Clues to Pathogenesis of Spondyloarthropathy Derived from Synovial Fluid Mononuclear Cell Gene Expression Profiles

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ABSTRACT. ***Objective.*** To use gene expression profiles of spondyloarthropathy (SpA) synovial fluid mononuclear cells (SFMC) to determine if there are transcripts that support the unfolded protein response (UPR) hypothesis, and to identify which cytokines/chemokines are being expressed and which cell fractions are involved.

Methods. Gene expression profiles were generated by microarray screening of SFMC of 5 patients with SpA, 5 patients with rheumatoid arthritis (RA), and peripheral blood mononuclear cells (PBMC) of 6 controls. Results were validated by reverse transcription polymerase chain reaction using samples from a larger panel of subjects.

Results. The repertoires of proinflammatory cytokines/chemokines expressed by SpA and RA SFMC were very similar: monocyte chemotractant protein 1 (MCP-1), interleukin 8 (IL-8), IL-1ß, endothelial-monocyte activating polypeptide II, interferon-γ, and tumor necrosis factor-α. MCP-1 was highly expressed in SpA SFMC. There was enhanced expression of immunoglobulin heavy chain binding protein (BiP) in SpA, which is compatible with the UPR hypothesis. BiP was most highly expressed in the adherent fraction of SpA SFMC.

Conclusion. Previous data postulating UPR in SpA are based on *in vitro* experiments with transfected cell lines. Our patient derived data suggest that it also occurs *in vivo* in the macrophages of SpA joints. (J Rheumatol 2002;29:2159–64)

Key Indexing Terms:
SPONDYLOARTHROPATHY MICROARRAY UNFOLDED PROTEIN RESPONSE

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There is little doubt that one important downstream mediator in spondyloarthropathy (SpA) is tumor necrosis factor-α (TNFα)[1]. It is likely that other cytokines, as well as chemokines, also participate. A number have already been identified in rheumatoid arthritis (RA)[2]. There is insufficient information to know if there are major differences between SpA and RA. In this study, we focused on synovial fluid mononuclear cells (SFMC). Compared to peripheral blood or synovial biopsies, SFMC constitute a reasonable target of study because they are exudative cells, and therefore the results are diluted to a lesser extent with bystander cells. To achieve minimal bias, we used a 1176 gene microarray. Because microarray technology is susceptible to tremendous imprecision, we used microarray only for screening. We compared the microarray results of SFMC to the peripheral blood mononuclear cells (PBMC) of normal individuals. PBMC from patients with RA were not used because in preliminary experiments, some of the cytokine/chemokine genes showed sporadically enhanced expression compared to healthy individuals. Hence, the interpretations of the results here are restricted to those of SF cells.

Our microarray screening generated completely unanticipated observations. It also led us to address the question of which factors are responsible for initiating the cascades of

SpA mediators. The only outstanding SpA related gene identified so far is HLA-B27. Several theories have been proposed as to how HLA-B27 causes arthritis. We have reported previously that expression of HLA-B27 in Hela cells leads to altered signaling and enhanced production of monocyte chemotractant protein 1 (MCP-1)[3]. The manner in which HLA-B27 modifies signaling events was only recently explained when it was reported that HLA-B27 has high potential of being misfolded while being assembled inside the endoplasmic reticulum (ER)[4]. The authors proposed that this might lead to 2 possible intracellular signaling events, known as the endoplasmic unfolded protein response (UPR) and the ER overload response[5]. Our previous MCP-1 finding and this innovative hypothesis are based entirely on studies using *in vitro* cell lines that are transfected with the HLA-B27 gene. There is as yet no corroborative evidence that such events occur in SpA. It is still unknown whether there is an increase in expression of MCP-1 or the hallmark of UPR, immunoglobulin heavy chain binding protein (BiP[6], also known as grp78 or 78 kDa glucose regulated protein) in SpA. In this study, we surveyed our microarray results for support of this novel hypothesis, and further tested it by measuring the BiP transcript, even though the BiP gene was outside our array.

MATERIALS AND METHODS

Patients and controls. Demographics of the study patients and controls are shown in Table 1. Only those controls tested by microarray are shown. Diagnosis and subclassification of arthritis were carried out according to the classification criteria for ankylosing spondylitis (AS), SpA, and RA[7-9]. All patients had active disease with inflammatory spinal pain (for SpA) or swollen peripheral joints (for RA). Medications are shown in Table 1. The total numbers tested included 13 controls providing PBMC, 11 patients with SpA, and 9 patients with RA providing synovial fluid (SF).

Preparation of samples and microarray. PBMC and SFMC were separated from heparinized blood and SF samples by standard Ficoll histopaque centrifugation. Adherent cells were separated from these by culturing on plates for 20 min at room temperature. More than 80% of adherent cells appeared to be macrophages under the inverted microscope. As reported, more than 85% of cells in the adherent cell fraction were CD14+ and

Table 1. Demographics of patients and controls providing samples for microarray.

Controls					
	Sex	Age	B27 Status		
1	M	45	Neg		
2	M	35	Not tested		
3	M	30	Pos		
4	F	31	Not tested		
5	M	29	Not tested		
6	M	25	Not tested		
Patients With SpA					
	Sex	Age	B27 Status	Diagnosis	Medications*
1	M	19	Pos	USpA	
2	M	22	Pos	AS	Sulf
3	M	16	Pos	AS	
4	M	17	Pos	ReA	Sulf
5	F	25	Pos	USpA	Sulf
6	M	34	Neg	ReA	Pred
7	F	37	Neg	USpA	Pred
8	M	32	Neg	AS + Crohn's	Pred
9	F	38	Neg	ReA	Sulf
10	M	40	Pos	USpA	Pred, MTX
11	M	31	Pos	USpa	
Patients With RA					
	Sex	Age	Rheumatoid Factor	Medications	
1	F	70	Pos	Plaquenil	
2	F	65	Neg		
3	F	78	Pos	Pred	
4	F	57	Pos	MTX, sulf, pred	
5	F	50	Pos	Plaquenil, sulf	
6	F	51	Pos	Pred	
7	M	45	Neg		
8	F	81	Pos	MTX	
9	F	55	Pos	Plaquenil	

Doses of prednisone were ≤ 10 mg per day. All patients with arthritis were taking NSAID. SpA: spondyloarthropathy; USpA: undifferentiated spondyloarthropathy; AS: ankylosing spondylitis; ReA: reactive arthritis; RA: rheumatoid arthritis; MTX: methotrexate; Sulf: sulfasalazine; Pred: prednisone. * Doses of prednisone: 7–20 mg/day.

showed light scattering characteristics of monocytes using flow cytometry[10]. Less than 10% of nonadherent fraction cells showed light scattering characteristics of monocytes. Extraction and purification of total RNA as well as microarray assays, using the 1176 gene Atlas Array 1.2 I, was performed following the protocols provided by the manufacturer (Clontech, Palo Alto, CA, USA). The genes in this Atlas 1,2 Array consisted of 200–600 bp cDNA fragments. For each array, 5 μg of total RNA was labeled with ^{32}P(dATP). Datum for each gene in each microarray test was recorded as intensity of signals. For comparison among samples, the intensities of all genes were normalized to that of G3PDH (glyceraldehyde-3-phosphate dehydrogenase).

Reproducibility of our microarray assays. To test the reproducibility of the microarray system, one sample of PBMC from a patient with AS was subjected to 2 microarray tests. The correlation coefficient of values between the 2 experiments was high (r = 0.97). In a second experiment, PBMC was prepared from a patient with RA one week apart, and microarray assays tested. The correlation coefficient of values between the 2 samples was also high (r = 0.91). In contrast, when the AS PBMC was compared to the RA PBMC, the correlation coefficient was much lower at 0.5. Because of this degree of reproducibility, and because we were using the microarray only as a screening tool, only one microarray assay was carried out in each of the samples described. The microarray screening threshold that we arbitrarily selected was sufficiently low to generate potentially false positives. In the initial analysis, no genes would be considered positively selected if the Bonferroni correction was taken into consideration in the threshold. Because of that, it was not applied in microarray analysis. As recommended by the manufacturer, a particular array result would not be considered definitive unless corroborated by another method of gene expression analysis. In our experiments, reverse transcription polymerase chain reaction (RT-PCR) was used for corroboration, and a microarray result was arbitrarily considered to be inaccurate if not verified by RT-PCR. Estimate of percentages of false positives will be described in the results section.

Semiquantitative RT-PCR and ELISA. Two methods were used for semiquantitative RT-PCR tests. The first method used an external standard to compare results of different samples. As reported, to construct an external standard, for each gene, serial dilutions of a sample of amplified cDNA were electrophoresed and measured, and their densitometry values plotted into a best-fit logarithmic standard curve using the Curve Expert 1.34 software (Starville, MS, USA)[11]. The other semiquantitative RT-PCR method used commercial competitive RT-PCR kits, following protocols provided by manufacturer (Maxim Biotech, San Francisco, CA, USA). Primer sequences were purchased from Clontech.

Statistical analysis. Comparison of gene expression data was carried out by Student's t test and Mann Whitney test. For RT-PCR results, differences were regarded as statistically significant if p values were less than 0.05. Correlation matrices were calculated by the classical parametric coefficients.

RESULTS

Screening with microarray and RT-PCR testing of cytokine/chemokine genes. Microarray tests were carried out with SFMC of 5 patients with SpA and 5 patients with RA. These were compared to PBMC of 6 controls. We then used the microarray data to screen for genes that might be expressed at higher levels than PBMC. To do this, we compared the mean values of SFMC to those of PBMC. To avoid false negatives, we set an arbitrarily low threshold of taking into consideration all those genes in which the p values were < 0.05 by Student's t test; the ratios between SFMC and PBMC values were more than 2-fold; and the differences in mean microarray values between patients and controls were higher than background values. As derived from the results shown in Table 2, our microarray threshold generated 17% false positives. In this first screening, we focused on only about 300 genes within the microarray. This panel contained those encoding cytokines/chemokines, growth factors and their receptors, participants of apoptosis/stress processes, and markers of monocyte/lymphocyte differentiation. From this preliminary screening, 23 genes were identified. Because this microarray threshold was an arbitrary one, these 23 genes were assayed independently with a semiquantitative RT-PCR. In this RT-PCR assay, the number of normal PBMC samples was increased to 13. Table 2 shows that the numbers of genes that were expressed higher in SpA and RA SFMC compared to normal PBMC were 17 and 20, respectively. The cytokine/chemokine genes that were expressed more highly in SFMC compared to normal PBMC were MCP-1, interleukin 8 (IL-8), IL-1ß, endothelial-monocyte activating polypeptide II (EMAPII), interferon-γ (IFN-γ), and TNF-α.

In SpA, the cytokine/chemokine gene with the highest

Table 2. Mean RT-PCR values of PBMC from controls and SFMC from patients with SpA and RA.

Genes	Control PBMC	SpA SFMC	p*	RA SFMC	p*
MNDA	2.7	117.9	0.001	32.7	0.001
MCP-1	0.8	54.4	0.001	103.9	0.028
IL-8	0.9	314.0	0.001	1110.4	0.001
IL1β	1.2	5.2	0.002	15.7	0.002
EMAPII	1.0	2.5	0.009	5.1	0.003
IFN-γ	0.7	1.3	0.016	1.7	0.001
TNF-α	1.0	17.4		11.2	0.01
PDGF	1.8	2.1		1.2	
IL-6	1.5	26.6		30.5	
S-L cytokine	1.1	1.1		1.0	
TGF-β	2.9	33.0	0.001	30.6	0.001
TG-β2	1.0	11.5	0.001	20.2	0.001
CCR1	1.1	25.6	0.002	46.0	0.012
IL-2Rα	1.0	7.0	0.003	11.1	0.003
CXCR4	6.0	24.9	0.005	45.8	0.004
TNFR2/p75	14.2	13.6		69.9	0.004
c-jun	9.2	98.8	0.004	178.0	0.001
JAK3	1.8	8.4	0.005	24.1	0.012
CD27L	3.0	7.4	0.009	6.6	0.009
CD44	1.9	10.7	0.002	14.2	0.003
LFA-1	90.1	142.9	0.300	144.4	0.034
Cathepsin D	18.3	43.2	0.016	61.8	0.001
HSP 90A	3.7	56.1	0.001	93.8	0.004

* p values are given when results are significantly different compared to control PBMC. Abbreviations for gene numbers (GenBank Accession numbers): MNDA: myeloid cell nuclear differentiation antigen (M8/750); MCP-1: monocyte chemotactic protein 1 (M24545); EMAPII: endothelial-monocyte activating polypeptide II (410117); PDGF: platelet derived growth factor (X06374); SL cytokine: FMS related tyrosine kinase 3 ligand (U04806); HSP90A: heat shock 90 kDa protein A (X07270); CD27L: CD27 ligand (S69339).

statistical significance was MCP-1. This is important because we have previously reported that out of a panel of 16 cytokines/chemokines, MCP-1 was the gene being upregulated by the presence of HLA-B27[3]. To validate the present MCP-1 data, we repeated the RT-PCR assay in a completely separate experiment. In this repeat assay, we extended the number of samples to include SFMC from 11 SpA and 9 RA patients. The results of the 2 separate RT-PCR assays were almost identical ($r = 0.97$, MCP-1 higher in SpA and RA SFMC compared to normal PBMC, $p < 0.0001$ and $p < 0.0001$ for SpA and RA, respectively). In all subsequent RT-PCR assays, this large sample panel was utilized.

Since MCP-1 is only one member of a C-C chemokine subfamily, we proceeded to assay the other members: MCP-2, MCP-3, and MCP-4. For MCP-4, no patient sample was statistically higher than normal PBMC. For MCP-2 and MCP-3, the values for SpA SFMC or RA SFMC samples were statistically higher than the corresponding values in PBMC ($p < 0.05$ and 0.0002 for SpA, $p < 0.018$ and 0.0002 for RA) (Figure 1). There was no statistically significant difference between the 2 arthritis groups.

All the above RT-PCR assays used external standards for measurement. To ensure the validity of the method, we selected 7 samples and assayed for MCP-1 using a competitive RT-PCR method. The results obtained with the 2 methods were almost indistinguishable ($r = 0.97$). Using samples outside this particular study, we have also compared results of the 2 RT-PCR assays for the genes TNF-α and IL-1ß. Their correlation coefficients also exceeded 0.8. In summary, the repertoires of statistically significant genes expressed in SpA and RA SFMC were almost identical, and MCP-1 was highly expressed in SpA.

Screening for genes that discriminate SpA SFMC. For additional clues, we attempted to use microarray results of the entire panel of 1176 genes to search transcripts that were significantly higher in the 5 SpA SFMC samples compared to the 5 RA SFMC samples. In this second screening, we set up the very high threshold criteria of selecting genes that were ≥ 100× higher in SpA, and for which the p values when comparing the means were < 0.05. The only gene that satisfied these criteria was proteasome component C2 (proteasome subunit alpha type 2). This gene was assayed with RT-PCR. We used a sample of PBMC from 13 controls, SFMC from 11 SpA and 9 RA patients, as with the previous MCP experiments. Results confirmed the conclusion derived from the microarray (mean ± SD of RT-PCR values in SpA SFMC and RA SFMC = 91.6 ± 36.7 and 18.5 ± 3.6, respectively; $p < 0.037$). This was our first clue that there was an ER UPR. As a negative control, we tested a proteasome component outside our array panel: low molecular protein 2 (LMP2), which is regulated by a separate process[12]. There was no difference in LMP2 between SpA SFMC and RA SFMC (30.5 ± 39.4 and 25.8 ± 31, respectively; $p = 0.46$).

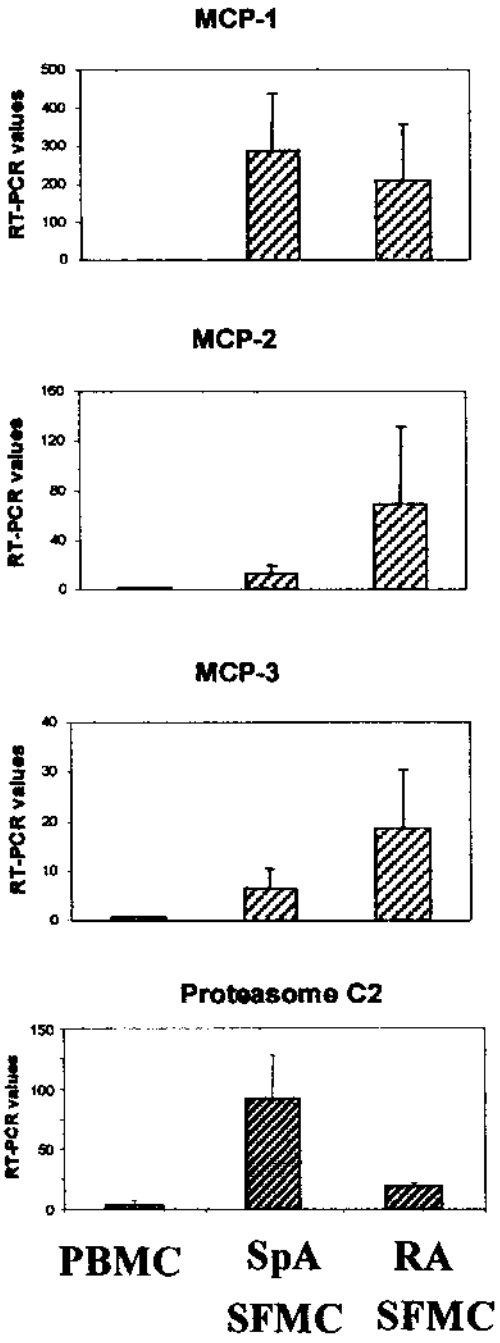

Figure 1. Mean RT-PCR values of MCP-1, MCP-2, MCP-3, and proteasome C2 in PBMC from controls, and also SFMC from patients with SpA and RA. Error bars represent standard errors. PBMC: peripheral blood mononuclear cells; SFMC: synovial fluid mononuclear cells.

Testing the expression of BiP, a measurement of the ER UPR. This clue led us to measure the expression of a gene that was again outside the array panel, BiP, which is invariably increased during an ER UPR[6]. In this experiment, we used the same samples as described for RT-PCR evaluation

of MCP and proteasome components. Strikingly, the RT-PCR values for BiP in SpA SFMC were also higher than those for RA SFMC (86.4 ± 111.3 and 18.5 ± 13, respectively; p = 0.044). To validate the BiP RT-PCR method, we tested the SFMC samples with a BiP competitive RT-PCR system. Again, the SpA SFMC values ($40.2 \pm 35.5 \times 10^{18}$ M) were significantly higher than the RA SFMC values ($9.1 \pm 7.7 \times 10^{18}$ M) ($p < 0.039$).

All studies were carried out with unfractionated SFMC. From 2 SpA and 2 RA patients, adherent as well as nonadherent fractions were available. Their BiP transcripts were assayed twice. Results of the 2 separate assays were identical. Figure 2 shows very clearly that the BiP values were high in SpA because of contribution by the adherent fraction. BiP in the SpA adherent fraction was 20× higher in the corresponding nonadherent fraction (p = 0.02), and also 20× higher than the RA adherent fraction (p = 0.038). Also in contrast to SpA, in RA, there was no statistically significant difference between adherent and nonadherent fractions.

DISCUSSION

Our study addressed 2 questions. First, we asked which transcripts encoding proinflammatory molecules are detectable in SFMC of SpA. Rather unexpectedly, we found that the repertoires in SpA and RA were identical. Surprisingly, in SpA, the proinflammatory chemokine that showed the highest degree of statistical difference from PBMC was MCP-1. We reported previously that expression of HLA-B27 modifies Hela cells to upregulate MCP-1[3]. Hence, the present MCP-1 observation might also be related to the pathogenesis of SpA.

Second, we asked what these SpA pathogenic factors might be. So far, the only gene clearly associated with SpA is HLA-B27. Under physiological conditions, an HLA-B27 is first generated in the ER as a heavy chain. The heavy chain then becomes complexed with a light chain ($ß_2$-microglobulin, $ß_2$m), and subsequently a polymorphic antigenic peptide, before acquiring a completely folded protein structure. A commonly recognized function of completely folded HLA-B allele molecules is to activate antigen-specific CD8+ T lymphocytes. Consequently, one widely held view is that HLA-B27 induces arthritis by presenting certain autoimmune arthritogenic peptides to disease-mediating CD8+ T lymphocytes[13,14]. In support of this is the observation that there is oligoclonal T cell activation in the joints of patients with reactive arthritis[15].

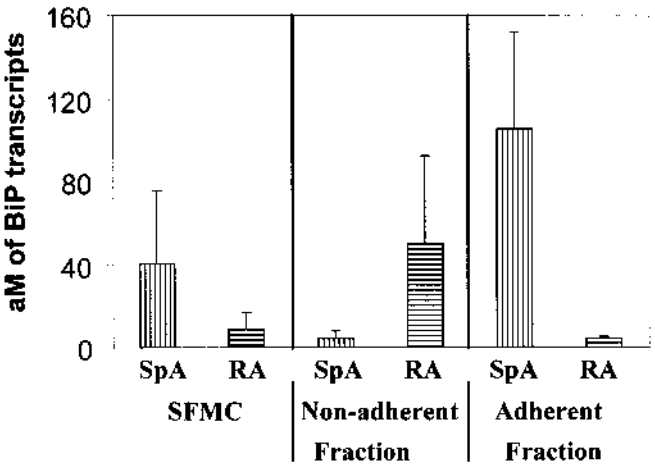

Figure 2. Results of BiP competitive RT-PCR in SFMC from patients with SpA and RA as well as in the adherent and nonadherent fractions. Error bars represent standard deviations. aM: 10^{18} M; PBMC: peripheral blood mononuclear cells; SFMC: synovial fluid mononuclear cells.

It is almost certain that SpA is induced by multiple factors, and that the arthritogenic peptide pathway is not the exclusive process. The arthritogenic peptide hypothesis would not explain why, for example, HLA-B27 transgenic mice would develop arthritis even in complete absence of mouse or human $ß_2$m[16]. Neither would it explain the *in vitro* experimental observations that a monocyte cell line transfected with HLA-B27 accommodates survival of arthritis-causing bacteria differently from control cells[17], and why Hela cells, when transfected with HLA-B27, appear to have additional signaling events when confronted with such bacteria[3]. Although intriguing, the major drawback with these challenging animal and *in vitro* observations is that there are no parallel experimental observations in patients with SpA. The several diversified observations have not been integrated into a unified concept, at least until recently. A new hypothesis is based on the observation that in culture cell lines, HLA-B27 proteins fold at a reproducibly slow rate, apparently because they have low affinity for stabilizing peptides. This slower rate leads to ER accumulation of misfolded and also partially folded heavy chain proteins[4]. In general, overloading of ER with misfolded proteins activates a large repertoire of genes, a process known as the UPR[18]. This new hypothesis postulates that the UPR from overloading of misfolded HLA-B27 heavy chains can also activate arthritis-mediating genes[5].

Until now there has been no published evidence that misfolding events occur *in vivo* in the joints of patients with SpA. Among the genes activated by UPR in yeast is SON1/RPN4[19]. This is a transcriptional regulator for genes encoding subunits of the proteasomes. Proteasomes are multiunit catalytic units responsible for protein degradation[12]. They are functionally linked with the ER, because misfolded ER proteins are dislocated from the ER into the cytoplasm where they are degraded by proteasomes. Taking all this into consideration, it is striking that among the genes we have found to be overexpressed in SpA compared to RA SFMC is a proteasome component. The proteasome finding led us to BiP, also designated as GRP78, which is a chaperone protein resident inside the ER. Because transcription of BiP is very sensitive to UPR[6], the level of BiP is commonly accepted as a reflection of the degree of UPR.

Remarkably, in our experiments, BiP is higher in SpA SFMC compared to RA SFMC.

In summary, we discovered that in SpA SFMC, there are increases in transcripts encoding MCP-1, proteasome subunit C2, and BiP. Increases in BiP suggest the existence of an ER UPR. However, SFMC consist of 2 major cell populations, macrophages and T lymphocytes. In preliminary experiments, we addressed whether the increase in BiP was restricted to only one cell type. Adherent and nonadherent cell fractions of RA SFMC samples were compared to SpA SFMC samples. The results showed clearly that BiP was much higher in SpA adherent cells, indicating that macrophages are the cell type involved. This is notable because, although not statistically significant, BiP expression in RA was higher than SpA in the nonadherent cell fraction. Enhanced expression of BiP in RA has already been reported and has been proposed as an autoantigen in RA[20,21].

The study of UPR in HLA-B27 SpA is only in its infancy. There are no reports in the literature to support its presence in patients with SpA, or to help investigators decide which patient cell types should be analyzed. The results of our study, although preliminary, not only support the hypothesis but also indicate that the rather neglected SpA SF macrophage cells should be targets of research.

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