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Characterization of Blood Mucosal Associated Invariant T (MAIT) cells in Axial Spondyloarthritis and of resident MAITs from control axial enthesis

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Running Title: Characterization of peripheral and entheseal IL-17 secreting Mucosal Associated Invariant T (MAIT) cells in Axial Spondyloarthritis

Accepted Article

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Accepted Article

Abstract

Objectives: The importance of IL-17A in the pathogenesis of Axial Spondyloarthritis (AxSpA) has been demonstrated by the success of IL-17A blockade. However, the nature of the cell populations producing this important pro-inflammatory cytokine remains poorly defined. Accordingly, we characterized major IL-17A-producing blood cell populations in AxSpA, with reference to entheseal biology, with a focus on mucosal associated invariant T-cells, (MAITs) a population known to be capable of producing IL-17.

Methods: IL-17A production from 5 sorted peripheral blood cell populations, namely MAITs, γδ T-cells, CD4+T-cells, CD8+T-cells and neutrophils was evaluated pre- and post-stimulation with PMA, the calcium ionophore A23187 and β1,3 glucan. IL-17A transcript and protein expression were determined using nCounter Technology and ultra-sensitive SimoA technology, respectively. MAITs from control human axial entheses (n=5) were further characterized by flow cytometric immunophenotyping, qPCR and IL-17 production assessed following stimulation.

Results: In blood, on a per cell basis, MAIT cells produced the highest amount of IL-17A compared to CD4+ ($p < 0.01$), CD8+ ($p < 0.0001$) and γδ T-cells ($p < 0.0001$). IL-17A was not produced by neutrophils. Gene expression analysis also showed significantly higher *IL17A* and *IL23R* expression in MAIT cells. Stimulation of blood MAIT cells with αCD3/CD28 and IL-7 and/or IL-18 induced strong expression of *IL17F*. MAITs were present in the normal enthesis and showed elevated *AHR*, *JAK1*, *STAT4* and *TGFB1* transcript expression with inducible IL-17A protein. IL-18 protein expression was evident in spinal enthesis digests.

Conclusion: Both blood MAITs and resident MAITs in axial entheses contribute to IL-17 production and may play important roles in AxSpA pathogenesis.

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Introduction

Within the last 15 years, a clear role of the IL-23/IL-17 axis underpinning physiopathology of the axial spondyloarthritis (axSpA) has emerged (1) as many of the genetic variants associated with AS susceptibility have been identified through Genome Wide Association Studies (GWAS), among which are genes in the IL-23/IL-17 pathway (*IL23R*, *IL12B*, *IL6R, JAK2, TYK2*) (2). As IL-17 is the terminal cytokine of this pathophysiological pathway, the development of new treatments has initially focused on blocking this cytokine (3, 4). To support this hypothesis and beyond the result of the genetic associations, an increased prevalence of T helper IL-17 cells (Th17) has been reported in the peripheral blood of AxSpA patients (5) and at the normal human enthesis (6). The γδ T-cells and MAITs cells were further described as alternative sources of IL-17A in the blood from AxSpA (7, 8). Appel *et al.* also suggested that neutrophils might be the main producers of IL-17A at the facet joint level in AS (9).

Il-23 plays a crucial role in maintaining the differentiation state of Th17. Nevertheless, recent studies have identified alternative pathways, independent of IL-23, that can stimulate IL-17 production, this includes TCR signaling via MR1 engagement by MAIT cells (10), IL-7 signaling in type 3 innate lymphoid cells (ILC3) (11), TGFβ and IL-1β signaling in iNKTs (12,13) and γδ T-cells (specifically the Vδ1 subset) (14), and recently the combination of IL-12 and IL-18 together with anti-CD3/CD28 triggering in MAIT cells (10). Such observations have substantial translational relevance given that antagonism of IL-23 has thus far shown no efficacy in ankylosing spondylitis (15,16).

Considering the growing body of evidence suggesting a role for IL17 in the pathogenesis of SpA, we wished to study the expression capabilities of IL17 by MAITs, CD4+, CD8+ and γδ T-cells in axSpA. We first analyzed cell-type-specific expression pattern of AS-associated genes in those cells, with a particular focus on genes belonging to the IL-23/IL-17 pathway. We further compared the respective IL-17 production capacity of these different cell subsets

from the adaptive and innate immune system and identified MAIT cells as potent IL-17 secreting cells in AxSpA. Considering that MAIT cells from healthy controls have been reported to express high level of IL7R and IL18R (17), we have shown their ability to produce IL17 independently of IL23 stimulation, in a context of TCR stimulation combined with IL-7 and IL-18. We further identified resident MAIT cells in the normal axial entheses which is the target tissue that leads to severe axial inflammation and later spinal fusion in AxSpA. Taken together, our data highlight the crucial role of MAIT cells in the pathophysiology of axSpA.

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Material and Methods

Patients and samples

AxSpA. Blood samples from 18 patients naïve from synthetic treatments and biologics with a clinical diagnosis of axial Spondyloarthritis (axSpA) fulfilling the Assessment of SpondyloArthritis international Society criteria (18) were included.

Enthesis Tissue from controls. Human interspinous process and matched peripheral blood were obtained from 5 non-axSpA patients who underwent elective spinal surgery for either decompression or scoliosis correction using methods previously reported (19).

All patients provided written informed consent before enrollment in the study as approved by the French Ethics Committee and by the North West-Greater Manchester West Research Ethics Committee. The clinical characteristics of the AxSpA patients and of the non-AxSpA patients who underwent spinal surgery are summarized in online supplementary table 1.

Cell sorting and stimulation

Isolation of cell populations from peripheral blood and entheseal samples was undertaken as previously described (8,20) and is further described in the supplementary material.

Gene expression analysis

Gene expression profiles from AxSpA patients sorted cells were assessed using the nCounter Autoimmune Discovery Consortium panel (NanoString Technologies®) and using a focused gene card on normal enthesitis. Transcriptional profiling of entheseal and blood MAITs (CD3+ CD45+ CD161+ TCRVα7.2+) was performed on non-AxSpA patients. Basal transcript expression of cytokines, chemokines, growth factors, signaling molecules, tissue residency

markers was assessed using a focused gene card. See supplementary materials for RNA preparation and gene expression analysis).

Protein expression analyses

IL-17A concentrations (in fg/ml) in cell culture supernatants from AxSpA patients were determined with Simoa IL-17A 2.0 Reagent kit (Quanterix corp. Lexington, MA 02421) using HD-1 Analyzer (Quanterix ®). Stimulated cells from PEB EMCs were intracellularly stained for TNF and IL-17 and analysed using flow cytometry gating system as before. Following 24hr LPS stimulation (100ng/mL) IL-18 protein was analysed in the supernatant using Legendplex inflammation panel (BioLegend) using the Cytoflex LX flow cytometer (BD) according to the manufacturer's instructions, see supplementary figure 5. Serum levels of IL-17A, IL-17F, IL-7, IL-18 were quantified using the Olink technology (see supplementary materials).

Statistical analysis

GraphPad Prism software was used for statistical analyses (GraphPad Software, USA).

For all graphs, significant results ($p<0.05$) were indicated: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p>0.0001$. Error bars represent the SE of the mean (SEM).

Detailed information regarding methods and statistical analyses is provided in the online supplementary material.

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Results

Genes associated with AS susceptibility are differentially expressed in innate and adaptive T-cell populations isolated from peripheral blood of AxSpA patients

The expression of 36 genes from a panel of 45 AS-associated genes was analysed in the four T-cell populations isolated from 9 AxSpA patients following PMA + A23187 + β-glucan stimulation. The expression pattern observed after hierarchical clustering showed a clear distinction between the innate and adaptive T-cell groups as shown in the heatmap (figure 1A). Gene clusters consisting of members of the MHC class-I mediated antigen processing & presentation - *NPEPPS* and *UBE2L3* - were upregulated in CD4+ and CD8+ T-cells. We observed genes expressed at relatively high levels in a specific cell type, e.g., *PTGER4* in CD4+ T-cells and *TYK2* in CD8+ T-cells.

MAIT cells expressed high levels of *IL23R* and the G protein-coupled receptors *GPR35* and *GPR65*. We also noted cell type-specific expression of several IL-23/IL-17 pathway genes (figure 1B). *IL17F* was expressed ~one log higher in CD4+ and MAIT cells, while *IL23R* was expressed two logs higher in MAIT and one log higher in γδ compared to CD4+ and CD8+ T-cells. *NFKB1*, *RELA* and *NFKBIA* were preferentially expressed in CD4+ T-cells, while several genes encoding cytokines and their receptors (*IL23A, IL23R, IL12RB1, IL18R1, IL18RAP, TNF* and *IFNG*) were expressed at higher levels in innate-like MAIT and γδ T-cells when compared to adaptive CD4+ and CD8+ T-cells. *IL1R1, TYK2* and *RUNX3* were expressed at high levels in CD8+ T-cells. Nevertheless, many other genes, not belonging to the IL-23/IL-17 pathway, participated in cell clustering suggesting that those different cell types were involved in AS susceptibility beyond their relative role in the IL-23/IL-17 pathway.

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Peripheral blood derived MAITs cells have a high potential for IL-17A and IL-17F secretion.

To better define the relative role of MAITs, CD4+, CD8+ and γδ T-cells in IL-17 expression, we further sorted these cells, together with neutrophils whose secretion of IL-17A is still controversial (21). Cell sorting was performed on samples from 18 AxSpA patients (see supplementary figure 1 for the gating strategy). IL-17A production by MAIT cells was significantly higher than CD4+ ($p < 0.05$), γδ ($p < 0.01$) and CD8+ ($p < 0.01$) T-cells as well as neutrophils ($p < 0.01$). Although lower than in MAITs (mean = 478.60 fg / 1000 cells), IL-17A production by CD4+ T-cells was significant (mean = 128.65 fg / 1000 cells). Whereas γδ T-cells produced a smaller amount of IL-17A (mean of 13.71 fg / 1000 cells), albeit in the same range as IL-17A production by CD8+ T-cells (mean = 4.66 fg / 1000 cells). β-glucan is the main component of *Aspergillus Fumigatus* hyphae and has been demonstrated to have a potential effect on the production of IL-17A by human neutrophils (22). Despite a strong β-glucan-associated stimulation, most neutrophils' expression for IL-17A in AxSpA did not exceed the detection limit (figure 2A). Gene expression analysis confirmed the protein data, with MAIT cells displaying the highest level of *IL17A* expression with CD4+ T-cells (no significant difference between MAIT and CD4+ T cells). A low level of *IL17A* expression was observed in γδ and CD8+ T-cells. For neutrophils, *IL17A* was undetectable, results that were consistent with protein data (figure 2B).

Next, we expanded our analysis to *IL17F*, *IL23R* and *IFNγ* expression. We observed that the expression level of *IL17F* displayed similar ranking to *IL17A* for the 5 cell populations (figure 3A), but there was no significant difference between CD4+ T-cells and MAITs. We also observed a 10-fold lower level of expression of *IL17F* compared with that of *IL17A* in all cell subsets. Regarding *IL23R* (figure 3B), MAITs had the highest level of expression, followed by γδ T-cells and CD4+ T-cells. Whereas CD8+ T-cells and neutrophils did not express significant

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levels of IL-23R. Contrary to *IL17A/IL17F*, the expression level of *IFNG* was high in all T-cell populations (figure 3C) suggesting cell-specific expression profiles for *IL17A/F*. Collectively, these data indicate that MAIT cells are the major producers of IL-17A at a per-cell level and express high levels of both *IL17F* and *IL23R* compared to the other IL-17A-producing cells in AxSpA.

IL-7 and IL-18 enhance *IL17F* expression in peripheral blood-derived MAIT cells.

We further assessed which stimulation conditions were able to induce IL-17A and IL-17F protein expression by MAIT cells. We stimulated sorted MAIT (see supplementary figure 2 for gating strategy) for 36 hours with anti CD3/CD28 alone or in combination with individual cytokines (IL-7, IL-18) or both IL-7 and IL-18 combined. We used CD4+CCR6+ T-cells ("Th17 like" T-cells) as control (figure 4). We observed that stimulation with CD3/CD28 in addition to IL-7 or IL-18 induced high expression of *IL17F* but not *IL17A* by MAIT cells (figure 4A and 4B). Furthermore, our results identified increased *IL-18R1* expression (figure 1B) by MAIT cells which supports our finding that IL-18 induces *IL17F* production. The combination of both cytokines (IL-7 and IL-18) with CD3/CD28 further increased *IL17F* expression. The expression of *IFNγ* was also remarkably high after stimulation by CD3/CD28 associated with IL-7 or IL-18 (figure 4C). Basal levels of expression of IL-18 were demonstrated in MAIT cells isolated from the normal human enthesis, (supplementary figure 5) which arguably supports the importance of IL-18 in MAIT cell modulation. In peripheral blood, the comparison between patients and healthy controls did not show a significant difference on the serum production of IL-17A, and IL-18 but a trend towards increased IL-17F and IL-7 expression in SpA patients was observed ($p=0.0508$ and $p = 0.06$, respectively) (supplementary figure 6). In MAIT cells, no significant difference in IL-17A secretion (supplementary figure 7) or IL-17A gene expression (supplementary figure 8) was observed.

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MAIT cells are present in human entheses

Considering that the hallmark of axial SpA pathogenesis is entheseal inflammation, we investigated and found the presence of MAIT cells within human axial enthesis (figure 5A). Within both EST and PEB, MAIT cells mainly expressed the CD69 resident memory marker compared to MAIT cells from the peripheral blood which were mainly CD45RA+ corresponding to a naïve/circulating phenotype (figure 5A).

Transcriptional profiling of entheseal MAIT cells compared to peripheral blood-derived MAIT cells

The comparison of blood and entheseal-derived (from both PEB and EST) MAIT cell transcripts (Vα7.2+ CD161+) identified EST MAIT cells with a phenotype compromising of higher *AhR, JAK1*, *STAT4* and *TGFβ1* transcript expression (figure 5B). Furthermore, transcripts indicative of circulating T-cells, such as *KLF2* and *T-bet* showed higher transcript expression in blood MAITs (figure 5B). Entheseal MAIT cells also showed higher expression of growth factors and molecules associated with tissue repair and homeostasis, such as *VEGFA* and *IL-10* when compared to matched peripheral blood (figure 5B).

In comparison to blood derived MAITs, entheseal-derived MAITs showed higher *CXCR3* and *CCR6* expression which could suggest that entheseal-derived MAIT's are better equipped to mediate pro-inflammatory signals and tissue migration.

TNF and IL-17 cytokine expression can be induced from entheseal MAITs

Following on previous results showing blood derived MAIT cells producing IL-17, we next investigated if entheseal MAITs had the ability to produce this cytokine. Following PMA/ionomycin stimulation, MAITs showed elevated expression of TNFα and IL-17A through intracellular flow (figure 5C). Overall, 3.2% of MAITs had inducible IL-17A and 17.9% inducible TNF (n=2). Given that IL-18 was shown to enhance IL-17 production, we

also investigated and confirmed that IL-18 was present in the human enthesis at basal levels (Supplementary figure 5).

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Discussion

The work presented herein suggests a potentially important role for MAITs in axial SpA. Gene expression profiles based on gene polymorphisms associated with AS showed substantial clustering in MAIT cells. MAIT cells had at least as much IL17A production capacity as CD4+ T cells. Furthermore, MAITs were able to produce *IL-17A* and *IL-17F* under IL-7 and IL-18 stimulation together with TCR triggering. Finally, MAITs were present in the spinal enthesis of healthy subjects where they mainly showed a resident memory cells phenotype with inducible IL-17A protein production. Collectively, these findings substantially add to the body of evidence incriminating innate-like lymphocytes in the pathogenesis of axial SpA.

We first analysed the gene expression profiles of 45 genes whose polymorphisms were significantly associated with the predisposition to AS in four T lymphocyte populations: CD4+ and CD8+ T-lymphocytes, γδ T-lymphocytes and MAITs. Eighty percent of these genes were expressed among these four cell types, with differential expression from one cell population to another leading to cell subset clustering. We observed higher expression levels of prostaglandin receptor EP4 (*PTGER4*) in CD4+ T-cells through which Prostaglandin E2 regulates Th17 cell differentiation and functions (23). This up-regulation of EP4 receptor in AxSpA could promote CD4 differentiation towards the Th17 pathway, as previously reported. *TYK2* was expressed at higher levels in adaptive CD8+ T cells, followed by innate-like γδ T cells. TYK2 is a crucial type 3 immunity mediator in SpA, and its inhibition can prevent disease progression by reducing the Th17 cell expansion in murine models of SpA (24). TYK2 inhibition as also showed promising results in a phase 2 trial in psoriasis (25). We observed an up-regulation of *IL18R1* and *IL18RAP* in MAIT cells. This was in accordance with the sensitivity of MAIT cells to IL-18 stimulation by for the induction of IL-17. High expression of IL-18R and IL-12R by MAIT cells has been shown to facilitate their activation in a TCR-independent manner, during

viral infections (26). The relatively high expression of the AS-associated G-protein coupled receptor (GPCR) genes, *GPR35* and *GPR65,* in MAIT cells could indicate their pathogenic role in AxSpA as increased expression of *GPR65* in GM-CSF^{+} CD4+ has been associated to "pathogenic" Th17 cells in SpA patients (27). These results could help us to design functional analysis specifically in MAIT cells.

To better decipher the relative contribution of MAITs in IL-17A/F expression compared with CD4+ T-cells, we assessed IL-17A secretion in cell culture supernatant under cell stimulation. MAIT frequency is relatively low compared to CD4+ and CD8+ T cells. However, on a per-cell basis, MAIT cells were the major producers of IL-17A in AxSpA compared to CD4+T-cells, CD8+T-cells and γδT-cells. The analysis on per cell basis allowed us to precisely characterize the production capacity of each cell type which is generally challenging for small cell subsets.

MAITs strongly expressed *IL23R* but our work shows that cytokines other than IL-23, such as the IL-7/IL-18 alone or in combination induced strong expression of *IL-17F* mRNA. We found particular high levels of *IL18R1* and *IL18RAP* in MAIT cells, suggesting that IL-18 is an important cytokine in the modulation of their function. Furthermore, we found IL-18 production at the basal level in unstimulated MAIT cells derived from PEB in non-axSpA, which supports the importance of IL-18 in MAIT cell regulation. Associated with IL-12 and IL-15, IL-18 induces IFNγ secretion by Th1 (28) and has been shown to synergize with IL-12 to promote IL-17A/F production by MAIT cells independently of IL-23 (10). Here we show that another cytokine combination (IL-7 and IL-18) particularly potentiates the production of IL-17F by MAIT. To our knowledge, this is the first time that a combination of cytokines has been identified to induce the expression of *IL-17F* by MAIT cells in AxSpA. IL-7 is a key cytokine of the adaptive immune system, especially for the development of T-cells and dendritic cells but also for the expansion and survival of immature B-cells. Whilst stromal and

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epithelial cells are known to be the main producers of IL-7, Ciccia *et al.* also showed that intestinal Paneth cells could also produce it (29). IL-7 is constitutively produced at low levels whereas elevated levels of IL-7 have been observed in sacroiliac joint fluid in patients with SpA (30). Recently IL-17F gained interest with the approval of bimekizumab in psoriasis (31) and promising results in AxSpA (32). Here, we didn't focus on the role of IL23R. Further studies are needed with a specific approach to better decipher the complexity of this interaction in the different IL17 producing cells.

In this work, the contribution of blood γδ and CD8+T-cells to IL-17A production was minimal but it is possible that tissue specific expression at target sites of disease is much higher. Whilst neutrophils have been previously described as IL-17 producing cells (22), this was not confirmed in our work with two other robust techniques (Nanostring and SimoA technologies), even using strong stimulation combining PMA, A23187 and β1,3 glucan, and supporting data that neutrophils do not substantially contribute to IL-17 production (21).

In the peripheral form of the disease, several publications suggest that MAITs are not the only IL-17 producing cells. Both iNKT and γδT-cells are increased in the synovial fluid of patients with SpA and contribute to IL-17 expression, but through an IL-23-dependent mechanism (13). This may be in part due to shared transcription factor PLZF (Promyelocytic Leukaemia Zinc Finger, encoded by *ZBTB16*) that is present in MAITs, iNKTs and γδ T-cells (33). Kenna *et al.* observed a high expression of IL-23R on the surface of γδ T-cells (6) but this cell population did not appear to be the main source of IL-17 in the axial form of the disease. ILC3's are also able to produce IL-17A but *Blijdorp et al.* (34) have recently shown that these cells produced IL-22 rather than IL-17A in the joints of patients with peripheral SpA.

This study had some limitations that could be addressed in future research is with regards to the assessment of a local production of IL-7 and IL-18 within entheses tissues among AxSpA

patients. It would be ideal to get matched MAIT cells from blood and enthesis in diseased patients, but tissue access is difficult.

In conclusion, this used data from GWAS studies to look at transcript expression in disease relevant cells in axial SpA with a focus on the IL-23/IL-17 pathway, especially given the recent unexpected translational therapeutics in SpA where IL-17, but not IL-23 inhibition, is effective. These data reinforce the hypothesis of the major involvement of conventional T-cells subsets and innate-like lymphocytes in the pathogenesis of SpA. However, blood MAIT cells were shown to be a main producer of IL-17A in AxSpA compared to CD4+, CD8+ and γδ T-cells. Moreover, IL-7/IL-18 combined stimulation was able to strongly induce *IL-17F* expression by MAITs cells. A trend towards an increased expression of IL-17F and IL-7 was also observed in the serum of SpA patients compared with controls. The importance of MAIT cells was highlighted by their identification within entheseal tissues where basal IL-18 protein expression was also found. Further studies are needed to assess whether similar findings hold true in the entheseal tissues from AxSpA patients and to what extent the *in-situ* production of IL-7 and IL-18 could induce IL-17F expression.

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Figure legends

Figure 1A: Heatmap showing the expression of genes associated with AS in T-cell subpopulations isolated from peripheral blood of AxSpA patients. The expression of 36 genes genetically associated with AS susceptibility in T-cells from AxSpA patients after 2h of stimulation by PMA (50ng/ml) + A23187(5μM) + β-glucan (50μg/ml). Cell populations are colour-coded as shown by the legend on the left. Columns in the heatmap represent patient samples (n=9) and the rows represent genes.

Figure 1B: The heatmap depicts the gene expression pattern of IL-23/IL-17 pathway genes in T-cell populations isolated from AxSpA patients as above. Genes associated with the IL-23/IL-17 pathway were selected from the molecular signature database (MSigDB). Shown are the mRNA levels of 29 genes after 2h of stimulation by PMA (50ng/ml) + A23187(5μM) + β-glucan (50μg/ml). Cell populations are colour-coded as shown by the legend on the left. Columns in the heatmap represent patient samples (n=9) and the rows represent genes.

The heatmaps are ordered by hierarchical clustering. Gene expression data are $\log_2$ transformed, centred to a mean value of zero and scaled to unit variance. The bar on the left denotes the scale for the gene expression levels- yellow higher- and blue lower levels of expression.

Figure 2: IL-17A protein production and *IL17A* gene expression by sorted cells from peripheral blood of Axial SpA patients.

(A) Left, IL-17A protein levels presented as femtograms per 1000 cells in sorted CD4+ T-cells (in yellow), MAIT cells (in red), CD8+T-cells (in blue), $\gamma\delta$ - cells (in purple) and neutrophils (in green) after 18h of stimulation by PMA (50ng/ml) + A23187(5μM) + βglucan (50μg/ml). P-values were calculated using a Mann-Whitney T test. Horizontal bars indicate the median and SD.

Differences are considered significant for P-values < 0.05, ****, P< 0.0001, ***, P<0.001, **, P<0.01, *, P<0.05, ns; not significant.

(B) Right, Transcripts levels of *IL17A* presented as normalized counts in sorted CD4+ T-cells (in yellow), MAIT cells (in red), CD8+T-cells (in blue), $\gamma\delta$ T-cells (in purple) and neutrophils (in green) after 2h of stimulation by PMA (50ng/ml) + A23187(5µM) + βglucan (50µg/ml). P-values were calculated using a Wilcoxon T test. Horizontal bars indicate the median and SD. Differences are considered significant for P-values < 0.05. ****, P< 0.0001, ***, P<0.001, **, P<0.01, *, P<0.05, ns; not significant

Figure 3: Gene expression analysis of sorted cells from peripheral blood of Axial SpA patients.

(A-C) Transcripts levels of *IL17F, IL23R, IFNG* presented as normalized counts in sorted CD4+ T-cells (in yellow), MAIT cells (in red), CD8+T-cells (in blue), $\gamma\delta$ T-cells (in purple) and neutrophils in green) treated by PMA (50ng/ml) + A23187(5µM) + βglucan (50µg/ml). P-values were calculated using a Wilcoxon T test and are indicated above the graph. Horizontal bars indicate the median and SD. Differences are considered significant for P-values < 0.05. ****, P< 0.0001, ***, P<0.001, **, P<0.01, *, P<0.05, ns; not significant.

Figure 4: Stimulation conditions for IL-17 expression in sorted MAIT cells and CD4+CCR6+ T cells.

(A-C) Transcripts levels of *IL17A, IL17F, IFNG* presented as normalized counts in sorted MAIT cells (left column) and CD4+CCR6+ T cells (right column) from donors (n=3) after 36 hours of stimulation in 6 different conditions: unstimulated (black), CD3/CD28 stimulation (blue),CD3/CD28 stimulation + IL-7(20ng/ml) (maroon), CD3/CD28 stimulation + IL-18 (50ng/ml) (orange), CD3/CD28 stimulation + IL-7 (20ng/ml)+ IL-18 (50ng/ml) (green).

Figure 5: Entheseal MAIT cells Transcriptional profiling and pro-inflammatory cytokines induction.

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MAIT cells were sorted from Entheseal Soft Tissue (EST), Peri-entheseal Bone (PEB) and Peripheral Blood (PB) defined by their expression of Vα7.2 TCR and CD161. Cells expressing tissue resident (R)/memory (M) markers were identified by CD69+ and naïve (N)/circulating (C) cells by CD45RA+. Results displayed as mean from n=5 (A). Basal expression of cytokines, chemokines, growth factors, signalling molecules, tissue residency markers was assessed. Colour coding refers to differentially expressed genes where values less than −1 indicate low expression and values greater than 1 indicate higher expression, those with grey boxes indicates no values, values displayed are log 10ΔCt relative to HPRT (n=7) (B). Significance between PB and PEB MAIT's in only CCR6, following an independent samples T-test p=0.038. Intracellular TNF and IL-17 cytokine expression with and without stimulation with PMA (50 ng/ml) and Ionomycin (1 μg/ml) for 3 hrs in the presence of Golgi Plug (BD) in PEB derived MAIT cells (n=2) (C).

Figure S1: Flow cytometry gating strategy 1. Flow cytometry gating strategy for the isolation of MAIT, γδ T-cells, CD4+ and CD8+ T-cells. For T cells, PBMCs were isolated from blood using lymphocyte separation medium (Eurobio®). After isolation, PBMCs were labeled with CD3 BUV395 (BD Biosciences®), CD4 VioBright FITC (Milteny Biotec®), CD8 PerCP vio700 (Milteny Biotec®), TCR Vδ PE (Milteny Biotec®), TCR Vδ2 PE (Milteny Biotec®), TCR Vα7.2 APC (BioLegend®), CD161 BV421 (Sony Biotecnology®).

Figure S2: Flow cytometry gating strategy 2. Flow cytometry gating strategy, after CD3 positive magnetic sorting, of MAIT and CD4+ CCR6+T-cells. The CD3 positive fraction was labeled with CD3 APC Vio770 (Milteny Biotec®) CD4 VioBright FITC (Milteny Biotec®), Vα7.2 APC (BioLegend®), CD161 BV421 (Sony Biotechnology®) and CCR6 BV786 (BD Biosciences®).

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Figure S3: Transcripts levels of *CD28* and *CLEC7A* presented as normalized counts in sorted CD4+ T-cells (in yellow), MAIT cells (in red), CD8+T-cells (in blue), $\gamma\delta$ T-cells (in purple) and neutrophils (in green) after 2h of stimulation by PMA (50ng/ml) + A23187(μM) + βglucan (50μg/ml).

Figure S4: Flow cytometry gating strategy for phenotypic identification of MAIT cells in entheseal tissues and peripheral blood cells. Doublet excluded EMCs (PEB and EST) or PBMCs were stained with zombie aqua (live/dead discrimination, Biolegend), anti-CD45 (to exclude non-leucocytes) and anti-CD3 (T-cell inclusion). MAIT cells were identified by CD161+ and Vα7.2 TCR+ staining. PEB EMCs were plated out at a minimum of $4x10^6$/mL in RPMI (containing 10% FCS, 1% penicillin/streptomycin) and stimulated with PMA (50 ng/ml) and Ionomycin (1 μg/ml) for 3 hrs in the presence of Golgi Plug (BD).

Figure S5: Basal production of IL-18 in MAIT cells from entheseal tissue
Protein expression of IL-18 (pg/mL) in unstimulated and LPS stimulated (100ng/mL) EMCs from PEB Mean ±SEM (n=6). PEB EMCs were plated out at $5x10^6$/mL in RPMI (containing 10% FCS, 1% penicillin/streptomycin) and stimulated with LPS (100ng/mL) for 24 hrs

Figure S6: Comparison of IL-17A, IL-17F, IL-7, IL-18 serum levels between 9 patients (in light blue) and 31 controls (in red). The p values were calculated using a Mann-Whitney test. The differences are considered significant for P values <0.05.

Figure S7: Comparison of IL-17A secretion by CD4 + T, CD8 + T, $\gamma\delta$ T, MAIT and neutrophils between patients and controls depending on cell type. (A) IL-17A protein expression levels are presented in femtograms per 1000 cells in sorted CD4 + T cells (in

orange), MAIT cells (in light blue), CD8 + T cells (in dark blue), $\gamma\delta$ T cells (in purple) and neutrophils (in green) after 18h of stimulation by PMA (50ng / ml) + A23187 (µM) + βglucan (50 µg / ml). The p values were calculated using a Mann-Whitney test. Horizontal bars indicate the median and standard deviation. The differences are considered significant for P values <0.05. ****, P <0.0001, ***, P <0.001, **, P <0.01, *, P <0.05, ns; not significant. (B) Diagram showing the distribution of IL17A secretion in patients and controls according to cell type.

Figure S8: Gene expression analysis in MAIT cells from 10 patients and 10 controls. (A) The principal component analysis was performed on the gene expression data of MAIT cells stimulated for 2 hours with PMA (50 ng / ml) + A23187 (5 µM) + βglucan (50 µg / ml). Each dot represents a sample colored according to group of patients (dark blue) or controls (yellow). (B) Heatmap showing the expression of genes differentially expressed by MAITs between patients and controls after 2 h of stimulation with PMA (50 ng / ml) + A23187 (µM) + βglucan (50 µg / ml). In the heatmap, the columns represent the samples belonging either to the patient group in blue (n = 10) or to the control group in yellow (n = 10) and the lines represent the genes differentially expressed between patients and controls, the samples are ordered by hierarchical clustering. T-test with false discovery rate correction q = 0.29. Yellow indicates high levels of expression and blue indicates low levels of expression.

The values for each gene were logarithmically transformed, centered on a mean value of zero, and adjusted to a variance of 1.

Table S1: Patients characteristics

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Table S2: Complete list of TaqMan Assays used for analysis of gene expression on non-SpA patients.

Table S3: List of antibodies used for flow cytometry (FC) and activation assays (AA) on non-AxSpA patients

Table S4: Log 10ΔCt values of basal MAIT cells derived from entheseal tissues

Table S5: Complete non AxSpA patients' cohort

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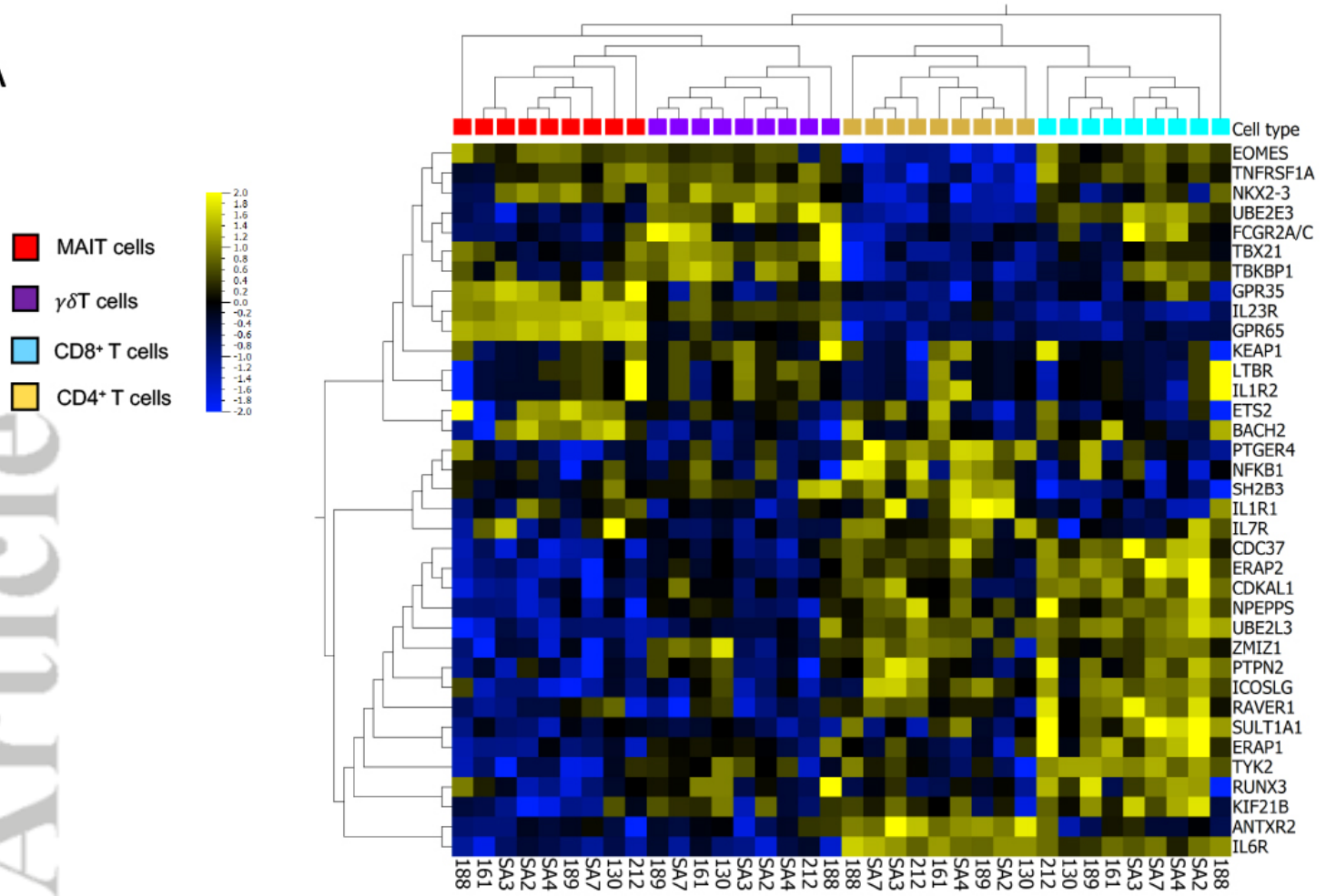

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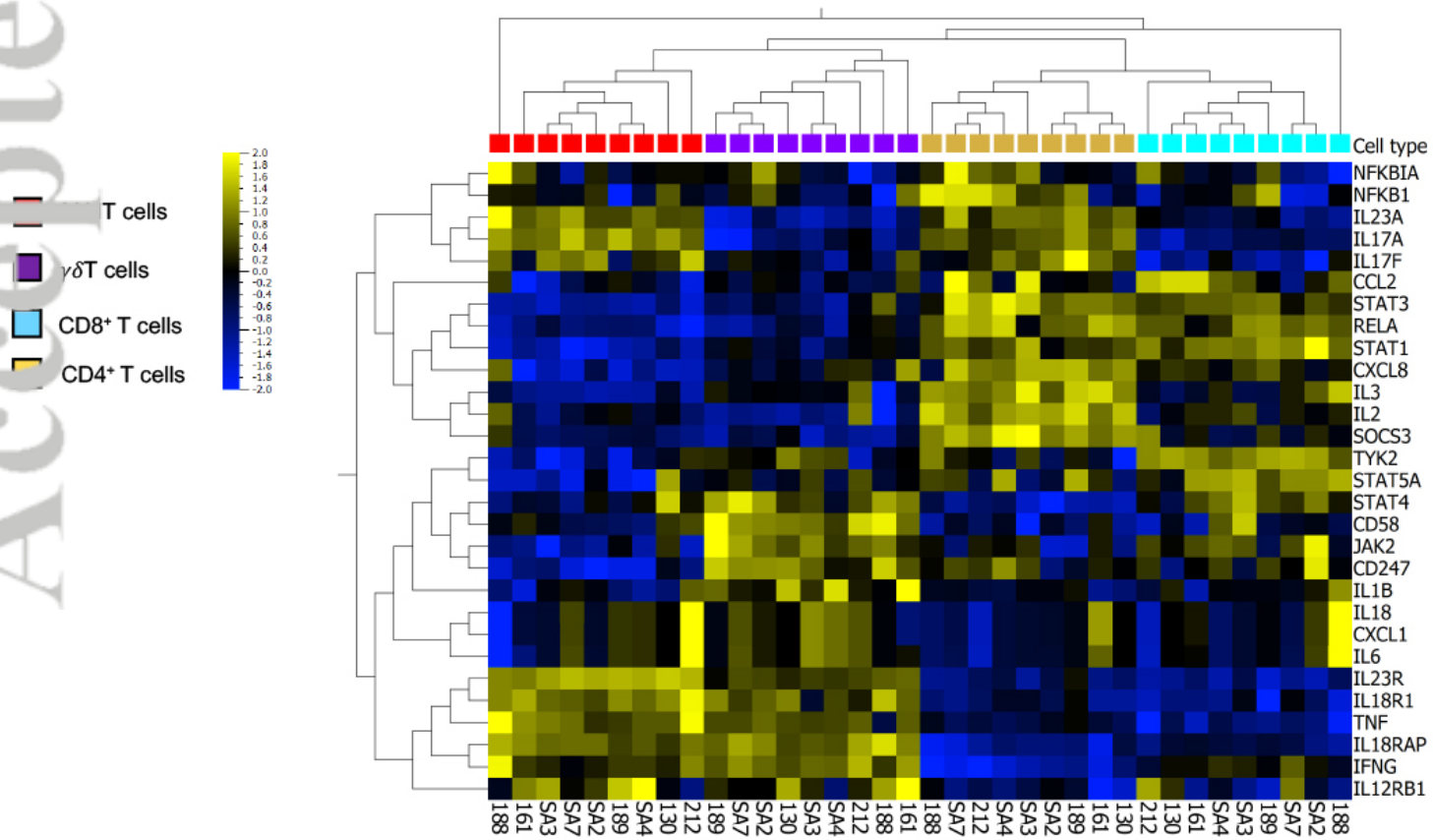

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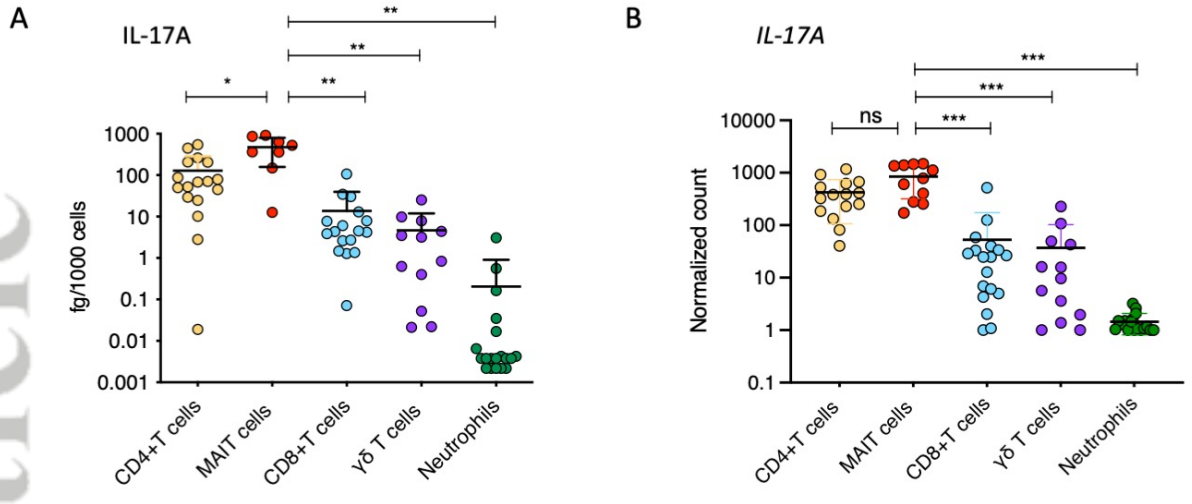

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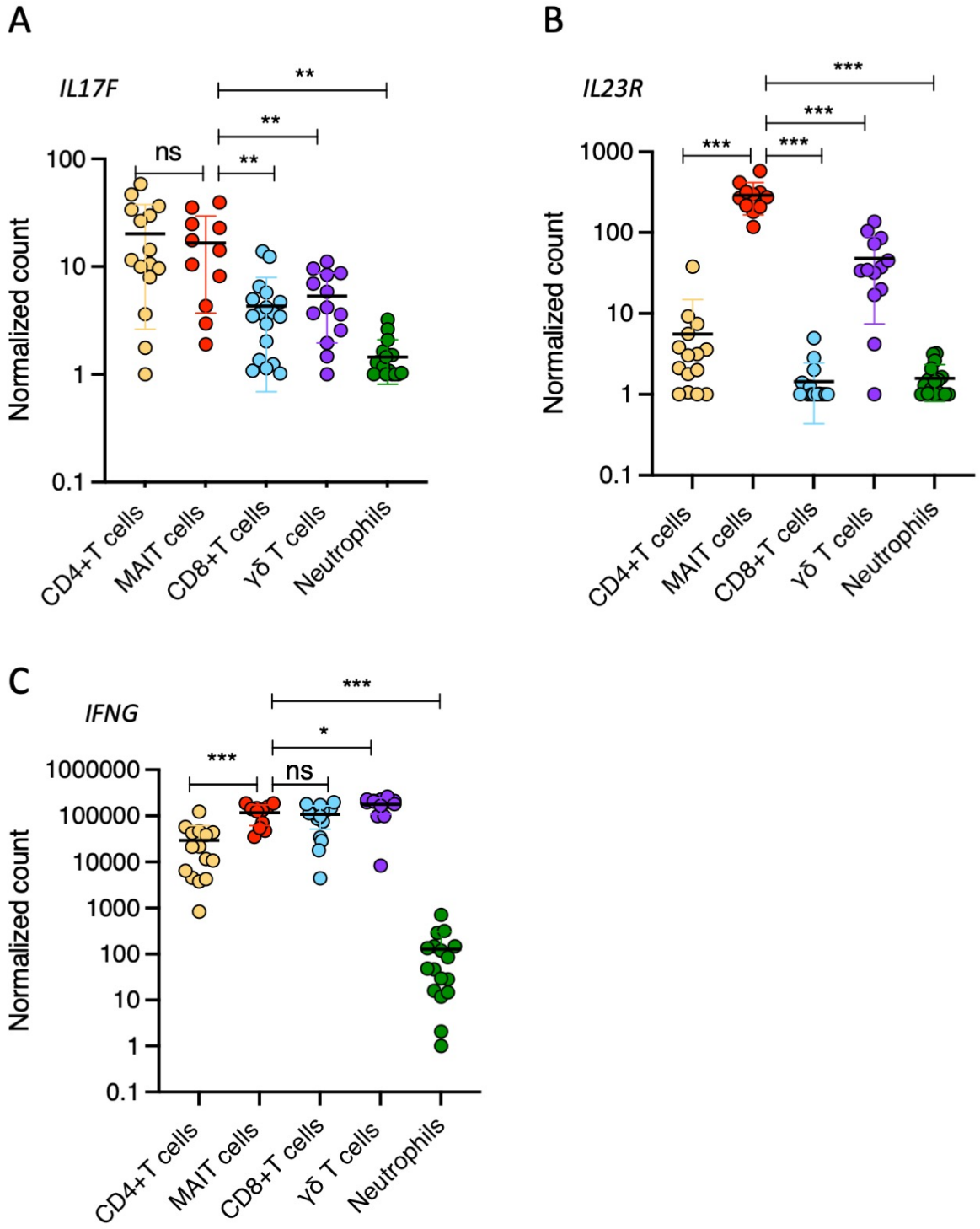

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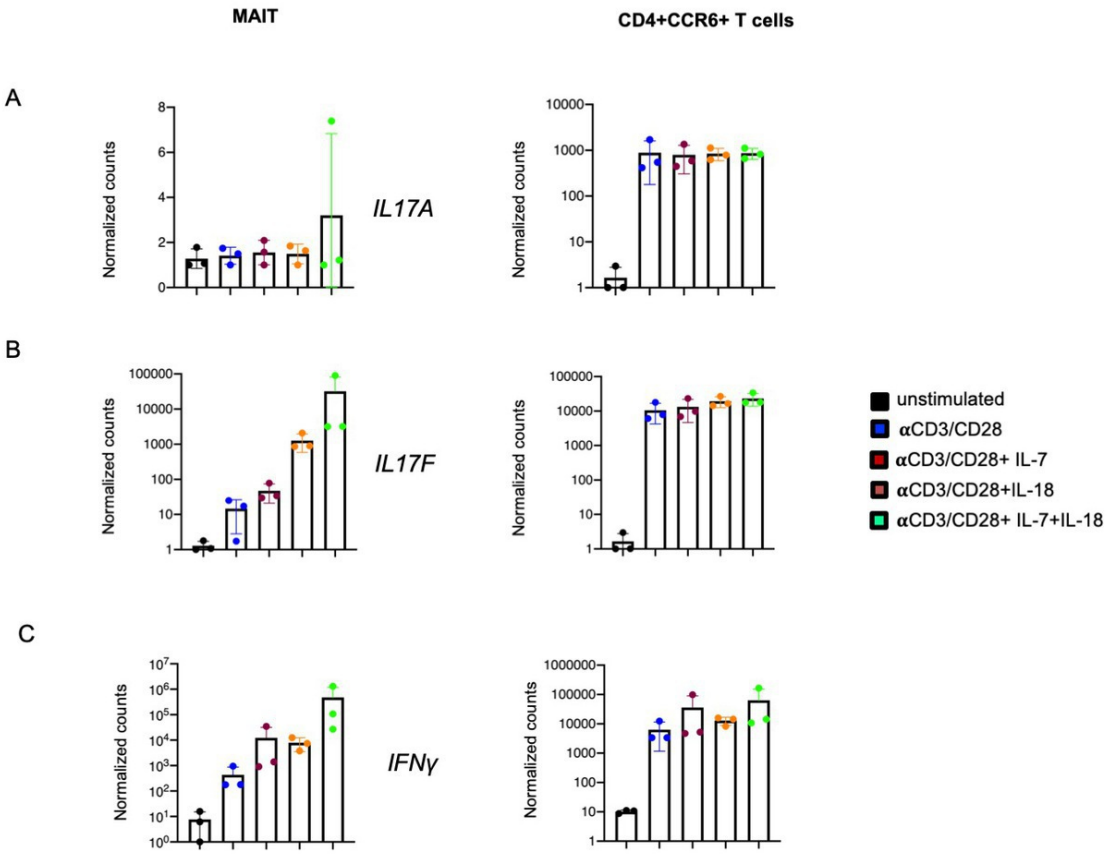

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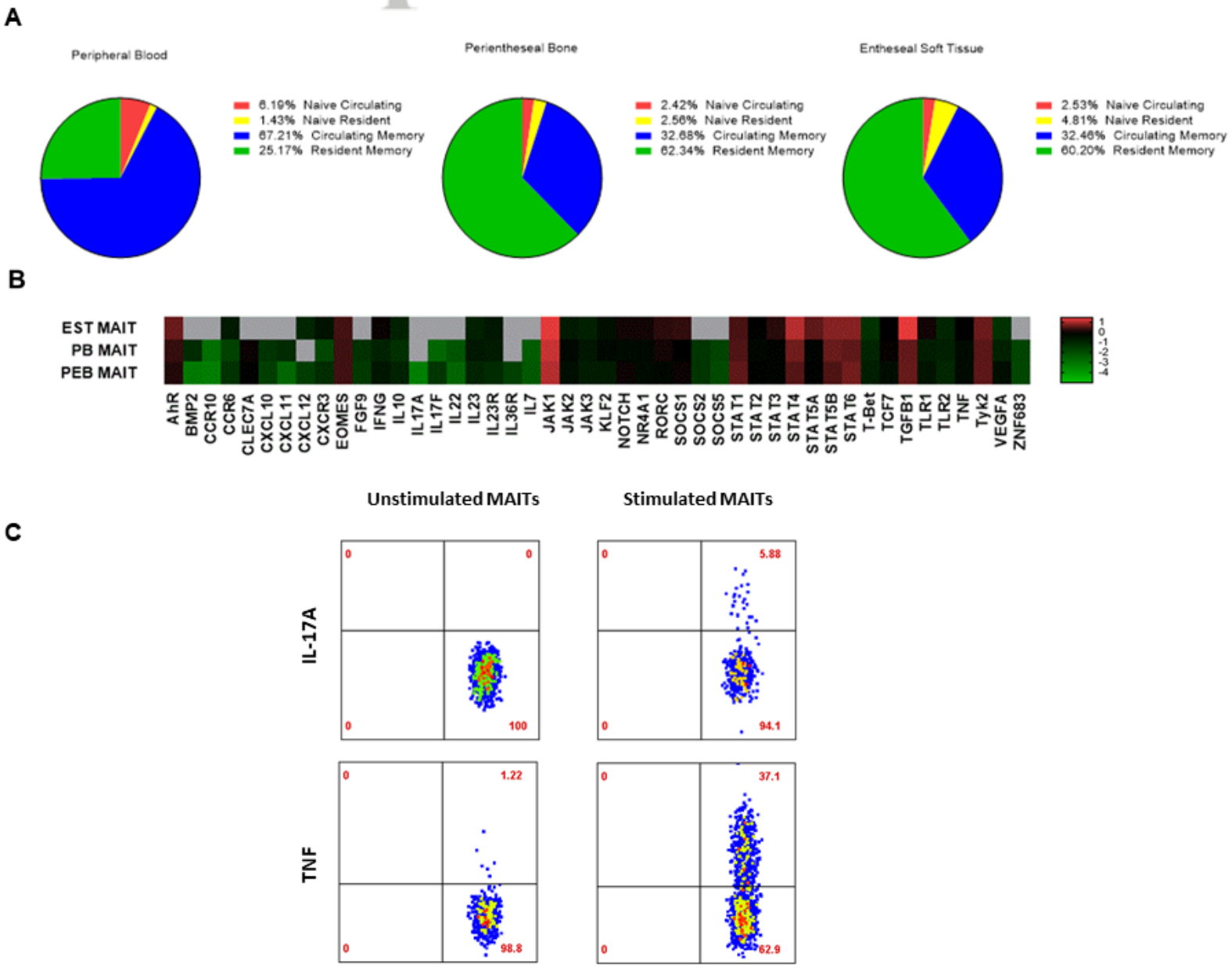
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