

Antiinflammatory and Chondroprotective Effects of Intraarticular Injection of Adipose-Derived Stem Cells in Experimental Osteoarthritis

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Objective. In experimental collagenase-induced osteoarthritis (OA) in the mouse, synovial lining macrophages are crucial in mediating joint destruction. It was recently shown that adipose-derived stem cells (ASCs) express immunosuppressive characteristics. This study was undertaken to explore the effect of intraarticular injection of ASCs on synovial lining thickness and its relation to joint pathology in experimental mouse OA.

Methods. ASCs were isolated from fat surrounding the inguinal lymph nodes and cultured for 2 weeks. Experimental OA was induced by injection of collagenase into the knee joints of C57BL/6 mice. OA phenotypes were measured within 8 weeks after induction. Histologic analysis was performed, and synovial thickening, enthesophyte formation, and cartilage destruction were measured in the knee joint.

Results. ASCs were injected into the knee joints of mice 7 days after the induction of collagenase-induced OA. On day 1, green fluorescent protein–labeled ASCs were attached to the lining layer in close contact with

macrophages. Thickening of the synovial lining, formation of enthesophytes associated with medial collateral ligaments, and formation of enthesophytes associated with cruciate ligaments were significantly inhibited on day 42 after ASC treatment, by 31%, 89%, and 44%, respectively. Destruction of cartilage was inhibited on day 14 (65%) and day 42 (35%). In contrast to early treatment, injection of ASCs on day 14 after OA induction showed no significant effect on synovial activation or joint pathology on day 42.

Conclusion. These findings indicate that a single injection of ASCs into the knee joints of mice with early-stage collagenase-induced OA inhibits synovial thickening, formation of enthesophytes associated with ligaments, and cartilage destruction.

Although it is generally accepted that osteoarthritis (OA) is a disease of the cartilage, other tissue types, such as subchondral bone and synovium, are thought to be involved in mediating joint destruction in this disease. Clinically, patients with OA have a variable degree of synovitis, as demonstrated by high levels of interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF α), and complement factors (1,2). A substantial subpopulation of patients with early symptomatic OA shows an increase in synovial lining layer thickness (3). Synovial biopsy specimens from OA patients show hyperplasia and an increased number of lining cells that mainly consist of CD68+ macrophages. Macrophages within these OA biopsy specimens exhibit an activated phenotype and produce proinflammatory cytokines, such as IL-1 β , TNF α , vascular endothelial growth factor (VEGF), and S100 damage-associated molecular patterns (DAMPs) (3,4), suggesting that these cells may play an important role in OA pathology (5).

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Dr. Casteilla holds patents for the use of adipose-derived stem cells in ischemic conditions and as an anticancer treatment.

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Synovial lining macrophages are crucial in joint destruction in experimental collagenase-induced OA in mice. In a previous study, we found that selective elimination of lining macrophages from the mouse knee joint prior to the induction of collagenase-induced OA strongly inhibited synovial thickening, cartilage destruction, and the development of new cartilage/bone, indicating that synovial macrophages mediate joint destruction within this model (6). Synovial macrophages are an important source of both catabolic and anabolic factors that contribute to either cartilage destruction or the formation of osteophytes (7).

Mesenchymal stem cells (MSCs), which reside in various tissue types in the body, have recently been shown to exhibit immunosuppressive characteristics. Although it has generally been accepted that the primary effect of stem cell treatment occurs through tissue-specific differentiation (8,9), new data suggest that the therapeutic potential of these cells might be related to their paracrine effect (10,11). Several studies have shown that MSCs can modulate the functions of cells of the adaptive immune system, such as T cells and B cells (12). Other studies have shown that these stem cells are also able to induce expression of antiinflammatory mediators, such as IL-10 and IL-12p40, in macrophages (13,14). Multiple factors secreted by MSCs that exert an immunosuppressive effect have been identified, e.g., IL-10, IL-1 receptor antagonist (IL-1RA), indoleamine 2,3-dioxygenase (IDO), transforming growth factor β (TGF β), and prostaglandin E₂ (PGE₂) (15). Although several contradictory results have been published, there is ample evidence that inflammatory molecules may provide the licensing signal for MSCs to deliver immunosuppressive signals, including PGE₂ (10,16,17).

Adipose tissue is known to play a key role in energy balance and metabolic disorders. A recent study demonstrated that adipose tissue hosts multipotent stem cells (18). These cells, named adipose-derived stem cells (ASCs), can easily be purified after digestion of fat and selection by adhesion onto plastic from the very heterogeneous crude stromal fraction. ASCs share numerous properties with bone marrow-derived MSCs (18). In adipose tissue, ASCs represent ~5% of nucleated cells in the normal vascular fraction, which is much higher than in the adult human bone marrow, where MSCs represent only 0.0001–0.01% of the nucleated cells. Both types are able to differentiate toward adipogenic, osteoblastic, and chondrogenic phenotypes. Moreover, they display immunosuppressive properties *in vitro* as well as *in vivo* (19). Because of their immunosuppressive phe-

notype, ASCs may be used to decrease local inflammation in rheumatic diseases (20).

In the present study, we investigated the effect of local administration of ASCs into the knee joints of mice with experimentally induced OA in which synovial involvement is evident.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from Janvier. Animals were between 12 and 14 weeks old and received a standard diet and tap water *ad libitum*. All experiments were approved by the local authority Animal Care and Use Committee and were performed by personnel certified by the Dutch Ministry of Health, Welfare, and Sports.

Induction of experimental OA. Experimental collagenase-induced OA was induced as previously described (21). Briefly, the right knee joint of each mouse was injected on days 0 and 2 with 1 unit of type VII collagenase from *Clostridium histolyticum* (Sigma-Aldrich) in 6 μ l of physiologic saline. Mice were killed on days 14 and 42 after treatment with ASCs. The injected collagenase causes instability of the joints by damaging ligaments and does not directly digest cartilage. This model is characterized by OA-like damage. Clear synovial activation, which is related to cartilage destruction, is observed in this model (22).

Adipose-derived stem cells. ASCs were isolated from adipose tissue surrounding the mouse inguinal lymph nodes. Next, they were cultured for 2 weeks according to standard procedures in Dulbecco's modified Eagle's medium–Ham's F-12 (Gibco) supplemented with 1% penicillin/streptomycin (Invitrogen), 0.5% amphotericin B (Invitrogen), 16 μ M biotin (Sigma-Aldrich), 18 μ M pantothenic acid (Sigma-Aldrich), and 100 μ M ascorbic acid (Sigma-Aldrich). After recovery, the cells were tested for 6 different stem cell markers. Consistent with the results of previous studies (18,19), the stem cells stained positive for Sca-1 (BD Biosciences) (86%), CD44 (BioLegend) (81%), and CD105 (eBioscience) (69%) and were negative for CD11b (BioLegend) (0%), c-Kit (BD Biosciences) (0%), and CD34 (eBioscience) (4%). A total of 20,000 cells (passage 2) in 6 μ l of mouse serum (Jackson ImmunoResearch) with 4% mouse albumin (Sigma-Aldrich) were injected intraarticularly into the mouse knee joint. The number of cells used was calculated based on the number of cells per kg body weight that would be used in a planned clinical trial in OA patients (5×10^6 cells/joint). Control animals were injected with mouse serum containing 4% mouse albumin only.

Immunodetection of green fluorescent protein (GFP)-labeled ASCs. To localize ASCs after intraarticular injection, ASCs were labeled with GFP prior to injection. After standard culture procedures, ASCs were transfected with a lentiviral construct containing GFP under a constitutively active PGK promoter. GFP expression was confirmed using fluorescence-activated cell sorting and fluorescence microscopy. Mice were injected intraarticularly with ASCs 7 days after the induction of collagenase-induced OA. Twenty-four hours after injection, mouse knee joints were isolated and processed for histologic analysis. After deparaffinization and rehydration, sections

were incubated for 2 hours at room temperature with 10 mM citrate buffer (0.1M citrate acid, 0.1M sodium acid [pH 8.0]) to unmask antigenic sites. Next, to avoid background staining, endogenous peroxidase activity was blocked using 1% H₂O₂ in methanol. Subsequently, the slides were incubated with a rabbit anti-GFP antibody (Cell Signaling Technology) overnight at 4°C. After incubation with a biotinylated goat anti-rabbit IgG secondary antibody (45 minutes at room temperature), peroxidase-labeled avidin–biotin complexes were incubated on the sections using an ABC kit (Vector). Diaminobenzidine (DAB; Sigma-Aldrich) was used to localize the peroxidase activity, and hematoxylin was used as a counterstain. Finally, the stained sections were mounted in Permount (Fisher Scientific).

Histologic analysis of OA progression. Mouse knee joints were dissected and fixed in 4% formalin. After fixation, the joints were decalcified in 4% formic acid buffered in phosphate buffered saline (PBS) and processed for histologic analysis. Paraffin-embedded knee joints were cut into sections measuring 7 μm. Six sections, spaced 140 μm apart, were mounted on glass slides and stained with Safranin O for analysis of cartilage damage and chondrocyte/osteocyte-associated ligaments (collateral/cruciate). For scoring of synovial activation, hematoxylin and eosin (H&E)-stained sections were used.

In this model of collagenase-induced OA, cartilage damage starts with surface fibrillation on day 21. Lesions were observed between days 35 and 42 in the present study and were scored using a modified form of the OA Research Society International grading system for histopathologic assessment of OA cartilage (23). Briefly, the depth of the cartilage damage as well as the extent of the damaged surface was scored in a blinded manner at 4 different locations in the mouse knee joint, i.e., the lateral and medial tibia and femur, using an arbitrary grading scale of 0–6 for the severity of cartilage destruction and of 0–5 for the extent of damaged cartilage surface. The OA score was defined as the product of the multiplication of these 2 scores.

Synovial thickness/activation was scored on H&E-stained sections using an arbitrary grading scale of 0–3, as previously described (21). In this OA model, synovial thickening starts on day 7 and is maximal on day 14, but is still significant on day 42. Briefly, scores ranged from 0, indicating no thickening of the lining layer (1 cell layer), to 3, indicating the maximal observed thickening of the synovial lining layer (10–12 cell layers).

Safranin O-stained sections were used to assess the size and number of chondrocytes/entheseocytes associated with ligaments. Chondrogenesis started between days 7 and 14, and structures were transformed into bone between days 35 and 42 after OA induction. The extent of chondrogenesis was scored in each joint by measurement of 3 sections, each spaced 140 μm apart. In each section, surface areas of chondrocytes/entheseocytes were measured in cruciate ligaments and medial collateral ligaments using an image analysis system (Leica QWin; Leica Microsystems). To assess cartilage formation in the cruciate ligaments, image analysis was used to calculate the percentage of the cruciate ligament surface that was positive for proteoglycans. In the medial collateral ligament, the cartilaginous and osseous structures that developed were defined as being chondrocytes or entheseocytes by hand. The mean

area per knee joint (3 sections) was calculated and expressed in micrometers squared.

Immunodetection of NITEGE epitopes. After deparaffinization and rehydration, mouse knee joint sections were treated with citrate buffer and 1% H₂O₂ in methanol as described above. Sections were then incubated with anti-NITEGE antibodies (provided by Dr. John Mort, Shriners Hospital for Children, Montreal, Quebec, Canada) in PBS containing 5% milk powder, 3% fetal calf serum, and 2% bovine serum albumin. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody. After incubation with peroxidase-labeled avidin–biotin complexes (Vector), DAB was used to localize the peroxidase activity. Sections were counterstained with hematoxylin for 1 minute and embedded in Permount.

Isolation of murine synovial specimens, preparation of RNA, and reverse transcriptase–polymerase chain reaction (RT-PCR). On day 14 after ASC treatment of the mouse knee joints (in which OA had been induced 7 days prior to ASC treatment), synovial specimens were isolated as previously described (9). Briefly, joint capsule specimens were isolated on the medial and lateral sides of the mouse patella with a biopsy punch. Synovial specimens were snap-frozen in liquid nitrogen and stored for RNA isolation. RNA was isolated from the synovium using TRIzol reagent according to the recommendations of the manufacturer (Invitrogen). Five micrograms of total RNA was reverse transcribed, and complementary DNA aliquots were subjected to PCR. RT-PCR was normalized to the transcriptional levels of GAPDH. Levels of messenger RNA (mRNA) for various cytokines (IL-1β, IL-6, and IL-10) were measured in the synovial specimens at various time points after the induction of collagenase-induced OA and were quantified using an ABI Prism 7000 sequence detection system.

Statistical analysis. Statistical differences were calculated by the Mann-Whitney U test, using GraphPad Prism software version 5. *P* values less than 0.05 were considered significant.

RESULTS

Decrease in synovial activation after ASC treatment. A thickened synovial lining layer comprising activated macrophages is prominent in collagenase-induced OA and has previously been shown to be involved in mediating cartilage destruction and osteophyte formation (7). Hyperplasia of the synovial lining layer is maximal on day 7 of collagenase-induced OA and remains high thereafter. To test whether ASCs can alter the synovial lining thickness, mice were given a single injection of ASCs (2×10^4 cells in 6 μl of mouse serum containing 4% mouse serum albumin) in the right knee joint on day 7 after induction of collagenase-induced OA. Synovial lining thickness was scored by 2 independent observers after injection of either ASCs or serum only. Figure 1A shows that the lining layer thickness was significantly lower in ASC-treated mice than in control-treated mice

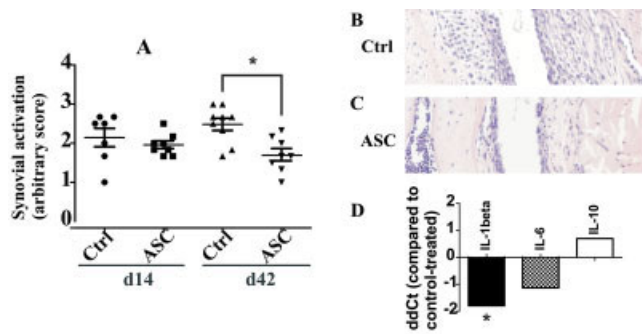


Figure 1. Decrease in synovial thickening in mice treated with adipose-derived stem cells (ASCs). **A**, Decreased thickening of the synovium in ASC-treated mice on day 14 and day 42 after treatment. The decrease in thickening versus control mice (31%) reached significance on day 42. Each data point represents a single mouse; horizontal lines and error bars show the mean \pm SEM ($n = 8\text{--}11$ mice per group). $* = P < 0.05$. **B** and **C**, Clear reduction in thickening of the lining layer in an ASC-treated mouse (**C**) compared to a control animal (**B**) on day 42 after treatment. Results are representative of 2 experiments. Original magnification $\times 250$. **D**, Significant reduction in the expression of mRNA for interleukin-1 β (IL-1 β), but not IL-6 or IL-10, in the osteoarthritic synovium of ASC-treated mice, as compared to controls, on day 14 after treatment. RNA from the synovium of 6 animals per group was used for the analysis of gene expression. Bars show the mean. $* = P < 0.05$.

on day 42 (31% lower) but not on day 14 (9% lower) after ASC treatment. Photomicrographs show that the synovial lining layer of ASC-treated mice was significantly less thickened, comprising lower numbers of macrophages (Figure 1C), as compared to control-treated mice (Figure 1B). Interestingly, on day 14 after ASC treatment, levels of mRNA for IL-1 β (but not for IL-6 or IL-10) were significantly lower in the OA synovium of ASC-treated mice than that of control-treated mice (Figure 1D).

Intraarticularly injected ASCs home to the subintimal synovial lining layer in mice. Since intraarticular injection of ASCs decreases the thickness of the synovial lining, we next used ASCs labeled with GFP to analyze whether ASCs directly interact with the synovium. In vitro, ASCs were transfected with a lentiviral GFP construct and additionally cultured for 24 hours in chamber slides. More than 85% of the ASCs expressed an abundant amount of GFP protein, as assessed by flow cytometry (data not shown) and fluorescence microscopy (Figure 2A).

Next, GFP-transfected ASCs were injected into the mouse knee joint 7 days after the induction of collagenase-induced OA. Twenty-four hours after injection and 5 days after injection, mouse knee joints were isolated and processed for histologic analysis.

When knee joint sections were immunostained using specific GFP antibodies 24 hours after injection, GFP-positive cells were clearly visualized in the cruciate ligament area and within the subintimal layer of the synovium (Figures 2B and C). A higher-magnification view shows that the ASCs were lying just below the intima lining layer, in close interaction with synovial macrophages (Figure 2D). On day 5 after injection, ASCs were no longer detected in the lining layer (data not shown).

Prevention of dislocation of the OA knee joint and prevention of new cartilage formation in the collateral and cruciate ligaments in ASC-treated mice. Synovial activation in collagenase-induced OA strongly contributes to damage of the collateral and cruciate ligaments, leading to destabilization and subsequent dislocation of the mouse knee joint. Investigating total knee joint sections, we observed that the induction of collagenase-induced OA caused dislocation of the knee joint in the majority (75%) of the mice. After a single injection of ASCs, cruciate ligament rupture was found in only 25% of the mice, suggesting that ASC treatment protects against damage to ligaments (data not shown).

Clinically, the formation of new cartilage and bone structures within ligaments impairs good functioning of the joint. During experimental OA, early pathologic changes comprise the formation of new cartilage (chondrocytes) in the collateral and cruciate ligaments; these chondrocytes subsequently change through ossi-

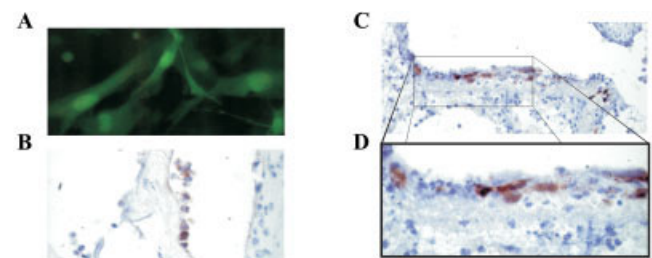


Figure 2. Intraarticularly injected adipose-derived stem cells (ASCs) home to cruciate ligaments and subintimal layers of the synovium in mice. Green fluorescent protein (GFP)-transfected ASCs were injected into mice on day 7 after the induction of collagenase-induced osteoarthritis. Twenty-four hours after the injection of ASCs, mouse knee joints were isolated and processed, and knee joint sections were stained with an anti-GFP antibody. **A**, Fluorescence microscopy, showing efficient transfection of ASCs with lentiviral GFP. **B**, GFP-positive cells close to the cruciate ligaments. **C** and **D**, Localization of clusters of GFP-positive cells in subintimal layers of the synovium. **D** shows a higher-magnification view of the boxed area in **C**. Original magnification $\times 1,000$ in **A**; $\times 250$ in **B** and **C**; $\times 400$ in **D**.

fication into enthesophytes (21). On day 14 after the induction of collagenase-induced OA, chondrogenesis was already observed in the collateral ligaments, partic-

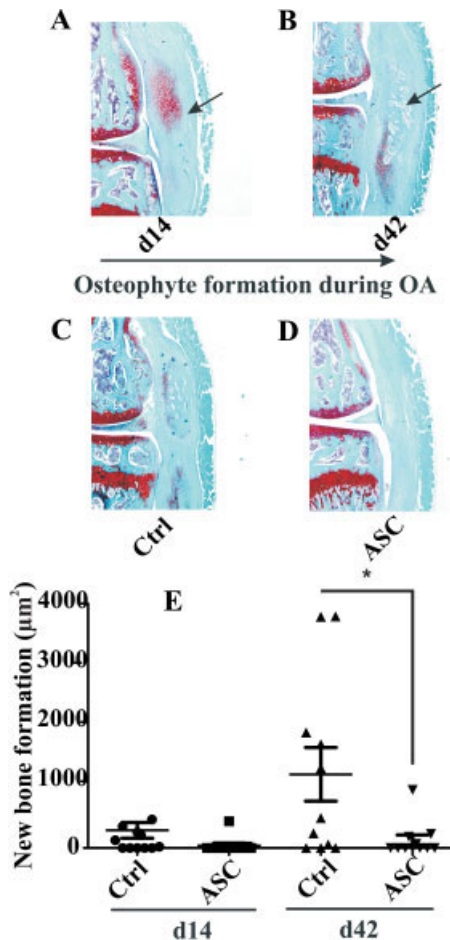


Figure 3. Prevention of chondrogenesis and enthesophyte formation in the medial collateral ligament in mice treated with adipose-derived stem cells (ASCs). On day 14 and day 42 after treatment, chondrogenesis and the size of newly formed bone structures were determined using a Leica QWin image analysis system. **A**, Chondrogenesis (arrow) within the mouse medial collateral ligament on day 14 in an untreated control animal. **B**, Transformation of structures into enthesophytes (arrow) through ossification by day 42 in an untreated control animal. **C** and **D**, Newly formed enthesophytes in a control animal (**C**) and an ASC-treated animal (**D**) on day 42 after treatment. Original magnification $\times 250$ in **A–D**. **E**, Decrease in the surface area containing proteoglycans (chondrogenesis) in ASC-treated animals compared to control animals on day 14 and decrease in the surface area of newly formed enthesophytes (by 89%) in ASC-treated animals compared to control animals on day 42. Each data point represents a single mouse; horizontal lines and error bars show the mean \pm SEM. Results are representative of 2 experiments ($n = 8–11$ mice per group). $* = P < 0.05$. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

ularly at the medial site (Figure 3A). On day 42 after OA induction, these structures had completely transformed into bone (enthesophytes) due to ossification (Figure 3B).

On day 14 after ASC treatment, chondrogenesis (measured as proteoglycan deposition) was mainly observed in the medial collateral ligaments in the majority of the control OA mice. In contrast, much less chondrogenesis (76% less) was observed in the ASC-treated mice ($P = 0.08$) (Figure 3E). Consistent with these observations, significantly smaller enthesophytes (size quantified using image analysis) were measured in the medial collateral ligaments on day 42 after treatment. The surface area decreased by 89% in ASC-treated animals compared to control animals ($P < 0.05$) (Figures 3C–E).

In addition to the collateral ligaments, the cruciate ligaments of the OA mouse knee joint showed chondrogenesis/enthesophytes, which were measured using image analysis (Figure 4A). Safranin O staining showed marked presence of proteoglycans on day 42, particularly at the sites of contact between the ligaments and the bone. The area that stained positive for proteoglycans was significantly smaller (44%) in ASC-treated mice (Figure 4C) than in controls (Figure 4B). Deposition of proteoglycans was seen in 35% of the ligaments of the ASC-treated mice compared to 63% of the ligaments of the controls ($P < 0.05$) (Figure 4D).

Synovial activation strongly up-regulates enzymes such as ADAMTS-4 and ADAMTS-5, members of the aggrecanase family that are major players involved in the formation and remodeling of cartilage. These enzymes cleave proteoglycans at a specific site, thereby leaving neoepitopes (ending at the amino acid sequence NITEGE), which can be detected using specific antibodies and immunolocalization, within the cartilage matrix. A strong staining of NITEGE neoepitopes was found within the cartilage-containing areas of the cruciate ligaments of the control OA mouse knee joint. A single injection of ASCs significantly inhibited NITEGE neoepitope expression, suggesting that aggrecanase activity is suppressed by ASC treatment (Figure 5). In vitro, ASCs expressed high levels of mRNA for tissue inhibitors of metalloproteinases (TIMPs), particularly TIMPs 1 and 3, when compared to macrophages, suggesting that they may be involved in inhibiting enzyme activity (data not shown).

Significant inhibition of cartilage destruction in mice with collagenase-induced OA after intraarticular injection of ASCs into the knee joint. Synovial activation in collagenase-induced OA also promotes

the destruction of cartilage, caused by a combined action of prolonged release of catabolic factors by the activated synovium and ligament biomechanical insuffi-

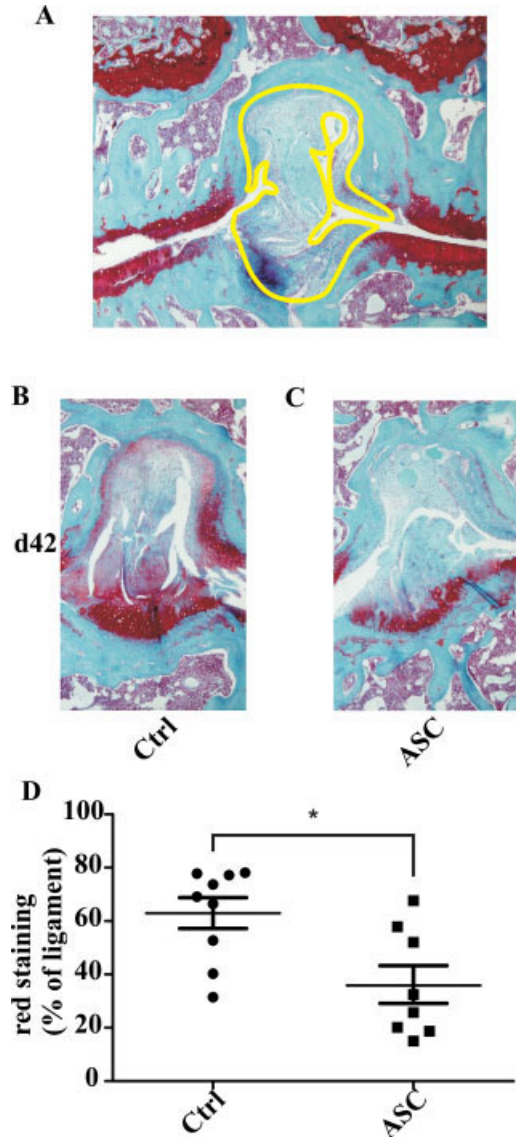


Figure 4. Chondrogenesis in the mouse cruciate ligaments during experimental osteoarthritis. **A**, Chondrogenesis in the mouse cruciate ligaments delineated as shown, quantified using a Leica QWin image analysis system. **B** and **C**, Proteoglycan deposition in a control animal (**B**) and an adipose-derived stem cell (ASC)-treated animal (**C**) on day 42 after treatment. Original magnification $\times 250$ in **A–C**. **D**, Significant decrease in proteoglycan deposition in ASC-treated animals compared to controls on day 42 after treatment. Each data point represents a single mouse; horizontal lines and error bars show the mean \pm SEM ($n = 8–9$ mice per group). $* = P < 0.05$. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

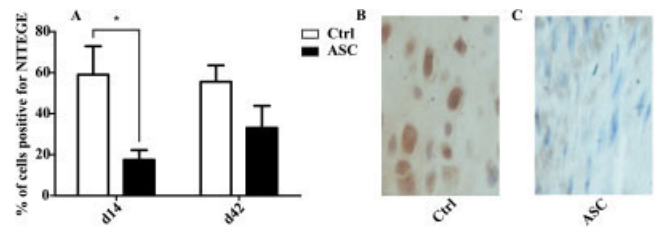


Figure 5. Decreased NITEGE expression in the cruciate ligaments of the knee joints of mice with collagenase-induced osteoarthritis treated with adipose-derived stem cells (ASCs). The neoepitope NITEGE is expressed upon ADAMTS activity during chondrogenesis/bone formation. The percentage of NITEGE staining regions in the mouse cruciate ligament was determined. **A**, Reduction of 69% and 35% on days 14 and 42, respectively, in NITEGE staining in mice treated with ASC compared to controls. Bars show the mean \pm SEM ($n = 8$ mice per group). $* = P < 0.05$. **B** and **C**, Hypertrophic, chondrocyte-like phenotype of cells in the cruciate ligaments of a control animal (**B**) compared to an ASC-treated animal (**C**). Original magnification $\times 250$. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

ciency induced by anabolic mediators. On day 42 after the induction of collagenase-induced OA, prominent cartilage destruction was observed in all 4 layers of the mouse knee joint. A single injection of ASCs into the mouse knee joint 7 days after the induction of OA inhibited cartilage damage on day 14 after ASC treatment. In particular, the cartilage in the lateral femur was protected against damage (65% lower OA score in ASC-treated mice than in control mice) ($P < 0.05$) (Figure 6A). On day 42 after treatment, the mean of the OA scores, indicating cartilage damage, for the 4 different locations within the knee joint was still significantly decreased (by 35%) in ASC-treated mice compared to controls ($P < 0.05$) (Figures 6B–D).

Lack of a significant effect of a single injection of ASCs at a later time point (day 14) on synovial thickness and joint destruction in mice with collagenase-induced OA. Next, we studied whether ASC treatment also affects synovial thickness and joint destruction when administered to mice on day 14 after the induction of collagenase-induced OA. At that time point, ligaments were already severely damaged, in contrast to day 7 after the induction of OA. On day 42 after a single injection of ASCs into the day-14 OA mouse knee joint, synovial thickness (Figure 6E), enthesophyte formation (Figure 6F), and cartilage destruction (Figure 6G), although somewhat lower, were not significantly reduced when compared to controls.

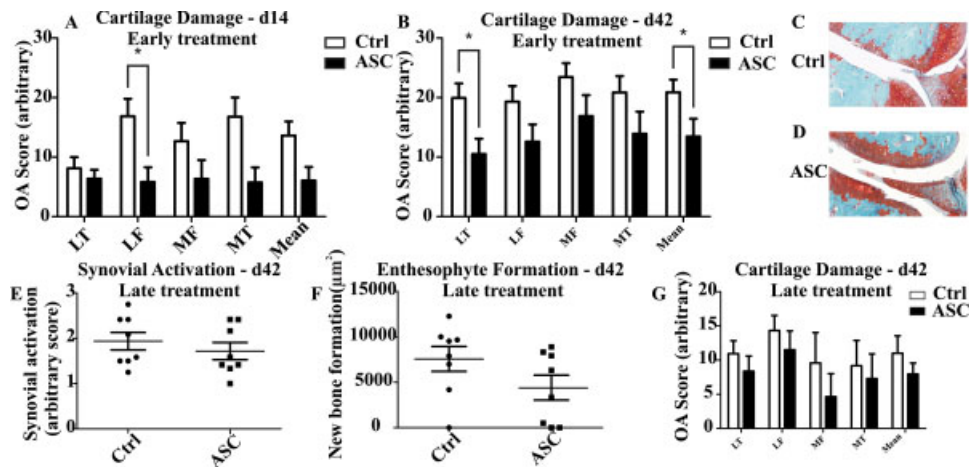


Figure 6. Inhibition of the development of cartilage destruction in mice treated with adipose-derived stem cells (ASCs) soon after the induction of collagenase-induced osteoarthritis (OA). ASCs were injected into the mouse knee joints on either day 7 or day 14 after the induction of collagenase-induced OA. Cartilage destruction was scored at 4 different locations in the knee joint, i.e., the lateral tibia (LT), lateral femur (LF), medial femur (MF), and medial tibia (MT). The mean OA score was calculated using the scores for the 4 individual locations. **A** and **B**, Reduction in cartilage damage scores on day 14 after treatment (**A**) and day 42 after treatment (**B**) in mice treated with ASCs 7 days after the induction of OA (early treatment) compared to control mice. The difference was significant for the lateral femur score on day 14 and for the lateral tibia score and mean score on day 42. Bars show the mean \pm SEM ($n = 8$ mice per group). $* = P < 0.05$. **C** and **D**, Cartilage damage on day 42 after treatment in a control mouse (**C**) and a mouse treated with ASCs 7 days after the induction of OA (**D**). Original magnification $\times 250$. **E–G**, Lack of a significant effect on synovial thickness (**E**), enthesophyte formation (**F**), or cartilage damage (**G**) on day 42 after treatment in mice treated with ASCs 14 days after the induction of OA (late treatment). In **E** and **F**, each data point represents a single mouse; horizontal lines and error bars show the mean \pm SEM. In **G**, bars show the mean \pm SEM ($n = 8$ mice per group). Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

DISCUSSION

In the present study, we found that a single injection of ASCs into the mouse knee joint given at an early time point (day 7) after the induction of collagenase-induced OA protects against synovial thickening, enthesophyte formation, and cartilage destruction.

Stem cells are used in cellular therapy for a broad spectrum of diseases. Although it is generally believed that the primary therapeutic effect of stem cells occurs through tissue differentiation (8,9), they may also act in a paracrine manner by the production of soluble anti-inflammatory factors. Previous studies in our laboratory (6,7) have shown that thickening of the synovial lining layer is a characteristic feature of collagenase-induced OA. This layer comprises mainly macrophages exhibiting an activated phenotype. Increased thickness of the lining started on day 3, reached its maximum on day 7, and persisted up to day 42 (24). We previously showed that selective elimination of the lining macrophages prior to the induction of collagenase-induced OA strongly inhibited synovial thickness but also osteophyte formation and cartilage destruction (6,24).

Synovial macrophages are important producers of chemokines that attract monocytes that subsequently differentiate into macrophages or induce proliferation of fibroblasts within the lining layer, thereby increasing its thickness. During OA, the cartilage layer is damaged and releases cartilage fragments. Small leucine-rich repeat proteins and small proteoglycans, such as biglycan and decorin, have been shown to stimulate macrophages via Toll-like receptor 4 (TLR-4), thereby releasing pro-inflammatory cytokines IL-1 (25), TNF α , or S100 DAMPs (S100A8/A9) (26), which may further stimulate the activation status of the lining layer.

Injection of ASCs on day 7 after the onset of collagenase-induced OA inhibited synovial thickening in the OA knee joint on day 42. Localization of GFP-labeled ASCs in the OA knee joint showed that 24 hours after injection, ASCs were detected within the synovium just below the lining layer. A close interaction between ASCs and macrophages within the intimal layer was observed. On day 5 after injection, virtually no GFP-labeled cells could be detected (data not shown), suggesting that the effects on the synovium are induced

within the first days after injection, initiating a prolonged long-lasting suppressive effect.

Chondrogenesis and the subsequent formation of enthesophytes within collateral/cruciate ligaments in this model are mediated by synovial macrophages (6,24) and hamper good functioning of the joint, which may further enhance joint destruction. Macrophage-derived factors involved in chondrogenesis include growth factors like TGF β , bone morphogenetic protein 2 (BMP-2), and BMP-4 (6). Production of these growth factors within the synovium was strongly diminished after macrophages were selectively depleted from the lining layer (24). During OA, dysregulated chondrogenesis is observed along the margins of the articular cartilage or within the ligaments, and most likely differentiate from chondrogenic precursor cells that reside within the periosteum or ligament. Stem cell treatment may suppress the production of growth factors by synovial macrophages, thereby preventing the proliferation of chondrogenic precursor cells.

Damage of the cruciate ligaments was strongly correlated to dislocation of the knee joint. In the cruciate ligament area, macrophages are also abundantly present and prolonged activation of these cells may further stimulate ligament destruction. Recent studies showed that enzymes like ADAMTS are involved in the degeneration of ligament structures (27) and are crucial for the destruction of cartilage in experimental OA (28). These enzymes cleave aggrecan, thereby inducing neoepitopes (NITEGE) which can be visualized with immunodetection (27). Stem cell treatment strongly inhibited expression of NITEGE neoepitopes, suggesting that ADAMTS activity is suppressed.

Cartilage destruction is a characteristic feature of OA that was also inhibited by local ASC treatment. ASCs may suppress macrophage activity and thereby the production of catabolic mediators, such as IL-1 β and TNF α . Recently, we showed that the alarmins S100A8 and S100A9, which are major products of macrophages, are key players in mediating cartilage destruction in collagenase-induced OA (28,29). These proteins stimulate matrix metalloproteinase (MMP) production in macrophages (30) and chondrocytes (29) via TLR-4 signaling. IL-1 β is expressed only during the first phase of collagenase-induced OA, whereas S100 proteins are released throughout the course of the disease, which may explain why S100 proteins are dominant within this model. Whether ASC treatment has an effect on IL-1 β , TNF α , and S100A8/A9 levels in the blood and synovium washouts is currently under investigation.

When ASCs were administered to mice intra-

articularly on day 14 after the onset of collagenase-induced OA, no significant protection against joint pathology was found. One explanation may be that collateral/cruciate ligaments are already irreversibly damaged by that time point, preventing successful recovery. Mediators like IL-1 β and MMPs have predictive value for ligament injury in animal models (31). Stem cells have been shown to produce soluble inhibitory factors, such as IL-1RA (32) and TIMPs (33), which may partly block the induction of IL-1/MMP-mediated ligament destruction (31). However, a previous study has shown that late delivery of autologous MSCs to caprine joints subjected to meniscectomy and complete resection of the anterior cruciate ligament still resulted in the regeneration of meniscal tissue and significant chondroprotection (34). In the present study, we injected only a small amount of ASCs into the mouse knee joint, which may be sufficient for inhibiting the induction of ligament damage but may be too low for the repair of already-damaged ligaments. Damaged ligaments can bind MSCs, thereby providing the basis for a biologic repair of the ligament (35).

A further explanation of why deposition of ASCs may not be effective when given on day 14 after the induction of collagenase-induced OA may be the minor release of cytokines by the synovium at that late time point. Proinflammatory cytokines, such as interferon- γ , IL-6, and TNF α , have been shown to enhance the immunosuppressive potential of ASCs (13,16,17,36). The release of proinflammatory factors is highest within the synovium during the first 7 days after the onset of collagenase-induced OA (28) and may drive the anti-inflammatory potential of ASCs. In reaction to pro-inflammatory stimuli, ASCs increase the intracellular enzymes IDO, heme oxygenase 1, and cyclooxygenase 2, leading to enhanced release of CO, biliverdin, and PGE₂, which suppress activated macrophages, thereby preventing OA pathology (13,37,38). This mechanism may further explain why stem cell treatment is so effective in inflammatory diseases such as arthritis (16) and ischemic heart disease (39). Stimulation of ASCs with proinflammatory cytokines prior to delivery to the OA joint may further enhance the efficacy of ASC treatment.

In the present study, we showed that ASCs, when given at an early phase of experimental OA, inhibit synovial lining thickening and protect against joint destruction by both anabolic and catabolic mediators. ASCs may therefore be a potent and safe tool for the treatment of a disease for which no cure is available to date.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. van Lent had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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