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Title:
Experimental Autoimmune Uveitis: An intraocular inflammatory mouse model

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Experimental Autoimmune Uveitis, C57BL/6J, Inflammatory Eye Disease, Immunisation, Fundoscopy, Angiography.

Summary:
Here, we present a protocol that allows the investigator to generate a mouse model of intraocular uveitis. More commonly referred to as experimental autoimmune uveitis (EAU), this established model captures many aspects of human disease. Herein, we will describe how to induce and monitor disease progression using several readouts.

Abstract:
Experimental Autoimmune Uveitis (EAU) is driven by immune responses to self-antigens. Many features of this non-infectious, intraocular inflammatory disease model recapitulate the clinical phenotype. EAU has been used reliably to study the efficacy of novel inflammatory therapeutics, their mechanism of action and to further investigate the mechanisms that underpin disease progression of intraocular disorders. Here, we provide a detailed protocol on EAU induction in the mouse – the most widely used model organism with high susceptibility to this disease. Clinical assessment of disease severity and progression will be monitored using fundoscopy, histological examination and fluorescein angiography. The induction procedure involves *subcutaneous* injection of an emulsion prepared from interphotoreceptor retinoid binding protein ($IRBP_{1-20}$), Complete Freund's Adjuvant (CFA) and supplemented with *Mycobacterium tuberculosis.* Injection of this viscous emulsion on the back of the neck is followed by a single intraperitoneal injection of pertussis. At the onset of symptoms (day 12-14) and under general anaesthesia, fundoscopic images are taken to assess disease progression through clinical examination. These data can be directly compared with those at a later timepoint and peak disease (day 20-22) with differences analysed. At the same time, this protocol allows the investigator to assess potential differences in vessel permeability and damages using fluorescein angiography. EAU can be induced in both wildtype mice or genetically modified and combined with novel therapies offering flexibility for studying drug efficacy and/or disease mechanisms.

Introduction

This protocol will demonstrate how to induce Experimental Autoimmune Uveitis (EAU) in the C57BL/6J mouse by a single subcutaneous injection of emulsified retinal antigen. Methods for monitoring and assessing disease progression will be detailed through fundoscopic imaging and histological examination, with measurement parameters outlined within. In addition, fluorescein angiography, a technique for examining the retina and blood vessels will be discussed.

This EAU model recapitulates central features of non-infectious posterior uveitis in human disease with regards to clinicopathologic characteristics and the basic cellular and molecular mechanisms that drive posterior uveitis. This disease is mediated by Th1 and/or Th17 subsets of self-reactive $CD4^+$T lymphocytes, as shown in adoptive transfer experiments and with IFNγ-depleted mice[1]. Much of the understanding of these cells in uveitis comes from studying EAU[2] where both Th1 and Th17 cells are detected within the retinal tissues[3]. Often, EAU is used as a preclinical model to assess the responsiveness of disease to novel therapies for efficacy testing. Therapeutic approaches that have successfully modulated EAU disease have often shown efficacy in the clinic and reached FDA approved status. Examples of these are groups of immunoregulatory drugs such as T cell–targeting therapies: cyclosporine, FK-506, rapamycin[4-6]. Therapies currently in development have used this model to explore a number of avenues for targeting; transcriptional regulation, chromatin readers applying Bromodomain Extra-Terminal (BET) proteins and P-TEFb inhibitors – which recently demonstrated suppression in EAU via modulation of effector Th17 $CD4^+$ cells[3]. In addition to these, targeting Th17 through the transcription factor, applying TMP778, a RORγt inverse agonist, has been found to significantly suppress EAU[7]. Furthermore, this model offers an opportunity to study chronic autoimmune inflammation and the accompanying underlying mechanisms such as lymphocyte priming.

The primary readouts for EAU preclinical studies are clinical assessment by performing retinal fundoscopy, Optical Coherence Tomography (OCT), histopathological evaluation and immunophenotyping of retinal cells by flow cytometry. Fundoscopy is an easy-to-use live imaging system that allows for rapid and reproducible clinical assessment of the whole retina. For immunohistochemical assessments, the techniques are based on preparation of retinal sections that allow us to study tissue architecture for degrees of inflammation and structural damage[8]. The assessment criteria and conventional scoring systems, for all techniques used, will be outlined within this protocol. The extent of damage recorded using fundoscopic imaging often closely correlates with histological changes. This dual approach to monitoring and assessing disease severity affords greater sensitivity and more reliable measurement outcomes.

EAU is a well-established, commonly used model for preclinical testing and investigation of immune-mediated eye disease. This model is reliable and will generate comprehensive data to be used for intraocular inflammatory diseases which leading causes of blindness worldwide[9].

Protocol:

All experiments were performed in accordance to the UK Animals (Scientific Procedures) Act of 1986, and institutional Animal Welfare and Ethical Review Body (AWERB) guidelines.

1. Mouse Preparation

1.1. Perform all experiments on adult, female C57BL/6J mice aged between 6-8 weeks old provided with food and water *ad libitum*.

2. $IRBP_{1-20}$ – CFA Emulsion Preparation

2.1. As emulsion preparation is essential to the reproducibility and incidence of disease, maintain consistency throughout the preparation process and across experiments.

2.2. When preparing the emulsion, note that there will be loss of the antigen emulsion that should be accounted for in the calculations of all reagent quantities beforehand. This loss can be approximately equivalent to 1.5x (or 50% extra of the volume prepared), based on the number of mice prepared for immunisation.

NOTE: To immunize 10 mice (10 x 1.5 = 15 mice), prepare 6.0 mg of peptide (100 µg peptide per 20 g mouse x 15 mice). Each mouse should receive 200 µL for immunization (prepare 3 mL total). The final volume comprises a 1:1 ratio of peptide solution and CFA, hence, 1.5 mL of peptide solution + 1.5 mL of CFA.

2.3. Prepare the following sterile solutions in a laminar flow cabinet.

2.4. Weigh out human $IRBP_{1-20}$ (LAQGAYRTAVDLESLASQLT) stored in a lyophilized form at -20 °C according to the desired amount (400 µg per 20 g mouse) and resuspend in a minimum volume in 100% DMSO to completely dissolve the powder.

2.4.1. To ensure the powder is fully dissolved, ensure that each flake comes into contact with the DMSO first. Then, add PBS in small portions, incrementally, to reach the final volume. Do not mix with a vortex; instead, use gentle agitation.

NOTE: The final concentration of DMSO should not exceed 1% of the total peptide preparation.

2.5. Add DMSO-PBS peptide solution at 1:1 v/v to Complete Freund's adjuvant which has been already supplemented with 1.5 mg/mL *Mycobacterium tuberculosis,* to give a final concentration of 2.5 mg/mL. Perform this dropwise, pipetting gently and frequently to form a viscous and evenly distributed emulsion.

2.6. Aerate the two solutions; use a 1000 µL pipette (set to 700 µL to prevent further loss) and pipette the solution to make a creamy thick substance. This technique involves using the pipette suction, up and down and repeated until reaching the desired consistency. For optimal results, ensure that the antigen solution and adjuvant are mixed thoroughly, under sterile conditions before injecting.

NOTE: Preparing the emulsion in a 20 mL plastic container with a tapered bottom should allow better accessibility of DMSO to the lypophilized powder.

2.7. In addition to the emulsion preparation, give each mouse a single i.p. injection of 1.5 µg of Pertussis toxin suspended in 100 µL of RPMI 1640 media and supplemented with 1% mouse serum.

NOTE: This injection should be performed before injecting the antigen, in order to avoid disturbances at the s.c. injection site.

3. Subcutaneous injection of IRBP emulsion and Intraperitoneal injection of Pertussis toxin

3.1. First, temporarily transfer each mouse to a separate cage to receive a single 100 µL i.p. injection of Pertussis toxin. Perform the injection with a sterile syringe and 23 G needle.

3.2. Next, inject the IRBP emulsion subcutaneously; a process that requires two animal handlers.

3.2.1. Have one trained personnel lightly restrain the mouse on top of the cage in a scruff-like position, with their stomach facing downwards whilst the other trained personnel pinches the skin to form a tent-like structure on the back of the neck where the needle can be threaded to slot between the finger and thumb.

3.2.2. Once the needle is positioned, inject 200 µL and apply pressure afterwards to prevent the emulsion from spilling out onto the skin. When removing the needle, rotate the needle head to close the skin before pulling out.

CAUTION: The emulsion must not make contact with the skin or fur as this may cause irritation and in more severe cases, a lesion to develop. If this occurs, the area must be wiped immediately and thoroughly using 70% ethanol then dried.

NOTE: If the draining lymph nodes are needed for examination at the end of the study, the injection site will be different. In this instance, inject 100 µL to both sides of the flank subcutaneously. This will generate a stronger response by the draining inguinal lymph nodes, which can be excised at the time of harvesting. However, if the intended outcome is solely to develop EAU, a single injection of 200 µL at the back of the neck is preferable to avoid discomfort from multiple injections.

4. Clinical Evaluation - Mouse Fundus Examination

NOTE: Clinical disease is to be scored using fundus examination, via bright-field live imaging using a fundoscope and Discover software used for visualisation.

4.1. At disease onset (day 12-14), sedate mice under general anaesthesia using a combination of both Ketamine (50 mg/mL) and Domitor (Medetomidine; 1 mg/mL). Dilute 1-part Domitor; 1.5 parts Ketamine and 2.5 parts sterile injectable water, then inject 100 µL per 30 g intraperitoneally. Use 1 mL sterile syringes and 23G needles for the above combination of anaesthesia.

4.1.1. Following this, monitor the mouse to ensure that all reflexes are lost and that it is unresponsive to stimuli.

4.2. Immediately after receiving the i.p. injection and whilst the mouse is still held in a scruff, apply 1% tropicamide and 2.5% phenylephrine topically to each eye pupil dilation. Aim to completely cover the cornea with both dilating solutions. It may take a few minutes before the pupil is fully dilated.

4.2.1. Afterwards, generously apply eye viscotears ointment and maintain throughout the imaging process in order to keep the eye fully lubricated.

4.3. In the meantime, open the software (e.g., Discover), and set the fundoscope (e.g., Micron III) to capture images with brightfield. Allocate each individual mouse a folder and label images with R or L according to each eye photographed.

4.4. Mount the mouse on a purpose-built stage for live visualization and position the microscope for full access to the retina.

4.4.1. To get an accurate representation of the disease, take images of the entire retinal area, covering all corners of the periphery in addition to the optic disc. To achieve this, adjust the eyepiece throughout. It is crucial for the eye to remain fully lubricated at all times throughout the imaging process; ensure this by topping up the eye ointment at a constant rate.

4.5. Refer to section 5 at this stage to perform fluorescent angiography.

4.6. Once all imaging is complete, dilute anaesthetic reversal anti-sedation (5 mg/mL Antisedan) in injectable water and administer at 0.1 mg/kg i.p. Return the mouse to a cage and place on a pre-heated mat with access to a wet-soaked diet until recovery. Complete recovery is characterized by whole body movement and walking around the cage with steady gait.

4.7. At the experimental endpoint (day 21-23), repeat steps 4.1–4.5 and take photographs of the entire retinal area again, covering the optic disc and all corners of the periphery to capture an accurate representation of disease.

5. Fluorescent angiography

5.1. To measure vessel leakage in these animals, whilst under anaesthesia, give each mice an injection of 2% fluorescein subcutaneously at the back of the neck and position such that the retina is centralized in the middle of the live image.

5.2. Set the fundoscope to a blue light excitation filter at 465-490 nm. The light captured from excited fluorescein is between 520-530 nm.

5.3. After 1.5 min, take a photograph per eye and repeat again at 7 minutes.

6. Clinical Disease Scoring

6.1. Base the clinical assessment on the severity of the following criteria: optic disc inflammation, retinal vessel cuffing, retinal tissue infiltrate and structural damage.

6.2. Award each of these parameters a score on a scale from 0 to 5 and the collective total is representative of clinical disease for the whole eye, with a maximum score of 20 obtainable per eye. **Table 1** can be used as a guide for scoring criteria.

7. Histology and Histological Scoring

7.1. Perform histological examination of eyes using standard protocols for Hematoxylin and Eosin (H&E) staining.

7.2. To enucleate the eyes, prise the eyelids apart for easy access to the entire eye.

7.3. Next, place curved forceps behind the globe with the intention of grasping the orbital connective tissue and optic nerve. Take care to avoid squeezing the globe.

7.4. For fixation, place the eye in 4% glutaraldehyde for a minimum of 15 minutes and then transfer to 10% formaldehyde for at least 24 h. 1-2 mL of fixative would give enough volume to cover two eyes.

7.5. Perform embedding in paraffin, sectioning on a microtome and staining according to the standard protocols. 3-4 µm section thickness is recommended for any type of staining.

7.6. Alternatively, assign scores on a scale of 0–4, according to the criteria for EAU scoring, based on the level of the immune cell infiltration and the degree of retinal damage, as previously described (Agarwal 2013) and summarized in **Table 3**[10].

Representative Results

Herein this protocol, we describe the induction methods for a model of experimental autoimmune uveitis by challenging mice with the retinal antigen IRBP. The model has provided valuable understanding of human non-infectious posterior uveitis and can be diagnosed using the same fundoscopic techniques. The first signs of EAU in C57BL/6J mice can be detected two weeks post-immunization and peak disease reached within three weeks as illustrated in **Figure 1**. Fundoscopic changes are classified during disease progression as inflammatory changes, which include retinal tissue, vascular and optic disc inflammation, retinal structural damage (**Figure 2**) in addition to histological changes based on infiltrating immune cells and structural impairments. These clinical and histo-pathological changes can be graded and scored for evaluation proposes to study disease progression and also in therapeutic intervention studies.

We are showing herein how the clinical and histological scoring systems (**Table 1** and **Table 2**) guide scientists to validate the efficacy of treatments and exploring the mechanism of drug action. Vascular leakage is also a pathological feature of the model and in human uveitis. We are showing examples of vascular leakage of fluorescein with focal leakages (**Figure 3**) as another readout of using this model.

Figure 1. Schematic timeline of clinical and histological disease progression in IRBP$_{1\text{-}20}$ induced EAU. A timeline outlining the important cellular events throughout

the progression of $IRBP_{1-20}$ induced EAU. From immunisation, the first signs of clinical disease, as detected by fundoscopic imaging and histopathological analysis, falls between days 12-14. The disease will then continue to progress, according to these parameters, until a peak is reached around day 21-23.

Figure 2. Representative fundoscopic images correlating with histological sections at different stages of $IRBP_{1-20}$ induced EAU disease in C57BL/6J mice. Clinical fundoscopic and corresponding tissue images of C57BL/6J immunised with $IRBP_{1-20}$ peptide. (A and B) fundoscopic images and histological sections of eye obtained from healthy and CFA injected mice. Retina has no sign of inflammation and corresponding histology sections show a preserved retinal layers. (C) Fundoscopic image of eye obtained from C57BL/6J mouse 14 days post immunization demonstrate classic signs of EAU, presenting with severe optic disc swelling in the early stage of disease, corresponding histology shows infiltrating immune cells into vitreous space. (D) Fundoscopic images of eye obtained from C57BL/6J mouse 21 days post immunization with inflamed vessels, retinal tissues and infiltrating immune cells throughout the retinal layers, retinal folding and structural damages observed in the corresponding histology.
V= vessel, O= optic disk, R=retina, L=lens, Vit=vitreous
iO= inflamed optic disk, iV= inflamed vessel, iR= inflamed retina, i= infiltrating cells in vitreous, RFs=retinal folds.

Figure 3. Representative images of fluorescent angiography taken using Micron III imaging system at peak disease. C57BL/6J mice were injected subcutaneously with 2% fluorescein and images taken at various timepoints after circulation of the tracer. (A) CFA only control mouse taken at 1.5 and 7 minutes post fluorescein administration. (B) Representative images of $IRBP_{1-20}$ immunized mice taken 1.5 and 7 minutes, respectively, after receiving fluorescein. White arrow indicates vessel leakage.

Table 1. Conventional clinical scoring scale for evaluating EAU clinical disease severity. Table showing criteria used to evaluate the extent of disease severity in mice immunised with $IRBP_{1-20.}$ Scores were allocated according to the hallmarks outlined above being visible on the fundus images, each eye was given a total score out of twenty. Table adopted with permission from Xu H., et al., 2008[7].

Table 2. Histologically scoring EAU
Table showing criteria used to evaluate the severity of EAU based on histopathological features of disease. Scores were allocated according to the hallmarks outlined above on H&E staining, each eye was given a total score out of four. Table adopted with permission from Agarwal et al. 2013[10].

Discussion

Experimental animal models are necessary tools for studying disease pathogenesis and preclinical testing of novel therapeutic paradigms. In the current protocol, we have discussed practical details for the experimental model of intraocular inflammatory uveitis (EAU). The main features of EAU in animals are retinal and/or choroidal inflammation, retinal vasculitis, photoreceptor destruction and loss of vision, all of which represent many essential clinicopathological features of human posterior uveitis[11]. Much of the understanding on the basic cellular and molecular mechanisms

involved in uveitis has derived from the induced EAU model as described herein. EAU can be induced in mice[12] and rats[10] by active immunization with retinal antigens that are recognized by lymphocytes. These retinal antigens take many forms; IRBP (for mice) or retinal soluble antigen (S-Ag) for rats. Inducing EAU on a C57BL/6J background generates a more chronic form of the disease, with peak pathology observed three weeks post-immunisation. By comparison, applying retinal antigen to a B10RIII background[13] induces an acute-monophasic EAU where peak pathology typically presents within two weeks of induction.

Different IRBP epitopes have been tested in C57BL/6J mice and $IRBP_{1-20}$ peptide has proven to be a reproducible model with a high incidence and severity levels. Recently a new uveitogenic epitope of IRBP, amino acid residues 651 to 670 of human IRBP have been reported to induce EAU with a higher clinical incidence and severe disease manifestation[10]. However, unpublished data from the laboratory showed no signs of disease development. Indeed, the impact of gut commensal microbiota and the activation of autoreactive T cell receptors (TCRs) is known to interfere with the onset[14]. Therefore, for beginners in this field, we advise both $hIRBP_{1-20}$ and $hIRBP_{651-670}$ peptides to be tested and titrated (300-500 µg) in order to achieve a reliable model in their own system.

There are a number of other models such as spontaneous uveitis that progresses in IRBP T cell receptor (TCR) transgenic (R161H) mice and spontaneously develops ocular inflammation by 5–6 weeks of age[15]. EAU can also be adoptively induced by transferring uveitogenic effector cells. Activated, IRBP-specific $CD4^+$T cells derived from primed mice can be used as a source of effector cells[3,10]. This model represents the effector phase of the disease while avoiding the complexities of using CFA in the inducible model.

Further to this, there are many advantages to using ocular inflammatory models as appropriate tools to investigate other inflammatory diseases, in particular, those with effector Th1 and Th17 subset pathology. The main advantages to using this model are the relatively simple methods to monitor disease development and progression, which are fundoscopy and angiography. These non-invasive imaging systems allow easy access to neuronal tissues, that would otherwise be concealed behind protective anatomical barriers. An additional method for monitoring disease progression involves the application of OCT imaging which is more sensitive than fundoscopic imaging in detecting cellular infiltrates, especially at the early stage of EAU onset. The technique allows multi-layer cross- and horizontal-sectional visualizations of retinal lesions longitudinally and in a non-invasive fashion. In vivo OCT imaging adds information that could not be obtained by fundoscopic and histological examinations such as retinal thickness[16]. Furthermore, the ability to dissect and isolate resident and infiltrating cell populations for deeper analysis of immunophenotypes using flow cytometry methods affords great opportunities for study.

There are a few established scoring systems based on clinical criteria obtained from fundoscopy[7,8,17]. Whilst these differ slightly between ophthalmology research centers, all are reliable and capable of accurately reflecting the severity of disease and correlate with histopathological features. In the current study, we refer to the scoring system developed by Xu et al.[7]. This system offers a more detailed assessment approach with greater numbers of clinical measurement parameters. It comprises a

maximum score of 20 which introduces a wider window for scoring than alternative systems limited to a maximum of 5. This is more important for further exploration within therapeutic approaches. EAU model has over 95% disease incidence when all procedures are performed according to the protocol outlined herein, and results in the development of chronic, monophasic EAU. The most critical parts of the preparation are antigen preparation and injection of the emulsion, both of which have been detailed in adequate amounts throughout.

Gender consideration in inducing autoimmune models has been discussed and connected to the cytokine milieu elicited by the physiological state of the individual[10]. However, we did not see any significant difference in the pilot studies. The issue for planning an experiment would be considering that males at the same age have higher body mass than females and need more reagents and antigen for inducing the disease. We also studied age-dependency of susceptibility in B10RIII mice and concluded that mice over 8 weeks of life have a lower incidence of EAU.

Animal models of intraocular disease have provided an invaluable tool to study human uveitis and aided in the development of experimental therapies. However, no animal model by itself reproduces the full spectrum of human uveitis, each has unique characteristics that make it suitable for studying particular aspects of the disease. This EAU model is induced by autoimmunity through the application of IRBP peptide supplemented with adjuvants (Mtb and pertussis) which trigger innate immune responses. However, we don't know if all idiopathic uveitis in humans are autoimmune and if antigenic mimicry is a triggering factor. In addition, we are still not sure about the involvement of any infections in triggering human uveitis.

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Disclosures:
The authors have no conflicts of interest to declare with this work.

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Figure 1

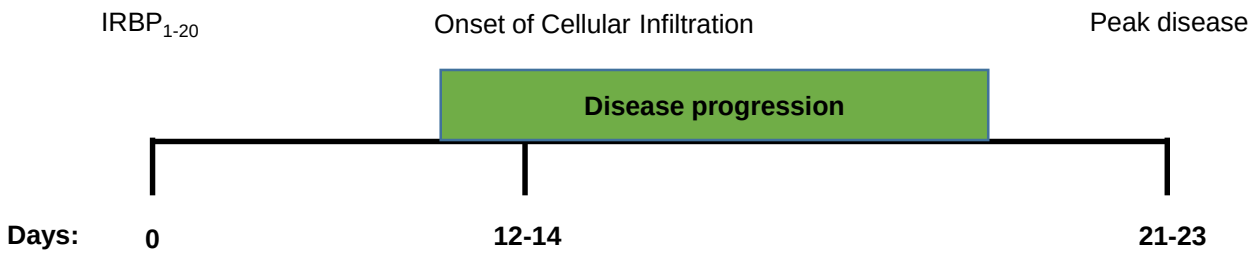
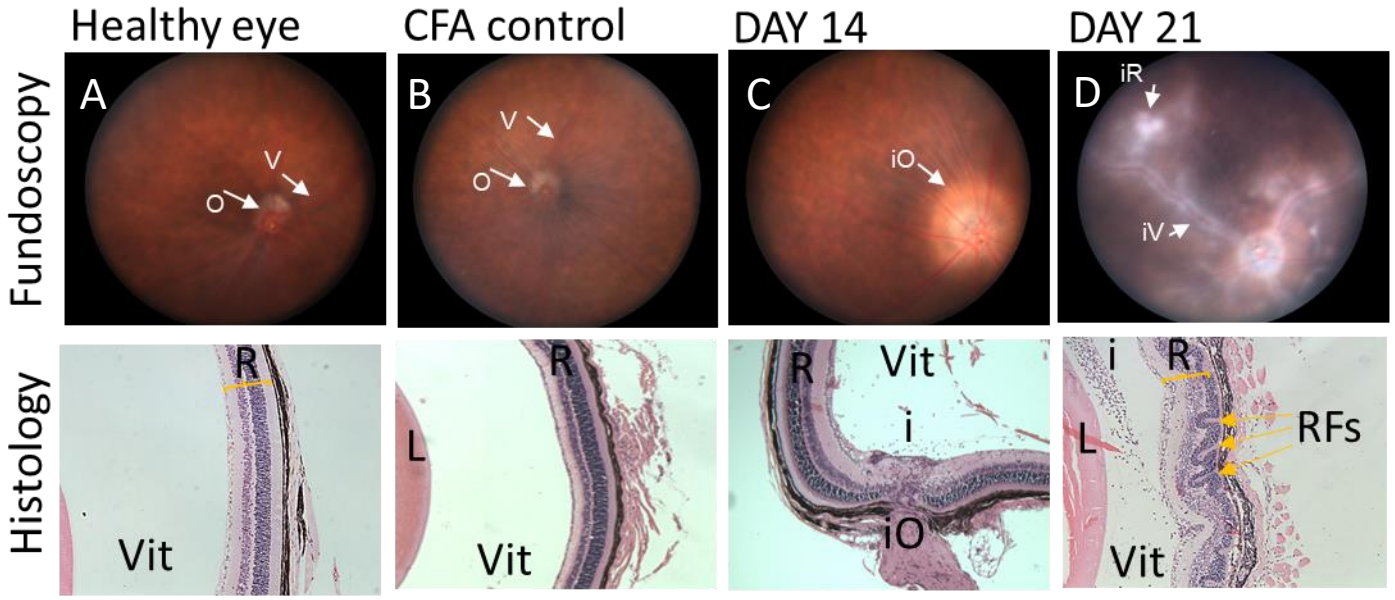


Figure 1. Schematic timeline of clinical and histological disease progression in $IRBP_{1-20}$ induced EAU. A timeline outlining the important cellular events throughout the progression of $IRBP_{1-20}$ induced EAU. From immunisation, the first signs of clinical disease, as detected by fundoscopic imaging and histopathological analysis, falls between days 12-14. The disease will then continue to progress, according to these parameters, until a peak is reached around day 21-23.

Figure 2



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V= vessel, O= optic disk, R=retina, L=lens, Vit=vitreous
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Figure 3

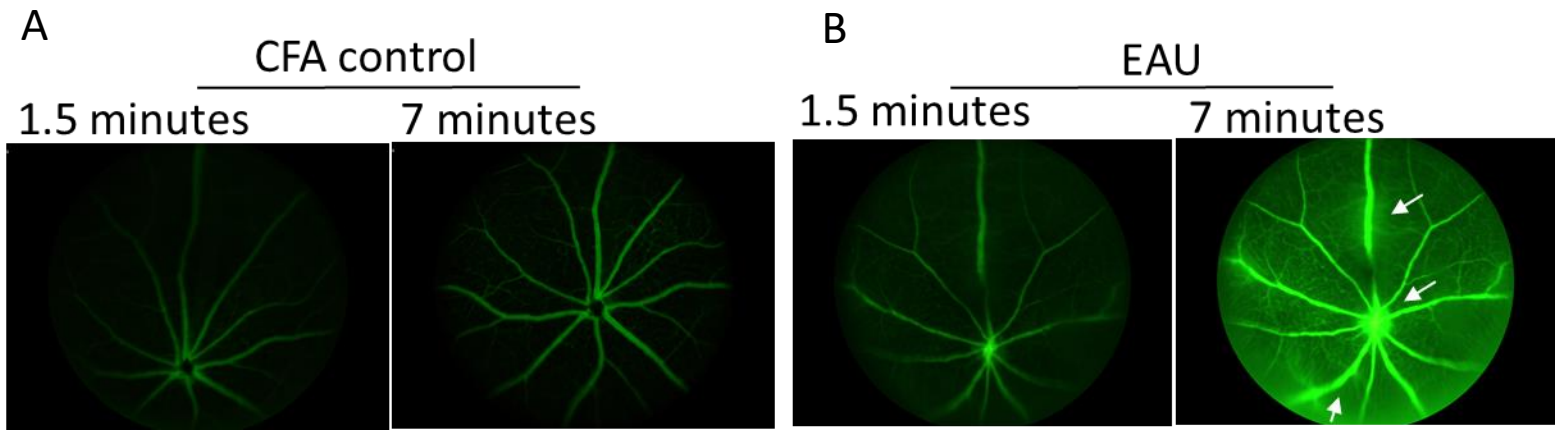

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Table 1

Score	Optic Disc	Retinal Vessels	Retinal Tissue Infiltrate
1	Minimal Inflammation	1-4 mild cuffings	1-4 small lesions or 1 linear lesion
2	Mild inflammation	>4 mild cuffings or 1-3 moderate cuffing	5-10 small lesions or 2-3 linear lesions
3	Moderate inflammation	>3 moderate cuffings	>10 small lesions or >3 linear lesions
4	Severe inflammation	>1 severe cuffing	Linear lesion confluent
5	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)	Not visible (white out or extreme detachment)

Structural Damage
Retinal lesions or retinal atrophy involving ¼ to ¾ retina area
Pan retinal atrophy with multiple small lesions (scars) or <3 linear lesions (scars)
Pan retinal atrophy with >3 linear lesions or confluent lesions (scars)
Retinal detachment with folding
Not visible

Table 2

Grade	Criteria
0	No change
0.5 (trace)	Mild inflammatory cell infiltration. No tissue damage
1	Infiltration; retinal folds and focal retinal detachments; few small granulomas in choroid and retina, perivasculitis
2	Moderate infiltration; retinal folds, detachments and focal photoreceptor cell damage; small to medium sized granulomas, perivasculitis and vasculitis
3	Medium to heavy infiltration; extensive retinal folding with detachments, moderate photoreceptor cell damage; medium sized granulomatous lesions; subretinal neovascularization

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
antisedan	ZOETIS, USA		for waking up
Complete Freund's Adjuvant; CFA	Sigma, UK	F5881	for immunisation
Domitor	Orion Pharma, Finland		for anesthesia
Flourescein	Sigma, UK	F2456	for Angiography
$IRBP_{1-20}$	Chamberidge peptide, UK		peptide;antigen
Ketamine	Orion Pharma, Finland		for anesthesia
Micron III	Phoenix Research, USA		for fundoscopy
Mouse Serum	Sigma, UK	M5905	for immunisation
Mycobacterium terberculosis	Sigma, UK	344289	for immunisation
Pertussis Toxin	Sigma, UK	P2980	for immunisation
phenylephrine hydrochloride 2.5%	Bausch & Lomb UK	PHEN25	for dilation
Tropicamide 1%	SANDOZ		for dilation
Viscotears	WELDRICKS Pharmacy, UK	2082642	for eye lubrication

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.

UCL INSTITUTE OF OPHTHALMOLOGY

14 September 2020

The Editor,
The Journal of Visualized Experiments

Re: Manuscript JoVE61832

Dear Editor,

We would like to thank the reviewers for their comments and critiques regarding our manuscript "**Experimental Autoimmune Uveitis: An intraocular inflammatory mouse model"**
We have given considerable considerations to all the reviewers' comments and have addressed the points raised by them. Accordingly, the paper has been revised in response to these comments.

We hope that with these responses to the queries, and the revisions that have been incorporated, our paper will now be considered for publication. We are grateful for the time and efforts of the editorial team and reviewers to critique and improve our work.

I look forward to hearing from you.

Yours sincerely,

M. EskanCo

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