EXTENDED REPORT

Mesenchymal stem cell therapy in proteoglycan induced arthritis

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ABSTRACT

Objectives To explore the immunosuppressive effect and mechanism of action of intraperitoneal (ip) and intra-articular (ia) mesenchymal stem cell (MSC) injection in proteoglycan induced arthritis (PGIA).

Methods MSC were administered ip or ia after establishment of arthritis. We used serial bioluminescence imaging (BLI) to trace luciferase-transfected MSC. Mice were sacrificed at different time points to examine immunomodulatory changes in blood and secondary lymphoid organs.

Results Both ip and local ia MSC injection resulted in a beneficial clinical and histological effect on established PGIA. BLI showed that MSC ip and ia in arthritic mice are largely retained for several weeks in the peritoneal cavity or injected joint respectively, without signs of migration. Following MSC treatment pathogenic PG-specific IgG2a antibodies in serum decreased. The Th2 cytokine IL-4 was only upregulated in PG-stimulated lymphocytes from spleens in ip treated mice and in lymphocytes from draining lymph nodes in ia treated mice. An increase in production of IL-10 was seen with equal distribution. Although IFN-γ was also elevated, the IFN-γ/IL-4 ratio in MSC treated mice was opposite to the ratio in (untreated) active PGIA.

Conclusions MSC treatment, both ip and ia, suppresses PGIA, a non-collagen induced arthritis model. MSC are largely retained for weeks in the injection region. MSC treatment induced at the region of injection a deviation of PG-specific immune responses, suggesting a more regulatory phenotype with production of IL-4 and IL-10, but also of IFN-γ, and a systemic decrease of pathogenic PG-specific IgG2a antibodies. These findings underpin the potential of MSC treatment in resistant arthritis.

INTRODUCTION

Mesenchymal stem or stromal cells (MSC), a subset of non-haematopoietic stem cells cultured from the plastic adherent cell fraction from bone marrow, exert a strong immunomodulatory effect both in vitro and in vivo.[1] Allogeneic MSC were first successfully used to rescue a patient from life-threatening steroid-refractory acute graft-versus-host-disease, an inflammatory complication of haematopoietic stem cell transplantation with high mortality rate (90%).[2] This case report was a major breakthrough, soon followed by a phase II study.[3] Trials with MSC are now underway in a broad spectrum of inflammatory diseases and are considered for rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA).

Studies in arthritis showed that MSC are present in synovial fluid (SF),[4 5] and their number in SF is much lower in RA patients than in osteoarthritis.[6] This low number of SF MSC in RA patients could be explained by an impaired MSC recruitment to the joint[7] and suppressed proliferation potential of MSC[8] associated with decreased telomere length.[9] However, the anti-proliferative quality of MSC on peripheral blood mononuclear cells is preserved in patients with RA.[10]

Earlier studies showed that RA patients in whom multiple drugs have failed can still respond favourably to a strong immune-ablative conditioning with autologous haematopoietic stem cells as rescue therapy, unfortunately with high morbidity and even mortality.[11 12] Treatment with MSC would not require such hazardous conditioning. Moreover, local MSC therapy could be helpful in therapy-resistant oligoarthritis for which there is currently limited availability of registered therapeutic agents.

To further examine the potential of MSC in RA and JIA, we explored the distribution of injected MSC and their effect on established arthritis after intraperitoneal (ip) or intra-articular (ia) administration. We chose to use proteoglycan induced arthritis (PGIA), one of the most extensive studied models of inflammatory arthritis. Once established, PGIA progresses in symmetrical polyarthritis with ankylosis of joints and no chance of full recovery.[13]

MATERIALS AND METHODS

Animals and experimental set-up

All animals were female BALB/c mice, retired breeders from Charles River (Sulzfeld, Germany). They were housed in standard conditions in our university's central animal laboratory. The treatment regimens were randomly placed in cages and blinded to researchers. The experiments were conducted with permission of the local Ethical Committee for Animal Experimentation and in compliance with national guidelines for laboratory animal use.

The mice (10 per treatment group) were injected twice ip with 0.4 mg human PG with 3-week intervals to induce arthritis as previously described.[14] At 7 and 36 days after final PG injection, 5×10^6 MSC ip or 1×10^6 MSC ia were subsequently administered as treatment. Ia injections were performed on the right knee; the left knee stayed untouched. The control group received phosphate-buffered saline (PBS) ip. Arthritis was scored until day 67 in a blinded fashion as described elsewhere by two independent persons (JS and FH).[14] The cumulative arthritis score consists of scoring four paws with a maximum of 4 per paw and obligatory sacrifice at a cumulative score of 12. Another experiment was performed for in vivo tracking with six

mice per treatment group. A separate study was performed to explore the effect of immunomodulation by MSC in which temporal sacrifice of four mice per treatment group was allowed.

MSC

Murine MSC were obtained by culture of single-cell suspensions in polystyrene flasks (Falcon, BD Bioscience, Bedford, Massachusetts, USA) at 5% CO_2 and 21% O_2 derived from femoral and tibial bone marrow of 7-week old BALB/c mice. Adherent cells were harvested by trypsinisation and subsequently passaged twice before use. MSC used were Sca1+, CD106+, CD90+, CD44+ as checked by flow cytometry (antibodies obtained from BD, and Caltag-Medsystems, Buckingham, UK). Multilineage differentiation was shown with adipocytes grown out of MSC in adipogenic stimulatory supplement (mouse) diluted 1:5 in Mesencult MSC Basal medium (both Stem Cell Technologies, Vancouver, Canada) as shown by positive oil-red-O staining and osteoblastic differentiation stained with *FAST* 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue-tetrazolium substrate (Sigma-Aldrich Chemie, Zwijndrecht, Netherlands). Prior to in vivo use, suppressive capacity of MSC was checked by suppression of in vitro proliferation with anti-CD3 stimulated carboxyfluorescein succinimidyl ester (CFSE) stained splenocytes.

Histology

Between 71 and 79 days after last PG injection, mice were sacrificed and knee joints removed. Joints were decalcified in 10% EDTA for 48 h and embedded in paraffin. Tissue sections were stained with H&E. For the ia and ip treated groups, eight paws per group were histologically scored and compared to nine paws from untreated mice. Joints were scored for presence or absence of cartilage erosions in a blinded fashion by an independent person.

In vivo MSC tracking

MSC were transduced with a GFP-luciferase retroviral vector as described elsewhere.[15] The transduced MSC used for bioluminescence imaging (BLI) showed 65% positivity for GFP-luciferase as assessed by flow cytometry. In six ia and six ip treated mice the presence and location of luciferase expressing MSC was monitored 1 and 4–5 weeks after injection of marked MSC as previously described.[15] With this BLI method we are able to detect in vivo clusters of 100 cells.

PG-specific IgG ELISA

Four ip and four ia treated mice received 2.5×10^6 and 1×10^6 MSC, respectively, at 7 and 10 days after the last PG injection. Sera were obtained 12 and 13 days after the last PG injection. Sera were diluted 1:400 with PBS and incubated in PG coated wells for 2 h at room temperature. IgG1 and IgG2a anti-mouse antibodies were detected with horseradish peroxidase anti-IgG1 (Clone X56, BD Bioscience) and anti-IgG2a (Clone R19-15, BD Bioscience) with 3,3′,5,5′-tetramethylbenzidine (MP-Biomedicals, Illkirch, France). Extinction was measured at 405 nm.

Cytokine measurement after ex vivo restimulation with PG of lymphocytes

Four ip and four ia treated mice received 2.5 and 1 million MSC, respectively, at 7 and 10 days after the last PG injection. On day 26 and 27 after the last PG injection these mice were sacrificed and their cells from spleen and draining lymph nodes (LN, inguinal and popliteal combined) were harvested with the LN separated for three ia mice according to injected or non-injected side. Splenocytes were lysed with shock buffer (1:10 KCl) for 10 min. Isolated spleen and LN cells were stimulated in a 96-well plate (2×10^5/well) with 10 µg/mL human PG or medium for 4 days. Cytokine concentrations (interferon (IFN)-γ, interleukin (IL)-10, IL-17, IL-1β, IL-4, IL-6, tumour necrosis factor (TNF)-α) in supernatants were measured with Bio-Rad (Veenendaal, Netherlands) mouse 7-plex cytokine assay and analysed with Bio-Plex Manager 4.1.

Statistics

Results were tested for significant differences with Graphpad Prism 5 for Windows. For dichotomous variables the χ^2 test was used, and for normally distributed variables, the parametric analysis of variance with Dunnett's post-hoc test; for the other variables we used the non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test. Significance at $p<0.05$ and $p<0.01$ was determined.

RESULTS

The murine MSC were shown to suppress lymphocyte proliferation in vitro (figure 1A). The set-up of our experiments is shown in figure 1B. The mice that were injected with PG developed arthritis at the expected incidence (mean 85.9% for all three studies), with a median onset of arthritis at 5.8 days after the second injection of PG.

Intraperitoneal administration of MSC results in a beneficial clinical and histological effect on PGIA

A protective effect of prophylactic MSC has previously been shown in collagen induced arthritis (CIA).[16] PGIA is a different model of arthritis, with a marked different immunopathogenesis in BALB/c mice which primarily exhibit a Th2-type immune response, whereas the DBA/1 mice in CIA respond in a Th1-dependent manner.[17] We first wanted to know whether in this non-collagen induced form of arthritis, MSC therapy has a beneficial effect. Indeed, repeated ip injection of 5×10^6 MSC in mice with established arthritis resulted in a stable arthritis score, compared to control mice which showed a marked increase in arthritis scores (figure 2A,B and see online supplementary figure S1). This effect did just not reach significance. Histology also showed that the incidence of cartilage erosions, which is the end stage of arthritic inflammation, decreased from 33.3% in the knee joints of control mice to 12.5% in the repeated ip treated mice, although it did not reach statistical significance ($p=0.3$) (figure 2C shows representative histology for eroded vs non-eroded joints). Thus, ip administration of MSC effectively suppressed arthritis.

Repeated intra-articular administration of MSC results in a beneficial clinical and histological effect on PGIA

We wanted to determine whether local ia injections had a similar suppressive effect on arthritis. Indeed, repeated ia injection of 1×10^6 MSC in the right knee suppressed the cumulative arthritis score (figure 2A,B and see online supplementary figure S1). Histology also showed a decrease of incidence of cartilage erosions from 33.3% in the joints of control mice to 12.5% in the ia mice, although it was not statistically significant ($p=0.3$). Thus, ia administration of 2×10^6 MSC effectively suppressed the arthritis.

MSC in arthritic mice survive several weeks at the region of injection without signs of specific migration

Interestingly, we noticed that repeated ia injection of MSC had a suppressive effect not only on the injected paw but also on the

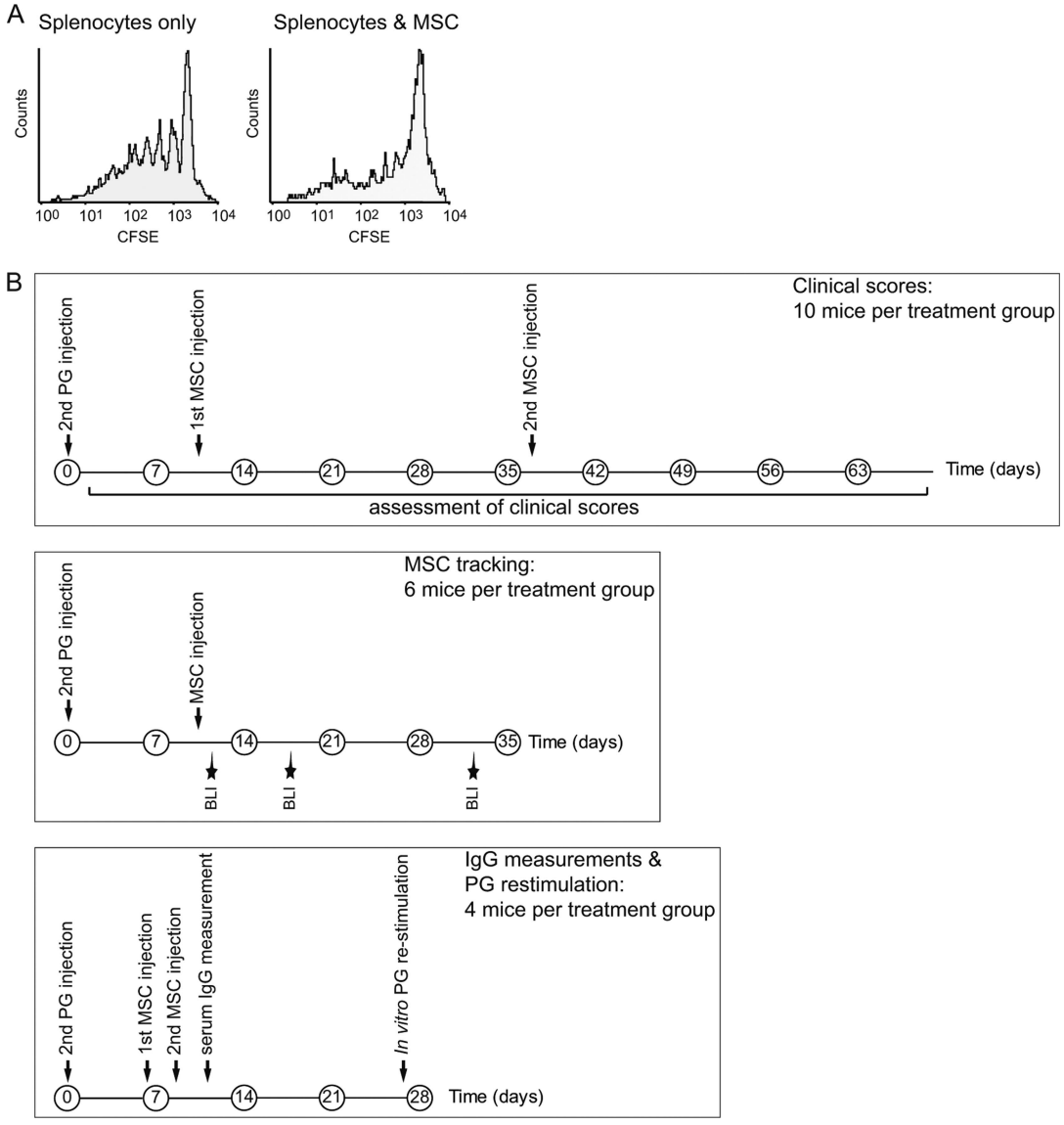

Figure 1 In vitro suppression of splenocytes by mesenchymal stromal cells (MSC) and experimental setup. (A) Proliferation of anti-CD3 stimulated carboxyfluorescein succinimidyl ester (CFSE)-stained lymphocytes was effectively suppressed from 100% (splenocytes only) to 54.2% when MSC were added in a 1:1 ratio. (B) Experimental setup. Mice were injected twice intraperitoneally (ip) with proteoglycan (PG) and received MSC ip (n=10) or MSC intra-articularly (ia; n=10) or phosphate-buffered saline ip (n=9) when arthritis had established at day 10 after PG injection and 4 weeks later. Arthritis was scored twice a week until 67 days after the first PG injection by two independent blinded observers. Mice (n=6 per treatment group) receiving luciferase transfected MSC were tested by bioluminescence imaging (BLI) on the indicated days. For specific PG-IgG measurement, mice (n=4 per treatment group) were sacrificed 5–6 days after first MSC treatment. For measuring cytokine production of PG-restimulated PG-specific lymphocytes, another four mice per treatment group were sacrificed 19–20 days after first MSC treatment. The lymphocytes from spleen and draining lymph nodes were studied separately in all treatment groups. In the ia treated mice the lymphocytes from the draining lymph nodes were additionally separated per injection side.

non-injected joints since the cumulative arthritis score of the four paws was suppressed. We questioned whether this was due to migration of MSC and explored this possible movement in vivo. We traced in vivo GFP-luc marked MSC using BLI with a sensitivity of detecting clusters of 100 cells, representing 0.002–0.01% of the injected cells in our study. This showed that many of the ia administered MSC are retained at the region of injection (figure 3), but were no longer detectable after 4 weeks in most mice and in two cases not even after 1 week. Likewise the ip administered MSC are largely retained in the peritoneal cavity for up to 5 weeks in the PGIA mice. Cells were at no point seen outside the peritoneal cavity (figure 3). The signal of luciferase positive cells weakened considerably when the 1-week images were compared to those made after 5 weeks. There was no clear relation between the arthritis scores and the luciferase signal over time. In conclusion, BLI of MSC did not show a distribution outside the injected regions within 4–5 weeks while the signal weakened considerably during that period. Thus, the observed systemic suppressive effect was not due to massive migration of MSC.

Reduction of PG-specific IgG2a antibodies in serum following MSC treatment

We next questioned what the effect of MSC injection was on the arthritis specific immune response. Since it is known that serum concentrations of PG-specific IgG2a isotype autoantibody correlate highly with the onset and severity of arthritis, suggesting a pathological role of this isotype,[18] we first studied whether

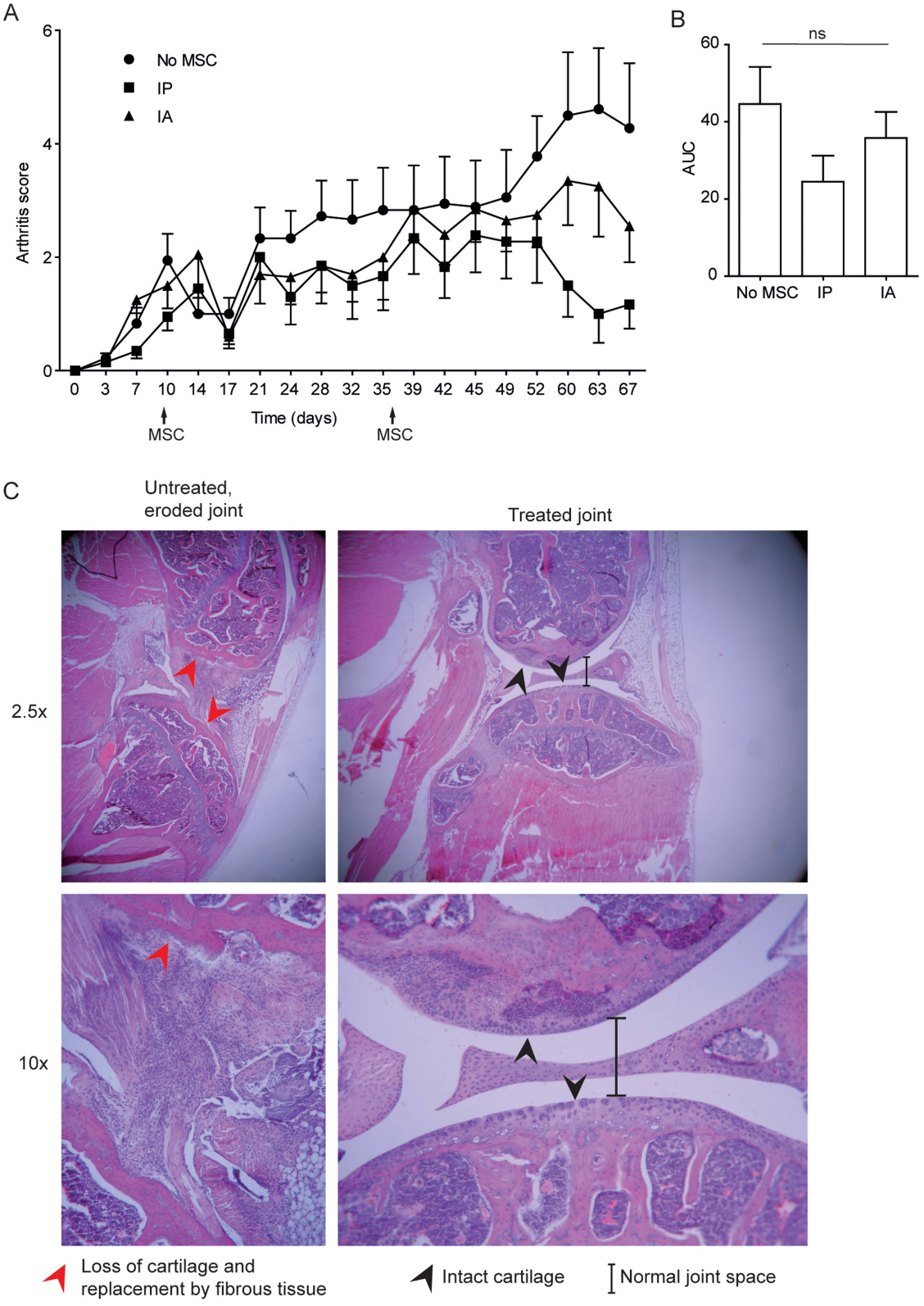

Figure 2 Intraperitoneal (IP) and intra-articular (IA) injected mesenchymal stromal cells (MSC) show systemic clinical effect. (A) Arthritis scores (mean±range) of mice from the second proteoglycan (PG) injection onward. Mice were scored twice a week. (B) Area under the curve (AUC) of A: AUC of each individual mouse was calculated. Graph shows mean AUC±SEM. Significance was tested with ANOVA. (C) Histology of the joints of mice sacrificed 71–79 days after the last PG injection was assessed with microscopy by a blinded independent observer. Representative pictures of eroded joints of an untreated mouse (left panels) and of an MSC-injected mouse (right panels) at different magnifications. Loss of cartilage (red arrows) is seen with replacement by fibrous tissue which fills the joint space.

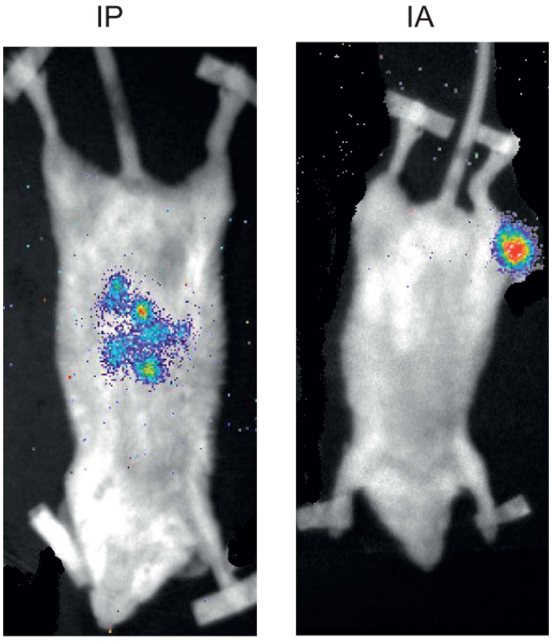

Figure 3 Bioluminescence imaging (BLI) in intraperitoneal (IP) and intra-articular (IA) treated mice. BLI was performed in a separate experiment with luciferase transfected mesenchymal stromal cells (MSC). Bioluminescence was observed only at the region of MSC injection, either IP (left) or IA (right). The figure shows mice 7 days after MSC injection.

MSC treatment influenced the early systemic PG-specific antibody levels. After induction of PGIA, mice injected with MSC showed reduced PG-specific IgG2a levels, which reached statistical significance for ia injected mice (figure 4). No effect of MSC on the non-pathogenic IgG1 levels was observed.

Thus, local MSC injection reduced systemically the PG-specific IgG2a antibody levels.

Shift in cytokine production of PG-specific T cells after MSC treatment

We next questioned whether MSC treatment can influence the cytokine production of PG-specific T cells. To investigate this, we isolated mononuclear cells from spleen and draining LNs, restimulated them with PG for 4 days and measured cytokine production in the supernatants by multiplex immunoassay.

A shift in immune activation in the lymphocytes of the spleen was only observed after ip infusion of MSC (figure 5). After PG restimulation we observed a significant increase in production of IL-4, IL-10 and IFN-γ by spleen-derived lymphocytes in the ip treatment group (figure 5). No shift in cytokine production of spleen derived lymphocytes was seen after local ia administration of MSC (figure 5).

To study the local effect of MSC, we compared draining LN cells between treatment groups. For the ia group we even separated the LN cells into those derived from the ia injection side and those from the contralateral non-injected side (figure 6). In the LN cells of ia treated mice, an upregulation of IL-4, IL-10 and IFN-γ was seen at the injection side only (figure 6). This upregulation was not found in LN cells of ip treated mice (figure 6).

Other cytokines were found in low amounts, but showed similar elevated local production at the region of injection of MSC only (figures 5 and 6).

Thus, in PGIA MSC have a local immune modulating capacity (figures 5 and 6), with a profound systemic effect (figures 2A and 4).

DISCUSSION

Even though promising new agents have become available for the treatment of JIA and RA, some arthritis patients fail to achieve even a modest improvement.[19] A promising new cellular therapy is the use of MSC which may provide an alternative to severely compromised children or as an adjuvant therapy earlier in the disease.[19]

This study shows for the first time the beneficial effect of MSC in a non-collagen induced RA model. PGIA is a chronic, progressive and self maintaining disease, where repeated inflammatory episodes eventually result in the complete deterioration of the articular cartilage. The MSC administered in established arthritis were able to decrease the arthritis score and the histological analysis confirmed this clinical observation. Our findings are in agreement with a study showing that in a CIA model, a single prophylactic ip injection of 5 million MSC can prevent exacerbation of the clinical severity and the occurrence of bone and cartilage erosions in the joints.[16] Other studies have shown that injection of 1–5 million xenogenic MSC intravenously or ip

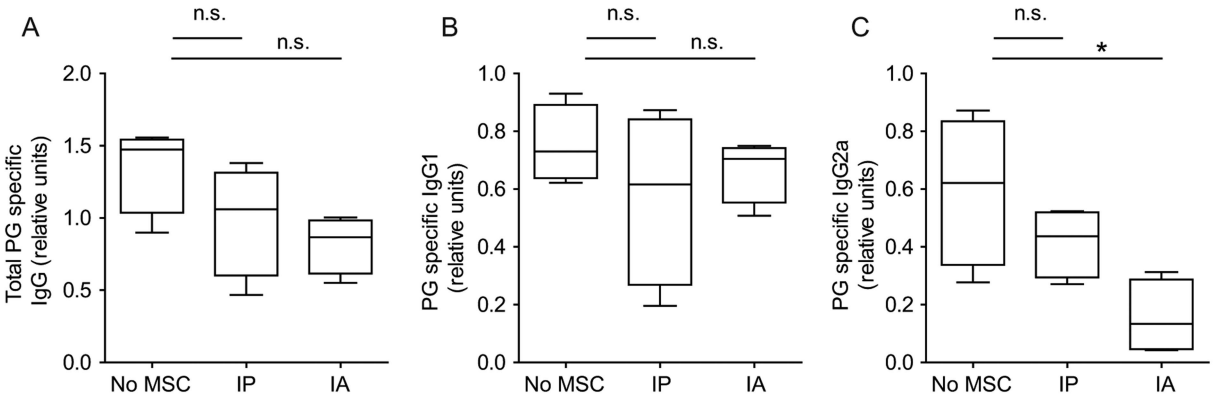

Figure 4 Mesenchymal stromal cells (MSC) treatment reduces the amount of proteoglycan (PG)-specific IgG2a. Serum was obtained from four mice of each treatment group. PG-specific antibody concentration was measured by ELISA 5–6 days after the first MSC treatment. Data are expressed as relative units obtained by extinction. Total PG-specific IgG (A), PG-specific IgG1 (B) and the pathogenic PG-specific IgG2a (C). Differences between treated and non-treated groups were tested using the Kruskal–Wallis test and Dunn's multiple comparisons for significance. *p<0.05; n.s., not significant. IA, intra-articular; IP, intraperitoneal.

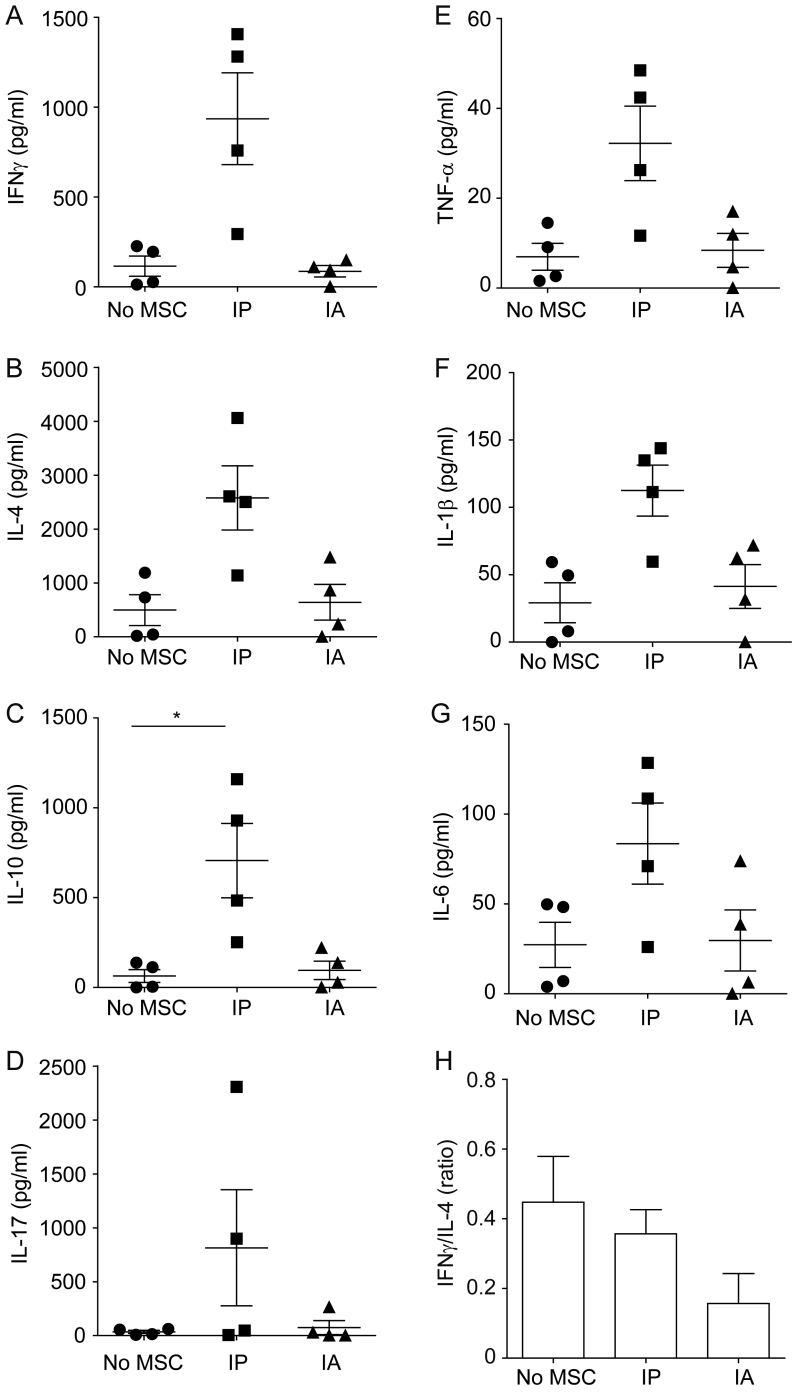

Figure 5 Lymphocytes in the spleen are activated only after intraperitoneal (IP) injection of mesenchymal stromal cells (MSC). For the IP and intra-articular (IA) treated and control mice splenocytes (n=4 per group) were isolated 19–20 days after the first mesenchymal stromal cells (MSC) injection and restimulated with proteoglycan (PG) for 4 days. Supernatant was tested for concentration of IFN-γ (A), IL-4 (B), IL-10 (C), IL-17 (D), TNF-α(E), IL-1β (F) and IL-6 (G). To investigate the effect of treatment on the ratio of Th1 and Th2 type responses, the IFN-γ/IL-4 ratio was calculated (H). Differences between treated and non-treated groups were tested using the Kruskal–Wallis test and Dunn's multiple comparisons for significance. *p<0.05.

prevents the occurrence or deterioration of CIA.[20 21] This effect depends on timing, dose and route of administration of MSC.[20] Immortalised MSC from the C3 cell line have been shown to worsen CIA,[22] whereas the same group showed beneficial effects on CIA when using freshly isolated MSC.[23]

The specific migration and engraftment of systemically administered MSC to locally damaged areas has been shown in several animal studies.[24–27] Therefore we expected to observe migration of at least a fraction of the MSC to the affected and damaged joints with BLI. With BLI, sensitive enough to locate clusters consisting of 0.002% of the ip and 0.01% of the ia injected cells, we did however not find indications that MSC home to the affected joint itself or to any other specific region when injected ip or ia. The MSC remained visible at the region of injection for up to 5 weeks. Our results are in agreement with a recent study showing with PCR that the majority of ia injected cells remained in the joints and after 3 months were still detectable in 20–30% of the mice.[28] In ip treated mice another group could not detect marked donor MSC in the (inflamed) joints at the end of their experiment.[16] These data all suggest that the effect of MSC on arthritis does not involve massive homing of MSC themselves towards the site of inflammation.

For PGIA it has been shown previously that inflammatory responses take place in spleen and draining lymph nodes more readily than in the joint itself.[29] It also appears to be more dependent on the availability of circulating IgG2 autoantibodies and neutrophils, rather than on the small population of T cells that migrate

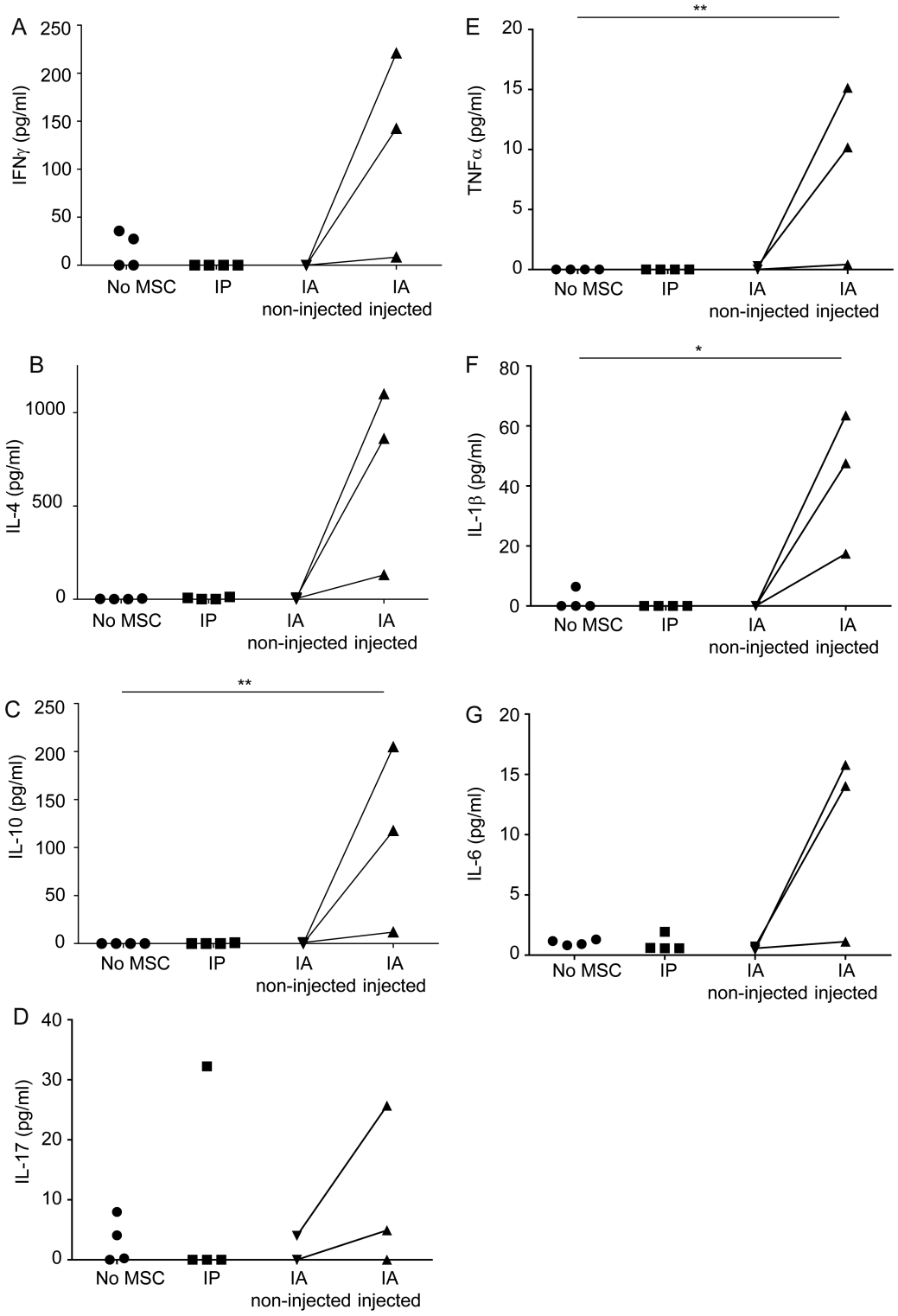

Figure 6 Lymphocytes in the draining lymph nodes are activated only at the side of intra-articular (IA) injection of mesenchymal stromal cells (MSC). For the intraperitoneal (IP) (n=4) and IA treated (n=3) and control mice (n=4), inguinal and popliteal lymph node cells were isolated 19–20 days after the first MSC injection and restimulated with proteoglycan (PG) for 4 days. Supernatant was tested for concentration of IFN-γ (A), IL-4 (B), IL-10 (C), IL-17 (D), TNF-α(E), IL-1β (F) and IL-6 (G). For the IA treated mice the lymph node cells were compared within a mouse between the MSC injection side and the non-injected (contralateral) side. The lines connect the results of cells from both sides within one mouse. Differences between treated and non-treated groups were tested using the Kruskal–Wallis test and Dunn's multiple comparisons for significance. *p<0.05; **p<0.01.

to the joints.[29] PG antibody production may be the primary event initiating activation of synovial macrophages and fibroblasts, which then may provoke and amplify the inflammatory process.[30] The systemic response we found in cumulative arthritis score could thus be a result of the reduced PG-specific IgG2 antibodies as we observed early in the serum of the treated mice.

The resulting immune activation of PG restimulated lymphocytes that we found despite the small group size, suggests a

deviation towards a more regulatory phenotype, with production of IL-4 and IL-10, but also of IFN-γ. The Th2 cytokine IL-4 was most strongly upregulated in PG-specific lymphocytes and only when drawn from the region of injection: in the ip group, only when derived from the spleen; and in ia treated mice, only when derived from draining LN at the injection side. A strong elevation in immunosuppressive IL-10 was seen with equal distribution. Although IFN-γ was also elevated, the much higher amounts of IL-4 resulted in an inversed IFN-γ/IL-4 ratio opposite to what is normally seen in active PGIA.[31] Interestingly, it has been shown that in IL-4 deficient mice, the PG-specific IgG2a concentration is six times higher than in wild-type mice and corresponds to increased severity of arthritis.[18] Furthermore, treatment with IL-4 prevents PGIA and induces a switch from Th1-type to Th2-type response.[31] IL-4 is even able to suppress arthritis when given at the time of maximum joint inflammation.[31]

MSC are required to be 'licensed' for their immunosuppressive activity. MSC appear to have dual immunomodulatory capacity; they have the ability to inhibit immune cell activation and proliferation, but also enhance immune responses via the secretion of pro-inflammatory factors and chemokines.[32] There is a possibility that the puncturing with a needle played a role in triggering the inflammation which activated the MSC to exert their immunosuppressive effect. The nature of the immunomodulatory effect of MSC depends on local immunological conditions, where in particular IFN-γ and TNF-α play a crucial role in inducing the immunosuppressive function of MSC.[32] IFN-γ, which has the potential to provoke PGIA,[33] is a very powerful inducer of MSC immunosuppressive activity (license for MSC to immunosuppress).[34] MSC interact with T lymphocytes, B lymphocytes, NK cells and dendritic cells through growth factors such as transforming growth factor-β and hepatocyte growth factor,[35] cytokines such as IL-10, and directly via adhesion molecules[36] with a general effect to skew the immune response towards anti-inflammatory or tolerant phenotypes, including a shift from Th1 towards Th2.[37] Furthermore, MSC can exert systemic effects without migration by means of MSC-derived extracellular vesicles,[38] potential mediators shedding peripheral tolerance towards auto-reactive cells via tolerogenic molecules and not visible by BLI, since our marked MSC contain luciferase in their DNA only.[38]

In conclusion, we showed for the first time the therapeutic suppressive effect of ip and ia MSC in a non-CIA model. In our opinion, this further supports the potential clinical and ia use of MSC in inflammatory arthritis since before clinical trials are started on the basis of available evidence from preclinical studies, it is not sufficient to rely on the evidence of benefit obtained in a single animal model,[39] and two different RA models are more predictive of clinical efficacy in human RA than data from either model alone.[40] Furthermore, we showed that the MSC are largely retained at the region of injection and an immune reaction took place, resulting in an early systemic decrease of PG-specific IgG2a antibodies with especially high IL-4 production by PG-specific lymphocytes in the region of injection.

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Contributors JFS: design, planning, conduct, analysis and interpretation of the data and reporting of the whole study (guarantor). SdeR: planning, conduct, analysis and interpretation of the data of immunological experiments (Exp 3). Revising article critically for important intellectual content. FMH: planning and conduct of effect on arthritis experiment (Exp 1) and conduct of immunological experiment (Exp 3). Contribution to drafting the article. HR: planning and conduct of bioluminescence imaging experiment (Exp 2). Revising article critically for important intellectual content. TvdB: planning and conduct of immunological experiments (Exp 3). Revising article critically for important intellectual content. PM: conduct of histology (Exp 1). FB: planning of arthritis experiment, preparation/purification of human proteoglycan and supervising the arthritis scores (Exp 1). Revising article critically for important intellectual content. FvW: planning and reporting of immunological experiments (Exp 3). Analysis and interpretation of the data and revising article critically for important intellectual content. WK: creation of the hypothesis, design and planning of the whole study. Interpretation of the data and revising article critically for important intellectual content. BJP: design, planning of the immunological experiments (Exp 3) and reporting the whole study. Analysis and interpretation of the data and revising article critically for important intellectual content. ACMM: design, planning of bioluminescence imaging (Exp 2), culturing mesenchymal stem cells and reporting the whole study. Analysis and interpretation of the data and revising article critically for important intellectual content. NMW: design, planning and reporting the whole study (guarantor). Analysis and interpretation of the data and revising article critically for important intellectual content. All contributors have finally approved this version to be published.

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Mesenchymal stem cell therapy in proteoglycan induced arthritis

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