We would like to thank all reviewers for taking the time to provide some extremely helpful suggestions. We have amended the manuscript accordingly and aimed to fulfil each request or otherwise provided an explanation. Please find responses to each request below:

Reviewers' comments:

Reviewer #1:

The authors introduced an intraocular inflammatory mouse model, Experimental Autoimmune Uveitis (EAU), that has served as a model of autoimmune uveitis in humans as well as a model for autoimmunity in a more general sense. The main phenotype of EAU is the inflammation and infiltrated inflammatory cells in the retina and choroid, which can be graded based on clinical scoring and histological scoring. The authors elaborated the method of EAU induction in terms of animals, reagents for immunization and technical details. The clinical assessment of EAU were monitored using fundoscopy and fluorescein angiography at day 14 and day 21 after immunization as well as the corresponding histological assessment. Animals injected with the solvent Complete Freund's Adjuvant (CFA) was used as the negative control. The manuscript is well-written and the protocol is clear. Here are my comments for further enhancement:

1. In the introduction, author mentioned many examples of immunoregulation drugs that were most related to T cells regulation. The EAU is a T cell (Th1 or Th17) driven disease that should be introduced prior to these drugs. In addition, there is no reference to justify to this model can be used to study age related macular degeneration and diabetic retinopathy.

Author's response: (1) The relevant information pertaining to Th1 and Th17 driven pathologies has now been added.

(2) The reference to AMD has now been removed.

2. In the introduction, on line 9 of the second paragraph, the abbreviation "BET" is duplicated.

Author's response: The text has now been amended

3. In section 7.1, please mention the desirable mouse body weight that would be suitable for EAU induction.

Author's response: All calculations based on an average 20g weight of female C57BL/6J, this information has now been added to the protocol

4. In section 7.2, it is confusing to illustrate the reagent lose in terms of immunizing 1.5X mice.

Author's response: In our lab, we routinely express excess reagent loss as (X the preparation) However, since this might appear confusing to others, we have now expressed the loss as a percentage (50%) as well.

5. In section 7.2, second sub-section, the last sentence should be ended with a full stop. And please start with a capital letter in the third sub-section. Also in the third sub-section, it was

mentioned that a pipette will be used to "whip" the solution. Please elaborate more on this technique. Dose it involve the pipette suction or simply stir the solution?

Author's response: This explanation has now been amended and elaborated on the technique.

6. In section 7.3, the skin and fur on back should be wipe using 70% ethanol before IRBP emulsion injection to prevent potential infection, which may interfere with the EAU induction. Also it is difficult to understand the meaning of "twist the needle head to close the skin".

Author's response: (1) Cleaning the skin after injection when there is a leak does not interfere with the development of EAU in our experience. However, if you leave the leak to dry on the skin there is a chance of developing a skin infection that almost always progresses to lesions development. These lesions are not easy to manage and depending on the animal licence you might have to terminate the experiment. (2) Rotating the needle head to allow the slant of the needle to be closest to the skin) before pulling out is standard procedure advised by our IACUC members and trainers.

7. In section 7.4, last sentence of the second sub-section, the word "through" should be "throughout". And in the fourth sub-section it was stated "the eyepiece should then be orientated to focus on centralising the optic disc in the image". However, the fundus images do not show the optic disc in the centre of the images. Also in this sub-section, "crucially important" is not a proper English expression as these are redundant words.

Author's response: (1) "through" has now been corrected to "throughout".

(2) The instructions to focus the eyepiece in one area have been removed and instead, emphasised the need to capture from multiple positions to ensure an accurate representation of disease along several parts of the retina.

(3) Have removed "crucially important" and re-expressed.

8. In section 7.6, the clinical scoring was based on the criteria of Xu (2008), which is new scoring system with maximum score of 20. There is another commonly used clinical scoring criteria report by Agarwal et al (reference 9 in the manuscript). I would suggest the authors to compare the two scoring systems and explain why they prefer to use the scoring system developed by Xu et. al.

Author's response: The scoring system developed by Xu et al. (2008) offers a greater range of measurement parameters, totalling 20, whereas the Agarwal method is limited to a score of 5. This greater range affords a more sensitive scoring panel for assessment, allowing the user to separate and focus in on potential anatomical differences in response to e.g. treatment. This method is particularly useful for judging disease suppression in translational studies as minor changes/certain aspects of disease alteration can be more easily detected. We have included these points in the discussion.

9. Please also include the histological scoring criteria as it is also an important scoring system for monitoring EAU.

Author's response: The histological scoring criteria has been added to the manuscript.

10. In figure 2, apart from the CFA injected group, the EAU group at the baseline (before IRBP injection) should also be shown as a self-negative control. In histology image, the structural damages in the retina and the infiltrated cells should be indicated. In addition, there is no label of A to F. And the fundus photo of the CFA control is not clear.

Author's response: We have added fundoscopic images and the corresponding histology from a naïve eye. Structural damages in histology have now been marked clearly. Text in the figure legend has been amended and the CFA fundoscopy images now have an improved resolution. The labelling has been changed to A to D.

11. In figure 3, the figure legend is very confusing. Micron III was used to take all the 3 images and not only B and C. But the legend would give an impression that only A was taken by Micron III. Also the abbreviation "p.i," should be explained. In addition, an image from CFA control at 1,5 minutes after fluorescence injection is needed for comparison. Furthermore, images B and C seem are not taken from the same retina as the number of vessels are very different. It is important to show images from the same retina for comparison.

Author's response: (1) The figure legend has been amended for clarity. (2) The abbreviation 'p.i.' has now been replaced with 'post-immunisation'. (3) The angiographic images have been changed and the same retina shown at both timepoints, 1.5 and 7 mins after fluorescein administration.

12. In section 10, the last sentence in first paragraph is not a complete sentence. In the paragraph 3, line 5, reference 13 is not published by Xu et al.

Author's response: (1) The last sentence has now been removed.

(2) The reference has been updated.

13. In the material table, there is a duplication of the "mouse serum".

Author's response: Duplicated "mouse serum" has been removed.

Reviewer #2:

Manuscript Summary:

Experimental autoimmune uveitis (EAU) model in mice is an excellent disease model for studying T cell mediated autoimmunity of central nervous system on a par with experimental autoimmune encephalomyelitis (EAE), without the bad effects of deteriorating body conditions, moribundity, and the demands of continuous monitoring and intensive care of experimental animals. Authors have tried to explain the protocol in detail through carefully

chosen wording to give a mental picture of step by step procedure. However, it is important to clarify certain points regarding this model and the procedure in the manuscript as this article mostly targets researchers who are new to the field of ocular immunology research. To broaden the applicability, it would be good to discuss that there are alternative protocols of disease induction, as the author's protocol is different fro the original publication (and cite appropriate refs).

It is equally important to mention in the 'Discussion' section other (better?) antigenic peptides for the same mouse strain, the different mouse strains (with their specific antigens) and also different models that are available for inducing EAU. The authors mention immunization-induced vs. spontaneous, but omit adoptive transfer, which is a widely used model to represent the effector phase of disease while avoiding the complexities of Complete Freund's Adjuvant.

Author's response: Relevant information pertaining to alternative EAU induction methods and antigenic materials has now been included in the discussion. Specifically, adoptive transfer and other antigenic peptides have been mentioned.

Major Concerns:

1. Section 7.1. Please clarify which sub-strain of C57BL/6 was used here? Please give the Vendor information. All C57BL/6 mice from vendors other than Jackson Lab are 'N' sub-strain that harbors 'rd8' mutation, which could be a confounding factor in the histological scoring of EAU severity. Please refer to Mattapallil et.al. 2012 (PMID: 22447858). C57BL/6J mice would be a better choice for EAU model. Please include this in the discussion. As a routine practice, investigators should genotype any genetically modified strains on C57BL/6 background for 'rd8' mutation before using them in EAU experiments. This will allow an informed decision to use the correct sub-strain as control group or to backcross the animals to 'J' sub-strain to breed out the mutation before using them in EAU experiments.

Author's response: The sub-strain has now been updated throughout the manuscript. C57BL/6 mice routinely used in our lab are C57BL/6J, purchased from Charles River, confirmed by our BSU manager, which were derived from the original colony from The Jackson Laboratory.

2. Section 7.1. Please clarify any age and gender considerations while choosing animals for EAU experiments.

Author's response: Suggestions taken into account, we have discussed the possibility of using males for inducing EAU. However, due to their increased body mass and subcutaneous fat, EAU in male mice of a similar age to females has a lower incidence level. In our experience, the most severe disease response and highest incidence occurs in female adult mice, 6-8 weeks of age. After 10 weeks' of age, the EAU disease incidence and severity decreases, despite adjusting the IRBP peptide levels to the increased body weight.

3. Section 7.2. Calculation for emulsion preparation - loss of 1.5 X when emulsion is prepared for a large number of animals (>15 mice) is an overestimation and could be a waste of antigen and other reagents. As a rule of thumb, preparing extra 10-20% would be better instead of preparing 1.5 X calculated by number of mice to be immunized.

Author's response: In our experience, 10-20% loss is not enough to confidently ensure all mice in the cohort will be immunised. This is especially true when preparing a viscous emulsion, such as the aim here. Often the emulsion is so thick the plunger will detach from the syringe, if this happens, what remains counts as loss. There is also loss in the syringe barrel which has to be changed every 2-3 mice.

4. Section 7.2.1. Selection of IRBP peptide for C57BL/6 strain - recently reported peptide 651-670 may be a better peptide for this strain, both in terms of lower quantity required to immunize, less Pertussis toxin, and in inducing a more severe EAU than peptide 1-20 (Mattapallil et.al. 2015 PMID: 26284549). That said, results may vary for different labs. Please include this in the Discussion.

Author's response: Unfortunately, multiple groups across our institute tried the most recently published antigenic peptide (651-670) and were unsuccessful in obtaining EAU, despite maintaining the same CFA, pertussis etc. These differences may be due to the source of mice, BSU facility practices and influences on the microbiome.

5. Section 7.3.2. Most investigators distribute the emulsion subcutaneously into several sites, so as to stimulate more lymph nodes and induce a stronger immune response. The authors chose to inject entire volume of 200µl into a single site. Was this procedure found better than distributing the antigen to several sites? Was this change made due to IACUC restrictions?

Author's comments: If the intended outcome is to examine lymph nodes then we have suggested both sites of the flank (100ul) could be injected. However, if the aim is solely EAU induction and study of the retina then a single bolus into the skin at the back of the neck is sufficient for disease to develop. Our best practice is to avoid multiple injection sites where necessary to reduce any discomfort to the mice and because, in some instances, emulsion can leak out which might lead to the development of skin lesions.

6. Section 7.3.2. Site of deposition of emulsion is mentioned as 'Flank' region in the abstract and as 'on the back' in section 7.3.2. Please clarify the exact location and position, keeping in mind that different locations along the trunk can drain to different lymph nodes.

Author's response: We have clarified and added more information regarding the injection sites.

7. Section 7.4.1. Domitor (Medetomidine) is known to be anti-inflammatory and hence would not be ideal for an inflammatory disease model that induces an immune response. Was this suggested by the IACUC? For systemic anesthesia it would be preferable to use Ketamine in combination with Xylazine to avoid the anti-inflammatory effect of anesthetic. Please clarify why Ketamine-Domitor was preferred over Ketamine-Xylazine.

Author's response: We have three main reasons for choosing Domitor (medetomidine) over Xylezine (1) Domitor provides a better sedation and analgesic and longer anaesthetic effect than xylazine[1], which is necessary when performing angiography and treatment such as intravitreal administration simultaneously (2) We were advised by our vet that Domitor and Xylazine belong to the same family of drugs "Alpha-2- adrenergic agonist tranquillizers" and have similar effects, however, Domitor is more specific and reported to have fewer adverse effects than Xylazine. (3) We achieve a high disease incidence and severity in our model system – no evidence of anti-inflammatory effects or disease suppression.

1 Taylor, P. Veterinary anaesthesia and analgesia: from chloroform to designer drugs. *Vet Rec* **174**, 318-321, doi:10.1136/vr.g2249 (2014).

8. Section 7.4.4. Fundoscopic images from Micron III are of poor quality and it is difficult to appreciate the infiltration of cells and inflammation even at day 21. It would be important to provide better quality images corresponding to various disease scores as per the scoring system explained in section 7.6 and Table 1.

Author's response: The fundoscopic image for day 21 has now been changed.

9. Histological sections: Since the criteria for scoring disease severity include histological lesions (structural damage), please explain how to collect eyes for histological sections and the fixative(s) used for processing.

Author's response: The information regarding sample preparation for histology, along with a conventional scoring system, have been added to the manuscript.

10. Discussion: Please include details of expected lesions or reaction at the site of injection and care if needed, the possibility of adjuvant-induced arthritis developing and its associated pain and distress issues, and any contra-indicated medications (any anti-inflammatory agents). Please include information about alternative mouse strains and antigens (whole protein or peptides) that can be used for inducing the EAU model.

Author's response: (1) We have included information relating to adverse skin reactions/lesion formation as a cautionary note in the methods section, including general advice on monitoring. We have avoided giving more specific instructions as this should be detailed within individual project licences. (2) In our EAU model system we have not experienced adjuvant-induced arthritis. (3) Alternative mouse strains and antigens have now been included in the discussion.

Minor Concerns:

1. Please check whether the correct references are cited in the text e.g., reference 13 is not Xu. et.al. ('Discussion' last paragraph) and reference 12 is on C57BL/6 strain and not on B10.RIII strain as cited in the text ('Discussion' first paragraph).
Author's response: The references have now been amended

2. Please check for spelling mistakes and repetitions in the list of 'Materials' on last page. Correct spelling is Tropicamide ('a' not 'o') and Domitor (no 'r' following 'o'). Mouse serum is repeated twice. Please include Mouse strain and vendor with stock number.
Author's response: The spelling errors have been corrected.

Reviewer #3:

This is a fairly straightforward synopsis of the most common mouse EAU model. There is nothing new in the paper except that it is perhaps more step by step methodological paper than many of the others available. I could see little wrong in the description of the method and their warnings about ensuring correct mixing of the adjuvant and its correct injection are critical. There are some minor comments below:

Intro

1. "This EAU model recapitulates central features of human disease with regards to clinicopathologic characteristics and the basic cellular and molecular mechanisms that drive uveitis"

Rev: they really should say posterior uveitis or retinochoroiditis as 'uveitis' includes anterior uveitis which as the authors know has a different pathogenesis.

Author's response: This is an important point which has now been clarified in the text.

2. "The primary readouts for EAU preclinical studies are: clinical assessment performing fundoscopy, histopathological evaluation and immunophenotyping of retinal cells. Fundoscopy is an easy-to-use live imaging system that allows for rapid and reproducible clinical assessment of the whole retina."

Rev: I am surprised the authors are not emphasizing the use of OCT and 'Spectralis'_type imaging/Micron imaging. Correction I indeed see later they do talk of Micron imaging

Author's response: We have now mentioned OCT in the discussion to inform the reader that this can be used as an additional readout for this model, although it is not described in our manuscript.

3. "This model is reliable and will generate complementary data to be used alongside intraocular inflammatory diseases such as age related macular degeneration (AMD) and diabetic retinopathy. The posterior retinal diseases that are, at present, leading causes of blindness worldwide7."

Rev: This is a big claim and an attempt to throw a big inclusive blanket over the AMD field which really is not necessary here - EAU is a model disease in young mice - ie no age and no macula - so to say it will aid AMD understanding is stretching a very long bow.

Author's response: This part has been removed from manuscript.