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Evidence that tissue resident human enthesis $\gamma\delta$ T-cells can produce IL-17A independently of IL-23R transcript expression

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ABSTRACT

Objectives Murine models of interleukin (IL)-23-driven spondyloarthritis (SpA) have demonstrated enthesal accumulation of $\gamma\delta$ T-cells which were responsible for the majority of local IL-17A production. However, IL-23 blockers are ineffective in axial inflammation in man. This study investigated $\gamma\delta$ T-cell subsets in the normal human enthesis to explore the biology of the IL-23/17 axis.

Methods Human spinous processes enthesal soft tissue (EST) and peri-enthesal bone (PEB) were harvested during elective orthopaedic procedures. Enthesal $\gamma\delta$ T-cells were evaluated using immunohistochemistry and isolated and characterised using flow cytometry. RNA was isolated from $\gamma\delta$ T-cell subsets and analysed by qPCR. Enthesal $\gamma\delta$ T-cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, anti-CD3/28 or IL-23 and IL-17A production was measured by high-sensitivity ELISA and qPCR.

Results Enthesal $\gamma\delta$ T-cells were confirmed immunohistochemically with V δ 1 and V δ 2 subsets that are cytometrically defined. Transcript profiles of both cell populations suggested tissue residency and immunomodulatory status. Enthesal V δ 2 cells expressed high relative abundance of IL-23/17-associated transcripts including IL-23R, RORC and CCR6, whereas the V δ 1 subset almost completely lacked detectable IL-23R transcript. Following PMA stimulation IL-17A was detectable in both V δ 1 and V δ 2 subsets, and following CD3/CD28 stimulation both subsets showed IL-17A and IL-17F transcripts with neither transcript being detectable in the V δ 1 subset following IL-23 stimulation.

Conclusion Spinal enthesal V δ 1 and V δ 2 subsets are tissue resident cells with inducible IL-17A production with evidence that the V δ 1 subset does so independently of IL-23R expression.

INTRODUCTION

The spondyloarthropathies (SpA) are a group of inflammatory diseases of which ankylosing spondylitis (AS) is often referred to as the prototypic member.¹ Mechanical strain is likely to be an important environmental risk factor² which may interact with various genetic elements to initiate disease. Although dysregulation of the interleukin (IL)-23 signalling pathway is accepted as an important risk factor for SpA development,^{3,4} the

Key messages**What is already known about this subject?**

- Enthesal resident $\gamma\delta$ T-cells drive enthesitis in an interleukin (IL)-23 overexpression animal model of spondyloarthritis (SpA) but very little is known about $\gamma\delta$ T-cells at the human enthesis.
- IL-17 but not IL-23 inhibition is effective in the treatment of ankylosing spondylitis.

What does this study add?

- This study identifies tissue resident populations of $\gamma\delta$ T-cells in the healthy enthesis that have transcript expression related to tissue repair and immunomodulation and identifies a subset of which is able to produce IL-17 independently of IL-23R expression.

How might this impact on clinical practice or future developments?

- First comprehensive evaluation of human enthesal $\gamma\delta$ T-cells, delivering insights into the mechanism underlying effective treatment of SpA.
- Highlights $\gamma\delta$ T-cells and IL-23 independent IL-17 production mechanisms as potential targets of future therapeutic strategies.

precise mechanism as to how IL-23 drives disease has not been elucidated. A crucial role for enthesitis in SpA pathogenesis is supported by observations in murine models where either TNF α or IL-23/17 pathway dysregulation leads to inflammation that spreads to adjacent synovium and bone.^{5–8} A poorly defined non-conventional population of lymphocytes was originally shown to be responsible for this murine enthesal pathology.⁶ It was subsequently shown that an enthesal resident population of $\gamma\delta$ T-cells were the primary source of IL-17A consequent to IL-23 overexpression.⁹

$\gamma\delta$ T-cells are non-conventional T-cells that express the $\gamma\delta$ form of the T-cell receptor (TCR) rather than the $\alpha\beta$ form expressed by most T lymphocytes.¹⁰ In comparison to $\alpha\beta$ T-cells, $\gamma\delta$ T-cells have a limited repertoire of V gene segment rearrangements and are thought to participate more extensively in innate immunity and homeostatic processes.^{10,11} In



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adults, $\gamma\delta$ T-cells expressing a V δ 1 domain constitute a minority of the blood $\gamma\delta$ T-cell population and are reported to recognise several unconventional MHC superfamily members which may present lipids or are stress induced.¹⁰ The V δ 2 subsets are by contrast the major subsets present in the blood and react to pyrophosphate molecules.¹² However, both subsets have been shown to be capable of producing IL-17¹³ and mouse studies have shown the importance of the IL-17 producing $\gamma\delta$ T-cells in wound healing and osteogenesis.¹⁴

Recent studies have pointed to the non-efficacy of anti-IL-23 therapy for ankylosing spondylitis,¹⁰ whereas anti-IL-17A therapy is effective in AS.^{15 16} A potential explanation for this surprising finding is that early experimental SpA, but not established disease, is dependent on IL-23,¹⁷ which points to the importance of innate immunity in disease onset. Therefore, this study investigated whether the normal human enthesis had resident entheseal $\gamma\delta$ T-cells, key cells in innate immune responses and focused on the IL-23/17 axis.

METHODS

Participants and samples

Human entheseal soft tissue (EST) and peri-entheseal bone (PEB) (figure 1A) were harvested as previously described¹⁸ from normal spinous process and interspinous ligament of 34 patients (14 men, 25 women, median age 53) undergoing spinal surgery at the Leeds General Infirmary for correction of scoliosis or spinal decompression of thoracic or lumbar vertebrae. Entheseal tissue donors were not known or suspected to have any systemic inflammatory condition including SpA.

Blood was collected from 20 patients with SpA (15 men, 5 women, median age 44) as well as 14 healthy controls (6 men, 8 women, median age 38). To determine whether $\gamma\delta$ T-cells might be present at sites of entheses damage, tissue was procured from the peri-entheseal rupture site of patients undergoing surgical repair of ruptured Achilles' tendons (n=3). The investigation was approved by North West-Greater Manchester West Research Ethics Committee and Leeds East Research Ethics Committee. Patients gave informed consent in accordance with the declaration of Helsinki.

Immunohistochemistry

Enthesis samples were fixed by incubation in 4% paraformaldehyde solution for 24 hours and then decalcified by incubation in 0.5 M EDTA solution. Histological sections of decalcified, entheseal tissue were stained using the Envision (Dako) immunohistochemistry staining kit. Slides were incubated with anti-TCR δ antibody (online supplementary table 1), diluted 1:20 in antibody diluent (Dako) and incubated for 1 hour at room temperature. Staining then proceeded as previously described.¹⁸

Cell staining prior to sorting and flow cytometry

EST was separated from PEB and both were enzymatically digested as previously described,¹⁸ following which, erythrocytes were removed by incubation in ammonium chloride buffer. Prior to incubation with antibodies, cells were incubated in a blocking buffer (10% mouse serum and 1% human IgG in PBS) for 15 min at room temperature. All antibody incubations were performed at room temperature for 15 min (online supplementary table 1). Cells were sorted using an Influx (BD) cell sorter directly into RNA extraction buffer supplied as a component of the PicoPure RNA isolation kit (Thermo Fisher), which was then used throughout for RNA isolation.

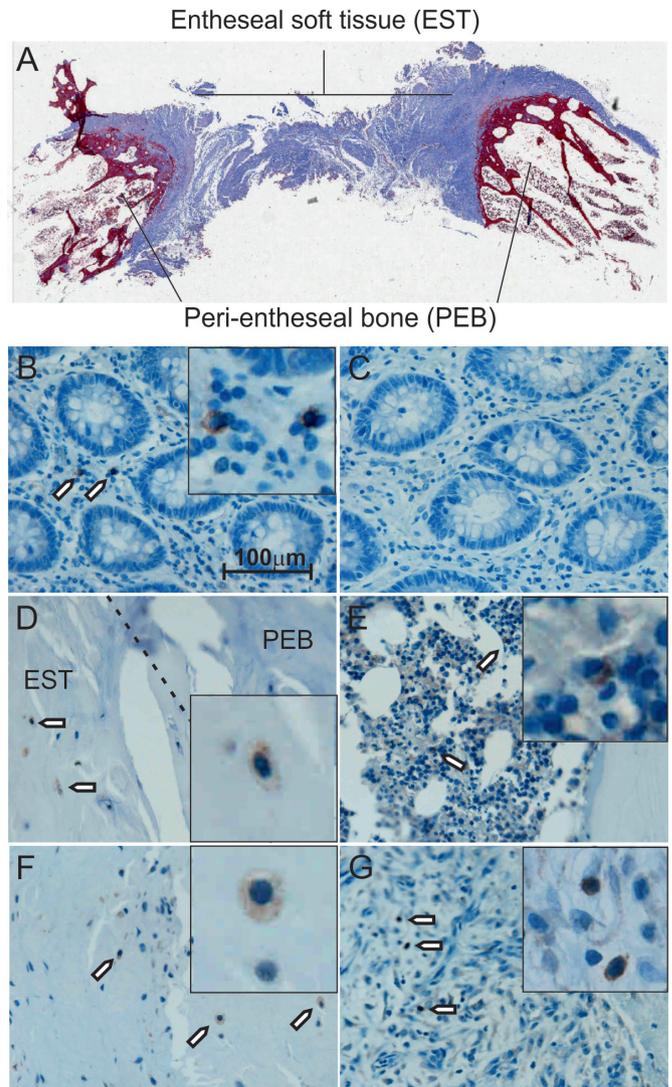


Figure 1 $\gamma\delta$ T-cell localisation and phenotyping. Masson's trichrome stained section showing the area of the spine harvested for analysis. Outer edges of the spinous process are labelled peri-entheseal bone (PEB) and the interspinous ligament labelled entheseal soft tissue (EST) (A). Immunohistochemistry showing $\gamma\delta$ T-cell receptor expression in human colon tissue, inset shows high power image (B). Staining is absent with omission of the primary antibody (C). Positive staining is observed in the border between the EST and the PEB (D) as well as in the haematopoietic bone marrow (E) and deeper within the EST (F). Inflammatory infiltrate of ruptured Achilles also contains positively stained cells (G). Brown colour and arrows indicate regions of positive staining.

For phenotypic characterisation, cells from each subset were categorised as naïve (CD45RA^{hi}, CD45RO^{lo}) tissue resident memory (CD45RA^{lo}, CD45RO^{hi}, CD69^{hi}, CD103^{hi}, CCR7^{lo}), effector memory (CD45RA^{lo}, CD45RO^{hi}, CCR7^{lo}) and central memory (CD45RA^{lo}, CD45RO^{hi}, CCR7^{hi}).

Magnetic bead enrichment and stimulation assays

For magnetic bead enrichment, mononuclear cells were isolated using Lymphoprep (Axis Shield) and blocked as previously described. Cells were incubated with biotinylated antibodies for 15 min at room temperature, then incubated with anti-biotin microbeads (Miltenyi) for 15 min at 4°C and isolated by two rounds of magnetic separation using MS columns (Miltenyi). For

post-enrichment activation, subsets were stimulated with PMA/ionomycin as above or with a combination of anti-CD3/CD28 or 100 ng/mL IL-23 for 48 hours.

Transcript analysis

Transcript analysis was performed by qPCR using the Biomark HD gene expression system (Fluidigm). Briefly, cDNA was reverse transcribed using reverse transcription master mix (Fluidigm) and then underwent pre-amplification (18 cycles) using a pre-amp master mix (Fluidigm) with a solution containing all primer sets. Gene expression was then measured using a dynamic array integrated fluidic circuit (Fluidigm), Taqman gene expression assays and universal Taqman master mix (both ThermoFisher). We focused on genes involved in tissue repair, cytokine signalling and signal transduction, pattern recognition and chemokine signalling. A subset of samples were also analysed for expression of genes associated with tissue residence (online supplementary table 2). Expression of all genes was measured relative to *HPRT*, and qPCR for PMA-induced cytokine expression was performed using a Quant Studio real-time PCR system (Applied Biosystems).

Measurement of IL-17A by high-sensitivity ELISA

The $\gamma\delta$ subsets ($\delta 1$ and $\delta 2$) were isolated as previously described. Cells were stimulated with PMA (25 $\mu\text{g/mL}$) and ionomycin (1 $\mu\text{g/mL}$) for 1 hour in standard culture conditions. The supernatant was removed and replaced with fresh media (RPMI 1640) and the cells were returned to standard culture conditions for 24 hours. Cell supernatant was tested for IL-17A protein using IL-17A high-sensitivity (0.25–16 pg/mL sensitivity range) ELISA (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol.

Statistics

An independent sample Mann-Whitney U test was used to detect differences in $\gamma\delta$ T-cell subset distribution between EST, PEB and peripheral blood (PB). The Kruskal-Wallis test was used to detect the differences between expression of tissue residency associated transcripts and IL-23/IL-17 axis transcripts in $\gamma\delta$ T-cell subsets from EST, PEB and PB. To compare the overall changes in transcript abundance between $\gamma\delta$ T-cells from enthesal tissue and PB, all $\gamma\delta$ T-cell subsets in both EST and PEB were grouped together and compared with all $\gamma\delta$ T-cell subsets in PB and a Mann-Whitney U test was used to detect statistical difference. A Wilcoxon signed rank test was used to detect statistical difference in intracellular cytokine protein expression and a Mann-Whitney U test was used to detect changes in cytokine transcript expression following PMA stimulation. Unless otherwise stated, all box plots display median—line, box—IQR and whiskers—range, all bar graphs display median—box, whiskers—IQR. All graphs were generated using Prism V.7 (GraphPad), and all statistical tests were performed using SPSS V.21 (IBM).

RESULTS

Immunohistochemistry

Positive staining for the $\gamma\delta$ TCR was observed in control human colon tissue in the stromal compartment (figure 1B), and $\gamma\delta$ T-cells were rare as is consistent with previously published research.¹⁹ The negative control (omission of the primary antibody) showed a total lack of positive staining (figure 1C). $\gamma\delta$ T-cells were observed in enthesal tissue at the bone/soft tissue border (figure 1D) as well as in the PEB anchoring region haematopoietic bone marrow (figure 1E) and in the soft tissue of the ligament (figure 1F). $\gamma\delta$ T-cells were also observed in inflammatory

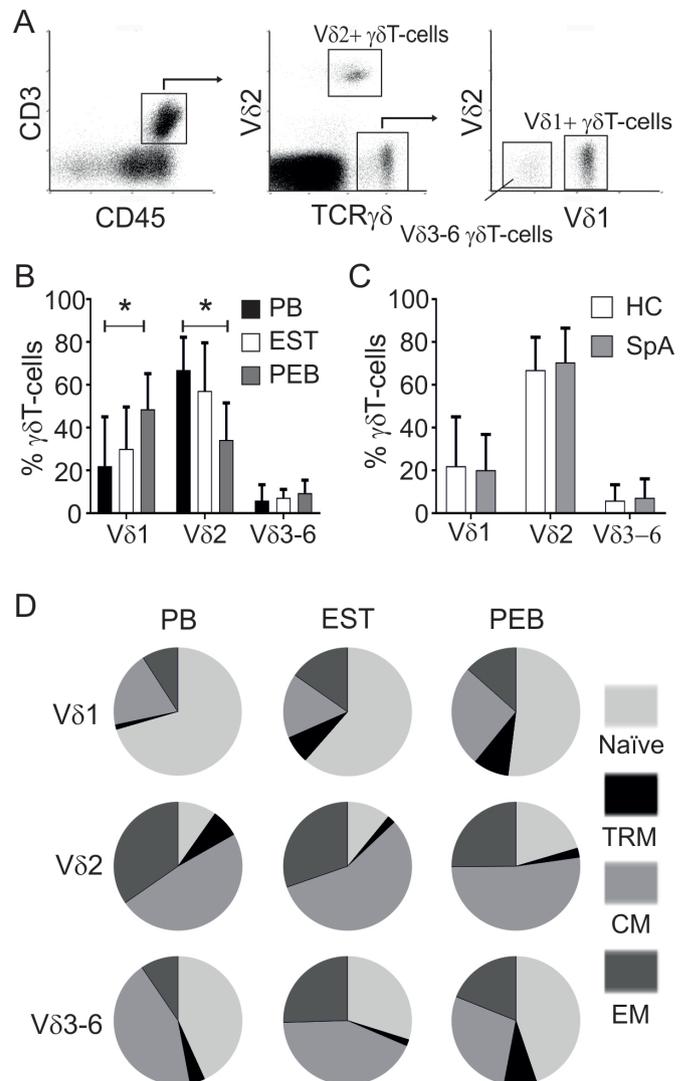


Figure 2 $\gamma\delta$ T-cell phenotyping in blood and enthesis using flow cytometry. $\gamma\delta$ T-cells were identified based on positive expression of CD45 and CD3 and positive expression of the $\gamma\delta$ T-cell receptor, and then subdivided based on the V δ isoform of the receptor expressed (A). In enthesal tissue (n=11), peri-enthesal bone contained a significantly higher proportion of the V δ 1 and a lower proportion of the V δ 2 expressing cells compared with healthy control blood (n=14) (B). There was no difference in subset proportion in spondyloarthritis patients (n=20) compared with healthy controls (C). Analysis of $\gamma\delta$ subsets showing naive, tissue resident memory (TRM), central memory (CM) and effector memory phenotypes (EM) (D). *P<0.05.

infiltrate in ruptured Achilles tissue, also indicating their presence at the sites of injury (figure 1G).

Phenotypic characterisation

For flow cytometry analysis, live cells were discriminated based on 7-aminoactinomycin D (7-AAD) exclusion. T-cells were selected based on positive expression of CD45 and CD3 with a pan- $\gamma\delta$ TCR antibody used in combination with V δ 1 and V δ 2 isoform-specific antibodies identified V δ 1⁺, V δ 2⁺ and V δ 3-6 (V δ 1, V δ 2 double negative) $\gamma\delta$ T-cell subsets (figure 2A). Healthy EST (n=11) contained a similar proportion of $\gamma\delta$ subsets (37% V δ 1 and 57% V δ 2) as was observed in peripheral blood (n=14) (22% V δ 1 and 67% V δ 2). PEB contained a higher proportion of the V δ 1 subset and a lower proportion of the

V δ 2 subset (49% and 34%, $p=0.031$ and 0.027 , respectively). The proportion of $\gamma\delta$ T-cells which were V δ 1, V δ 2 double negative was consistently low ($\sim 7\%$) with no significant difference observed between groups (figure 2B). Blood from patients with PsA or AS ($n=20$) showed no difference in the proportion of each subset compared with healthy controls (figure 2C). Similarly no significant difference was observed in patients' blood with active disease compared with those in remission (data not shown).

Additional phenotypic characterisation for markers of naïve and memory T-cell subsets showed differences between $\gamma\delta$ T-cell subsets and changes associated with tissue origin. The V δ 1 subset had a far greater proportion of cells with a naïve phenotype compared with the V δ 2 irrespective of tissue origin ($p=0.001$) and the V δ 1 subset from PEB contained a greater proportion of the tissue resident memory phenotype compared those from blood ($p=0.43$) (figure 2D).

Transcriptional profile for tissue residency and immunomodulatory status

Analysis of transcripts associated with tissue residency showed significantly increased expression in enthesal derived V δ 1, V δ 2 and V δ 3–6 subsets ($n=12$) compared with those derived from peripheral blood ($n=6$). *TGF β 1* was increased on average 5.4-fold ($p=0.010$, 0.012 and 0.014 , respectively) and *NR4A1*, 7.4-fold ($p=0.005$, 0.016 and 0.041 , respectively).²⁰ This pattern was reversed in genes with reported lower expression in tissue resident T-cells,²⁰ with lower expression in enthesal derived V δ 1, V δ 2 and V δ 3–6 subsets of *KLF2*, 533-fold ($p=0.004$, 0.034 and 0.009 , respectively) and *TBX21* 6.9-fold ($p=0.036$ and 0.011 , respectively) although in this case only in enthesal derived V δ 1 and V δ 2 subsets (figure 3A).

Transcriptional analysis of all $\gamma\delta$ T-cell subsets derived from enthesal tissue ($n=12$) compared with those derived from blood ($n=6$) showed increased expression of growth factor transcripts including *BMP-2* and *VEGFA* ($p=0.008$ and <0.001), as well as immunomodulatory factors including *IL-10*, *TGF β* and aryl hydrocarbon receptor (*AHR*) (all $p<0.001$). All subsets strongly expressed JAK/STAT signal transduction genes and had little or undetectable expression of *IL-17A*, *IL-17F* and *IL-22* (Figure 3B and online supplementary figure 1).

Different IL-23/17 axis transcriptional profile between V δ 1 and V δ 2 cells

STAT3 expression was significantly increased in V δ 1, and V δ 3–6 tissue derived subsets in comparison with age matched blood (both $p=0.003$) with a strong trend were observed in the V δ 2 subset ($p=0.065$, figure 4A). Expression of *RORC*, *IL-23R* and *CCR6* (figure 4B–D) was consistently higher in the V δ 2 subset compared with V δ 1. Although loss of detectable expression in low expressing subsets rendered statistical analysis problematic, significance was achieved in *CCR6* expression in PEB ($p=0.004$). *IL-23R* expression was consistently detected in the V δ 2 subset but was largely absent in enthesal derived V δ 1 and V δ 3–6 subsets. *IL-23R* transcript was detected at a low level in 1 of 12 samples in each case in EST and in one sample in the V δ 3–6 subset in PEB (figure 4C).

IL-17 production in $\gamma\delta$ T-cell subsets

Next, the ability of enthesal $\gamma\delta$ T-cell subsets to produce the pro-inflammatory cytokines IL-17A, IL-17F, IL-22 and TNF α was assessed using ELISA and qPCR. In PMA and ionomycin stimulated $\gamma\delta$ T-cell subsets, TNF α transcript expression was

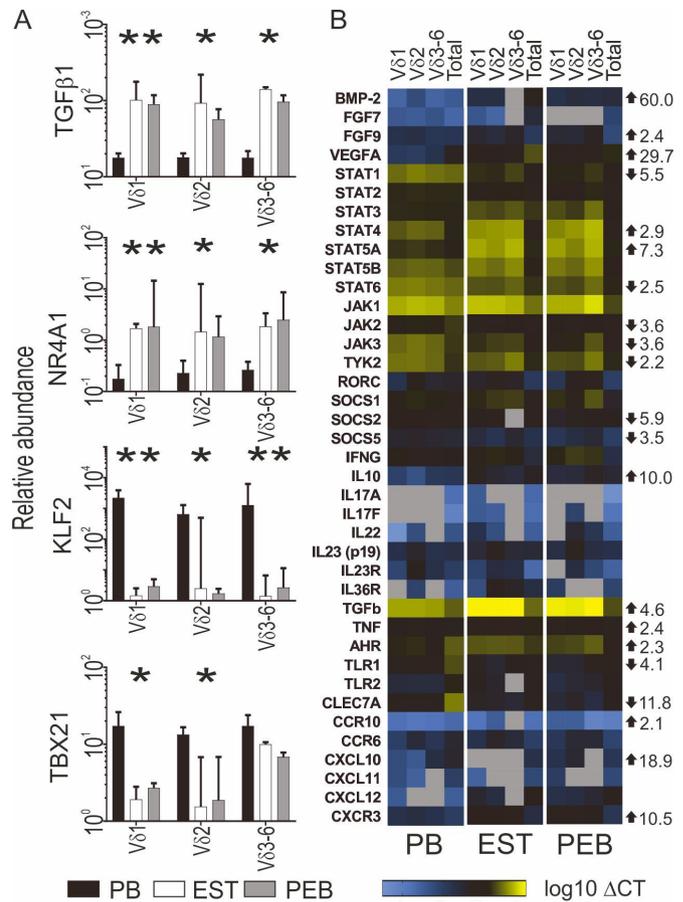


Figure 3 $\gamma\delta$ T-cells in enthesis and blood are transcriptionally distinct. Unmatched enthesal tissue derived subsets were compared with healthy blood derived cells. They had significantly higher expression of transforming growth factor β 1 (TGF β 1), nuclear receptor subfamily 4 group a member 1 (Nr4a1) and lower expression of Kruppel-like factor 2 (KLF2) and T-box 21 (TBX21) (A). All $\gamma\delta$ T-cell subsets expressed high levels of signal transduction molecules and immunomodulatory genes. Expression of IL-23/IL-17 axis cytokines was low or absent. Colour denotes relative expression to HPRT blue-low, black-equal, yellow-high, grey-below detection. Arrows indicate higher expression in $\gamma\delta$ T-cells (all subsets) from enthesal tissue (EST and PEB) compared with blood. Numbers show difference in median relative abundance. The 'un-sorted' category represents gene expression in an unsorted mixture of all cells released from enthesal digests (B) (PEB $n=12$, EST $n=12$, PB $n=6$). * $P<0.05$, ** $P<0.01$. EST, enthesal soft tissue; PEB, peri-enthesal bone.

significantly increased in V δ 1 and V δ 2 subsets ($p=0.001$ and 0.002 , respectively) (figure 5A). IL-17A was not detected without stimulation but was detected following stimulation (figure 5A) in both V δ 1 and V δ 2 subsets. Additionally, high-sensitivity ELISA confirmed an increase in IL-17A production in both subsets on PMA/ionomycin stimulation in the V δ 1 fraction, the mean basal level was 0.70 pg/mL and this rose to 1.60 pg/mL (2.28-fold). In the V δ 2 fraction, basal level was 15.56 pg/mL and this rose to 23.30 pg/mL (1.49-fold) (figure 5B). However, the low number of cells made accurate determination of cell number problematic, therefore although the number of input cells used in stimulated and unstimulated conditions was comparable within subsets, caution should be used in comparing IL-17A concentrations between V δ subsets.

Next we assessed the impact of more physiological stimuli on both V δ 1 and V δ 2 subsets expression of IL-17A, IL-17F and

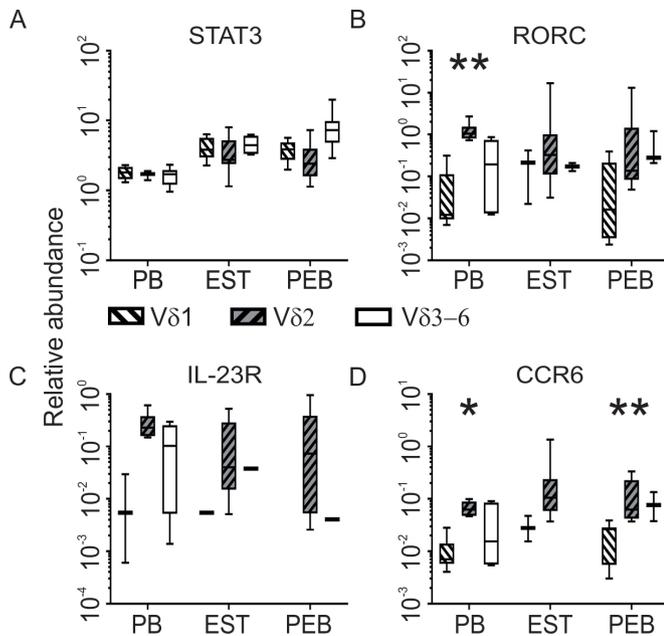


Figure 4 The Vδ2 subset expressed higher levels of genes involved or associated with IL-23-driven IL-17 signalling. Enthesal tissue derived subsets had generally higher expression of STAT3 compared with blood (A) and the Vδ2 subset had the highest expression of RORC (B), IL-23R (C) and CCR6 transcript (D) (PEB n=12, EST n=12, PB n=6). *P<0.05, **P<0.01. EST, enthesal soft tissue; PEB, peri-enthesal bone.

IL-22 cytokine transcripts. Following stimulation with a combination of anti-CD3/CD28, robust detection of IL-17A, IL-17F and IL-22 transcripts was observed in Vδ1 and Vδ2 subsets

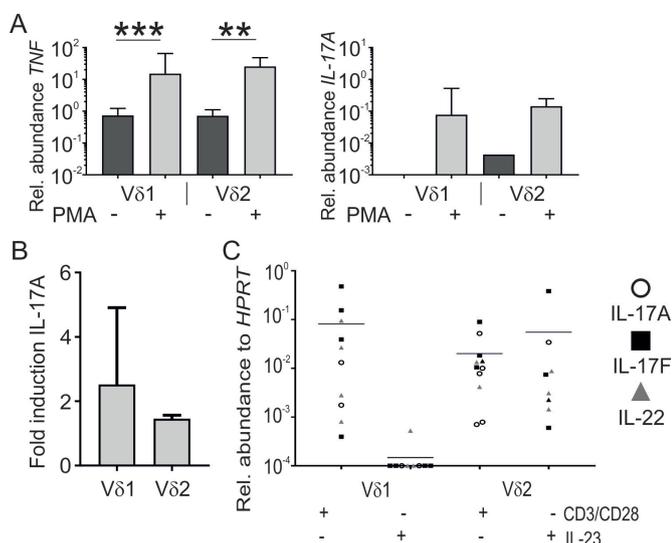


Figure 5 TNF α and IL-17A are produced by both $\gamma\delta$ T-cell subsets quantitative PCR showing TNF α and IL-17A transcript expression in $\gamma\delta$ T-cell subsets with or without stimulation (n=8) (A). Fold induction of secreted IL-17A protein following PMA/ionomycin stimulation compared with unstimulated fraction of purified Vδ1 and Vδ2 subsets (n=3) bar shows that mean whiskers represent 1 SD (B). Transcript expression of IL-17A, IL-17F and IL-22 following 48-hour stimulation of Vδ1 and Vδ2 subsets with combined anti-CD3/CD28 or IL-23 stimulation (n=10). Line denotes mean expression (all genes combined) (C). Genes for which a housekeeping value was obtained, but for which a target value was not, were assigned a value of 0.0001, as 0 cannot be plotted on a log scale. *P<0.05, **P<0.01, ***P≤0.001.

(figure 5C). In contrast, IL-23 stimulation had almost no effect in the Vδ1 subset but caused a marked increase in the Vδ2 subset (figure 5C). Finally, we ascertained whether this equated with measurable IL-17A protein detection using the highly sensitive ELISA. Unlike PMA stimulation, we could not detect changes in IL-17A protein using either CD3/CD28 or IL-23 stimulation (online supplementary file 5)).

DISCUSSION

The $\gamma\delta$ T-cell population is responsible for the majority of the IL-17A produced at the enthesis in IL-23 dependent murine models of SpA.^{6,9} Herein, we describe $\gamma\delta$ T-cell populations at the normal and injured human enthesis by immunohistochemistry and flow cytometry and provide evidence supporting their enthesal residency. We defined both Vδ1 and Vδ2 enthesal resident cells but only Vδ2 subset consistently expressed high levels of transcripts associated with IL-17/IL-23 axis cytokine signalling, namely RORC, IL-23R and CCR6.²¹ To the best of our knowledge, this is the first description of enthesal resident $\gamma\delta$ T-cells in man and fits in with the knowledge of their role in skin and gut disease in general and other recent studies from peripheral blood and synovial fluid in spondyloarthritis pointing to a key role in disease.²²

Following PMA stimulation, IL-17A production was also detected in both the Vδ1 and Vδ2 populations by high-sensitivity ELISA and this was confirmed by qPCR. Furthermore, qPCR on both subpopulations stimulated with anti-CD3/CD28 or IL-23 confirmed that IL-17A, IL-17F and IL-22 production could be driven in the absence of IL-23 and supports the assertion that the Vδ1 subset do not express IL-23R under basal conditions in enthesal tissue. Since IL-23R transcript expression was absent in 11 of 12 Vδ1 subset isolates, this suggests a potential for this population to produce IL-17A in an IL-23 independent manner. We demonstrated IL-17A protein production in both populations using PMA/ionomycin although this is a non-physiological stimulus. Nevertheless, PMA been used extensively to demonstrate the functional characteristics of cells although we acknowledge that the potential functional drives in vivo in man need to be defined.

Also, in the present work, we noted low level IL-23R transcript expression in the peripheral blood Vδ1 subset. It has also been previously shown that IL-23R expression is upregulated following anti-CD3/CD28 stimulation of Th17⁺ cells, thus meaning any potential IL-17 secretion independent of IL-23 is hard to prove in the current setting.²³ This highlights the potential plasticity of these cells and may explain differences observed between circulating and enthesal resident $\gamma\delta$ T-cells. It also suggests that IL-23 cannot be excluded from driving IL-17A, IL-17F and IL-22 production in these cells in the disease environment.

A lower proportion of the Vδ2 subset was observed in enthesal tissue compared with blood suggesting potential enrichment of a tissue homing phenotype, since $\gamma\delta$ T-cells are known to migrate and reside in tissues based on specific isoform expression.¹¹ Alternatively, this may represent the expansion of a resident population. We did not observe a change in the distribution of subsets in the blood of SpA patients where the subset distribution was similar to healthy controls (figure 2). Analysis of enthesal $\gamma\delta$ T-cell transcripts that have been suggested to correlate with tissue localisation of T-cells in general²⁰ showed clear disparities from those isolated from blood. *TGF β 1* and *NR4A1* (nuclear hormone receptor *NUR/77*) were more highly expressed in enthesal $\gamma\delta$ T-cell subsets compared with those isolated from

blood. The expression of *KLF2* (Krupple-like factor 2) was more than two orders of magnitude lower in enthesal derived cells, similarly *TBX21* (T-bet) expression was greater in V δ 1 and V δ 2 subsets but interestingly not in the V δ 3–6 subsets perhaps reflecting a stronger Th1-like phenotype in these cells.²⁴ The modulation of these genes on tissue residence has largely been elucidated in the context of conventional T-cells.^{25–28} However, the striking changes observed in this study suggest that these findings can be applied more broadly, including to $\gamma\delta$ T-cells and add additional verification that the $\gamma\delta$ T-cells derived from enthesal tissues are a distinct tissue resident population.

Further transcriptome analysis showed increased expression of genes relevant to bone repair including *BMP-2*²⁹ and angiogenesis, *VEGFA*³⁰ in enthesal $\gamma\delta$ T-cell populations compared with those isolated from blood. *IL-17A*, *IL-17F* and *IL-22* are expressed at low levels or absent and cytokines associated with an immunomodulatory phenotype, *TGF β* and *IL-10*^{31 32} are increased in enthesal cells. Following in vitro activation of these cells TNF α and IL-17A protein were readily detectable.

It has previously been shown that the $\gamma\delta$ T-cells produced IL-17A in the absence of IL-23R. The importance of IL-23 independent IL-17 production was first highlighted in the colon where inhibition of IL-17A but not IL-23 led to loss of barrier integrity, an effect that was attributed to IL-23 independent IL-17A production in $\gamma\delta$ T-cells.³³ Analogous to this study, this work suggests that IL-17A protein expression can occur independently of IL-23 in spinal derived tissue. However, the in vivo basis for this in axial SpA remains conjectural but could involve activation of different pattern recognition receptors at entheses which needs further exploration. Noting that there is evidence for IL-23 inhibition efficacy in peripheral psoriatic arthritis,^{34 35} the putative differences between spinal and peripheral entheses that might underscore this observation need exploration.

Given the recent reports that IL-17A inhibition works in AS but IL-23 inhibition does not,^{15 36} these findings point towards a potential IL-23 independent innate immune pathway that may provide insights into understanding the clinical scenario. Indeed, the demonstration that IL-23 plays a role in innate (disease initiation) but not adaptive immunity in experimental SpA supports this view.¹⁷ Further studies looking at peripheral entheses and enthesal spinal or sacroiliac tissue from active SpA are needed. However, these findings highlight a potentially pivotal role for $\gamma\delta$ T-cells in spinal innate immunity in SpA.

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