



SARS-CoV-2 Infection Induces Psoriatic Arthritis Flares and Enthesis Resident Plasmacytoid Dendritic Cell Type-1 Interferon Inhibition by JAK Antagonism Offer Novel Spondyloarthritis Pathogenesis Insights

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Objective: Bacterial and viral infectious triggers are linked to spondyloarthritis (SpA) including psoriatic arthritis (PsA) development, likely via dendritic cell activation. We investigated spinal enthesal plasmacytoid dendritic cells (pDCs) toll-like receptor (TLR)-7 and 9 activation and therapeutic modulation, including JAK inhibition. We also investigated if COVID-19 infection, a potent TLR-7 stimulator triggered PsA flares.

Methods: Normal enthesal pDCs were characterized and stimulated with imiquimod and CpG oligodeoxynucleotides (ODN) to evaluate TNF and IFN α production. NanoString gene expression assay of total pDCs RNA was performed pre- and post- ODN stimulation. Pharmacological inhibition of induced IFN α protein was performed with Tofacitinib and PDE4 inhibition. The impact of SARS-CoV2 viral infection on PsA flares was evaluated.

Results: CD45+HLA-DR+CD123+CD303+CD11c- enthesal pDCs were more numerous than blood pDCs ($1.9 \pm 0.8\%$ vs $0.2 \pm 0.07\%$ of CD45+ cells, $p=0.008$) and showed inducible IFN α and TNF protein following ODN/imiquimod stimulation and were the sole enthesal IFN α producers. NanoString data identified 11 significantly upregulated differentially expressed genes (DEGs) including TNF in stimulated pDCs. Canonical

pathway analysis revealed activation of dendritic cell maturation, NF- κ B signaling, toll-like receptor signaling and JAK/STAT signaling pathways following ODN stimulation. Both tofacitinib and PDE4i strongly attenuated ODN induced IFN α . DAPSA scores elevations occurred in 18 PsA cases with SARS-CoV2 infection (9.7 ± 4 pre-infection and 35.3 ± 7.5 during infection).

Conclusion: Enteseal pDCs link microbes to TNF/IFN α production. SARS-CoV-2 infection is associated with PsA Flares and JAK inhibition suppressed activated enteseal plasmacytoid dendritic Type-1 interferon responses as pointers towards a novel mechanism of PsA and SpA-related arthropathy.

Keywords: plasmacytoid dendritic cells, interferon alpha, psoriatic arthritis, COVID-19, enthesitis

INTRODUCTION

Entheses are locations where tendons or ligaments attach to the bone and inflammation of the enthesitis (enthesitis) is an important and frequent manifestation of inflammatory musculoskeletal disease, especially the Spondyloarthritis (SpA) group of diseases, which includes ankylosing spondylitis (AS) and psoriatic arthritis (PsA) (1–3). Plasmacytoid dendritic cells (pDCs), are a rare immune cell subset that was first functionally characterized in the late 1990s (4) that upon toll-like receptor (TLR)-7 and TLR-9 stimulation, which recognize RNA and DNA respectively, are known to secrete type-I interferons (IFN) and other inflammatory cytokines such as TNF (5, 6). Moreover, pDCs have been implicated in psoriasis immunopathology whereby cutaneous pDCs orchestrate disease development through IFN α induction culminating in psoriasis development (4, 7, 8). Bacterial or viral infection or vaccinations have been linked to PsA and SpA like disease (7, 9, 10).

The IL-17 axis and TNF cytokine pathways are central to the pathogenesis of PsA and SpA, but the emerging JAK inhibition in PsA and AS do not directly impact on the TNF/IL-17 pathways (11). However, the JAK-STAT pathway is a critical regulator of pDC cytokine production, including TNF and interferon. Nevertheless, direct evidence for this pathway as a potential human SpA initiator is lacking. During the global COVID-19 virus pandemic, arthralgia has been reported, raising the possibility of a poorly defined enthesial mechanism (12, 13).

We previously found evidence for a population of enteseal resident pDCs by phenotypic criteria (14). Herein, we investigated immune responses of enteseal pDCs following TLR7 and TLR9 stimulation and the potential modulation of this response by emergent SpA therapeutics and also whether the SARS-CoV-2 RNA virus, with potent TLR7 agonist properties was associated with PsA disease flares.

MATERIALS AND METHODS

Enthesis Samples

Normal interspinous process was obtained from 54 patients (20 men and 34 women; age 53.6 ± 23.2 years) who were undergoing

spinal decompression or corrective scoliosis surgery. Peripheral blood was also collected from these patients and healthy volunteers (n=5). The enthesitis was subsequently separated into peri-enteseal bone (PEB) and the enteseal soft tissue (EST). PEB was chosen for the following experiments because cells were more numerous in PEB than in ST. Mononuclear cells were isolated from PEB by mechanical digestion, as previously indicated (14, 15). The study protocol of the current investigation was approved by North West-Greater Manchester West Research Ethics Committee.

Phenotyping of Enteseal pDC and Intracellular Measurement of TNF Following ODN or Imiquimod Stimulation

Following digestion, 5×10^6 PEB cells were incubated in RPMI (Sigma-Aldrich) containing 10% FBS (Gibco) and 1% p/s (Penicillin-Streptomycin). Cells were stimulated with CpG oligodeoxynucleotides (ODN 2216, InvivoGen, 20 μ g/mL) or imiquimod (30 μ M, Cayman Chemical) for 2 hr. Cells were subsequently incubated for 12 hr in the presence of Golgi Plug (BD Biosciences). Following this, cells were blocked in 10% mouse serum and 1% human IgG buffer prior to incubation with antibodies. Cells were then stained extra and intracellularly using the Intraprep staining kit (Beckman Coulter) according to the manufacturer's protocol. Cells were stained using anti-CD45, CD3, CD14, CD19, CD56, CD11c, HLA-DR, CD123, CD303 and TNF (additional details on the antibodies and processes used available upon request from the corresponding author). Cells were analyzed using LSRII (BD Biosciences) and FlowJo software (Tree Star Software).

Isolation of pDC and Tomographic Microscopy

Following digestion, pDCs were selected using CD304 (BDCA-4)-Biotin (Miotenyl Biotec, 2 μ l per 10×10^6 cells) using LS columns (Miotenyl Biotec) according to the manufacturer's protocol. Cells were subsequently separated into pDCs and non-pDCs. Isolated enteseal pDCs were seeded into 35-mm tissue culture dish at 15,000 cells/dish. The dish was then placed on a holotomographic microscope (3D Explorer, NanoLive, Lausanne, Switzerland) equipped with a 60X objective, and images were taken at $\times 600$ magnification.

Stimulation of pDCs and Quantification of IFN α by ODN

Due to sample size variance and resultant cell yields, different pDC numbers were isolated and seeded per experiment (between 12000 and 50000 per well) with IFN α secretion being expressed as pg/cell. During method optimization we noted that ODN was a much more potent inducer of enthesal pDC IFN α production than imiquimod (**Supplementary Figure 1B**). Consequently, further *in vitro* studies were carried out on ODN stimulated cells whereby cells were stimulated with 20 μ g/ml ODN for 24 hrs. Following this, supernatant was harvested. IFN α (all isoforms) were measured using ELISA (PBL Assay Science). The cells were harvested and RNA was isolated using PicoPure RNA isolation kit (ThermoFisher) and complementary DNA was synthesized using a high-capacity reverse transcription kit (ThermoFisher). Quantitative real-time polymerase chain reaction (PCR) with an ABI 7500 thermocycler (Applied Biosystems) was performed to measure IFN α gene expression using Taqman gene expression assay and universal Taqman master mix (both ThermoFisher). Expression levels of target gene were calculated relative to expression of the housekeeping gene HPRT1.

Antagonism of IFN α Secretion

After digestion, 1×10^5 cells/ml enthesal cells were seeded into 96-well plate. Cells were stimulated with ODN as before, with and without tofacitinib 1 μ M (Pfizer), PDE4i/Rolipram 100 μ M (Cayman Chemical) or Methotrexate 5 mg/ml (Cayman Chemical) for 24 hr. DMSO 0.1% served as a solvent control. IFN α was quantified by ELISA as before.

Nanostring Analysis of Enthesal and Blood pDCs Upon ODN Stimulation

Post ODN stimulation, RNA was extracted from enriched enthesal pDCs and matched blood cells as described above (each n=5). Immune related gene expression was assessed using a predesigned human autoimmune profiling panel established by Nanostring consisting of 770 immune related (genes involved in autoimmune, chronic inflammatory, and aberrant immune response diseases) and 20 housekeeping genes. All RNA samples were quality controlled using Nanodrop 8000. 100ng of total RNA from each sample was used for setting up the NanoString probe PCR hybridization (24 hrs) at 65°C. Following hybridization, excess probes were removed using nCounter Prep Station and magnetic beads, hybridized mRNA/probe were immobilized on a streptavidin-coated cartridge. The processed cartridges were scanned using an nCounter digital analyzer platform (nCounter[®] FLEX Analysis System) for generation of the raw data with a high-resolution scan (555 fov). Raw data were processed with nSolver Analysis Software (V.4.0), imaging quality control (QC), mRNA positive control QC and normalization QC checked, and all the samples were within the quality parameters of NanoString gene expression assays. Differential gene expression analysis was performed using nSolver advance analysis module V. 2.0.

Bioinformatics Analysis

Nanostring data was analyzed using nSolver 4.0 and plot using Graphpad 8.0. Log_2 |fold change| >1 and Benjamini-Yekutieli p-value (FDR) <0.05 were considered statistically significant. Protein-protein interaction (PPI) network was analyzed using online STRING database (<https://string-db.org/>). Pathways mapping was generated by ingenuity pathway analysis (IPA, QIAGEN Inc.) (16). IPA was used to predicted possible upstream regulators of the proteins in this study, which were assigned as inhibited or activated according to Z-score, a statistical result of differential gene expression according to the fold changes (17). Enrichment is determined by significance overlap of genes known to be in this pathway according to the knowledge base and genes that are regulated in the dataset. Significance is measured by Fisher exact test by p-value.

Assessment of Disease Flares in PsA Post COVID-19 Infection

SARS-CoV2 virus is a single-stranded RNA virus that stimulates TLR7 pathways. To provide further support for the potential relevance of viral infection in SpA related disease, 18 PsA patients (Classification Criteria for Psoriatic Arthritis (CASPAR) score ≥ 3) with a confirmed COVID-19 diagnosis by nasopharyngeal PCR were evaluated for PsA disease flare between February and April 2020. The disease activity in psoriatic arthritis (DAPSA) scores including swollen and tender joints count, were collected at three times points: T0-last most recent measurement before infection; T1-during infection, whilst the patient was stable; T2-post infection, following 2 negative nasopharyngeal specimen tests. Patient demographic is reported in **Table 1 (Supplementary Material)**.

Statistical Analysis

Statistical significance was calculated using 2-tailed paired t-test unless stated. The analysis was performed using Prism software (GraphPad Software8.0). Error bars represent the standard error of the mean (SEM). $p < 0.05$ was considered statistically significant.

RESULTS

Human Enthesal pDCs Secrete TNF and IFN α After ODN Stimulation

Following enthesal digestion, pDCs were confirmed in PEB by flow cytometry by a CD45+HLA-DR+CD123+CD303+CD11c-Lin- phenotype (**Figure 1A**) and morphology is shown in **Supplementary Figure 1A**. Flow cytometry showed that pDCs were more numerous in PEB than in peripheral blood ($1.86 \pm 0.77\%$ vs $0.21 \pm 0.07\%$ among CD45+ cells, n=5, $p=0.008$, **Figure 1B**). Enthesal cells stimulated with ODN showed upregulation of intracellular TNF (**Figure 1A**). TNF was also induced following imiquimod stimulation as determined by intracellular flow cytometry (n=4, **Figure 1C**).

Following isolation of both enthesal pDCs and non-pDCs and subsequent ODN stimulation, basal IFN α transcripts were detectable in unstimulated pDC (relative expression $1.552 \pm$

TABLE 1 | Basic demographic information for PsA patients.

Gender	Age (years)	BMI (kg/m ²)	PsA duration in years	Current treatment	COVID treatment	Time between COVID infection and flare (days)
M	65	28	8	Ustekinumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Prednisone 25 mg	13
M	53	29	11	Secukinumab	Amoxicillin 1000mg twice per day, Paracetamol 1000mg, Oxygen, Lopinavir-ritonavir 400mg twice	19
F	48	31	16	Secukinumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Doxycycline 100 mg, Azithromycin 500 mg	21
M	67	27	14	Etanercept	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die	17
F	61	28	12	Ustekinumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Prednisone 25 mg	19
F	53	31	17	Adalimumab	Amoxicillin 1000mg twice per day, Paracetamol 1000mg, Oxygen, Hydroxychloroquine, Doxycycline 100	23
F	58	27	11	Ustekinumab	Doxycycline, Hydroxychloroquine, Lopinavir-ritonavir 400mg twice	14
M	45	26	13	Adalimumab	Oxygen, Lopinavir-ritonavir 400mg twice, Amoxicillin 1000mg, Prednisone 25 mg	12
M	57	27	12	Ustekinumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Prednisone 25 mg	18
F	66	28	17	Adalimumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Doxycycline	21
M	43	26	15	Etanercept	Hydroxychloroquine, Amoxicillin	15
F	54	28	16	Ustekinumab	Lopinavir-ritonavir 400mg twice, Hydroxychloroquine	19
F	69	27	9	Secukinumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Amoxicillin 1000mg x2	16
M	45	28	6	Ixekizumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Prednisone 25 mg	17
F	37	28	13	Adalimumab	Oxygen, Lopinavir-ritonavir 400mg twice, Hydroxychloroquine 800mg/die, Prednisone 25 mg	14
F	71	29	7	Adalimumab	Hydroxychloroquine, Doxycycline	16
M	60	27	17	Ustekinumab	Hydroxychloroquine, Doxycycline	15
M	48	30	6	Adalimumab	Hydroxychloroquine, Doxycycline	17

M, male; F, female. BMI, body mass index.

0.747, **Figure 1D**) but this was significantly higher following ODN stimulation (relative expression 3.053 ± 1.070 , $p=0.01$, **Figure 1D**). At the protein level, IFN α was only detectable from the pDC stimulated fraction (0.089 ± 0.038 pg/cell, $p=0.031$, **Figure 1E**). Noting that total IFN α was secreted by enthesal pDCs, further experiments were undertaken on unsorted cells.

We investigated the effect of SpA disease-relevant compounds and their potential to modulate IFN α secretion. Both Tofacitinib (0.003 ± 0.002 pg/cell, $p=0.008$) and PDE4i (0.008 ± 0.004 pg/cell, $p=0.002$) significantly reduced ODN induced IFN α . Methotrexate however, had no effect on IFN α production (0.050 ± 0.016 pg/cell, $p=0.24$) (**Figure 1F**).

DEG Pattern of Stimulated Enthesal pDCs and TNF Pathway

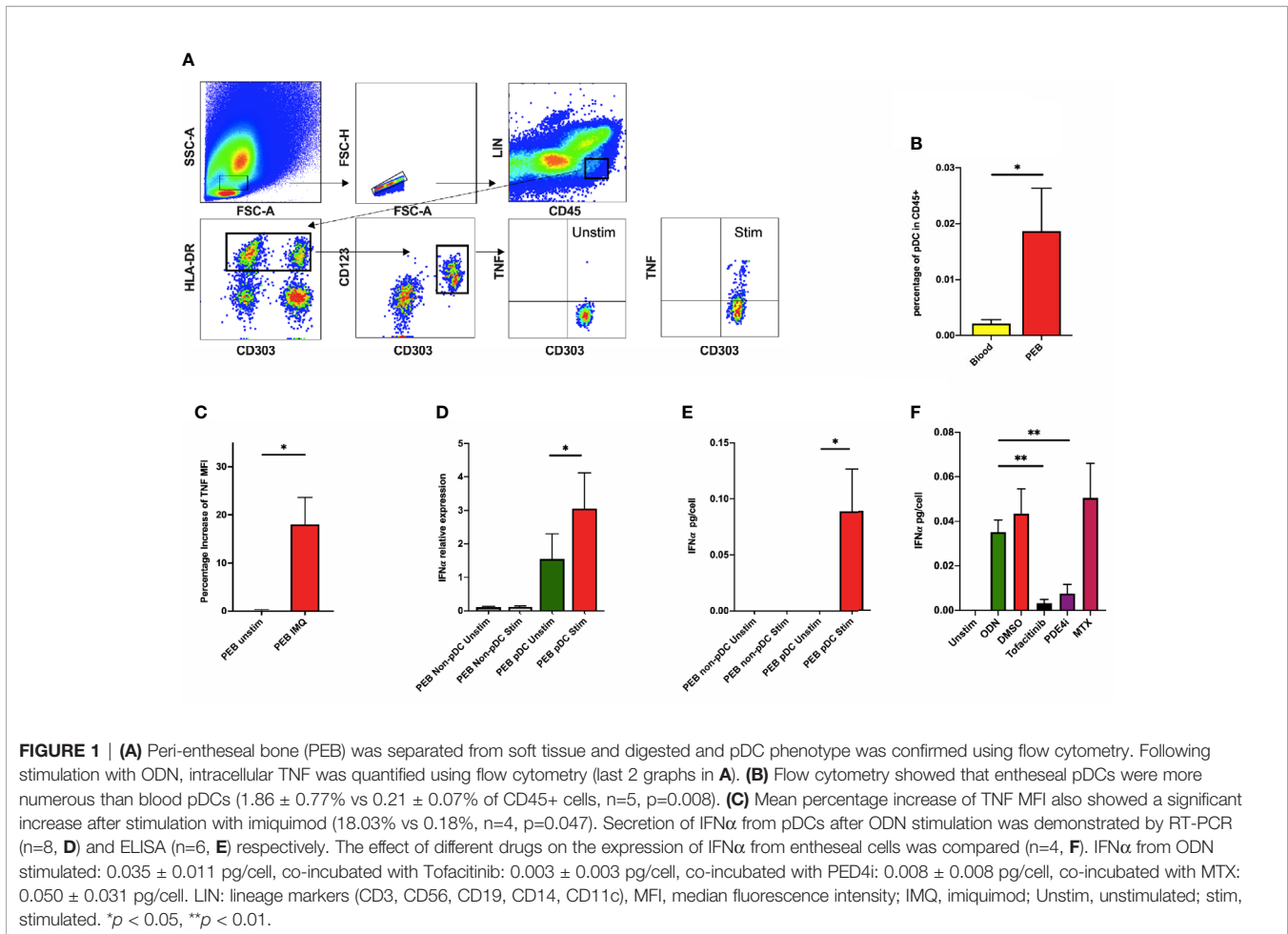
Following ODN stimulation of enthesal pDCs, using NanoString, hierarchically clustered heat map representation of global gene expression profiles showed that stimulated pDCs could be differentiated from the unstimulated samples, although heterogeneity might exist (**Figure 2A**). Eleven DEGs were significantly upregulated in the stimulated pDCs, including TNF (\log_2 fold change=1.95, $p=0.042$, **Figure 2B**). Protein-protein interaction (PPI) network was constructed from the 11 DEGs including 4 isolated nodes and 1 main connection component (**Figure 2C**). There were a total of 8 edges

identified and PPI enrichment p -value was 0.004. The main component consisted of ZBP1, RIPK3, TNF, ITGB2 and TLR7. RIPK3 and TNF had the greatest degree of interaction (**Figure 2C**).

Canonical pathway analysis of the enthesal pDC genes upon ODN stimulation using IPA showed that pathways related to dendritic cell maturation (22 genes, $z=4.416$, $p=1.59E-19$, **Supplementary Figure 2**), NF- κ B signaling (19 genes, $z=3.3$, $p=5.5E-16$, **Supplementary Figure 3**), toll-like receptor signaling (14 genes, $z=2.714$, $p=1.65E-15$, **Figure 2D**) and JAK/STAT signaling (8 genes, $z=2.828$, $p=3.05E-07$, **Figure 2E**) were significantly activated.

Increased DAPSA Following Sars-Cov-2 Infection

18 PsA patients with confirmed COVID-19 showed increased DAPSA score following COVID-19 (**Figure 3A**, 9.7 ± 4.06 vs 35.3 ± 7.47 pre- and during infection, respectively, $p<0.0001$). Even post-infection, DAPSA scores remained higher (**Figure 3B**, 9.7 ± 4.06 vs 18.8 ± 4.39 for pre- and post-infection, respectively, $p<0.0001$). With respect to swollen and tender joint count, statistically significant increases were shown for both during and post-infection (**Figure 3C**, 0.9 ± 0.9 , 1.9 ± 0.8 , 1.5 ± 0.6 for pre-, during, and post-infection, respectively. $P=0.0005$ for during- vs. pre-infection and $p=0.002$ for post- vs. pre-infection).



DISCUSSION

We report the presence of an enriched pDC population at the normal human enthesis. Upon relevant TLR pathway stimulation, we demonstrated inducible TNF and IFN α protein production and IPA analysis showed this operated through NF κ B or MYD88 pathways, respectively. The enthesis pDC TNF induction by TLR7/9 is interesting since this is a critical cytokine in enthesitis (18). Although TNF is produced by many cells, pDCs was the sole enthesal source of TLR9 induced IFN α . The pDCs production of type-I interferons is a quintessential component of viral defense (19). Bacterial triggers are well defined in reactive arthritis and in experimental SpA and a role for viral infection is also reported in reactive arthritis and several epidemiological surveys have shown a link between viral triggers and PsA onset (20–22), but viral initiation is not commonly thought of as important in axial disease including in AS. In keeping with this, we report that SARS-CoV-2 infection resulted in PsA disease flares. Also, pDCs have previously been reported to be more abundant in PsA synovial fluid when compared to osteoarthritis or rheumatoid arthritis (23, 24). Blood pDCs are decreased in peripheral blood of PsA and showed chemotaxis towards PsA synovial fluid (24).

We also investigated several SpA and PsA disease relevant compounds for their ability to modulate enthesal IFN α induction. In agreement with previous blood derived pDC studies, both PDE4i and Tofacitinib attenuated ODN induced IFN α (25, 26); however, methotrexate did not. As TLR stimulation does not directly signal *via* JAK-STAT pathway, Tofacitinib attenuation of enthesal IFN α production could be due to reduced pDC TLR mRNA receptor expression, thus leading to decreased IFN α production as previously reported (25).

The limitations of this study include the relatively small sample numbers and the fact that functional analysis could only be done on the spinous process peri-enthesal bone. However, it still highlighted that upregulation of TNF and IFN α in stimulated enthesal pDCs that could be blocked with JAK and PDE4 inhibition. Studies of pDCs from sites of active enthesitis would also be desirable. Second, the time length of post-infection was different for each PsA patient so we cannot use a specific time period to indicate how long symptoms persisted after infection.

In conclusion, enthesal pDCs may represent a novel population that bridges SpA enthesis immunopathology and may be a population modified by JAK and PDE4 inhibition

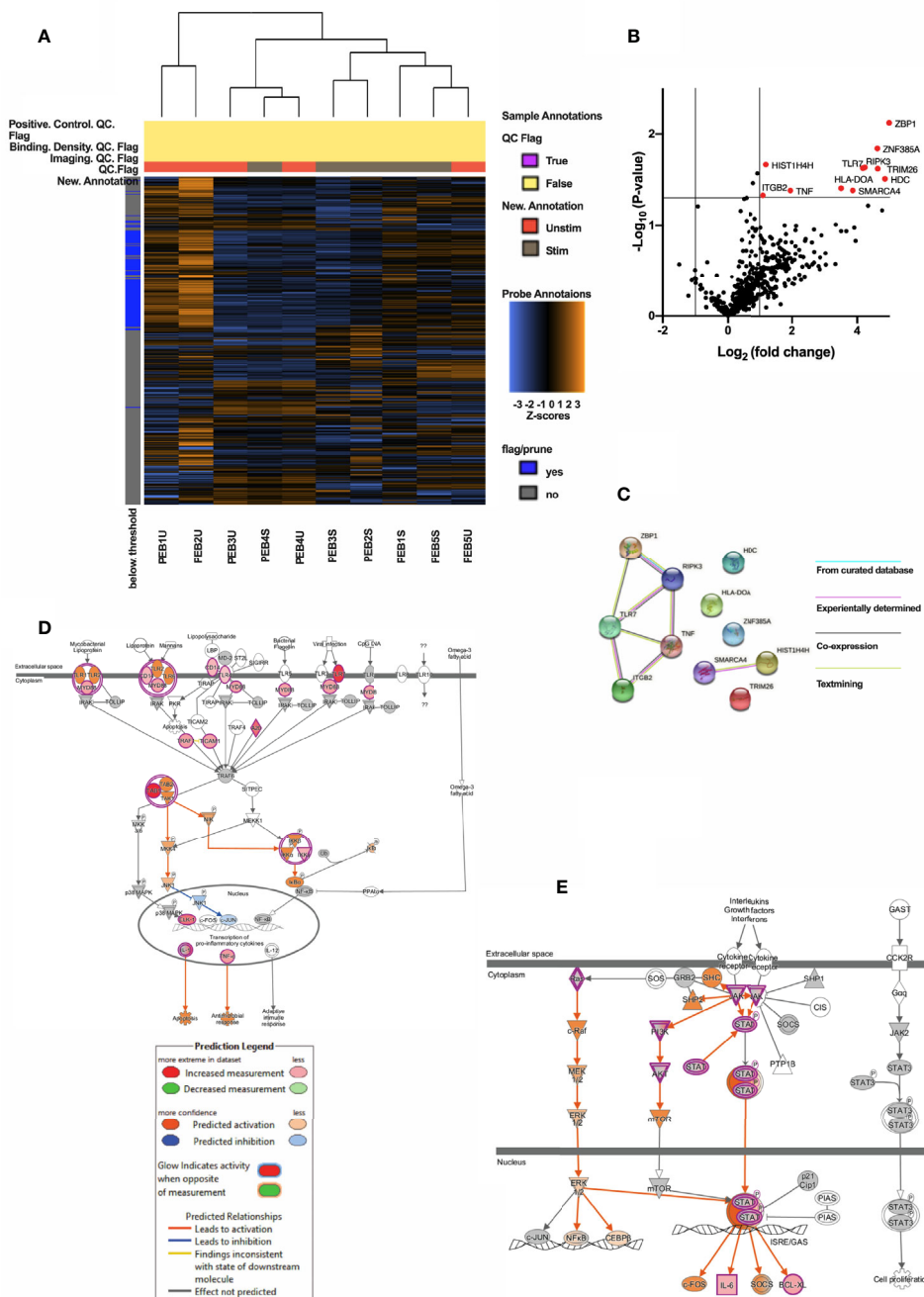
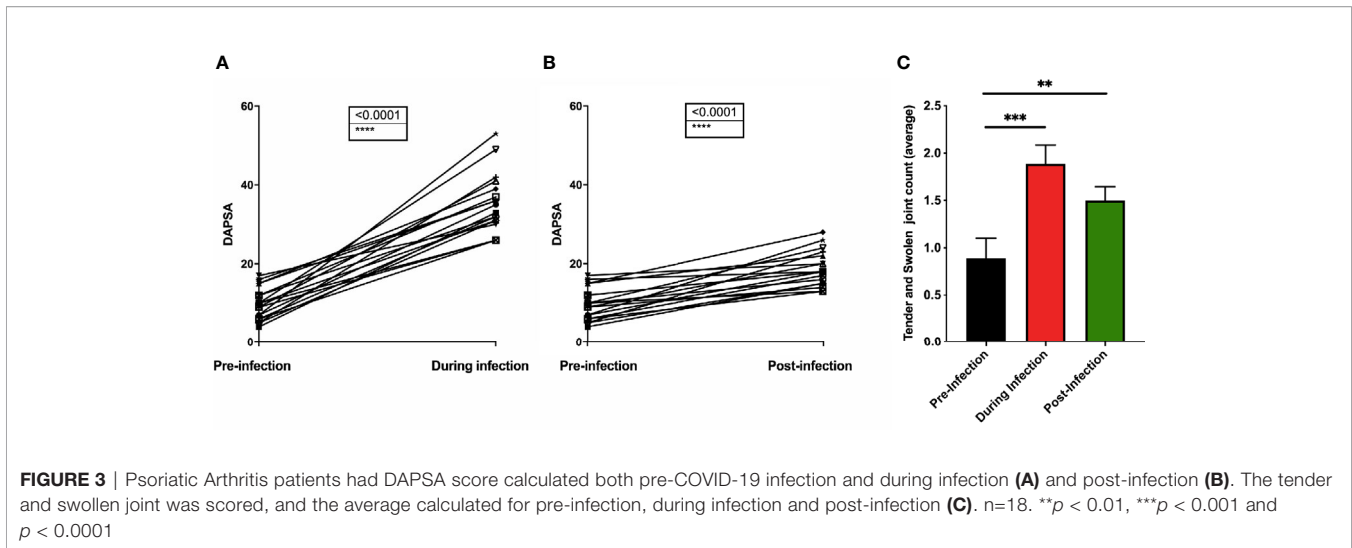


FIGURE 2 | (A) Heatmap showing clustering of PEB stimulated pDCs and PEB unstimulated pDCs. **(B)** Volcano map showing 11 significantly upregulated genes (red, $\text{Log}_2(\text{fold change}) > 1$ and $p < 0.05$) in stimulated vs unstimulated enthesal pDCs. Gene symbols were displayed if $|\text{Log}_2(\text{fold change})| > 1$. **(C)** PPI network of the 11 DEGs showing a main component consisted of 4 nodes and 4 isolated nodes. Lines connecting two nodes represent protein-protein associations. Pathway mapping analysis of pDCs generated by IPA revealed the toll-like receptor signaling **(D)** and JAK/STAT signaling **(E)** among the topmost canonical pathways enriched upon treatment with ODN. Stimulation of TLRs leads to activation of MYD88 and finally transcription of pro-inflammatory cytokines including TNF **(D)**. Activation of JAK leads to activation of STAT or PI3K pathway, which is predicted by IPA **(E)**. Prediction legend is shared by 2D and 2E. Red & pink represents upregulation and intensity represents the relative magnitude of change in gene expression. Predicted activation indicated by orange color and blue for the predicted inhibition. Direct and indirect interactions are indicated by solid and dashed lines, respectively.

and also provides a novel mechanistic link between infection, including viral triggers and SpA associated arthropathies. Numerical and functional abnormalities have already been

reported in other rheumatic diseases including systemic lupus erythematosus and rheumatoid arthritis but this is the first study to show resident pDCs at the normal entheses, which offers new



insights into how infectious triggers may precipitate SpA pathology (27, 28).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol of the current investigation was approved by North West-Greater Manchester West Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QZ, CB and DM had substantial contributions to study conception and design. QZ, JV, YE-S, CB, and DM had

substantial contributions to analysis and interpretation of data. All the authors had substantial contributions to acquisition of data, drafting the article or revising it critically for important intellectual content and final approval of the version of the article to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.635018/full#supplementary-material>

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