Video Article

Intra-articular injection of murine MSC for the treatment of inflammation and tissue degradation in Antigen-induced Arthritis.

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Abstract

Antigen-induced Arthritis (AIA) is a valuable tool for modeling Rheumatoid Arthritis (RA) *in vivo*. AIA displays the pathophysiology of acute inflammatory arthritis through an accumulation of exudate in the joint cavity, leukocyte infiltration into the synovial sublining and hyperplasia in the synovial lining with chronic degradation of cartilage in the extracellular matrix. Herein we introduce a protocol to establish AIA in a preclinical mouse model and evaluate mesenchymal stem cells (MSC) as a therapeutic treatment for RA. Arthritis is induced via intra-articular injection using methylated bovine serum albumin (mBSA) and complete Freund's Adjuvant (CFA), with a serum-free medium vehicle solution injection in control mice. In both conditions, the contralateral joint is used as a control (PBS injection) to normalize results. Therapeutic effectiveness is measured through reduction in inflamed joint diameter, histological analysis using H&E and Toluidine Blue stains and serum expression of TNFα.

Inflammatory response is assessed through measurement of swelling in the articular joint, showing significant reductions in MSC-treated mice. Structural changes and cartilage damage are measured from histological sections. AIA responses show decreased proteoglycan present in cartilage and increases in exudate, leukocyte infiltration and hyperplasia. Treatment with MSC delivered by intra-articular injection demonstrates a significant reduction in symptoms of RA and decreases in TNFα in blood serum.

These results demonstrate the therapeutic effect of MSCs injected in the joints of mice with AIA.

Introduction

Rheumatoid arthritis is the most common inflammatory joint disorder and second most common form of arthritis in the UK, affecting more than 400,000 people with around 6200 new cases diagnosed every year[1,2]. It commonly causes joint pain and swelling, stiffness and fatigue and leads to increased morbidity and mortality. Recent research for novel treatments has led to the introduction of biological agents such as anti-Tumor Necrosis Factor Alpha (anti-TNFα), antibodies against interleukin-1 (IL-1) and interleukin-6 (IL-6) that target and neutralize the function of cytokines linked with inflammation. These therapeutics have made a significant impact on the alleviation of symptoms in RA sufferers however the treatment is not successful in all cases and 50% of those prescribed the treatment discontinue use of the drug after 2 years[3]. Consequently it is imperative to develop treatments that will be effective in those who do not respond to the therapies currently available.

Animal models provide an insight into the in vivo response to new therapies prior to clinical trials on humans. AIA is a model of inflammatory arthritis with many histopathological and clinical similarities to RA[4,5,6]. The AIA model is used with BALB/c and C57BL/6 mice as an efficient model of immunologically induced joint inflammation. This model has been shown in a rabbit model to bear similarities to RA in synovial histopathology, the responsiveness of antibodies and T cells to cartilage, and the levels of mediators of inflammation in diseased joints[7]. The AIA model for RA typically sees a peak in inflammation at around 24 hours post induction with the acute phase response lasting 1-5 days and a gradual reduction in inflammation over time[8,9]. Animal models are vital in gaining an insight into pathophysiological disorders and their response to novel treatments.

AIA displays the pathophysiology of chronic inflammatory arthritis through an accumulation of exudate in the joint cavity, leukocyte infiltration into the synovial sublining and hyperplasia in the synovial lining as well as chronic degradation of cartilage through the loss of proteoglycan[8,9]. These signs are symptomatic of a rheumatoid knee joint and therefore the AIA model provides an opportunity to study these processes and evaluate potential treatments for their therapeutic effects.

Adult stem cells, including Mesenchymal Stem Cells (MSC) have a high capacity for self-renewal whilst retaining an ability to differentiate down multiple lineages. The ultimate fate of differentiating MSCs is largely restricted to cells present in tissues of the musculoskeletal system such as adipocytes, chondrocytes, osteocytes and cells of muscle, skin and tendon[10]. MSCs provoke minimal immune response in a graft host owing to a lack of expression of Major Histocompatibility Complex (MHC-II) on the cell surface but also have immunomodulatory properties exhibited both during *in vitro* culture and *in vivo*[11]. An infusion of MSC into a damaged joint can aid tissue repair through increasing repair and reducing continual damage[32,33]. The consequence of this is the *de novo* synthesis of tissue specific structures at the site of damage. They have also

been show to secrete biologically active molecules termed cytokines that modulate inflammation and immune response in the host, aiding the reduction of swelling and edema in the arthritic joint[12].

Recent advances in the use of MSC in cartilage repair and the treatment of inflammatory disorders present MSC cell therapy as a potential therapeutic option. MSCs have been shown to be effective in clinical treatments for active rheumatoid arthritis confirming the safety and efficacy of MSC in cell therapy[13]. With the increased interest in transplantation of MSC as a therapeutic technique, it becomes imperative to develop a standardized, efficient protocol to describe the investigative administration of these cells into damaged joints in an appropriate animal model for RA. The delivery of MSC through intravenous or intraperitoneal injection has been reported with wide variations in the therapeutic outcomes[11,14]. There is therefore no clear evidence to support these administration methods for MSC therapies. The intra-articular delivery of MSC has been shown to encourage localization of the therapeutic cells within the synovial membrane[8]. It is surmised that, rather than directly generating *de novo* cartilage through synthesis of matrix components, such as aggrecan or hyaluronic acid, these cells produce biologically active molecules that may affect the immune response directly within the joint capsule.

Inflammation can be assessed in the AIA model during disease progression through measurement of joint diameter in comparison to an untreated (PBS control) contralateral control knee on the same animal. Evaluation of the successful induction of arthritis is made through histological analysis using H&E and Toluidine Blue stains to examine structural and tissue changes and the loss of proteoglycans from cartilage. Results show decreased proteoglycan present in cartilage and increases in exudate, leukocyte infiltration and hyperplasia. Combined data from these measures confirm the model as a preclinical representation of RA responses induced in vivo. These results demonstrate the successful induction of a short-term inflammatory arthritis with histopathological and clinical similarities to RA.

In contrast, intra-articular administration of mMSC has been shown to alleviate the listed symptoms, reducing breakdown of aggrecan or loss of proteoglycans from the cartilage matrix, reduced swelling through decreases in accumulation of exudate and leukocytes/mononuclear cells, and reduced hyperplasia in the synovial lining[8].

A complete, peer-reviewed and visualized protocol for the induction and evaluation processes in the AIA model system has not been previously available. This protocol serves to provide an efficient system with reliable induction of the rheumatoid pathology in model organisms. Furthermore, the demand for novel therapeutics for inflammatory or autoimmune arthritis requires assessment of treatments in a preclinical model such as AIA. MSC may be applied to treat disorders such as RA and in particular as a treatment for cartilage degradation through surgical intervention in processes such as Autologous Chondrocyte Implantation (ACI).

Protocol

All procedures were performed in accordance with UK Home Office approved project license PPL 40/3594. All animal handling should be performed following correct safety procedures and with the appropriate PPE (laboratory coat, gloves, eye protection when necessary).

1. Animal Selection

1. Select C57Bl/6 mice aged 7-8 weeks, weighing approximately 25 g for use. Select a minimum of 5 mice per control/test group (10 mice total).
    1. For this study, select all male mice due to availability. If possible, gender match all mice to reduce the impact of this as a variable; however male or female mice could be selected for testing. Reduced variation in response to mBSA and anti-mBSA production is noted in male mice which favors this gender selection.
    2. Use animals aged 8-10 weeks at selection. Immune system development is variable in mice younger than 8 weeks and the responses to mBSA inoculation may vary dependant upon immune development at this age[34,35].
2. Randomly allocate animals to test or control groups and mark using ear clip or tail marks for identification purposes.
3. Isolate murine MSCs (mMSCs) from C57Bl/6 mice (n = 5) as previously described[15].
    1. Briefly, collect bone marrow cells by flushing them out of femurs and tibiae and plate cells out in cell isolation media (CIM) (RPMI-1640) with 9% fetal bovine serum, 9% horse serum, and 1% penicillin-streptomycin at 37 °C in 5% CO_2.
    2. After 24 hours, remove non-adherent cells. 4 weeks later, re-plate cells at 100 cells per cm^2 in complete expansion media (CEM) (Iscove Modified Dulbecco Medium (IMDM)) supplemented with 9% fetal bovine serum, 9% horse serum, and 1% penicillin-streptomycin for MSC expansion.
    3. Examine cells for their ability to differentiate into chondrocytes (using pellet cultures), osteocytes and adipocytes, as described[15]. Inject MSC prior to passage 5 to ensure cells had not been altered by extended time in culture. Characterize cells undergoing continual culture following injection to verify injected MSCs.

2. Preparation of mBSA in Complete Freund's Adjuvant (CFA).

1. Store all stock solutions at 4 °C until preparing the mBSA in CFA emulsion. Mix base suspension of 1 mg/mL of methylated bovine serum albumin (mBSA) using 25 mg of mBSA in 25 mL of PBS.
2. Set suspension to stir for ≥ 20 min at room temperature to thoroughly mix.
3. Emulsify mBSA with vortexed complete Freund's adjuvant (CFA), at a concentration of 1 mg/mL *Mycobacterium tuberculosis* (H37Ra, ATCC 25177), for total volume of e.g. 2 mL by adding 1 mL of 1 mg/mL mBSA to 1 mL of CFA, using a 5 mL syringe with a 19G needle, giving concentrations of 0.5 mg/mL each of mBSA and *Mycobacterium tuberculosis*. Prepare sufficient mixture for 100 µL per animal with a 10% excess for contingency.
4. Use a 19G syringe needle and 5 mL syringe to mix the solution by drawing and expelling from the syringe ten times. Finally agitate the solution using a laboratory vortex to emulsify. Ensure the final solution for injection has a thickened consistency and presents resistance during injection in order to avoid too rapid dispersal from the injection site. Some variation in viscosity is permissible and it is not necessary to perform a droplet test for viscosity.

5. Administer the primary immunization of 100 µL mBSA/CFA emulsion subcutaneously (S/C) into the lower back of each mouse adjacent to the base of the tail but not into the tail itself. Anaesthetic is not required for this S/C injection.

3. Preparation and administration of *Bordetella Pertussis* Toxin (*P* Tx)

1. Use personal protective equipment (gloves, eye protection) to handle *pertussis* toxin (*P*Tx) as it is a contagious pathogen (whooping cough). *P*Tx is used to boost the anti-mBSA Th immune response by increasing the systemic expression of cytokine IL-23.
2. Mix 0.2 mg/mL PTx stock (10X) into 1:10 solution with PBS, making sufficient volume for 100 µL to be administered per animal with a 50% contingency volume for waste, e.g., For 12 animals, mix 1800 µL of solution. This concentration will deliver a dose of 160 ng of *P*Tx per animal.
3. Load *P*Tx solution into 1 mL syringe with 25G needle. Ensure waste is properly disposed of into a sharps bin.
4. Perform an intraperitoneal (I/P) immunization of 100 µL of heat-inactivated *P*Tx in PBS per animal using 25G needle. Anesthetic is not required for this injection.

4. Preparation and administration of booster injection of mBSA with Freund's adjuvant only

1. 7 days later, prepare a repeat immunization to boost immune response with 100 µL of 1 mg/mL mBSA and CFA in 1:1 ratio as described in steps 2.1 - 2.4.
2. Administer 100 µL of this mBSA in CFA mixture via S/C injection into the scruff of the neck to avoid aggravating the previous injection site (do not administer PTx at day 7). Anesthetic is not required for this injection.

5. AIA Day 1 – Antigen Induction of Arthritis

1. 21 days following the initial immunizations, anesthetize the mouse using 5% isoflurane for induction of anesthesia in an anesthesia induction box and subsequently 3% isoflurane for maintenance using a nose cone. Confirm anesthesia using the withdrawal reflex (leg, toe).
2. Once anaesthetized, locate the knee joint by close examination of the location of the patella. The patella is visible as a visible white region on the outer knee joint. Flexing the joint aids in locating the patella. The joint cavity is located on the proximal side of the patella.
3. Measure the knee joint diameter with a dial indicating outside caliper (resolution 0.005 mm). The caliper plates are in contact when closed. Gently apply pressure to open the calipers and briefly allow to settle on either side of the knee join, located using the patella as a guide. Ensure the calipers are not in contact with any surface other than the knee joint.
    1. Obtain a static reading. Record the knee joint diameter for both right (R) and left (L) knees for each animal prior to injection.

4. Induce AIA with the intra-articular administration of 10 µL of a 10 mg/mL mBSA in PBS solution directly into the synovial cavity of the right (stifle) joint.
    1. As a control, inject an equal volume of PBS into the left synovial cavity. Inject intra-articularly using 0.5 mL monoject (29 G) insulin syringe through the patellar ligament into the right knee joint. Stretching of the hindleg facilitates the intra-articular injection.
    Note: Following injections, swelling will occur. Swelling is visible on treated mice and animals may have a visible limp due to effects on mobility, however no visible discomfort is observed through behavior or pain assessments. Swelling will typically cause an increase in knee diameter in the range of 0.6-1.4 mm.

6. AIA Day 2 - Experimental intra-articular injections of characterized mMSC

1. Prepare experimental treatments for intra-articular injections using the following step.
    1. Load 0.5 mL monoject (29 G) insulin syringes with either mMSC suspension ($5.0x10^7$ cells/mL) in serum-free Iscove's Modified Dulbecco's Medium (IMDM) (test) or serum-free IMDM vehicle solution (control) to deliver 10 µL treatment per mouse.
    Note: Contralateral control joints treated with an injection of PBS instead of mBSA will not exhibit the swelling and histological changes and are therefore again used as a baseline control.

2. Anesthetize mice as per step 5.1.
3. Measure and record knee diameter as in steps 5.2-5.3.
4. hours post-induction of arthritis, administer the primary knee (R) with novel treatment therapeutic of $5.0x10^5$ mMSC delivered in 10 µL serum-free Iscove's Modified Dulbecco's Medium (IMDM) (test) or serum-free IMDM vehicle solution (control). Perform injections as described in step 5.4

7. AIA Immune response – Measurement of swelling and dissection of joints for histological analysis

1. Anesthetize mice as per step 5.1 and measure the knee diameter (swelling) of mice at days 1, 2, 3, 5, 7, 14, 21 and 28 post induction of arthritis as described in steps 5.2 – 5.3.
2. For examination, euthanize mice at days 3, 7, 14 and 28 using 1 minute of carbogen followed by a programmed control of rising CO^2 gas for 4 minutes in a veterinary chamber as per UK Home Office guidelines. Death is confirmed using foot withdrawal and blink reflexes.
3. Perform a cardiac puncture to remove minimum 1 mL of blood. Insert a 23G needle through the chest cavity directly into the heart and remove the available volume of blood (~1 mL) by syringe. Transfer the aspirated blood to a microcentrifuge tube and allow to clot for 10 minutes whilst completing dissection.
4. Confirm death using cervical dislocation then remove euthanized mice to sterile dissection area.
5. Spray animal with a 70% ethanol (EtOH) in sterile water for aseptic dissection.

6. Make an incision at the lower back above pelvis and separate the dermis from underlying muscle along the leg. Trim excess tissue and remove muscle and tendon leaving the synovial joint intact. Remove the knee joint by cutting mid-femur to mid-tibia using surgical scissors.
7. Fix knee joints in 10 mL of 10% neutral buffered formalin for a minimum of 24 hours and store at 4 °C until processing.

8. Measurement of TNFα in blood serum

1. Centrifuge extracted blood (from step 7.3) at 1,500 x g for 10 minutes to separate the cellular component from blood serum.
2. Remove serum supernatant and transfer to a 1 mL microcentrifuge tube.
3. Test serum for the presence of TNFα using a mouse TNFα ELISA kit according to the manufacturer's instructions or freeze at -80 °C for later testing.

9. Histological scoring of AIA in knee joints.

1. Decalcify joints with 10% formic acid at 4 °C by immersion for between 24 and 72 hours (depending upon thickness and density of bone). Complete decalcification will result in a bone flexible enough to bend without fracture occurring. This will enable histological sectioning whilst maintaining joint morphology within each section.
2. Paraffin embed joints for sectioning.
3. For histological analysis, cut mid-sagittal sections of 5 µm thickness from paraffin embedded knee joints and stain with haematoxylin and eosin (H&E)[16].
4. Make an initial assessment of H&E for synovial hyperplasia, cellular exudate and cartilage depletion scoring from 0 (normal) to 3 (severe) whilst scoring synovial infiltrate 0 to 5 (scoring system as described in Nowell *et al*[15]).
    1. In brief, assess synovial hyperplasia by locating the synovial membrane and performing a count of cell thickness (i.e. the number of cells deep in the membrane) along a wide length of the membrane. The length will be determined by the size of the knee joint.
        1. Allocate scores for cell depths of 1 cell (score 0), 2 cells (score 1), 3 cells (score 2) or >3 cells (score 3). Assess the presence of cellular exudate by thorough examination of the synovial cavity on H&E stained sections. Exudate will appear as cells present in the joint cavity between the cartilage surface and the synovial membrane. Outcomes are no exudate (score 0), a small trace of exudate (score 1), several small or single mid-size region of exudate (score 2) or clear presence of substantial cellular exudate (score 3).
    2. Assess synovial infiltrate by examining the cellular component contributing to joint swelling in the synovial membrane, between the membrane surface and the outer capsule of the knee. As the degree of swelling can be variable, it may be necessary to refer to the contralateral knee with this, and all, measures to assess the "normal" state prior to assessing damage. A large volume of cellular infiltrate between the synovial membrane and the outer articular capsule, such that the spaces commonly visible in the adipose tissue are obscured, will be scored as a 5. Normal tissue will give clear appearance of the adipose tissue and will be scored as a 0. Between these extremes, a graded scoring system is used. Example images are shown in Figure 4.
5. Cut further sections from joints previously assessed with H&E and stain using Toluidine Blue to assess loss of proteoglycan in cartilage as a measure of damage. Deparaffinise and rehydrate sections by immersing for 5 minutes each in 100% xylene (x2) and 100% Iso-2-propyl (x2) followed by 5 minutes in Dulbecco's Phosphate Buffered Saline.
6. Stain sections with 1% Toluidine Blue solution for 20 seconds as per manufacturer's instructions, immediately rinsing with water and finally immersing for 5 minutes in water. Allow the slides to air dry on a laboratory bench.
    1. Follow this with 5 minutes 100% xylene exposure and mounting with DEPEX mounting medium for microscopic examination. Score this on a scale of 0 to 3 as per Nowell et al.[15].
    Note: A score of zero will be given where no cartilage depletion is visible on patella, femoral condyle or tibial plateau. Cartilage depletion is indicated by a decreased intensity of stain in the outer layer of cartilage. Comparisons to contralateral controls should be made to assess a baseline for comparison. A score of 3 will be given where a full thickness decrease in stain intensity can be seen on one or more cartilage surfaces across the length of the surface. Example images are shown (Figure 4).
7. Calculate an "arthritis index" by summing the individual scores (steps 9.4, 9.6) for each individual mouse, then, for each group of mice within a condition, calculate the mean ± Standard Error of Means (SEM). Increased score is representative of more severe arthritis response[15]. Assess comparisons between results as described in section "Statistical Analysis".

10. Statistical Analysis

1. Perform statistical analysis using statistical software according to manufacturer's protocol, e.g. GraphPad. *P* values less than 0.05 being deemed as significant. Assess swelling measurements and serum TNFα measurements for normality, as this directs the choice of statistical test.
2. In this case, analyze results using Mann-Whitney *U* test with Bonferroni correction post hoc. Assess histological scores for Arthritis Index using Students *T*-test.

Representative Results

All of the following representative example results shown here were previously published by Kehoe *et al* 2014[8]. RA was successfully modeled in C57BL/6 mice with increased swelling in affected knee joints, increased cartilage degradation, increased leukocyte infiltration into synovial sublining, increased exudate in the joint cavity and evidence of hyperplasia in the synovial lining. Administration of therapeutic mMSC via intra-articular injection produced significant alleviation of the symptoms of RA.

Swelling measurements

Primary results take the form of knee joint diameters (swelling) measurements following induction of AIA (step 5.1).

Typical results are shown from right knee joints of C57BL/6 mice 48 hours post induction of arthritis. Peak swelling occurs at 24 hours post induction. This time point is therefore selected for the administration of any RA treatments to be investigated, and matched vehicle control injections, and a further 24 hours allowed for the initial assessments to be made. Swelling measurements are individually normalized against diameter measurements for contralateral (non-arthritic) knees, giving a final figure for each mouse representing the increase in knee diameter. Averaged swelling measurements are expressed as mean ± SEM in millimeters.

Swelling measurements taken at 2 days post-induction in a vehicle solution "control-treated" AIA joint immunized with tissue culture medium averaged 1.27 ± 0.06 mm whilst at this stage MSC-treated AIA joint showed significantly reduced inflammation at 0.98 ± 0.06 mm ($p = 0.0009$). At 7 days post-induction, swelling had reduced in both joints, with vehicle control-treated measuring 0.74 ± 0.09 mm and MSC-treated measuring 0.34 ± 0.06 mm ($p = 0.0009$) (Figure 1). At later time points, no significant differences were found between treated and untreated joint swelling.

Histological analysis

Histologically AIA can be characterized by the presence of synovial hyperplasia of the synovial lining layer, infiltration of the synovial sublining by leukocytes, exudate in the joint cavity (H&E), and loss of proteoglycans from the articular cartilage (Toluidine Blue). This damage is observed using haematoxylin/eosin and toluidine blue stained sections (Figure 2).

Histological examination of sections of day 3 joints showed a reduction in exudate volume present in joints containing leukocytes including neutrophils ($p = 0.0004$). Similarly, cartilage depletion was reduced in MSC-treated joints ($p=0.0003$) and the measure of "arthritis index" to represent overall AIA severity was reduced in MSC-treated joints ($p=0.0009$). These effects continued to be present at day 7 post-injection, with maintained reductions in exudate ($p=0.024$), cartilage depletion ($p=0.035$), synovial hyperplasia ($p=0.01$) and overall arthritis index ($p=0.013$) (Table 1). Significant differences were not found at 14 day or 28 day time points in any of these measures.

Serum measurements for TNFα

Serum measurements showed a reduction in circulating TNFα of animals receiving MSC treatment in AIA joints compared to non-treated AIA at each time point, day 3, day 7 and day 14 ($p = 0.024$, $p=0.001$, $p = 0.008$ respectively, Mann Whitney U test for grouped analysis) (Figure 3).

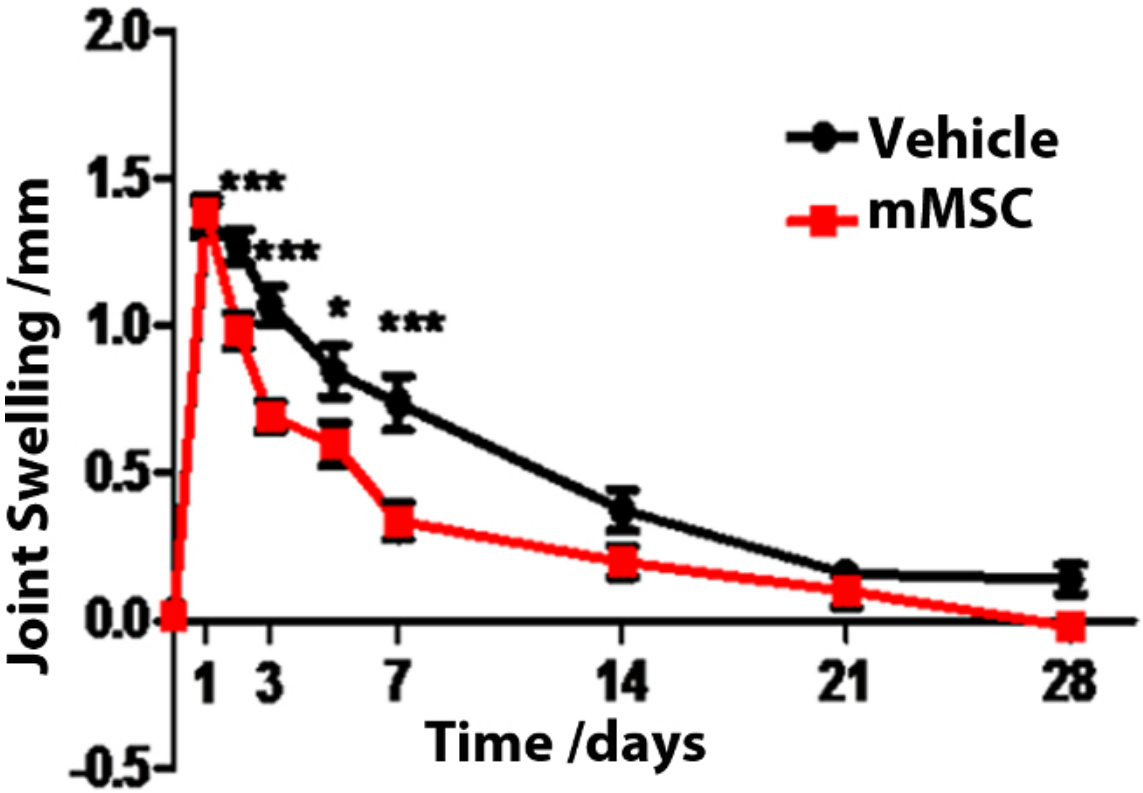

Figure 1: The effects of intra-articular injection of murine MSCs (mMSCs) into knee joints of mice with antigen-induced arthritis[8]. Inflammation assessment by measurement of joint diameter at 2 days after arthritis induction was significantly reduced in mMSC treated mice compared to vehicle control-treated mice (0.98 ± 0.06 mm versus 1.27 ± 0.06 mm; $p = 0.0009$) and continued up to ~7 days (0.34 ± 0.06 mm versus 0.74 ± 0.09 mm; $p = 0.0009$). No significant differences were seen at days 14 and 21 ($p>0.05$). Data are means ± SEM for right knee after subtraction of left knee control; n = 6 mice with mMSCs and vehicle control without mMSCs. *$p < 0.05$, ***$p < 0.001$ compared with corresponding time point.

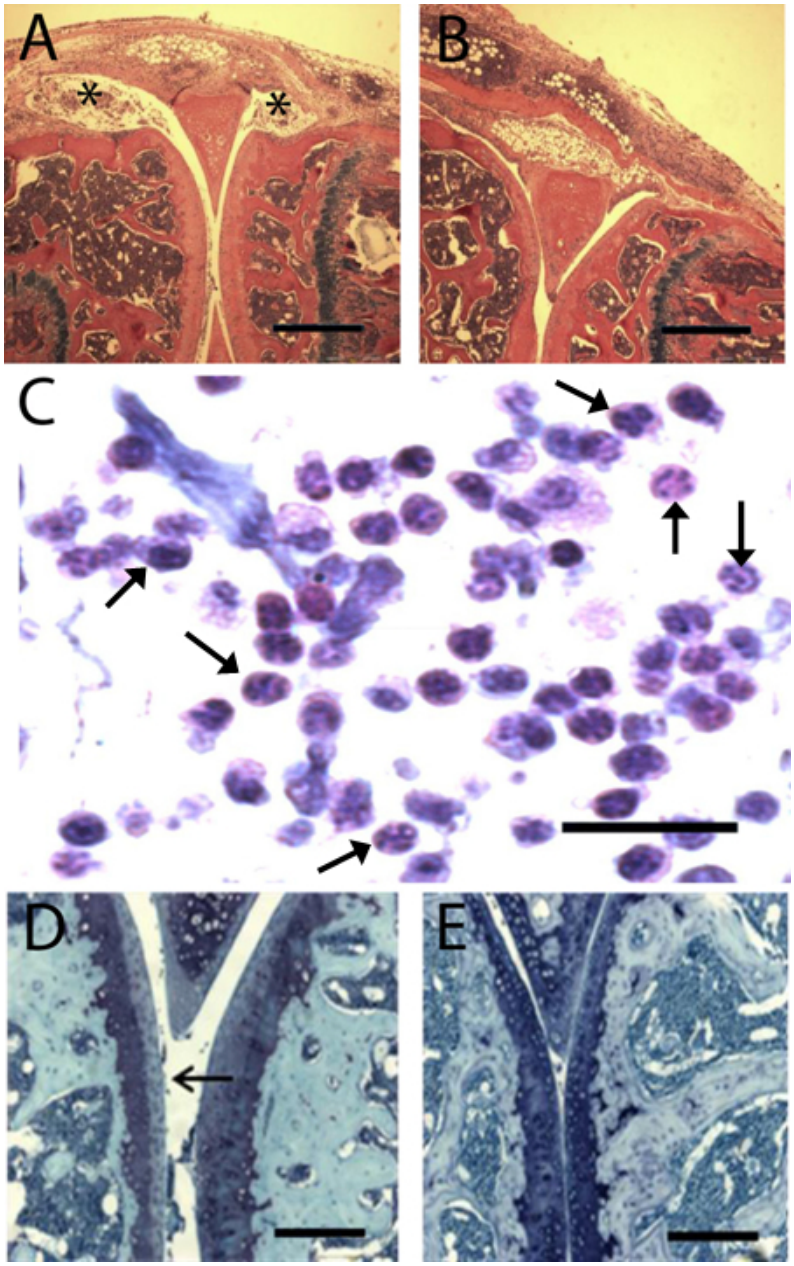

Figure 2: Histology of joints in mouse antigen-induced arthritis 3 days after induction[8]. Arthritis was induced by intra-articular injection of methylated BSA in the right knee (stifle) joint (t = 0 in A). For a control, PBS was injected into the left knee joint. Murine MSCs were isolated from bone marrow and expanded in culture. 5.0 x 10^5 mMSCs in serum free medium were injected at the peak of joint swelling (after 1 day) into the knee joints of mice with arthritis. Injection of serum free medium vehicle alone was control. (A-E) Histology of joints in mouse antigen-induced arthritis 3 days after induction. (A) Arthritic knee joint with marked leukocyte exudates in the joint space (*). (B) is the same as A except MSCs have been injected. Note the lack of exudates. (C) detail of exudate in the joint cavity in the absence of MSCs comprising leukocytes, particularly neutrophils (arrows show examples). (D) arthritic knee joint with loss of proteoglycan staining in the surface regions of tibial and femoral articular cartilage (arrow). E is the same as D except the joint was injected with mMSCs and there is less cartilage degradation. A, B and C were stained with haematoxylin and eosin and D and E with toluidine blue. Scale bar =500 µm in A and B, 20 µm in C and 200 µm in D and E.

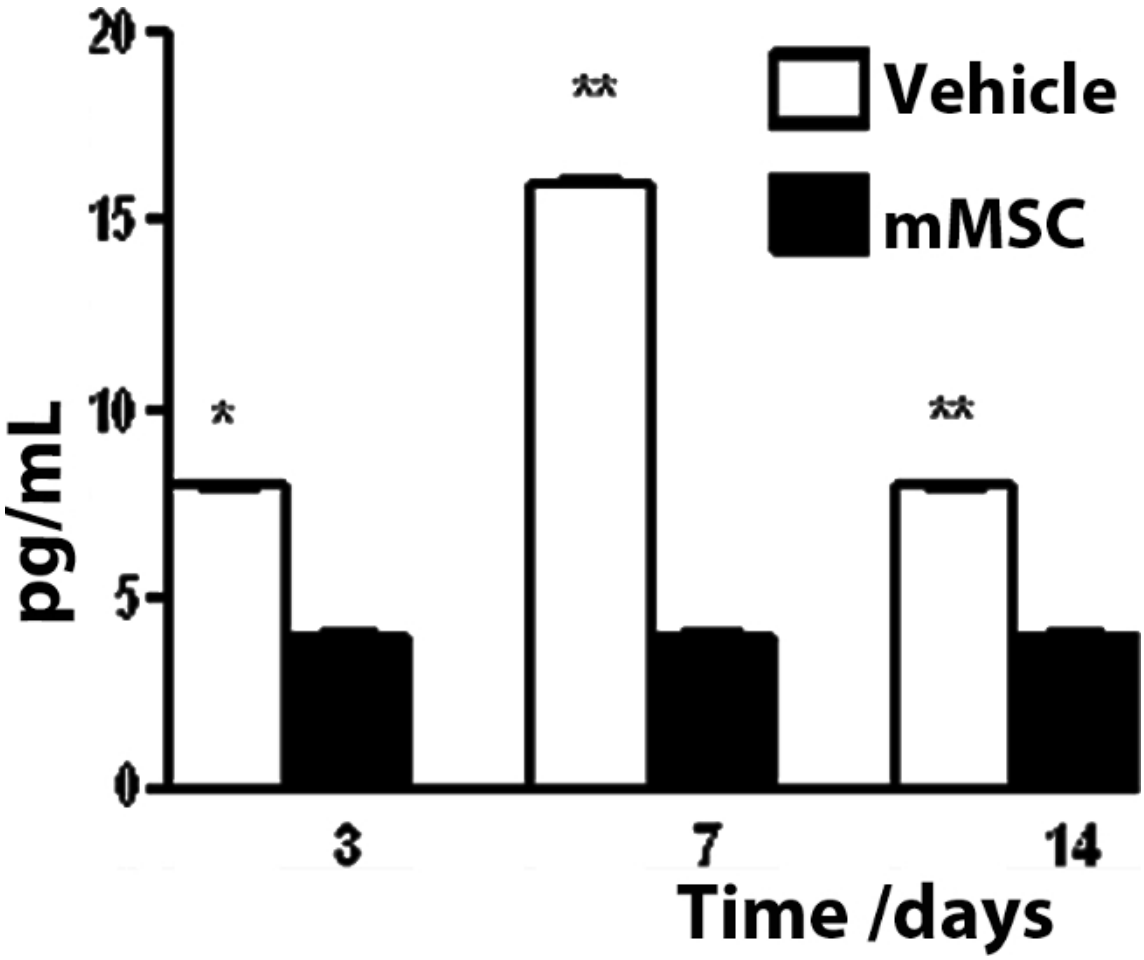

Figure 3: Serum concentration of TNFα at the indicated time points (day 3, 7 and 14), analysed by enzyme-linked immunosorbent assay[8]. In MSC treated mice with AIA, serum levels of TNFα were significantly lower than in non-treated (vehicle control) mice at day 3 ($p = 0.024$), day 7 ($p = 0.001$) and day 14 ($p = 0.008$). Data are means ± SEM; n = 6 mice with MSCs and vehicle control without MSCs. *$p < 0.05$, **$p < 0.01$.

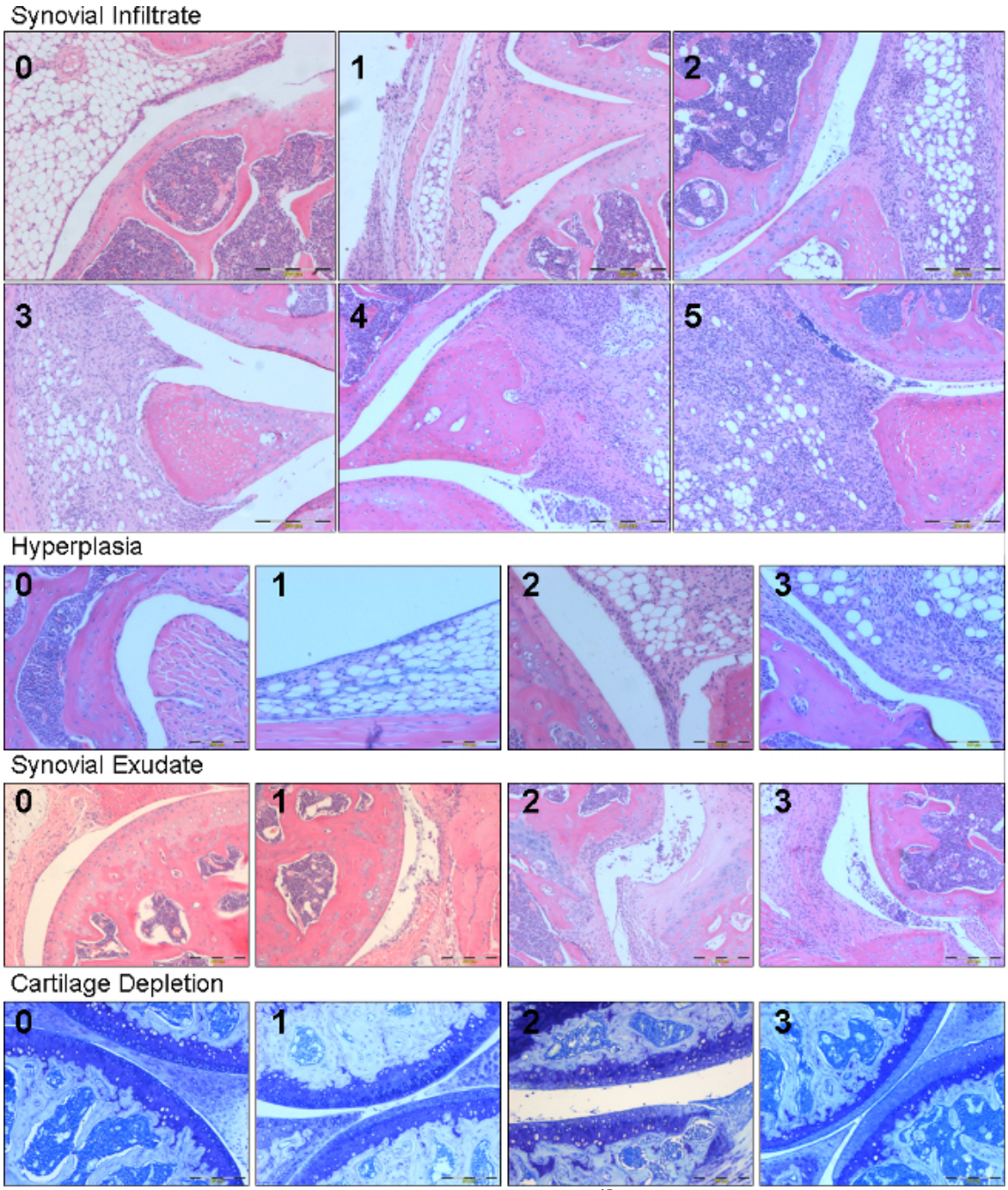

Figure 4: Sample images for histological scoring for arthritis index[15]. H&E sections are scored for Synovial Infiltrate, Hyperplasia and Synovial Exudate and Toluidine Blue staining for cartilage depletion. Representative images are shown for each score. Scores for each section are summated to give an overall 'Arthritis Index' score from 0 to 14. Scale bars are 100 µm (Hyperplasia) or 200 µm (Synovial Infiltrate, Synovial Exudate, Cartilage Depletion).

Species	Days	Hyperplasia	Synovial Infiltrate	Exudate	Cartilage depletion	Arthritis index
Control animals	3	1.75 ±0.29	3.56 ±0.40	2.78±0.15	1.78±0.33	9.89±0.88
	7	2.43±0.23	3.93±0.39	2.14±0.46	1.71±0.52	10.2±1.46
	14	1.67±0.67	1.67±1.20	1.17±0.92	1.50±0.87	6.00±3.61
MSC treated animals	3	1.70±0.17	2.83±0.25	1.13±0.32	0.33±0.14	5.96±0.57
				***	***	***
	7	1.19±0.28	2.69±0.43	0.75±0.31	0.44±0.29	5.06±1.09
		*		*	*	*
	14	1.00±0.00	1.00±0.41	0.00±0.00	0.00±0.00	2.00±0.41

Table 1: Joint inflammation and cartilage damage on day 3, 7 and 14 of antigen-induced arthritis as assessed by histological scoring[8]. Synovial hyperplasia of the lining layer, synovial infiltration of the sublining by leukocytes, exudate in the joint cavity, and loss of proteoglycan from the articular cartilage observed in haematoxylin/eosin and toluidine blue stained sections. Quantified differences between MSC-treated and non-treated mice at day 3 showed a significant reduction of amount of exudates ($p = 0.0004$), cartilage depletion ($p = 0.0003$) and arthritis index (representing overall disease severity) ($p = 0.0009$). Significant differences were found at day 7 post intra-articular injection of mBSA, for exudate ($p = 0.024$), cartilage depletion ($p = 0.035$), synovial hyperplasia ($p = 0.01$) and arthritis index ($p = 0.013$). At day 14, all parameters were reduced in the presence of MSCs and these only approached significance (e.g. $p = 0.09$ for cartilage depletion). At day 28 post intra-articular injection of mBSA, there were no significant differences between MSC-treated and vehicle solution control-treated animals for all parameters. *$p < 0.05$; and ***$p < 0.001$ of the parameter compared to control animals at the same point, using unpaired t test. n = 6 mice per condition and per time point.

Discussion

This study describes the process for induction of arthritis using antigen immunization to present the pathophysiological symptoms of acute and chronic arthritis in inbred mice strains. In this study, recognition of the pathology of RA is achieved through assessing hyperplasia of the synovial lining, synovial infiltration of the sublining by neutrophils and mononuclear cells, increased presence of exudate in the joint cavity, and cartilage degradation marked by loss of proteoglycan. An evaluation is made through histological examination using H&E and Toluidine Blue and by direct measurement of inflammation through increased joint diameter when normalized against untreated contralateral control joints. Inflammation through swelling is caused by endothelial cells of blood vessels in inflamed synovium becoming leaky, causing edema and allowing release of leukocyte and mononuclear cells. This may be due to opening of adherens junctions (VE-cadherin and β-catenin) in response to cell signals[17] and these signals are increased in the rheumatoid condition. Cartilage destruction has been examined and the initial loss of proteoglycan observed may be due to enzymatic degradation of aggrecanase. It is thought that the breakdown of aggrecan may be due to the action of a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS), e.g., ADAMTS5, or Matrix Metalloproteinases (MMP), e.g., MMP1, MMP3, MMP13[18]. In particular, interest focuses on ADAMTS5 as research has shown that cartilage degradation is reduced in mBSA induced arthritis models using transgenic mice with ablated ADAMTS5 activity[19].

In studies performed using this protocol[8], and ongoing work, the efficiency of the AIA protocol has been ~100%. Some observations should be observed in the completion of the protocol. A dorsal injection site is used for the initial S/C injection of mBSA and CFA (step 2.5) as in some cases CFA can cause localized inflammation, pain and necrosis around the injection site. A dorsal location is selected to allow the handling and treatment of the animal without the risk of pain and/or suffering. An accompanying injection of *Pertussis* toxin is given with the initial mBSA and CFA injection (step 3.4). The *Pertussis* serves to enhance the immune response to inoculations of mBSA.

AIA is successful in producing a strong inflammatory response with an acute and chronic phases[25]. The acute phase occurs within the first week post intra-articular injection and is characterized by swelling/edema and synovial infiltrate with granulocytes and mononuclear cells[38]. Cartilage and bone erosion occurs and synovitis characteristic of RA[36].

Mediating bone and joint damage through regulation of osteoclast differentiation in bone marrow and signaling molecules for osteoclast production is achieved through regulation of the production of T-lymphocytes. AIA has been shown to act through CD4+ T lymphocytes and is therefore also a useful model for cytokine production in RA[20,25]. RA development and progression has multifaceted etiology with the interaction of a complex combination of antigens and biological signaling molecules leading to the varied symptoms of the disease. Consequently, recent advances in therapeutic treatments, such as anti-TNFα, target specific molecules but do not provide remedy for all sufferers. It is advantageous in treating multifaceted disorders to utilize a multifaceted therapy and MSC infusion appears to offer one such remedy. TNFα is a proinflammatory cytokine important in the regulation of IL-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor. This cytokine, referred to as a “master regulator”, has upregulated expression in RA and has therefore been identified as a therapeutic target for novel treatments[20]. The technique described has demonstrated that administration of MSCs results in reduced levels of TNFα in the circulation. This may contribute strongly to the anti-inflammatory effects observed due to this therapy. Further investigation of stem cell injection is required to evaluate whether this treatment option functions through the same mechanism as anti-TNFα biological treatments in clinical use or if MSCs have a wider impact on the symptomatic expression of arthritis.

The use of animal models for preclinical testing garners some debate. The size, density and biomechanical properties of joint tissues and the type of pressures sustained by a mouse knee joint does not identically match that of human knee joints. However, rodent models of rheumatoid arthritis are useful in demonstrating mechanisms of action and improving our understanding of the pathogenesis of arthritic disorders. As an individual model, AIA has certain limitations and disadvantages that may make selection of a different preclinical model for arthritis more

favorable. AIA pathology comprises an acute inflammatory response mediated by immune complexes followed by an articular T-cell mediated response. In AIA, immunological self-tolerance is not breached in the manner observed when testing with a CIA model[25]. One limitation of AIA is the acute nature of severe allergic arthritis. Spontaneous resolution, or significant remission, from AIA occurs in a period of 14 to 28 days, with reduced swelling, synovial exudate and hyperplasia over this time course and retention of a less severe but chronic arthritis condition[22]. This can be beneficial in modeling the remission and exacerbations symptomatic in RA. It is, however, possible to increase the chronicity of AIA by sustaining the presence of antigen in the knee joint. Antibody trapping varies between animal species. In rabbits, antibody levels raised to mBSA antigens are high, giving increased retention and a more chronic form of arthritis than seen in mice[35]. Injection of mBSA antigens prompts only modest antibody response in mice and it has been shown that different murine species will respond with different levels of severity and chronicity. C57Bl/6 mice are recommended owing to the more reliable response and good severity and chronicity of disease. Other common options, such as BALB/c will show variations that may be beneficial dependent upon the focus of the research, e.g. BALB/c produce more granulocyte rich infiltrate during inflammatory response[36]. As stated earlier, males of ages 8-10 weeks are recommended here to minimize variations between animals.

Increased duration of symptomatic response be achieved through repeated injection or retention of antigen in the knee capsule, or through a positive and sustained T-cell response against the introduced mBSA. AIA relies on T-cell response to generate arthritis symptoms and AIA will not work in animals with compromised T-cell function[34]. Retention may also be improved by increasing the antigen bonding within the knee joint. Cartilage and ligaments are negatively charged, so a positively charged antigen will bind with tissue surfaces. It is for this reason that BSA is applied in a methylated form, as mBSA has increased positive charge for enhanced tissue bonding and retention (increase pI from 4.5 in BSA to 8.5-9.0 in mBSA). Due to this, mBSA is retained at the cartilage surface rather than penetrating deeply into the tissue[37]. Care must be taken, however, as an excessive increase in isoelectric point (pI) can cause positively charged proteins (cations) to irritate the joint, causing cell irritation rather than immune activation[35]. Furthermore, AIA is a localized model for RA, isolated to articular joints at the site of injection. Unlike the systemic nature of CIA, systemic injection of mBSA occurs only in preparatory stages and the induction of arthritis occurs due to a local intra-articular injection that does not affect the mouse beyond the injection knee joint. AIA cannot be used to induce systemic rheumatoid arthritis, and it is therefore not possible to use method to model a spread of RA from joint to joint[25].

Other models are available for RA in mice and each model brings its own advantages and disadvantages in use. A commonly studied alternate model for RA is the Collagen-induced arthritis (CIA) model. This model is achieved through a similar mechanism to AIA. An autoimmune response to an emulsion of collagen II and CFA, inducing autoimmune arthritis in knee joints of mice. In CIA susceptibility of mice is linked to expression of MHC class II genes[9]. The symptomatic development of CIA matches that of AIA. Unlike AIA, CIA relies on both B cell and T cell immune response to type II Collagen and is restricted in mice to susceptible strains, in which efficiency of induction is 80-100% making CIA more difficult to achieve and study than AIA[21]. In CIA, severity of arthritis is sometimes assessed by measuring morphological changes in the paw, such as increased paw volume measured using a plethysmometer[21]. In contrast, AIA as used in the protocol described is a localized form of arthritis that is induced through intra-articular injection and affects only the knee joint at the site of injection. This is beneficial as it allows the contralateral knee joint to be used to demonstrate non-arthritic conditions within the same animal. This gives a more accurate representation of the subject specific baseline that using joints of entirely untreated animals. Further antigen-induced mouse models for RA in common use include proteoglycan-induced arthritis (PGIA) and streptococcal cell-wall arthritis[22,23,24]. Other means of inducing arthritis in murine models include, but are not limited to, oil-induced (chemical) arthritis and spontaneously induced arthritis in TNFα transgenic mouse (induced through the transgenic over-expression of TNFα), K/BxN T-cell receptor transgenic mouse model arthritis and SKG model mice[24,25,26,27]. The advantages of AIA over other models are noteworthy. Whilst CIA produces a larger antibody response (anti-CII) measurable in blood, the efficiency of AIA is greater and the range of susceptible strains is wider. CIA susceptibility is a complex issue, integrally tied to MHC expression type and also may be regulated in response to, or dependent upon expression of, IL-4, IL-12, Th1 and Th2[28]. In terms of measured response to novel therapeutics, a major area of research for degenerative disorders such as RA, the application of common strains to investigations makes results more widely understandable.

MSCs are relatively large sized cells which can lead to them becoming lodged in narrow vascular tissue such as the lungs[29]. The described protocol favors the intra-articular injection route over intra-peritoneal, subcutaneous or intra-vascular methodologies. It is observed that intra-articular injection localizes cells to the synovium[8]. Consequently, the risk of impeding vascular routes, or of stroke are minimized. Inducing enhanced homing ability would allow the alternate methods for introduction of cell therapy and may become more feasible however the results of studies using intra-articular routes show positive outcomes with minimal risk of *in vivo* impediments. As discussed, edema may present as a response to increased permeability of endothelial cells in blood vessels and MSC produce biological molecules that preserve adherens junctions[17,30]. The described model demonstrates the response to MSCs in the knee joint is a reduction in edema and exudates in the joint. Previous studies have shown inhibition of endothelial cell permeability in response to MSC presence through the preservation of adherens junction proteins VE-cadherin and β–catenin[30,31].

The technique described examines the use of MSCs delivered through a single intra-articular injection as a therapeutic strategy for RA. The technique has demonstrated these cells as therapeutic, reducing the severity of an antigen-induced model of arthritis in mice. The injection of 5.0 x 10^5 MSC given at the peak of joint swelling is demonstrated to be sufficient to prevent the occurrence of severe cartilage damage, and reduce joint inflammation and exudate in the joint cavity. The technique can be applied further to give insight into the development of RA and investigate the mechanism by which MSC reduce the severity of RA in mice.

Disclosures

The authors have nothing to disclose.

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