Rare IL-17A+ Lymphocytes in the Pathogenesis of Spondyloarthritis

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Table of Contents

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Abstract

Spondyloarthritis is a family of rheumatic diseases characterized by inflammation in the spine, peripheral joints, skin, intestine and other organs. Systemic overexpression of IL-23 via hydrodynamic minicircle injection in adult B10.RIII mice induces a spondyloarthritis-like disease with high expression of IL-17A, a pro-inflammatory cytokine downstream of IL-23. A previous study identified a rare subset of CD3+CD4-CD8- Double Negative (DN) tissue-resident T cells as the major source of IL-17A in this model. Here we characterize these DN T cells further as γδ T cells and DN αβ T cells and show that they require co-stimulation with IL-1β and IL-23 for IL-17A induction. IL1R1-/- and IL23R-/- mice had largely similar frequencies of γδ and DN αβ T cells compared with wild type controls, demonstrating that these cytokine receptors control the function but not the development of γδ and DN αβ T cells. In γδTCR-/- mice, IL-17A secreting DN αβ T cells were expanded, consistent with numerical and functional compensation. C57BL/6 mice, in contrast to B10.RIII mice, did not develop spondyloarthritis upon IL-23 minicircle injection. C57BL/6 and B10.RIII mice had similar frequencies of γδ and DN αβ T cells but IL-17A production by DN αβ T cells was significantly higher in disease-prone B10.RIII mice. Oral treatment of B10.RIII mice with broad-spectrum antibiotics also prevented the development of IL-23 minicircle-induced disease. While antibiotics had no major impact on the frequency of γδ and DN αβ T cells, IL-17A secretion by γδ and DN αβ T cells was significantly reduced. Further investigations are required to elucidate how intestinal microbiota control IL-17A secretion by γδ and DN αβ T cells, which may lead to novel therapies for patients with spondyloarthritis.

Introduction

Spondyloarthritis (SpA) encompasses several rheumatic illnesses that are characterized by inflammation in the spine, peripheral joints, skin, intestine and other organs. Approximately 0.9 - 1.4 % of the adult population in the United States have SpA (1). Variants include ankylosing spondylitis, psoriatic arthritis and SpA associated with inflammatory bowel disease. One of the prominent features of SpA is enthesitis, inflammation at the attachment sites of tendons or ligaments to bone. Enthesitis causes pain and stiffness and may result in abnormal bone formation. In ankylosing spondylitis, entheseal inflammation at the edges of vertebral bodies leads to bony fusion of the spine, resulting in limited mobility and increased fracture risk. The specific mechanisms driving entheseal inflammation and pathological bone formation in SpA are not well understood.

Multiple lines of evidence suggest a role for the IL-23/IL-17A axis in SpA pathogenesis. IL-23 is a heterodimeric cytokine composed of IL-23p19 and IL-12/23p40 chains. IL-23 is produced by myeloid cells such as macrophages and dendritic cells. The IL-23 receptor is composed of IL-23R and IL-12Rβ1 chains and signals via the JAK-STAT pathway (2). Many lymphocyte subsets express the IL-23 receptor including Th17 cells, γδ T cells, and subsets of innate lymphoid cells (3). One of the genes induced by IL-23 signals is IL-17A, a pro-inflammatory cytokine whose major function is to attract neutrophils and macrophages to sites of inflammation via induction of chemokines in non-immune cells (4). Genome-wide association studies have revealed polymorphisms in multiple genes involved in IL-23 signaling to be associated with SpA (5). Increased levels of IL-23 and IL-17A levels have been found in the peripheral blood of patients with SpA and, most importantly, antibodies blocking either IL-23 or

IL-17A have been introduced into the clinic as therapies for SpA. Secukinumab, an antibody that inhibits IL-17A, recently received approval for ankylosing spondylitis and psoriatic arthritis. Ustekinumab, which targets the p40 subunit of IL-23, has been approved for psoriatic arthritis. Other agents that target the p19 subunit of IL-23 (Tildrakizumab, Guselkumab), IL-17A or the IL-17 receptor (Briakinumab) are under development for SpA. While the importance of the IL-23/IL-17A axis in SpA is clear, many open questions remain. For instance, it is not known which signals drive the expression of IL-23 and which immune cell subsets produce IL-17A in response to IL-23 stimulation in SpA. These questions need to be answered in order to find a cure or prevent SpA in the future.

An important mouse model to study the pathogenesis of SpA is the IL-23 minicircle model introduced a few years ago by Sherlock et al. (6) This model utilizes hydrodynamic injection of IL-23 minicircle plasmids to achieve high systemic levels of IL-23. Hydrodynamic injection is the injection of a large volume (equal to 10% of the mouse's body weight) in a span of 4 - 8 seconds into the tail vein of the mouse (7). Minicircles are a form of plasmid devoid of most bacterial DNA, and through hydrodynamic injection, are taken up by the liver resulting in stable long-term expression (8). Adult B10.RIII mice injected with IL-23 minicircles develop extensive paw swelling, enthesitis, synovitis and psoriasis-like skin disease. Sherlock et al. identified a rare subset of tissue-resident CD3+CD4-CD8- (DN) T cells that appears to play a critical role in the pathogenesis of this model. They isolated these DN T cells from entheses, a signature area of inflammation in SpA and showed that they expressed the IL-23R and secreted IL-17A in response to stimulation with IL-23 *in vitro*. However, it was not addressed whether these cells belonged to αβ or γδ T cell subsets.

Rapid secretion of cytokines in response to cytokine stimulation without the need for TCR stimulation is a hallmark of innate-like lymphocytes. A number of studies have demonstrated that IL-23 stimulation alone is insufficient to induce IL-17A secretion by innate-like lymphocytes. For instance, Sutton et al. demonstrated that two cytokines, IL-1β (or IL-1α) and IL-23, were required for IL-17A expression by γδ T cells (9). Whether the DN T cells described by Sherlock et al. follow similar rules has not been studied.

In the original description of the IL-23 minicircle model, several strains of mice were used including B10.RIII and C67BL/6. The latter strain is the most commonly used inbred mouse strain and many knockout mice have been developed on this genetic background. However, we found in preliminary experiments that C57BL/6 mice were protected from the development of SpA over an observation period of up to 6 weeks, whereas B10.RIII mice developed disease as expected. This contrasts with the report by Sherlock et. al. who demonstrated that both B10.RIII and C67BL/6 mice developed disease. B10.RIII mice have been shown to be more susceptible to the induction of autoimmune or inflammatory diseases in a variety of models (10). We hypothesized that differences in the number or function of the tissue-resident DN T cells might explain the variance in susceptibility to disease induction between the C67BL/6 and B10.RIII mice.

Differences in the microbial environment in animal facilities are a major factor for variable experimental results between laboratories (11). The impact of microbiota on inflammatory diseases including SpA has gained much attention over the last few years. Several studies have found that inflammatory diseases in mice including B10.RIII mice are microbiota-dependent (12-14). In a preliminary experiment we found that B10.RIII mice who had a cocktail

of broad-spectrum antibiotics added to their drinking water were protected from the development of IL-23 minicircle-induced disease. The IL-23 minicircle model thus represents an opportunity to analyze the effects of intestinal microbiota on tissue-resident lymphocytes. What distinguishes this from other SpA models is the fact that IL-23 overexpression bypasses upstream mechanisms of immune activation in antigen presenting myeloid cells, which are known to express microbial pattern recognition receptors.

Based on these published studies and preliminary data, the goals of this study were:

1. Determine T cell receptor (TCR) utilization and MHC dependence of the entheseal DN T cells.
2. Confirm the requirement for IL-1β + IL-23 co-stimulation to induce IL-17A secretion by γδ and DN αβ T cells. Ascertain if γδ and DN αβ T cell frequency or function are affected in IL23R-/- and IL1R1-/- mice.
3. Test the hypothesis that the frequency of γδ and DN αβ T cells or their ability to produce IL-17A differ between B10.RIII and C57BL/6 mice.
4. Test the hypothesis that the frequency of γδ and DN αβ T cells or their ability to produce IL-17A differ between antibiotic-treated and control B10.RIII mice.

Materials and Methods

Mice. C57BL/6 wild type, B10.RIII wild type, C57BL/6 Tap1-/-, C57BL/6 MHC II-/-, C57BL/6 IL1R1-/-, and C57BL/6 γδTCR-/- mice were obtained from the Jackson Laboratory. C57BL/6 IL23R-GFP mice were a gift from Vijay Kuchroo, Harvard Medical School. Lines were maintained in the animal facility at the Harvard T.H. Chan School of Public Health and in the Building for Transformative Medicine at Brigham and Women's Hospital. For all experiments, approximately 8 week old male mice were used. Disease was induced by hydrodynamic tail vein injection of 3 μg of IL-23 minicircle DNA (System Biosciences). Antibiotics were given in the drinking water starting 2 weeks prior to analysis or disease induction (VMNA = Vancomycin 0.5 g/l, Metronidazole 1 g/l, Neomycin 1 g/l, Ampicillin 1 g/l) (15).

Lymphocyte Isolation. Mice were sacrificed by carbon dioxide asphyxiation. Blood was extracted from the heart via cardiac puncture and added to 100 μl of 50 mM EDTA to prevent coagulation. Mononuclear cells were then isolated via density centrifugation with Histopaque 1.083 RT (Sigma). Single cells from the hind foot skin and achilles tendon enthesis were isolated by digesting the tissue with an enzyme mix consisting of collagenase P (Roche), dispase II (Sigma), hyaluronidase (Sigma), and DNAase I (Sigma). Lymphocytes from the spleen were prepared by filtering the smushed organ through a cell strainer. The liver was dissociated using a *gentleMACS Dissociator* (Miltenyi Biotec) and mononuclear cells were isolated by 40/75 % Percoll density gradient centrifugation (Sigma). Blood, liver and spleen cells were further purified with red blood cell lysis buffer to eliminate leftover red blood cells.

Flow cytometry. The following antibodies were obtained from Biolegend: CD4 (PE-Cy7, BV421), CD8a (BV510, PE-Cy5), CD45 (PerCP-Cy5-5), CD11c (APC-Cy7, BV510), CD11b (APC-Cy7, BV711), CD19 (PE-Dazzle, APC-Cy7), αβTCR (APC-Cy7, PE), γδTCR (BV605, FITC), MHC II I-A/I-E (BV786), IL-1R1 (PE) and IL-17A (APC). Surface marker staining was performed in 96 well plates. The cells were first stained with a fixable viability dye (FVD-450 UV)(eBioscience) in PBS. Subsequently, cells were incubated with Fc block (Biolegend) to inhibit non-specific binding to Fc receptors found on white blood cells. Cells were then stained with fluorescently labeled antibodies and re-suspended in staining buffer (PBS with 2 mM EDTA and 0.5 % BSA). A Canto II or Fortessa flow cytometer (BD Biosciences) was used for data collection and FlowJo FACS Software was used for analysis.

In vitro **stimulation and intracellular staining.** Single cell suspensions from the spleen ($2x10^6$ cells per well) were added to 96 well plates with RPMI 1640 cell culture medium (Cellgro) supplemented with 10% fetal calf serum (Atlanta Biologicals), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin (100 U/ml/100 µg/ml), 50 µg/ml gentamicin (all Cellgro) and 50 µM β-mercaptoethanol (Sigma). In some experiments, anti-IL-1R1 (10 µg/ml), anti-IL-23R (10 µg/ml), or isotype control antibodies were added and plates were placed in a 37° C incubator for 20 minutes prior to stimulation. Cells were stimulated with either IL-1β (10 ng/ml, Biolegend), IL-23 (10 ng/ml, R&D), IL-1β + IL-23, or PMA/Ionomycin (50 ng/ml/1 µM, BD Biosciences). Golgi Stop (1 µl/well, BD Biosciences) was added to inhibit vesicle transport of cytokines. After four hours of incubation, cells were harvested and stained

for surface markers as described. After washing with staining buffer, the cells were fixed with IC Fixation Buffer (eBioscience) for 30 minutes at room temperature. Anti-IL-17A (APC, Biolegend) in permeabilization buffer (eBioscience) was added for intracellular staining for another 30 minutes. Followed by a series of washes with permeabilization buffer, cells were re-suspended in staining buffer and analyzed.

Statistics. Statistical analysis and graphing were done with *GraphPad Prism* software.

Results

Further characterization of DN T cells distinguishes subsets of γδ and DN αβ T cells

Sherlock et al. did not report whether the enthesis-resident DN T cells expressed αβ or γδ TCRs. In order to analyze TCR utilization by DN T cells in wild type mice, a flow cytometry gating strategy was devised, which is illustrated in Figure 1. First, a lymphocyte gate was set based on forward scatter vs side scatter values. Next, the cells were gated based on being viable and CD45+. Alive and functional cells can pump out the viability dye, whereas dead or damaged cells cannot. CD45 is a glycoprotein found on hematopoetically derived cells, which includes T cells but excludes stromal cells. Cells were further gated by excluding macrophages, dendritic cells and B cells using the lineage markers CD11b, CD11c, and CD19 markers, respectively. CD45+Lineage- cells were then plotted based on expression of CD4 and CD8 to identify the DN subset, which was then analyzed for expression of αβ or γδ T cell receptors.

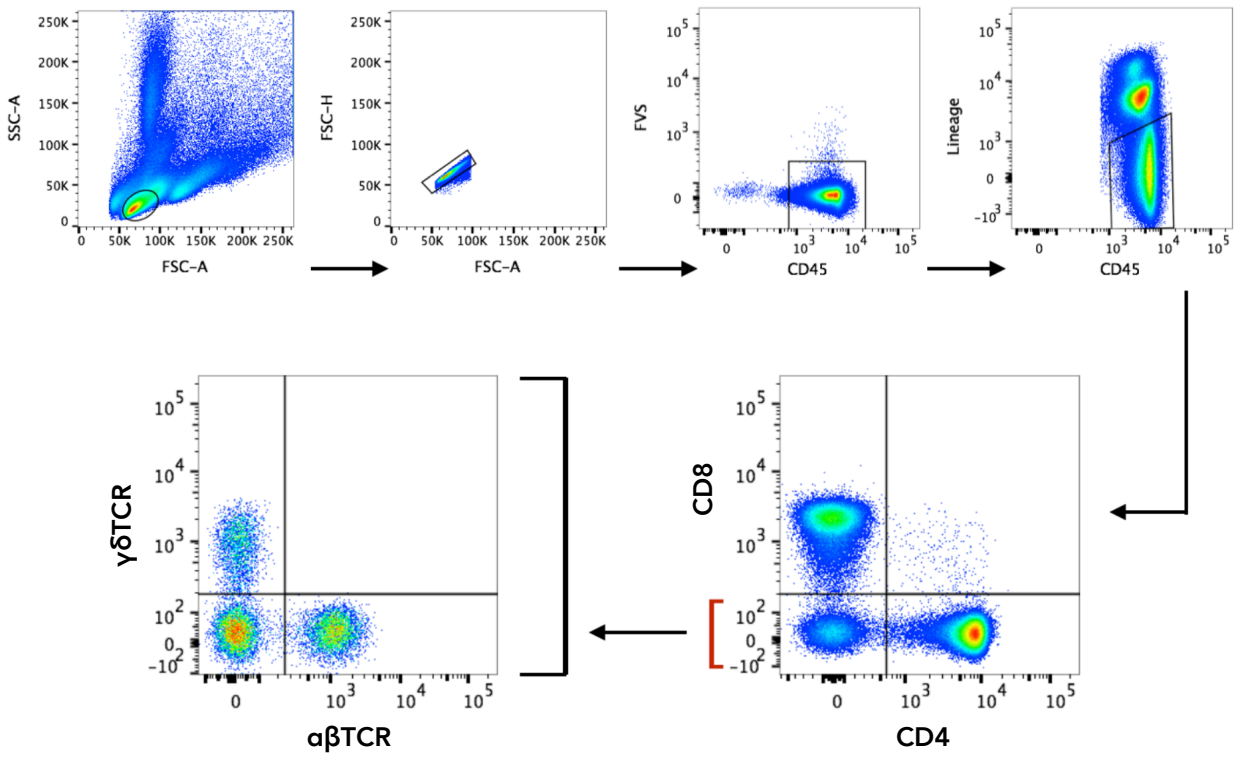

Figure 1 | Flow cytometry gating strategy for identifying γδ and DN αβ T lymphocytes. C57BL/6 splenocytes were first gated based on forward scatter and side scatter values. Cell doublets were then excluded by comparing forward scatter height and forward scatter area values. Then, viable cells (FVS negative) of hematopoietic lineage (CD45+) were gated in. Furthermore, a lineage cocktail consisting of antibodies against macrophage (CD11b), dendritic cell (CD11c) and B cell (CD19) markers was used to exclude these cell types. Subsequently, CD4-CD8- (DN) cells were analyzed for αβ and γδ T cell receptor expression.

Data in Figure 2 show the analysis of single cell suspensions prepared from the achilles tendon enthesis, blood, liver, skin and spleen of C57BL/6 wild type mice. Flow cytometry was performed to measure γδ and αβ TCR expression on DN T cells using the gating strategy explained in Figure 1. Analysis of cells from the achilles tendon enthesis (Figure 2A) shows that the CD45+Lineage- DN cell populations included both γδTCR+ and αβTCR+ cells. The former cells represent γδ T cells, the latter cells will be subsequently referred to as DN αβ T cells. At this site, γδ T cells and DN αβ T cells had similar frequencies among the CD45+Lineage- cells. Analysis in other locations also showed the presence of both the γδ and the DN αβ T cell subsets (Figure 2B). The skin showed a much higher frequency of γδ T cells than the DN αβ subset. The

ratio here was approximately 7:1, which is consistent with the previously reported unique cell profile in the mouse skin (16). Furthermore, in the blood, liver and spleen, the DN T cells were present in much lower frequencies. γδ T cells and the DN αβ subsets together accounted for approximately 20% of the CD45+Lineage- cells isolated from the achilles tendon enthesis. The same measurements for the blood, liver and spleen were 3.1%, 6%, and 3.2% respectively. In summary, the DN T cells located at the achilles tendon enthesis contain both γδ and αβ T cells. Both subsets of cells are present in all locations at variable ratios.

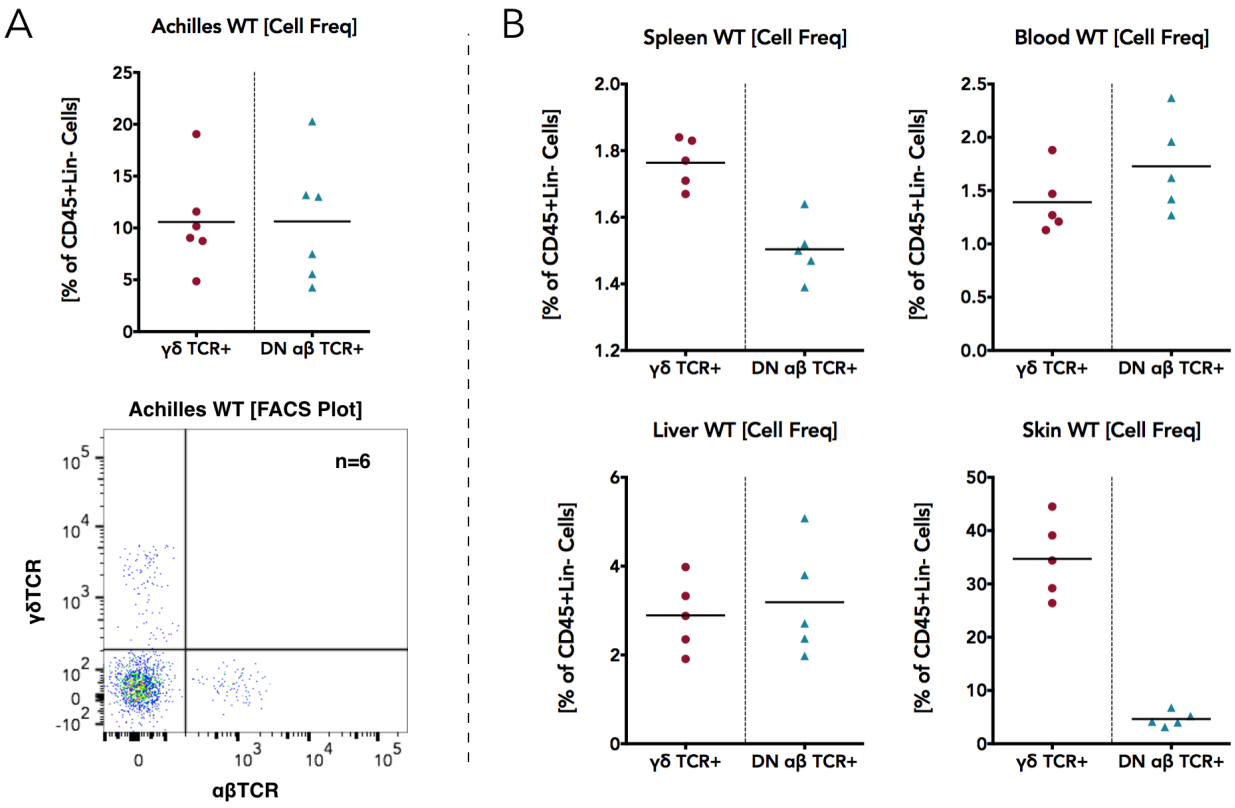

Figure 2 | Entheseal CD4-CD8- DN T cells in wild type mice are comprised of γδ and DN αβ T cells. (A) Single cell suspensions prepared by digestion of achilles tendon entheses samples were stained and gated as in Figure 1. Both γδ and αβ TCR+ cells could be detected within the CD4-CD8- DN lymphocytes subset. (B) The same analysis for cells prepared from spleen, blood, liver and skin.

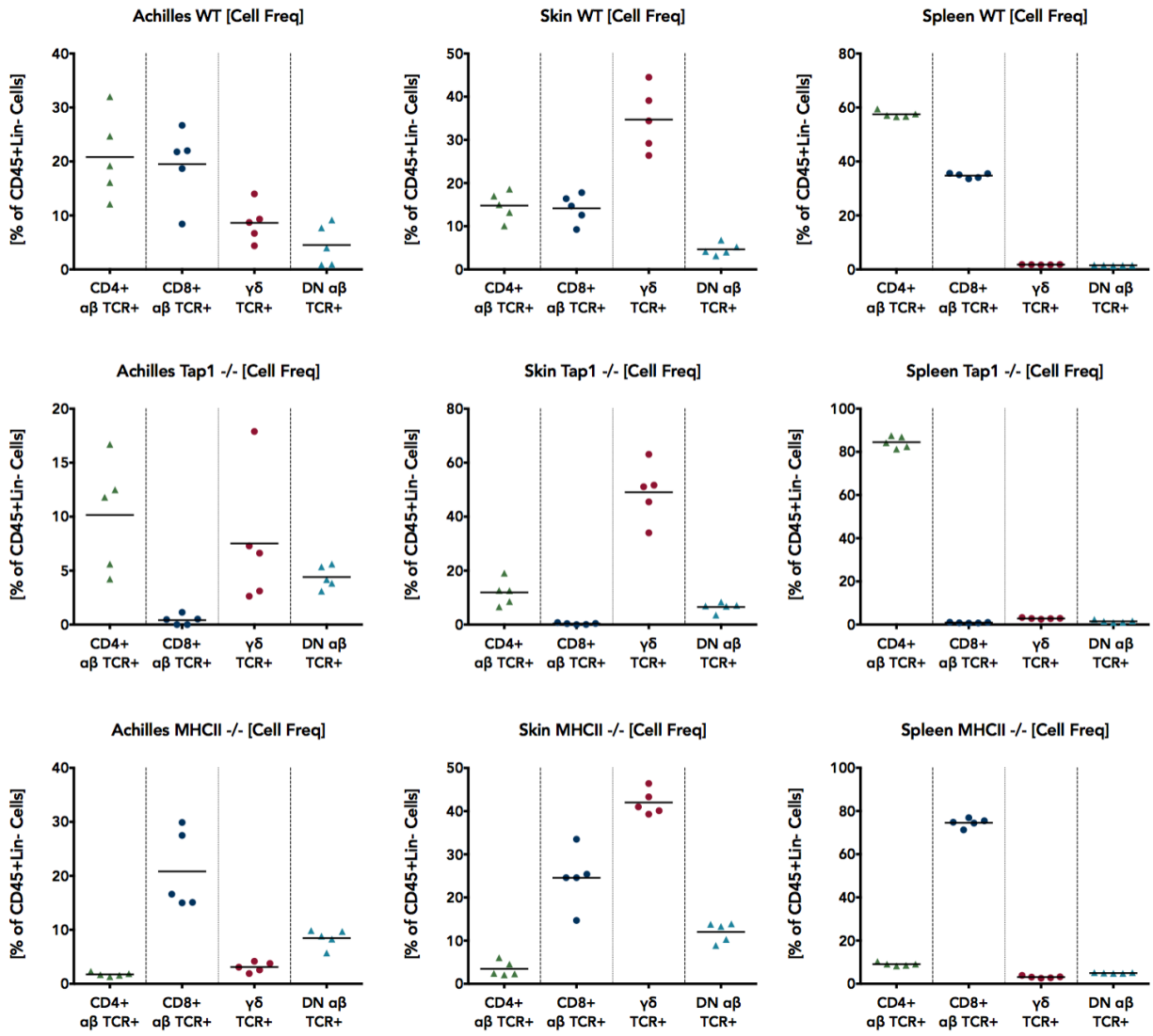

Figure 3 | γδ and DN αβ T cell development does not depend on MHC expression. Single cell suspensions were prepared from the achilles tendon enthesis, skin and spleen of wild type, Tap1-/-, and MHCII-/- mice (n=5 for each genotype). Across all locations, Tap1-/- mice, have severely depleted CD8+ populations and MHCII-/- mice have diminished CD4+ populations. γδ and DN αβ T cell frequencies are largely unaffected in these knockout mice when compared to wild type.

γδ and DN αβ T cell development is independent of MHC expression

After determining that γδ and DN αβ subsets exist in multiple tissues and organs in wild type mice, the effect of MHC Class I and Class II expression on these cells was ascertained. Two knockout mouse lines were used in this experiment: Tap1-/- and MHCII-/-. Tap1-/- mice represent functional MHC Class I knockout mice (17). In the absence of Tap1 (Transporter associated with Antigen Processing1), antigenic peptides cannot enter the rough endoplasmic

reticulum, which is a prerequisite for the assembly of functional MHC Class I complexes. MHC Class 1 complexes are important for the positive selection of CD8+ T cells in the thymus and for the initiation of cytotoxic T cell responses in the periphery. Knocking out MHC Class II impairs the positive selection of CD4+ cells during thymocyte development (18). While γδ T cells have been shown not to require MHC Class I or MHC Class II signals for their development, this is unclear for DN αβ T cells. In addition to γδ and DN αβ T cells, the CD4+ αβ T cells and CD8+ αβ T cells were analyzed as controls (Figure 3).

MHCII-/- deficient mice were expected to have deficient CD4+ populations and similarly, Tap1-/- mice were expected to be deficient in CD8+ T cells. This was confirmed for the cells isolated from the achilles tendon enthesis. Importantly, in both MHCII-/- and Tap1-/- mice, the γδ TCR+ DN and αβ TCR+ DN T cells populations were present. The mean frequencies for DN αβ T cells in the wild type, MHCII-/- and Tap1-/- mice were 4.0%, 8.0%, and 4.4%, respectively. γδ T cell frequencies for the same strains were 8.6%, 3.1%, and 7.5%, respectively. Similar patterns were found in the skin and spleen. In summary, both γδ and DN αβ T cells are present in the achilles tendon enthesis of Tap1-/- and MHCII-/- mice. Their development is thus unaffected by MHC deficiency.

Both IL-1β and IL-23 signals are required for IL-17A Production by γδ T Cells and DN αβ T Cells.

To investigate the dependency on IL-1β and IL-23 signals in the induction of IL-17A, splenocytes were isolated from wild type mice and stimulated with IL-1β alone, IL-23 alone, or a combination of IL-1β and IL-23. Antibodies against IL-1R1 or IL-23R were added under single

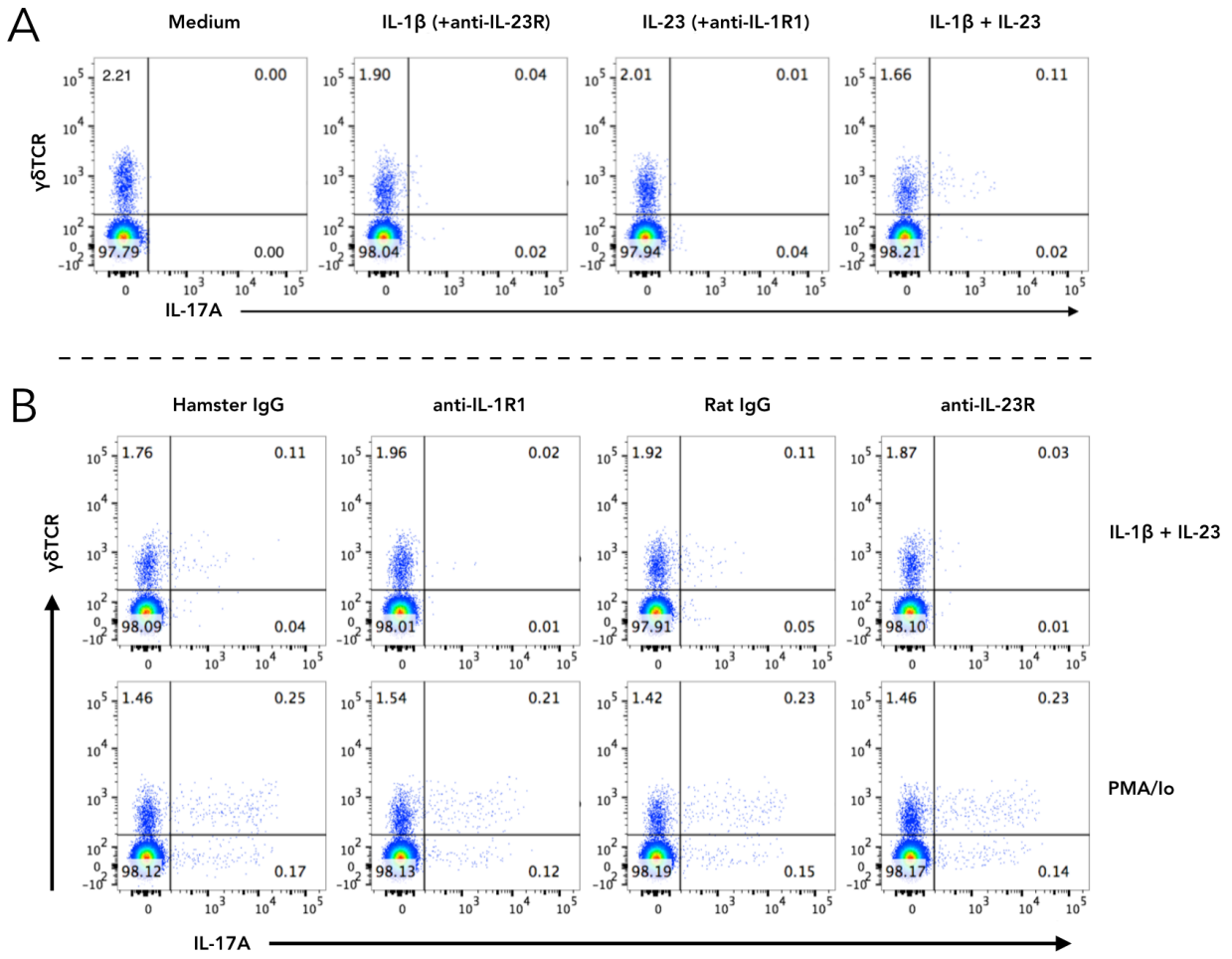

Figure 4 | γδ T cells are the main producers of IL-17A in response to stimulation with IL-23 *in vitro* and require IL-1 as second signal. (A) Splenocytes isolated from C57BL/6 wild type mice were stimulated with IL-1β, IL-23, IL-1β + IL-23 (all 10 ng/ml). Receptor blocking antibodies (10 µg/ml) were added as indicated to assure the absence of a second signal. IL-17A production was measured by intracellular staining after 4 hours of stimulations. (B) Splenocytes were stimulated with IL-23 + IL-1β or PMA/Ionomycin in the presence of hamster IgG isotype, anti-IL-1R1, rat IgG isotype, or anti-IL-23R (all 10 µg/ml).

cytokine conditions to block potential confounding signals from cytokines produced by cells in the culture. Only stimulation with both IL-1β and IL-23, but not stimulation with either IL-1β or IL-23 alone, resulted in substantial induction of IL-17A (Figure 4A). Most of the IL-17A+ splenocytes were γδ T cells with minor contributions from DN αβ T cells.

PMA/Ionomycin is frequently used in immunology research to activate lymphocytes and analyze their cytokine secretion patterns. PMA/Ionomycin stimulation induced the production of IL-17A by γδ T cells and DN αβ T cells (Figure 4B). We asked whether this induction of IL- 17A

by PMA/Ionomycin might be mediated indirectly via IL-1β/IL-23 signals. First, wild type splenocytes were stimulated with IL-1β and IL-23 plus either Rat IgG, Hamster IgG, anti-IL-1R1, or anti-IL-23R. In wells with isotype control antibody (Rat IgG or Hamster IgG), 0.15 % of the CD45+Lineage- cells (as defined by the gating strategy in Figure 1) produced IL-17A. Addition of either anti-IL-1R1 or anti-IL-23R severely reduced the number of IL-17A producing cells demonstrating that these antibodies were functional (Figure 4B). In parallel, wild type splenocytes were stimulated with PMA/Ionomycin with addition of the same antibodies. Here, the frequency of IL-17A+ cells within the CD45+Lineage- gate was approximately 0.37 % across all conditions. Thus, PMA/Ionomycin induced IL-17A production was not affected by addition of anti-IL-1R1 or anti-IL-23R antibodies. This suggests that PMA/Ionomycin stimulation bypasses the IL-1β and IL-23 signaling pathways to induce IL-17A expression.

IL23R-/- and IL1R-/- γδ and DN αβ T cells fail to produce IL-17A upon in vitro stimulation with IL-1β + IL-23

The requirement for IL-1R1 and IL-23R for the induction of IL-17A was further analyzed in IL1R1-/- and IL23R-/- mice. In the first comparison, splenocytes were isolated from IL23R+/- and IL23R-/- mice and stimulated with IL-1β + IL-23 or PMA/Ionomycin. IL23R-/- γδ T cells and DN αβ T cells stimulated with IL-1β + IL-23 failed to secrete IL-17A above background. PMA/Ionomycin-induced IL-17A secretion however, was equivalent in both IL23R+/- and IL23R-/- mice (Figure 5). Similar functional assays were conducted by stimulating wild type vs. IL1R1-/- mice under the same conditions. Comparisons between both IL-17A+ γδ and IL-17A+

DN αβ in the IL-1β + IL-23 stimulation show higher mean IL-17A secretion in the wild type compared to IL1R1-/- mice (Figure 6).

In both IL23R-/- and IL1R1-/- mice, IL-1β + IL-23 failed to induce IL-17A, whereas PMA/Ionomycin stimulation did. This demonstrates that both IL-23R and IL-1R1 are required for γδ and DN αβ T cells to respond to IL-1β + IL-23 stimulation and thus secrete IL-17A.

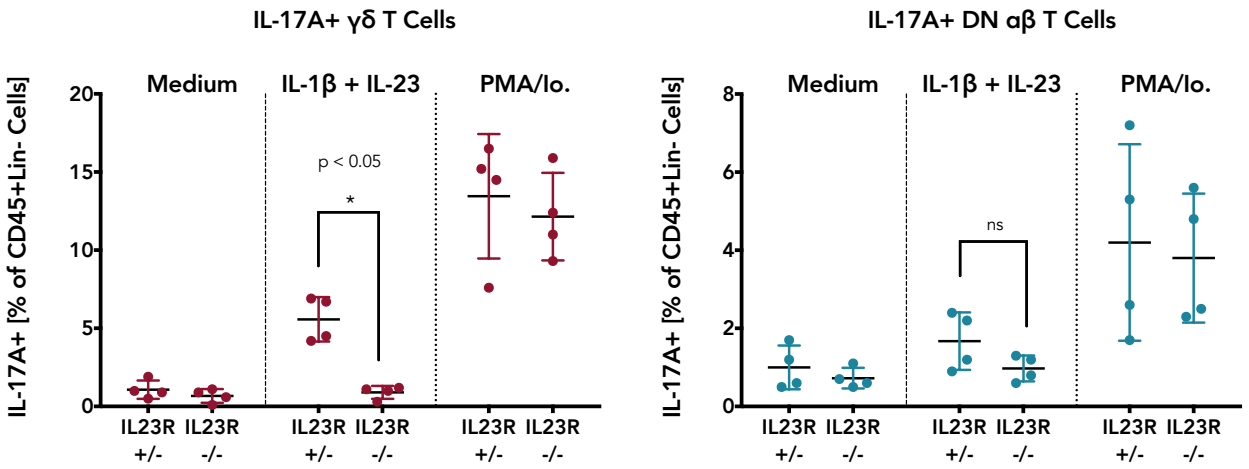

Figure 5 | Stimulation with IL-1β + IL-23 fails to induce IL-17A secretion by γδ T and DN αβ T cells from IL23R-/- mice. Splenocytes isolated from IL23R+/- and IL23R-/- mice were stimulated with medium, IL-1β + IL-23 or PMA/Ionomycin. There was no induction of IL-17A+ above background upon stimulation with IL-1β + IL-23 in γδ T and DN αβ T cells from IL23R-/- mice, whereas PMA/Ionomycin-induced IL-17A production by cells from IL23R+/- and IL23R-/- mice was equivalent.

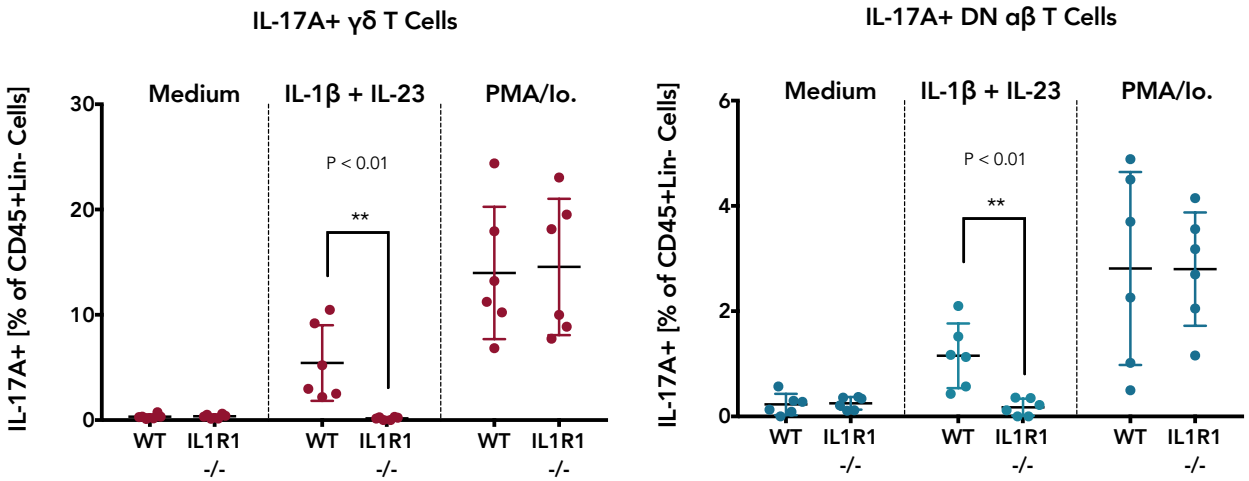

Figure 6 | Stimulation with IL-1β + IL-23 fails to induce IL-17A secretion by γδ T and DN αβ T cells from IL1R1-/- mice. Splenocytes isolated from wild type and IL1R1-/- mice were stimulated with medium, IL-1β + IL-23 or PMA/Ionomycin. There was no induction of IL-17A+ above background upon stimulation with IL-1β + IL-23 in γδ T and DN αβ T cells from IL1R1-/- mice, whereas PMA/Ionomycin-induced IL-17A production was equivalent in cells from wild type and IL1R1-/- mice.

γδ and DN αβ T cell development is independent of IL-23R or IL-1R1 expression

To further understand the role of IL-23R and IL-1R1, we investigated whether the presence or absence of these receptors impacted the development of γδ and DN αβ T cells. Cells were isolated form the achilles tendon enthesis, blood, liver, skin and spleen to determine the frequency and absolute numbers of γδ and DN αβ T cells at these locations. Comparisons were first made between IL23R+/- and IL23R-/- mice. γδ and DN αβ T cells compared between these two genotypes showed no statistically significant differences in their cell frequencies except in the skin and in the liver (Figure 7). Frequencies of γδ T cells in the skin were significantly higher in the IL23R+/- mice compared with IL23R-/- mice ($p < 0.0001$). γδ T cell frequencies in the liver were also slightly higher in the IL23R+/- mice compared with IL23R-/- mice ($p < 0.01$).

Similar comparisons were made between wild type and IL1R1-/- mice. The only statistically significant differences here were found in the spleen (Figure 8). Both γδ and DN αβ T had higher frequencies in the wild type than the IL1R1-/- mice ($p < 0.01$).

The γδ and DN αβ T cell frequencies of IL23R-/- and IL1R-/- mice suggests that the presence or the absence of IL-23R or IL-1R are not critical for the development of these rare lymphocytes.

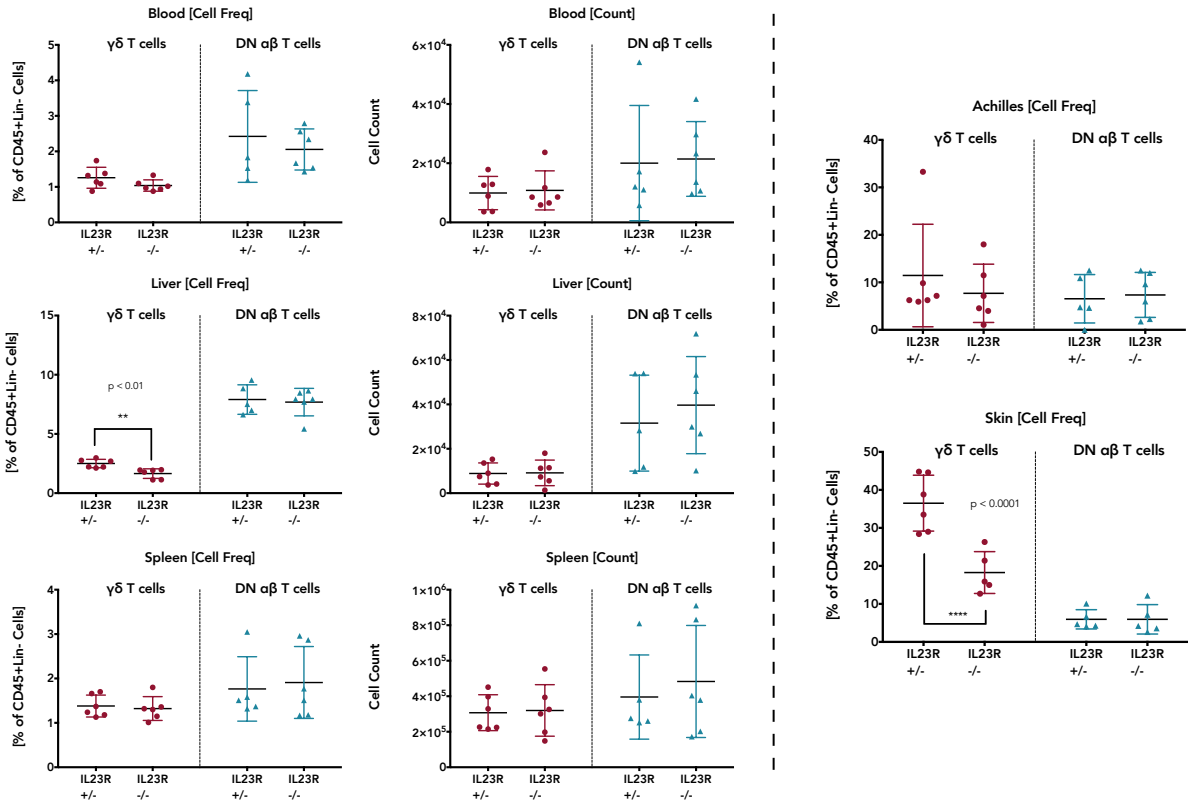

Figure 7 | Frequency and number of γδ T cells and DN αβ in tissues is largely independent of IL-23 signals. Frequencies and absolute counts of γδ T cells and DN αβ T cells were compared between IL23R+/- and IL23R-/- mice in blood, liver, spleen, achilles tendon enthesis and skin (n=6 mice for each genotype). There were no statistically significant differences between the two genotypes except for the frequency of γδ T cells in the skin ($p < 0.0001$ by T test) and the frequency of γδ T cells in the liver ($p < 0.01$ by T test).

DN αβ T cells undergo numerical compensation in γδTCR-/- mice

Since γδ T cells produced more IL-17A *in vitro* than DN αβ T cells (Figure 4), we questioned the effects of eliminating this γδ lymphocyte subset. Cells were isolated from the achilles tendon enthesis, blood, liver, skin, and spleen from both wild type and γδTCR-/- mice. As expected, γδ T cell were absent in γδTCR-/-, however, this was accompanied by an increase in DN αβ T cell frequency (Figure 9A). When the total (γδ + DN αβ) number of DN T cells was calculated for both the wild type and the γδTCR-/- mice, there were no statically significant differences between the two genotypes, suggesting that an expanded population of DN αβ T cells compensated for the lack of γδ T cells in γδTCR-/- mice (Figure 9B).

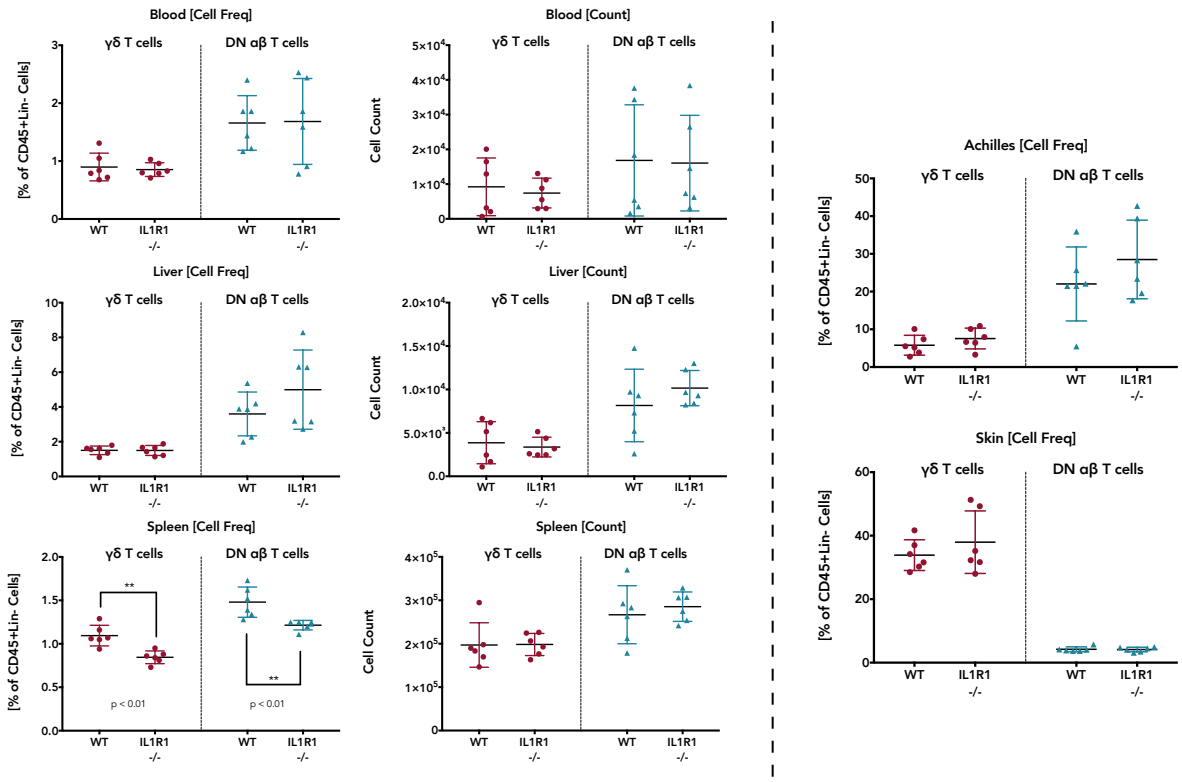

Figure 8 | Frequency and number of γδ T cells and DN αβ in tissues is largely independent of IL-1 signals. Frequencies and absolute counts of γδ T cells and DN αβ T cells were compared between wild type and IL-1R-/- mice in blood, liver, spleen, achilles tendon enthesis and skin (n=6 mice for each genotype). There were no statistically significant differences between the two genotypes except for the frequency of γδ and DN αβ T cell frequencies in the spleen (p value < 0.01 by T test).

IL-1β + IL-23 and PMA/Ionomycin stimulations induce different IL-17A+ populations in γδTCR-/- mice.

To understand the functional effects of this compensatory mechanism, splenocytes from wild type and γδTCR-/- were stimulated with either IL-1β + IL-23 or PMA/Ionomycin *in vitro*. Upon IL-1β + IL-23 stimulation, γδ T cells were the prominent IL-17 producing cell type in wild type splenocytes. With PMA/Ionomycin stimulation, robust IL-17A induction was also seen in the non-γδ T cell population of wild type mice (Figure 10A). Interestingly, there were no significant differences in the total number of IL-17A producing cells between the wild type and γδTCR-/- splenocytes upon stimulation with either IL-1β + IL-23 or PMA/Ionomycin (Figure

10B), suggesting that subsets of non-γδ T cells compensate for the lack of γδ T cells in the knockout mice (Figure 10).

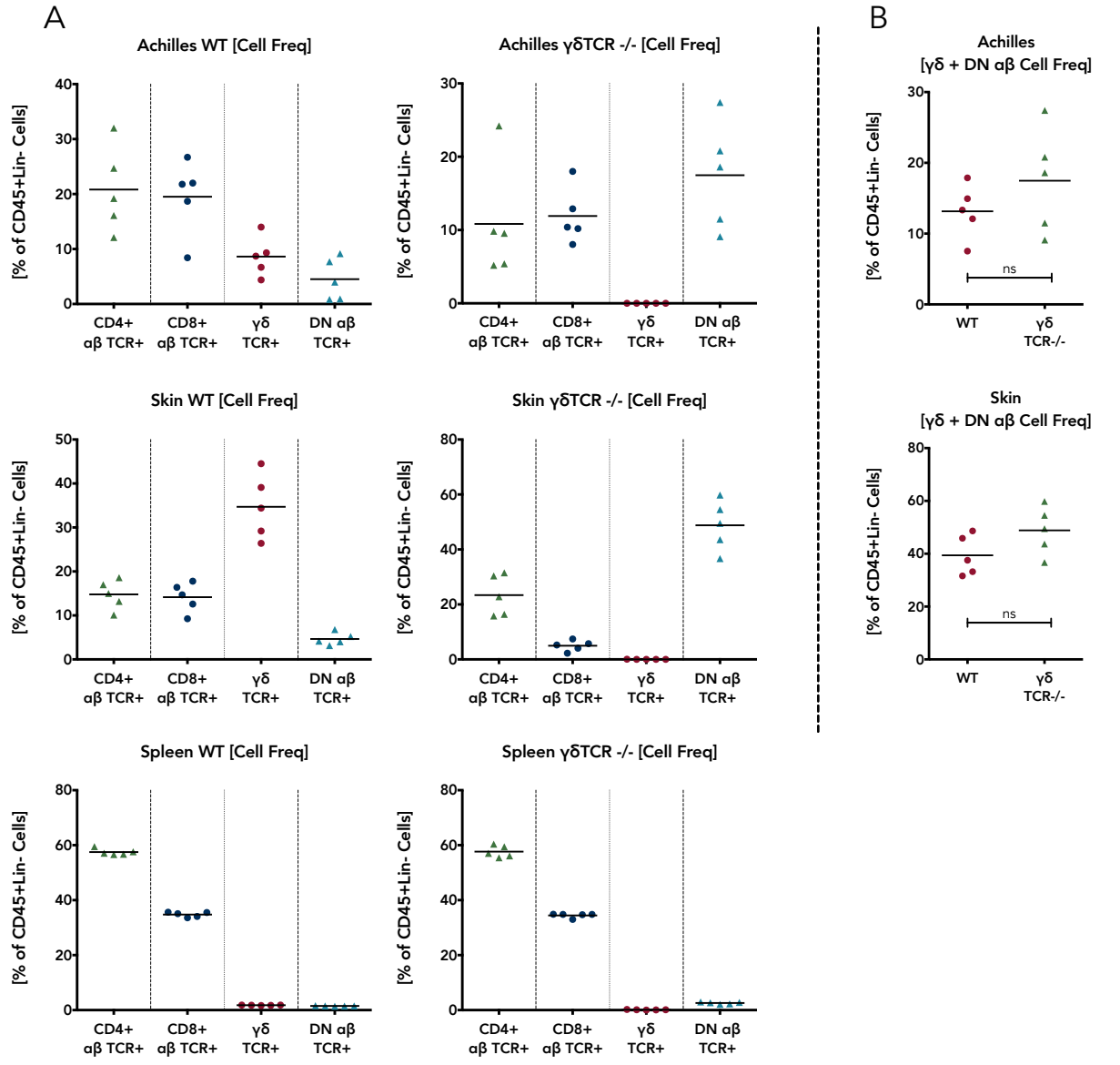

Figure 9 | DN αβ T cell frequencies increase in the absence of γδ T cells in γδTCR-/- mice. (A) Single cell suspensions were prepared from the achilles tendon enthesis, skin and spleen of wild type and γδTCR-/- mice (n=5 for each genotype). γδ T cells were absent across all locations in γδTCR-/- mice, whereas the frequency of DN αβ T cells was increased. CD4+ and CD8+ populations were largely unaffected by genotype, except for fewer CD8+ T cells in the skin of γδTCR-/- compared to wild type. (B) DN αβ T cells compensate numerically for the absence of γδ T cells in γδTCR-/- mice.

The identity of the IL-17A producing cells in γδTCR-/- spleens required further clarification. We therefore gated on the IL-17A+ cells and analyzed the expression of αβTCR, CD4, and CD8 on these cells. Almost all of the IL-17A+ cells in γδTCR-/- spleens were αβTCR+ (not shown). We then plotted the CD4 vs CD8 parameters for these cells (Figure 11A). Upon IL-1β + IL-23 stimulation, most of the IL-17+ cells in γδTCR-/- spleens were CD4-CD8- DN T cells, i.e. DN αβTCR+ cells. In contrast, CD4+CD8- T cells were responsible for the majority of the γδTCR-producing IL-17A cells after PMA/Ionomycin stimulation (Figure 11B).

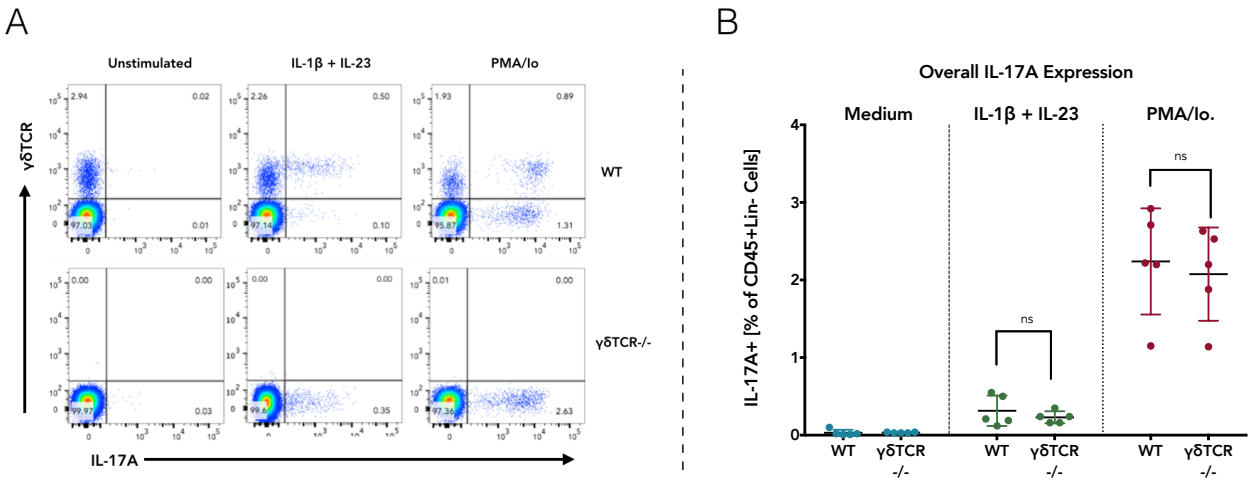

Figure 10 | IL-17A secreting non-γδ T cells compensate for the lack of γδ T cells in γδTCR-/- mice upon stimulation of splenocytes with IL-1β + IL-23 or PMA/Ionomycin in vitro. Splenocytes from wild type and γδTCR-/- mice (n=5 mice per genotype) were stimulated with IL-1β + IL-23 or PMA/Ionomycin. There were no statistically significant differences in the percentages of IL-17A+ cells between wild type and γδTCR-/- mice.

C57BL/6 mice do not develop arthritis upon hydrodynamic IL-23 minicircle injection.

Having established 1) the need for IL-1 co-stimulation for IL-23-induced secretion of IL-17A by DN T cells and 2) the numerical and functional compensation by DN αβ T cells for the lack of γδ T cells in γδTCR-/- mice, we wanted to test these knockout mice in the IL-23 minicircle model *in vivo*. All knockout strains introduced earlier are on a C57BL/6 genetic background. We therefore tested the induction of IL-23 minicircle-induced SpA in C57BL/6 wild

type mice. Adult male B10.RIII or C57BL/6 mice were hydrodynamically injected with 3 µg IL-23 minicircle plasmid. Animals were monitored weekly for the development of arthritis. Paw swelling was measured with calipers and clinical arthritis severity was assessed using a semi-quantitative scoring system. Data in Figure 12 demonstrate that unlike B10.RIII mice, C57BL/6 mice failed to develop arthritis after injection with IL-23 minicircles. They also did not develop the psoriasis-like skin disease observed in B10.RIII mice There was no difference in the production of IL-23 between the two strains (data not shown).

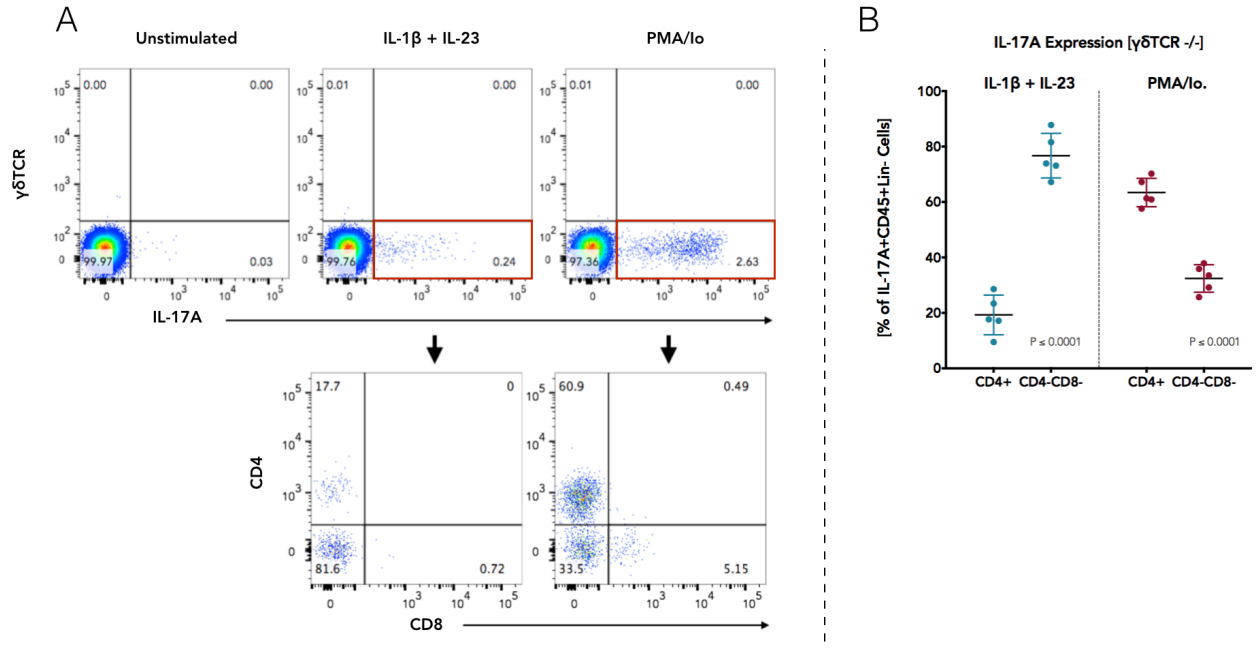

Figure 11 | DN αβ T cells largely compensate for the lack γδ T cells in γδTCR-/- mice upon *in vitro* stimulation with IL-1β + IL- 23. Gating on IL-17A+ T cells from γδTCR-/- mice after stimulation with IL-1β + IL- 23 reveals that most of these cells are αβTCR+ and CD4-CD8-, whereas the majority of IL-17A+ T cells after PMA/Ionomycin stimulation are αβTCR+ and CD4+.

γδ and DN αβ T cell frequencies and numbers are similar between C57BL/6 and B10.RIII mice

To analyze why B10.RIII are susceptible to disease induction but C57BL/6 mice are not, we compared the frequency and numbers of γδ and DN αβ T cells between C57BL/6 and B10.RIII mice. Cells were isolated from the achilles tendon enthesis, blood, liver, spleen and

skin and analyzed by flow cytometry. There were no statistically significant differences in the frequency of γδ and DN αβ T cells between C57BL/6 and B10.RIII mice at any of the 5 analyzed locations (Figure 13).

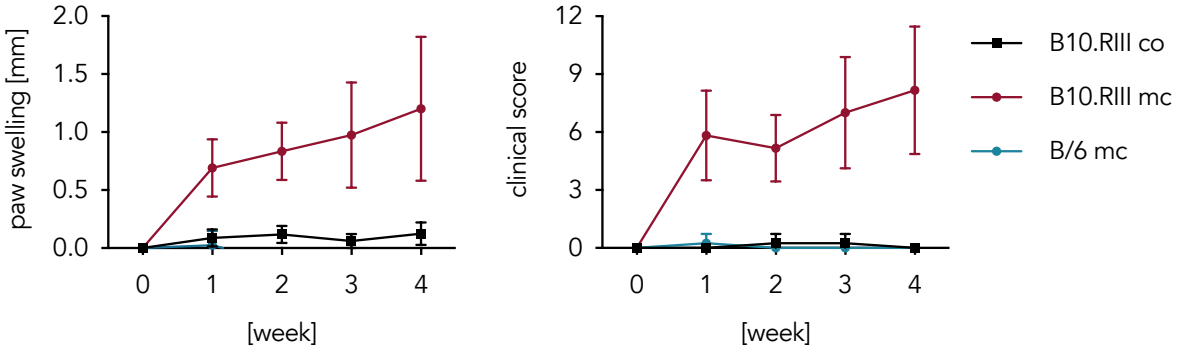

Figure 12 | IL-23 mini circle injection induces spondyloarthritis-like disease in B10.RIII mice but not in C57BL/6 mice. Adult male B10.RIII or C57BL/6 (B/6) mice were hydrodynamically injected with 3 μg IL-23 minicircle plasmid. Animals were monitored weekly for the development of arthritis. Paw swelling was measured with calipers and clinical arthritis severity was assessed using a semi-quantitative scoring system. In contrast to B10.RIII mice, minicircle-injected C57BL/6 mice failed to develop arthritis.

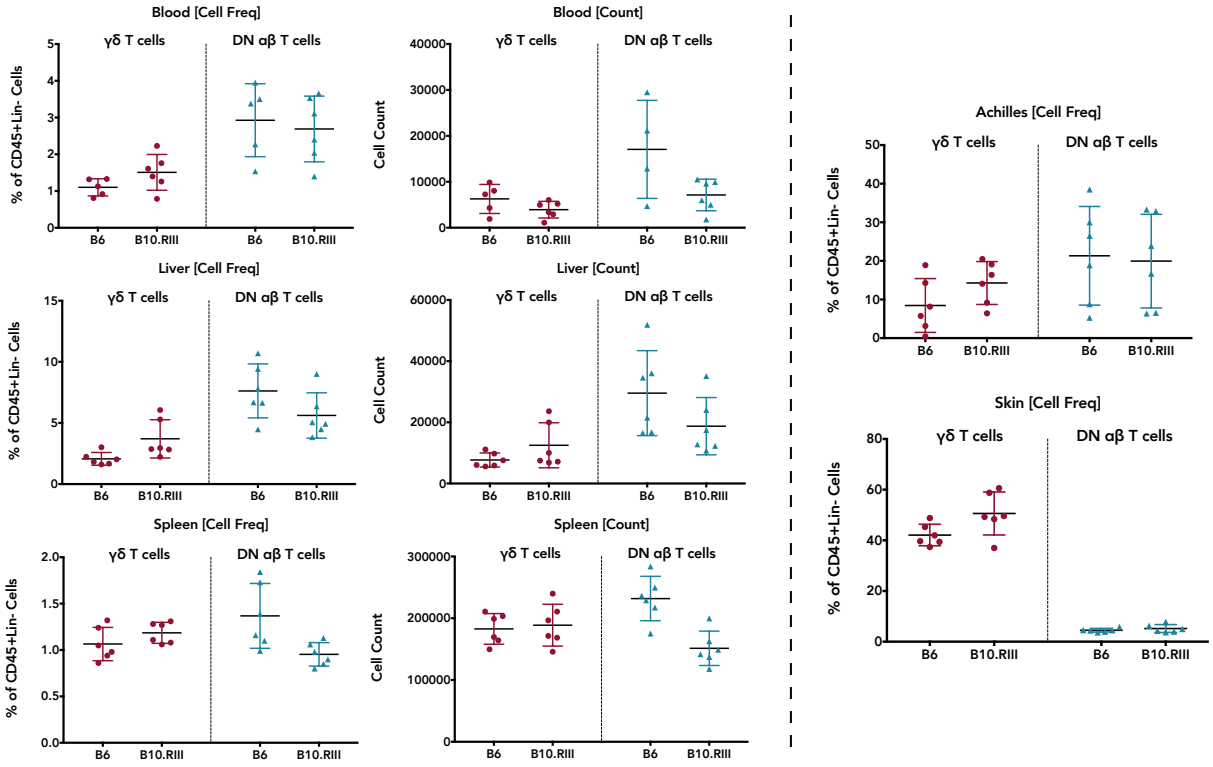

Figure 13 | Numerical differences in γδ and DN αβ T cell population between C57BL/6 and B10.RIII mice are minimal. Frequencies and absolute counts of γδ T cells and DN αβ T cells were compared between C57BL/6 and B10.RIII mice in blood, liver, spleen, achilles tendon enthesis and skin (n=6 mice for each strain). There were no statistically significant differences between the two strains, except that DN αβ T cells are more frequent in B/6 mice ($p < 0.01$).

IL-17A+ DN αβ T cells are more abundant in B10.RIII mice after in vitro stimulation with IL-1β + IL-23 or PMA/Ionomycin

If γδ and DN αβ T cell frequencies do not differ between C57BL/6 and B10.RIII mice, it would be important to uncover any potential differences in functional capacity to produce IL-17A. Splenocytes from both strains were stimulated with IL-1β + IL-23 or PMA/Ionomycin *in vitro*. Interestingly, data in Figure 14 show that the fraction of IL-17A+ DN αβ T cells was significantly higher in B10.RIII mice compared with C57BL/6 mice after stimulation with either IL-1β + IL-23 or PMA/Ionomycin ($p < 0.0001$). No statically significant differences were found in the IL-17A+ γδ T cell populations between the two strains (Figure 14). This suggests functional differences (IL-17A production) in the DN αβ T cell population as a potential explanation for the differential disease susceptibility after IL-23 minicircle induction between C57BL/6 and B10.RIII mice.

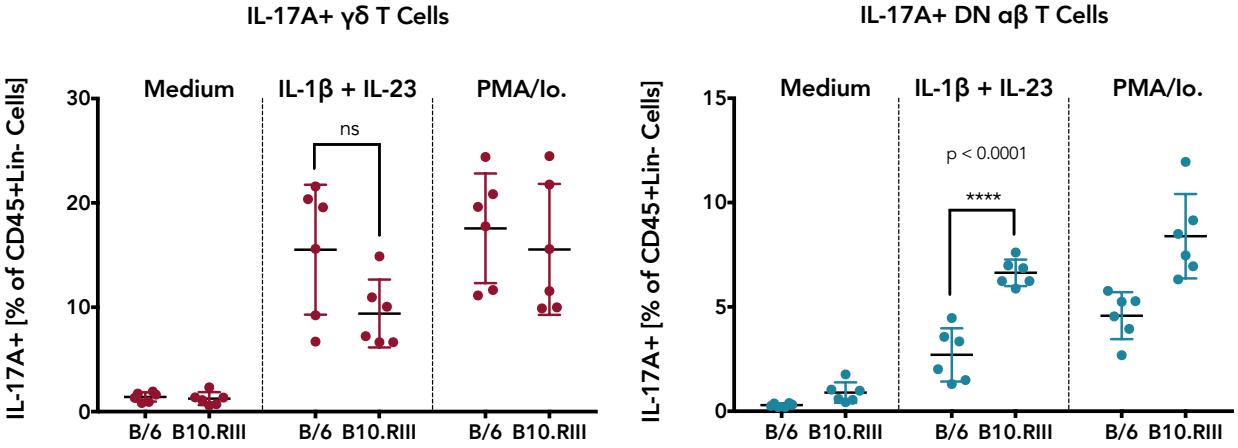

Figure 14 | Upon in vitro stimulation, a higher frequency of DN αβ T cells produces IL-17A in B10.RIII mice compared with C57BL/6 mice. Splenocytes were stimulated with medium, IL-1β + IL-23 or PMA/Ionomycin (n=6 mice for each genetic background). Comparing the frequency of IL-17A+ γδ T cells, there were no statistically significant differences between B10.RIII and C57BL/6 mice. However, DN αβ T cells from B10.RIII mice produced significantly more IL-17A than DN αβ T cells from C57BL/6 mice ($p < 0.0001$) both after stimulation with IL-1β + IL-23 and with PMA/Ionomycin.

Oral treatment with broad-spectrum antibiotics prevents IL-23 minicircle-induced disease in B10.RIII mice

The lack of IL-23 minicircle-induced disease in our colony of C57BL/6 mice contrasts with the publication by Sherlock et al., who observed IL-23 minicircle-induced disease in C57BL/6 mice. Differences in disease phenotypes between different laboratories are not uncommon and often related to differences in the microbial environment (11). The role of microbes in the pathogenesis of SpA and other autoimmune and inflammatory diseases has received much attention over the last few years. To globally assess the contribution of bacteria in IL-23 minicircle-inducee disease, we pre-treated B10.RIII mice in their drinking water with a broad-spectrum antibiotic cocktail containing Vancomycin, Metronidazole, Neomycin and Ampicillin (VMNA) using an established protocol (15). Control mice received regular drinking water. After 2 weeks on antibiotics, IL-23 minicircles were injected and animals were monitored weekly for the development of arthritis. Antibiotics were continued throughout the experiment. Data in Figure 15 demonstrate that VMNA-treated animals were protected from disease development.

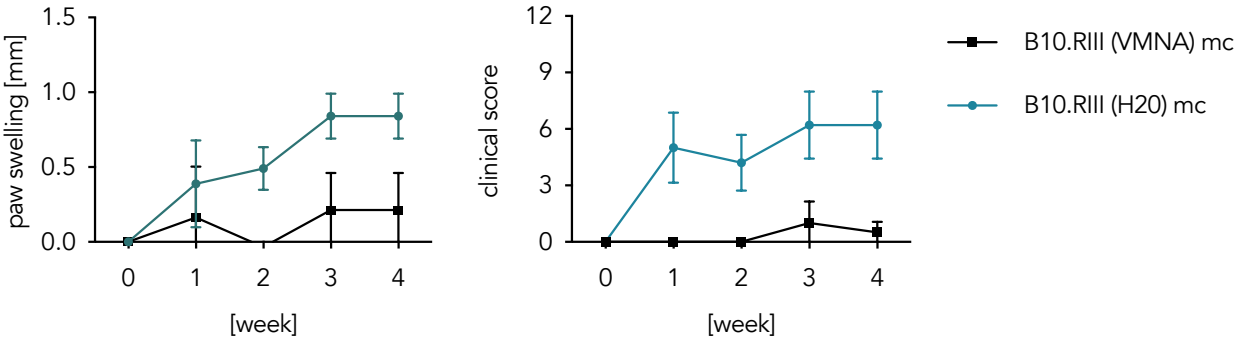

Figure 15 | Treatment with broad-spectrum antibiotics protects B10.RIII mice from IL-23 minicircle-inducee spondyloarthritis-like disease. Adult male B10.RIII mice were pre-treated with the VMNA cocktail (Vancomycin, Metronidazole, Neomycin, Ampicillin) in their drinking water for two weeks prior to hydrodynamic injection with 3 µg IL-23 minicircle plasmid. Animals were monitored weekly for the development of arthritis. Paw swelling was measured with calipers and clinical arthritis severity was assessed using a semi-quantitative scoring system. Antibiotics were continued throughout the experiment. In contrast to control animals receiving regular drinking water, antibiotic-treated mice were protected from arthritis development.

VMNA treatment reduces the γδ and DN αβ T cell populations in the liver and impairs IL-17A secretion of γδ and DN αβ T cell in the spleen

Cells were then isolated from the achilles tendon enthesis, blood, liver, spleen and skin from B10.RIII mice treated with H_20 or VMNA for 2 weeks and analyzed by flow cytometry (Figure 16). Treatment with antibiotics resulted in lower frequency and absolute number of γδ T cells in the liver ($p < 0.05$). There were no significant differences between VMNA-treated mice and controls in other locations.

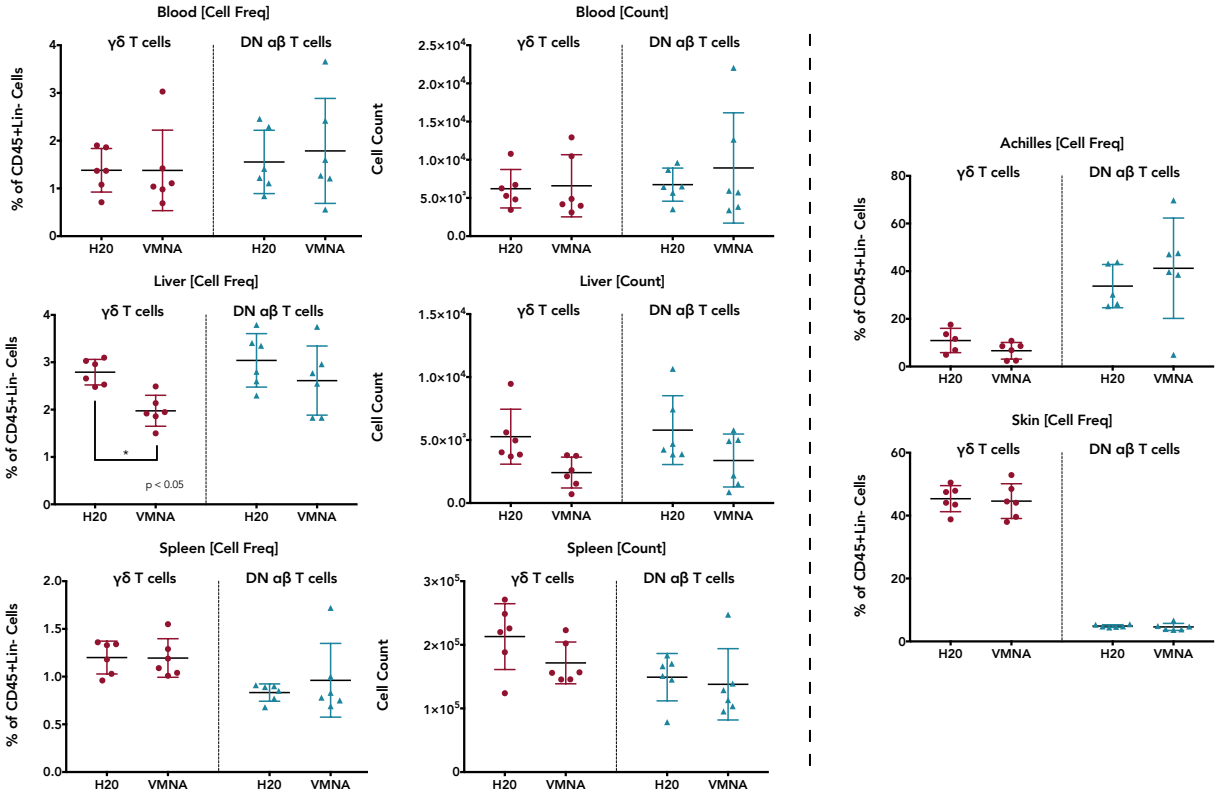

Figure 16 | No significant differences in γδ and DN αβ T cell populations between antibiotic-treated mice and control animal except in the liver. Frequencies and absolute counts of γδ T cells and DN αβ T cells were compared in blood, liver, spleen, achilles tendon enthesis and skin between VMNA-treated B10.RIII mice and control B10.RIII mice on regular drinking water (n=6 mice for each group). There were no statistically significant differences between the two groups except for the frequency of γδ T cells in the liver ($p < 0.05$ by T test).

Splenocytes from the same mice were stimulated with IL-1β + IL-23 or PMA/Ionomycin *in vitro*. Both γδ and DN αβ T cells isolated from VMNA-treated mice exhibited reduced capability to secrete IL-17A with statically significant reductions of the frequency of IL-17 positive γδ and DN αβ T cells upon stimulation with IL-1β + IL-23 or PMA/Ionomycin (Figure 17).

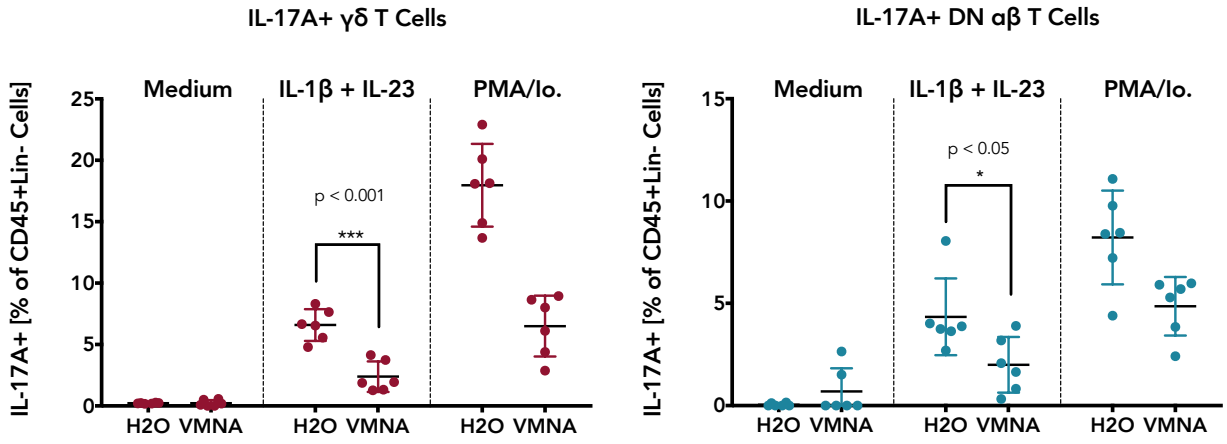

Figure 17 | VMNA-treatment reduces the frequency IL-17A producing γδ and DN αβ T cells in the spleen. Splenocytes from VMNA-treated mice or water-treated controls (n=6 mice per group) were stimulated with nothing, IL-1β + IL-23 or PMA/Ionomycin. VMNA-treated mice had significantly fewer IL-17A+ γδ T cells and DN αβ T cells upon stimulation with IL-1β + IL-23 ($p < 0.001$ for γδ T cells, $p < 0.05$ DN αβ T cells) or PMA/Ionomycin ($p < 0.0001$ for γδ T cells, $p < 0.01$ DN αβ T cells) when compared to the water-treated controls.

Discussion

In this study, entheseal DN T cells were investigated with regard to their potential role as effector cells in IL-23 minicircle-induced SpA. Results can be summarized as follows:

1. Entheseal DN T cells are comprised of γδTCR+ or αβTCR+ subsets and their development is independent of MHC Class I and II expression.
2. IL-17A secretion by γδ and DN αβ T cells requires IL-1β + IL-23 cytokine stimulation. γδ and DN αβ T cell are present in IL23R-/- and IL1R1-/- mice with minimal differences in cell frequency compared to wild type mice.
3. Expanded DN αβ T cells compensate in number and IL-17A secretion for the absence of γδ T cells in γδTCR-/- mice.
4. B10.RIII and C57BL/6 mice have similar γδ and DN αβ T cell frequencies. DN αβ T cells from disease-prone B10.RIII mice secrete significantly more IL-17A *in vitro*.
5. B10.RIII mice treated with antibiotics have similar γδ and DN αβ T cell frequencies compared with H_2O treated controls mice. γδ and DN αβ T cells from VMNA treated mice secrete significantly less IL-17A compared with γδ and DN αβ T cells from H_2O-treated control animals.

In the IL-23 minicircle model of SpA, disease is thought to result from high systemic levels of IL-23 that cause activation of IL-23R expressing tissue-resident lymphocytes. Enthesitis and skin inflammation are prominent features of the disease (Supplementary Figure 1). In the analysis of DN T cell subsets we therefore focussed on the achilles tendon enthesis and skin. The liver is the location of gene transcription of IL-23 minicircles (5). Therefore, it is highly likely

that lymphocytes in the liver are affected by high local IL-23 levels. Lymphocytes from the spleen were isolated because of its role as a secondary lymphoid organ and its harboring of many immune cells. Analysis of the blood was done because lymphocytes travel through the circulatory system to aid in immune responses elsewhere. Lymphocytes might become activated in the liver or spleen and subsequently travel through the blood to target tissues like the achilles tendon enthesis or skin.

Human SpA is strongly associated with the MHC class I variant HLA-B27 (19). It was therefore important to analyze whether the development of the rare entheseal γδ or DN αβ T cells is influenced by MHC expression. It has been shown that γδ T cells develop independently of MHC (20). Whether DN αβ T cells require MHC is controversial. Some data suggest that DN αβ T cells are previously activated peripheral CD8+ T cells that have down-regulated expression of CD8 (21). Based on these studies one would expect that DN αβ T cells are severely reduced in MHC class I deficient mice. As expected we found that γδ T cells were present in MHC deficient animals. There were also no significant differences in the frequency of DN αβ T cells in MHC Class I or MHC Class II deficient mice. Considering that CD8+ T cells were essentially absent in MHC Class I deficient Tap1-/- mice, this makes it unlikely that DN αβ T cells are progeny of peripheral CD8+ cells.

To further understand the functional capabilities of these rare lymphocytes, IL-17A was induced *in vitro* by not only using IL-23, but also IL-1β, acting as stimulatory molecules. Sutton et al. have previously shown that both cytokines are required to induce IL-17A in γδ T cells. Our results confirm this finding and demonstrate that DN αβ T cells behave similarly. Results are consistent between experiments with wild type splenocytes stimulated with IL-1β and IL-23

either individually or in combinations and with splenocytes from receptor knockout mice stimulated with the combination of IL-1β + IL-23.

PMA/Ionomycin was used as an alternative stimulus in our *in vitro* experiments. PMA activates Protein Kinase C, a kinase downstream of ligand-receptor activation (22). This allows for signaling without ligand-to-receptor binding. Ionomycin is a calcium ionophore that releases calcium from intracellular stores into the cytoplasm thereby activating NFAT signaling. PMA/ Ionomycin is often used to mimic TCR activation. Our results demonstrate that IL-1β + IL-23 and PMA/Ionomycin stimulation induce different subsets of lymphocytes to secrete IL-17A. γδ T cells and DN αβ T cells respond strongly to stimulation with IL-1β + IL-23, whereas IL-17A secretion by CD4+ T cells in response to IL-1β + IL-23 stimulation is negligible. A subset of CD4+ T cells has the potential to produce IL-17A as demonstrated by PMA/Ionomycin stimulation. However, they do not express the required receptors to respond to stimulation with IL-1β and IL-23. Our data demonstrate further that PMA/Ionomycin-induced IL-17A secretion is independent of signals through the IL-1 or IL-23 receptors.

After understanding the functional importance of the IL-23R and IL-1R1 for γδ and DN αβ T cells, we examined whether these receptor might play a role in the development of γδ and DN αβ T cells. Lymphocytes from the aforementioned 5 locations were isolated and quantified. Largely, for IL23R-/- and IL1R1-/- mice, there were no significant differences in γδ and DN αβ T cell frequencies when compared to their respective wild type counterparts. This suggests that despite the dependence of IL-23-induced IL-17A secretion by γδ and DN αβ T cells on IL-23R and IL-1R, these receptors do not play a role in the development of these rare lymphocytes.

Notable differences in γδ T cell frequencies were found in the skin and liver between IL23R+/- and IL23R-/- mice.

Data from the *in vitro* experiments heavily favored γδ T cells as the predominant producers of IL-17A after stimulation with IL-1β and IL-23. With the idea in mind to inject γδTCR-/- mice with IL-23 minicircles, we analyzed IL-17A secretion by splenocytes from these mice. Interestingly, the total number of IL-17A producing lymphocytes upon stimulation with IL-1β + IL-23 or PMA/Ionomycin was not different between γδTCR-/- mice and wild type controls. The lymphocytes in γδTCR-/- mice that produced IL-17A in response to IL-1β + IL-23 were almost all DN αβ T cells. We also demonstrated that DN αβ T cells were expanded in the tissues of γδTCR-/- mice. The experimental data presented are thus consistent with a scenario where DN αβ T cells capable of secreting IL-17A compensate for the lack of γδ T cells in γδTCR-/- mice *in vivo* and *in vitro*. γδ T cells have been shown to seed peripheral tissues during embryonic development. It is feasible that DN αβ T cells enter this niche in the absence of γδ T cells and then fulfill similar functions. Together with the finding that MHC Class I deficiency has no impact on the development of DN αβ T cells, these data strongly argue that DN αβ T cells are innate lymphocytes similar to γδ T cells and not descendants of conventional MHC Class I restricted CD8+ T cells. Moreover, we would hypothesize that γδTCR-/- mice would develop IL-23 minicircle-induced disease similar to wild type mice. This hypothesis is not currently testable as γδTCR-/- are not available on a B10.RIII genetic background.

C57BL/6 is the most commonly used inbred strain and provides the genetic background for the various knockout mice investigated in this study. We therefore tested disease induction with hydrodynamically injected IL-23 minicircles in C57BL/6 mice. Contrary to a previous

report (6), C57BL/6 mice in our colony did not develop arthritis or skin inflammation, whereas B10.RIII mice did. This is an unfortunate finding, because it means that the various knockout strains including IL1R1-/- mice and γδTCR-/- mice cannot be tested in the disease model *in vivo*. At the same time, it raises the interesting questions of what causes the difference in susceptibility between the two mouse strains. B10.RIII mice have been found to be more permissive to disease induction than C57BL/6 mice in other disease models, but the mechanisms are not fully understood (23). Here we analyzed the frequency and function of γδ and DN αβ T cells between the two strains. We found no major differences with regard to the number of γδ and DN αβ T cells in 5 analyzed tissues. We then hypothesized that there might be a functional difference in the cells producing IL-17A. *In vitro* stimulation with IL-1β + IL-23 (or PMA/Ionomycin) demonstrated a significantly higher number of IL-17A+ DN αβ T cells in the B10.RIII mice compared with disease-resistant C57BL/6 mice. The reason for this difference still remains to be discovered, however, it provides a potential explanation why B10.RIII mice develop IL-23 minicircle-induced disease whereas C57BL/6 mice do not.

In a separate experiment, disease susceptibility in antibiotic treated mice was measured in B10.RIII mice. These IL-23 minicircle injected mice were either given H_20 or a broad-spectrum antibiotic cocktail, VMNA. Interestingly, mice with the antibiotic treatment were protected against disease induction. Differences in γδ and DN αβ T cell frequencies were minimal between the two treatments, but IL-17A secretion was significantly lower for both γδ and DN αβ T cells in VMNA treated mice. The reason for this difference is not well understood. However, it can be speculated that due to the role of antibiotics in targeting bacterial populations, disease induction may involve the microbiome and may influence pathogenesis.

This investigation sought to further characterize the tissue-resident DN T cells identified by Sherlock et al. as mediators of IL-23 minicircle-induced SpA in mice. We have shown that these cells comprise γδ and DN αβ T cell subsets and ascertained that they require a signal through the IL-1 receptor in addition to IL-23 for the secretion of IL-17A. We found that DN αβ T cell compensate for the lack of γδ T cells in γδTCR-/- mice, both numerically *in vivo* and functionally *in vitro*. Moreover, differences in IL-17A production by γδ and DN αβ T cell were identified that might explain the differential disease susceptibility to IL-23 minicircle induction in two scenarios, comparing disease-prone B10.RIII with resistant C57BL/6 mice, and comparing antibiotic-treated B10.RIII mice who do not develop disease with H_2O-treated controls. However, many open questions remain. For instance, all functional *in vitro* assays in this work were performed with splenocytes. It is likely but needs to be confirmed that γδ and DN αβ T cells isolated from entheses behave similarly. These are challenging experiments considering the low numbers of cells that can be isolated from the mouse achilles tendon enthesis. Moreover, the *in vitro* data presented in this thesis suggest that IL-23 minicircle-induced SpA is critically dependent on IL-1 receptor signals. This should be formally demonstrated *in vivo* with reagents that block IL-1α, IL-1β or IL-1R1. *In vivo* experiments with IL1R1-/- mice would not be informative given the lack of disease in C57BL/6 mice upon IL-23 minicircle injection.

The most exciting direction for further research would be to explore in more detail the relationship between intestinal microbiota and SpA susceptibility and the role of γδ and DN αβ T cells therein. The first experiment would be to pretreat B10.RIII mice with antibiotics that have a more limited activity spectrum, targeting only target gram-positive or gram-negative bacteria,

and then inject them with IL-23 minicircles and repeat the functions studies of γδ and DN αβ T cells. Future experiments could involve the generation of germ-free B10.RIII mice and reconstitution experiments with specific types of bacteria.

Most importantly, but this goes far beyond the scope of the research presented in this work, one would like to analyze whether similar cells exist at entheses in humans and whether they contribute to SpA pathogenesis by secreting IL-17A in response to IL-1β and IL-23.

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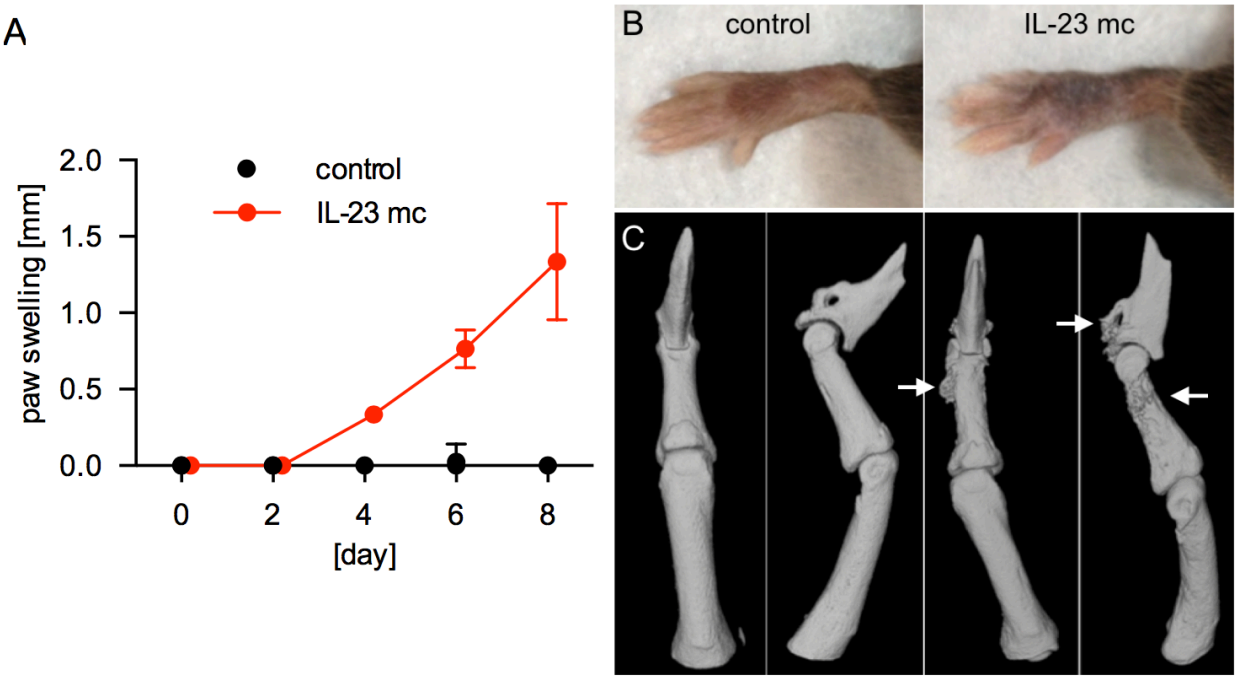
Appendix



Supplementary Figure 1 | Hydrodynamic injection of IL-23 minicircles induces SpA in adult mice. 12 week-old male B10.RIII mice received IL-23 minicircles (3 µg per animal) or PBS on day 0 via hydrodynamic injection into the tail vein. (A) The thickness of the front paws and ankles was measured with calipers every two days. Paw swelling represents the sum of the change from baseline for all four paws (mean ± SD, n=3 mice per group). (B) Photographs taken on day 24 demonstrate paw swelling, dactylitis, and hyperkeratosis in the recipient of IL-23 minicircles.