Ann Rheum Dis 84 (2025) 1995–2007

Contents lists available at ScienceDirect

Annals of the Rheumatic Diseases

journal homepage: https://www.sciencedirect.com/journal/annals-of-the-rheumatic-diseases

Axial spondyloarthritis

Healthy human enthesis stromal cells mediate immunoregulation via the CD39/CD73 adenosine ectonucleotidase pathway

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ARTICLE INFO

Article history:
Received 20 May 2025
Received in revised form 1 September 2025
Accepted 2 September 2025

Handling editor Josef S. Smolen

ABSTRACT

Objectives: Entheseal inflammation (ligament and tendon insertions) and subclinical intestinal inflammation are both hallmarks of the seronegative spondyloarthropathy (SpA) diseases. While regulatory T cells (Tregs) are key to intestinal homeostasis, entheseal homeostatic mechanisms are poorly understood in humans.

Methods: Single-cell RNA sequencing of entheseal tissue, comparative transcriptomic analysis with intestinal tissue datasets, and functional analysis of entheseal stromal populations were undertaken. Functional immunomodulation by entheseal mesenchymal stromal cells (MSCs) was evaluated via coculture with activated T cells. CD39/CD73 pathway involvement was confirmed using pharmacological inhibition and transcriptional profiling.

Results: Single-cell RNA sequencing of 27,348 entheseal cells revealed a lower frequency of Tregs in the T cells of the enthesis (2.60% ± 0.36%) compared to those of the ileum (7.37% ± 4.47%) and colon (18% ± 8.59%). We found that entheseal fibroblasts expressed key immunomodulatory markers, including CD39 (*ENTPD1*) and CD73 (*NT5E*), which were further upregulated upon coculture with activated T cells. Entheseal MSCs significantly suppressed T cell proliferation (up to 89%, $P < .0001$) in an adenosine-dependent manner. Transcriptional profiling revealed that entheseal MSC cocultured with activated T cells upregulated genes of known functional importance in Treg, including *IL10*, *IDO1*, *PTGS2*, *HLA-G*, and *CD274*. In this assay, dual CD39/CD73 inhibition restored T cell proliferation by ~48% ($P = .0004$), confirming that entheseal MSC-mediated immunomodulation acts in part through an adenosine-mediated mechanism.

Conclusions: The normal spinal enthesis harbours an immunoregulatory cellular environment related to entheseal MSCs that utilises the CD39/CD73 adenosine ectonucleotidase axis that may help maintain local immune homeostasis, while the same adenosine ectonucleotidase immunoregulatory pathway is likely dependent on Treg function in the intestine. This has broad implications for understanding the cellular bases of immune dysregulation in the gut-joint axis and could help guide tissue-specific therapy in SpA.

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https://doi.org/10.1016/j.ard.2025.09.001

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Entheseal inflammation and subclinical intestinal inflammation are both hallmarks of the seronegative spondyloarthropathy diseases.
- The intestine is a site rich in immune cells, whereas entheseal soft tissue anchorage sites are mostly composed of fibroblasts and are subject to high biophysical stress.
- How immune regulation occurs at the enthesis is poorly understood.

WHAT THIS STUDY ADDS

- We built a single-cell RNA sequencing atlas of human entheseal tissue. We found that the healthy human enthesis has fewer regulatory T cells than are present in healthy human intestinal tissue.
- We showed that entheseal fibroblasts are capable of robustly inhibiting T cell proliferative responses. Native entheseal fibroblasts robustly expressed CD39 (*ENTPD1*) and CD73 (*NT5E*), which mediate the adenosine ectonucleotidase immunoregulatory pathways—a key element of Treg immunomodulation.
- Entheseal fibroblast cocultured with activated T cells further upregulated CD39 (*ENTPD1*) and CD73 (*NT5E*), and pharmacological inhibition of these factors significantly reduced immunomodulation.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The normal spinal enthesis harbours a unique immunoregulatory cellular environment that is distinct from the intestine.
- Entheseal fibroblasts utilise molecular immunoregulatory mechanisms that are shared with regulatory T cells, and some of these could be tractable for future development of therapy for enthesitis.

INTRODUCTION

The seronegative spondyloarthritis (SpA) family constitutes a cluster of inflammatory diseases predominantly afflicting musculoskeletal structures, skin, eye, and the intestine. While substantial progress has been made in unravelling the cellular make-up and the pathogenic mechanisms within the skin and intestine [1,2], the immune dynamics of the enthesis—a central site of musculoskeletal inflammation in SpA—remain largely uncharted [3]. Of particular note, subclinical intestinal inflammation is common in SpA, but there is a therapeutic disconnect between drug efficacy in the intestinal and axial skeleton. In the axial skeleton, interleukin (IL)-17 antagonism is effective but may simultaneously be associated with nonefficacy or even the precipitation of intestinal inflammation [4–8]. This suggests that fundamental differences may exist between normal spinal and intestinal immunity in SpA.

The healthy human spinal enthesis contains fibroblasts also known as mesenchymal stromal cells (MSCs) due to the ability of such cells to differentiate into osteoblast, adipocyte and chondrocyte lineage cells (ref our MSC enthesis paper). Furthermore, myeloid cells, conventional T cells, and various innate immune cell populations, including $\gamma\delta$ T cells and innate lymphoid cells (ILCs), have been reported [9]. Given that entheses are sites of high biophysical stress, microdamage and microinflammation, identification of the cellular mechanism(s) that govern normal entheseal immunoregulation and immune homeostasis is of central importance for understanding the etiopathology and informing the targeted treatment of human SpA [10].

Regulatory T cells (Tregs) are important for peripheral tolerance in many tissues with their absence in the gut resulting in potentially fatal neonatal colitis [11]. Moreover, it is noteworthy that subclinical colitis and dysbiosis frequently occur in SpA spectrum diseases [7,12]. The basis for the presence of both entheseal and intestinal inflammation in SpA spectrum disorders remains incompletely understood. The reason why intestinal inflammation typically remains subclinical in SpA, in contrast to that of axial and peripheral joints, is unknown. If a common disease process is assumed, it is possible that, commensurate with it being a barrier site, the gut harbours different immunomodulatory mechanisms than are found in healthy entheses, which are sterile tissues.

In a previous report, using bulk RNA-seq and cytometric analysis, we noted a paucity of Tregs in the normal soft tissue and bony anchorage of the spinal enthesis [9], suggesting that other cell types may provide immune tolerance and tissue homeostasis. One of the key shared pathways between Tregs and stromal cells for mediating immunosuppression is the CD73/CD39 adenosine pathway [13–15]. This mechanism relies on the enzymatic actions of CD39 (ectonucleoside triphosphate diphosphohydrolase 1 [E-NTPDase1]) and CD73 (ecto-5′- nucleotidase [Ecto5′Ntase]). CD39 catalyses the hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into 5′-adenosine monophosphate, which is subsequently converted into adenosine by CD73 [15]. This process plays a critical role in immune regulation by producing adenosine, a potent immunosuppressive molecule [16,17]. Notably, both entheseal soft tissue MSCs (EST-MSCs) and perientheseal bone MSCs (PEB-MSCs) are known to exhibit surface expression of CD73 [18].

Here, we generated the first single-cell atlas of the human spinal enthesis to assess the presence of immunomodulatory cell populations in the soft tissue and bony anchorage of the enthesis. We propose that the reduced frequency of Tregs in the healthy spinal enthesis compared to intestinal tissues is compensated for by shared specialised regulatory functions of entheseal MSCs. In keeping with this concept, we demonstrate that entheseal MSCs are capable of suppressing activated T cells via the adenosine pathway.

METHODS

Approval was obtained from the Northwest-Greater Manchester West Research Ethics Committee (16/NW/0797). Tissue was obtained from patients undergoing elective spinal surgery for either scoliosis correction or lumbar decompression. Samples included interspinous ligament enthesis and adjacent bone anchorage tissue from regions macroscopically free of inflammation or gross pathology. The interspinous ligament is anatomically distant from primary degenerative or deformity-related changes and is not typically involved in these disease processes. Full details of sample processing and generation of single-cell data using the 10× Genomics Chromium platform are described in the Supplementary Methods. Single-cell data analysis was processed using cellhub pipelines (https://github.com/sansomlab/cellhub) and the downstream computational analyses performed as described in the Supplementary Methods. T cell assays, immunoassay, cytokine measurements, bulk RNA identities sequencing, CD73/CD39 antagonism assays, and statistical analyses were performed as described in the Supplementary Methods.

RESULTS

Generation of a single-cell atlas of human spinal enthesis

We used single-cell RNA sequencing to investigate the cellular basis of immunomodulation in the enthesis (Fig 1A). After quality control (see Methods), we clustered 27,348 single-cell transcriptomes from 4 paired peri-entheseal bone (PEB) and entheseal soft tissue (EST samples, identifying 17 transcriptionally distinct immune cell subsets (Fig 1B,C). These comprised myeloid cells (including progenitors, mature neutrophils, and monocytes), B cells at different stages of differentiation, T cells, erythroid cells including precursors and MSCs (Supplementary Fig S1B,C). The presence of differentiating B and myeloid cells, along with nucleated erythroid cells, is likely attributable to the inclusion of bone marrow in the digested samples.

Comparative composition analysis of the EST and PEB samples showed that most cell subsets were present at both anatomical sites, with the exception of endothelial cells and fibroblasts, which were found almost exclusively in the soft tissue entheseal region (Fig 1D, Supplementary Fig S1A). This shift in MSC composition aligns with known ligamentous soft tissue structure, where mechanical fibroblast lineage cells are more abundant compared to mineralised zones and the underlying trabecular regions that are rich in marrow lineage cells but have a paucity of fibroblasts. Conversely, plasma cells, early B cells (pre-B and pro-B cells) and erythroid cells were more abundant in the bone-associated region (reflecting the presence of bone marrow tissue in the sampled enthesis bony anchorage region).

Extraction and reanalysis of myeloid cells revealed the presence of distinct monocyte and neutrophils clusters (Fig 1D), arranged along a continuum suggestive of a potential differentiation trajectory (Supplementary Fig S1D). These led to (i) 2 *VCAN* and *S100A4* expressing monocyte populations (monocyte 1,2) and (ii) 3 subsets of neutrophils, which were characterised by expression of S100A8/9 cells (neutrophils 1) and *CD24* and *LCN2* (neutrophils 2 and 3). The higher-resolution clustering of the myeloid space also allowed the separation of common myeloid progenitors (characterised by *ELANE*, *MPO*), pro-myelocytes (*DEFA3*, *CD24*) and mast cells (*CPA3*, *HDC)* (Fig 1D and Supplementary Fig S1E). To investigate which myeloid cell populations might contribute to cytokine production in the enthesis under inflammatory conditions such as SpA, we examined the expression of known inflammatory mediators (Fig. 1C). Tumour necrosis factor (*TNF)*, *IL1B,* and *IL6* expression were found predominantly in mature monocytes. Neutrophils, in contrast, were the principal myeloid source of *CXCL8* (encoding IL-8) in the healthy spinal enthesis, as previously observed [19], while *MIF*, an emerging inflammatory mediator in SpA [20], was broadly expressed by monocytic subsets (Fig 1E). Lastly, *PTGS2*, encoding COX-2, was found in both mature neutrophils and monocytes.

T cell diversity and reduced Tregs in the spinal enthesis

To characterise the entheseal T and natural killer (NK) cell populations, we extracted and reanalysed these cells separately, adopting a bespoke strategy to integrate T cells from the bone and soft tissue (see Methods, Supplementary Fig S2A). We identified 11 T cell and NK subpopulations, including rare, specialised lymphocyte subsets previously described in entheseal studies, such as $\gamma\delta$ T cells, ILCs, and tissue-resident memory cells (Fig 2A,B; Supplementary Fig S2B).

A small population of Tregs was also detected (Fig 2A-C). Given our previous finding of a paucity of conventional (CD4 + CD25 + FOXP3 +) Tregs in the spinal enthesis [9], we compared the immunomodulatory phenotype and frequency of cells from the enthesis with those identified in a aingle-cell RNA sequencing (scRNA-seq) study of the intestine [2], another site of inflammatory involvement in SpA [21] (Fig 2D,E).

At the transcriptional level, entheseal and intestinal Tregs showed similar expression of *FOXP3*, *TIGIT*, *IL2RA*, and *CTLA4*. *IKZF2*, which encodes Helios, was detected in a much larger fraction of entheseal Tregs, indicating that a greater proportion of entheseal Tregs were thymic-derived, while the lower levels of *IKZF2* in the intestinal Treg population were consistent with the large numbers of peripherally induced Treg known to be present in the gut [22] (Fig 2D). Entheseal Tregs also showed more frequent expression of *TGFB1* [23]. Within the total T cell population, Tregs were present at a lower frequency in the T cell population of the healthy enthesis (2.60% ± 0.36%) compared to that of the healthy ileum (7.37% ± 4.47%) and healthy colon (18% ± 8.59%) (Fig 2E). For an annotation-free assessment of the regulatory potential of T cell in the enthesis, we scored the expression of Treg 'signature genes' in all T cells from the enthesis and intestine [24]. While most T cells in both tissues showed low regulatory scores, a subset of healthy intestinal T cells had high Treg signature scores (>0.5). In contrast, no T cells from the healthy enthesis showed high Treg signature scores (Supplementary Fig S2C). The entheseal T cell populations showed a lower cytotoxicity scores than T cells from the gut [25], potentially explaining the reduced frequency of Tregs present in the enthesis (Supplementary Fig S2C). To help confirm our findings, we also examined T cells from a murine enthesis dataset [26]. This analysis was limited by low T cell numbers, but consistent with our observations of low Treg frequency in the human enthesis, we did not detect Foxp3 messenger RNA (mRNA) or identify any cell predicted to be a Treg by automated annotation.

We hypothesised that the paucity of Tregs (and absence of T cells with high Treg signature scores) in the stromal compartment of the enthesis, compared to the intestine, might reflect the differing immunomodulatory requirements at these sites. Unlike the enthesis, the gut is a barrier site in which cytotoxic lymphocytes are important for host defence. Intestinal Tregs are essential for maintaining homeostasis and orchestrating tolerogenic responses to the microbiota [27,28]. In contrast, we reasoned that, at the enthesis, immunomodulation of inflammatory signals resulting from sterile physical stress and microdamage may rely on MSCs to maintain immune homeostasis rather than Tregs.

Enthesis-derived MSCs suppress T cell proliferation and modulate cytokine levels in a dose-dependent manner

To evaluate the immunomodulatory potential of EST-MSCs and PEB-MSCs, a direct contact coculture assay was conducted. Stimulated T cells were cocultured with EST-MSCs or PEB-MSCs at ratios of 1:1, 1:2, 1:4, and 1:8 (MSCs to stimulated T cells) for 5 days. Unstimulated T cells were negative controls, and stimulated T cells without MSCs were positive controls. On day 5 of coculture, T cells in suspension were collected from the supernatants and the suppression of their proliferation was assessed using flow cytometry, with gating on the CD45 + CD90−CD4 + CD25- population (Fig 3A,B). The proliferation index was then calculated and normalised to that of stimulated T cells. Coculturing with EST-MSCs and PEB-MSCs significantly

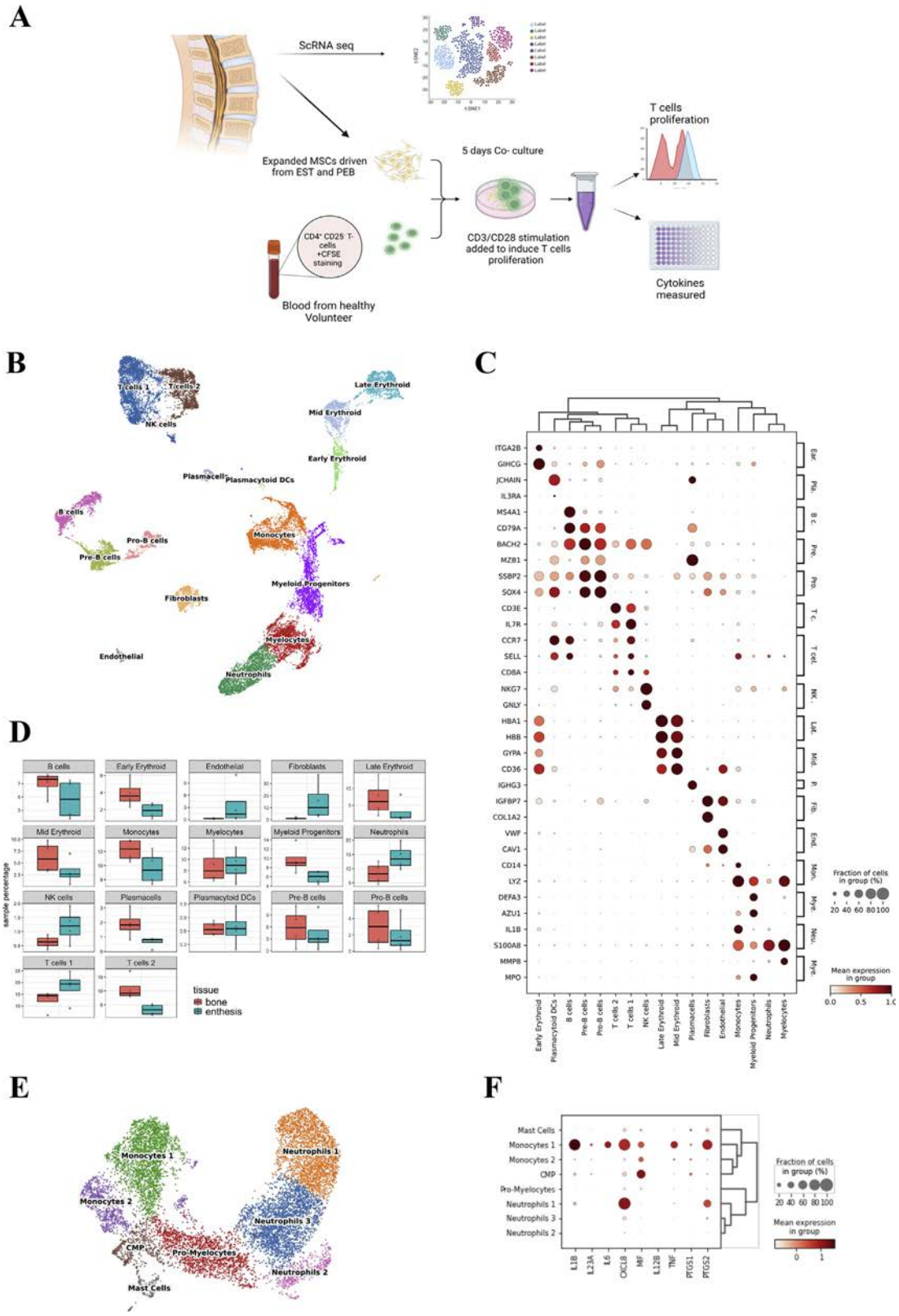

Figure 1. Single-cell atlas of the spinal enthesis: (A) schematic diagram outlining the study methodology. (B) The Uniform Manifold Approximation and Projection (UMAP) shows the annotated map of 27,348 single cells from peripheral enthesis bone (PEB) and entheseal soft tissue (EST). (C) Dot plot showing marker gene expression across identified cell clusters, with colour intensity indicating normalised expression and dot size representing the fraction of expressing cells. (D) The boxplots show the cellular composition by anatomical site within the enthesis complex. (E) The UMAP shows visualisation of myeloid cell subpopulations. (F) The dot plot shows the expression levels of key inflammatory mediators in the entheseal myeloid cell populations. CMP, common myeloid progenitor; MSC, mesenchymal stromal cell.

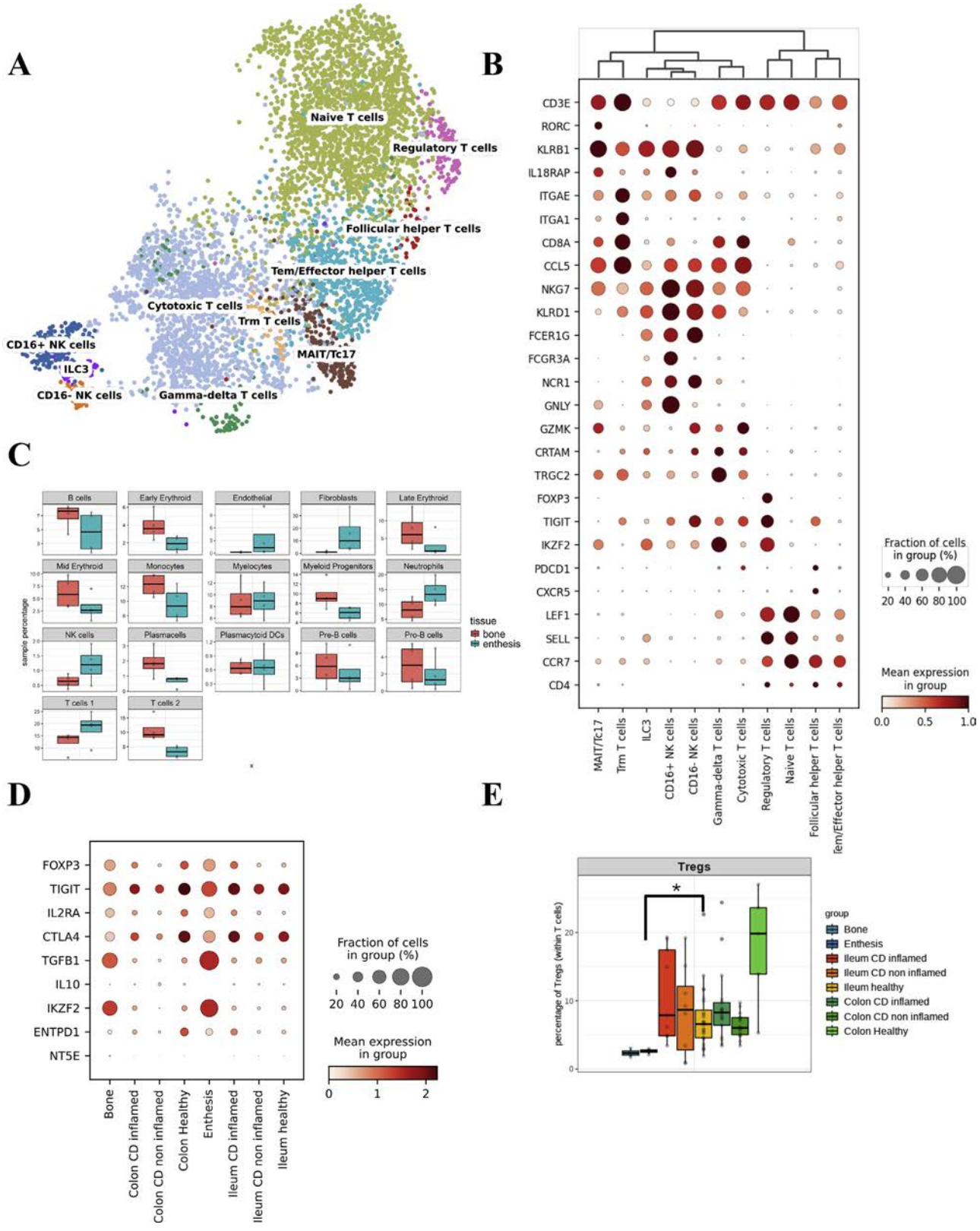

Figure 2. T cell subpopulations in the spinal enthesis: (A) UMAP visualisation of T cell transcriptomes from the spinal enthesis, showing distinct subpopulations. (B) Dot plot displaying marker gene expression across identified T cell clusters. (C) Cluster percentages across entheseal soft tissue and bone regions. (D) Regulatory marker expression in FOXP3+ regulatory T cells (Tregs) from enthesis tissue and intestinal sites (Crohn's disease and healthy controls). (E) Proportion of Tregs in total T cell populations across different anatomical sites (bone/enthesis n = 4, ileum n = 8-24, colon n = 5-17). Tregs were significantly lower in the enthesis compared to the intestine (Wilcoxon rank sum test, $P = .002$).

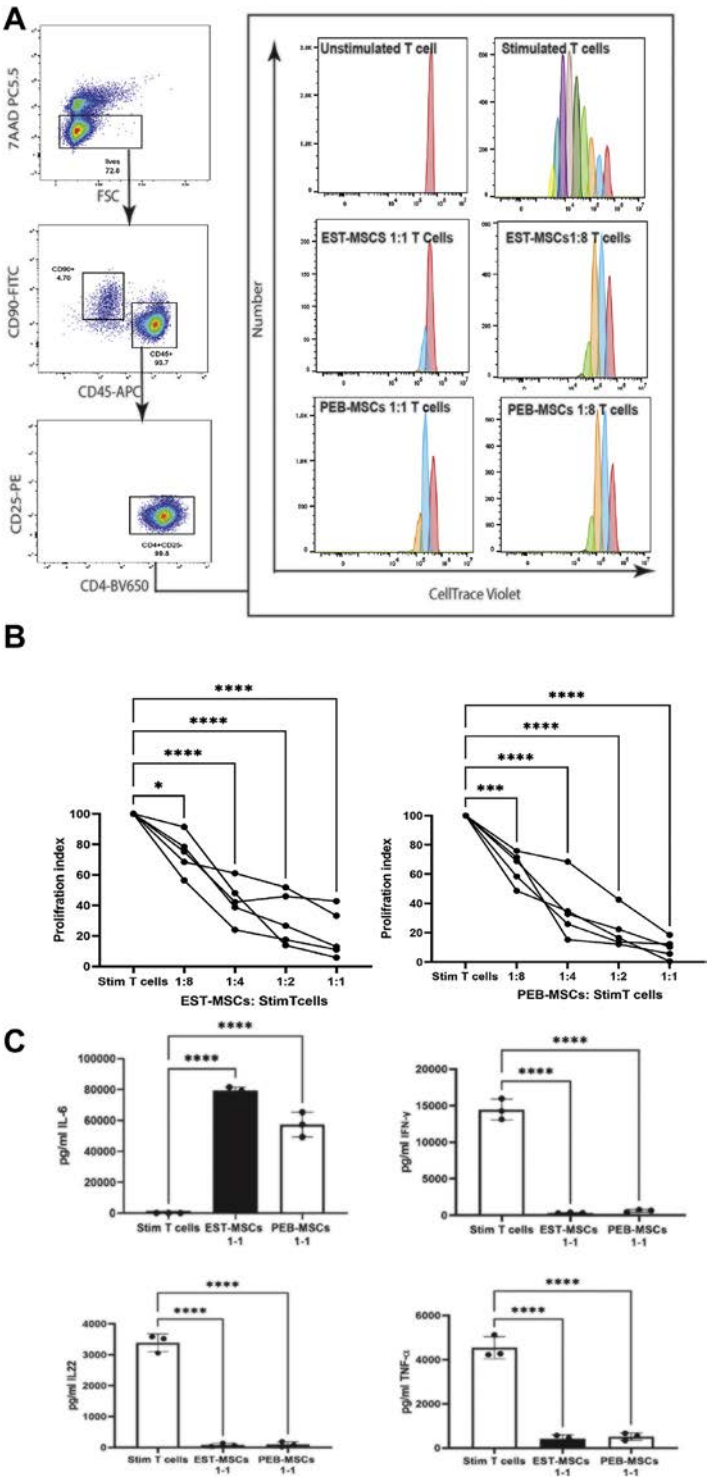

Figure 3. Immunomodulatory effects of EST-MSCs and PEB-MSCs on stimulated T cells: (A) T cells were cocultured with EST-MSCs or PEB-MSCs for 5 days, stained with CD90, CD45, CD4, CD25, and 7AAD for viability assessment. CD45 + CD90−CD4 + CD25−T cells were gated and analysed for proliferation. Histograms show proliferation suppression across increasing T cell ratios (n = 5). (B) Comparative analysis of T cell proliferation suppression by EST-MSCs (left) and PEB-MSCs (right) at varying ratios. Stimulated T cells alone serve as a positive control. (C) Cytokine levels (TNF-α, IFN-γ, IL-22, IL-6) in coculture supernatants were quantified (n = 3). Statistical significance was assessed using 1-way ANOVA ($P < .05$, $P < .001$, $P < .0001$). Error bars represent mean ± SD. FSC, forward scatter; ANOVA, analysis of variance; EST, entheseal soft tissue; IL, interleukin; IFN, interferon; MSC, mesenchymal stromal cell; TNF, tumour necrosis factor; PEB, peri-entheseal bone.

suppressed the proliferation of stimulated T cells. The most substantial suppression was observed at a 1:1 ratio of MSCs:T cells, with a proliferation index of 21.2% for EST-MSCs ($P < .0001$) and 9.5% for PEB-MSCs ($P < .0001$). As the MSCs:T cells ratio decreased, the proliferation index increased (Fig 3B): 31.2% ($P < .0001$), 42.9%($P < .0001$), and 74.1% ($P = .0205$) for EST-MSCs and 21.5% ($P < .0001$), 35.5% ($P < .0001$), and 64.7% ($P = .0006$) for PEB-MSCs at ratios of 1:2, 1:4, and 1:8, respectively. No significant difference was found between the abilities of EST-MSCs and PEB-MSCs to suppress T cell proliferation ($P = .746$), suggesting that EST-MSCs and PEB-MSCs suppressed T cell proliferation in a dose-dependent manner.

Additionally, cytokine levels were measured in the supernatants of cocultured cells at the 1:1 ratio, where the most significant suppression of T cell proliferation was observed. The T cell proliferation-related cytokines TNF-a, interferon-y, and IL-22 were significantly reduced with both EST-MSCs and PEB-MSCs ($P < .0001$). On the other hand, IL-6 was significantly increased at 1:1 ratio with both EST-MSCs and PEB-MSCs ($P < .0001$), consistent with its known abundant production by fibroblasts and also potentially its known immunoregulatory role in T cells [30] (Fig 3C).

Spinal enthesis-derived MSCs suppress T cell activation through CD39/CD73 axis

To explore the immunosuppressive mechanism mediated by spinal enthesis MSCs, we assessed the surface expression of the

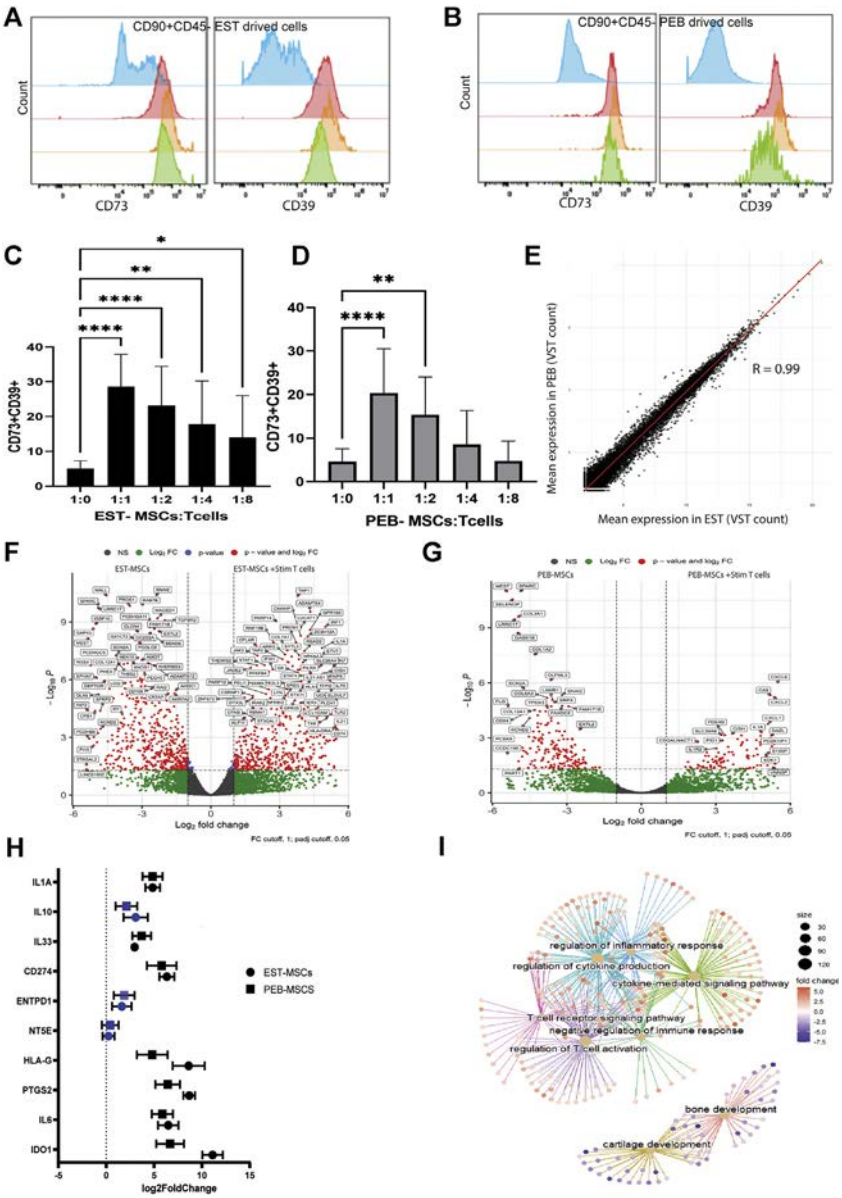

Figure 4. Mechanism of T cell Suppression by EST-MSCs and PEB-MSCs: (A, B) Flow cytometry histograms illustrating CD73 and CD39 expression on native CD90 + CD45− EST and PEB-MSCs. Blue histograms represent unstained controls, while red, orange, and green indicate stained cells (n = 3). (C, D) CD73 and CD39 expression on EST-MSCs and PEB-MSCs after coculture with stimulated T cells for 5 days at varying ratios (n = 5). Statistical significance assessed via 1-way ANOVA, with $P < .05$ considered significant. Error bars represent mean ± SD. (E) Pairwise correlation of average gene expression between EST and PEB-MSCs. Each point represents 1 gene. Pearson correlation coefficient (r > 0.99) (F, G) Volcano plots displaying differentially expressed genes in EST-MSCs and PEB-MSCs (n = 3) after coculture with stimulated T cells (1:1 ratio), compared to controls. (H) Log fold change of differentially expressed genes in cocultured EST-MSCs and PEB-MSCs with stimulated T cells, compared to MSCs without inhibitors (n = 3). (I) Cnet plot illustrating upregulated and downregulated pathways via gene set enrichment analysis (GSEA). Expression intensity is colour-coded (blue = upregulated, red = downregulated) relative to controls (MSCs only). VST, variance stabilizing transformation; ANOVA, analysis of variance; EST, entheseal soft; MSC, mesenchymal stromal cell; TNF, tumour necrosis factor; PEB, peri-entheseal bone.

CD39/CD73 axis markers on resting entheseal MSCs and on MSCs expanded in culture after their coculture with stimulated T cells.

Cells derived from uncultured CD90 + CD45− EST and PEB showed expression of CD73 and CD39 (Fig 4A,B). A significant difference was observed in the coexpression of CD73 + CD39 between EST CD90 + CD45− cells and PEB CD90 + CD45− cells, with proportions of 2.08 and 51.93, respectively ($P = .0143$) (Supplementary Fig S3). We next investigated whether the expression of CD39 and CD73 by entheseal MSCs could be induced by stimulated T cells. Coculture of EST-MSCs and PEB-MSCs with stimulated T cells led to an elevation in the expression levels of CD39 and CD73, with the highest expression observed at a 1:1 ratio compared to the negative control (MSCs without T cells) ($P < .0001$) (Fig 4C,D). In keeping with their comparable immunosuppressive function, the transcriptomes of EST and PEB-MSCs were highly similar, showing a Pearson correlation of r > 0.99 (Fig 4E), and a differential expression analysis (DESeq2, Benjamini−Hochberg adjusted $P < .05$, $\log_2$ fold change > 1) identified no genes with significant differential expression between the 2 populations (Supplementary Fig S3B).

To further delineate the mechanism of T cell proliferation suppression, we performed bulk RNA-seq profiling of cocultures of spinal enthesis MSCs and T cells. MSCs cocultured with stimulated T cells had a significantly different transcriptomic profile to the MSCs with no coculture. In EST-MSCs, coculture with stimulated T cells upregulated 390 and downregulated 306 genes (DESeq2, *P*adj < .05, |log2FoldChange| > 2) (Fig 4F; Supplementary File S1). Key significantly upregulated transcripts included those encoding *IDO1* (11-fold, *P*adj < .0001), *PTGS2* (8.7-fold, *P*adj < .0001), *IL1A* (4.9-fold, *P*adj < .0001), *IL6* (6.5-fold, *P*adj < .0001), and *IL33* (3.0-fold *P*adj < .0001). While these latter cytokines are well known for their proinflammatory functions, their expression in both fibroblasts and MSCs has also been associated with context-specific immunoregulatory effects, including modulation of T cell responses and support of immune homeostasis [29,30]. Immune checkpoint regulators such as *HLA-G* (8.6-fold, *P*adj < .0001) and CD274 (6.3-fold, *P*adj < .0001) were also significantly upregulated. Transcripts from the genes encoding CD39 and CD73 (*ENTPD1* and *NT5E*) showed small, nonsignificant increases compared to MSCs with no coculture (Fig 4H).

Table
Functional enrichment analysis results using gene set enrichment analysis

		EST-MSCs		PEB-MSCs	
		EST		PEB	
ID	Description	NES	*P*adj	NES	*P*adj
GO:0050727	Regulation of inflammatory response	1.869168673	1.36834E−06	1.790646268	1.19929E−05
GO:0019221	Cytokine-mediated signalling pathway	2.495508595	1E−10	2.568040444	1E−10
GO:0050863	Regulation of T cell activation	2.374466468	1E−10	2.457049726	1E−10
GO:0060348	Bone development	−1.957946691	1.63464E−06	−1.815266647	4.20194E−05
GO:0051216	Cartilage development	−1.920879981	5.70038E−06	−1.924765499	9.65674E−06

EST, entheseal soft; MSC, mesenchymal stromal cell; PEB, peri-entheseal bone.
This table presents the identified upregulated and downregulated pathways obtained from gene enrichment set analysis, including the normalised enrichment score (NES) and corresponding *P*adj.

In PEB-MSCs, 90 transcripts were upregulated and 119 were downregulated (Fig 4G; Supplementary File S2). Similar trends were observed, with significant upregulation of IDO1 (6.7-fold, *P*adj < .0001), PTGS2 (6.4-fold, *P*adj < .00001), IL1A (4.8-fold, *P*adj < .0001), IL6 (5.9-fold, *P*adj < .0001), and CD274 (5.7-fold, *P*adj < .05). As for the PEB-MSCs, *ENTPD1* and *NT5E* showed small, nonsignificant increases (Fig 4H).

Gene set enrichment analysis revealed shared enrichment in key immunosuppressive pathways in both MSC types. These included 'regulation of inflammatory response' (normalised enrichment score [NES] = 1.87, *P*adj < .0001 for EST-MSCs, NES = 1.79, *P*adj < .0001 for PEB-MSCs), 'Cytokine-mediated signalling' (NES = 2.49 *P*adj < .0001 for EST-MSCs, 2.56 *P*adj < .0001 for PEB-MSCs), and 'Regulation of T cell activation' (NES = 2.37 *P*adj < .0001 for EST-MSCs, 2. *P*adj < .0001 for PEB-MSCs) (Table). Downregulated pathways included 'Cartilage development' (NES = −1.92 for both MSC types) and 'Bone development' (NES = −1.95 *P*adj < .0001 for EST-MSCs, −1.81 *P*adj < .0001 for PEB-MSCs), indicating a shift from differentiation to immunoregulation (Fig 4I, Table).

Several genes known to be upregulated in *in vitro* Treg suppression assays were also significantly increased in EST-MSCs and (2.1-fold, *P*adj = .008) in PEB-MSCs) including *IDO1, PTGS2,* CD274, *IL10,* and *HLA-G* in addition to *IL33* (3.0-fold, *P*adj < .00001), further highlighting the role of these MSCs in immunomodulation associated with Treg function (Fig 4G).

Taken together, these findings confirm that MSCs from both EST and PEB upregulate multiple transcripts associated with Treg-mediated suppression when cocultured with activated T-cells, with EST-MSCs showing consistently higher expression levels.

Entheseal ENTPD1 derived immunoregulatory MSCs express CXCL12

To identify whether native entheseal MSCs exhibited regulatory functions, we performed a separate analysis of these from our single-cell atlas, which predominantly originated from the soft tissue entheseal portion of each sample, consistent with the higher cellularity of this location. High-resolution clustering revealed 7 groups, including tendon cells (TMND+ fibroblasts) and myofibroblasts (*MYL9*, *ACTA2*) (Fig 5A,B). Among the remaining clusters, we hypothesised that the CXCL12+ cells, expressing *ENTPD1* and *LEPR* [31] (Fig 5C), and at least in part *NGFR* (encoding CD271 that is a marker for native MSCs), likely contained cells with immunoregulatory transcript expression. CXCL12+ cells were enriched in the bone marrow entheseal anchorage locations compared to soft tissue, in keeping with the known role of CXCL12 (which encodes for SDF-1) in regulating haematopoiesis (Fig 5D). Conversely, *NT5E* expression was more diffuse across various MSC cell subsets. Given that contact with activated T cells upregulates these markers (Fig 4C,D), it is plausible that more than 1 subtype of MSCs may be involved in maintaining entheseal integrity and promoting immune homeostasis. To help confirm our findings, we examined 2 publicly available single-cell RNA-seq datasets from murine entheseal tissue [26,32]. While we did not find an identical counterpart to the human CXCL12+ regulatory MSCs, both *Nt5e* (CD73) and *Entpd1* (CD39) were expressed in the adult murine entheseal samples from both datasets (Supplementary Fig S4). Together, these data support the conclusion that fibroblast populations capable of immunomodulatory functions are conserved in human and mice entheses. While TNMD+ SCX+ MKX+ cells are canonically associated with tendon/ligament, their enrichment in PEB-derived clusters may reflect either true anatomical extension into adjacent bone as small entheses have a relatively paucity of fibrocartilage compared to large ones, thus making microanatomical dissection technically challenging and resulting in partial tissue overlap during dissection. Other hitherto undefined factors may be involved, but this limitation does not affect our broader immunoregulatory findings, which were validated in anatomically separated cultures. Future studies incorporating spatial validation techniques such as immunohistochemistry (IHC) or Visium-based transcriptomics are warranted to map the tissue distribution of tenogenic and stromal subsets with greater anatomical resolution. Further, in one of the samples, we noted the presence of a cluster of cells that co-expressed *Entpd1*, *Nt5e*, *Cxcl12*, and *Tgfb1* (GSE182997 cluster 2; Supplementary Fig S4). Together, these data support the conclusion that fibroblast populations capable of immunomodulatory functions are conserved in human and mice entheses.

MSC-mediated adenosine pathway involvement modulates T cell proliferation

Given that CD73 and CD39 were both expressed by EST-MSCs and PEB, we tested the capacity of entheseal MSCs to mediate immunomodulation via the adenosine pathway. EST-MSCs and PEB-MSCs were treated with specific inhibitors targeting CD73 and CD39 (APCP and POM-1, respectively). These cells were subsequently cocultured with stimulated T cells at both 1:1 and 1:4 ratios, and single and double blockage experiments were conducted, and the impact on the suppression of T cell proliferation was determined at day 5.

To verify adenosine production and inhibition, we quantified the total adenosine production in the supernatant and observed

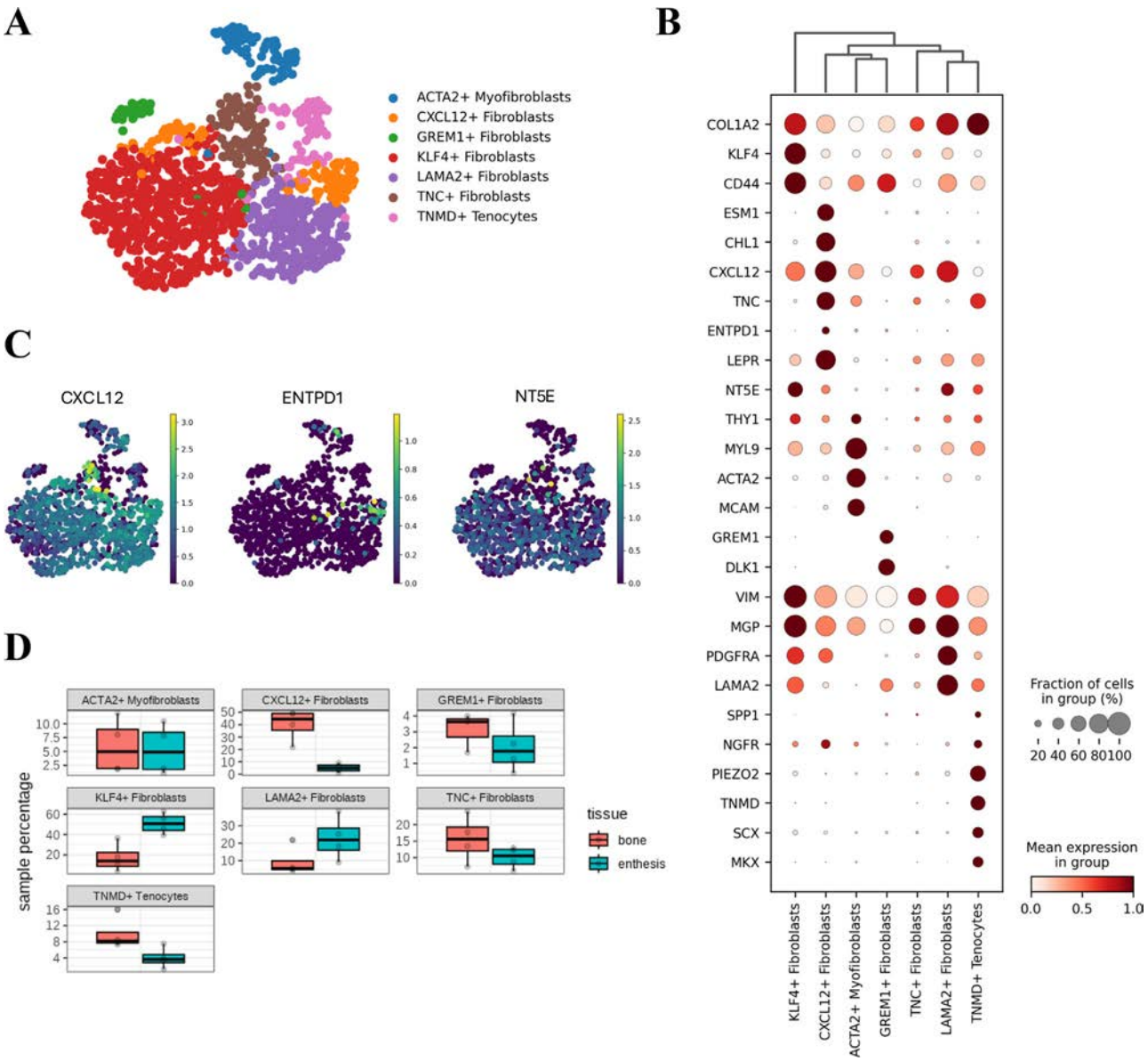

Figure 5. Stromal cells in the enthesis express CXCL12 and ENTPD1: (A) UMAP visualisation of stromal cells from the spinal enthesis, displaying distinct cellular clusters. (B) Dot plot showing marker gene expression across identified stromal cell subsets. (C) Expression of markers of CXCL12+ fibroblasts. (D) Cluster percentages across tissue sites.

a significantly increased adenosine production at a 1:1 coculture compared to MSCs alone ($P < .0001$) (Fig 6A). However, the blockade of CD39 and/or CD73 on MSCs cocultured with stimulated T cells substantially reduced adenosine production ($P < .0001$) compared to untreated cocultured MSCs.

Next, we assessed the impact of adenosine inhibition on the ability of MSCs to control the proliferation of stimulated T cells. We observed an increase in the proliferation index of T cells by 18% ($P = .2934$) and 35% ($P = .2934$) with EST-MSCs and PEB-MSCs pretreated with the CD73 blocker APCP. Similarly, at a 1:1 ratio, the proliferation increased by 12% ($P = .8397$) and 26% ($P = .8397$) with EST-MSCs and PEB-MSCs pretreated with the CD39 blocker POM-1, compared to cocultured T cells with untreated MSCs. Dual blockade of CD39 and CD73 led to a 47% and 48% increase in T cell proliferation with EST-MSCs ($P = .0004$) and PEB-MSCs ($P = .0004$) (Fig 6B,C). Similar comparisons were made at a 1:4 ratio, for which the pretreatment of EST-MSCs and PEB-MSCs with APCP increased T cell proliferation by 29% ($P = .3542$) and 20% ($P = .3542$), respectively. Likewise, T cell proliferation increased by 39% and 20% following POM-1 treatment with EST-MSCs and PEB-MSCs, respectively ($P = .1639$). However, pretreatment of EST-MSCs and PEB-MSCs with both APCP and POM-1 resulted in the loss of the ability to suppress T cell proliferation, as evidenced by a 90% and 87% increase in the proliferation index of T cells cocultured with EST-MSC and PEB-MSCs, respectively ($P < .0001$) compared to cocultured T cells with untreated MSCs (Fig 6B,C).

To explore the effects of adenosine pathway inhibition on MSC-mediated immunoregulation, we performed bulk RNA-seq on EST- and PEB-derived enthesis MSC cocultured with activated T cells in the presence or absence of CD73 and CD39 inhibitors (APCP and POM-1, respectively).

To visualise transcriptomic changes following dual CD39/CD73 blockade, we generated a volcano plot comparing EST-MSCs/PEB-MSCs cocultured with activated T cells ± APCP and POM-1 combined (Fig 6D). Several immunomodulatory transcripts of interest—including *IDO1, HLA-G, IL1A, IL6, PTGS2, IL10, NT5E, ENTPD1,* and *CD274*—were consistently

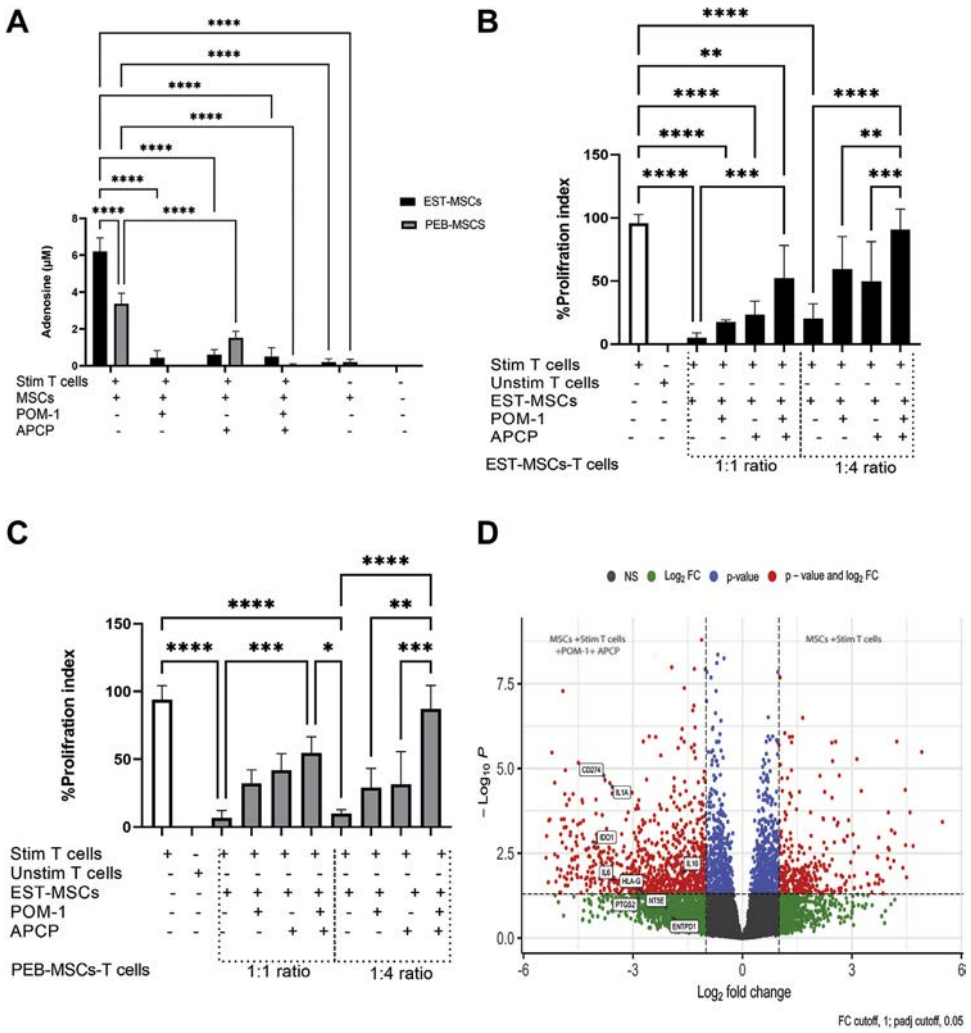

Figure 6. Impact of adenosine pathway inhibition on MSC-mediated immunosuppression of activated T cells: (A) total adenosine levels in supernatants from EST-MSCs and PEB-MSCs pretreated with CD73 inhibitor (APCP) and CD39 inhibitor (POM-1), followed by coculture with stimulated T cells at varying ratios (n = 3). (B, C) T cell proliferation index after coculture with EST-MSCs and PEB-MSCs pretreated with 50 μM APCP and 100 μM POM-1 at 1:1 and 1:4 ratios (n = 5). (D) Volcano plot showing differentially expressed genes in MSCs following coculture with activated T cells versus MSCs treated with APCP and POM-1. Red dots represent significantly upregulated genes (adjusted $P < .05$; $|\log_2 FC| > 1$) (n = 3). EST, entheseal soft; MSC, mesenchymal stromal cell; PEB, peri-entheseal bone; POM-1, sodium polyoxotungstate; APCP, α, ß-methylene ADP.

downregulated with fold changes exceeding the $\log_2$(FC) threshold of −1. MSCs, transcripts were downregulated following inhibition including *IDO1* (−4.07 fold, *P*adj = .002), *PTGS2* (-2.8 fold, *P*adj = .05), *IL1A* (−3.6-fold, *P*adj < .0001), *IL6* (−3.4fold, *P*adj < .05), *HLA-G* (−2.6 fold, *P*adj < .05), and *CD274* (−3.7-fold, *P*adj < .0001) (Supplementary File S3 and Supplementary Fig S3C).

DISCUSSION

The enthesis is a biomechanically dynamic interface between tendons or ligaments and bone. It constantly undergoes microdamage and repair and is a key site of pathology in SpA [33,34]. The existence of a gut-joint and a gut-enthesis axis in SpA is also well-recognised but the immunological mechanisms underlying shared entheseal and intestinal immune homeostasis remain poorly understood [35]. This study provides the first detailed single-cell atlas of healthy human spinal enthesis, offering new insights into its cellular architecture and immunoregulatory dynamics. A key finding from our study was the paucity of FOXP3+ Tregs that we observed in the enthesis relative to the gut [2,9]. This low entheseal Treg presence contrasts with the robust immunosuppressive environment maintained at the enthesis, implicating MSCs as the key contributors to local immune regulation, a function that complements, rather than replaces, canonical Treg-mediated tolerance seen in other tissues. Given that MSCs are especially abundant in the EST adjacent to fibrocartilage in the soft tissue side of the enthesis further supports a key role of this pathway at that location. By leveraging multiple pathways, including the CD73/CD39 adenosine axis, which is also operative in stromal cells from other tissues, MSCs appear to be to contribute to the regulation of immune responses in the enthesis, potentially compensating for the paucity of entheseal Tregs in health, ensuring immune homeostasis in this mechanically stressed tissue [17,18]. Nevertheless, it is plausible that the relative absence of Tregs at entheseal sites may help to explain their susceptibility to inflammation and damage in SpA. It is tempting to speculate that the immunosuppressive capacity of MSCs can be overcome by the infiltration of 'arthritogenic' self-reactive T cells into these normally sterile tissues [36].

The CD73/CD39 adenosine axis plays a vital role in MSC-mediated immunoregulation [14]. This pathway catalyses the conversion of extracellular ATP and ADP, released during tissue

stress or injury, into immunosuppressive adenosine [17,37]. Our findings demonstrate that both EST-MSCs and PEB-MSCs express CD73 and CD39, with further upregulation observed in the presence of activated T cells. While *ENTPD1* (CD39) and *NT5E* (CD73) transcripts showed only modest, statistically insignificant, changes compared to MSCs without coculture, the increased surface protein levels (Fig 4C) suggested that the surface levels of these factors are regulated post-transcriptionally. The translation efficiency and stability of CD39/CD73 proteins may be modulated independently of mRNA levels, leading to increased surface expression [38]. Another possibility is intracellular trafficking, where CD39/CD73 might have an intracellular reservoir that is trafficked to the membrane under coculture conditions, contributing to elevated surface levels [39]. Additionally, protein recycling and shedding could be involved, with CD39/CD73 undergoing rapid cycling between intracellular compartments and the plasma membrane. Additionally, a reduction in protein turnover could lead to accumulation on the surface [40]. This dynamic response underscores the adaptive capacity of MSCs to modulate inflammation in response to local signals. However, in SpA, this pathway may become dysregulated or overwhelmed by chronic inflammation and elevated levels of proinflammatory cytokines [12,14].

Consistent with a role of this pathway in immunosuppression, inhibition of CD73/CD39 led to a significant reduction in adenosine levels, a 50% increase in T cell proliferation, and downregulation of key immunosuppressive transcripts, including *IDO1*, *PTGS2*, *IL10*, and *HLA-G*. Although we observed partial reversal of MSC-mediated suppression with pharmacologic CD39 and CD73 blockade, we acknowledge that these inhibitors —particularly POM-1—can have off-target effects on other nucleotidases. Our approach mitigated this by applying inhibitors exclusively to MSCs and confirming that T cell only viability and proliferation were unaffected. While siRNA or blocking antibodies could provide complementary validation, transfection efficiency in primary MSCs is low and donor-to-donor variability further limits standardisation. This limitation of our study could be addressed in future by the use of genetic or antibody-based strategies to inhibit the adenosine pathway. We acknowledge that pharmacologic inhibition, even transient, may reprogramme MSC transcriptional or metabolic states, and thus the observed effects on T cells may not solely reflect acute adenosine pathway blockade. Gene knockdown or clustered regularly interspaced short palindromic repeats (CRISPR)-based deletion of *ENTPD1* and *NT5E* in MSCs would provide complementary mechanistic validation and is a priority for future work.

Our observation that CD73/CD39 inhibition led to approximately a 50% increase in T cell proliferation indicates that EST/PEB-MSCs employ additional immunosuppressive mechanisms that complement the CD73/CD39 axis, and we found evidence that MSCs can utilise multiple immunomodulatory pathways to suppress inflammation and promote immune tolerance. Transcriptomic profiling revealed upregulation of several key molecules during MSC-T cell interactions [41,42]. These molecules collectively contribute to the suppression of T cell proliferation, modulation of cytokine production, and promotion of tolerogenic immune phenotypes. Notably, EST-MSCs demonstrated consistently higher expression of these molecules compared to PEB-MSCs, reflecting site-specific functional adaptations at the entheseal soft tissue and bone anchorage. The upregulation of *IDO1*, a key enzyme in tryptophan metabolism, highlights its role in creating an immunosuppressive microenvironment, while the elevation of PGE2 (mediated by *PTGS2*) underscores the importance of lipid mediators in MSC-driven immune modulation [30,43].

The observed overexpression of checkpoint molecules such as CD274 and HLA-G further indicates the convergence of MSCs and Tregs on shared immunoregulatory mechanisms, supporting the hypothesis that MSCs act as surrogate immune regulators in the enthesis. Although transcriptomic profiling revealed consistent upregulation of key immunomodulatory genes (eg, *IDO1*, *PTGS2*, *IL6*, *HLA-G*, *CD274*), we recognise that post-transcriptional regulation may alter protein output. Similar to our validation of CD39 and CD73 levels by flow cytometry, assessment of these additional immunomodulatory factors using protein-based techniques (eg, ELISA or immunoblotting) will be important for future studies of their roles in entheseal immunomodulation. We noted enthesis MSC upregulation of several cytokine transcripts widely known for their proinflammatory roles, including *IL6*, *IL1A*, and *IL33,* following T cell coculture. The functions of these factors is however known to be context-specific, and when produced by stromal cells they have been shown to promote Treg induction, control local inflammation, and support tissue tolerance [44,45] In particular, MSC-derived IL-6 has been shown to support Treg stability, promote the development of myeloid-derived suppressor cells, to contribute to immune resolution in barrier tissues and to suppress T cell proliferation [30,46,47], in support of the concept that enthesis MSC-derived IL-6 may contribute to local immune homeostasis.

MSCs have emerged as pivotal orchestrators of immunomodulation in addition to their well-recognised multilineage differentiation capacity in skeletal tissues [48] Within the spinal enthesis, 2 distinct subpopulations of multipotential MSCs have been previously identified: a PEB-derived stroma (PEB-MSCs) and interspinous ligament EST-derived stroma (EST-MSCs), as defined according to ISCT guidelines ref. Both populations share the ability to undergo multilineage differentiation into skeletal tissue elements, including bone [49]. It is known that MSCs exert multifaceted immunosuppressive effects on various immune cells, including B and T cells, NK cells [50]. These effects are mediated by the secretion of soluble factors such as IDO1, PGE2, TGF-β, IL-10, and IL-6, as well as by direct T cell −cell interactions, including via PD-L1.

While MSCs with immunomodulatory potential were first described in culture-expanded bone marrow fractions and thereafter in other tissues, our data provide the first description of these cells in the context of enthesis immunobiology [48,51]. While this property is not exclusive to the enthesis, its presence in a tissue with a low Treg frequency and known susceptibility to inflammation is notable. The immunosuppressive capacity of MSCs/stromal cells is well-documented in other tissues, including the skin and bone marrow, and has been therapeutically exploited in conditions such as graft-vs-host disease using MSC-based therapies (eg, Ryoncil). Indeed, dermal fibroblasts and other tissue-resident stromal cells can suppress immune responses via similar mechanisms [51]. Therefore, our findings extend these principles to the spinal enthesis and support the concept that resident MSCs help maintain local immune homeostasis in mechanically stressed, immune-sparse environments. Future comparative studies using fibroblasts from other uninflamed tissues (eg. skin or lung) may further delineate tissue-specific versus shared features of stromal immunoregulation. Finally, we unexpectedly noted that TNMD+ SCX+ MKX + cells, which are canonically associated with tendon/ligament, were more enriched at the bony anchorage side of the enthesis but both EST and PEB-derived cells had broadly similar immunoregulatory capabilities. Future studies incorporating spatial validation techniques such as IHC or Visium-based transcriptomics are warranted to map the tissue distribution of these stromal subsets with greater anatomical resolution

Our findings have significant implications for the pathophysiology and treatment of SpA [52]. Dysregulation of MSC pathways, particularly the CD73/CD39 axis, may underpin the immune imbalance observed in SpA, providing a rationale for targeting the enhancement adenosine levels via CD73/CD39 pathway manipulation. The identification of regulatory MSC subsets also opens new avenues for precision targeting of specific niches within the enthesis. However, as in murine arthritis models, it is recognised that during the induction of enthesitis that Treg and other cells may migrate from the intestine to the enthesis and thus may assume increased importance during inflammation [53]. Some TNF transgenic models specifically exhibit intestinal and an enthesitis immunopathology [54]. When this TNF-dependent SpA model is crossed with the depletion of Tregs (DEREG) mice, Tregs accumulate in arthritic joint [55]. Nevertheless, the translocation of Tregs to inflamed joints in human SpA, and their possible functional contribution, if present, remains to be investigated.

In conclusion, our findings reveal an immunoregulatory function for MSCs in the human enthesis, extending observations made in other tissue-resident stromal cells, and support their potential role for these cells in normal enthesis immune homeostasis. The dynamic interplay between MSCs and immune cells, mediated by pathways such as CD73/CD39, underscores the adaptive nature of enthesis immune regulation. Future research should focus on comparing healthy and SpA-affected entheses at single-cell resolution, elucidating the mechanisms by which inflammation disrupts MSC function, and explore innovative MSC-based therapies. In summary, compared to the intestine, we found a paucity of entheseal Tregs but evidence that entheseal MSCs subsumed functional attributes of Tregs. These findings have implications for a better understanding of the translational immunology of human SpA, particularly in relation to tissue-specific stromal–immune interactions.

Funding

This research was supported by funding from the Medical Research Council MR/S035850/1.A.A was funded by Medical Research Council MR/S035850/. D.S. was supported by funding from Versus Arthritis #22252. S.N.S is supported by funding from the Kennedy Trust for Rheumatology Research #KENN222309. DM is supported by NIHR Leeds Biomedical Research Centre.

Competing interests

All authors declare they have no competing interests.

CRediT authorship contribution statement

Ala Altaie: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Davide Simone:** Writing – original draft, Visualization, Formal analysis. **Nicole McDermott:** Data curation. **Heather Owston:** Data curation. **Moustafa Attar:** Data curation. **Liying Jin:** Data curation. **Chi Wong:** Data curation. **Peter R. Loughenbury:** Resources. **Borse Vishal:** Resources. **Tristan McMillan:** Resources. **Christopher D. Buckley:** Writing – review & editing. **Stephen N. Sansom:** Writing – review & editing, Supervision, Conceptualization. **Dennis McGonagle:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Patient consent for publication

Written informed consent was obtained from all participants.

Ethics approval

Ethical approval for the study was approved by the North-west-Greater Manchester West Research Ethics Committee (REC:16/NW/0797). And were in accordance with the Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

The single-cell RNAseq data generated in this study have been deposited in the GEO database under accession code GSE292866 (raw data) and Zenodo database under accession 15090823 (processed data).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.09.001.

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