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Disease-specific and inflammation-independent stromal alterations in spondyloarthritis synovitis

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Abstract

Objective: The molecular processes driving the distinct patterns of synovial inflammation and tissue remodelling in spondyloarthritis (SpA) versus rheumatoid arthritis (RA) remain largely unknown. Therefore, we aimed to identify novel and unsuspected disease-specific pathways in SpA by a systematic and unbiased synovial gene expression analysis.

Methods: Differentially expressed genes were identified by pan-genomic microarray and confirmed by quantitative PCR and immunohistochemistry using synovial tissue biopsies of SpA (n=63), RA (n=28) and gout (n=9) patients. The effect of inflammation on the gene expression was assessed by stimulating fibroblast-like synoviocytes (FLS) with synovial fluid and by analysis of synovial tissue samples at week 0 and 12 of treatment with etanercept.

Results: Using very stringent statistical thresholds, microarray analysis identified 64 upregulated transcripts in SpA versus RA synovitis. Pathway analysis revealed a robust myogene signature in this gene set. The myogene signature was technically and biologically reproducible, was specific for SpA, and was independent of disease duration, treatment and SpA subtype (non-psoriatic versus psoriatic). Synovial tissue staining identified the myogene expressing cells as vimentin(+), 4-prolyl-hydroxylase-ß(+), CD90(+) and CD146(+) mesenchymal cells that were significantly overrepresented in the intimal lining layer and synovial sublining of inflamed SpA synovium. Neither in vitro exposure to synovial fluid from inflamed SpA joints nor in vivo TNF blockade modulated the SpA-specific myogene signature.

Conclusion: These data identify a novel and disease-specific myogene signature in SpA synovitis. The fact that this stromal alteration appeared not to be downstream of local inflammation warrants further analysis of its functional role in the disease pathogenesis.

Introduction

Spondyloarthritis (SpA) is a polygenic chronic inflammatory arthritis which affects mainly young adults and is characterized by inflammation of the axial skeleton, oligoarticular peripheral arthritis, and extra-articular manifestations such as inflammatory bowel disease (IBD), uveitis and psoriasis (1). Unlike the other major form of chronic inflammatory arthritis, rheumatoid arthritis (RA), SpA does not display the classical features of T and/or B cell driven autoimmune diseases such as: female predominance, presence of autoantibodies, common lymphocyte-related genetic risk factors such as SNPs in PTPN22 and CTLA-4, and clinical response to T and B cell targeted therapies (2). In contrast, the clinical features as well as the role of bacterial and mechanical danger signals strongly suggest an autoinflammatory origin of SpA (3;4).

The fact that the origin and pathophysiology of tissue inflammation is fundamentally different in SpA versus RA is supported by two additional lines of evidence. Firstly, comparative immunopathology has demonstrated marked differences in histological features, inflammatory cell populations, and cytokine milieu at the primary site of inflammation, the synovial membrane. Whereas RA synovitis displays features of antigen-specific lymphocyte activation (5-9), alterations of innate immune system appear to be more prominent in SpA synovitis. This includes a significant increase in CD163+ alternatively activated macrophages (10;11) which is associated with altered macrophage-derived cytokine levels in the synovial fluid (12), increased expression of TLRs (13), and a marked increase in IL-17 expressing mast cells and neutrophils (14). In addition, the antigen presenting role of HLA-B27 in the disease pathophysiology has been severely challenged in experimental HLA-B27 associated disease (15) and

alternative hypotheses propose that HLA-B27 directly modulates innate immune responses through triggering of NK cells by HLA-B27 homodimers (16) and endoplasmic reticulum stress responses caused by HLA-B27 misfolding (17). In conjunction with the genetic associations with molecules of the innate immune system (18), these data indicate a predominant role for the innate rather than acquired immune system in SpA.

Secondly, a fundamental difference in pathophysiology is also supported by the distinct structural outcome in SpA versus RA. Whereas chronic inflammation in RA leads to cartilage and bone destruction almost without repair, SpA is characterized by excessive new cartilage and bone formation leading to syndesmophytes and eventually ankylosis. Additionally, anti-tumour necrosis factor (TNF) drugs effectively suppress the progression of cartilage and bone destruction in RA but the currently available evidence suggests that these drugs do not inhibit osteoproliferation and ankylosis in experimental ankylosing enthesitis (19) as well as human SpA (20-22).

Taken together, these findings indicate that distinct inflammatory and tissue remodelling pathways are operative in these two different forms of chronic inflammatory arthritis. However, the precise molecular processes driving these distinct pathways remain unknown. Gene expression analysis by microarray has been developed and validated as a powerful tool to perform an unbiased screening for unsuspected mediators of complex diseases. In early studies, we have used this approach to analyze peripheral blood and synovial fluid mononuclear cells of SpA patients (23;24) and demonstrated that this technology can be used to identify thus far unsuspected mediators of inflammation in synovial tissue (24;25). The aim of the present study was to identify

novel and unsuspected cellular and/or molecular disease-specific pathways in SpA by a systematic and unbiased comparative gene expression analysis of actively inflamed synovial tissue in SpA versus RA.

Material and methods

Patients and samples

The study included a total of 100 patients with either SpA according to the ESSG classification criteria (26) (n=63), RA according to the ACR 1987 classification criteria (27) (n=28), or gout (n=9). The SpA cohort consisted of patients with non-psoriatic SpA (n=35), psoriatic arthritis (n=18), as well as 10 patients in which biopsies were obtained at week 0 and week 12 of etanercept treatment. None of the other patients was treated with a biological drug. All patients gave written informed consent to participate in the study as approved by the local Medical Ethics Committee before inclusion in the study. The demographic and clinical data of the different patient cohorts are given in Table 1. The first cohort was used for microarray analysis and technical confirmation by quantitative RT-PCR, the second cohort was used for an independent confirmation of the biological validity of the gene expression signature, the third cohort was used for confirmation at the protein level by immunohistochemistry, and the fourth cohort was used to investigate the impact of TNF blockade on the genes of interest in SpA. In all cohorts, a total of 20-25 synovial tissue biopsies per patient were obtained from clinically inflamed knee or ankle joints and processed as described earlier (28;29): 6-8 biopsies were pooled and used for RNA isolation and 6-8 additional biopsies were used for immunohistochemistry. In some cases, additional biopsies were use to obtain fresh cells for flow cytometry or culture.

Microarray analysis

RNA was obtained from 6-8 snap frozen synovial tissue biopsies per patient. The RNA isolation, cDNA synthesis, and labelling was carried out using Agilent's Two-Colour Microarray-Based Gene Expression Protocol according to the manufacturer's instructions (G4140-90050, Version 5.7, Agilent Technologies, Palo Alto, CA, USA). To correct for gene-specific dye biases, we used a reverse labelling (dye swap) design with common reference (the Universal Human Reference, Agilent Technologies). Labeled samples were hybridized to 4x44K Human Whole Genome microarrays (Agilent Technologies). Data were extracted using Agilent Feature Extraction software (Version.10.1, Agilent Technologies) and analyzed using the Limma package (30) of the Bioconductor project (www.bioconductor.org). We performed background correction according to the "offset" method in order to avoid negative intensity values after background subtraction, and within array normalization using global loess normalization. Subsequently, we applied between-array normalization using scale normalization (31). The normalized data were then fitted within a linear model and Limma was used to moderate the standard errors of the estimated log ratios (32). The expression data in SpA versus RA synovitis were then compared using *t* –statistics. The false discovery rate was set to 0.01 and probes with *P* values less than 0.001 as adjusted for multiple hypothesis testing were judged as differentially regulated. Hierarchical clustering and heat-map representation of gene expression were performed and visualized with the *hclust and heatmap.2* functions of the R gplots package.

Quantitative RT-PCR

Total RNA was isolated from synovial tissue biopsies or PBMCs using RNA Stat-60 (Tel-Test Inc, Friendswood, Tx, USA) or a GenElute mammalian total miniprep RNA kit (Sigma-Aldrich, St Louis, Missouri, USA), treated with DNase I (Invitrogen, Carlsbad, CA, USA), and reverse transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). RNA concentration was determined with the Nanodrop (Nanodrop Technologies, Wilmington, DE USA). Quantitative real-time PCR was performed in duplicate using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green PCR Master Mix (Applied Biosystems) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. The oligonucleotide primers were designed by Real-time PCR (TaqMan) Primer Design (Genscript) and obtained from Invitrogen. Additionally, pre-designed TaqMan probe and primer sets for ACTA1 (Hs00559403_m1), MYH2 (Hs00430042_m1), MYH7 (Hs01110632_m1), CKM (Hs00176490_m1), IL-8 (Hs00174103_m1) and GAPDH (4310884E) were purchased and assayed according to the manufacturer's protocol (Applied Biosystems).

Immunohistochemistry and double immunofluorescence

Synovial biopsy samples (8 per patient) were snap-frozen and mounted in Jung tissue freezing medium (Leica Instruments, Nussloch, Germany). Frozen tissue sections were used for immunohistochemical staining with monoclonal mouse anti-human alpha smooth muscle actin (α-SMA) (clone 1A4, Dako, Glostrup, Denmark), monoclonal mouse anti-human alpha actin (clone AC-40, Sigma-Aldrich, Bornem, Belgium) and monoclonal mouse anti-human Smooth Muscle Myosin Heavy Chain (SMMHC) (clone

SMMS-1, Dako). Parallel sections were incubated with irrelevant isotype- and concentration-matched monoclonal antibody as negative control. The staining protocol and quantification have been extensively described and validated previously (6;10). Briefly, sections were sequentially incubated with the primary antibodies, a biotinylated second antibody, a streptavidin–horseradish peroxidase link, and finally aminoethylcarbazole substrate as chromogen (all from Dako). Expression in the different synovial compartments (blood vessels, intimal lining layer, and synovial sublining) was quantified on a semiquantitative four-point scale by two independent observers blinded for diagnosis. Double staining of alpha actin positive cells was performed with biotinylated monoclonal antibodies against macrophages (CD68, clone Y1/82A, Biolegend, San Diego, CA; and CD163, clone GHI/61, Biolegend), T lymphocytes (CD3, clone UCH-T1, Thermo Scientific Pierce Products), lining layer fibroblasts-like synoviocytes (CD55, clone MEM-118, Scientific Pierce Products), plasma cells (CD138 clone B-A38, Abcam), leukocytes (CD45, clone HI30, Biolegend), pericytes (CD146; clone P1H12, Abcam), fibroblasts/myofibroblast (CD90, clone 5E10 Biolegend, vimentin, (D21H3) XP™ Rabbit mAb, Cells Signaling, Frankfurt a. M., Germany, 4-prolyl-hydroxylase-ß, rabbit polyclonal, Abcam, Camebridge, UK) and endothelial cells (von Willebrand factor, rabbit polyclonal, Dako). Incubation with the primary antibodies was carried out overnight at 4°C, followed by incubation with a secondary Alexa Fluor 555-or 488 conjugated goat anti-mouse secondary antibody and streptavidin-Alexa Fluor 488 (in case of staining with von Willebrand factor, vimentin and 4-prolyl-hydroxylase-ß secondary Alexa Fluor 488 or 594-conjugated goat anti-rabbit antibody was used). Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed on

a fluorescent imaging microscope (Leica DMRA, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, the Netherlands).

Cell isolation, flow cytometry and cell culture

Single-cell suspensions were prepared from synovial tissue using the modified protocol (33). Briefly, tissue biopsy samples were finely minced and digested with 1000 units/ml hyaluronidase (Sigma-Aldrich), Liberase TM (125 µg/ml) and DNase I (200 µg/ml; both from Roche, Basel, Switzerland). After filtering through a 70-µm nylon mesh or steel strainer, the cell suspension was directly stained with anti-α-SM actin-PE (R&D Systems, Abingdon, UK), anti-CD90-Alexa700 (Biolegend) and anti-CD146-PercP-Cy5.5 (BD Pharmingen, San Diego, CA) and analyzed by flow cytometry. In other experiments, the cells were put into culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics, L-glutamine, and 10% fetal bovine serum. FLS lines were obtained from the adherent cell fraction and used at passage 3 and 4 for stimulation with a combination of TGFβ1 (2 ng/ml) and PDGF (10 ng/ml) (both from R&D Systems, Abingdon, UK) or pooled SF from either 20 RA patients or 20 SpA patients. FLS were recovered after different time points and RNA was isolated for qPCR as described previously.

Pathway analysis

Gene ontology analysis was performed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA, USA, http://www.ingenuity.com), analysis of pro-fibrotic

markers was performed based on defined by Pathway Central (The Ultimate Resource for Pathway Research, Pathways For Extracellular Matrix and Adhesion Molecules, QIAGEN, http://www.sabiosciences.com/ECM_CellAdhesion.php).

Statistics

Results from qPCR and immunohistochemistry were analyzed using non-parametrical statistics with the Kruskal-Wallis test, the Mann-Whitney U test and the Spearman's rank correlation for correlation between data. $p<0.05$ was considered statistically significant.

Results

Identification of a SpA-specific synovial myogene expression signature by microarray analysis

In order to identify novel and unsuspected cellular and molecular pathways involved in the pathogenesis of SpA, we performed a pan-genomic expression analysis of SpA versus RA synovitis. To minimize biases related to patient and sample heterogeneity, we selected synovial samples (Table 1, cohort 1) based on a) a homogeneous population of non-psoriatic SpA patients, b) exclusion of any disease-modifying antirheumatic drugs (DMARD) or biological treatments, and c) a matched degree of local tissue inflammation as assessed by synovial infiltration with T and B lymphocytes on histology. Using very stringent data analysis criteria (more than two-fold difference in expression, p value less than 0.001), we identified 296 transcripts that were differentially regulated in SpA versus RA synovitis: 64 transcripts were upregulated in SpA versus RA synovium whereas 232 transcripts were downregulated in SpA. Focusing for the remainder of the study on the genes that were overexpressed in SpA, the obtained gene signature was robust as indicated by three additional observations. Firstly, hierarchical clustering of the synovial samples according to this set of differentially regulated genes grouped all SpA synovial samples under one node, with four samples showing high homology and the seven other samples showing an even higher degree of similarity (data not shown). The RA samples had a more heterogeneous expression profile with two of them clustering together with the SpA samples. Secondly, the fold expression in SpA versus RA was high: of the 64 transcripts with a more than twofold increase, 22 transcripts had a more than 3 fold increase and 10 transcripts a 4 to 15 fold increased expression. Thirdly, a large majority

(33 out of 64) of the genes that were overexpressed in SpA synovitis related to muscle/myocyte/myofibroblast biology (Table 2). These transcripts were particularly enriched in the top genes: 21 out of the 22 transcripts with a more than 3-fold upregulation in SpA belonged to this myogene-group. This was furthered confirmed by gene ontology analysis, which revealed calcium signalling ($p=5.72^{-12}$), actin cytoskeleton signalling ($p=1.77^{-09}$), integrin linked kinase signalling ($p=1.92^{-08}$), and hepatic fibrosis/hepatic stellate cell activation ($p=3.40^{-06}$) as the canonical pathways being overrepresented in the SpA signature dataset. Taken together, these data indicate a robust myogene expression signature in SpA synovitis.

Technical and biological validation of the myogene expression signature by quantitative RT-PCR

In order to confirm the obtained results by an independent technique on the same set of samples, we selected 8 known transcripts with an at least 3-fold upregulation in SpA synovitis and reassessed their expression by quantitative real-time RT-PCR (qPCR). As shown for ACTA1, MYH7, MYH2, and ACTN2 in Figure 1A, the overexpression in SpA versus RA synovitis was confirmed for all 8 genes ($p<0.05$) by qPCR with some of these genes showing a more than 100-fold upregulation in SpA versus RA synovitis. In order to verify the biological validity of the gene signatures, we subsequently reanalysed 7 of these genes by qPCR in an independent set of synovial tissue samples obtained from 10 non-psoriatic and 13 psoriatic SpA, 11 RA patients, and 9 gout patients (Table 1, cohort 2). These patients were specifically selected for short (less than one year) disease duration in order to exclude biases due to disease duration. In this independent sample

set, 5 of the 7 genes were confirmed to be significantly overexpressed in SpA versus RA synovitis, as shown for ACTA1, MYH7, MYH2, and ACTN2 in Figure 1B. Additionally, this analysis revealed a similar upregulation of these target genes in non-psoriatic and psoriatic SpA (Figure 1B). Finally, the expression of these genes was very low and similar to RA in the gout samples (Figure 1B). Accordingly, all tested genes were also significantly upregulated in SpA versus gout, indicating that their differential expression reflects a disease-specific increase in SpA synovitis. Cumulatively these qPCR data demonstrated that the identified myogene signature was technically and biologically reproducible, was SpA specific, and was independent of disease duration and clinical SpA subtype.

Identification and phenotyping of alpha actin-positive cells in SpA synovitis

We next investigated whether the overexpression of myogene transcripts also translated in altered expression of the encoded proteins in SpA synovitis. Immunohistochemistry on a large set of SpA and RA synovial biopsies revealed the expression of α-SMA and SMMHC not only around blood vessels, as in healthy synovium, but also in the intimal lining layer and the synovial sublining (Figure 2A). α-SMA positive cells were significantly increased in SpA versus RA in the intimal lining layer ($p=0.003$) as well as in the synovial sublining ($p=0.035$) despite similar staining around vessels (Figure 2B). Similarly, SMMHC positive cells were significantly increased in SpA versus RA in the synovial sublining ($p=0.018$) whereas the expression around vessels was lower in SpA than in RA synovitis ($p=0.031$) (Figure 2C). These data confirmed the gene signature at the protein level and indicated that the increase in myogene expression can not be solely

explained by the increased vascularity in SpA versus RA synovitis (6;34). Further phenotyping of the synovial α-SMA positive cells by double immunofluorescence did not reveal any colocalization with CD45, CD3, CD68, CD163 and CD138 (data not shown), indicating that these cell are not of hematopoietic origin. Analyzing markers of mesenchymal cells, α-SMA did not co-localize with von Willebrand factor (VWF), a marker for endothelial cells, but did show substantial co-localization with CD146 and CD90 (Figure 2D), which are markers of myofibroblasts, pericytes and mesenchymal stem cells. These cells also expressed the fibroblast markers vimentin and 4-prolyl-hydroxylase-ß indicating their mesenchymal nature (data not shown). The results of these stainings were further confirmed by flow cytometric analysis of two SpA synovial tissue cell suspensions: the percentage of cells expressing α-smooth muscle actin was enriched in the CD90 and CD146 positive cell fractions compared to the CD90 and CD146 negative cell fractions, respectively (17.9±4.8% versus 0.4±0.1% for CD90; 11.2±5.8% versus 0.9±0.2% for CD146). Taken together, these data indicate that the myogene signature of SpA synovitis is associated with the increased presence of α-SMA expressing CD90+ and CD146+ cells in the intimal lining layer and synovial sublining.

The SpA-specific myogene signature is not induced by local inflammatory mediators

We next aimed to investigate whether the specific myogene signature was a primary synovial feature of SpA rather than a secondary phenomenon induced by local joint inflammation. First, we analyzed the expression of genes related to the TGFß, PDGF (35) and Wnt pathways in SpA versus RA synovitis as these pathways are known to be involved in the expression of myofibroblast markers such as α-SMA. Analysis of

microarray gene expression data revealed that a substantial number of genes encoding pro-fibrotic mediators associated with TGFß/BMP Signaling Pathway, WNT Signaling Pathway and Fibrosis Pathway as defined by Pathway Central (The Ultimate Resource for Pathway Research, M&M, Pathway analysis) were significantly increased in the synovial tissue of SpA patients (Table 3). Of particular interest was the significant upregulation of PDGF alpha and, to a lesser extend, beta, as these soluble factors could induce the myogene expression. To assess the contribution of such soluble factors to the observed SpA myogene signature, we performed in vitro experiments with FLS as a substantial fraction of these cells (10-15%) express α-SMA as assessed by flow cytometry (Figure 3A). In agreement with this data indicating that factors such as TGFβ1 and PDGF promote the trans-differentiation of fibroblasts towards myofibroblasts, TGFβ1/PDGF stimulation of FLS (n=11) induced a two-fold upregulation of ACTA1 messenger expression (p=0.019) (Figure 3B). In contrast, incubation with pooled synovial fluid (SF) from either 20 SpA samples or 20 RA samples resulted in a suppression rather than induction of ACTA1 expression by FLS (Figure 3B), suggesting that the myogene signature is not induced by the local soluble and/or inflammatory mediators in the SpA joint. To confirm this in vivo, we analyzed the expression of ACTA1, MYH2, MYH7 and CKM in paired synovial biopsies obtained before and 12 weeks after treatment with etanercept. Despite clear clinical and histological improvement of the arthritis upon treatment (36), none of the myogenes was significantly down-modulated by TNF blockade whereas expression of pro-inflammatory mediators such as IL-6 (p=0.031) and IL-8 (p=0.039) was significantly decreased by this treatment (Figure 3C). Taken together with the presence of the myogene signature in early disease

(Figure 1B), these in vitro and in vivo data concord to indicate that the SpA-specific stromal alterations identified in this study are not downstream of local inflammation.

Discussion

Genetic, immunopathological and clinical evidences indicate that, despite common downstream pathways of inflammation such as macrophage-derived TNF, the synovial inflammation is driven and maintained by different cellular and molecular mediators in SpA versus RA (1;2). Accordingly, we have proposed that SpA is an autoinflammatory disease driven by innate immune cells rather than a genuine autoimmune disease triggered by self-reactive T and/or B lymphocytes (4). In this study, we aimed to confirm and refine the concept that SpA and RA are driven by clearly distinct pathophysiological pathways by performing a broad and unbiased screening for novel and disease-specific molecular signatures of the inflamed primary target organ. Using the combination of the unique availability of synovial tissue biopsies from untreated patients with active disease and the power of cutting-edge microarray analysis, we describe here for the first time a unique set of genes that are specifically overexpressed in SpA synovitis versus RA as well as gout synovitis. The obtained gene signature was particularly robust as indicated by the high fold difference in expression levels and by the strong clustering of the overexpressed genes in particular biological pathways as indicated by Ingenuity analysis. Moreover, we demonstrated that this disease-specific molecular profile was technically and biologically reproducible by performing qPCR and immunohistochemical analyses on different sample sets. Finally, the SpA gene signature appeared to be independent of treatment, disease duration, and clinical phenotype. The latter observation is in line with

previous studies indicating that there is a high cellular and molecular resemblance between non-psoriatic and psoriatic SpA synovitis (12;14;34;37).

The most striking finding of the study was the fact that a large majority of the genes that were specifically overexpressed in SpA synovitis did not classify into immune or inflammatory pathways but were directly related to muscle/myofibroblast biology. The stromal rather than hematopoietic origin of the signature was confirmed by double immuno-stainings which did not reveal any co-localization with a panel of leukocyte markers. Synovial tissue is not known to contain muscle cells with exception of smooth muscle cells around the blood vessels. As SpA synovitis is characterized by a more pronounced hypervascularity than RA synovitis (6), we explored whether this may explain the myogene signature by assessing separately the different compartments in the synovial tissue by immunohistochemistry. The presence of α-SMA and SMMHC positive cells was not increased in the blood vessels or their immediate vicinity in SpA versus RA synovium. In contrast, however, we observed an increase of single α-SMA and SMMHC positive cells in the intimal lining layer and the synovial sublining in SpA, indicating the presence of a distinct stromal cell population expressing these myogenes in SpA. Double immunofluorescence phenotyping indicated that these cells were positive for the stromal markers CD90, CD146, vimentin and 4-prolyl-hydroxylase-ß. Although exact phenotyping of stromal cell populations remains a challenging issue, these markers suggest that these cell are closely related to myofibroblasts, pericytes or their immediate precursors, mesenchymal stromal cells (MSCs) (38;39).

The specific presence of these myofibroblast-related cells reinforces the concept that synovial stromal cells represent a heterogeneous mix of different specialized cell types

and raises the question of the origin and reason for accumulation of these cells in SpA synovium. A first possibility is that synovial fibroblasts trans-differentiate locally into myofibroblasts under influence of cytokines and growth factors released during joint inflammation. Factors such as TGFβ1, PDGF and CTGF have a well established role in myofibroblast differentiation and can be secreted by macrophages, other inflammatory cells, epithelial cells and mesenchymal cells during joint inflammation (40). In line with this hypothesis, gene expression analysis of pro-fibrotic pathways in SpA versus RA revealed a significant upregulation of not only genes involved in Wnt and TGF beta signaling, but also a substantial upregulation of PDGF alpha and beta. However, the failure of SpA SF to upregulate myogenes in FLS and the failure of TNF blockade to downregulate the myogene signature in vivo despite clinical efficacy do not support the concept that soluble factors such as PDGF or local inflammatory mediators drive this disease-specific stromal alteration. Alternatively, the synovial α-SMA expressing cells may derive from circulating fibrocytes, a small bone marrow-derived cell population which represent 0.1 to 0.5% of the circulating cells (41;42). Several chemoattractants, such as CCL2 (43), CXCL12 (44;45), PDGF beta (44) and CCL21 (46), have been shown to have a role in fibrocyte migration into tissues, where they differentiate further towards specialized stromal cells such as myofibrobasts and pericytes. Whereas additional studies are required to analyze in depth the presence and role of fibrocytes in SpA, gene expression analysis of peripheral blood failed to detect the same difference in myogene expression between SpA and controls as found in the synovial compartment (data not shown). These data thus do not support the idea of a systemic origin of these cells and emphasize the importance of direct analysis of the target tissues.

Besides originating from local fibroblasts by trans-differentiation or from circulating fibrocytes, we should also consider the possibility that these cells represent genuine mesenchymal stem cells as such distinct pluripotent cell population has already been described in the synovial tissue by several groups (47-49). This possibility, which should be further tested by selective sorting and in vitro testing of the osteogenic and chondrogenic potential, is of particular interest in SpA as it may directly relate to the remodeling nature of the disease. In this context, it is interesting to note that we recently observed that osteoproliferation did not start from the bone or cartilage in the axial and peripheral joints of HLA-B27 transgenic rats but originated from the connective stromal tissue which, similarly to human SpA synovium, contains α-SMA+ cells (Van Duivenvoorde unpublished data) . Although a link between the myogene expressing cells identified in this study and remodeling in SpA remains purely speculative at this stage, the fact that this signature is specific for SpA synovitis and is not influenced by TNF blockade emphasizes their potential pathophysiological relevance and warrants detailed investigation of the exact function of these cells in both human SpA and relevant animal models.

From a clinical perspective, finally, two important issues need to be further addressed. Firstly, the microarray and qPCR analyses showed a very clear upregulation of these synovial myogenes in a majority of SpA samples in comparison to RA and gout controls. Analysis of larger patient cohorts are planned to determine whether this signature can be used as a diagnostic biomarker in early, undifferentiated arthritis and/or as a biomarker of a specific subset of SpA patients as not all SpA samples displayed this upregulation. Secondly, this study was performed on tissue samples obtained from inflamed peripheral

joints. Although challenging because of the lack of available biopsies, it will be important to determine whether a similar signature is found in other articular manifestations of SpA, in particular in enthesitis and in axial disease, as this may help us to define more precisely whether this signature is related to structural remodeling and new bone formation. Histological or transcriptional analysis of human samples in combination with functional experiments in vitro and in relevant animal models (50) will allow us to decipher the role of this disease-specific stromal pathway in SpA.

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Table 1: **Demographic and clinical data of the patient cohorts**. Data are represented as mean and standard deviation for age (years), disease duration (years), number of swollen joints, and C-reactive protein serum levels (mg/L). Gender is given as male/female. In cohort 4, paired samples were obtained at week 0 and week 12 of treatment with etanercept. RA = rheumatoid arthritis. SpA = spondyloarthritis. PsA = psoriatic spondyloarthritis. SJC = swollen joint count. CRP = C-reactive protein. RF = rheumatoid factor. ACPA = anti-citrullinated protein antibodies. DMARD = disease modifying anti-rheumatic drugs.

	Cohort 1		**Cohort 2**				**Cohort 3**		**Cohort 4**
	RA (n=8)	**SpA (n=12)**	**RA (n=11)**	**SpA (n=10)**	**PsA (n=13)**	**Gout (n=9)**	**RA (n=9)**	**SpA (n=18)**	**SpA (n=10)**
Age	56 ± 15	44 ± 17	56 ± 13	35 ± 14	47 ± 12	69 ± 16	52 ± 16	46 ± 16	38 ± 13
Gender	4/4	8/4	4/7	7/3	9/4	6/3	7/2	9/9	7/3
Disease duration	3.7 ± 2.9	6.8 ± 5.8	0.5 ± 0.3	0.6 ± 0.4	0.9 ± 0.8	2.7 ± 2.3	13.9 ± 12.2	6.7 ± 5.8	9.8 ± 7.8
SJC	10 ± 5	3 ± 3	12 ± 10	2 ± 1	2 ± 3	2.7 ± 2.6	9 ± 3	2.4 ± 2.3	3 ± 3
CRP	45 ± 22	43 ± 47	34 ± 48	21 ± 39	12 ± 13	15 ± 19	29 ± 33	30 ± 44	11 ± 9
RF or ACPA	4	0	9	0	0	0	6	0	0
DMARD	0	0	0	3	2	0	7	7	0
Anti-TNF	no	no	no	no	no	no	no	no	yes

Table 2: List of genes upregulated in SpA versus RA.

Gene symbol	Gene name	Fold change
ACTA1	Actin, alpha 1, skeletal muscle	15.31
MYH2	Myosin, heavy chain 3, skeletal muscle, embryonic	12.49
CKM	Creatine kinase, muscle	12.08
MYH7	Myosin, heavy chain 7, cardiac muscle, beta	8.36
MB	Myoglobin	5.43
MYL1	Myosin, light chain 1, alkali; skeletal, fast	5.01
MYH1	Myosin, heavy chain 3, skeletal muscle, embryonic	4.69
CSRP3	Cysteine and glycine-rich protein 3 (cardiac LIM protein)	4.33
ACTN2	Actinin, alpha 2	4.25
TNNI2	Troponin I type 2 (skeletal, fast)	3.96
NRAP	Nebulin-related anchoring protein	3.81
MYLPF	Fast skeletal myosin light chain 2	3.69
CMYA3	Xin actin-binding repeat containing 2	3.64
MYBPC1	Myosin binding protein C, slow type	3.52
KBTBD10	Kelch repeat and BTB (POZ) domain containing 10	3.52
MYL2	Myosin, light chain 2, regulatory, cardiac, slow	3.31
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	3.29
TTN	Titin	3.27
SMPX	Small muscle protein, X-linked	3.24
TCAP	Titin-cap (telethonin)	3.20
CA3	Carbonic anhydrase III, muscle specific	3.14
TNNC2	Troponin C type 2 (fast)	2.98
TNNC1	Troponin C type 1 (slow)	2.77
TNNT3	Troponin T type 3 (skeletal, fast)	2.75
TPM1	Tropomyosin 1 (alpha)	2.71
ANKRD23	Ankyrin repeat domain 23	2.69
TNNT1	Troponin T type 1 (skeletal, slow)	2.67
NEB	Nebulin	2.57
APOBEC2	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	2.56
MYLK2	Myosin light chain kinase 2, skeletal muscle	2.56
RPS4Y2	Ribosomal protein S4, Y-linked 2	2.52
RPS4Y1	Ribosomal protein S4, Y-linked 1	2.52
ENO3	Enolase 3 (beta, muscle)	2.44
SLN	Sarcolipin	2.41
MYOZ1	Myozenin 1	2.40
MYBPC2	Myosin binding protein C, fast type	2.40
TRIM63	Tripartite motif-containing 63	2.38
MYOT	Myotilin	2.35
C8orf22	Chromosome 8 open reading frame 22	2.34
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	2.33
GJB2	Gap junction protein, beta 2, 26kDa	2.32
C9orf61	Chromosome 9 open reading frame 61	2.32
SYNPO2L	Synaptopodin 2-like	2.31
ENST00000284767		2.30

Table 2: List of genes upregulated in SpA versus RA. *Continued*

Gene symbol	Gene name	Fold change
ASB5	Ankyrin repeat and SOCS box-containing 5	2.28
ADCY1	Adenylate cyclase 1 (brain)	2.27
GJB6	Gap junction protein, beta 6, 30kDa	2.26
HBA2	Hemoglobin, alpha 2	2.25
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	2.24
DUSP13	Dual specificity phosphatase 13	2.23
BC015836	Homo sapiens, clone IMAGE:4293240, mRNA	2.23
TMOD4	Vacuolar protein sorting 72 homolog (S. cerevisiae)	2.22
IRF5	Interferon regulatory factor 5	2.20
RFX2	Regulatory factor X, 2 (influences HLA class II expression)	2.20
MYF6	Myogenic factor 6 (herculin)	2.18
KIAA0672	Rho-type GTPase-activating protein RICH2	2.16
ENST00000332074		2.15
HSPB3	Heat shock 27kDa protein 3	2.15
PDLIM3	PDZ and LIM domain 3	2.12
CORO6	Coronin 6	2.12
RRAD	Ras-related associated with diabetes	2.09
DUSP14	Dual specificity phosphatase 14	2.08
PYGM	Phosphorylase, glycogen; muscle (McArdle syndrome, glycogen storage disease type V)	2.07
A2BP1	Ataxin 2-binding protein 1	2.01

Table 3. The number of genes encoding pro-fibrotic mediators is significantly (p<0.001) increased in synovial biopsies from patients with SpA versus RA

Pathway	Gene	Gene name	Fold change
Human TGFß Signaling Targets	PDGFA	platelet-derived growth factor alpha polypeptide	1.76
	CEBPB	CCAAT/enhancer binding protein	1.61
	PTHLH	parathyroid hormone-like hormone	1.55
	RARA	retinoic acid receptor, alpha	1.39
	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.38
	RHOB	ras homolog gene family, member B	1.37
	GLI2	GLI-Kruppel family member GLI2	1.31
	ATF3	activating transcription factor 3	1.30
	HES1	hairy and enhancer of split 1	1.27
	MYC	v-myc myelocytomatosis viral oncogene homolog	1.27
	SMAD3	SMAD, mothers against DPP homolog 3	1.22
	BHLHB2	basic helix-loop-helix domain containing, class B, 2	1.20
TGFß/BMP Signaling Pathway	TGFB1I1	transforming growth factor beta 1 induced transcript 1	1.40
	GSC	goosecoid	1.39
	BMP1	bone morphogenetic protein 1	1.35
	MYC	v-myc myelocytomatosis viral oncogene homolog	1.27
	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	1.26
	SMURF1	SMAD specific E3 ubiquitin protein ligase 1	1.22
	SMAD3	SMAD, mothers against DPP homolog 3	1.22
WNT Signaling Pathway	PITX2	paired-like homeodomain transcription factor 2	1.93
	NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	1.57
	KREMEN1	kringle containing transmembrane protein 1	1.55
	ACTB	actin, beta	1.49
	CTNNBIP1	catenin, beta interacting protein 1	1.38
	FZD5	frizzled homolog 5	1.38
	LRP5	low density lipoprotein receptor-related protein 5	1.34
	DVL1	dishevelled, dsh homolog 1	1.30
	MYC	v-myc myelocytomatosis viral oncogene homolog	1.26
	FBXW4	F-box and WD-40 domain protein 4	1.26
	AXIN1	axin 1	1.25
Fibrosis	PLAT	plasminogen activator, tissue	1.89
	PDGFA	platelet-derived growth factor alpha polypeptide	1.76
	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	1.71
	CEBPB	CCAAT/enhancer binding protein	1.61
	EGF	epidermal growth factor (beta-urogastrone)	1.49
	CAV1	caveolin 1, caveolae protein	1.44
	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.27
	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	1.26
	ILK	integrin-linked kinase	1.25
	SMAD3	SMAD, mothers against DPP homolog 3	1.22

Figure 1. Technical and biological validation of the SpA-specific synovial myocyte/myofibroblast gene signature. The expression of four genes (ACTA1, MYH2, MYH7, ACTN2) that were strongly upregulated in SpA versus RA synovitis as assessed by microarray analysis were confirmed by quantitative PCR analysis (qPCR). **A.** Gene expression analysis by qPCR on the same SpA and RA samples that were used for the original microarray analysis (cohort 1). **B.** Gene expression analysis by qPCR on independent samples from patients with non-psoriatic (filled circles) and psoriatic (open circles) SpA, RA (filled squares) and gout (filled triangles), all having a short disease duration (cohort 2). Data represent the mRNA expression of the gene of interest relative to GAPDH and bars show the median-interquartile range. ***p<0.001 **p<0.01, *p<0.05. In panel B, statistical significance between the 3 groups was first assessed by Kruskal-Wallis (KW) non-parametric ANOVA test followed by Dunns' multiple comparison analysis.

Figure 2. Immunohistochemical analysis of myogene expressing cells in SpA synovitis. A. Representative pictures of isotype control (IC), α-SM actin (α-SMA) and smooth muscle myosin heavy chain (SMMHC) staining on frozen sections of SpA synovial tissue. **B**. Semi-quantitative scores for α-SMA expression in the intimal lining layer, synovial sublining, and vessels of synovial membrane biopsy specimens obtained from SpA and RA joints. Data are shown as a mean +/- SD. **C**. Semi-quantitative scores for SMMHC expression in the intimal lining layer, synovial sublining, and vessels of synovial membrane biopsy specimens obtained from SpA and RA joints. Data are shown as mean +/- SD. **D.** Double immunofluorescence staining of α-SMA positive cells with Von Willebrand factor (α-SMA red, vWF green, DAPI blue), CD146 (α-SMA red,

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Figure 3. In vitro and in vivo analysis of the effect of inflammation on myogene expression in SpA. A. Flow cytometric analysis of the expression of α-SM actin by cultured fibroblast like synoviocytes. The histogram represents the geometric mean fluorescence intensity (gMFI) of positively stained cells (black line) compared to the isotype control (solid gray). **B**. Expression of ACTA1 by fibroblast like synoviocytes (n=11) that were not stimulated (negative control), were stimulated with TGFβ1/PDGF (positive control), or were stimulated with pooled synovial fluid from SpA or RA patients. Data are presented as median ± interquartile range mRNA expression of ACTA1 relative to GAPDH. **C**. Expression of myogenes and proinflammatory cytokines in paired synovial biopsy samples obtained at week 0 and 12 of TNF blockade with etanercept in SpA patients. Data are resented as mRNA expression of the gene of interest relative to GAPDH. *$p<0.05$.

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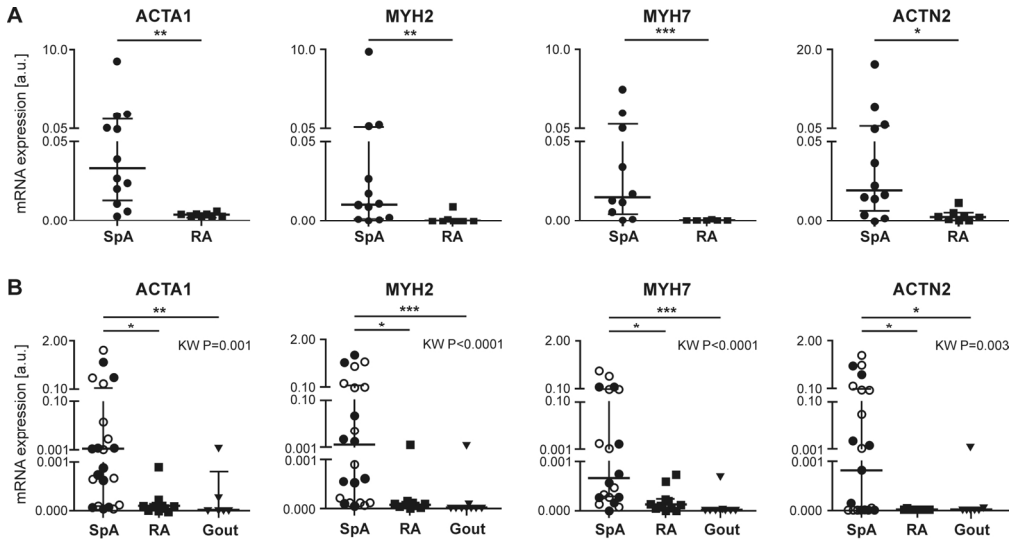

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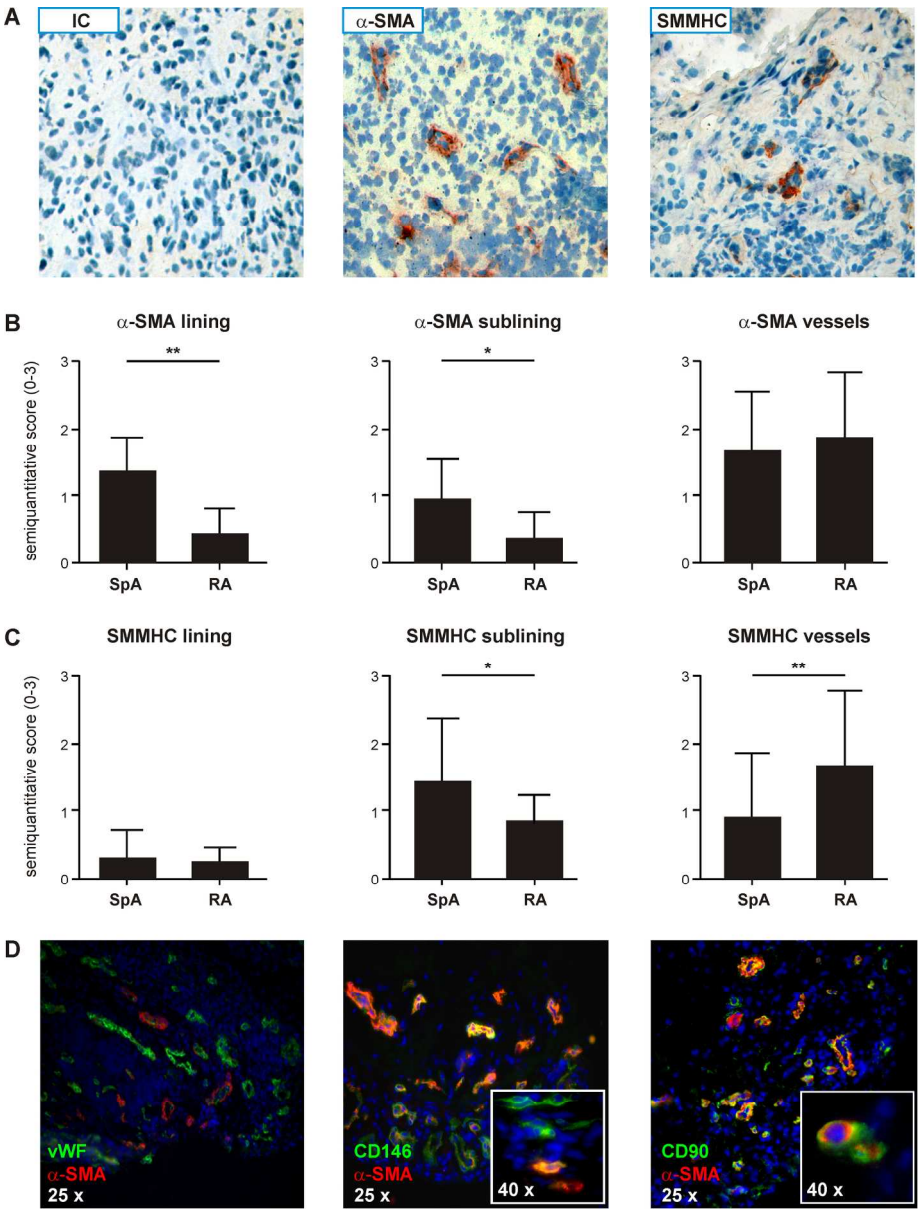

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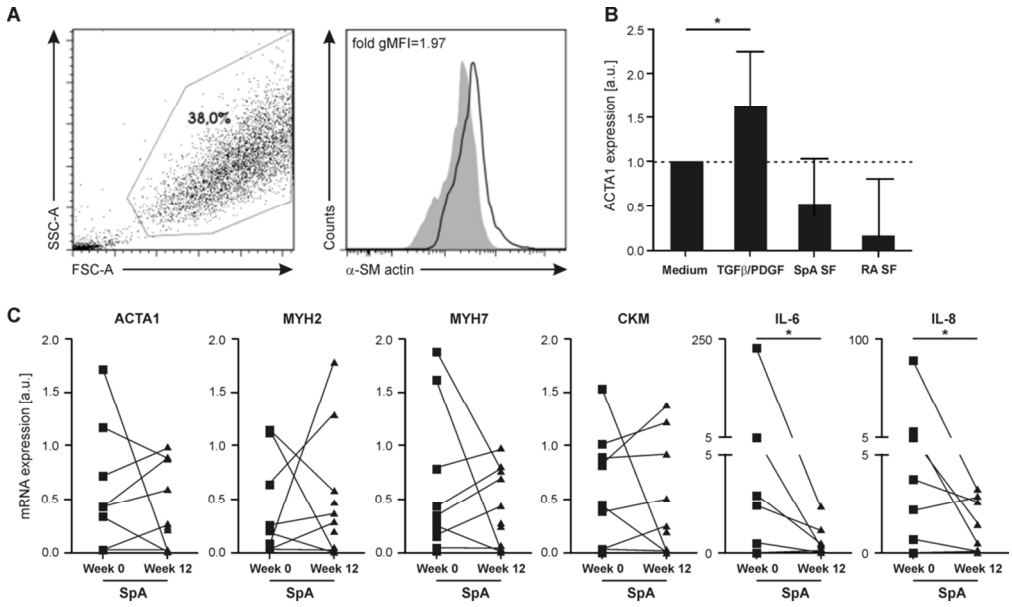

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