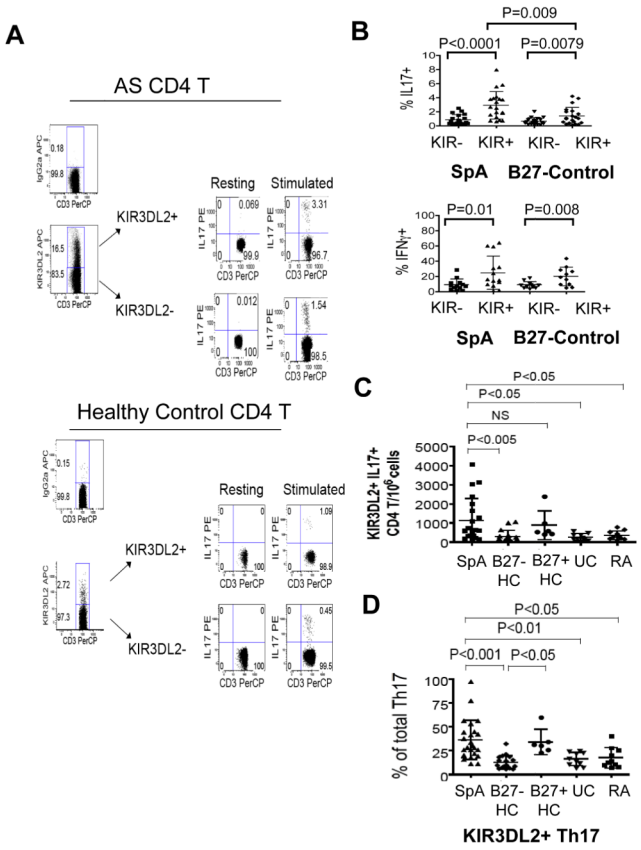

Figure 3. KIR3DL2+ CD4 T cells from AS PBMC show enhanced IL17 production

A. Representative FACS plot showing IL-17 production by KIR3DL2+ and - CD4 T lymphocytes from an Ankylosing Spondylitis (AS) patient (upper) and B27− control (lower panels), both resting and following PMA/ionomycin stimulation. **B.** (Upper panel) Percentage of KIR3DL2+ (KIR+) and KIR3DL2− (KIR−) CD4+ T cells producing IL17 (IL17+) in patients with spondyloarthritis (SpA) (n=26) compared to B27− healthy controls (n=18). (Lower panel) Percentage of KIR3DL2+ (KIR+) and KIR3DL2− (KIR−) CD4 T cells producing IFNγ in SpA patients and B27-controls. P values calculated using a students T test. **C.** Total number of KIR3DL2+ IL17+ CD4 T cells/10^6 peripheral blood mononuclear cells in SpA patients (n=26) compared to B27− (n=18) and B27+ (n=6) healthy controls (HC) and inflammatory disease controls with Ulcerative colitis (UC) (n=10) or Rheumatoid Arthritis (RA) (n=10). IL17+ CD4 T cells were enumerated following PMA/ionomycin stimulation. SpA 1141/10^6 PBMC +/− 1010; B27-HC 286+/−322; B27+HC 952+/−758; RA 352+/−235; UC 264+/−180. Individual datapoints and mean ± SD are shown in B and C. with ANOVA. **D.** Proportion of total Th17 expressing KIR3DL2 in SpA patients and healthy B27− (HC), healthy B27+ (HC), RA and UC controls. (Mean values+/−SD with ANOVA).

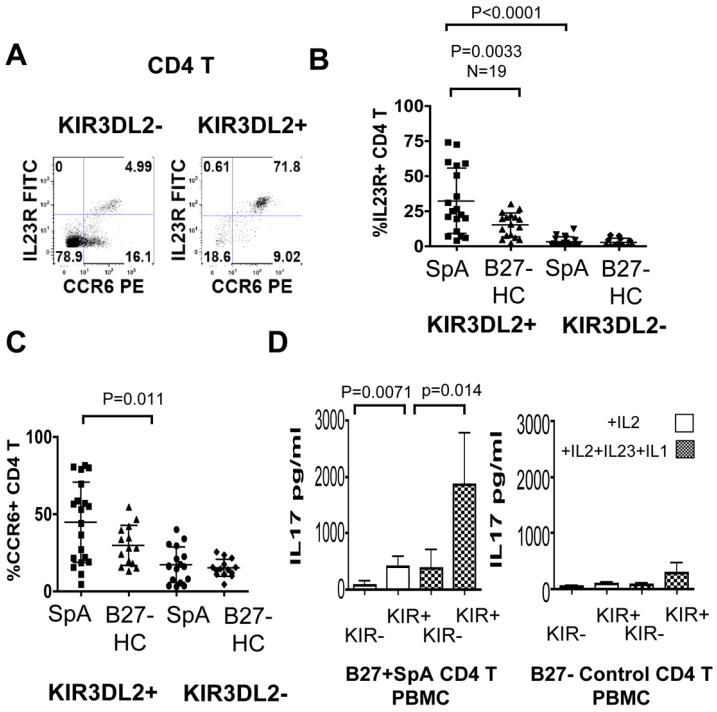

Figure 4. KIR3DL2+Th17 cells from SpA patients express high levels of IL23 receptor and CCR6 and respond to IL23 and IL1

A. FACS staining of PBMCs from an AS patient for CCR6 and IL23R expression. **B.** 32.2±23.2% 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 KIR3DL2+ and 2.5+/−2.9 KIR3DL2− CD4 T cells from healthy B27− healthy controls (HC) express IL23R (n=17). (All data presented as mean+/−SD). **C.** 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% KIR3DL2− CD4 T cells from B27− healthy controls 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 IL17 than equivalent numbers of purified KIR3DL2− CD4 T (KIR−) (left hand panel) and KIR3DL2+CD4 T (KIR+) from healthy B27− healthy controls (right hand panel). IL17 secretion by KIR3DL2+ CD4 T is enhanced by co-culture with IL23 and IL1 to a greater degree than secretion by KIR3DL2− CD4 T (1750±386.6 pg/ml/50,000 cells vs 294.6 ±135.3 pg/ml/50,000 cells; p values calculated using a students T test. Data from 7 patients with AS and 7 healthy controls. All data presented as mean+/−SD).

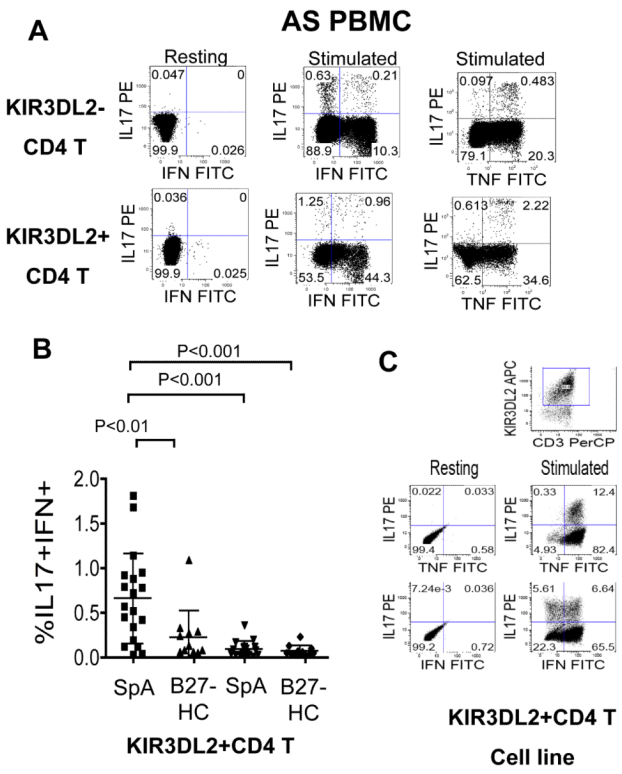

Figure 5. SpA KIR3DL2+ Th17 produce TNFα and IFNγ in addtion to IL17

A. Representative *ex vivo* FACS stain of TNFα, IFNγ and IL17 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 controls producing IL17 and IFNγ (%IL17+IFNγ+). Data presented as mean+/−SD with ANOVA. **C.** TNFα, IFNγ and IL17 production by a KIR3DL2+ CD4 T cell line from a ReA patient activated with PMA and ionomycin. (Representative of results from 4 different cell lines with 2 B27+AS and 2 B27+ ReA patients).

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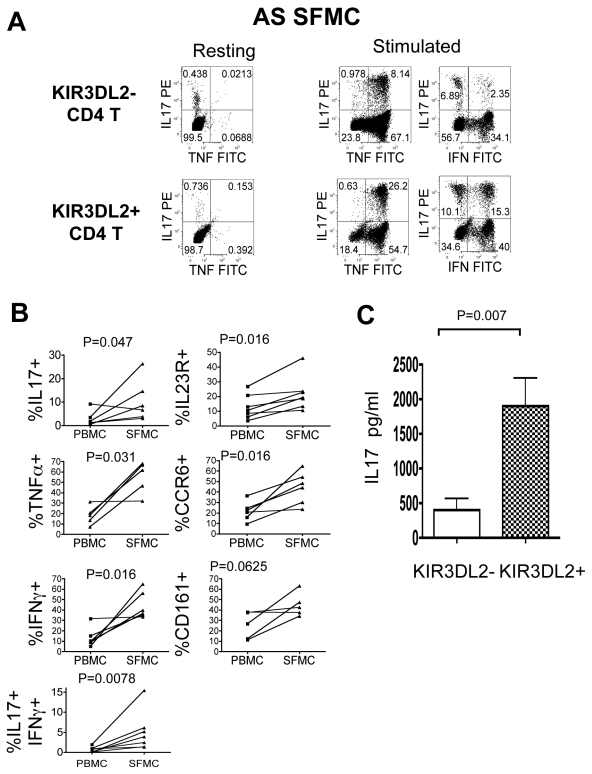

Figure 6. SpA patients with active joint disease have increased numbers of KIR3DL2+ Th17 cells in synovial fluid

A. FACS comparison of IL17, TNFα and IFNγ production by PMA ionomycin activated KIR3DL2+ and KIR3DL2− CD4 T of from synovial fluid leucocytes from an AS patient. **B**. Plot of comparative data from matched peripheral blood (PBMC) and synovial fluid mononuclear cell (SFMC) samples from 3 AS and 3 reactive arthritis (ReA) HLA-B27+ patients, showing expression of CCR6, CD161 and IL23R and production of IL17, TNFα and IFNγ and proportions of IL17+IFNγ+cells by synovial fluid KIR3DL2+ CD4 T compared to peripheral blood KIR3DL2+ CD4 T. Significance calculated using a wilcoxon signed rank test. **C**. Equivalent numbers of FACS-purified synovial fluid KIR3DL2+CD4 T cells secreted more IL17 than KIR3DL2− CD4 T (1904+/−401 pg/ml/50,000 cells vs 403+/−164 pg/ml respectively; mean ±SD of values for 3 patients). Resting KIR3DL2− cells produced <50pg/ml (data not shown).

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