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Mesenchymal stem cells in rheumatoid synovium: Enumeration and functional assessment in relation to synovial inflammation level

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Abstract

Objective. Achieving joint regeneration in rheumatoid arthritis (RA) represents a future challenge. Autologous synovial mesenchymal stem cells (MSCs) could be therapeutically exploited. However, the inflammatory milieu in the RA synovium could adversely affect endogenous MSC function. To test this hypothesis, the frequency and multipotency of RA synovial MSCs was evaluated in relation to existing synovial inflammation.

Methods. Synovial inflammation was measured using arthroscopic visual analogue score (VAS) and further validated using immunohistochemistry and flow cytometry. Highly-proliferative clonogenic *in vivo* MSCs were enumerated following fluorescence-activated cell sorting and expansion for 20 population doublings. MSC multipotency was quantified following standard *in vitro* culture-expansion and trilineage differentiation assays. Real-time PCR, flow cytometry and ELISA were used to evaluate pro- and anti-chondrogenic molecules in standard polyclonal synovial MSCs.

Results. Arthroscopic visual score of inflammation (VAS) significantly correlated with synovial macrophage infiltration. In RA, synovial MSC chondrogenesis was inhibited in direct relation to VAS (r=-0.777, p<0.05) and reduced compared to control OA-MSCs (p<0.05). *In vivo* MSCs resided in the synovial fibroblastic/stromal fraction ($CD45^-CD31^-$) and were reduced in frequency in relation to VAS (r=-0.695, p<0.05). In RA-MSCs, CD44 levels correlated negatively with inflammation and positively with chondrogenesis (r=-0.830 and r=0.865, respectively). Cytokine production and Sox9 expression was similar between RA- and OA-MSCs.

Conclusions. Our findings demonstrate a negative relationship between synovial MSC chondrogenic and clonogenic capacities and the magnitude of synovitis in RA. Effective suppression of joint inflammation is therefore necessary for the development of autologous MSC therapies aimed at cartilage regeneration in RA.

Key words: mesenchymal stem cells, synovium, inflammation, chondrogenesis

INTRODUCTION

Current therapies have greatly reduced the degree of joint destruction in rheumatoid arthritis (RA); however achieving cartilage regeneration in RA remains a challenge. Regenerative medicine strategies, including autologous chondrocyte implantation, have already shown efficiency in the treatment of solitary chondral injuries. [1-2] Distinct chondral lesions in osteoarthritis (OA) are the next therapeutic target. [3-8] Developing similar strategies in RA is however much more challenging. RA afflicts multiple joints, is associated with widespread tissue inflammation and is characterised by a global denudation of articular cartilage. Classical tissue engineering approaches are therefore impractical necessitating the development of alternative strategies.

Together with articular chondrocytes, multipotential mesenchymal stromal cells, commonly termed mesenchymal stem cells (MSCs), are recognised sources of regenerative cells for cartilage repair. [6, 9] Originally discovered in the bone marrow (BM), MSCs have now been found in several joint tissues, including the synovial membrane [7, 10], synovial fluid (SF) [11, 12], tendon [13], periosteum [14] and joint fat. [15, 16] Distinct niche-specific "identities" of BM- and joint MSCs have been recognised. [7, 14, 17, 18] This has challenged the original concept proposing systemic mobilisation of BM-MSCs, their circulation and homing to the joint in response to injury. [19] Alternatively, recent studies have highlighted an important role of local synovium-derived MSCs in joint repair responses. [12, 18] Direct recruitment of synovial cells into chondral defects [20] and their homing to injured sites [12] has been demonstrated *in vivo*. These findings provide a rationale for the study of autologous synovial MSCs in RA and suggest that redirecting *in vivo* MSC trafficking from synovium towards denuded cartilage could be explored as a potential '*in situ*' cartilage regeneration strategy in RA.

The synovium has a common embryological origin with articular cartilage [21], and synovial MSCs show good intrinsic chondrogenic activity in health. [7] However, the chondrogenic commitment of synovial MSCs is not stable in a non-chondrogenic environment *in vivo*. [22] With respect to RA, it is also critical to consider the effects of the disease-associated inflammatory milieu, since inflammatory cytokines have previously been shown to suppress differentiation of chondrocytes and BM-MSCs. [23-25] This study therefore tested the hypothesis that the pro-inflammatory environment associated with RA interferes with the normal function of synovial MSCs. We compared the capabilities of MSCs from RA synovium with control non-inflamed OA synovium and with normal BM MSCs. The extent of MSC abnormalities in RA was examined in relation to the magnitude of synovial inflammation, which was assessed by arthroscopy, immunohistochemistry and flow cytometry.

METHODS

Detailed methods of synovial MSC expansion, differentiation, qPCR, ELISA and statistical analysis are presented in Supplementary Methods. Supplementary Tables 1, 2 and 3 detail patient sample distribution, primary antibodies and PCR primers, respectively.

Patient cohorts and arthroscopy

Ethical approval was obtained from Leeds Teaching Hospitals NHS Trust Ethics Committee. Synovial tissue biopsies from the suprapatella pouch (median weight 82 mg) were obtained from 45 RA and 5 OA patients using 3.5mm grasping biopsy forceps under direct vision using a Hopkins 2.7mm 300 arthroscope. Visual Analogue Score (VAS) was used as a measure of macroscopic joint inflammation. Scores (scale between 0 and 100) were established upon joint inspection based on visual impression of vasculature (vessels

and redness due to hyperemia). [26] Additional OA synovial tissues (used in flow cytometry, clonogenicity and gene expression experiments) were obtained during knee arthroplasty (*n*=6). Due to the large number of experiments performed, tissue samples were distributed amongst different studies (Supplementary Table 1). BM-MSCs (*n*=6) were generated from posterior iliac crest aspirates of normal donors.

Assessment of synovial inflammation by immunohistochemistry and flow cytometry

Unless otherwise indicated, all chemicals were purchased from Sigma (Poole, UK). Anti-CD3 and CD68 staining was performed on 4μm-thick serial cryostat sections and visualized using Chemmate HRP (Dako, High Wycombe, UK). [27] For scoring, both semi-quantitative [28, 29] and digital [30] methods were used. For flow cytometry, synovial tissue was first digested with collagenase (Stem Cell Technologies, Vancouver, Canada) for 4 hours at 37°C, to release single cells. 10^6 cells from synovial tissue digests were stained with CD45-FITC, CD14-PE and propidium iodide (PI). The proportion of resident monocytes ($CD45^+CD14^+$) was calculated in relation to total live (PI^-) cells. Data acquired by BD FACScan. [11, 31]

Enumeration of highly clonogenic *in vivo* MSCs in synovial tissue digests

To accurately enumerate *in vivo* synovial MSCs prior to culture-expansion, synovial tissue digests (10^6 cells/test) were stained with CD45-FITC, CD31-PE and PI. Haematopoietic ($CD45^+PI^-$) and endothelial cells ($CD45^-CD31^+PI^-$) were removed by cell-sorting (MoFlo cell sorter). Fibroblastic/stromal cells ($CD45^-CD31^-PI^-$) were seeded into 96 well plates (50 cells/plate) to generate single-cell derived clones, which were expanded in standard MSC expansion media until senescence. The number of population doublings (PDs) was calculated based on a formula $\log_2(N)$, where N is the ultimate number of cells at senescence. *In vivo* MSC frequency was calculated as the number of clones grown for 20 PDs/total $CD45^-CD31^-$ cells seeded)x100%.

RESULTS

Arthroscopic VAS as an *in situ* measure of synovial inflammation

Throughout this study, RA synovial MSC functions were correlated with the inflammatory status of the synovium at the time of arthroscopy. Due to ethical constraints, the size of biopsy had to be minimal and within guidelines for best clinical practice. Consequently, arthroscopic VAS was selected as a primary measure of synovial inflammation, since establishing such a score required no collection of extra tissue, whilst offering more tissue for live cell experimentation, including tissue culture and cell sorting. VAS is a rapid and reliable measure of *in situ* synovial inflammation [32], with particularly high inter-observer reliability. [26] To further justify its use in this study, VAS was validated against well-known clinical and tissue indicators of inflammation. In the RA group, the VAS score correlated positively with C-reactive protein (CRP), a marker of systemic inflammation, although not to a significant degree with DAS28 (Figure 1A); a composite score including a more global measure of patients' general health. Synovial tissue macrophages have been reported as sensitive biomarkers of response to treatment in patients with RA. [33] VAS correlated significantly with sublining layer CD68 and CD3 staining, determined by immunohistochemistry using semi-quantitative scoring (Figure 1B and C). For CD68, both semi-quantitative and digital scoring were performed demonstrating good correlation with each other (r=0.889, p=0.0001, Supplementary Figure).

To confirm the link between VAS and the extent of inflammatory cell infiltration into the synovium, a flow cytometric test on synovial digests was developed. Viable cells were gated by PI exclusion and a standard combination of CD45/CD14 was used to identify synovial monocytes (Figure 1D). A direct positive correlation was observed between VAS

and the percentage of infiltrating monocytes (Figure 1D), consistent with the immunohistochemistry data. The proportion of monocytes in a sample of normal human synovium (generous gift from Prof Cosimo De Bari) was 10%.

In summary, these data demonstrated that VAS correlated well with several objective measurements of inflammatory cell infiltration into the synovium and hence was a reliable measure of synovial inflammation.

Characterisation of standard polyclonal synovial MSC cultures in RA

Following synovial tissue digest, one million cells from 8 RA and 5 OA patients were used to initiate synovial MSC cultures (Table 1). Data from representative RA and OA patients are shown on Figure 2A. Compared to skin fibroblasts, all MSC cultures possessed mesenchymal tri-potentiality (Figure 2B). However, the chondrogenic potential of passage 3 (p3) RA-MSC cultures (measured by sulfated glycosaminoglycan (sGAG)/pellet) was ~3-fold lower compared to OA (p=0.027). Moreover, a direct inverse relationship was observed between chondrogenesis of RA cultures and level of synovial inflammation assessed by VAS (Figure 2B, top panel) or by the proportion of infiltrating monocytes (n=6, r=-0.807, p<0.05). No significant differences between RA and OA were found in adipo- or osteogenic assays (Figure 2B, middle and bottom panels). Therefore, synovial inflammation specifically affected synovial MSC chondrogenesis in RA.

Table 1. Synovial MSC differentiation experiments: Patient characteristics

	RA (n=8)	**OA** (n=5)
Age	62 (31-73)*	62 (42-76)
Sex	7 Female/1 Male	4 Female/1 Male
VAS	43 (0-68)	5 (0-9)
Current medication	Infliximab/MTX, n=4 Infliximab/MTX/NSAIDs, n=1 Etanercept, n=3	NSAIDs, n=2 No treatment, n=3

* - all values are shown as median (range), MTX – methotrexate, NSAIDs – non-steroid anti-inflammatory drugs.

Enumeration of *in vivo* MSCs in RA synovium

Although MSC definitions vary, most groups agree that *in vivo* MSCs are highly proliferative, clonogenic cells that reside within tissue stroma and proliferate for at least 20 PDs. [31, 34] To enumerate in vivo MSCs directly in RA and OA synovium, tissue digests were subjected to FACS sorting for haematopoietic ($CD45^+$), endothelial ($CD45^-CD31^+$) and fibroblastic/stromal ($CD45^-CD31^-$) fractions. Colony-forming cells were exclusively present in the fibroblastic/stromal fraction (Figure 3A). Therefore *in vivo* MSC frequency was measured within this fraction, by seeding the sorted $CD45^-CD31^-$ cells at a low density and expanding single colonies for at least 20 PDs. Altogether, 54 clonal cultures were generated – 33 from OA and 21 from RA synovium (n=9 and 7 patients, respectively). All nine randomly selected clonal cultures possessed MSC tripotentiality (data not shown).

The proportion of *in vivo* MSCs was 1.5-fold higher in OA compared to RA, but the differences failed to reach statistical significance (Figure 3B). A significant negative correlation was however observed between the *in vivo* MSC content of RA stroma and VAS (Figure 3B, right panel). The frequency of less proliferative progenitors (capable of a maximum of 13 and 17 PDs prior to senescence) was similarly found to decline with VAS, albeit less significantly (r=-0.501 and r =-0.680, respectively). Furthermore, clonal RA-

MSCs were more heterogeneous and on average expanded slower than clonal OA-MSCs (p=0.026, Figure 3C). Overall, synovial inflammation was associated with a reduced frequency of rapidly-growing *in vivo* MSCs within the RA stroma.

The marker phenotype of RA-MSCs and uncultured fibroblasts

Phenotyping was performed to confirm the MSC nature of standard polyclonal (p3) and clonal synovial cultures and to identify potential markers associated with MSC chondrogenicity in RA. Both RA- and OA-derived cultures exhibited a classic MSC phenotype (positive for CD73, CD105, CD90) and no significant differences in the expression of these markers were found (Figure 4A). The expression of potential chondrogenic markers (CD44, CD151, CD166) [8, 35], measured as mean fluorescence intensity, was also investigated in relation to VAS in RA. No correlations were found, with the exception of CD44, which declined significantly with VAS (Figure 4B). Importantly, CD44 expression levels correlated positively with cultures' chondrogenesis (Figure 4C). These data confirmed previous findings describing CD44 as a potential chondrogenic marker. [18, 35]

Marker expression was compared between RA-MSCs (p3 and clonal) and fibroblastic/stromal cells ($CD45^-CD31^-$) in fresh tissue digests (Figure 4D). MSCs had significantly higher proportions of $CD166^+$ and $CD105^+$ cells compared to uncultured fibroblastic cells (~64- and 4-fold for p3-MSCs and 81- and 4-fold for clonal clutures, respectively), confirming previous findings that CD166, in particular, could be considered as a potential marker for further enrichment of synovial MSCs. [36]

Molecular phenotype and cytokine production by synovial RA-MSCs

To explore the molecular basis of the reduced chondrogenesis of RA MSCs, the expression of several pro- and anti-chondrogenic molecules was assessed by real-time PCR (Figure 5). The expression of the chondrogenic "master switch" Sox9 [37] was similar between RA and OA p3-MSCs. No correlation was observed between Sox9 and sGAG/pellet or between Sox9 and VAS (data not shown). NF-κB is a molecule implicated in inhibiting MSC chondrogenesis [23, 38]; the expression of its pro-inflammatory subunit p65 did not differ between RA and OA (Figure 5). Molecules involved in cartilage turnover (MMPs and TIMPs) [39], also showed no significant differences in expression between RA- and OA-MSCs (MMP9 expression was below detection). Furthermore, markers of the "destructive" phenotype of RA fibroblasts (Galectin 3 and SUMO) [40-42], did not differ between RA- and OA-MSCs.

Pro-inflammatory cytokines could be produced by MSCs from inflamed environments and therefore interfere with their chondrogenesis. Consistent with previous findings [43, 44], all p3 synovial MSCs released IL-6, with no difference between RA and OA (average 38 and 37 pg/ml, respectively). IL-1β production was below detectable levels in all MSCs. TNF-α was also below detection, both at mRNA and protein level. Therefore, no link could be established between expression of these molecules in standard polyclonal MSCs and synovial inflammation levels.

DISCUSSION

To date, only a few studies have explored MSCs in RA and these were focussed on BM-MSCs. [45, 46] This study is the first investigation of the regenerative "fitness" and the MSC content of RA synovium. It shows a negative relationship between the magnitude of synovial inflammation and the regenerative capacity of RA synovium, both in terms of the numbers of resident *in vivo* MSCs and their ability to generate multipotent cultures competent in chondrogenesis.

Arthroscopic VAS was validated as a suitable measure of synovial inflammation. A flow cytometry method was also developed for the analysis of different cell fractions present in synovial tissue digests; this demonstrated an increased influx of immune cells into the RA synovium. Whilst consistent with the immunohistochemistry data, flow cytometry had the additional advantage of enabling us to sort different synovial cell fractions and assess their *in vivo* MSC content. By sorting haematopoietic, endothelial and stromal/fibroblastic cells from tissue digests we showed that *in vivo* MSCs resided exclusively in the stromal/fibroblastic fraction, representing a small proportion of these cells (0.1-0.8%). To the best of our knowledge no other study to date had enumerated synovial MSCs prior to culture-expansion.

In RA, *in vivo* MSCs showed an inflammation-related reduction in numbers. Extensive proliferation leading to synovial hyperplasia [47] could explain this reduction. Clonal RA-MSCs were more heterogeneous in their proliferative capacity and on average grew slower than clonal OA-MSCs. This could be explained by variable premature telomere shortening previously observed for RA BM-MSCs. [45] Functional heterogeneity of clonal synovial MSCs was first described by De Bari et al in 2001. [10] Our data suggest that this heterogeneity in RA may be exacerbated by ongoing inflammatory processes. Despite an apparent reduction, MSCs were however present even in the most inflamed synovial tissues, potentially explaining clinical observations of apparent synovial regeneration after synovectomy. [48]

We observed inflammation-related down-regulation of chondrogenesis in standard polyclonal synovial RA-MSCs. Low-VAS cultures were as chondrogenic as control BM-MSCs whereas high-VAS cultures (VAS>40) yielded very low amounts of sGAG. The cellular and molecular mechanism of this down-regulation is likely to involve several complex processes. A direct role for TNF-α in the inhibition of MSC chondrogenesis has been reported using similar pellet cultures [23, 25], but we did not detect any TNF-α production in RA-MSCs. Expression of NF-κB, as well as of several mediators of cartilage degradation also did not differ between RA and OA. Finally, no relationship could be established with the expression of Sox9, the inducer of chondrogenesis. [37] Despite the fact that no direct link between these molecules and VAS prior to differentiation was found, their abnormal regulation during the chondrogenic differentiation of RA-MSCs remains a possibility and merits further investigation.

The "*in vitro* age" of synovial OA-MSCs has been associated with reduced chondrogenesis. [8] The most inflamed RA tissues contained fewer culture-initiating MSCs, which could have consequently led to "older" proliferative age of cultures at p3. However, calculated PDs at p3 revealed only small differences (10.6 versus 12.8 PDs for VAS 0 and 62, respectively), both within control BM-MSC range (10-13 PDs), arguing against this hypothesis.

Extensive *in vitro* cultivation is likely to reduce the environmental effects of joint inflammation on MSC function. However, emerging data on MSCs from other tissues suggests that epigenetic pre-programming of undifferentiated MSCs is maintained during prolonged expansion and differentiation *in vitro* [49], explaining the preservation of "niche-specific" propensities of single MSCs after extensive cultivation [7, 18, 31]. It could therefore be speculated that a "memory" of the inflamed niche, from which MSCs were extracted, could be imprinted in cultured RA-MSCs. To address this issue, a comparison

between uncultured *(in vivo)* and expanded synovial RA-MSCs would be necessary. In this respect, our study provides an essential step forward towards the purification of *in vivo* synovial MSCs, by describing their residence and frequency within the $CD45^{-}CD31^{-}$ fraction. CD166 [36] and CD105 [50] could be used as additional selection markers in view of the high expression observed on standard polyclonal and, particularly, clonal MSCs compared to synovial fibroblasts.

Importantly, we detected one surface molecule which could be linking RA synovial inflammation and MSC chondrogenesis. CD44 levels on RA-MSCs were more variable compared to OA-MSCs and correlated positively with RA-MSC' chondrogenesis. The role of CD44 in chondrogenesis could include its ability to anchor and retain the proteoglycans produced by differentiating chondrocytes [35] or MSCs. [18] CD44 expression in RA-MSCs also correlated negatively with VAS. These data are concordant with previous reports showing reduced CD44 expression in RA fibroblasts compared to health. [51] Investigating the regulation of CD44 on RA-MSCs may unravel the potential mechanism of inflammation-related decrease in their chondrogenesis.

Finally, considerations were given to ongoing drug therapy. Although our chondrogenesis cohort was relatively small, all subjects were treated with anti-TNF agents (Infliximab or Etanercept). The fact that synovial MSC chondrogenesis in this cohort correlated with residual inflammation suggested that the key determinant in suppressing chondrogenesis was the degree of ongoing synovitis. However, we acknowledge that NSAIDs, which were administered to some patients, could also have affected synovial MSC chondrongenesis *in vivo*. [52]

Spontaneous joint regeneration following effective suppression of inflammation has recently been documented in clinical trials [53] and using *in vivo* animal models. [54] Our findings, demonstrating a negative effect of synovial inflammation on MSC function, offer a potential mechanism behind these observations. They further support the notion that joint inflammation not only leads to joint destruction, but also inhibits the intrinsic joint regeneration potential involving local MSCs. Therefore, any therapeutic intervention using autologous synovial MSCs to induce neo-chondrogenesis in RA should include effective suppression of local inflammation as a necessary first step.

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FIGURE LEGENDS

Figure 1. **Arthroscopic synovitis visual analogue score (VAS) correlates with known measures of systemic and local inflammation in RA.** A - Correlation between VAS and CRP (mg/l) (left panel) and lack of significant correlation between VAS and DAS28 (right panel). B - Correlations between VAS and semi-quantitative scoring of CD68 (left panel) and CD3 (right panel) staining assessed by immunohistochemistry. C – CD68 and CD3 staining of tissue biopsies of a representative RA patient with low VAS (left) and high VAS (right). Original magnification x100. D - Flow cytometry-based assessment of infiltrating monocytes in synovial tissue digests. Left panel - representative plot illustrating monocyte enumeration strategy, right panel - positive correlation between VAS and a percentage of monocytes. A cohort of 37 RA patients participated in this study and specimens were re-distributed between different aspects of this work depending on the size of biopsy.

Figure 2. **Inflammation reduces the chondrogenic potential of synovium-derived mesenchymal stem cell (MSC) cultures.** A – Representative OA and RA patients: macroscopic appearance of synovium (far left panel), chondrogenesis (Toluidine Blue staining, middle left panel), osteogenesis (alkaline phosphatase staining, middle right panel) and adipogenesis (Oil Red staining, far right panel). Original magnification x100. B – Quantitative differentiation assays showing tri-potentiality of standard polyclonal (passage 3, p3) synovial MSCs from OA and RA patients and positive (BM) and negative (Fib) controls of differentiation. Right panels – Relationships between synovial MSCs chondro-, osteo- and adipogenesis and arthroscopic visual analogue score (VAS) of synovitis. Only chondrogenesis shows a direct inverse relationship with VAS. Open triangles=RA, dark triangles =OA. * - $p<0.05$.

Figure 3. **Inflammation affects the frequency of highly-proliferative *in vivo* MSCs in RA synovium.** A – An assessment of the *in vivo* MSC frequency of synovial tissue digests. Left panel – sorting strategy: PI-positive dead cells/debris were gated out (R1). Hematopoetic cells (R1+R3), endothelial cells (R1+R2+R4) and fibroblastic stromal cells (R1+R2+R5) were sorted separately. Right panel: colony-forming cell assay (1000 cells/flask) confirmed the presence of *in vivo* MSCs in the fibroblastic stromal fraction. B – Frequencies of *in vivo* MSCs in this fraction in RA and OA (left panel) or in RA in relation to VAS (right panel). C – Average growth rates (measured as days/PD) of clonal MSC cultures from RA and OA patients. * - $p<0.05$.

Figure 4. **The marker phenotype of synovial MSC cultures and synovial stromal fibroblasts in RA**. A – Expression of putative MSC markers on standard polyclonal (p3) synovial MSCs from RA (empty bars, n=9) and OA (dark bars, n=5) patients. B – Linear relationship between CD44 mean fluorescence intensity (MFI) and VAS in polyclonal RA-MSCs. C – Linear relationship between chondrogenesis of polyclonal RA-MSCs and CD44 MFI. D - Expression of putative MSC markers on RA synovial fibroblastic/stromal ($CD45^-$ $CD31^-$) cells (light grey bars, n=5), polyclonal RA-MSCs (empty bars, n=9) and clonal RA-MSCs (dark grey bars, n=4). ** indicate markers differentially expressed between both types of MSCs and synovial stromal fibroblasts, $p<0.01$.

Figure 5. **Expression of pro- and anti-chondrogenic molecular markers as well as markers of "aggressive" RA fibroblasts in RA and OA MSCs measured by real-time PCR.** Real-time PCR was performed on polyclonal p3 cultures; cells were lysed for RNA extraction upon reaching confluence. RA – empty triangles, OA – filled triangles.

Supplementary Figure. **Direct correlation between semi-quantitative and digital analysis of synovial biopsy staining for CD68.** Semi-quantitative score (between 0 and 4) is assigned as described in [29]. Quantitative analysis is adapted from [30]. Quantification and analysis was performed using NIS-Elements BR software (Nikon Instruments Inc.). Five fields of view (x200) were photographed randomly, lining layer was removed digitally and areas of positive (DAB) stained cells were converted into binary data, before the total highlighted area was measured. This was repeated for the areas of positive cells plus negative cells. The score is shown as a ratio: area of positive (DAB) stain/(area of positive (DAB) stain + area of negative cells), roughly equating to the ratio of positive cells in relation to total cells.

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SUPPLEMENTARY METHODS

Trilineage differentiation of standard expanded synovial MSC cultures

For primary culture, 10^6 cells from synovial tissue digests were plated in 25cm^2 flasks in standard media, containing DMEM, antibiotics and 10% FCS, optimised for MSC growth (Mesencult, Stem Cell Technologies). Positive control BM-MSCs were established from BM mononuclear cells (10^7/25cm^2 flask) following separation with Lymphoprep (Axis-Shield, Dundee, UK). Negative control skin fibroblasts were purchased from ATCC (Rockville, MD, USA). Flow cytometry, colony-forming unit-fibroblast (CFU-F), differentiation assays, real-time PCR and ELISA were performed on passage 3 (p3) cultures. Passaging was performed by standard trysinization [11, 16] of nearly-confluent cultures (1:2 splits). *In vitro* "age" of control BM MSCs at p3 was 10-13 population doublings (PDs) based on the number of seeded CFU-Fs.

Osteogenic and adipogenic differentiation was induced by placing cells in standard osteo- and adipoinductive conditions as previously described [11, 16]. The extent of osteogenesis was measured as the amount of calcium produced at the end of the culture (day 21), using a commercial calcium kit (DLC, Charlottetown, Canada). Adipogenesis was quantified as a percentage of fat-laden cells (Oil Red-positive) in relation to all nucleated cells (following counterstaining with Harris's Haematoxylin). Chondrogenic assay was performed as previously described, with $2.5x10^5$ cells used to initiate each pellet, in triplicate [11, 16]. Chondroinductive media contained high-glucose DMEM (Invitrogen), 100 μg/ml sodium pyruvate, 40 μg/ml proline, 50 μg/ml ascorbic acid-2-phosphate, 1 mg/ml BSA, 1x insulin-transferrin-selenium plus, 100 nM dexamethasone (all from Sigma) and 10 ng/ml transforming growth factor β3

(R&D Systems, Abingdon, UK). Pellets were harvested at the end of the three-week culture period and digested with 100 µl papain solution. The amount of sulphated glycosaminoglycans (sGAG) (in µg/pellet) was measured by Alcian blue binding assay (IDS, Boldon, UK) [11, 16]. Assay detection range was 12.5-400 µg of sGAG/ml.

Flow cytometry and real-time PCR on standard expanded synovial MSC cultures

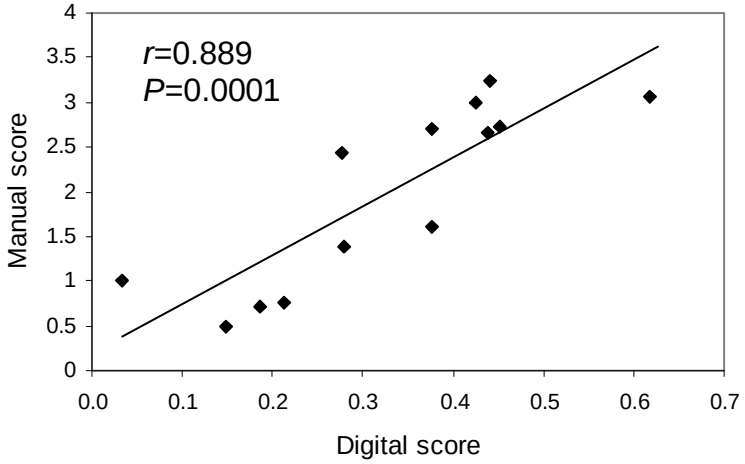
Flow cytometry was performed using standard methods as described previously (11, 31). RNA was extracted [32] and cDNA synthesized using 400U Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, Warrington, UK) in the presence of SYBR-green or Taqman. Expression was normalized using a house-keeping gene GAPDH.

ELISA

Cytokine production (Tumour necrosis factor-α (TNFα), interleukin (IL)-6 and IL-1β) in culture supernatants was measured by ELISA using HS Quantikine kits according to manufacturer's instructions (R&D Systems). Assay sensitivity was 0.039, 0.057 and 0.106 pg/ml for IL-6, IL-1β and TNFα, respectively. MSCs ($5x10^4$ cells) were seeded in 12-well plates in 2 ml of MSC expansion media and supernatants collected for measurements 48 hours later.

Statistical analysis

Spearman's rank correlation coefficient was used to correlate two variables. Mann-Whitney U test for 2 independent samples was used to compare groups. $p<0.05$ and $r>0.6$ were considered significant.



Direct correlation between semi-quantitative and digital analysis of synovial biopsy staining for CD68.

Supplementary Table 1: RA and OA patient sample distribution into different parts of the study

Type of study	Diagnosis	n*
Differentiation assays on polyclonal MSC cultures	RA OA	8 5
CRP/DAS28 analysis** and immunochemistry	RA	37
Flow cytometry on synovial tissue digests	RA	20
Flow cytometry on expanded synovial MSCs (polyclonal and clonal)	RA OA	9 5
Cell sorting for the *in vivo* MSC enumeration	RA OA	7 9
Gene expression and ELISA	RA OA	6 4

* - tissues from the same patient were often distributed amongst several studies

** - data obtained from clinical notes

Supplementary Table 2. List of antibodies used in different parts of this study

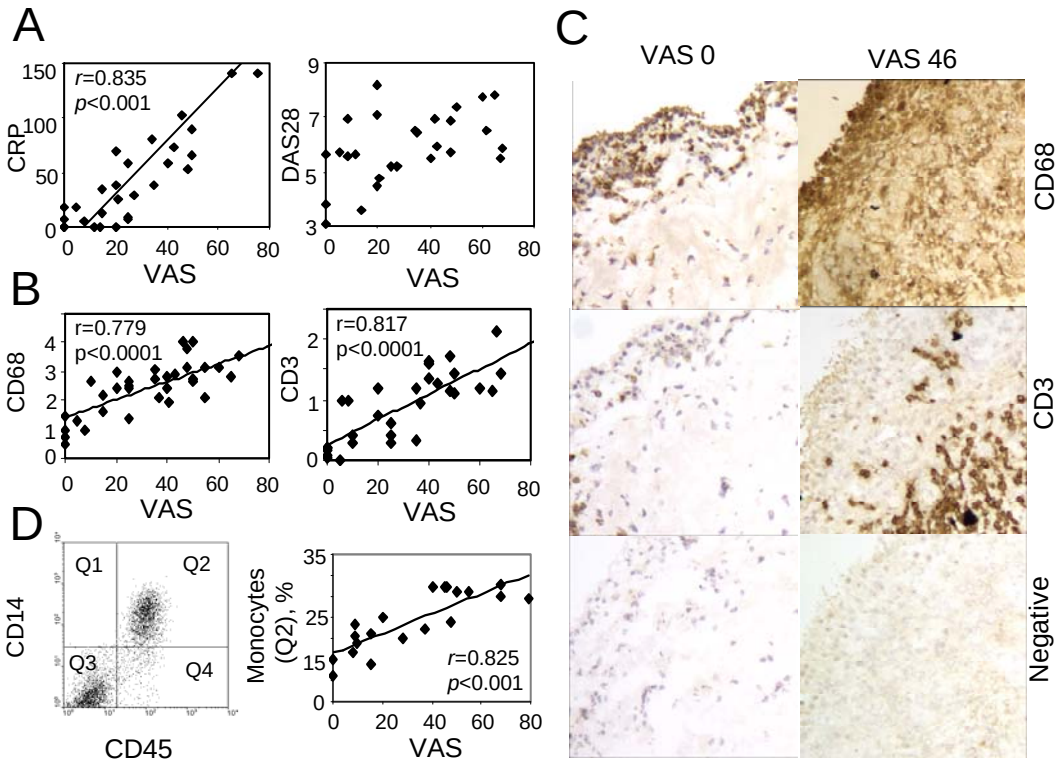
Antibody	Conjugate	Manufacturer	Study
CD45	FITC	Dako	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD14	PE	Gift from Dr R Jones (clone UCHM1)	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD31	PE	Caltag/Invitrogen	Flow cytometry on synovial tissue digests
CD31	FITC	Serotec	Flow cytometry on expanded synovial MSCs
CD44	PE	BD Biosciences	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD73	PE	BD Biosciences	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD151	PE	BD Biosciences	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD166	PE	BD Biosciences	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD90	PE	Serotec	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD105	PE	Serotec	Flow cytometry on synovial tissue digests and expanded synovial MSCs
CD3	pure	Gift from Dr R Jones (clone UCHT1)	Immunohistochemistry on synovial tissue biopsies
CD68	pure	Dako	Immunohistochemistry on synovial tissue biopsies

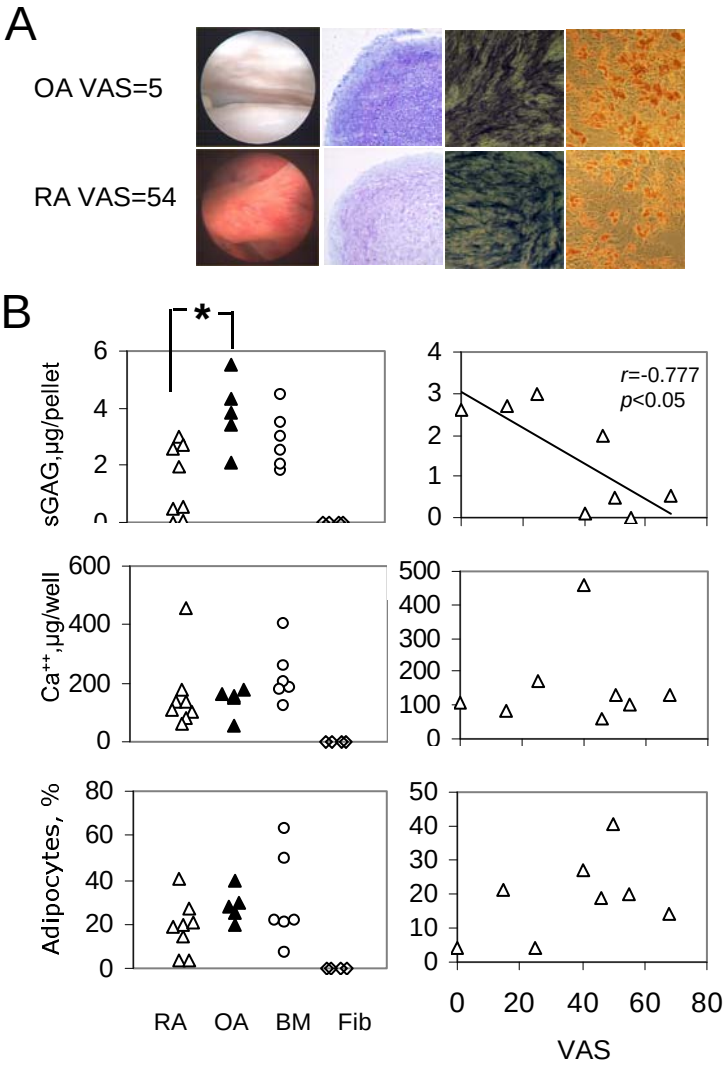
Company addresses: Dako - High Wycombe, UK, Caltag/Invitrogen – Paisley, UK, Serotec - Kidlington, UK, BD Biosciences - Oxford, UK. Dr R Jones is from HMDS, Leeds, UK. All isotype-specific negative controls were from Serotec.

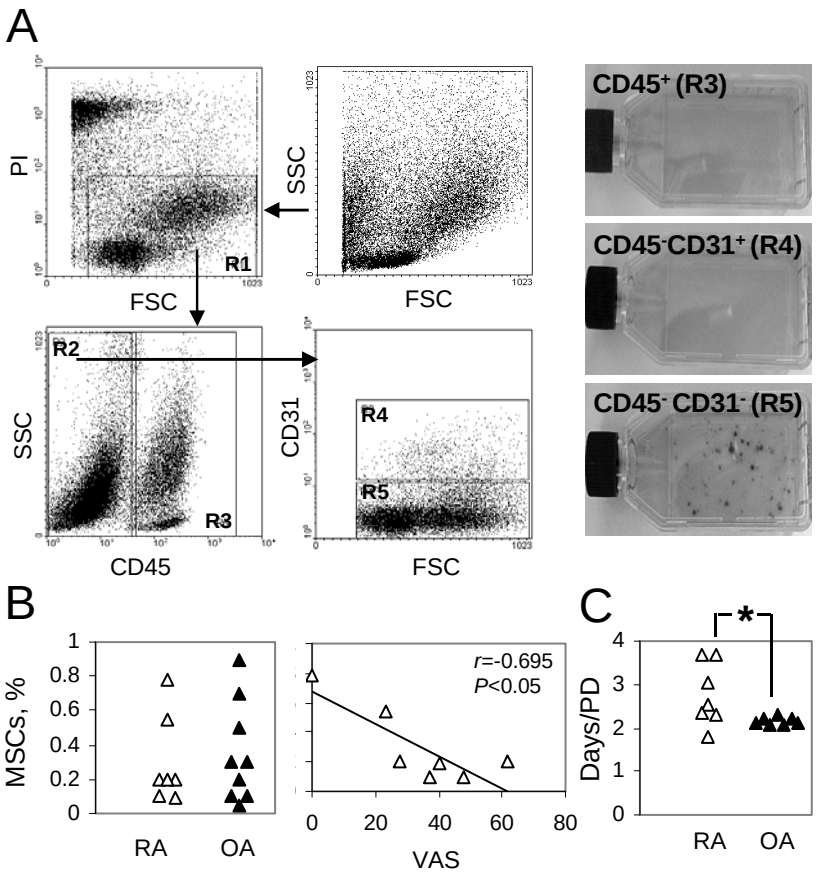
Supplementary Table 3. Primers used for real time PCR.

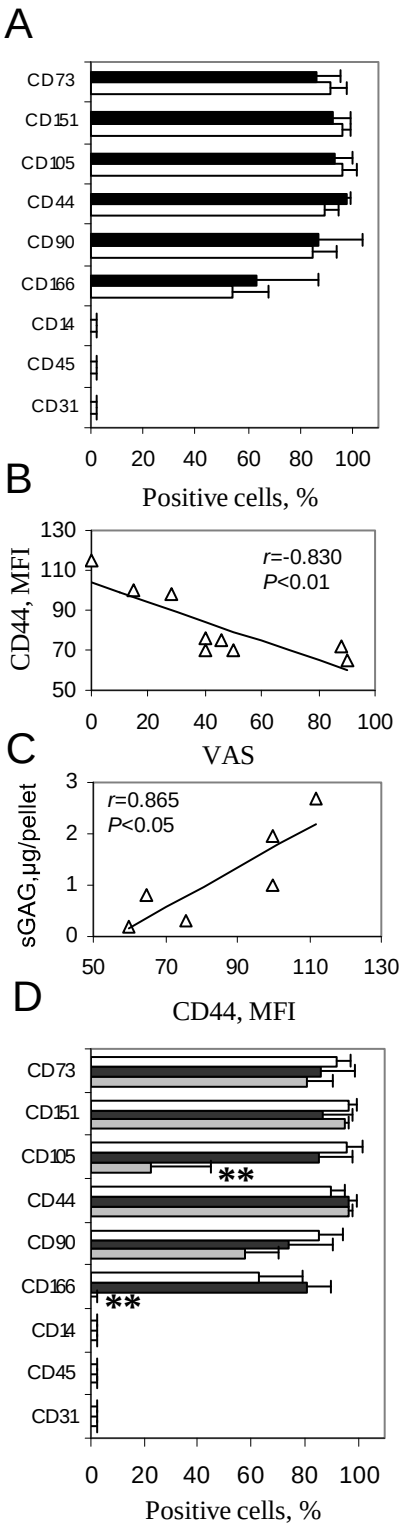
Primer	Forward primer (5'- 3')	Reverse primer (5'- 3')
GAPDH	aacagcgacacccactcctc	cataccaggaaatgagcttgacaa
Sox9	gacacctctggggtcccttc	tcctcaaggtcgagtgagctgt
NF-κB p65	atccacagtttccagaacctgg	ttgttggtctggatgcgct
IκBa	tgaaggctaccaactacaatggc	agcacccaaggacaccaaaa
MMP1	agtccggtttttcaaagggaa	gccaaaggagctgtagatgtcc
MMP2	actcctgagatctgcaaacagga	ccgccaaatgaaccggt
MMP3	ggaaaacccaccttacatacagga	ttcccagactttcagagctttctc
MMP9	gaggcgctcatgtaccctatgt	agggcgaggaccatagaggt
TIMP1	ttctgcaattccgacctcgt	ttggtcatcttgatctcataacgc
TIMP2	ctggaaacgacatttatggcaa	tctcaggccctttgaacatctt
Galectin3	tgaagcccaatgcaaacaga	tgttgttctcattgaagcgtgg
Osteopontin	catcacctgtgccataccagtta	ggccacagcatctgggtattt
SUMO1	gtgtcaaagacagggtgttcca	cttcttcctccattcccagttgt

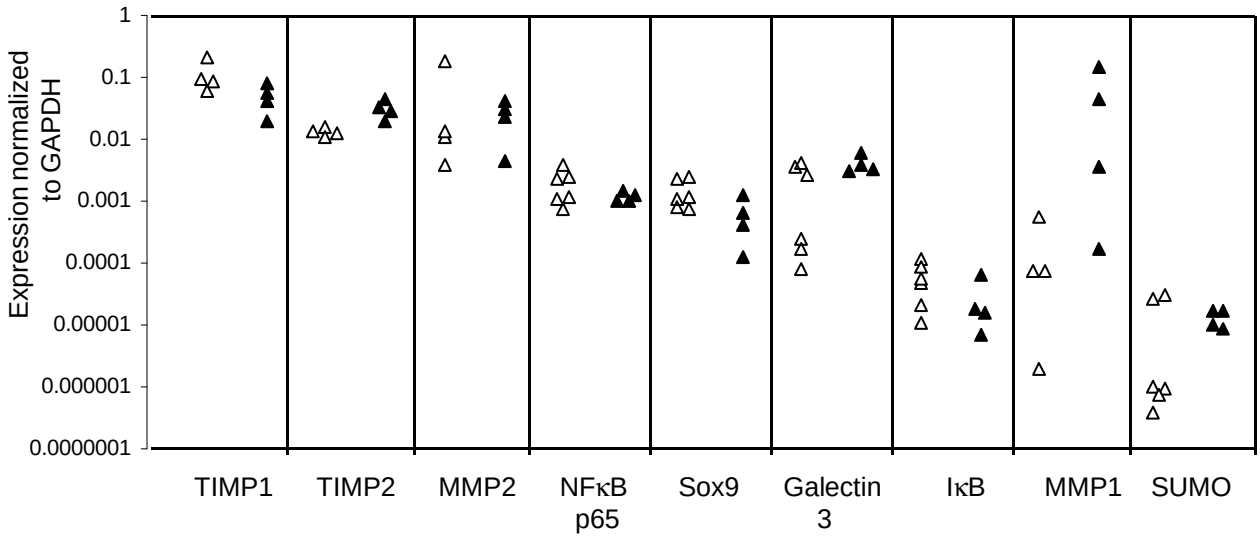
Taqman assays were used for TNFα normalised to GAPDH (Hs00174128 and Hs99999905, respectively, Applied Biosystems). All other primers (0.5 pmol/μl) were designed using Primer Express 2. Efficiencies calculated using the log-linear phase of dilution and were found to be approximately equal. All reactions were performed in triplicate and a melting curve analysis was used to ensure specificity of each PCR product.











Mesenchymal stem cells in rheumatoid synovium: Enumeration and functional assessment in relation to synovial inflammation level

E Jones, S M Churchman, A English, M H Buch, E A Horner, C H Burgoyne, R Reece, S Kinsey, P Emery, D McGonagle and F Ponchel

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Corrections

The department of one of the authors who co-authored all of the below papers has found that the affiliations were not correct. The correct affiliations for Professor P Emery, for all of the below articles, are: [1]Section of Musculoskeletal Disease, Leeds Institute of Molecular Medicine, University of Leeds; [2]NIHR Leeds Musculoskeletal Biomedical Research Unit, Leeds Teaching Hospitals Trust, Leeds, UK.

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