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

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Abstract:	<p>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.</p> <p>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.</p> <p>These results demonstrate the therapeutic effect of MSCs injected in the joints of mice with AIA.</p>

Author Comments:	The invited manuscript follows on from a publication by Kehoe et al 2014 in J Trans Med. All previously published figures and legends, and the data included in the representative results section, are from or modified from this publication and permission is being sought for reuse.
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Institute for Science and Technology in Medicine at RJA Orthopaedic Hospital
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19th November 2014

Dear Sirs,

Please find attached the invited manuscript "Intra-articular injection of murine MSC for the treatment of inflammation and tissue degradation in Antigen-induced Arthritis."

This manuscript describes an established pre-clinical model for Antigen-Induced Arthritis (AIA) and the application of intra-articular injection of a therapeutic agent, in this case mesenchymal stem cells (MSC), for treatment of inflammation and tissue degradation. The technique is a valuable pre-clinical test to follow in vitro research and lead into clinical testing or larger animal models.

Protocols currently exist for AIA however a visualized protocol is not currently available and would be a useful addition for researchers attempting to establish a preclinical model for Rheumatoid Arthritis. The use of MSC in the treatment of musculoskeletal disorders is increasing and the prospects of the discovery of an effective and novel therapeutic agent for the treatment of this incurable inflammatory immune disorder is of huge value to the field.

I hope you find the manuscript appropriate for JoVE and look forward to hearing from you further.

Yours faithfully,

Alasdair Kay, PhD

TITLE:

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

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KEYWORDS:

Rheumatoid Arthritis, Mesenchymal Stem Cells, Inflammation, Murine Antigen-induced Arthritis model, Cartilage degradation

SHORT ABSTRACT:

Here, we present a protocol to induce inflammatory arthritis *in vivo* using antigen response, termed Antigen-induced Arthritis (AIA), an effective tool for research into Rheumatoid Arthritis (RA) research. Additionally we demonstrate the therapeutic effects of infusion of MSC to reduce inflammation, cartilage degradation and expression of TNF α .

LONG 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 TNF α .

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 TNF α 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-TNF α), 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 years³. 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 joints⁷. 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 tendon¹⁰. 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 shown 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 joint¹².

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 therapy¹³. 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 membrane⁸. 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 lining⁸.

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

1.1. 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).

1.1.1. 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.

1.1.2. 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}.

1.2. Randomly allocate animals to test or control groups and mark using ear clip or tail marks for identification purposes.

1.3. Isolate murine MSCs (mMSCs) from C57Bl/6 mice (n = 5) as previously described¹⁵.

1.3.1. 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% CO₂.

1.3.2. After 24 hours, remove non-adherent cells. 4 weeks later, re-plate cells at 100 cells per

cm² 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.

1.3.3. Examine cells for their ability to differentiate into chondrocytes (using pellet cultures), osteocytes and adipocytes, as described¹⁵. 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).

2.1 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.

2.2 Set suspension to stir for ≥ 20 min at room temperature to thoroughly mix.

2.3 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.

2.4 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.

2.5 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 (PTx)

3.1 Use personal protective equipment (gloves, eye protection) to handle *pertussis* toxin (PTx) as it is a contagious pathogen (whooping cough). PTx is used to boost the anti-mBSA Th immune response by increasing the systemic expression of cytokine IL-23.

3.2 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 PTx per animal.

3.3 Load PTx solution into 1 mL syringe with 25G needle. Ensure waste is properly disposed of into a sharps bin.

3.4 Perform an intraperitoneal (I/P) immunization of 100 μ L of heat-inactivated PTx 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

4.1 7 days later, prepare a repeat immunization to boost immune response with 100 μ L of 1 mg/mL mBSA and CFA in 1:1 ratio as described in steps 2.1 - 2.4.

4.2 Administer 100 μ L 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

5.1 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).

5.2 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.

5.3 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 joint, located using the patella as a guide. Ensure the calipers are not in contact with any surface other than the knee joint.

5.3.1. Obtain a static reading. Record the knee joint diameter for both right (R) and left (L) knees for each animal prior to injection.

5.4 Induce AIA with the intra-articular administration of 10 μ L of a 10 mg/mL mBSA in PBS solution directly into the synovial cavity of the right (stifle) joint.

5.4.1. 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

6.1 Prepare experimental treatments for intra-articular injections using the following step.

6.1.1. Load 0.5 mL monoject (29 G) insulin syringes with either mMSC suspension (5.0×10^7 cells/mL) in serum-free Iscove's Modified Dulbecco's Medium (IMDM) (test) or serum-free IMDM vehicle solution (control) to deliver 10 μ L 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.

6.2 Anesthetize mice as per step 5.1.

6.3 Measure and record knee diameter as in steps 5.2-5.3.

6.4 20 hours post-induction of arthritis, administer the primary knee (R) with novel treatment therapeutic of 5.0×10^5 mMSC delivered in 10 μ L 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

7.1 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.

7.2 For examination, euthanize mice at days 3, 7, 14 and 28 using 1 minute of carbogen followed by a programmed control of rising CO² gas for 4 minutes in a veterinary chamber as per UK Home Office guidelines. Death is confirmed using foot withdrawal and blink reflexes.

7.3 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.

7.4 Confirm death using cervical dislocation then remove euthanized mice to sterile dissection area.

7.5 Spray animal with a 70% ethanol (EtOH) in sterile water for aseptic dissection.

7.6 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.

7.7 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 TNF α in blood serum

8.1 Centrifuge extracted blood (from step 7.3) at 1,500 x g for 10 minutes to separate the cellular component from blood serum.

8.2 Remove serum supernatant and transfer to a 1 mL microcentrifuge tube.

8.3 Test serum for the presence of TNF α using a mouse TNF α ELISA kit according to the manufacturer's instructions or freeze at -80 °C for later testing.

9. Histological scoring of AIA in knee joints.

9.1 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.

9.2 Paraffin embed joints for sectioning.

9.3 For histological analysis, cut mid-sagittal sections of 5 μ m thickness from paraffin embedded knee joints and stain with haematoxylin and eosin (H&E)¹⁶.

9.4 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).

9.4.1. 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.

9.4.1.1. 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).

9.4.2. 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.

9.5 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.

9.6 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.

9.6.1. 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).

9.7 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 \pm Standard Error of Means (SEM). Increased score is representative of more severe arthritis response¹⁵. Assess comparisons between results as described in section “Statistical Analysis”.

10. Statistical Analysis

10.1. 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 TNF α measurements for normality, as this directs the choice of statistical test.

10.2. 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* 2014⁸. 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 \pm 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 TNF α

Serum measurements showed a reduction in circulating TNF α 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 arthritis⁸. 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 \pm 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 induction⁸. 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. 5.0×10^5 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 μ m in A and B, 20 μ m in C and 200 μ m in D and E.

Figure 3: Serum concentration of TNF α at the indicated time points (day 3, 7 and 14), analysed by enzyme-linked immunosorbent assay⁸. In MSC treated mice with AIA, serum levels of TNF α 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 \pm 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 index¹⁵. 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 μ m (Hyperplasia) or 200 μ m (Synovial Infiltrate, Synovial Exudate, Cartilage Depletion).

Table 1: Joint inflammation and cartilage damage on day 3, 7 and 14 of antigen-induced arthritis as assessed by histological scoring⁸. 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 signals¹⁷ 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, MMP13¹⁸. 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 activity¹⁹.

In studies performed using this protocol⁸, and ongoing work, the efficiency of the AIA protocol has been $\sim 100\%$. 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 phases²⁵. 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 cells³⁸. Cartilage and bone erosion occurs and synovitis characteristic of RA³⁶.

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-TNF α , 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. TNF α 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 treatments²⁰. The technique described has demonstrated that administration of MSCs results in reduced levels of TNF α 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-TNF α 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 model²⁵. 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 condition²². 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 mice³⁵. 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 response³⁶. 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 function³⁴. 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 tissue³⁷. 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 activation³⁵. 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 joint²⁵.

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 genes⁹. 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 AIA²¹. In CIA, severity of arthritis is sometimes assessed by measuring morphological changes in the paw, such as increased paw volume measured using a plethysmometer²¹. 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 TNF α transgenic mouse (induced through the transgenic over-expression of TNF α), 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 Th2²⁸. 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 lungs²⁹. 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 synovium⁸. 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 5.0×10^5 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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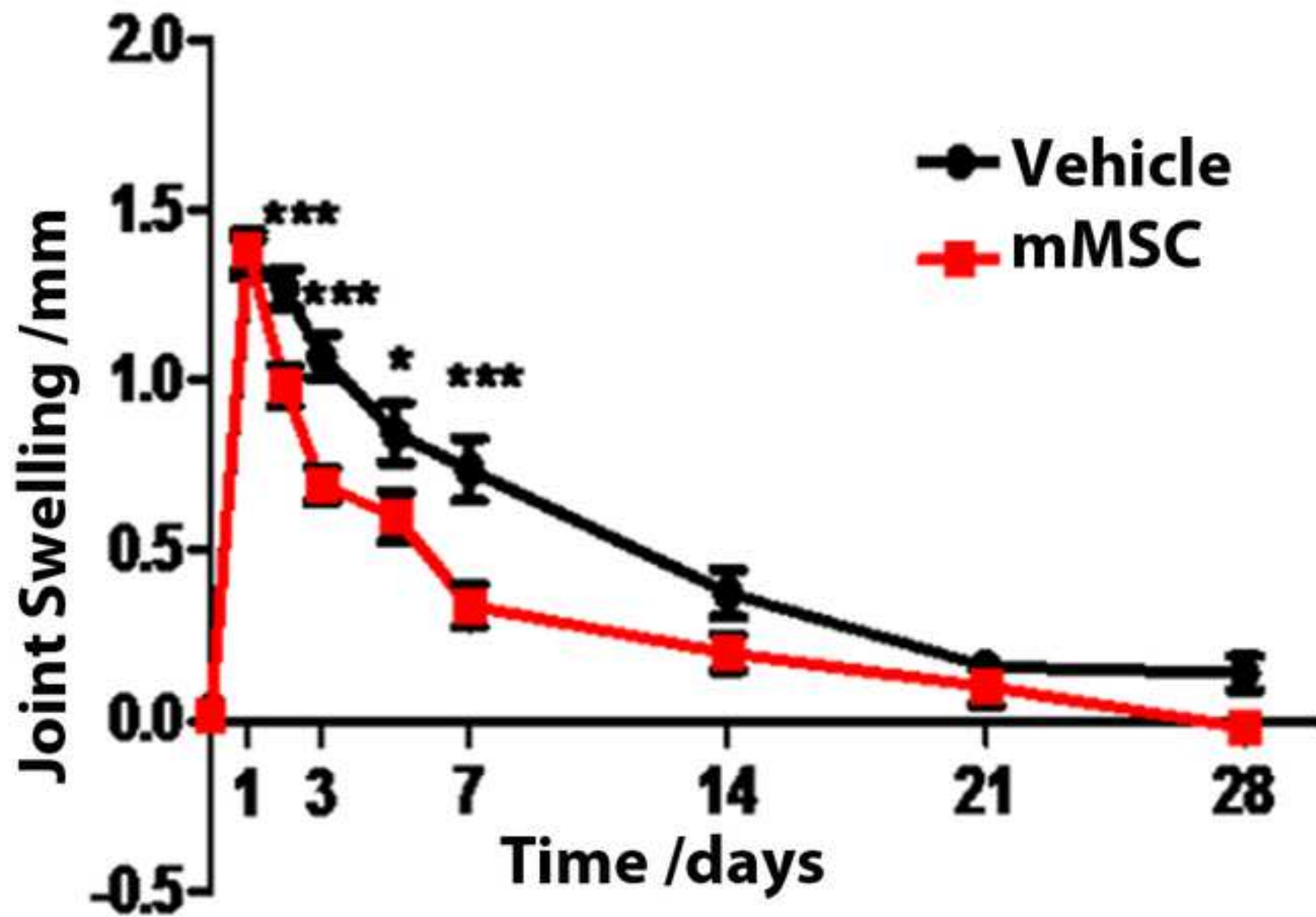


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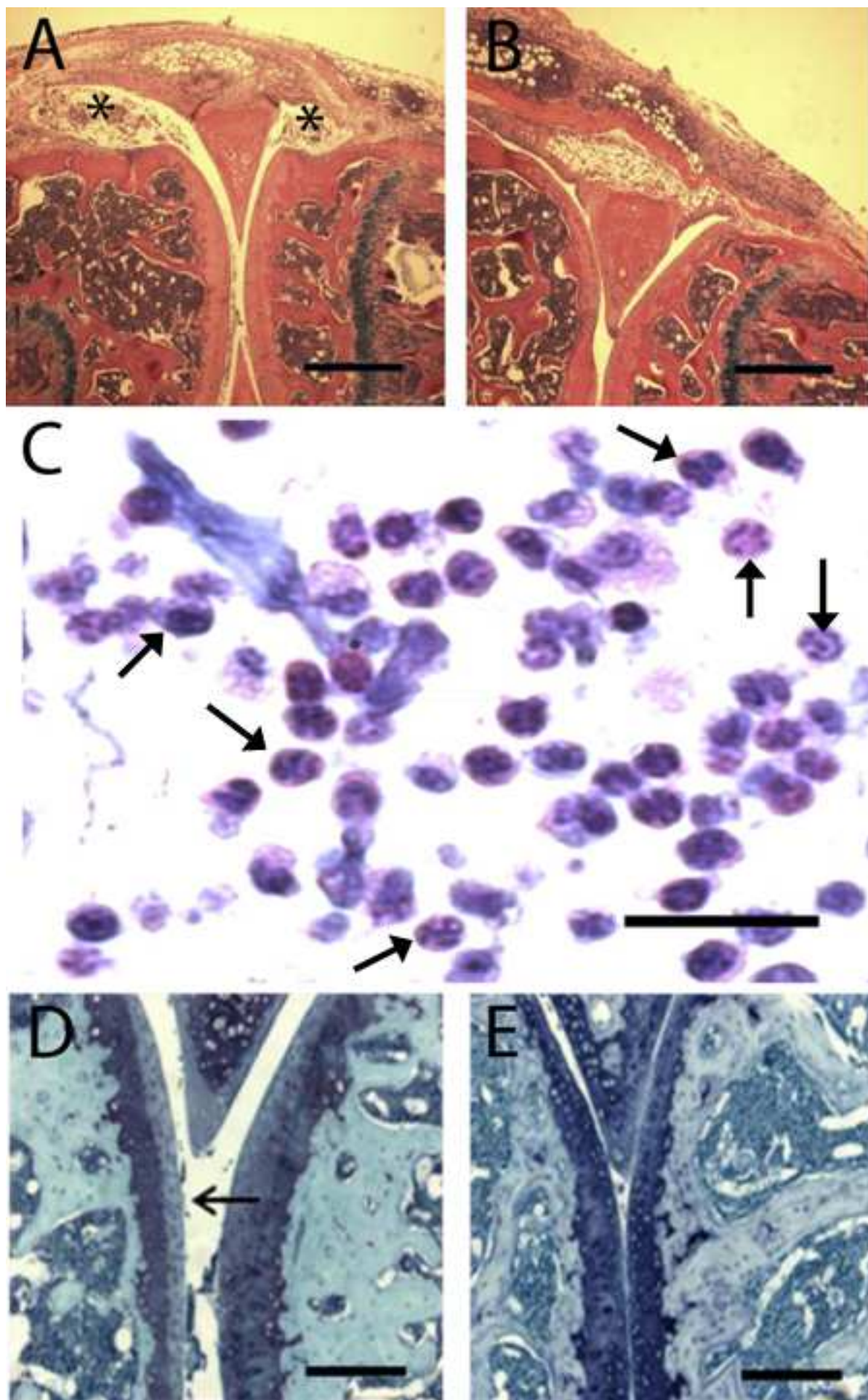


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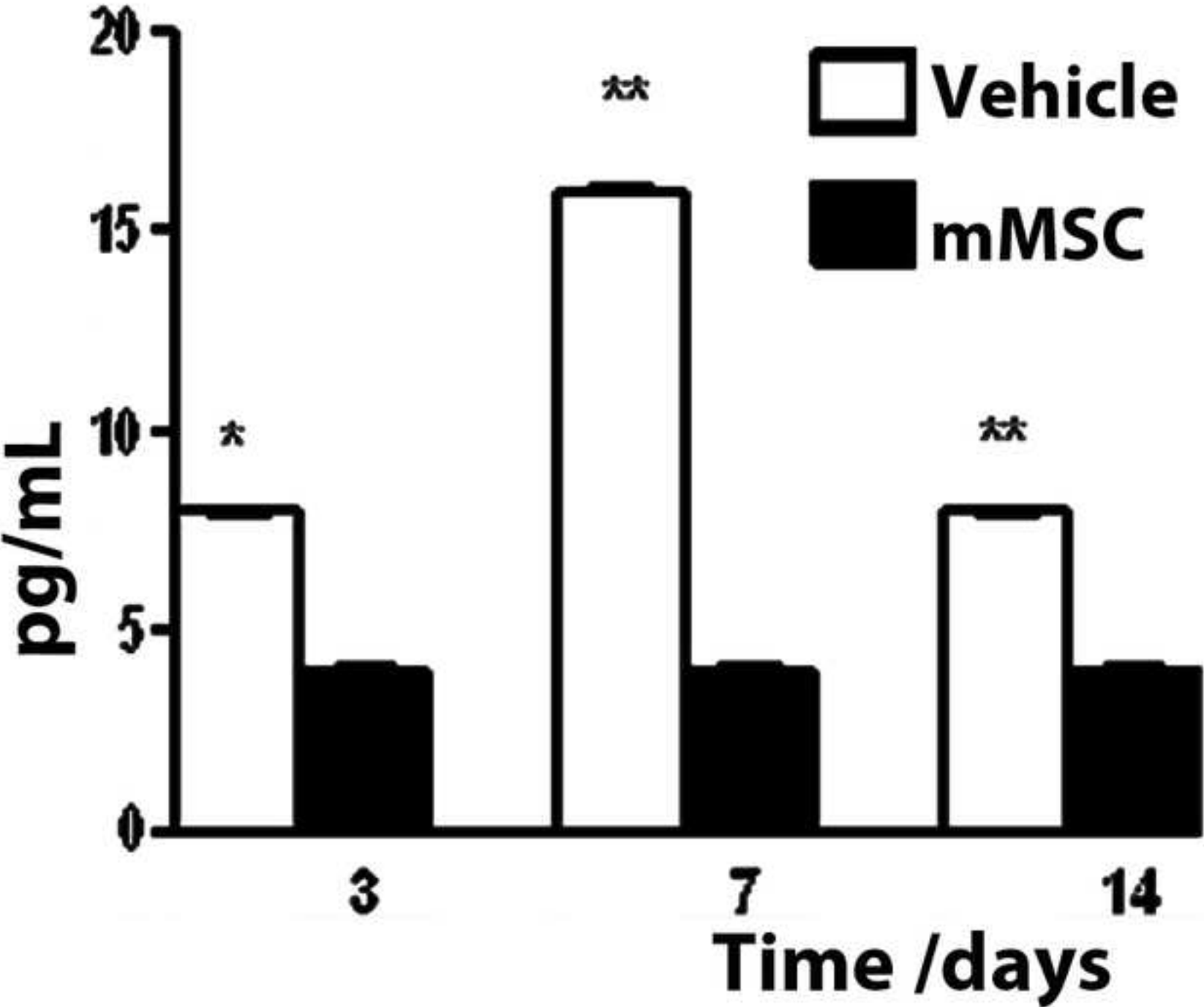
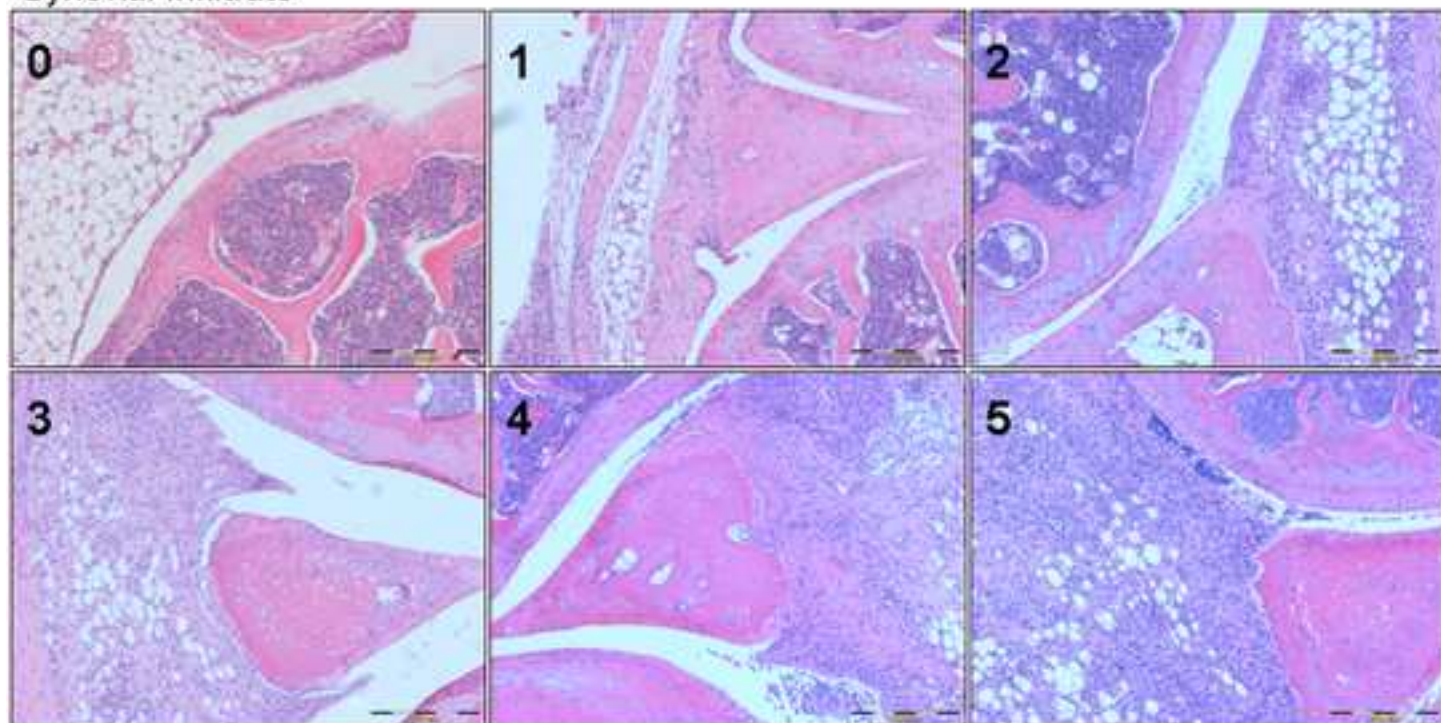
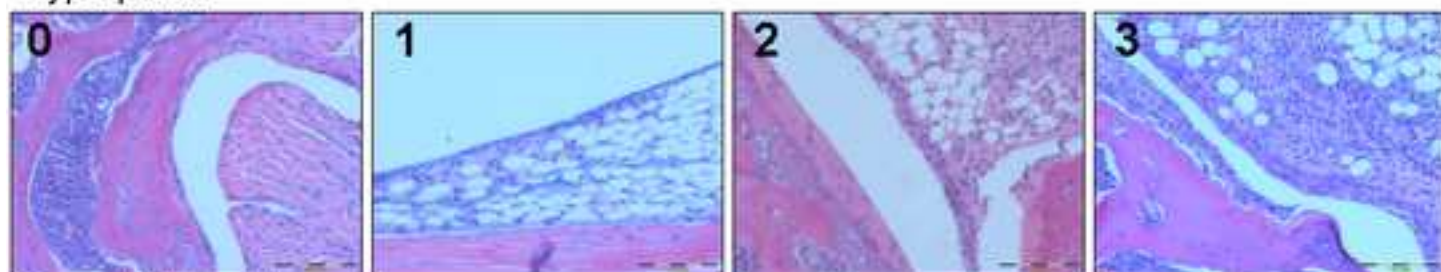


Figure 4
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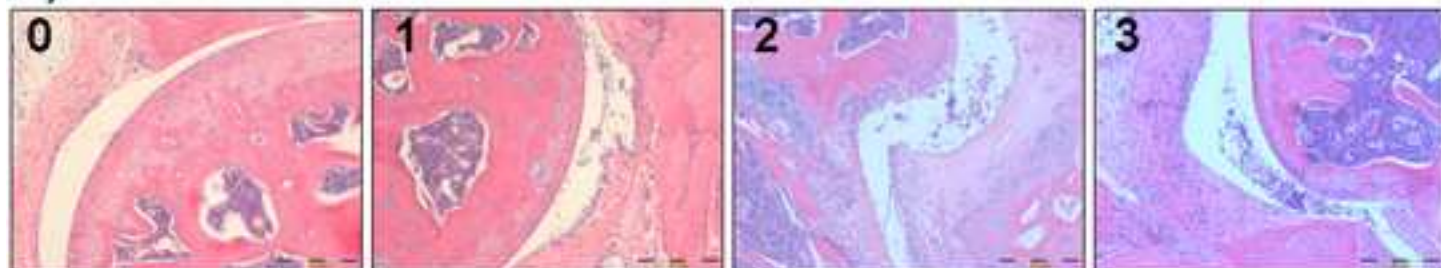
Synovial Infiltrate



Hyperplasia



Synovial Exudate



Cartilage Depletion

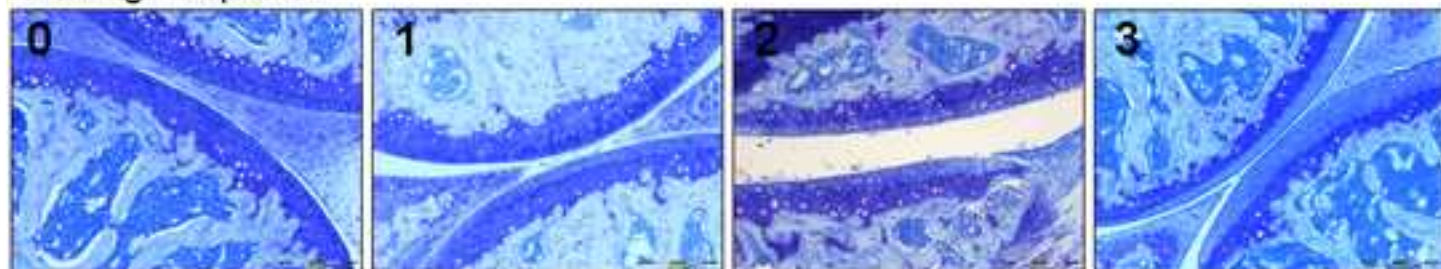


Table 1
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Species	Days	Hyperplasia	Synovial Infiltrate	Exudate	Cartilage depletion	Arthritis index
Control animals	3	1.75 ±0.29	3.56 ±0.40	2.78±0.15	1.78±0.33	9.89±0.88
	7	2.43±0.23	3.93±0.39	2.14±0.46	1.71±0.52	10.2±1.46
	14	1.67±0.67	1.67±1.20	1.17±0.92	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

Name of Reagent/ Equipment	Company	Catalog Number
Complete Freund's Adjuvant	Sigma-Aldrich	F5881
methylated Bovine Serum Albumin	Sigma-Aldrich	A1009-1G
Dulbecco's Phosphate Buffered Saline (DPBS)	Invitrogen	14190-086
heat-inactivated Bordetella pertussis toxin	Sigma-Aldrich	P2980
Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen	12440-061
mouse TNFα ELISA Ready-SET-Go! Kit (eBioscience)	eBioscience	88-7324-22
Eosin	SLS	CHE1916
Haematoxylin (Harris)	Atom Scientific	RRSP67-D
Toluidine Blue	Atom Scientific	RRSP146-B
Syringe Needle 19G	BD Microlance 3	301750
Syringe Needle 21G	BD Microlance 3	304432
Syringe Needle 23G	BD Microlance 3	300700
Syringe Needle 25G	BD Microlance 3	300600
Dial indicating outside calliper	Kroeplin GmbH	D4R50

Comments/Description

10ml Each mL contains 1 mg of Mycobacterium tuberculosis (H37Ra, ATCC 25177), heat killed and dried, 0.85 mL paraffin oil and 0.15 mL mannide monooleate.

Methyl ester of albumin prepared per Mandell, J.D. and Hershey, A.D., *Anal. Biochem.* , **1**, 66 (1960).

100ml, without calcium, magnesium or phenol red

Aseptically filled solution in 50% glycerol, containing 50 mM TRIS, 10 mM glycine, 0.5 M NaCl, pH 7.5

With L-glutamine, phenol red, without α -thioglycerol, 2-mercaptoethanol

Recognition of mouse TNF alpha by this assay is not interfered with by 1000x excess soluble TNF alpha

Eosin Y 24050 C I 4538025g - mixed with Harris Haematoxylin for H&E staining

An alum haematoxylin similar to Delafield's formulation, but which uses chemical ripening to produce

A 1% aqueous working solution of Toluidine blue O which can be used to demonstrate Nissl substance and mast cell granules.

The one in use is no longer currently available so catalog number for an equivalent item has been given



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Authors response to reviewers:

The authors feel the points raised by the reviewers are valid. An attempt has been made to address all of the points listed in the revised version of the manuscript, with the following notes:

1. Use of anaesthetic: Mice are anaesthetised for swelling measurements and intra-articular injections, and not for I/P or S/C injections as these can be performed with minimal discomfort to the mouse.
2. A "statistical analysis" section has been inserted. Consequently, to avoid repetition, all descriptions of which statistical tests are performed have been placed in this section. Multiple comparisons were used for serial data using Mann Whitney U.
3. The contralateral knee histology ideally matches that of unaffected joints (i.e. a score of 0 in all aspects of the scoring system). Consequently, whilst a panel of representative images for scoring has been included, individual images of contralateral knees were not.
4. A detailed breakdown of the scoring system is now included.
5. The decalcification procedure was performed by the histology department at RJA, as noted in the acknowledgments. The details given of this procedure were supplied by this group.

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52955_R1_120514.docx) is located in your Editorial Manager account. Please download the .docx file and use this updated version for any future revisions.

Changes made by the Science Editor:

1. There have been edits made to the manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. The Long Abstract should focus less on the results of the treatment and more on the protocol.
3. A number of areas require additional detail:
 - 7.2: How are mice euthanized?
 - 9.4: Please define "IMS."
 - 9.5: How are samples dried before xylene exposure?
4. Figure 2 Legend is unclear. Which panels go with which treatments? Which arrows are they referring to in the first sentence? Why is there only one scale bar?
5. Discussion: What are the limitations of the method?

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper submitted is a description of the methodology used to initiate a resolving model of RA and a therapeutic intra-articular administration method. Whilst not entirely novel, this is the first, to my knowledge, methods paper explicitly explaining the process from start to finish. This submission appears to have been written to support a prior publication by the group "Intra-articular injection of

mesenchymal stromal cells leads to reduced inflammation and cartilage damage in murine antigen-induced arthritis" (Kehoe et al. Journal of Translational Medicine 2014, 12:157), in which the data used in this manuscript is taken from.

Whilst a good manuscript, I feel there are several issues that must be addressed and other minor issues relating to style rather than content.

Major Concerns:

Line 180 - Section 2.5 - To perform the SC injections at the base of the tail, do the animals need to be anaesthetised. In our lab, this type of SC injection is performed under inhalational, recovery anaesthetic.

Line 206 - Section 5 - Intra articular (IA) injection. - Major comments

1. When performing the IA injection, are the mice anaesthetised? What gauge needle is used for the injection? Are there any welfare issues from the injection (e.g. severe effects on mobility)?

a. Listing needle gauges required would be useful in your materials table too

2. The actual IA injection is not well described, which is somewhat disappointing as, to me, it is the focus of the article. In ref 8 (Kehoe et al) the process is described better: "intra-articularly (0.5 ml monoject (29 G) insulin syringe, BD Micro-Fine, Franklyn Lakes, USA) through the patellar ligament into the right knee joint. Stretching of the hindleg facilitated the intra-articular injection." These kinds of comments always help methods papers.

3. Are the knee diameters measured before or after the injections or both? Is there a noticeable size effect following injection?

4. Mice are anaesthetised for measurements, but not for injections. Is this correct?

Most of the comments for section 5 are equally applicable to section 6, however, most would only need to be stated once.

Line 292 - Representative Results - please begin this section with "all of the following results ... Kehoe et al 2014" (line 297 - 298). Also, "et al" should be italicised.

1. Results section would benefit from either explicitly describing statistical analyses performed, or alternatively having a section describing statistical analyses in the methods section.

Line 327 - "two independent observers" - why isn't this stated in the methods section, if this is how it was done. Furthermore, I assume the mean of the two independent scores are used for an individual section, this can lead to observer error causing an error in the mean. This should be accounted for, but does require more than two observers.

Lines 331 - 335 - p values are reported with no explanation of statistical tests used. Please state somewhere in the text how these were performed - this applies throughout.

Regarding statistics - many of the comparisons drawn in this document can be considered multiple comparisons from one data set - particularly the swelling measurements as these are serial data. As a result corrections to significance values should be performed or a more robust test used.

Figure 2 Legend - This legend needs re-writing. In Kehoe et al (ref 8), from which the data is taken, the images displayed are from control treated and MSC treated arthritic knees. Whilst the exact same images are used, in the figure legend in this manuscript, panels B and D are referred to as contralateral (when they should be MSC treated). Please correct this.

Regarding figure 2: with the above comments in mind, it would be good to include images of contralateral knee sections from both treated and untreated mice, to demonstrate the differences occurring from the arthritis.

Line 387 - "a detailed breakdown is given ..." - I do not think this is the case in this manuscript. You have given 2 H&E images, but with no reference to score. A detailed breakdown would be an image for each "score" for each parameter - or at the very least examples of these. A figure demonstrating this I feel is highly beneficial in a methods paper such as this. Furthermore, Nowell et al (ref 15) do not go into any detail either, making it more important to set the standard.

Minor Concerns:

Line 106-109 - The authors state "...infusion of MSC into a damaged joint can aid tissue repair through establishing new cell types appropriate to damaged tissues. The consequence of this is de

novo synthesis..." (Line 106-109) This statement either needs to have a suitable reference that demonstrates this sufficiently, or should be removed. There is still controversy in the field over whether therapeutic administration of stem cells leads to direct repair or instead allows for self-repair. Most mechanisms linked to SC therapy suggest a degree of modulation of immune systems (and other cell types) not direct repair

Line 116 - missing full stop following ref 13.

Line 123 - 125 The authors state the MSC are only functioning by producing immunomodulating molecules, and not affecting *de novo* (which should be italicised) matrix. In ref 8 they only surmise this, and do not demonstrate it

Line 149 - "ACI" - please expand

Line 164 - Section 2: Could you please give a recommendation with regards to excess (as has been done for section 3)? E.g. how many ml for 12 mice?

Line 169 - Section 2.2 - Done at room temperature or on ice?

Line 177 - Section 2.4 - How do you ensure a "thickened consistency"? Method papers written for collagen induced arthritis (that uses a collagen/CFA emulsion) recommend using a "droplet test", whereby 20ul of emulsion placed onto water should hold together for >25s before dispersal. Would you recommend this for your emulsion?
Also, are these materials temp sensitive? Would it be better to be done on ice?

Line 180 - Section 2.5 - I would recommend altering 2.5 to read "100ul of mBSA/CFA emulsion" instead of repeating "mBSA in CFA at ratio of 1:1".

Line 183 Section 3 - "Pertussis" should be italicised - it is a species name

Line 194 - Italicise "Bordetella pertussis" - it is the genus and species

Line 201 - Section 4.1 - "...described in steps 2.1 - 2.5" Consider changing 2.5 to 2.4, as site of injection is now different. Very minor comment.

Address in comments -- Line 241 - Section 7.2 - Mice are euthanized and then a cardiac puncture (CP) performed. Just to confirm, do you not CP under anaesthesia and then confirm death by cervical dislocation?

Line 253 - Section 7.6 - "neutral buffered formal saline" should be "neutral buffered formalin". Also, what concentration NBF is used? Is it bought in complete, or is it made? Please state and put into materials table. Also, by "store as necessary" what do you mean? Please clarify. Have you seen any issues with extended storage in formalin? Varied reports on issues with both under- and over-fixation. Please clarify.

Line 256 - "TNF" to "TNF α " - very minor comment

Line 264 - please be explicit with regards to freezing and storage of serum - do you freeze at -20C or -80C?

Line 269 - 9.1 - I think it would be better to split into two points - firstly decalcifying of fixed tissue then paraffin embedding.

Line 276 - 9.2 - Be more explicit that scoring system comes from ref 15 - unnecessary in title of section, would be better here.

Line 279 - 9.4 - "matching sections" - does this mean matched to contralateral, or across all mice? Minor, picky point, but could be made clearer.

Line 281 - expand IMS. Please also state concentrations of xylene and IMS.

Line 289 - 9.6 - "Calculate an arthritis index (mean \pm SEM) by summing all scores" - I am not sure what this means. I think that you are saying that the AI is calculated by summing the individual scores for an individual mouse, then, for each group of mice you calculate a mean \pm SEM. Please could you clarify this. Also, are these scored blind, and/or by multiple observers? Would this not be a better procedure? Finally, SEM should be expanded.

change made – comment? Line 302 - "... following induction of AIA" - please expand to explain that this is AIA Day 1 (Step 5)

Line 308 - "normalised" - may be better to explain that by normalised the diameter measurements are the difference between swollen knee and contralateral.

Line 309 - "results are expressed as mean \pm SEM" - which results?

Line 313 - the error measurements reported seem very small. What is the smallest accurate measurement that can be made using the calipers? These errors are only applicable if greater than the smallest measurement capable of being read on the calipers

Line 330 - change "Histological examination showed ..." to "Histological examination of day 3 sections showed ..." or something similar.

Line 352 - Colon missing from figure legend

Line 363 - Colon missing from figure legend

Line 382 - "N=6 mice" - is this N=6 mice per time point or spread through the 3 time points? Please clarify

Line 402 - it may be good to have a hypothesis regarding how MSC are reducing the cartilage damage, although not strictly necessary

Line 434 - 464 - The authors discuss CIA with regards to causing arthritis in the knee joints - in our experience, CIA predominantly affects paws and ankles/wrists over the knee joints. Although, there are still strong signs of inflammation at the knee (with regards to synovial infiltrate), I will not contradict that(!), but I think a comment regarding the effects on paws should be made.

Paragraph (Line 432 - 451) - During this paragraph the merits of AIA and other models are discussed, as are the downfalls of the other models. I feel this passage would be strengthened by including some of the shortfalls of the AIA model too (for example, the spontaneous resolution of the model). It does not diminish the model, but can strengthen reasons to choose AIA over other models.

Figure 1 - Change "control" to "vehicle" - please make the figures consistent.

Figure 1 - x axis legend should be changed to something like "Time post AIA induction, days". At the very least, please change to be consistent in terms of highlighting units (in the y axis you separate label and units with a comma)

Figure 2 - C, D and E have no scale bars (and yet are referred to in the legend)

Figure 3 - y axis needs relabeling - requires TNF α in the label. Similarly x axis requires a label e.g. timepoint or days. Also, change "non-treated" to "vehicle treated" please.

Table 1 - The layout needs improving. Better layout in Kehoe paper of the same table. Please remove the colour from the header.

Reagent/equipment table - incomplete, please have a completed list - see earlier comments. Furthermore, please clarify the comment "recognition of mouse TNF α by this assay is not interefered with by 1000x excess soluble TNF α " - I think that you have missed "... TNF α Receptor type I or II" from the data sheet.

Additional Comments to Authors:

Overall, it is good to see a methods paper outlining this kind of experiment from start to finish. However, I feel that in some areas detail is lacking. Furthermore, notes and comments on steps of the procedure that have been gained from experience of doing the procedure tend to be very welcome in methods papers, and indeed, it would be the case here.

I strongly feel that a section on data analysis would also be beneficial - how one analyses data gained from an experiment is as important as the procedure.

Reviewer #2:

Manuscript Summary:

The methods described in this article will be useful for scientists interested in testing the effects of MSC treatment in autoimmune disease. However, there are sections in the protocol (i.e. section 9) that would benefit from being in the video recording while other sections (section 4 and section 8) that do not need to be recorded as they are well established protocols. Additionally, there are several key points in the protocol that are not clearly explained and require further explanation (e.g. section 2.) to ensure that someone who is new to the technique can easily follow it. Furthermore, in some places abbreviations are given for scientific terms without giving the full term initially while in other places this information is provided. Also, the authors do not state the post-test used to do the statistical analysis for figures 1 and 3 in the paper. The discussion while well written and sufficiently detailed was nearly as long as the protocol itself which further indicates that the protocol requires more detail.

Major Concerns:

1. Sections of the protocol are not detailed enough for a methods paper. For example on line 226 "6.1. Prepare experimental treatments in the form of intra-articular injection" is very vague. Further details on example experimental treatments would be useful here.
2. No detail is provided on the MSCs used. Information about passage number, cell viability, culture media used, source of MSCs, initial yield of cells from the tissue source and detachment of cells are essential for this protocol. The authors stress the importance of standardising the AIA model but the isolation and expansion of MSC is also a critical part of this process that would need to be detailed as variations in source and culture of MSC has previously been shown to alter their immune regulatory properties. The MSC culture would not need to be part of the video protocol but should be included in the text.
3. The statistical post-test used in figures 1 and 3 are not stated. Authors only explain that the data represents the mean \pm SEM but the post-test used is not detailed. This will need to be provided.
4. Line 314 and 316: P-values for the data set shown in figure 1 are found in the text. However, no information about the post-test used and the n number is provided. This information can only be found in the figure legend. The p-values should be removed from the text and the authors should refer to figure 1 on line 314.
5. All of the reagents used in the protocol are not listed in the table of materials.

Minor Concerns:

1. Lines 140 and 148: the word "though" is used when it should be "through".
2. Line 149: The abbreviation ACI is given without stating what it stands for.
3. Line 159: Should recommend the number of mice to use for each treatment group.
4. Lines 171-174: Section 2.3. This needs to be broken down into more sub-sections (i.e. 2.3.1. and 2.3.2.) with a better step-wise explanation on how to perform that part of the procedure.
5. Line 176: Section 2.4. It states that you need to mix (x10) although it is unclear what x10 is referring to. Additionally, it states that the sample needs to be vortexed but information on speed and duration of the vortexing is not provided.
6. Line 185: The abbreviation PTx is given without stating what it stands for.
7. Line 241: Section 7.2. It states that at the end of the experiment the mice were euthanized at days 3, 7, 14 and 28. However, is that really the end of the experiment?
8. Line 247: Section 7.4. The abbreviation EtOH is given without stating what it stands for.
9. Section 8.1 and 2 do not need to be part of the video protocol as it is a very straight forward procedure.
10. Line 270: It states that the duration of the decalcification depends on the thickness and density of bone but measurements are not provided. An example, including images, of the appropriate thickness is needed to ensure that the protocol is carried out optimally.

11. Line 277: The authors refer to another paper for assessing the arthritis index of the mice. However, it would be useful for them to explain it in detail in this protocol for the ease of the reader including images.
12. Line 325-328: Should be in the methods section as it is not a result but a key aspect of the experimental procedure.
13. Figure 2: Images of the control mice should come in panels A and C with B and D showing experimental treatment. In the results section the images shown are not explained in enough detail. The arrows and lines used to indicate loss of proteoglycans and leukocyte infiltration respectively are not clear enough. For someone who is unfamiliar with histological sections of this kind will find it difficult to interpret this data. More description is needed in the results section about the figure and clearer indications of the different structures in the joint are needed on the figure for it to be clearly understood.
14. Table 1: Sufficient detail are not provided on how to assess the hyperplasia of the synovial lining, infiltration by neutrophils, exudate and cartilage depletion. In the protocol the authors refer to Nowell et al. but they themselves do not provide any detail on how to measure this. This needs to be clearly outlined in order for the protocol to be clear and easy to follow.
15. Line 387-389: The authors state that they give a "detailed breakdown for the recognition and assessment of the pathology of RA". However, no such detail is provided. This needs to be clearly outlined for readers attempting to replicate this assay.
16. Line 454-455: The authors did not compare subcutaneous or intra-vascular injection methods with intra-articular injection and therefore cannot state that intra-articular administration is a more favourable route. It would be nice to include comparative data in this study to truly highlight the benefit of this route of administration of MSC compared to others.
17. Line 470: State that 500,000 MSCs were administered. Whereas on line 231 they state that 5x10⁵ MSCs were used. They should use the same format throughout the paper.

Reviewer #3:

Manuscript Summary:

The AIA model is well established however an updated and well detailed protocol to reflect current directions in the development of therapies to RA would be of interest to specialists, both immunologists and stem cell biologists.

Major Concerns:

1. Long abstract, the second last paragraph (line 59 onwards) is somewhat repetition of the first paragraph and could be condensed better.
2. Introduction Line 102 The sentence "The ultimate fate of differentiating MSCs is restricted to cells...". This is not strictly true as MSCs have been shown to differentiate into other lineages, perhaps state as "largely restricted to". And in the next sentence "owing to reduction in..." - it is a "lack of" expression of MHCII rather than "reduction" as most studies show no MHCII expression by MSCs.
3. Protocol section, Line 153 - correct this to "UK Home Office approved..."

Step 1.1 - It might be useful to note in the Table of Materials where the mice strains can be sourced from. And also specify whether these can be a mix of males/females in the groups.

What numbers should be used per group and can the authors indicate how a power analysis might be performed by a reader interested in testing a novel MSC therapy to determine the correct/adequate number of mice per group?

Step 2.5 - The handling of the mice requires further detail of a reference for correct procedures to be applied at each stage i.e. is the injection or other manipulations require anaesthesia and so on.

Steps 3.1 to 3.3 - The PTx abbreviation needs to be consistent e.g. in 3.3 the PTx is introduced differently as Bordetella PTx. Is this the same PTx described in 3.1?

Step 5.1 - Specify which needle gauge to use.

Would be helpful to add "knee joint or patellar joint" along with "stifle" as this term is used later.

Would be useful to add a line in the discussion to indicate the expected behaviour of the mice during the 21 days and potential adverse reactions that might indicate that an animal cannot be used further in the study.

Step 5.3 - What features should an operator be looking for to identify the patella?

Step 5.4 - The diameter and swelling of the knee joint is an important quantitative measure in the AIA mouse but the measurement is subject to many operator variations. Further, the differences between contralateral (normal knees) and treated/controls can be very small. Therefore the use of the digital micrometer procedure needs more detail on its correct use so that reliable statistical analysis can be made. E.g. how to set the pressure of the grips consistently, how to obtain a static reading, is the knee diameter measured in more than one position around the knee, is an average taken of multiple reads and so on.

Step 6.1 - It is outside the scope of the protocol to detail the isolation and expansion of the mMSCs, however a reference to the preparation/expansion of mMSCs would be useful. Is there a best passage number to use? Why is this dose (500,000) of cells used? Can a dose response be investigated?

Step 8.3 - What is the best storage temperature and is there a "shelf-life" before which to measure the cytokine?

Line 319 - In the histology analysis, can the mMSCs be identified? Do they survive in the numbers injected, proliferate, or are depleted in numbers?

Line 413 - The IL-6 description is irrelevant as this cytokine was not assayed in the protocol. The first 2 sentences are not required.

Line 445 onwards - The authors argue the advantages of the AIA model. However, as the authors note, RA is a complex disease and as no model is truly representative of the human condition (which varies anyway), these other models do offer other advantages over the AIA which likely are useful for development of new therapeutics.

Minor Concerns:

Long Abstract line 59, add "in the articular joint" to better describe "measurement of swelling in the articular joint,".

Mesenchymal Stem Cells does not require the first letter to be capitalised.

Step 7.2 - What needle gauge size is used? Add "allow to clot" in a microcentrifuge tube.

There are a number of typographical errors (not listed here) that the authors need to correct.