**Response to Reviewers' comments:**  
**Editorial comments:**  
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.

**Authors’ response:** We have checked carefully the whole manuscript to avoid language issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**Authors’ response:** Copyright permission has been obtained and the document has been uploaded.

3. Figure 1: Please label the molecular weight of the bands.

**Authors’ response:** Revised as requested.

4. Figure 2: Please combine all panels of one figure into a single image file or split it into several figures.

**Authors’ response:** Fig. 2 is now a single image.

5. Figure 2A: Please change ml to mL. Please include a space between 30 and mV (i.e., 30 mV).

**Authors’ response:** Corrected as requested.

6. Figure 2B: Please line up the panels better. Some panels are off-set in the Figure.

**Authors’ response:** Revised as requested.

7. Figure 2C: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

**Authors’ response:** Scale bars have been provided.

8. Figure 2D: Please define error bars in the figure legend.

**Authors’ response:** The error bars have been defined.

9. Figure 3: Please include a space between numbers and their units (i.e., 2 h, 8 h, 24 h).

**Authors’ response:** Corrected.

10. Please provide an institutional email address for each author.

**Authors’ response:** The institutional email address for each author has been provided.

11. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

**Authors’ response:** Corrected.

12. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Brookhaven ZetaPlus, etc.

**Authors’ response:** Revised.

13. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

**Authors’ response:** Ethics statement has been included in the revised ms.

14. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

**Authors’ response:** Revised as requested.

15. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

**Authors’ response:** Revised as requested.

16. 1.1.1: What type of water is used? How to dissolve under the protection of N2? Where is this step performed?

**Authors’ response:** Revised as requested. No special environment is needed to perform this step.

17. 1.3.2: How to perform purification by using 100k ultrafiltration tube? Does 100k mean 100 kDa?

**Authors’ response:** Revised.

18. 1.3.3: Please describe how this is done.

**Authors’ response:** Revised.

19. 2.2: Please specify the source of siRNA. Is it purchased or isolated in lab?

**Authors’ response:** The source of siRNA used in this manuscript can be found in the Table of Materials. We don’t think it is necessary to specify the source of siRNA at this step because viewers should, depending on their experimental purposes, use siRNA which may differ from those used in this manuscript. Our protocol should be suitable for delivery of any siRNAs, whatever their sequences.

20. 2.4: Please break up into sub-steps.

**Authors’ response:** Done as requested.

21. 3.3: How long does it take for the majority of the cells to detach?

**Authors’ response:** Specified.

22. 3.9: Does step 7 refer to step 3.7?

**Authors’ response:** We thank the editor for careful reading of our ms. This has been corrected.

23. 4.1: Please specify the age, sex, and strain of rats.

**Authors’ response:** Done as requested.

24. 4.3: Are rats constrained before injection? Please describe how to assess biodistribution or therapeutic effect.

**Authors’ response:** Relevant info has been provided in the revised ms.

25. What happens to rats at the end of experiment?

**Authors’ response:** In Section 4 of the protocol text, we describe how to deliver siRNA to macrophages in vivo using rats with adjuvant arthritis as a disease model. Because the sequence of siRNA used in this section is not provided, we don’t think it is suitable here to mention what happens to rats at the end of experiment. Moreover, the therapeutic effect may vary, depending on siRNA specificity, its silencing activity, the choice of an appropriate target, dose, etc. Failure in obtaining expected therapeutic benefits does not necessarily mean infeasibility of the PEI-SPION-based siRNA transfection method.

26. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

**Authors’ response:** The essential steps for the video are highlighted in red.

27. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

**Authors’ response:** Done as requested.

28. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Authors’ response:** Done as requested.

29. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique  
**Authors’ response:** We have completely rephrased the Discussion following these instructions.

Reviewer #1:  
  
Comments:  
The authors introduced many reported researches. Some errors and inappropriate statements (including English writings and experimental data) should be corrected. Specifically, the authors should address the following points:  
  
1. The authors reported that "we presented a method for synthesizing SPIONs whose surface was modified with low-molecular-weight, branched PEI (PEI-SPION)". However, the molecular weight of PEI used in this research was not mentioned in the manuscript, although in section of preparation of PEI-SPIONs, the resultant solution was purified by 100K ultrafiltration tube, which seems like the cut-off molecular weight of free PEI was by less than 100kDa;  
**Authors’ response:** In our initial submission the molecular weight of PEI had been described in the Table of Materials. Anyway, in the revised manuscript we also indicate the molecular weight of PEI at step 1.3.1.

2. Mulens-Arias.V et al (2015) reported that Polyethylenimine-coated SPIONs activate macrophage through TLR-4 signaling and ROS production and modulate podosome dynamics. Then, is PEI-SPION for siRNA delivery to macrophage suitable?

**Authors’ response:** We thank the reviewer for pointing out the potential effect of PEI-SPION on macrophage activity. Nanoparticles may induce immune response, depending on their surface modification, surface charge, size, shape and even the methodology used to synthesize them. For instance, according to the paper of Mulens-Arias et al (2015), PMag 25Br-Zonyl FSA nanoparticles (a kind of PEI- SPION) synthesized at 90 °C dose-dependently increased IL-12 secretion when incubated with THP1 macrophages, while those synthesized at room temperature did not. Our method of PEI-SPION synthesis differs significantly from that of Mulens-Arias et al., and therefore whether PEI-SPIONs prepared based on our protocol could trigger macrophage activation awaits further investigation. However, since we previously demonstrated that PEI-SPION-mediated in vivo delivery of IL-2/IL-15R siRNA to macrophages was efficacious for treating experimental arthritis, a disease model in which abnormal activation of macrophages is a key pathological event, our PEI-SPIONs might have a negligible effect on macrophage activation. To address the referee’s concern, in the Discussion of the revised manuscript we advocate incorporation of PEI-SPION (vehicle only) treated cells or animals as a control group.

3. For siRNA binding assay, Fe:siRNA ratio was calculated. This referee suggests the authors provide exact nitrogen:phosphate (N/P) ratio, which is the more accurate information in terms of reproducibility;  
**Authors’ response:** The PEI-SPION we used is not a commercial product. The person who would like to try this protocol has to synthesize the nanoparticle de novo. Even if vehicle:siRNA ratio is given in the form of N/P, pilot experiments for optimizing N/P ratio remain necessary. Due to its simplicity Fe:siRNA weight ratio was used to measure the amount of PEI-SPIONs needed for siRNA binding.

4. The author said that zeta potential of PEI-SPION over +37 mV can be toxic and suggested that PEI-SPION with an average zeta potential not higher than +37 mV be used for siRNA delivery. In additionally, the authors noted that the surface charge and hydrodynamic size of nanoparticles can be controlled within a desired range by adjusting the PEI content. But PEI-SPION was prepared at only WFe: WPEI =1:3 ratio. Is this the actual ratio for siRNA delivery in this manuscript? This referee suggests that the authors mention the zeta potential and the size of this ratio, with and without siRNA loading;  
**Authors’ response:** The WFe to WPEI ratio of 1:3 can serve as a starting point for synthesizing PEI-SPION suitable for siRNA delivery. We found that even for a given WFe to WPEI ratio the size and charge of PEI-SPIONs prepared in independent experiments may vary to some extent. The PEI-SPIONs used in this manuscript were synthesized in separate experiments and the actual WFe to WPEI ratio fluctuated about 1:3, depending on the charge and size wanted. Although the physicochemical properties of PEI-SPIONs in this manuscript differ to certain extent, these particles showed similar siRNA binding ability and transfection efficiency except that highly positively charged particles can be toxic.

At the suggestion of the referee we provided the zeta potential and size of PEI-SPIONs in the revised figure legends. We did not provide the charge and size of the particles loaded with siRNA. We recommend performing gel retardation assay to determine the ratio of PEI-SPION (Fe content) to siRNA because using this assay siRNA binding to PEI-SPIONs can be presented in a straightforward, clear manner. Measurement of charge and size of PEI-SPION/siRNA is optional, as increase in size and decrease in zeta potential upon siRNA binding do not indicate to what extent siRNA is loaded onto PEI-SPIONs.

5. In line 271-272 and 278-279, the authors said that this figure was modified from ref. 17 and the authors are awaiting permission from the publisher. But ref.17 is not related to the present protocol. This referee suggests that the authors double check the references used in the manuscript;  
**Authors’ response:** We thank the referee for pointing out the mistakes. The two mistakes have been corrected.  
  
  
Reviewer #2:  
  
Manuscript Summary:  
With the main objective of using macrophages as target for autoimmune diseases, the authors describe an interesting protocol in with PEI-SPIONs are synthesized for targeted delivery of siRNA to macrophages. In general, the manuscript is clear and copes with the aim of the journal. However, there are some points to be addressed before its acceptance.  
  
Major Concerns:  
  
1. There is a lack of information about the physicalchemistry characteristics of the nanoparticles, mainly data about size and size distribution.  
**Authors’ response:** In the Results section we rephrased the opening sentence and provided the size and charge range of PEI-SPIONs used in this manuscript.

2. Figure legends are not clear. In addition, Fig 2 and 3, need permisión from the Publisher but they correspond to an old reference. Why do not construct these figures with the results obtained by the authors?  
**Authors’ response:**, In the revised figure legends we indicated the size and charge of PEI-SPIONs for each experiment. Fig. 2D and Fig. 3 were modified from our previous publication because these experiments, particularly Fig. 3, are time consuming and expensive.   
  
Minor Concerns:  
References should be revised.

**Authors’ response:** Checked and mistakes corrected.