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Human *in vitro*-induced IL-17A+ CD8+ T-cells exert pro-inflammatory effects on synovial fibroblasts

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Abstract

IL-17A+ CD8+ T-cells, termed Tc17 cells, have been identified at sites of inflammation in several immune-mediated inflammatory diseases. However, the biological function of human IL-17A+ CD8+ T-cells is not well characterized, likely due in part to the relative scarcity of these cells. Here, we expanded IL-17A+ CD8+ T-cells from healthy donor PBMC or bulk CD8+ T-cell populations using an *in vitro* polarization protocol. We show that T-cell activation in the presence of IL-1β and IL-23 significantly increased the frequencies of IL-17A+ CD8+ T-cells, which was not further enhanced by IL-6, IL-2, or anti-IFNγ mAb addition. *In vitro*-generated IL-17A+ CD8+ T-cells displayed a distinct type-17 profile compared with IL-17A– CD8+ T-cells, as defined by transcriptional signature (*IL17A*, *IL17F*, *RORC*, *RORA*, *MAF*, *IL23R*, *CCR6*), high surface expression of CCR6 and CD161, and polyfunctional production of IL-17A, IL-17F, IL-22, IFNγ, TNFα, and GM-CSF. A significant proportion of *in vitro*-induced IL-17A+ CD8+ T-cells expressed TCRVα7.2 and bound MR1 tetramers indicative of MAIT cells, indicating that our protocol expanded both conventional and unconventional IL-17A+ CD8+ T-cells. Using an IL-17A secretion assay, we sorted the *in vitro*-generated IL-17A+ CD8+ T-cells for functional analysis. Both conventional and unconventional IL-17A+ CD8+ T-cells were able to induce pro-inflammatory IL-6 and IL-8 production by synovial fibroblasts from patients with psoriatic arthritis, which was reduced upon addition of anti-TNFα and anti-IL-17A neutralizing antibodies. Collectively, these data demonstrate that human *in vitro*-generated IL-17A+ CD8+ T-cells are biologically functional and that their pro-inflammatory function can be targeted, at least *in vitro*, using existing immunotherapy.

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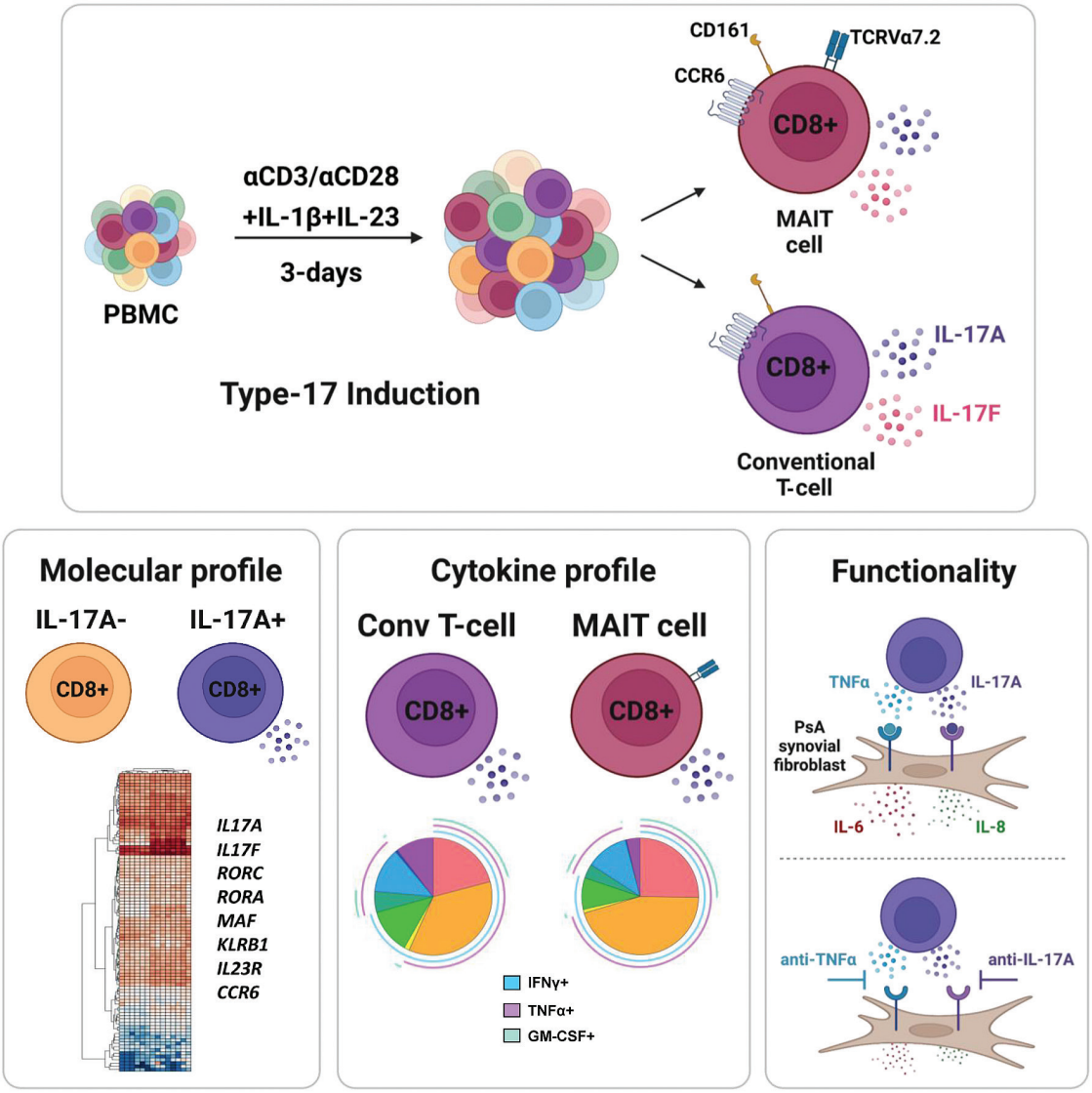
Graphical Abstract



Keywords: fibroblasts, IL-17A, IL-17F, MAIT cells, psoriatic arthritis, Tc17 cells

Abbreviations: MAIT cells: mucosal-associated invariant T-cells; PsA: psoriatic arthritis; Tc17 cells: IL-17A+ CD8+ T-cells.

Introduction

Interleukin-17A (IL-17A) was originally identified as an effector cytokine produced by T-helper (Th) CD4+ T-cells [1] and, together with fellow family member IL-17F, has come to characterize a distinct T-cell lineage termed Th17 cells. Multiple studies have described the factors and conditions that drive the differentiation of murine and human Th17 cells as well as their involvement in both host-protective and pathological immune responses [2, 3]. In addition, robust evidence implicates IL-17A (either independently or synergistically with other pro-inflammatory mediators [4, 5]) to have a pivotal function in the pathogenesis of immune-mediated inflammatory diseases such as psoriasis, psoriatic arthritis (PsA), and axial spondyloarthritis for which IL-17A is now a therapeutic target [6, 7].

In vivo and *in vitro* studies have highlighted IL-17A production is not restricted to Th17 cells; instead, a collection of conventional and unconventional innate-like T-cell types serve as sources of IL-17A and IL-17F either in health or disease-relevant tissues (reviewed in [8, 9]). In particular, IL-17A+ CD8+ T-cells, often referred to as Tc17 cells, are known to share phenotypic and functional features of Th17 cells (reviewed in [10]). These include expression of the lineage-committing transcription factors retinoic acid-receptor (RAR)-related orphan receptor (ROR)γt and musculoaponeurotic fibrosarcoma (c-MAF), surface expression of the typical type-17 markers CD161, CCR6, and IL-23R, and concomitant expression of Th17-related cytokines IL-17A, IL-17F, IL-21, IL-22, and GM-CSF, as well as cytotoxic CD8+ Tc1-related cytokines, such as IFNγ and TNFα. The presence of Tc17 cells has been described at different tissue sites in a variety of human infectious, autoimmune and inflammatory diseases, including tuberculosis [11], multiple sclerosis [12], inflammatory bowel disease [13], and psoriasis [14–16]. Our own lab previously identified an enrichment of Tc17 cells in the joints of patients with PsA that correlated with clinical parameters of disease activity [17, 18].

While phenotypic and transcriptomic *ex vivo* profiles of Tc17 cells from patients with inflammatory disease have been reported, functional studies remain limited due to the relative paucity of these cells [19, 20]. *In vitro* expansion of Tc17 cells could overcome this limitation and allow phenotypic and functional studies of this cell population. To date, several studies in mice have demonstrated that Tc17 cell differentiation can be achieved *in vitro* by applying Th17 polarizing protocols, largely inclusive of a combination of TGFβ, IL-6, IL-1β, IL-21, IL-23, anti-IL-4, and anti-IFNγ (reviewed in [10]). Analogous to Th17 cells, murine Tc17 cells were shown to be a heterogeneous population including non-pathogenic and pathogenic subtypes, the latter typically defined by enhanced cytotoxicity with dual IL-17 and IFNγ expression promoted by IL-23 [21]. Studies on human Tc17 cells offer a consensus in that IL-1β, IL-6, IL-23 without or with TGFβ, and anti-IFNγ can direct Tc17 cell responses to varying degrees [11, 13, 22–26]. In addition, evidence for involvement of cellular drivers comes from reports that activated monocytes from tumour sites rather than non-tumour tissues more potently induced IL-17A+ CD8+ T-cells *in vitro* [27] and that pleural mesothelial cells from patients with tuberculosis infection significantly enhanced IL-17 production by patient blood-derived CD8+ T-cells, in a cell–cell contact-dependent manner [11].

To address the gap in knowledge regarding the function of human IL-17A+ CD8+ T-cells, we optimized a protocol to polarize and increase the frequency of human IL-17A+ and IL-17F+ CD8+ T-cells from either human PBMC or purified CD8+ T-cells. We used this protocol to characterize these cells in terms of their phenotypic and molecular profile as well as their function. Our findings show that human *in vitro*-induced IL-17A+ CD8+ T-cells comprise both conventional T-cells as well as mucosal-associated invariant T (MAIT) cells. We show these cells are biologically active and elicit disease-relevant pro-inflammatory responses from PsA patient-derived synovial fibroblasts. Importantly, we show that the pro-inflammatory function of these cells can be counteracted by the addition of neutralizing antibodies against TNFα and IL-17A. These data indicate that Tc17 cells are biologically relevant contributors to inflammation in diseases where an enrichment of these cells is found.

Materials and methods

Samples and cell isolation

Human peripheral blood samples were obtained from healthy adult volunteers at King's College London following written informed consent (Research Ethics Committee (REC) references 06/Q0705/20 and 17/LO/1940). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep™ (Axis-Shield). CD8+ T-cells were negatively isolated from PBMC by magnetic separation using the EasySep Human CD8+ T-Cell Enrichment Kit (Stemcell Technologies; average purity 93%, $n = 16$).

Synovial fibroblast cell lines were derived from patients with PsA. Some lines were kindly provided by Professor Anca Catrina (Rheumatology Unit, Karolinska Institute, Sweden), other lines were generated in-house from PsA synovial membrane tissue obtained during knee replacement surgery at Guy's Hospital Rheumatology Department (REC reference 07/H0809/35). In brief, to isolate synovial fibroblasts, tissue explants were cultured as 1–2 mm^3 pieces in plates coated with 0.1% bovine gelatin (Sigma–Aldrich) with DMEM (ThermoFisher Scientific) supplemented with 20% heat-inactivated foetal calf serum (FCS), 1% penicillin/streptomycin, 2% L-glutamine, and 1 mg/ml amphotericin B (all ThermoFisher Scientific). Tissue explants were incubated at 37°C in an atmosphere of 5% CO_2; supplemented DMEM medium was replenished every 3 days. Synovial fibroblasts that had migrated out of the synovial tissue were collected and maintained in T175 flasks for cell line generation. Fibroblasts were passaged with 1× trypsin (Sigma–Aldrich) once they reached 80% confluency and were used in cultures when between passages 3 and 7.

In vitro type-17 polarization

For induction of IL-17+ CD8+ T-cells, freshly isolated or cryopreserved human PBMC or purified CD8+ T-cells (1×10^6) were cultured in complete culture medium (RPMI 1640 (Gibco) supplemented with 10% FCS, 1% penicillin/streptomycin/L-glutamine) at 37°C in an atmosphere of 5% CO_2. Cell cultures were stimulated for 3 days with either 100 ng/ml soluble or 1.25 μg/ml immobilized (plate-bound) anti-CD3 mAb (clone OKT3, BioLegend) in combination with 1 μg/ml soluble anti-CD28 mAb (clone CD28.2, BD Biosciences) in the absence or presence of human recombinant (hr) IL-1β (10 ng/ml, Peprotech) and hrIL-23 (20 ng/ml, R&D systems). In some experiments, hrIL-6 (20 ng/ml), hrIL-2 (20 U/ml, both Peprotech), neutralizing anti-IFNγ, or isotype control (mouse IgG2b, both 5 μg/ml, R&D Systems) was added to cultures. Cell culture supernatants were collected on day 3 before cells were re-stimulated with PMA/ionomycin without or with GolgiStop.

Flow cytometric analysis

For intracellular cytokine staining, PBMC or CD8+ T-cells were stimulated, either *ex vivo* or following 3-day culture, with PMA (50 ng/ml) and ionomycin (750 ng/ml, both Sigma–Aldrich) for 3 hours at 37°C in the presence of GolgiStop (monensin, according to manufacturer's recommendation, BD Biosciences). Cells were washed and labelled with fixable viability dye (LIVE/DEAD eFlour780, eBioscience) for 15 minutes at 4°C. PBMC samples were then FcR-blocked using 10% human AB serum (Invitrogen) in FACS buffer for 15 minutes at room temperature (RT). Where applicable, cells were stained with human MR1-5-OP-RU or MR1-6-FP (supplied by the NIH Tetramer Core Facility) for 40 minutes at RT followed by incubation at 4°C for 30 minutes with a combination of mAbs against cell surface markers including: CD8, CD14, CD19, CD161, CCR6, and TCRVα7.2 (full details are described in Supplementary Table S1). Cells were fixed with 2% paraformaldehyde for 15 minutes at RT then permeabilized using 0.5% saponin (Sigma–Aldrich) and stained for 30 minutes at 4°C for the following intracellular markers: CD3, CD4, IL-17A, IL-17F, IFNγ, TNFα, GM-CSF, granzyme A and B (full details are given in Supplementary Table S1). Samples were acquired using either a FACS Canto II or LSRFortessa (BD Biosciences), and data were analysed using FlowJo software (v10.7.1, TreeStar Inc.). CD8+ T-cells were gated as shown in Supplementary Fig. S1. FM control stainings were used to aid determination of cytokine-expressing cell populations. Boolean gating strategy of IFNγ, TNFα, and GM-CSF expression by IL-17A+ Vα7.2– CD8+ and IL-17A+ Vα7.2+

CD8+ T-cell subsets was imported into SPICE software (v5.1) for visualization of cells expressing polyfunctional or monofunctional cytokine combinations.

IL-17A cytokine secretion assay

For transcriptional and functional assessments, IL-17A-producing CD8+ T-cells were isolated post-polarizing culture using an IL-17A cytokine secretion assay (CSA) (Miltenyi Biotech, according to manufacturer's recommendations) combined with FACS sorting. Purified CD8+ T-cells were cultured for 3 days with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence of hrIL-1β and hrIL-23. After 3 days, cells were re-stimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) for 1.5 hours before cells were counted with trypan blue. Cells were washed, then re-suspended in cold complete culture medium and labelled with the IL-17A catch reagent for 5 minutes on ice. Cell suspension was diluted with warm complete culture medium and incubated in a continuous motion using the MACSmix rotator (Miltenyi Biotec) for 45 minutes at 37°C (5% CO_2) to allow secretion and capture of IL-17A on the cell surface-bound catch antibody. After cells were washed with cold PBS containing 0.5% EDTA, they were labelled for viability, CD3, CD4, and CD8, with CD19 as an exclusion marker, as well as with the IL-17A-detection antibody (PE or APC conjugated) for 20 minutes on ice. Once washed, IL-17A–secreting (IL-17A+) and non-IL-17A–secreting (IL-17A–) CD8+ T-cells were immediately FACS sorted on a BD FACS ARIA II. In some experiments, mAbs against the semi-invariant TCR Vα7.2 (with pan γδTCR and CD56 as exclusion markers) were included in the panel to sort IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ cells from CD8+ T-cell cultures. Sorted T-cell subsets were either stored in TRIzol® Reagent (ThermoFisher) at –80°C for later qPCR array analysis or used directly for functional assessments. For the latter, sorted T-cells were either added to fibroblasts as described below or cultured for 20 hours at 37°C (5% CO_2) in complete culture medium for supernatant generation.

PsA synovial fibroblast co-cultures

PsA synovial fibroblasts (1×10^4 per well) were seeded in a flat-bottomed 96-well plate in supplemented DMEM medium and incubated for 24 hours at 37°C, 5% CO_2. Following supernatant removal, fibroblasts were cultured in supplemented DMEM in the absence or presence of 20% (v/v) cell culture supernatants from FACS-sorted CD8+ T-cell populations for a further 24 hours, after which supernatants were collected and analysed for IL-6 and IL-8 production. Alternatively, fibroblasts were co-cultured for 24 hours with CSA-FACS sorted IL-17A+ or IL-17A– CD8+ T-cells at a 1:2.5 fibroblast to T-cell ratio, in the absence or presence of anti-IL-17A mAb (secukinumab, Novartis) and/or anti-TNFα-blocking antibodies (adalimumab, Abbott Laboratories) or isotype control mAb (all human IgG1 and added at 5 μg/ml).

Cytokine detection

The presence of IL-17A in T-cell culture supernatants and of IL-6 and IL-8 in fibroblast culture supernatants was quantified by ELISA according to manufacturer instructions (deluxe or standard kits, respectively, all BioLegend). Plates were read at 450 nm using a Spark 10M (Tecan). Cytokine secretion profiles of supernatants from FACS-sorted IL-17A+ Vα7.2– CD8+, IL-17A+ Vα7.2+ CD8+, and IL-17A– CD8+ T-cell subsets were assessed by custom magnetic Luminex (Bio-Techne). Luminex plates were analysed on a Luminex FlexMap 3D platform. IL-17A and IL-17F were measured on separate assay plates due to the cross-reactivity of the magnetic beads with IL-17AF.

RNA extraction and cDNA synthesis

Total RNA was extracted from healthy donor CSA-FACS sorted IL-17A+ CD8+ and IL-17A– CD8+ T-cell subsets (cell numbers indicated in Supplementary Table 2) using the TRIzol® Reagent phenol–chloroform extraction method in combination with Phasemaker tubes (ThermoFisher). To increase total RNA precipitation, during the isopropanol step, samples were kept at –80°C for 24 hours and 1 μl GlycoBlue™ Coprecipitant (ThermoFisher) was added. RNA yield and integrity (mean RIN = 8.9) were assessed by Bioanalyzer (Waterloo Genomics Centre, KCL), and RNA was stored short term at –80°C. Amount of RNA input for complementary DNA (cDNA) transcription was standardized within donor-paired samples by adjusting RNA concentrations to the total extracted from the IL-17A+ CD8+ T-cell subset (indicated in Supplementary Table S2). First-strand cDNA synthesis was performed as a 20 μl reaction using the LunaScript® RT SuperMix Kit (NEB, following the manufacturer's protocol), and cDNA was stored short-term at –20°C before transcriptional assessment.

Quantitative real-time polymerase chain reaction

Gene expression profiles of *in vitro*-generated IL-17A+ CD8+ and IL-17A– CD8+ T-cells were assessed by bespoke TaqMan® quantitative polymerase chain reaction (qPCR) array (all 96 primer assays, including 3 endogenous controls and 93 targets, were selected from ThermoFisher, details listed in Supplementary Table S3). Array cards and sample cDNA templates were prepared according to manufacturer's instructions using the TaqMan® Fast Advanced PCR Master Mix (ThermoFisher). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using a QuantStudio™ 7 Flex System (Applied Biosystems) with the following amplification conditions: hold at 50°C for 2 minutes then hold at 92°C for 10 minutes followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Housekeeping genes 18S ribosomal RNA (*18S*), beta-2-microglobulin (*B2M*), and peptidylprolyl isomerase A (*PPIA*) were screened for stability using the web-based tool RefFinder and R-based package NormFinder. Expression of each target gene was normalized to the expression of *B2M* and *PPIA* within a sample, performed using the comparative threshold cycle (Ct) method calculated as: ΔCt = Ct(gene of interest) – Ct(geometric mean (*B2M* + *PPIA*)). Ct was defined as 40 for the ΔCT calculation when the signal was under detectable limits. The relative fold change in target mRNA expression levels was assessed in IL-17A+ CD8+ versus IL-17A– CD8+ T-cells and calculated with the formula $2^{-\Delta\Delta CT}$ where for a given gene ΔΔCT = ΔCt(IL-17A+) – ΔCt(IL-17A–).

Data analysis and statistical testing

Graphs were constructed and statistical tests performed using GraphPad Prism v9.1. Sample sizes with $n < 8$ or when the population assumed a non-normal distribution were tested non-parametrically using a Wilcoxon signed-rank matched

pairs test. Data sets with $n > 8$ were tested for normality using the D'Agostino and Pearson omnibus normality test then tested for significance using the appropriate parametric or non-parametric test as stated in the figure legends. For transcriptional data analysis, undetectable genes (*ZBTB32* and *CD5L*) were filtered from initial principal component analysis and heatmap generation; both were computed in R using prcomp and pheatmap functions. Genes that were identified to have very low/negligible expression in both IL-17A+ CD8+ and IL-17A– CD8+ T-cells with reference to the geometric mean of *CD4* normalized expression were excluded from statistical testing and relative fold change analysis (*IL17B*, *IL17C*, *IL17D*, *IL25*, *IL17RC*, and *CCR9*). Differential statistical analysis was performed on normalized expression values using a parametric paired Student's *t*-test with Holm-Šídák multiple comparisons test with adjusted *P* values ($P < 0.05$) reported.

Results

In vitro expansion protocol for human IL-17+ CD8+ T-cells

First, we determined the *ex vivo* frequencies of IL-17A+ and IL-17F+ CD8+ T-cells in human peripheral blood. Freshly isolated or cryopreserved healthy donor PBMC were stimulated for 3 hours with PMA/ionomycin in the presence of GolgiStop followed by intracellular cytokine staining. CD8+ T-cells were gated as shown in Supplementary Fig. S1A. Low frequencies of IL-17A+ cells (median: 0.080%), and very low frequencies of IL-17F+ cells (median: 0.007%), were detected within the CD8+ T-cell population *ex vivo* (Fig. 1A and B), while higher frequencies of IL-17A+ and IL-17F+ cells were detected in CD4+ T-cells (median: 0.75 and 0.04%, respectively, Supplementary Fig. S2A and B).

To investigate whether IL-17+ CD8+ T-cells could be expanded *in vitro*, freshly isolated or cryopreserved PBMC from

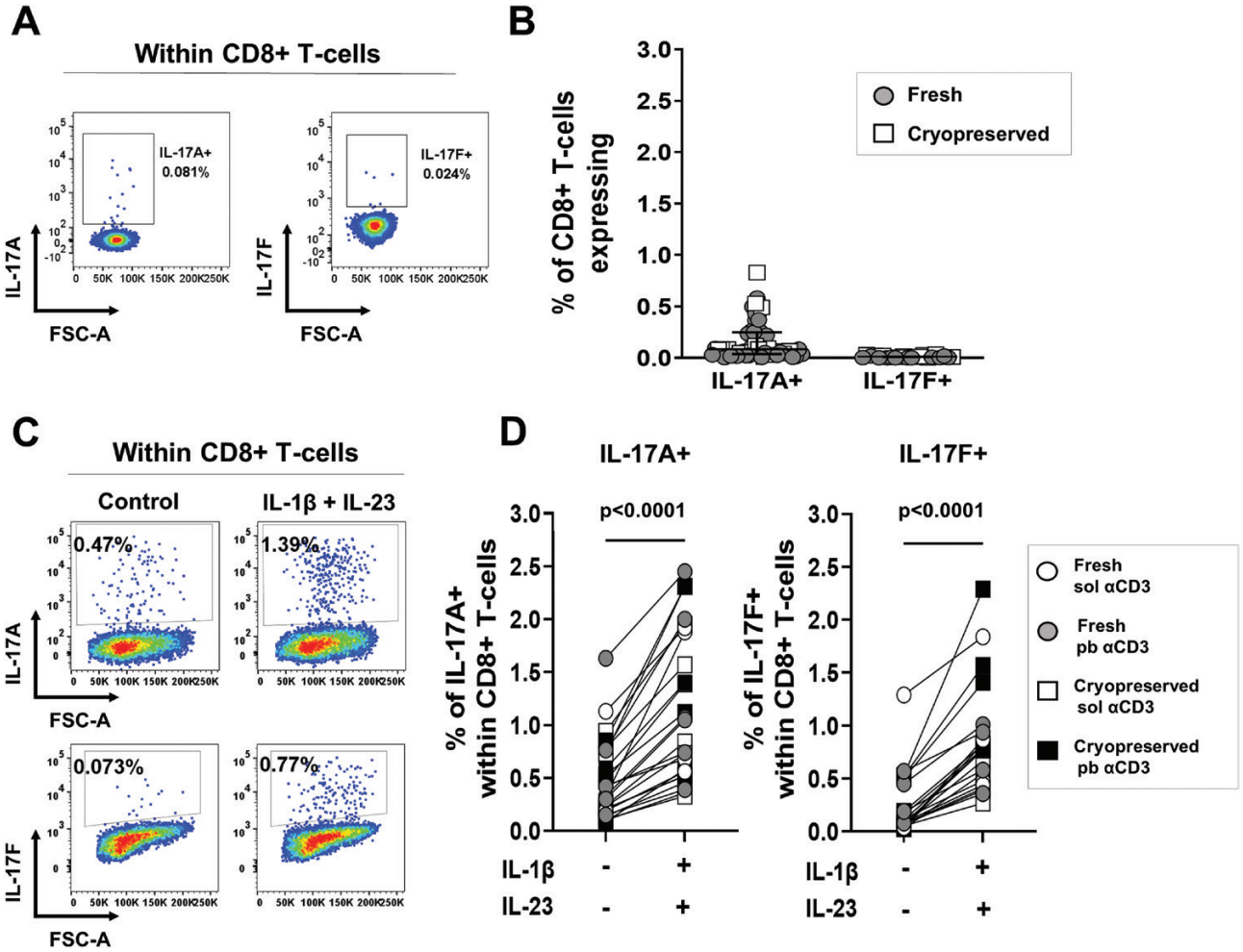


Figure 1. Frequencies of IL-17A+ and IL-17F+ CD8+ T-cells in healthy donor PBMC *ex vivo* and upon expansion. (A, B) Freshly isolated (circles) or cryopreserved (squares) healthy donor PBMC were stimulated *ex vivo* for 3 hours with PMA/ionomycin in the presence of GolgiStop for assessment of intracellular IL-17A and IL-17F cytokine expression by flow cytometry. Representative staining plots (A) and cumulative data (B) show frequencies of IL-17A+ and IL-17F+ cells within live CD3+CD8+ T-cells from independent donors ($n = 51$ and $n = 23$, respectively). Data plotted as median ± IQR. (C, D) Freshly isolated (circles) or cryopreserved (squares) healthy donor PBMC were cultured for 3 days with either plate-bound (filled symbols) or soluble (open symbols) anti-CD3 mAb and soluble anti-CD28 mAb in the absence (control) or presence of hrIL-1β (10 ng/ml) and hrIL-23 (20 ng/ml). After 3 days, cells were re-stimulated with PMA/ionomycin in the presence of GolgiStop as detailed earlier. Representative dot plots (C) and cumulative data (D) show the frequencies of *in vitro*-induced IL-17A+ and IL-17F+ cells within live CD3+ CD8+ T-cells from independent donors ($n = 50$ and $n = 22$, respectively). Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

healthy donors were stimulated using plate-bound or soluble anti-CD3 mAb with soluble anti-CD28 mAb in the absence or presence of the well-established human Th17-promoting cytokines IL-1β and IL-23. After 3 days, cells were re-stimulated with PMA/ionomycin and Golgistop for intracellular cytokine assessment by flow cytometry (CD8+ T-cell gating strategy shown in Supplementary Fig. S1B). Frequencies of IL-17A+ and IL-17F+ cells were significantly increased when PBMC were cultured in the presence of IL-1β and IL-23, as compared with cell cultures with only anti-CD3/CD28 stimulation (3.2-fold and 7-fold higher, respectively, both $P < 0.0001$) (Fig. 1C and D). As expected, culturing PBMC under type-17 polarizing conditions led to a significant increase in IL-17A+ and IL-17F+ CD4+ T-cells also ($P < 0.0001$, Supplementary Fig. S2C and D).

In vitro induction of IL-17+ CD8+ T-cells upon anti-CD3/CD28 stimulation in the presence of IL-1β and IL-23 is not further enhanced by addition of IL-6, IL-2, or anti-IFNγ

Given that the presence of other immune cell subsets in whole PBMC cultures may have contributed additional type-17 promoting and/or inhibitory factors, we sought to explore the direct effect of IL-1β and IL-23 on IL-17A+ CD8+ T-cell induction. For this, CD8+ T-cells were purified by magnetic bead separation from freshly isolated healthy donor PBMC and cultured for 3 days with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the absence or presence of IL-1β and IL-23 (purity assessment and representative gating strategy shown in Supplementary Fig. S1C and D). Akin to whole PBMC cultures, a statistically significant increase in IL-17A+ cells was observed when CD8+ T-cells were stimulated in the presence of type-17 polarizing cytokines as opposed to anti-CD3/CD28 stimulation alone (median: 1.44 versus 0.28%, 5.1-fold increase, $P = .0005$, Fig. 2A). This was confirmed at the cytokine secretion level, with significantly elevated levels of IL-17A detected in cell culture supernatants of CD8+ T-cells cultured under type-17 polarizing conditions without PMA/ionomycin restimulation (median: 432 pg/ml versus 84 pg/ml, 4.1-fold increase, $P = 0.0039$) (Fig. 2B).

Previously, human and murine studies have shown that IL-6 can promote Th17 and IL-17A+ CD8+ T-cell polarization, while IFNγ can antagonize this [28, 29]. However, the addition of hrIL-6 or of IFNγ blocking antibodies to our purified CD8+ T-cell culture system did not further increase the frequency of IL-17A+ cells or the production of IL-17A by CD8+ T-cells (Fig. 2C–E). Addition of hrIL-2 and extending the culture system to 6 days also did not achieve any further consistent additive effect on the frequency of induced IL-17A+ CD8+ T-cells (Supplementary Fig. S3A and B). The addition of hrIL-6 or anti-IFNy mAb did not affect the frequencies of single IFNy+ or IL-17A+IFNy+ co-expressing CD8+ T-cells either (Supplementary Fig. S3C and D).

In vitro-induced IL-17A+ CD8+ T-cells are characterized by a core type-17 transcriptional and phenotypic signature

We next sought to determine whether human *in vitro*-generated IL-17A+ CD8+ T-cells display a type-17-related gene profile compared with IL-17A– CD8+ T-cells. Purified CD8+ T-cells were polarized for 3 days as described earlier, after which highly pure IL-17A-secreting (IL-17A+) and non-IL-17A-secreting (IL-17A–) CD8+ T-cells were FACS sorted using an IL-17A CSA (representative gating strategy shown in Supplementary Fig. S4A). Accuracy of the combined CSA-FACS sorting method was validated by assessment of secreted IL-17A in cell culture supernatants from sorted IL-17A+ and IL-17A– CD8+ T-cell subsets, as well as by determining expression of *CD8A*, *CD8B*, and *CD4* for each population (Supplementary Fig. S4B and C).

We investigated the expression of a broad range of type-17-associated genes using a bespoke qPCR array. First, we applied PCA to globally evaluate transcriptomic profiles of the sorted *in vitro*-induced T-cell subsets. IL-17A+ CD8+ T-cells from all seven independent donors clustered separately from IL-17A– CD8+ T-cells (upper panel Fig. 3A). Several hallmark type-17 genes were identified among the top 20 genes that contributed to Component 1, which accounted for most of the variation described by the PCA plot (PC1, 44.2%) (lower panel Fig. 3A). Heatmap analysis further highlighted that a type-17-related gene signature was enriched in all donor IL-17A+ versus IL-17A– CD8+ T-cells (Fig. 3B). Quantitative assessment deduced that a total of 29/85 genes were statistically more abundant in *in vitro*-polarized IL-17A+ versus IL-17A– CD8+ T-cells (select genes shown in Fig. 3C, all 29 listed in Supplementary Table S4). Importantly, a large proportion of these enriched genes are characteristic of the type-17 program including: cytokines and chemokines *IL17A*, *IL17F*, *IL26*, and *CCL20*; migratory, lineage-defining, and cytokine-signalling receptors *CCR6*, *CXCR6*, *KLRB1* (encoding CD161), and *IL23R*; and the transcription factors *RORC*, *RORA*, and *MAF* (encoding RORγt, RORα, and c-MAF, respectively). We also detected slightly increased expression of *IL2RA*, *CTLA4*, *PDCD1* (encoding programmed cell death 1 (PD-1)) and *GZMB* in polarized IL-17A+ CD8+ T-cells versus IL-17A– counterparts. Our analysis did not identify significant differences in mRNA expression of additional type-17 effector molecules *IL21*, *IL22*, and *CSF2* (encoding GM-CSF) or in *IFNG* or *TNF* which both displayed high mRNA levels in each T-cell subset. Transcript levels of *TCF7* (encoding T-cell factor 1 (TCF-1)), a recently identified murine Tc17 cell transcriptional regulator [30] was variable among donor IL-17A+ CD8+ T-cells and not significantly lower than levels detected in IL-17A– CD8+ T-cells. Evaluation of other T-cell lineage-specific markers, namely *TBX21* (encoding T-bet), *CXCR3*, *GATA3*, *FOXP3*, and *IL10*, showed no selective expression in either subset.

We identified 25/29 IL-17A+ CD8+ T-cell signature genes to be differentially expressed with a relative fold change ≥1.5 compared with IL-17A– CD8+ T-cells (Fig. 3D). Of these, *IL17A* and *IL17F* were the most significantly differentially regulated genes (9.18-fold and 5.86-fold, respectively, both $P < 0.0001$). Expression levels of IL-23 and IL-1β cognate receptor subunits *IL23R* and *IL1R1* were also significantly increased (3.43-fold, $P < 0.0001$; and 1.94-fold, $P < 0.05$, respectively) in IL-17A+ compared with IL-17A– CD8+ T-cells. *ZBTB16* (encoding PLZF), which was identified as the leading PC1 parameter in our primary PCA analysis, was further revealed as a signature type-17 gene differentially expressed in *in vitro*-generated IL-17A+ CD8+ T-cells (3.29-fold, $P < 0.01$).

We confirmed the molecular data for the type-17 T-cell markers CCR6 and CD161 at the protein level by flow cytometry, which showed that a high proportion of *in vitro*-induced IL-17A+ CD8+ T-cells co-expressed CCR6 and CD161 on their surface (median: 88.1 and 93.4%, respectively), as compared with a much lower proportion of IL-17A– CD8+ T-cells (median: 10.6 and 18.7%, respectively) (Fig. 3E and F).

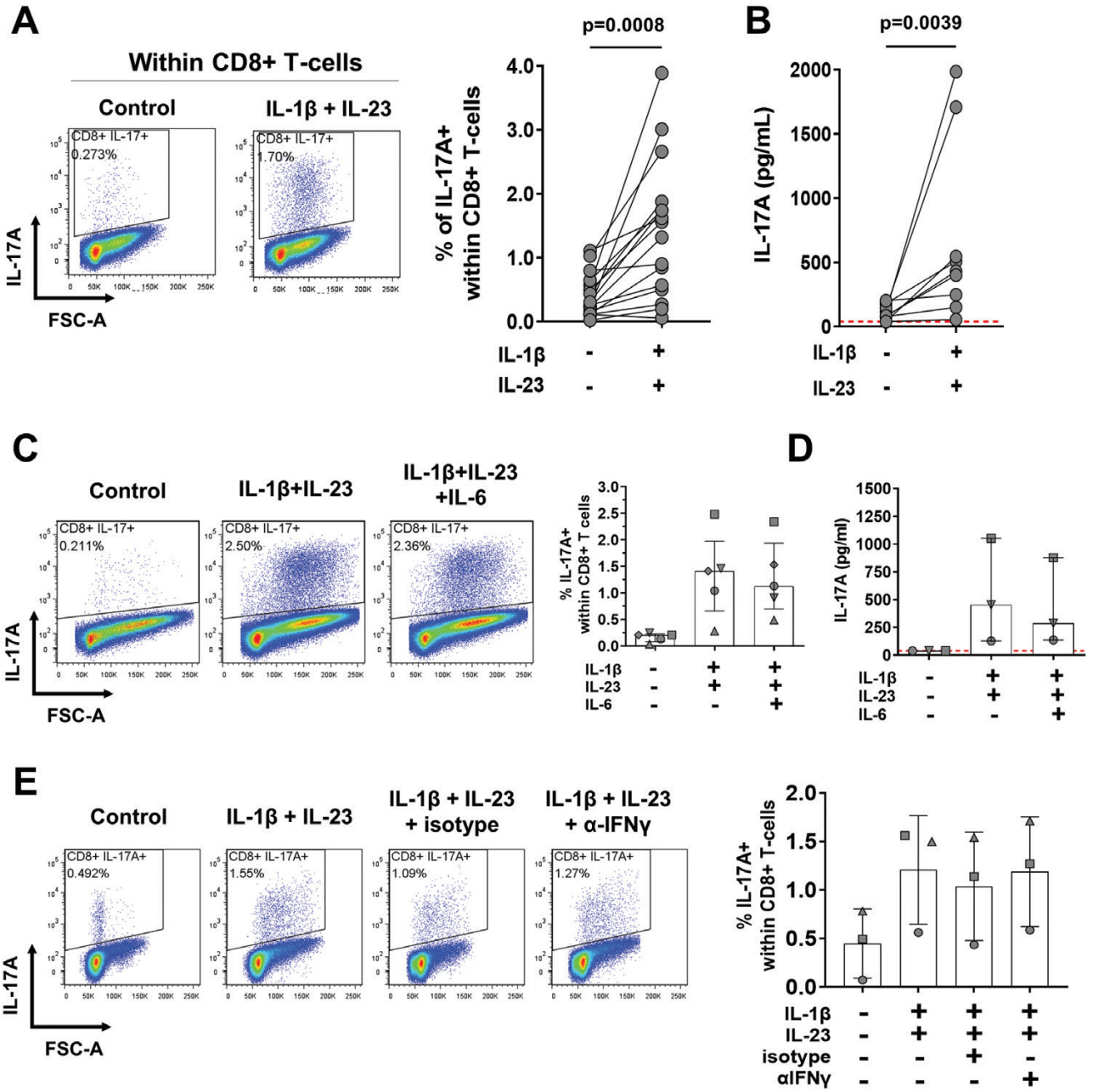


Figure 2. Anti-CD3/CD28 stimulation in the presence of IL-1β and IL-23 is sufficient to expand IL-17A+ CD8+ T-cells from purified CD8+ T-cell cultures. Purified CD8+ T-cells from freshly isolated PBMC were cultured in the presence of plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the absence (control) or presence of hrIL-1β and hrIL-23 for 3 days. On day 3, culture supernatants were removed and remaining cells were re-stimulated for 3 hours with PMA/ionomycin in the presence of GolgiStop for detection of intracellular IL-17A expression by flow cytometry. (A) Representative staining plots and cumulative data show frequencies of IL-17A+ cells within live CD3+ CD8+ T-cells (n = 16). (B) Levels of IL-17A detected in 3-day culture supernatants were measured by ELISA (n = 9). Statistical analysis was performed using a paired t-test (A) or Wilcoxon matched-pairs signed rank test (B). (C–E) Bulk CD8+ T-cells were cultured under type-17 polarizing conditions with or without hrIL-6 (20 ng/ml) (C, D) or in the absence or presence of neutralizing anti-IFNγ or isotype control mAb (5 μg/ml) (E) for 3 days. Representative stainings and cumulative data (n = 3–5, each symbol correspond to an independent donor) show frequencies of IL-17A+ cells within live CD3+ CD8+ T-cells as detected by flow cytometry (C and E), or (D) indicate levels of IL-17A detected by ELISA in culture supernatants collected prior to re-stimulation (n = 3). Data are plotted as median ± IQR.

Both IL-17-expressing CD8+ conventional T-cells and unconventional MAIT cells are induced *in vitro* upon type-17 polarization

The transcriptional profiling revealed that *in vitro*-generated IL-17A+ CD8+ T-cells were endowed with prototypical type-17 markers; however, several genes including *ZBTB16*, *ABCB1* (encoding multidrug resistance 1 (MDR-1)), *IL12RB2*, *IL18R1*, *DPP4* (encoding CD26), *GZMB*, and *KLRB1*, together with constitutively high surface expression of CD161, are also associated with MAIT cell identity. This prompted us to investigate whether a proportion of *in vitro*-generated IL-17A+ CD8+ T-cells were comprised of MAIT cells. We stimulated PBMC with anti-CD3/CD28 mAbs in the absence or presence of IL-1β and IL-23 and assessed the expression of the MAIT cell-associated TCRVα7.2. Frequencies of IL-17A+ and IL-17F+ cells within Vα7.2– and

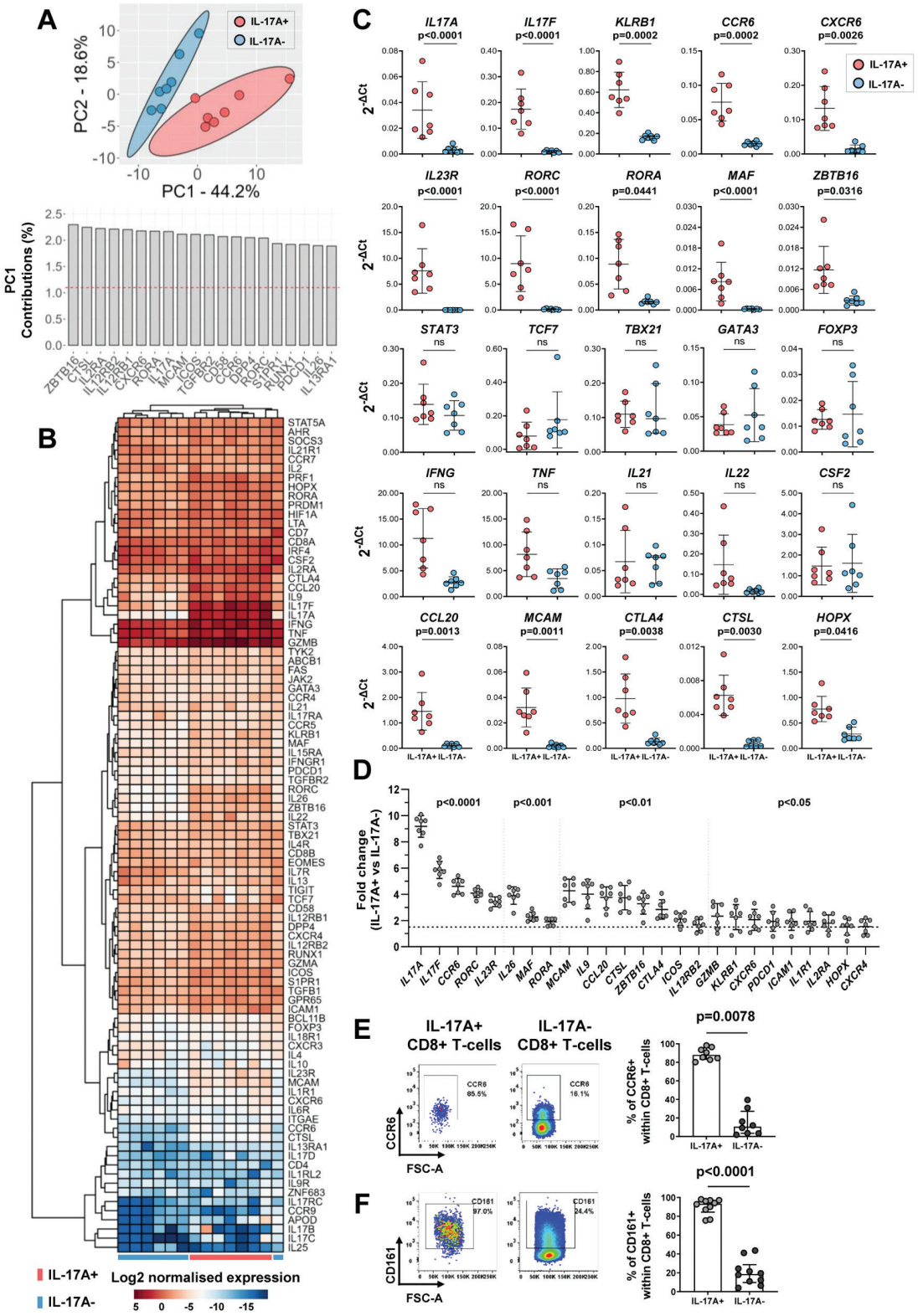


Figure 3. Transcriptional analysis of *in vitro*-generated IL-17A+ CD8+ T-cells. Bulk CD8+ T-cells were isolated from healthy donor PBMC and cultured for 3 days with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence of hrIL-1β and hrIL-23. On day 3, cells were re-stimulated for 1.5 hours with PMA/ionomycin and stained with an IL-17A cytokine secretion assay followed by FACS sorting. (A–D) Gene profiles of matched donor IL-17A+ CD8+ and IL-17A– CD8+ T-cell subsets were assessed by custom qPCR array (n = 7 independent donors). Each target gene expression was normalized to endogenous controls *B2M* and *PPIA*. (A) Principal-component analysis (PCA) of IL-17A+ (red) and IL-17A– (blue) CD8+ T-cell transcriptional profiles plotted on components 1 and 2 (upper panel); bar plot of the top 20 PCA parameter contributions (%) for component 1 (lower panel). (B) Heatmap showing gene expression profiles in IL-17A+ compared with IL-17A– CD8+ T-cells with donors assigned to columns and the log2 transformed normalized gene expression to rows. Unbiased hierarchical gene and sample clustering indicated by dendrograms. (C) Scatter plots depicting absolute mRNA expression levels ($2^{-\Delta Ct}$) of a selection of T-cell lineage specific genes in IL-17A+ and IL-17A– CD8+ T-cells. (D) Scatter plot showing relative fold

Vα7.2+ CD8+ T-cells were quantified by intracellular cytokine staining. This analysis showed that IL-17A+ and IL-17F+ T-cells were significantly increased under type-17 polarizing conditions within both Vα7.2– and Vα7.2+ CD8+ T-cells as compared with control conditions (Fig. 4A–C). Notably, in all 12 donors tested, the frequencies of IL-17A and IL-17F expressing cells were significantly greater within Vα7.2+ compared with Vα7.2– CD8+ T-cells (median: IL-17A+ 0.56 versus 0.18%; IL-17F+ 0.46 versus 0.16% in Vα7.2+ versus Vα7.2– CD8+ T-cells respectively, both $P = 0.002$).

Recent literature has shown that TCRVα7.2 bearing T-cells that do not express CD161 are transcriptionally distinct from CD161^{hi} Vα7.2+ MAIT cells [31]. We, therefore, sought to further characterize the Vα7.2+ CD8+ T-cell compartment induced under type-17 polarizing conditions by inclusion of CD161 co-stain. Within IL-17A and/or IL-17F expressing CD8+ T-cells, three populations were defined by TCRVα7.2 and CD161 expression: conventional Vα7.2– T-cells, Vα7.2+ CD161+ MAIT cells and Vα7.2+ CD161- T-cells. *In vitro*-induced IL-17A+IL-17F–, IL-17F+IL-17A–, and IL-17A+IL-17F+ cells comprised mostly of MAIT cells (median: 57.7, 58.6, and 63.5%) followed by conventional Vα7.2– CD8+ T-cells (median: 34.1, 28.6, and 22.3%) (Fig. 4D). In contrast, IL-17A and/or IL-17F producing cells only rarely contained Vα7.2+ CD161– CD8+ T-cells (median: 2.4, 0.9, and 0%). We confirmed this finding using the MR1-5-OP-RU loaded tetramer, which unequivocally identifies MAIT cells [32] (Supplementary Fig. S5). Again, we found that upon type-17 polarization IL-17A+ and IL-17F+ cells were induced in both MR1-tetramer^{neg} conventional CD8+ T-cells and in MR1-tetramer^{pos} MAIT cells, with a statistically significant dominance of MAIT cells amongst the IL-17-expressing cells (Supplementary Fig. S6). We investigated if the preferential expansion of IL-17A+ cells within the MAIT cells was due to increased proliferation of MAIT cells during the culture period using CTV and Ki67 staining; however, no substantive differences were observed between the MAIT and conventional CD8+ T-cell populations (Supplementary Fig. S7).

In vitro-induced IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ CD8+ T-cells share a polyfunctional, pro-inflammatory phenotype

Having identified that unconventional Vα7.2+ MAIT cells represented around 60% of IL-17A and/or IL-17F expressing CD8+ T-cells within *in vitro*-induced cultures, we sought to compare whether the IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ CD8+ T-cell subsets displayed similar cytokine expression profiles. Comparable frequencies of IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ CD8+ T-cells co-expressed GM-CSF (median: 22.8 versus 24.5%), IL-17F (median: 48.6 versus 43.6%) and IFNγ (median: 73.8 versus 78.6%), with only the frequency of TNFα significantly higher in the IL-17A+ Vα7.2+ compared with IL-17A+ Vα7.2– CD8+ T-cell subset (median: 80.1 versus 89.9%, $P = 0.0313$) (Fig. 5A). Applying a Boolean gating strategy and the visualization software SPICE, we explored the mono- and polyfunctional cytokine profiles of IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ CD8+ T-cells (Fig. 5B and C). This revealed that both *in vitro*-induced IL-17A+ CD8+ T-cell subsets predominantly comprised polyfunctional cells that express multiple pro-inflammatory cytokines (triple-positive for IL-17A+IFNγ+TNFα+, orange pie segment; or quadruple-positive for IL-17A+IFNγ+TNFα+GM-CSF+, red pie segment). We only observed a statistically significant difference in the proportion of single-positive IL-17A+ cells which was higher in IL-17A+ Vα7.2– compared with IL-17A+ Vα7.2+ CD8+ T-cells (purple pie segment, $P = 0.0104$) (Fig. 5B and C). In line with our molecular profile, we found that a high proportion of *in vitro*-generated IL-17A+ Vα7.2– as well as Vα7.2+ CD8+ T-cells harboured intracellular protein stores of granzyme B ($n = 2$, median: 94.6 versus 97.9%, respectively) and to a lesser extent granzyme A ($n = 2$, median: 49.1 and 69.4%, respectively) suggestive that both cell types also shared cytotoxic potential (Supplementary Fig. S8A). In addition, IL-17A-expressing CD8+ T-cell subsets showed much higher cytotoxic potential than IL-17A– CD8+ T-cells (Supplementary Fig. S8B).

Using a Luminex® assay, we measured the production of the cytokines IL-17AA/AF, IL-17FF/AF, IFNγ, TNFα, GM-CSF, IL-21, IL-22, and IL-10 by sorted IL-17A+ Vα7.2–, IL-17A+ Vα7.2+, or IL-17A– CD8+ T-cell subsets (representative gating strategies shown in Supplementary Fig. S4D). While not statistically significant, in the majority of donors unconventional IL-17A+ Vα7.2+ CD8+ T-cells showed higher production of IL-17AA/AF, IFNγ, and TNFα compared with conventional Vα7.2– counterparts (median IL-17AA/AF: 1861 pg/ml versus 840 pg/ml; IFNγ: 3894 pg/ml versus 2202 pg/ml; TNFα: 514 pg/ml versus 106 pg/ml) (Fig. 5D). More variation was observed in the production of IL-17FF/AF, GM-CSF, IL-22, and IL-10, with Vα7.2+ IL-17A+CD8+ T-cells showing more consistent production of these cytokines than Vα7.2– IL-17A+CD8+ T-cells. Neither subset produced IL-21. *In vitro*-induced IL-17A– CD8+ T-cell cultures were assessed as a negative control, which showed no production of the type-17-associated cytokines IL-17AA/AF, IL-17FF/AF, IL-21, and IL-22 but rather secreted IFNγ, TNFα, and GM-CSF only.

In vitro-induced IL-17A+ CD8+ T-cells are functional, with capacity to induce pro-inflammatory cytokine production by PsA synovial fibroblasts

As our final step, we sought to determine the functional contribution of *in vitro*-induced IL-17A+ CD8+ T-cells, by investigating their ability to promote clinically relevant pro-inflammatory cytokine production in an *in vitro* model of joint inflammation. For this, cell culture supernatants were collected from *in vitro*-induced and then CSA-FACS-sorted IL-17A+ or IL-17A– CD8+ T-cells. Supernatants were added

change ($2^{-\Delta\Delta Ct}$) in type-17 signature genes identified as significantly differentially expressed with fold change ≥1.5 (marked by the horizontal dashed line) in IL-17A+ compared with IL-17A– CD8+ T-cells. Data are plotted as mean ± SD and significance was analysed using paired Student's *t*-test with Holm-Šídák multiple comparisons, adjusted *P* values are reported. (E, F) After 3-day stimulation in the presence of hrIL-1β and hrIL-23, CD8+ T-cell cultures were re-stimulated for 3 hours with PMA/ionomycin in the presence of GolgiStop for identification of *in vitro*-induced IL-17A+ and IL-17A– CD8+ T-cell subsets by intracellular flow cytometry staining. Representative dot plots and cumulative data plotted as median ± IQR show surface expression of CCR6 (E) and CD161 (F) on IL-17A+ or IL-17A– cells gated within CD8+ T-cells ($n = 8$–10). Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

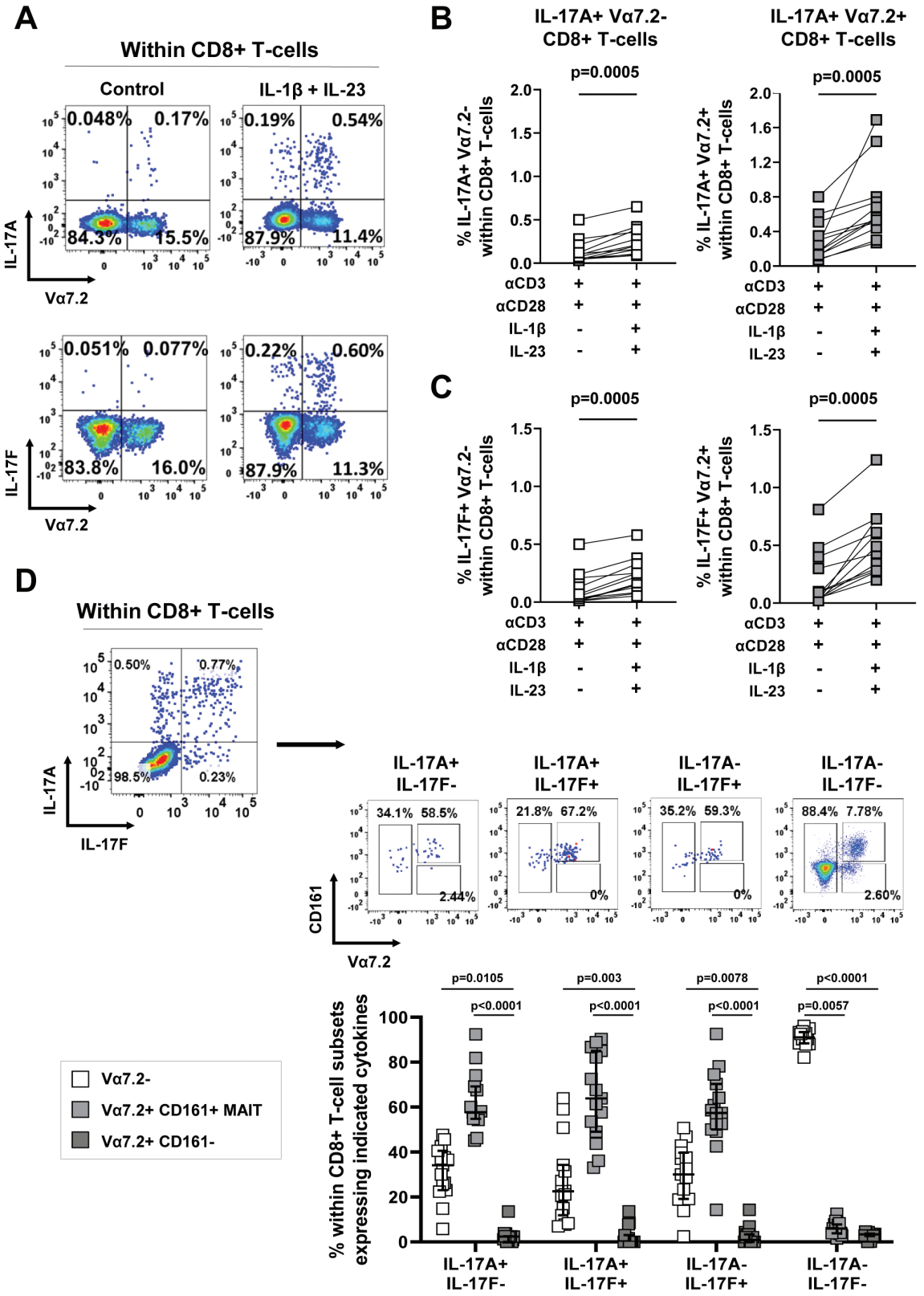


Figure 4. Type-17 *in vitro* polarization increases the frequencies of both conventional and MAIT IL-17A+ CD8+ T-cells. Healthy donor PBMC were cultured with soluble anti-CD3/CD28 mAbs in the absence or presence of hrIL-1β and hrIL-23 for 3 days, then re-stimulated for 3 hours with PMA/ionomycin in the presence of GolgiStop for assessment of intracellular IL-17A and IL-17F expression. Representative flow staining plots (A) and cumulative data (B, C) show frequencies of IL-17A+ (A, B) or IL-17F+ (A, C) cells within conventional Vα7.2- (white squares) and unconventional Vα7.2+ (grey squares) CD8+ T-cells (n = 12). (D) Representative dot plots showing the identification of IL-17A+IL-17F−, IL-17A−IL-17F+, IL-17A+IL-17F+ and IL-17A−IL-17F− cells within total CD8+ T-cells after culture in the presence of anti-CD3/CD28 stimulation with hrIL-1β and hrIL-23, and the proportions of CD161 and/or Vα7.2 expressing cells in each of these subsets. Cumulative data (n = 15) are plotted as median ± IQR. Statistical analysis was performed using Friedman test with Dunn's multiple comparison.

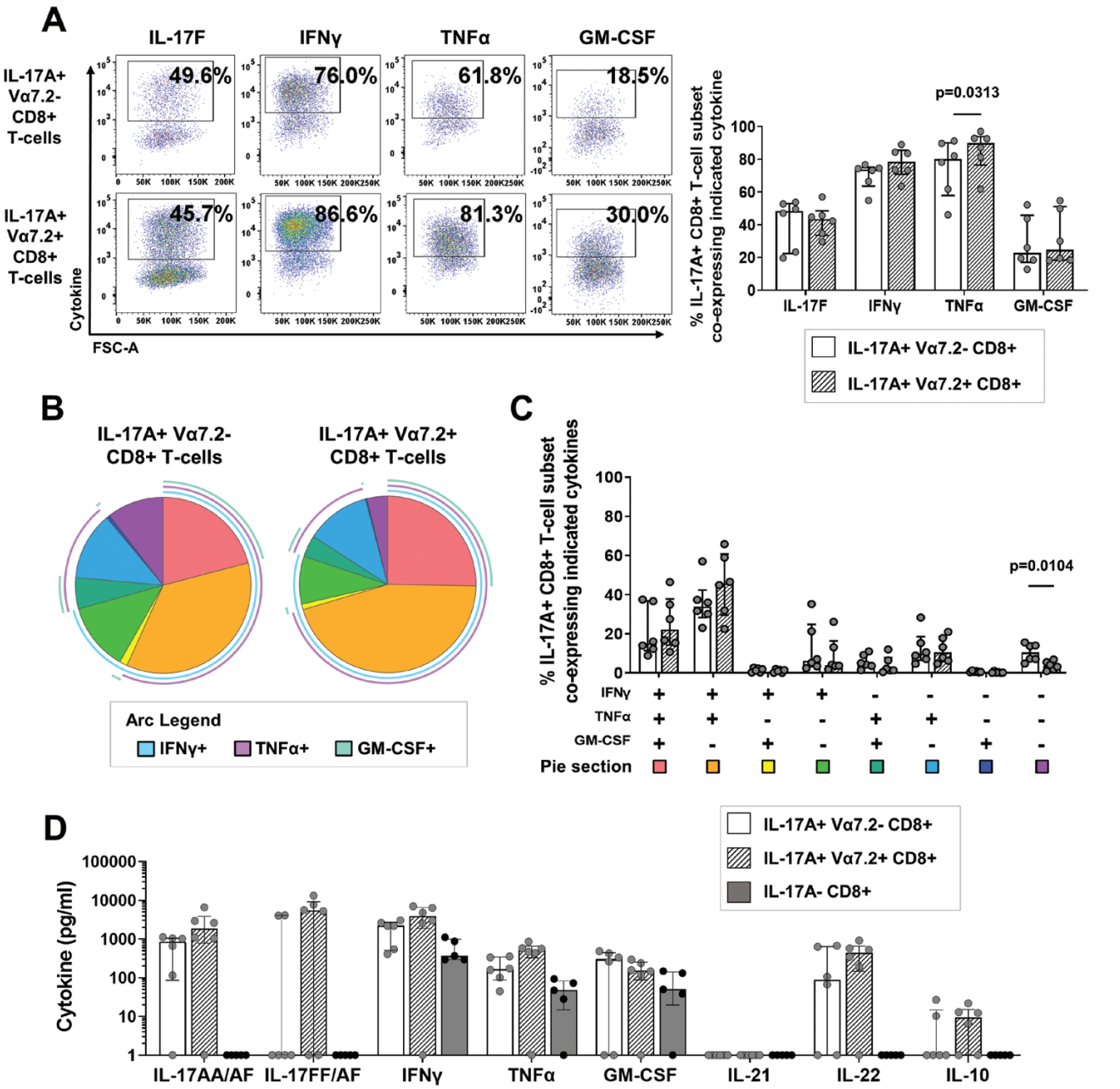


Figure 5. *In vitro*-induced IL-17A+ Vα7.2− and IL-17A+ Vα7.2+ CD8+ T-cell subsets have a similar type-17 cytokine profile. Bulk CD8+ T-cells isolated from healthy donor PBMC were cultured for 3 days with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence of hrIL-1β and hrIL-23. On day 3, cells were re-stimulated for 3 hours with PMA/ionomycin in the presence of GolgiStop for assessment of intracellular cytokine expression. (A) Representative flow cytometric staining and cumulative data (n = 6) show the frequencies of IL-17A+ Vα7.2− (white bars) and IL-17A+ Vα7.2+ (hashed bars) CD8+ T-cell subsets that express IL-17F, IFNγ, TNFα, or GM-CSF. (B, C) Polyfunctional cytokine data were analysed using SPICE software. Pie charts (B) depict cytokine profiles of IL-17A+ Vα7.2− (left) and IL-17A+ Vα7.2+ (right) CD8+ T-cells with the individual and overlapping arcs indicating the double, triple, or quadruple cytokine combinations produced by each proportion of cells. (C) Cumulative frequencies of each pie section within IL-17A+ Vα7.2− (white bars) and IL-17A+ Vα7.2+ (hashed bars) are plotted as median ± IQR (n = 6). (D) CD8+ T-cells were FACS sorted using an IL-17A cytokine secretion assay into IL-17A+ Vα7.2− (white bars), IL-17A+ Vα7.2+ (hashed bars), or IL-17A- (grey bars). Cells were cultured for 20 hours and supernatants collected for detection of IL-17AA/AF, IL-17FF/AF, IFNγ, TNFα, GM-CSF, IL-21, IL-22, and IL-10 by Luminex. Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

(20% v/v) to synovial tissue fibroblasts from patients with PsA. After 24 hours, fibroblast culture supernatants were collected for analysis of the pro-inflammatory cytokines IL-6 and IL-8. Addition of IL-17A+ CD8+ T-cell culture supernatant led to a significant increase in IL-6 and IL-8 production by PsA fibroblasts as compared with fibroblasts cultured in medium alone, while no significant increase in either IL-6 or IL-8 secretion was observed when PsA fibroblasts were cultured in the presence of IL-17A− CD8+ T-cell supernatants (Fig. 6A).

We determined whether there were differences in these pro-inflammatory responses when fibroblasts were cultured in the presence of secretory products from *in vitro*-induced IL-17A+ Vα7.2− conventional or IL-17A+ Vα7.2+ MAIT CD8+ T-cells. Addition of supernatants from either cell type

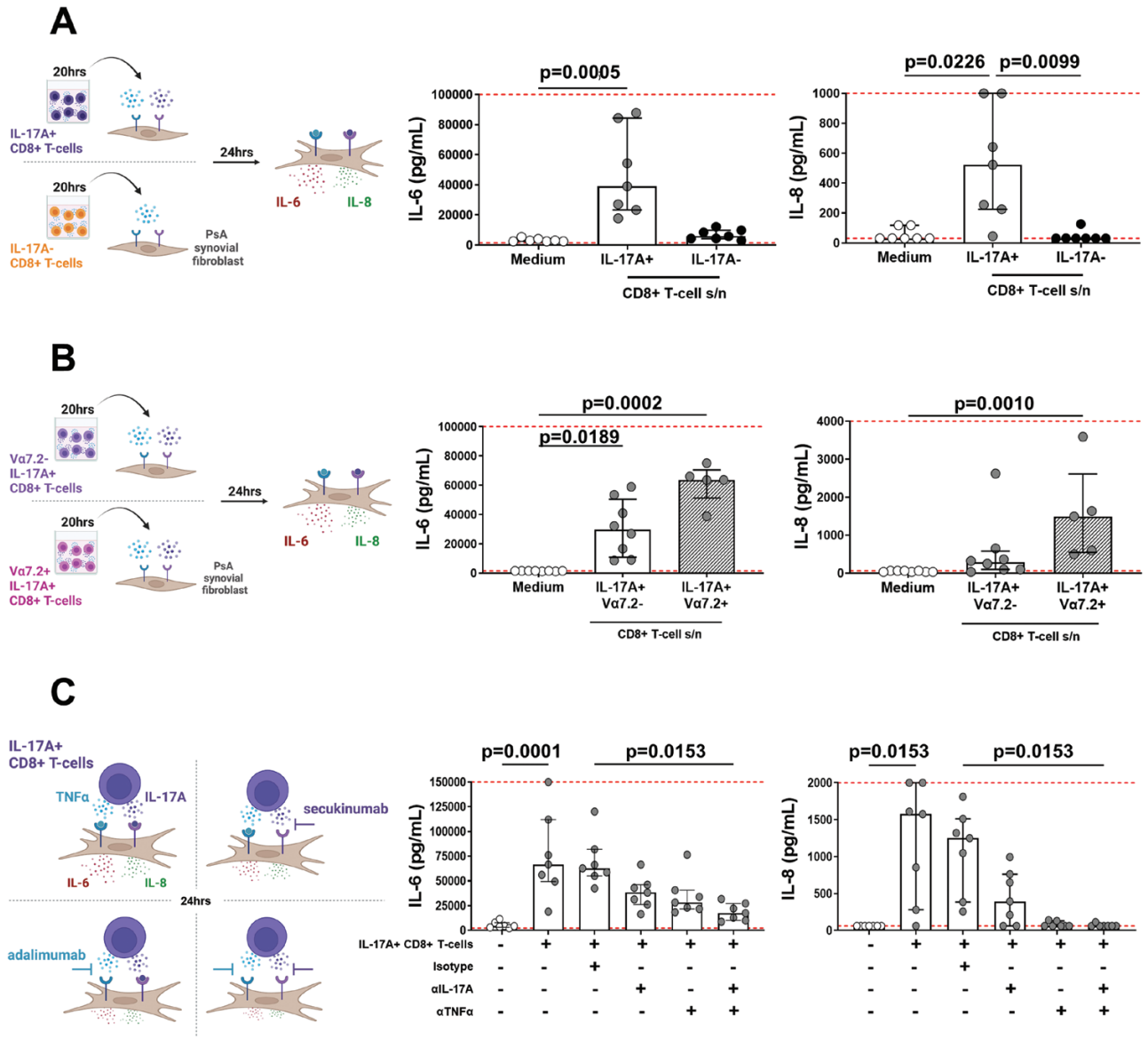


Figure 6. *In vitro*-induced IL-17A+ CD8+ T-cells are biologically functional. Bulk IL-17A+ and IL-17A– CD8+ T-cells or IL-17A+ Vα7.2– and IL-17A+ Vα7.2+ CD8+ T-cells were FACS sorted using an IL-17A cytokine secretion assay following 3-day culture with plate-bound anti-CD3, soluble anti-CD28 mAbs in the presence of hrIL-1β and hrIL-23. The T-cells or their post-sort 20-hour cell culture supernatants (20% v/v) were added to PsA synovial fibroblasts (1×10^4) for 24 hours. IL-6 (left panels) and IL-8 (right panels) production in fibroblast culture supernatants were measured by ELISA. Schematics depict experimental workflows. Cumulative data show IL-6 and IL-8 production from fibroblasts cultured with (A) supernatants from sorted IL-17A+ or IL-17A- CD8+ T-cells, (B) supernatants from IL-17A+ Vα7.2– or IL-17A+ Vα7.2+ CD8+ T-cells, or (C) total IL-17A+ CD8+ T-cells (2.5×10^4) in the absence or presence of isotype control, anti-IL-17A or anti-TNFα neutralizing antibodies. Data are plotted as median ± IQR with dashed lines indicating lower and upper ELISA detection limits. Statistical analysis was performed using a matched-paired Friedman test (A, C, $n = 7$) or unmatched Kruskal Wallis test (B, $n = 5$–7) with comparison to medium (A–C) and isotype (C) by Dunn's Multiple Comparisons test.

induced a significant increase in IL-6 production compared with fibroblasts cultured in medium alone. IL-8 levels were significantly increased in the presence of cell culture supernatant from IL-17A+ Vα7.2+ CD8+ T-cells but not from IL-17A+ Vα7.2– CD8+ T-cells. While the median IL-6 and IL-8 production was higher in presence of cell culture supernatant from IL-17A+ CD8+ MAIT cells compared with conventional T-cells (median: 63 700 pg/ml versus 29 500 pg/ml and 1500 pg/ml versus 280 pg/ml, respectively), this difference did not reach statistical significance (Fig. 6B).

IL-17A is known to act synergistically with TNFα to promote pro-inflammatory cytokine production by stromal cells [4, 5]. Given our observation that *in vitro*-induced IL-17A+ CD8+ T-cell subsets actively secreted IL-17A and TNFα (Fig. 5), we investigated the effect of single and dual blockade of these cytokines in co-cultures of PsA fibroblasts and FACS-sorted *in vitro*-induced IL-17A+ CD8+ T-cells (1:2.5 cell ratio). First, we identified that as with T-cell supernatants, fibroblasts co-cultured with *in vitro*-induced IL-17A+ CD8+ T-cells produced significantly higher levels of IL-6 and IL-8 compared with fibroblast monocultures ($P = 0.0001$ and $P = 0.0153$, respectively) (Fig. 6C). Addition of an isotype control mAb to the co-cultures did not affect IL-6 or IL-8 production. IL-6 and IL-8 production was lower in the presence of either secukinumab (anti-IL-17A) or adalimumab (anti-TNFα) and was significantly reduced upon combined blockade of IL-17A

and TNFα (P = 0.0153). Collectively, these data demonstrate that *in vitro*-induced IL-17A+ CD8+ T-cells and their secretory products are biologically active, with capacity to significantly increase pro-inflammatory IL-6 and IL-8 production by synovial fibroblasts from patients with PsA.

Discussion

Our findings show that human IL-17A+ and/or IL-17F+ CD8+ T-cells that are induced *in vitro* upon anti-CD3 and anti-CD28 stimulation in the presence of IL-1β and IL-23 display typical type-17 phenotype, cytokine and transcriptional profiles. Furthermore, we show that *in vitro* polarization induces both conventional IL-17A+ CD8+ T-cells as well as unconventional IL-17A+ CD8+ MAIT cells. Our findings demonstrate that these two induced cell types have a similar phenotype and are functionally active, with the capacity to drive biologically relevant pro-inflammatory cytokine production from PsA synovial tissue-derived stromal cells.

Our study confirms previous reports that detected low *ex vivo* frequencies of IL-17A+ CD8+ T-cells in healthy human peripheral blood with a rare presence of IL-17F+ CD8+ T-cells [30, 33–36] and adds weight to the few studies that reported on the expansion of human IL-17-expressing CD8+ T-cells [22, 23]. We also demonstrate that equivalent IL-17A+ CD8+ T-cell induction can be achieved using either cryopreserved or freshly isolated PBMC, indicating that our protocol can be used with bio-banked samples. Kondo et al. [22] previously showed a limited percentage of Tc17 cells (0.11%) were differentiated upon culture of human naïve CD8+ T-cells with anti-CD3/CD28 mAbs in the presence of TGFβ, IL-6, IL-1β, and IL-23 for 5 days and supplemented with IL-2 for a further 4 days. Gras et al. [23] instead stimulated bulk CD8+ T-cells with anti-CD3/CD28 mAbs, TGFβ and IL-6 for 3 days and measured secreted IL-17A and IL-17F in culture supernatants by ELISA as readouts of Tc17 cell induction. The authors reported elevated secretion of IL-17A and IL-17F compared with the control condition (anti-CD3/CD28 stimulation without TGFβ and IL-6). These studies each tested a very small sample size (n = 2–3) and presented limited assessment on either the frequency or immunophenotype of IL-17A and/or IL-17F+ CD8+ T-cells. Two recent studies reported the use of *in vitro* polarizing cultures to investigate pharmacological or cytokine-directed modulation of Tc17 cell responses [13, 26]. The study by Li et al. [26] reported a 20-fold increase in Tc17 cell frequency of healthy and colorectal cancer patient naïve CD8+ T-cell cultures upon 7-day culture in IMDM media using anti-CD3/CD28 mAb stimulation with IL-2, IL-1β, IL-6, IL-23, TGFβ, anti-IL-4, and anti-IFNγ. Globig et al. reported a maximum induction of ~0.6% IL-17A+ CD8+ T-cells within healthy donor PBMC cultured with T-cell activator in the presence of IL-1β, IL-6, IL-23, and TGFβ [13]. Neither study performed extensive Tc17 cell immunophenotyping post-culture as we report here. Our study adds to these previous reports by showing a 3–5-fold increase in the frequencies of IL-17A+ CD8+ T-cells as well as a 7-fold increase in IL-17F+ CD8+ T-cells upon anti-CD3/CD28 mAb stimulation in the presence of IL-1β and IL-23. Our data suggest IL-1β and IL-23 are sufficient to induce an increase in IL-17 expression in human CD8+ T-cells, as no additive effect was observed upon supplementation with IL-6, IL-2, or anti-IFNγ. This is in line with previous studies that showed that IL-6 is not a requirement for human Th17 cell differentiation that IL-2 might be inhibitory for IL-17A production [37] and that IFNγ neutralization could either promote or antagonize Th17 cell polarization depending on the timing of administration [38, 39].

Transcriptional analysis revealed that our *in vitro* polarizing culture confers a strong type-17 signature in induced IL-17A+ CD8+ T-cells distinct from IL-17A– CD8+ T-cells. This signature was characterized by high expression of type-17-related genes including *IL17A*, *IL17F*, *RORC*, *RORA*, *MAF*, *CCR6*, *CXCR6*, *KLRB1*, and *IL23R*, which is concordant with previous immunophenotyping reports of human Tc17 cells in health and at sites of inflammation [18, 30, 40]. In addition, some of the more novel markers associated with human Th17 and/or Tc17 cells were found more abundantly expressed in our IL-17A+ CD8+ T-cells including *CTSL* (encoding cathepsin L), *HOPX* (encoding homeodomain-only protein homeobox), *MCAM* (melanoma cell adhesion molecule), and *GPR65* (G protein-coupled receptor 65) [41–45], as was *CTLA4* (encoding cytotoxic T-lymphocyte-associated protein 4), which has been described as a regulator of murine Tc17 cell differentiation and stability [46]. Both sorted subsets showed similar levels of mRNA expression for a number of surface receptors (*IL6R*, *IL18R1*, *IL21R*), transcription factors (*TBX21*, *EOMES*, *STAT3*, *IRF4*, *BCL11B*), and effector molecules (*IFNG*, *TNF*). We confirmed by flow cytometry that *in vitro*-induced IL-17A+ CD8+ T-cells predominantly co-expressed the hallmark type-17 surface markers CCR6 and CD161. A small proportion of IL-17A– CD8+ T-cells also expressed CCR6 and CD161 (also at lower transcript levels than IL-17A+ counterparts), which is consistent with literature highlighting that these surface markers are not exclusively restricted to human IL-17-expressing T-cells [28, 47]. This finding reiterates the need for identification of additional lineage-specific surface markers to better facilitate identification of Tc17 cells and type-17 cells more broadly. Taken together, these analyses validate the CSA approach for specifically purifying type-17 cells that secrete bioactive IL-17A.

The leading transcriptional parameter defining segregation of IL-17A+ from IL-17A– CD8+ T-cells was identified as *ZBTB16*, which is commonly associated with directing type-17 effector function in unconventional rather than conventional T-cells [48, 49]. Our analysis indeed revealed that *in vitro* polarization did not only induce IL-17A and IL-17F expression in conventional CD8+ T-cells, but also in Vα7.2+/MR1-tetramer^{pos} MAIT cells. MAIT cells are an innate-like T-cell subset defined by high expression of CD161 and their semi-invariant αβ T-cell receptor (TCR) restricted to Vα7.2-Jα33/Jα12/Jα20 [50]. Expression of TCRVα7.2 restricts MAIT cells to the non-polymorphic MHC class Ib molecule MHC-related protein 1 (MR1), which presents microbial-derived metabolites of riboflavin (vitamin B2) biosynthesis including 5-(2-oxopropylideneamino)-6-ribitylaminouracil (5-OP-RU) [51]. These unconventional T-cells are abundant in human tissues such as the gut and liver as well as in peripheral blood where they are predominantly CD8+ (enriched for CD8αα). Owing to their innate-like capacity, MAIT cell activation is elicited through either a TCR-dependent, TCR-independent, or synergistic manner, with the local cytokine milieu purportedly enhancing their effector function [52, 53]. MAIT cells can express several type-17-associated markers including IL-23R, CCR6, RORγt and are potent producers of IL-17A and IL-17F that together with

IFNγ, TNFα, and granzymes, rapidly orchestrate protective anti-microbial responses (reviewed in [54]). Moreover, and akin to classical Tc17 cells, MAIT cells (notably of a type-17 phenotype) have been implicated in several inflammatory diseases including psoriasis and spondyloarthritis [55–57]. Interestingly, the largest fraction of *in vitro*-induced IL-17A/F expressing cells was contained within the MAIT population. One explanation could be that there was preferential expansion of IL-17A/F-producing MAIT cells upon *in vitro* culture; however, our preliminary data did not reveal substantive differences in proliferative capacity between IL-17A+ CD8+ MAIT and conventional T-cells.

It was interesting to note that our PBMC polarizing conditions led to a relative greater fold increase in IL-17F than IL-17A (7-fold versus 3-fold). In addition, both conventional and MAIT IL-17A+ CD8+ T-cells comprised IL-17A or IL-17F single and double positive cells. We have previously demonstrated that CD28 co-stimulation differentially regulates IL-17F versus IL-17A expression in CD4+ T-cells [5]. Furthermore, a previous study showed a 10-fold higher production of IL-17F compared with IL-17A upon anti-CD3/CD28 stimulation of CD8+ T-cells [23], and IL-17F was found to be the dominant isoform when MAIT cells were stimulated in the presence of IL-12 and IL-18 [58]. Collectively, these findings suggest differential regulation of IL-17A and IL-17F production in human T-cells.

Even though *in vitro* polarization induced two distinct populations of IL-17A+ CD8+ T-cells, our data indicate that Vα7.2– conventional and Vα7.2+ MAIT IL-17A+ CD8+ T-cell types share a common polyfunctional profile and actively secrete a combination of pro-inflammatory cytokines including IL-17A, IL-22, GM-CSF, IFNγ, and TNFα. Of note, IL-17A+ Vα7.2+ CD8+ T-cells appeared to induce a more potent pro-inflammatory response in PsA-derived synovial fibroblasts, on average inducing greater levels of IL-6 and IL-8 compared with IL-17A+ Vα7.2– conventional CD8+ T-cells. This may be attributable to elevated levels of TNFα, and for some donors more IL-17AA/FF, IFNγ and/or IL-17FF/AF, in IL-17A+ Vα7.2+ CD8+ T-cells compared with the IL-17A+ Vα7.2– CD8+ T-cell counterparts. These subtle differences in our findings strengthen the importance to distinguish between classical and unconventional cell types, which few studies to date have done when reporting on IL-17-producing CD8+ T-cells. This may explain research discrepancies, and it is, therefore, important that in future protective or pathological contributions of individual IL-17A producing CD8+ T-cell subsets are clearly ascribed to conventional or unconventional T-cell types.

By addition of secukinumab and adalimumab in IL-17A+CD8+ T-cell–fibroblast co-cultures, we reaffirmed co-operation of IL-17A with TNFα in driving pro-inflammatory cytokine production in the context of PsA. While IL-17F has reduced potency relative to IL-17A, we previously showed IL-17F can similarly synergize with TNFα to elicit significant inflammatory responses in synovial fibroblasts from PsA and RA patients [5]. Dual blockade of IL-17A and IL-17F by bimekizumab also more effectively reduced IL-17-driven secretion of IL-6 and IL-8 by synovial fibroblasts compared with blockade of IL-17A alone [5, 58, 59]. An IL-17F CSA is currently not commercially available; however, it would be of interest to isolate IL-17A and IL-17F single and double positive T-cell subsets to investigate further their individual versus combined functional roles.

Our data clearly indicate that *in vitro*-generated IL-17A+ CD8+ T-cells have a pro-inflammatory effect on synovial fibroblasts, implying that Tc17 cells contribute to PsA joint inflammation. A pathogenic function of human Tc17 cells has been demonstrated previously in adoptive transfer models. Hu et al. [24] showed that adoptive transfer of human *in vitro*-generated CAR-directed type-17 T-cells (a mixture of Th17 and Tc17) primed with a RORγt agonist-mediated potent anti-tumour responses in a murine melanoma model. Mechanistically, they showed that co-transfer of Th17 cells with Tc17 cells-mediated robust and long-lived anti-tumour immunity, consistent with previous publications, which showed that Th17 cells can augment the activation of CD8+ T-cells [60, 61]. Hinrichs et al. [62] also reported that type 17-polarized CD8+ T-cells mediated enhanced anti-tumour immunity and demonstrated greater persistence than non-polarized CD8+ T-cells. An in-depth study by Gartlan et al. [63] using fate mapping reporter mice also showed that mouse Tc17 cells differentiate during GVHD culminating in a highly plastic, hyperinflammatory, poorly cytolytic effector population, which they termed ‘inflammatory iTc17’. Targeted depletion of these inflammatory iTc17 cells resulted in protection from lethal GVHD.

Taken together, these *in vivo* data together with our *in vitro* data strongly suggest that Tc17 cells are biologically relevant contributors to inflammation in diseases where an enrichment of these cells is found. In addition, our *in vitro* induction approach has important potential to improve mechanistic understanding of how Tc17 cells contribute to pathogenic, as well as homeostatic, immune responses, which could offer novel translational insights into therapeutic targeting of these cells.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

Ethical approval for the study and collection of healthy donor peripheral blood and PsA patient synovial membrane tissue samples following informed consent was obtained from Bromley Local, Bexley & Greenwich, and London – Harrow Research Ethic Committees (REC refs: 06/Q0705/20, 07/H0809/35, and 17/LO/1940, respectively).

Conflict of interests

LST has previously received speaker fees and/or research support from GSK, Novo Nordisk A/S, UCB, and Novartis, outside this work. BWK has received research support from Eli-Lilly, Novartis, Roche Pharmaceuticals, and UCB Pharma, and has been an advisor or received speaker fees from Eli-Lilly, Janssen, and Novartis, outside this work. The other authors declare no commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

L.T., B.K., and K.S. contributed to study conception and supervision. L.T., U.S., and E.G. designed experiments that were equally performed, analysed, and interpreted by U.S. and E.G. L.D. and S.L. aided with CSA-FACS sorting experiments, and A.C. kindly provided some synovial tissue fibroblast lines for functional assessments. B.K. coordinated PsA patient recruitment and sample collection during clinic at the Rheumatology Department, Guy's hospital. U.S. wrote an initial draft of the manuscript, which was re-worked and finalized by E.G. with editing by L.T. All authors except A.C., who sadly passed away during completion of the project, were involved in critically revising the article and approved the final submitted version.

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