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 RJAH, 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 you 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 showed 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 "TNFα" - 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 ± 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 ± 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 ± 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 TNFα 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 TNFα by this assay is not interefered with by 1000x excess soluble TNFα" - I think that you have missed "… TNFα 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 ± 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.