Osteoarthritis and Cartilage

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Treatment efficacy of adipose-derived stem cells in experimental osteoarthritis is driven by high synovial activation and reflected by S100A8/A9 serum levels

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S U M M A R Y

Objective: Synovitis is evident in a substantial subpopulation of patients with osteoarthritis (OA) and is associated with development of pathophysiology. Recently we have shown that adipose-derived stem cells (ASC) inhibit joint destruction in collagenase-induced experimental OA (CIOA). In the current study we explored the role of synovitis and alarmins S100A8/A9 in the immunomodulatory capacity of ASCs in experimental OA.
Method: CIOA, characterized by synovitis, and surgical DMM (destabilization of medial meniscus) OA were treated locally with ASCs. Synovial activation, cartilage damage and osteophyte size were measured on histological sections. Cytokines in synovial washouts and serum were determined using Luminex or enzyme-linked immunosorbent assay (S100A8/A9), mRNA levels with reverse-transcriptase (RT)-qPCR.
Results: Local administration of ASCs at various time-points (days 7 or 14) after DMM induction had no effect on OA pathology. At day 7 of CIOA, already 6 h after ASC injection mRNA expression of pro-inflammatory mediators S100A8/A9, interleukin-1beta (IL-1β) and KC was down-regulated in the synovium. IL-1β protein, although low, was down-regulated by ASC-treatment of CIOA. S100A8/A9 protein levels were very high at 6 and 48 h and were decreased by ASC-treatment. The protective action of ASC treatment in CIOA was only found when high synovial inflammation was present at the time of deposition which was reflected by high serum S100A8/A9 levels. Finally, successful treatment resulted in significantly lower levels of serum S100A8/A9.
Conclusion: Our study indicates that synovial activation rapidly drives anti-inflammatory and protective effects of intra-articularly deposited ASCs in experimental OA which is reflected by decreased S100A8/A9 levels.

Introduction

Osteoarthritis (OA) is primarily a disease of the cartilage. However, synovitis is thought to contribute to aggravation of joint pathology as well[1,2]. Up to 50% of OA patients have synovitis, which was demonstrated by magnetic resonance imaging, ultrasonography and arthroscopy[3–5]. Histologically, inflammation in the synovial membrane is characterized by high levels of CD68+macrophages expressing an activated phenotype and presence of pro-inflammatory cytokines like IL-1β and TNFα[3,6,7].

Synovial lining macrophages contribute to joint destruction in murine collagenase-induced osteoarthritis (CIOA). When lining macrophages were selectively depleted from a mouse knee joint prior to induction of CIOA, a strongly diminished thickening of the synovium was observed which coincided with inhibition of cartilage degradation and formation of cartilage/bone within ligaments[8]. Synovial macrophages produce both catabolic and anabolic factors that contribute to degradation and new formation of cartilage[9]. Major catabolic factors produced by activated macrophages are alarmins or damage associated molecular patterns (DAMPs) S100A8 and

S100A9[10]. These are found in large amounts in the serum and synovium of OA patients and we recently showed that S100A8/A9 predict development of cartilage destruction in the Dutch CHECK cohort of early symptomatic OA patients and have catabolic effects on OA chondrocytes via Toll-like receptor (TLR)-4[11,12].

Mesenchymal stem cells (MSCs) have been shown to exhibit immunosuppressive characteristics[13,14], next to their effect on tissue specific differentiation[15]. MSCs can secrete multiple immunosuppressive factors, like IL-10, IL-1 Receptor Antagonist (IL-1RA), Indoleamine 2,3-DiOxygenase (IDO), TGFβ and ProstaGlandin E2 (PGE2)[16] and they can induce anti-inflammatory effects in macrophages[17,18]. Moreover, evidence is gathering that MSCs need an inflammatory milieu to deliver immunosuppressive signals[13,19,20]. Next to bone marrow, adipose tissue also hosts multipotent stem cells. These adipose-derived stem cells (ASCs) share multiple properties with bone marrow-derived MSCs[21], but are more accessible and abundant (5% vs 0.0001–0.001% of nucleated cells). Both types have adipogenic, osteogenic and chondrogenic differentiation potential and display immunosuppressive properties *in vitro* as well as *in vivo*[22].

Recently, we described that local administration of ASCs in the knee joint in early stage CIOA inhibited synovial activation and largely protected against new cartilage/bone formation within collateral and cruciate ligaments[23]. Furthermore, cartilage damage was reduced in various layers of the knee joint. We localized GFP-labeled ASCs within the synovial lining layer in close contact to synovial macrophages[23]. The immunosuppressive capacity of the ASCs may thereby be stimulated by the pro-inflammatory cytokines released by macrophages during the early phase of CIOA.

In the present study we investigated whether the suppressive effect of ASCs on experimental induced OA is driven by synovitis. Furthermore, we explored the role of S100A8/A9, major products during synovitis, in this anti-inflammatory effect of ASCs.

Materials and methods

Animals

A total of 120 C57BL6J mice were obtained from Janvier. Animals were male and between 12 and 14 weeks old (average weight 24.5 g SD 0.3), housed in filter-top cages with up to 10 animals per cage and received a standard diet and tap water *ad libitum*.

Induction of experimental OA

We used two different models of experimental OA, CIOA and DMM, because they differ in synovial inflammation. Experimental CIOA (which has relatively high synovial inflammation) was induced as previously described[24]. Briefly, right knee joints of mice were injected with 1 U collagenase type VII from *Clostridium histolyticum* (Sigma–Aldrich) at day 0 and day 2, causing disruption of the ligaments and local instability of the knee joint. To achieve a CIOA with less synovial activation, only one time 1 U collagenase was injected. Experimental DMM OA (destabilized medial meniscus, a model where synovial activation is scant) was induced by transection of the medial anterior meniscotibial ligament[25].

For the DMM-study eight mice/group were used on day 7 and 10 mice/group for day 14. For the effects of ASCs on synovium in CIOA, six mice/group were used on day 2, 14 and 42, eight mice/group after 6 h. For the comparison between high and low synovial activation in CIOA, eight mice/group were used.

Adipose stem cells

ASCs were isolated from adipose tissue surrounding the inguinal lymph nodes of mice and cultured for 2 weeks according to standard procedures in DMEM/F12 (Gibco) supplemented with 1% penicillin/streptomycin (Invitrogen), 0.5% amphotericin B (Invitrogen), 16 μM biotin (Sigma–Aldrich), 18 μM pantothenic acid (Sigma–Aldrich), 100 μM ascorbic acid (Sigma–Aldrich). ASCs stained positive for Sca-1, CD44 and CD105 (BD Bioscience; Biolegend and eBioscience) and negative for CD11b, cKit and CD34 (Biolegend; BD Bioscience and eBioscience). 20.000 cells in 6 μL mouse serum (Jackson Immunoresearch) with 4% mouse albumin (Sigma–Aldrich) were injected intra-articularly into the knee joint. Control animals were injected with only mouse serum containing 4% mouse albumin.

Isolation of murine synovial specimens

At various time points (6 h, 2, 14 and 42 days) after induction of OA, synovial specimens were isolated as described previously[11]. Briefly, joint capsule specimens were isolated on the medial and lateral sides of the patella with a biopsy punch. Synovial specimens were snapfrozen in liquid nitrogen and stored for RNA isolation.

In vitro cross-talk between adipose stem cells and activated macrophages

ASCs (passage 2) were stimulated for 24 h with 10 ng/ml IFNγ and IL-1β and 1 μg/ml recombinant murine S100A8. Bone marrow cells were harvested from C57Bl6J mice and 1×10^6 were differentiated into macrophages with M-CSF for 6 days and activated with 10 ng/ml LPS for 24 h. Consequently, these macrophages were incubated for 24 h with IFNγ- and IL-1β- stimulated ASC supernatant. Effect on gene expression of suppressive factors in both ASCs and macrophages was investigated using reverse transcription (RT)-qPCR.

Preparation of RNA and RT–qPCR

RNA from murine synovium was isolated by first disrupting synovial tissue with the MagNA Lyser (Roche) 5 times for 20 s and then isolating the RNA using the RNeasy-kit (Qiagen) according to the manufacturers protocol. RNA from cultured ASCs and macrophages was isolated using TRI-reagent (Sigma–Aldrich). RNA was reverse transcribed to cDNA and qPCR was performed with specific primers and the SYBR Green Master mix in the ABI Prism 7000 Sequence Detection System (Applied Biosystems/Life Technologies). Expression levels were normalized to GAPDH. Primer sequences were designed on exon–exon transition of murine genes and can be found in Table I.

Histological analysis of OA progression

Knee joints were dissected and fixed in 4% formalin. After fixation, joints were decalcified in 4% formic acid buffered in PBS and processed for histology.

Paraffin embedded knee joints were cut in sections of 7 μm, stained with Safranin O (Saf-O) and counterstained with Fast Green for analysis of cartilage damage and chondrophyte/osteophyte associated ligaments (collateral/cruciate). Cartilage damage was scored blinded using a modified form of the Pritzker Osteoarthritis Research Society International (OARSI) OA score, which takes into account the grading and staging components[26]. Five sections for each specimen were evaluated by two blinded investigators. Minimal score is 0, indicating no cartilage pathology whatsoever, whereas 30 is the maximum score, indicating highest grade (6) and stage (5). For scoring of synovial activation, Hematoxylin Eosin (HE) staining was used. Synovial thickening/activation was scored using an arbitrary score from 0 to 3 as previous described[27]. Saf-O stained

Table I
Primers used for RT-qPCR

Gene	Forward primer	Reverse primer
Arginase	GAAAGTTCCCAGATGTACCAGGAT	CGATGTCTTTGGCAGATATGCA
IDO	CAAAGCAATCCCCACTGTATCC	CTATGTCGTGCAGTGCCTTTTC
IL-1β	GGACAGAATATCAACCAACAAGTGATA	GTGTGCCGTCTTTCATTACACAG
IL-1RA	CAAGATGCAAGCCTTCAGAATCT	CACCATGTCTATCTTTTCTTCTAGTTTGA
IL-6	CAAGTCGGAGGCTTAATTACACATG	ATTGCCATTGCACAACTCTTTTCT
IL-10	ATTTGAATTCCCTGGGTGAGAA	ACACCTTGGTCTTGGAGCTTATTAA
iNOS	CGTTTCGGGATCTGAATGTGA	GGGCAGCCTGTGAGACCTT
KC (CXCL1)	TGGCTGGGATTCACCTCAA	GAGTGTGGCTATGACTTCGGTTT
S100A8	TGTCCTCAGTTTGTGCAGAATATAAAT	TTTATCACCATCGCAAGGAACTC
S100A9	GGCAAAGGCTGTGGGAAGT	CCATTGAGTAAGCCATTCCCTTTA
TGF-β1	GCAGTGGCTGAACCAAGGA	AAGAGCAGTGAGCGCTGAATC

Primer sequences for target genes for RT-qPCR spanned exon–exon transitions of murine genes. IDO = indoleamine 2,3-dioxygenase, IL = interleukin, RA = receptor antagonist, iNOS = inducible nitric oxide synthase, TGF = transforming growth factor.

sections were used to measure the size of chondrophytes/osteophytes using an image analysis system (Leica Application Suite, Leica).

Measurement of S100A8/A9 in the serum of mice with experimental OA

S100A8/A9 concentrations were determined in synovial washouts of murine knee joints by a sandwich enzyme-linked immunosorbent assay (ELISA) specifically for murine S100A8/A9 as described previously[28].

Measurement of TGFβ-activity

TGFβ-activity was determined by adding 1:5 dilution of synovial washouts overnight to 3T3 fibroblasts transduced with adenoviral CAGA-luciferase (CAGA-luc) with a multiplicity of infection (MOI) of 10 (10 plaque-forming units) per cell, after which luminescence was measured. The plasmid was kindly provided by Dr. Ten Dijke (Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands). The CAGA-boxes in the vector are transcribed by Smad3/4 through active TGFβ, resulting in luciferase activity[29].

Ethical considerations

All animal experiments were approved by the local authority Animal Care and Use Committee and local ethics committee of the Radboud university medical center (RU-DEC 2011-101/2012-247) and were performed by personnel certified by the Dutch Ministry of WVC.

Statistical analysis

Data were statistically evaluated using the Mann–Whitney U test or Student t test using Graph Pad Prism 5 (GraphPad Software). Differences were called significant with P-value <0.05 (*), <0.01 (**) or <0.0005 (***).

Results

ASC treatment has no effect on development of joint destruction in DMM experimental OA with low synovial activation

In a previous study we found that a single injection of ASCs into a mouse knee joint of CIOA inhibited development of cartilage destruction, chondrogenesis in ligaments and osteophyte formation[23]. CIOA is characterized by thickening and activation of the synovial layer containing activated macrophages. To investigate further whether synovial macrophage activation may drive the anti-inflammatory activity of ASCs, ASCs were tested in the DMM model, in which synovial macrophage activation is scant[11]. A single injection of 20.000 ASCs was given into the right knee joint either at day 7 or day 14 [Fig. 1] or days 14 and 21 (data not shown) after induction of DMM. In contrast to CIOA, no effect of ASC treatment was found in DMM on cartilage destruction, osteophyte formation or chondrogenesis in ligaments. These results suggest that the healing capacity of ASC is related to synovitis.

Intra-articularly applied ASCs rapidly suppress the activation status of the synovial lining layer during CIOA

To investigate the effect of the intra-articularly injected ASCs on the inflammatory status of the synovium, we injected 20.000 ASCs in the knee joint at day 7 after induction of CIOA and subsequently isolated synovium 6 h, 2, 14 and 42 days thereafter. Various macrophage derived cytokines (IL-1β, IL-6, TNFα, IL-10, KC, S100A8, S100A9) were measured using RT-quantitative polymerase chain reaction (qPCR). Pro-inflammatory mediators S100A8, S100A9, IL-1β, IL-6 and KC were highest 6 h after control injection and declined later on, which is in line with earlier studies showing highest synovial activation in early stages of CIOA[11]. Interestingly, ASCs significantly downregulated S100A8, S100A9 [Fig. 2(A)], IL-1β and KC mRNA [Fig. 2(B)] already 6 h after ASC injection. Although mRNA levels were still lower at day 14, this did not reach significance. IL-6 mRNA levels were significantly reduced at day 14, and lower at day 2, although not significantly [Fig. 2(B)]. In addition, protein levels of various cytokines (IL-1β, IL-6, IL-10, KC, IFNγ) and S100A8/A9 were measured in synovial washouts using Luminex or ELISA (for S100A8/A9). Low levels of IL-1β [Fig. 3(B)] and very high levels of S100A8/A9 [Fig. 3(A)] were detected 6 h and 2 days after ASC or control injection. IL-1β protein levels were found to be lower at day 2, 14 and 42 after ASC treatment (44, 45 and 34% lower, respectively) [Fig. 3(B)]. Moreover, S100A8/A9 was significantly downregulated on protein level 6 and 48 h after ASC injection (48 and 58% lower, respectively) [Fig. 3(A)]. No effect of ASC treatment was found on protein levels of IL-6 and KC [Fig. 3(B)]. These results indicate that the suppressive effect by ASCs is rapidly initiated in the synovium. Apart from lowering catabolic cytokines, ASC treatment may also affect anabolic growth factors like TGFβ and BMP-2, crucial during osteophytosis[30]. However, no effect of ASCs was found on mRNA levels of TGFβ and BMP-2 (data not shown). Measuring TGFβ activity in synovial washouts using a CAGA-luc assay, no differences were found at 6 h, 2, 14 or 42 days after ASC and control treatment [Fig. 3(C)].
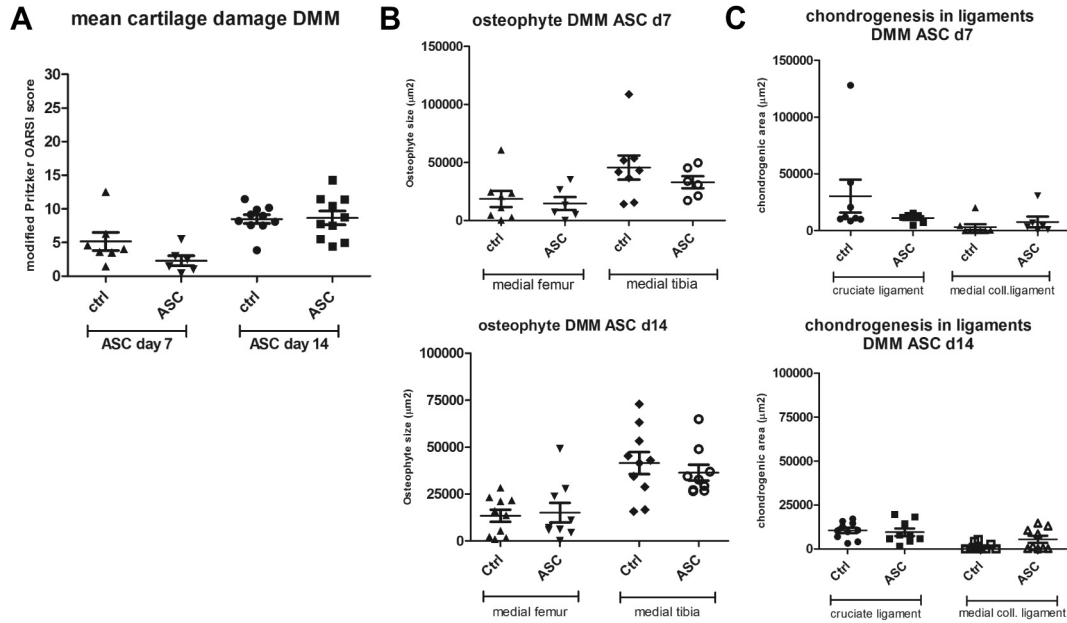
Fig. 1. ASC treatment has no effect on development of joint destruction in a DMM model in which synovial activation is scant. DMM OA was induced by transection of the medial anterior meniscotibial ligament and 20.000 ASCs were injected intra-articularly at day 7 or 14. Mice were sacrificed at day 56 and whole knee joints were prepared for histology. **A**. No difference was found on mean cartilage damage as assessed by the modified Pritzker OARSI score. **B**. Injection of ASCs on neither day 7 or 14 changed osteophyte size as measured at the margins of the medial femur and tibia by image analysis software. **C**. Similarly, chondrogenic area in cruciate and medial collateral ligaments was not changed by ASC injection at day 7 or 14 of DMM, measured by redness in Safranin-O stained sections. $N = 8$ mice/group in experiment with ASC injection at day 7, $n = 10$ at day 14.

Interaction between cytokine stimulated ASCs and macrophages

In various studies it has been shown that pro-inflammatory cytokines, and in particular IFNγ, directly stimulate the anti-inflammatory capacity of ASCs[31–33]. First, we confirmed that IFNγ could directly induce anti-inflammatory mediators arginase, IL-1RA and iNOS in ASCs on mRNA level [Fig. 4(A)].IL-1β also

induced higher levels of arginase and iNOS mRNA in ASCs, whereas S100A8 did not affect any anti-inflammatory genes in ASCs [Fig. 4(A)]. We next studied the *in vitro* effect of IL-1β and IFNγ on the cross talk between ASCs and activated macrophages. To do this, murine bone marrow derived macrophages (BMM) were activated with LPS and consequently stimulated with supernatant of IL-1β- or IFNγ-stimulated ASCs. The supernatant of IFNγ-stimulated, but not
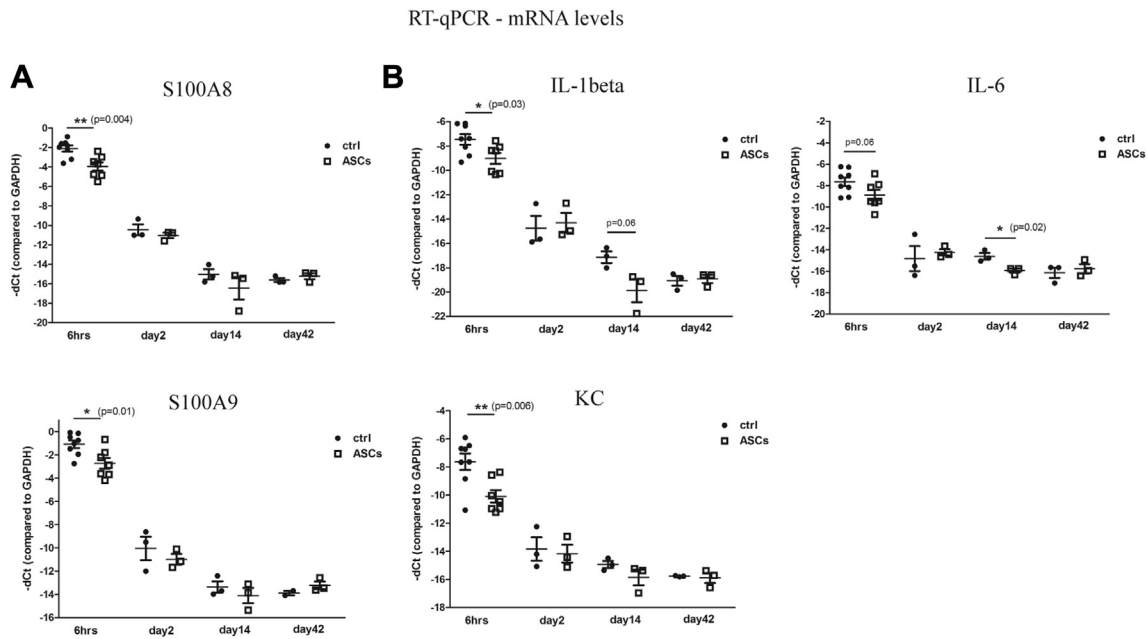

Fig. 2. Intra-articularly applied ASCs rapidly suppress synovial mRNA-expression of pro-inflammatory mediators during collagenase-induced OA ASCs (20.000) were injected at day 7 of CIOA, and 6 h, 2, 14 and 42 days thereafter synovial RNA was collected. mRNA levels of alarmins S100A8, A9 (**A**) and pro-inflammatory cytokines/chemokines IL-1β, IL-6 and KC (**B**) were measured on RT-qPCR and were significantly lowered after 6 h (except for IL-6), however not at later timepoints. $N = 6$-8 mice/group (2 synovia pooled at timepoints 2, 14, 42 days). * = $P < 0.05$, ** = $P < 0.01$ as measured by Student t test.
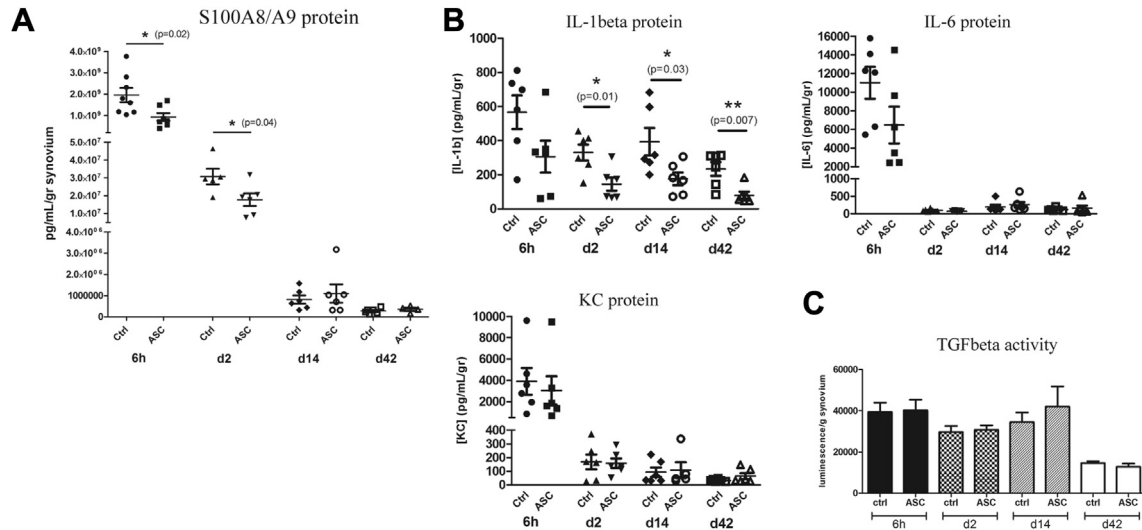
Fig. 3. Intra-articular ASC-injection reduces synovial protein levels of pro-inflammatory S100A8/A9 and IL-1β during collagenase-induced OA ASCs (20.000) were injected at day 7 of CIOA, and 6 h, 2, 14 and 42 days thereafter synovial washouts were collected. Protein levels of S100A8/A9 were measured with a specific ELISA (**A**) and IL-1β, IL-6 and KC proteins levels were measured on Luminex (**B**). S100A8/A9 levels were significantly lowered 6 h and 2 days after ASC treatment, IL-1β at day 2, 14 and 42. **C**. TGFβ-activity, measured by CAGA-luc in synovial washouts, was not changed after ASC treatment. $N = 6$ mice/group * $= P < 0.05$, ** $= P < 0.01$ as measured by Student t test.

IL-1β-stimulated ASCs strongly increased IDO mRNA expression in BMM (25-fold increase compared to unstimulated ASCs or IFNγ alone), while also upregulating iNOS (83- or 6-fold increase compared to unstimulated ASCs or IFNγ alone). Supernatant of unstimulated ASCs already upregulated arginase mRNA levels in BMM (8-fold increase). No effect was found on IL-1RA, IL-10 and TGFβ mRNA levels [Fig. 4(B)].

The efficacy of local ASC treatment in the early phase of OA is related to synovial thickness and is reflected by S100A8/A9 levels in the serum

To investigate whether the efficacy of ASC treatment is related to synovial thickness, we compared CIOA with high and somewhat lower synovial inflammation by injecting 1U collagenase either

once or twice. The synovial thickening of CIOA on day 42 with one time injection was 65% lower compared to two times injection (2.48 vs 1.5). However, this is still higher than observed in the DMM model (synovial activation score at endpoint, day 56: 0.7, data not shown). In the experiment with high synovial activation, day 7 ASC treatment significantly suppressed synovial thickening (32% lower). In addition, osteophyte size was also greatly reduced in the ASC treated group (72% lower) [Fig. 5(A)]. In contrast, in the CIOA experiment with low synovial inflammation, no suppressive effect of ASC treatment on synovial thickening, nor on osteophyte size [Fig. 5(B)] was found. These data suggest that the inflammatory status of the synovium drives the anti-inflammatory capacity of the ASC.

During synovitis release of high amounts of pro-inflammatory and catabolic S100A8/A9 may leak from the joint into the
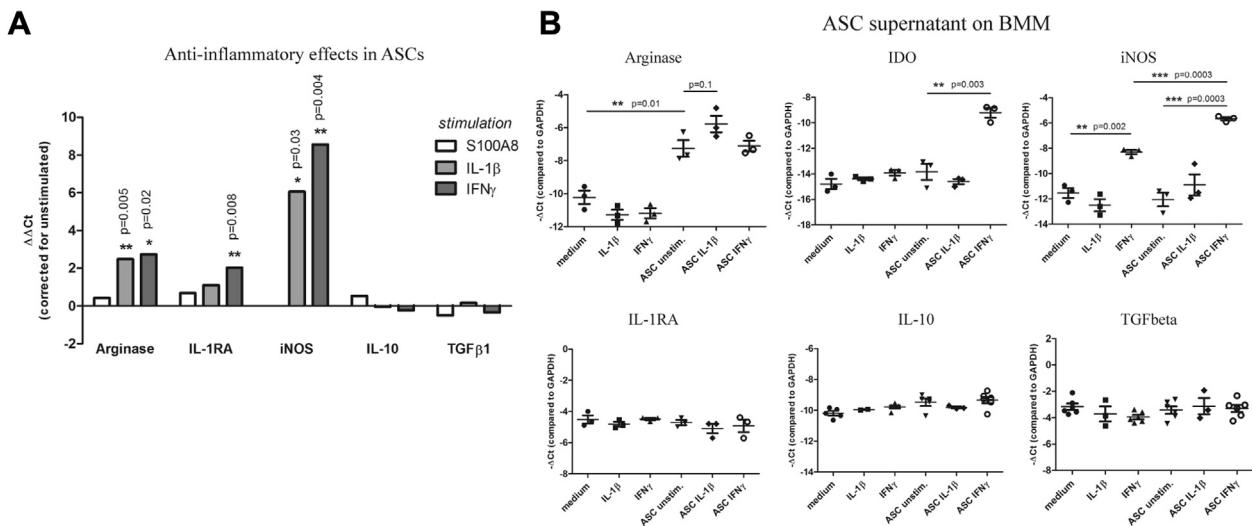

Fig. 4. Cytokine stimulated ASCs induce anti-inflammatory effects in activated macrophages ASCs were stimulated with pro-inflammatory cytokines IFNγ, IL-1β and S100A8 and the supernatants were used to stimulate activated murine bone-marrow derived macrophages (BMM). **A**. Direct effects of IFNγ, IL-1β and S100A8 on the mRNA expression of anti-inflammatory mediators by ASCs, showing upregulation of arginase, IDO, IL-1RA and iNOS. **B**. Supernatants of unstimulated ASCs already upregulated arginase mRNA in BMM, while supernatant of IFNγ-stimulated ASCs upregulated IDO and iNOS mRNA. IL-1RA, IL-10 and TGFβ mRNA levels in BMM were not affected. Values in **A** are represented as ΔΔCt, corrected for GAPDH and unstimulated. Values in **B** are represented as −ΔCt, corrected for GAPDH. Results are representative of two independent experiments. * $= P < 0.05$, ** $= P < 0.01$, *** $= P < 0.005$ as measured by Student t test.
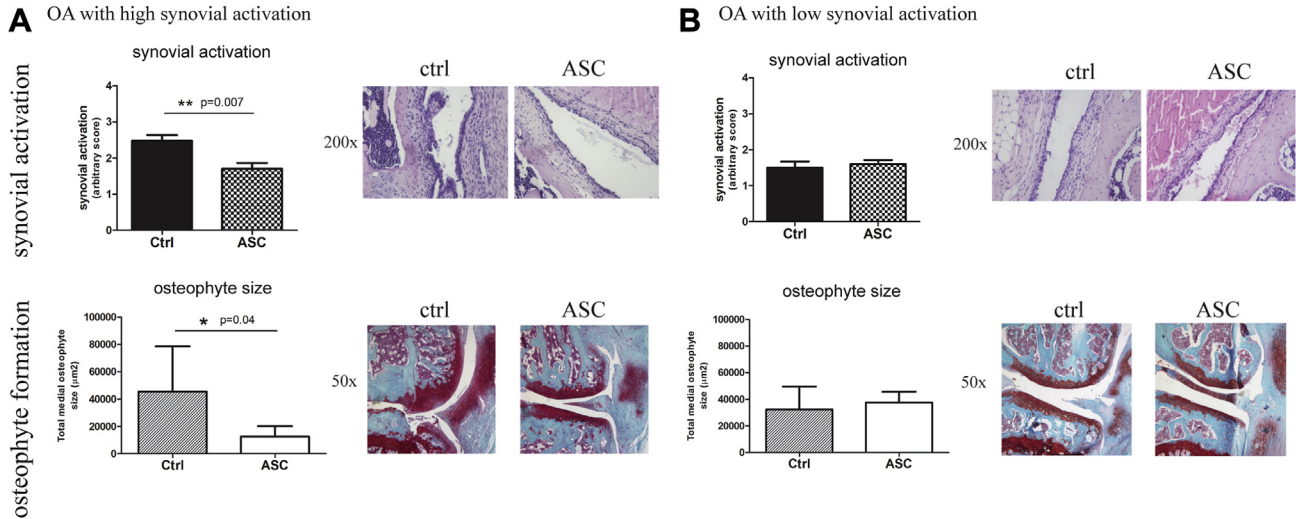
Fig. 5. The efficacy of local ASC treatment in the early phase of collagenase-induced OA depends on synovial activation. Two collagenase-induced OA experiments differing in synovial thickening/activation (1.7 fold difference) at day 42 were compared on the effect of intra-articular ASC treatment at day 7. Local ASC treatment only reduces synovial activation and osteophyte formation at the medial femur in OA with high synovial activation (**A**), compared to OA with low synovial activation (**B**). Right panels show representative histological pictures of hematoxylin-eosin stained knee sections (synovial activation, top panels) or Safranin-O stained knee sections on the medial side (osteophyte formation, bottom panels). Magnification, 50× or 200×, is indicated. * = P-value <0.05, ** = P-value<0.01. Significance of osteophyte size was calculated using Student t test, synovial activation with Mann−Whitney U. n = 6 mice/group.

bloodstream[11]. Serum was isolated at 14 and 42 days after ASC-treatment in the two CIOA experiments differing in synovial activation. In the experiment with high synovial activation, serum levels of S100A8/A9 levels were very high at day 14 (497 ng/ml, 371% of non-arthritic C57Bl6 mice, "naïve") and at day 42 still clearly above naïve (282 vs 134 ng/ml) [Fig. 6(A)]. In the experiment showing less synovial thickening, serum levels of S100A8/A9 levels were much lower at day 14 (272 vs 497 ng/ml) and returned to control levels at day 42 [Fig. 6(B)]. Interestingly, in the first experiment, local application of ASC at day 7 after OA induction dramatically inhibited S100A8/A9 serum levels (80% at day 14 and 44% at day 42 after treatment) [Fig. 6(A)]. In contrast, in the experiment with low synovial activation, S100A8/A9 levels were not significantly changed by ASC treatment at day 14 whereas at day 42 a 58% decrease was observed [Fig. 6(B)].

Discussion

In the present study we find that synovitis is essential in mediating the immunosuppressive effects of ASCs in experimental OA. S100A8/A9, as marker of this synovitis, could be used as predictor for successful ASC-treatment. Furthermore, locally administered ASCs rapidly suppress the activation status of the synovial lining layer possibly via a suppressive effect on macrophages.

Thickening of the synovial lining layer is a characteristic feature of CIOA, paralleling large subgroups of OA patients that show synovitis[1]. The synovial layer comprises mainly activated macrophages and selective elimination of these macrophages prior to induction of CIOA strongly inhibited synovial thickening, chondrogenesis/osteophyte formation and cartilage destruction[8]. However, elimination of lining macrophages in destabilization of the medial meniscus (DMM), in which synovitis is scant, has no effect on OA pathology. In the present study we find that when ASCs were injected into a DMM OA joint, no effect was found on development of joint destruction. Furthermore, ASC treatment is most efficient when given in a CIOA with high, not low synovitis. These data further support the importance of the activated synovial lining and macrophages in mediating the anti-inflammatory ASC effect.
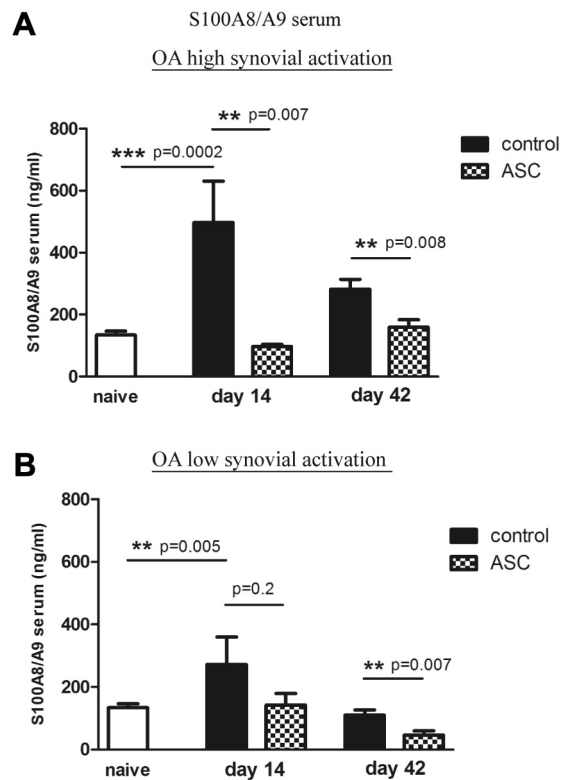

Fig. 6. Efficacy of ASC treatment can be measured by serum levels of S100A8/A9. Systemic S100A8/A9 levels in collagenase-induced OA differing in synovial activation was compared and the effect of ASC treatment at day 7 examined. **A.** Serum S100A8/A9 levels were very high in OA with higher synovial activation (807 ng/ml at day 14, compared to 134 ng/ml in non-arthritic mice "naïve") and were significantly lowered at day 14 and 42 when ASCs were given intra-articularly at day 7. **B.** In contrast, S100A8/A9 serum levels were much lower in the experiment with low synovial activation (272 vs 807 ng/ml at day 14). Treatment with ASCs did not significantly decrease S100A8/A9 levels at day 14, only at day 42. S100A8/A9 was measured using ELISA. n = 8 mice/group, except naïve (n = 32) * = P < 0.05, ** = P < 0.01 as measured by Student t test.

ASCs injected during the first phase of CIOA encounter high levels of pro-inflammatory cytokines released by activated synovial lining macrophages. During early phase CIOA, levels of IL-1β and S100A8/A9 are strongly upregulated in the synovium[11]. In the present study we find that injection of ASCs into day 7 CIOA joints caused a rapid downregulation of both IL-1β and S100A8/A9 levels and this effect was prolonged up to day 42 after treatment (for IL-1β). Also, five days after injection, no ASCs could be detected anymore within the synovium (data not shown). Apparently, a short pulse is sufficient for giving a prolonged ameliorating effect. An explanation may be that the ASC imprint on synovial macrophages may result in transformation into suppressive macrophages producing lower amounts of IL-1β or S100A8/A9 cytokines.

Although we show clear anti-inflammatory effects of ASCs in CIOA, we cannot rule out that cell-types other than MSCs could also induce immunomodulatory capacities. Indeed, we have shown earlier that skin fibroblasts exert anti-inflammatory effects in collagen-induced arthritis[34]. However, we believe that the use of ASCs is superior to other cell-types. First, they have the ability to differentiate into cell-types that can contribute to joint repair, such as chondrocytes and osteoblasts[15]. Furthermore, ASCs have an advantage of use in OA compared to fibroblasts, because the anti-inflammatory effect of the latter is largely attributed to inhibition of T-cell proliferation and activation with T-cells playing no role in OA pathogenesis. Finally, MSCs, to which ASCs belong, are known for their low immunogenicity and can be easily used in therapeutic approaches, without the need of autologous application[35].

It is becoming increasingly accepted that MSCs secrete many factors including growth factors, cytokines, chemokines, metabolites and bioactive lipids which orchestrate multiple interactions with the surrounding microenvironment[36]. Pro-inflammatory cytokines, like IFNγ, IL-6, TNF-α and DAMPs, present during the first phase of CIOA, are capable of inducing an immunosuppressive phenotype in ASCs/MSCs[17,19,20,32]. In reaction to these stimuli, ASCs release factors like IDO, cyclo-oxygenase (COX)-2 and PGE2 which are able to suppress activated macrophages, although subtle species differences exist between mouse and man[17,37]. In line with that we show that ASCs are able to induce expression of anti-inflammatory mediators arginase and iNOS after IL-1β or IFNγ stimulation and upregulate IL-1RA after IFNγ stimulation. Moreover, several *in vitro* and *in vivo* studies suggest that MSCs can decrease inflammation by changing the macrophage phenotype from M1 (classically activated) to M2 (alternatively activated)[38–40]. Previously, we already found that ASCs, when injected into the knee joint, interact with synovial lining macrophages[23]. We now find that supernatant of IFNγ stimulated ASC strongly upregulated arginase, IDO and iNOS in activated macrophages which is characteristic for transformation into an M2 signature. We speculate that both IL-1β and IFNγ could stimulate ASCs *in vivo* during (experimental) OA, thereby activating their anti-inflammatory phenotype, possibly via macrophages. IL-1β is frequently studied and detected in OA synovium[41,42]. Although data on IFNγ in OA is more scarce, it has been shown to be present in OA synovium by immunohistochemistry[41,43] and IFNγ positive CD4 T-cells were found in OA synovium[44,45]. Finally, IFNγ (and to a lesser extent IL-1β) could also be used as tool to activate the ASCs *ex vivo* to generate a larger anti-inflammatory potential.

Chondrogenesis within collateral/cruciate ligaments hampers a good functioning of the joint and may further enhance cartilage destruction. In previous studies performed in CIOA, we showed that activated synovial macrophages mediate chondrogenesis/osteophyte formation[9,46]. Macrophage derived factors involved in new cartilage formation include growth factors like TGFβ and BMP-2[9,47]. Production of these growth factors within the synovium was strongly diminished after macrophages were selectively depleted from the lining layer[46]. However, ASC treatment did not suppress mRNA levels of TGFβ and BMPs or active TGFβ within the synovium, suggesting that ASCs mostly act via inhibition of pro-inflammatory cytokines (IL-1β) or alarmins S100A8/A9, rather than via growth factors.

Comparing efficacy of ASC treatment in two CIOA experiments differing in synovial activation we found that ASC treatment was anti-inflammatory particularly when injected in joints with high but not with low synovitis, indicating that a certain threshold of pro-inflammatory factors is needed for induction of the anti-inflammatory effect of ASCs. In line with this is that ASC treatment when given at later phases (day 14 or day 21) after CIOA is also not effective[23] and that treatment of DMM with ASCs is also ineffective [Fig. 1]. High synovial activation in CIOA joints was reflected by high S100A8/A9 levels in the serum [Fig. 6]. S100A8/A9 produced in the synovium could leak out of the joint cavity thereby reflecting inflammatory status in the serum[48]. S100A8/A9 serum levels may thus be used firstly as a marker for determining synovitis which is a licensing signal for using local ASC treatment. Secondly, local stem cell treatment of CIOA joints lowered levels of S100A8/A9 levels within the serum and may therefore also be used as a marker for measuring the efficacy of ASC treatment.

In the present paper we show that intra-articular deposition of ASCs inhibit thickening/activation of the synovial lining layer and protect joint destruction in experimental OA with high synovial activation. ASCs had an immunomodulatory effect on the synovium by suppressing catabolic factors like IL-1β and S100A8/A9 produced by synovial macrophages. *In vitro,* ASCs upregulated anti-inflammatory factors in activated macrophages. Finally, S100A8/A9 could be used both as marker to determine feasibility of ASC-treatment in experimental OA as well as read-out to determine its efficacy.

ASCs may be a potent and safe tool to combat joint destruction and inflammation in OA patients with high synovial activation.

Author contributions

Conception and design of study: RS, JR, CJ, WvdB, PvdK, AB, PvL, FvdL.

Acquisition of data: RS, SvD, MtH, AS, TV, AB.

Analysis and interpretation of data: RS, MtH, TV, DN, PvdK, AB, PvL, FvdL.

Drafting the article: RS, PvL, MtH.

Revising article critically: SvD, AS, JR, TV, DN, CJ, WvdB, PvdK, AB, FvdL.

Final approval of the submitted manuscript: RS, SvD, MtH, AS, JR, TV, DN, CJ, WvdB, PvdK, AB, PvL, FvdL.

Conflict of interest statement

None of the authors had financial or personal relationships with people or organizations that could inappropriately influence the bias of the presented work.

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