In vivo multi-modal imaging of experimental autoimmune uveoretinitis in transgenic reporter mice reveals the dynamic nature of inflammatory changes during disease progression

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Abstract

Background: Experimental autoimmune uveoretinitis (EAU) is a widely used experimental animal model of human endogenous posterior uveoretinitis. In the present study, we performed in vivo imaging of the retina in transgenic reporter mice to investigate dynamic changes in exogenous inflammatory cells and endogenous immune cells during the disease process.

Methods: Transgenic mice (C57Bl/6 J Cx3cr1GFP/+, C57Bl/6 N CD11c-eYFP, and C57Bl/6 J LysM-eGFP) were used to visualize the dynamic changes of myeloid-derived cells, putative dendritic cells and neutrophils during EAU. Transgenic mice were monitored with multi-modal fundus imaging camera over five time points following disease induction with the retinal auto-antigen, interphotoreceptor retinoid binding protein (IRBP1–20). Disease severity was quantified with both clinical and histopathological grading.

Results: In the normal C57Bl/6 J Cx3cr1GFP+ mouse Cx3cr1-expressing microglia were evenly distributed in the retina. In C57Bl/6 N CD11c-eYFP mice clusters of CD11c-expressing cells were noted in the retina and in C57Bl/6 J LysM-eGFP mice very low numbers of LysM-expressing neutrophils were observed in the fundus. Following immunization with IRBP1–20, fundus examination revealed accumulations of Cx3cr1-GFP+ myeloid cells, CD11c-eYFP+ cells and LysM-eGFP+ myelomonocytic cells around the optic nerve head and along retinal vessels as early as day 14 post-immunization. CD11c-eYFP+ cells appear to resolve marginally earlier (day 21 post-immunization) than Cx3cr1-GFP+ and LysM-eGFP+ cells. The clinical grading of EAU in transgenic mice correlated closely with histopathological grading.

Conclusions: These results illustrate that in vivo fundus imaging of transgenic reporter mice allows direct visualization of various exogenously and endogenously derived leukocyte types during EAU progression. This approach acts as a valuable adjunct to other methods of studying the clinical course of EAU.

Keywords: Experimental autoimmune uveoretinitis, Reporter mice, Clinical imaging, Retinal inflammation, Microglia, Neutrophils, Dendritic cells

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**Introduction**

Uveitis is the fourth leading cause of blindness in the working age population in developed countries [1,2]. Endogenous posterior uveoretinitis makes up 22% of uveitis cases [3]. The aetiology of non-infectious uveoretinitis is unknown in most cases and has been considered to have an autoimmune basis [4,5]. Well-established animal models of experimental autoimmune uveoretinitis have provided a valuable experimental platform for improving our understanding of the disease pathogenesis and mechanisms of autoimmune uveoretinitis [4-8].

Experimental autoimmune uveoretinitis (EAU) is an organ-specific, T-cell-mediated disease that can be induced in susceptible mouse strains by direct immunization with retinal antigens, including interphotoreceptor retinoid binding protein (IRBP) or arrestin (retinal soluble antigen, S-antigen), in complete Freund’s adjuvant and a simultaneous intraperitoneal injection of pertussis toxin. Alternatively, EAU can be induced indirectly by adoptive transfer of retinal antigen-specific effector T cells [4]. Disease usually develops around 10 to 14 days after immunization and is clinically evident within the retina as inflammatory cell infiltration which will include macrophages [9], dendritic cells (DCs) [10], neutrophils [11] and T cells [9,11]. In addition to the influx of blood-derived leukocytes, the resident myeloid-derived macrophages, retinal microglia, are also activated during EAU [7,8].

The Cx3cr1gfp/+ transgenic knock-in mouse [12] has enabled exquisite in vivo and ex vivo visualization of the dynamic changes in microglia in steady and diseased states in the eye [13-17], as well as in the brain [18,19], and in particular their rapid responsiveness to injury and presence of noxious stimuli [14,15]. The study of Cx3cr1-bearing brain microglia in vivo in these reporter mice requires the surgical creation of a defect or window in the calvaria and two-photon microscopic examination of the superficial cortex [20]. By contrast, the eye offers unique advantages for direct in vivo visualization of infiltrating and resident immune cells in reporter mice with minimal experimental manipulation [16,21-23]. However, to date there has been limited use of reporter mice to investigate the dynamics of infiltrating leukocytes and resident myeloid cells in an ocular model of autoimmunity.

In the present study, we demonstrate that in vivo fundus examination of transgenic reporter mice facilitates monitoring of the dynamic changes in various exogenously and endogenously derived cells of myeloid lineage during EAU progression. In particular we chose to examine EAU in transgenic mice (C57Bl/6 J Cx3cr1GFP/+, C57Bl/6 CD11c-eYFP, and C57Bl/6 J LysM-eGFP) in which promoter elements of the myeloid-specific Cx3cr1, CD11c and lysM genes are expressed alongside a specific fluorescent reporter in an attempt to characterize the relative temporal pattern of resident and infiltrating myeloid cells, DCs, and neutrophils, respectively. Whilst we appreciate that none of these transgenic reporter mice provide definitive identification of any of the above myeloid cell subsets and thus have their limitations [24], our results do provide novel insights into the cell mediated immune events in this model of human endogenous posterior uveoretinitis and allow accurate clinical grading of disease severity that correlates with histopathological changes.

**Materials and methods**

**Mice**

Transgenic reporter mice in which the myeloid-specific promoter of the Cx3cr1, CD11c, and lysM genes drives the expression of fluorescent reporters (C57Bl/6 J Cx3cr1GFP/+, C57Bl/6 N CD11c-eYFP, and C57Bl/6 J LysM-eGFP mice, respectively) were used in this study at an age of between 8 and 10 weeks. The details of the myeloid promoter and reporter mice used in this study are summarized in Table 1. C57Bl/6 J Cx3cr1GFP/+ mice were created by crossing homozygous C57Bl/6 J Cx3cr1GFP/GFP mice, originally obtained from Professor Steffen Jung [12], to wild-type

| Transgenic reporter mouse line | Description | Specificity | Research use |
|--------------------------------|-------------|-------------|--------------|
| C57Bl/6 J Cx3cr1GFP/+ | Knock-in mouse line where one copy of the Cx3cr1 gene is replaced by the GFP reporter gene [12]. | Cx3cr1 (GFP) is expressed in monocytes, subsets of natural killer and T cells, DCs, and microglia. | Extensively used to study monocytes during normal and diseased state in gut [72,73], kidney [74,75], brain [76,77], and the retina of the eye [8,78]. |
| C57Bl/6 N CD11c-eYFP | Reporter mouse line that expresses eYFP under the CD11c (Itgax) promoter [25]. | CD11c is a cell surface molecule expressed on myeloid cells, lymphocytes, natural killer cells and DCs [79]. In C57Bl/6 N CD11c-eYFP mice, DCs are characterized by CD11c-eYFP while B and T cells are CD11c-eYFP. | Extensively used to study distribution of DCs in numerous organs including the brain [31,32,80], lung [34], skin [35], gut [36], and the cornea of the eye [33]. |
| C57Bl/6 J LysM-eGFP | Reporter mouse line that expresses eGFP under the LysM promoter [26]. | LysM is expressed in neutrophil granulocytes (LysM-eGFP) and macrophages (LysM-eGFP). | Widely used to study neutrophil extravasation during infection [81,82] and inflammation [37,83]. |

DC, dendritic cell; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; eYFP, enhanced yellow fluorescent protein; LysM, Lysosome M.
C57BL/6 N CD11c-eYFP mice express enhanced yellow fluorescent protein (eYFP) under the promoter for the Itgax (CD11c) gene and are widely regarded as a valuable model for investigating the distribution of DCs with the caveat that other cells are capable of limited expression of CD11c [25]. C57BL/6 N CD11c-eYFP breeding pairs were kindly provided by Associate Professor Michael Hickey (Monash University, Clayton, VIC, Australia) with permission from Professor Michel Nussenzweig (The Rockefeller University, New York, NY, USA) from whom the original mice were obtained [25]. C57BL/6 N Cx3cr1^{GFP/+} and C57BL/6 N CD11c-eYFP mice were bred at the Monash Large Animal Facility (Monash University). C57BL/6 N LySM-eGFP mice were kindly provided by Associate Professor Michael Hickey. C57BL/6 N LySM-eGFP mice were created by cloning enhanced green fluorescent protein (eGFP) into the lysozyme M (LySM) locus where LySM is expressed in myelomonocytic cells (macrophages and neutrophil granulocytes) [26]. C57BL/6 N Cx3cr1^{GFP/+} and C57BL/6 N LySM-eGFP mice were screened by PCR [22] and determined to be negative for retinal degeneration 8 (rd8) mutation in the crumbs 1 (Crb1) gene (data not shown). C57BL/6 N CD11c-eYFP mice were found to carry the rd8 mutation and this has been described in detail elsewhere [22]. All experimental animals were maintained under 12:12-hour light/dark cycle with ad libitum access to food and water. All procedures in this study were approved by the Monash Animal Ethics Committee (MARP/2011/094) and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of experimental autoimmune uveoretinitis**

EAU was induced using a standard protocol, previously described by others [8]. Briefly mice received a subcutaneous (s.c.) injection of a total of 100 μl emulsion, containing a 50:50 mix of 400 μg human IRBP peptide 1–20 (IRBP1–20GPTHLFQPSLVDMAKVLDDL; China Peptides, Shanghai, China) in complete Freund’s adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA) supplemented with Mycobacterium tuberculosis H37RA (2.5 mg/ml; BD PharMingen, San Diego, CA, USA), distributed between the base of the tail and the right flank of each mouse. Mice simultaneously received an intraperitoneal (i.p.) injection of 1.5 μg pertussis toxin (Sigma-Aldrich) in phosphate-buffered saline (PBS). Control mice received s.c. injection of a total of 100 μl emulsion containing 50:50 mixture of CFA and PBS (no IRBP) and i.p. injection of pertussis toxin.

**Clinical examination of eyes**

Mice were clinically examined on day (d) 0, 14, 21, 28 and 35 post-immunization (p.i.). Mice were anesthetized by an i.p. injection of a mixture of ketamine (80 mg/kg; Troy Laboratories, Glendenning, NSW, Australia) and xylazine (10 mg/kg; Troy Laboratories). The pupils were dilated with 0.5% tropicamide (Mydriacil, Alcon Laboratories, Vilvoorde, Belgium), and the cornea was kept moist with the application of sterile lubricant GenTeal gel (Novartis, North Ryde, NSW, Australia). The fundus was examined using the Micron III camera (Phoenix Research Laboratories, Pleasanton, CA, USA) with StreamPix 5 software. Examination consisted firstly of capturing short 5 seconds videos with 100 frames in brightfield, followed by a sequence of GFP+ or YFP+ cells using the green fluorescent barrier filters, and lastly by a short sequence following a 20 μl s.c. injection of 10% fluorescein isothiocyanate (Alcon Laboratories, Frenchs Forest, NSW, Australia) to visualize the retinal vasculature. The brightness and contrast of all fundus images were adjusted equally using ImageJ 1.48C software [27].

**Clinical assessment of experimental autoimmune uveoretinitis**

The severity of disease was graded based on examination of the three modes of clinical fundus images (brightfield, fluorescent, and fluorescein angiography) by two masked observers using a modification of a previously described clinical grading scheme for EAU [6]. The modifications to the grading criteria (Table 2) were primarily a consequence of having both traditional brightfield fundus views augmented by the fluorescent capability offered by the Micron III camera.

**Histological assessment of experimental autoimmune uveoretinitis**

Mice were euthanized on d35 p.i. with an i.p. injection of sodium pentobarbitone (Lethabarb; Virbac, Milperra, NSW, Australia) and were perfused with 1% heparin in PBS followed by 4% paraformaldehyde (PFA) in PBS. Eyes were enucleated and stored in 4% PFA at 4°C. The right eye from each animal was post-fixed with Karnovsky fixative (4% PFA: 1% glutaraldehyde) for 48 hours and processed for resin histology. Tissues were embedded in Technovit glycol methacrylate resin (Heraeus Kulzer, Wehrheim, Germany), sectioned at 5 μm thickness through the optic nerve-pupalillary axis and stained with haematoxylin and eosin. Sections from three different levels of each eye separated by a minimum distance of 50 μm were examined by light microscopy and disease severity was scored by a masked observer using the previously published histopathology grading system for mouse EAU [28].

**Results**

**Imaging of experimental autoimmune uveoretinitis disease progression in C57BL/6 J Cx3cr1^{GFP/+} mice**

In vivo clinical examination of eyes in C57BL/6 J Cx3cr1^{GFP/+} mice prior to immunization revealed a normal fundus using
brightfield ophthalmoscopy (Figure 1A), and revealed the distribution of the retinal microglia network in green fluorescent mode (Figure 1B) and normal retinal vasculature following fluorescein angiography (FA; Figure 1C). The time course of EAU was monitored in C57Bl/6 J Cx3cr1GFP+/− mice immunized with IRBP, over four time points (d14, d21, d28, and d35 p.i.). EAU disease was first observed on d14 p.i. (Figure 1D-F), was maximal at d21 p.i. (Figure 1G-I) and d28 p.i. (Figure 1J-L), and was only slightly reduced in severity at d35 p.i. (Figure 1M-O). All IRBPimmunized mice developed EAU while adjuvant treated control mice appeared normal at all time points (Additional file 1: Figure S1A-C).

Clinical examination of C57Bl/6 J Cx3cr1GFP+/− mice during experimental autoimmune uveoretinitis reveals different disease severities

EAU disease severity can vary in IRBPimmunized C57Bl/6 J Cx3cr1GFP+/− mice from mild (Additional file 1: Figure S1D-F), moderate (Additional file 1: Figure S1G-I) to severe (Additional file 1: Figure S1J-L) at all time points. Mild disease is characterized by swelling of the optic nerve head and mild vasculitis or perivenular cuffing (Additional file 1: Figure S1D), both evident due to accumulations of Cx3cr1-GFP+ myeloid cells (Additional file 1: Figure S1E). However, no fluorescein leakage was noted in FA at this point although only the early filling phase was examined (Additional file 1: Figure S1F). Moderate grades are characterised by multiple small focal retinal lesions with moderate vasculitis affecting all retinal vessels evident in brightfield (Additional file 1: Figure S1G) and as Cx3cr1-GFP+ perivenular infiltrates (Additional file 1: Figure S1H) with an absence of fluorescein leakage (Additional file 1: Figure S1I). Despite the presence of Cx3cr1-GFP+ perivenular infiltrates, Cx3cr1-GFP+ cellular infiltrates were absent in some focal retinal lesions seen in brightfield (Additional file 1: Figure S1G). Severe EAU can occasionally be detected as early as d14 p.i. and in the eyes where EAU disease was seen to be severe, multiple large retinal lesions and vasculitis affecting all retinal veins was noted (Additional file 1: Figure S1I). Marked Cx3cr1-GFP+ peri-vascular infiltrates (Additional file 1: Figure S1K) sometimes correlated with leakage of fluorescein (Additional file 1: Figure S1L). The short 5 second brightfield fundus video also demonstrated rolling and migration of Cx3cr1-GFP+ cells and non-Cx3cr1-GFP+ cells in the retinal veins (Additional file 2: Supplementary video 1).

To eliminate the possibility that the hyperfluorescent perivascular infiltrates in C57Bl/6 J Cx3cr1GFP+/− mice are due to autofluorescence inflammatory cells we have provided clinical fundus images of IRBPimmunized Wildtype C57Bl/6 J taken at d21 p.i. (Additional file 3: Figure S2).

Clinical grading strongly correlates with histopathological grade of experimental autoimmune uveoretinitis disease in C57Bl/6 J Cx3cr1GFP+/− mice at day 35 post-immunization

EAU clinical disease scores from all clinical imaging time points were determined by two masked observers using the grading criteria (Table 2). All adjuvant controls appeared normal at all time points (Figure 2A) and no histopathological changes were observed at d35 p.i. (Figure 2C). In IRBPimmunized C57Bl/6 J Cx3cr1GFP+/− mice EAU began to develop around d14 p.i. with grade 1.86 ± 0.33 (mean ± SEM) disease then gradually progressed over time and remained at grade 2.81 ± 0.24 until d35 p.i. (Figure 2A).

Histopathological disease scores in C57Bl/6 J Cx3cr1GFP+/− mice at d35 p.i. revealed a mean infiltrative grade of 2.38 ± 0.60 and a structural grade of 2.00 ± 0.38. Total histopathological score shows a significant positive correlation (r = 0.73; P = 0.019) with the clinical scores (Figure 2B). C57Bl/6 J Cx3cr1GFP+/− mice immunized with IRBP displayed classical histopathological features of EAU (Figure 2D-F), which have been described previously by several authors [6,28-30].
Clinical examination of experimental autoimmune uveoretinitis in C57Bl/6 N CD11c-eYFP mice

The C57Bl/6 N CD11c-eYFP mouse has been extensively used to map putative DCs in a variety of tissues [25,31-36]. We have recently described our discovery of the presence of the rd8 mutation in the Crb1 gene in the C57Bl/6 N CD11c-eYFP mice [22]. Multiple retinal lesions (Figure 3A) were observed in the fundus of naïve C57Bl/6 N CD11c-eYFP mice and we illustrated that these lesions co-localized with CD11c-eYFP+ cells (Figure 3B) [22]. At the time of our experiments to investigate the course of EAU in C57Bl/6 N CD11c-eYFP mice we were unaware of the rd8 mutation. It was during clinical examination of d0 naïve animals that we became suspicious of a baseline pathological status. Despite the presence of retinal degeneration, EAU was readily induced and typical EAU changes were noted in C57Bl/6 N CD11c-eYFP mice on d14 and d21 p.i. By d14 p.i. severe vasculitis was seen on brightfield.

Figure 1 Time-course monitoring of experimental autoimmune uveoretinitis disease with multi-modal imaging in one representative C57Bl/6 J Cx3cr1GFP/+ mouse. Fundus images of a C57Bl/6 J Cx3cr1GFP/+ mouse immunized with IRBP1-20 in brightfield (BF) (A,D,G,J,M), fluorescent (Fluor) (B,E,H,K,N), and fluorescein angiography (FA) (C,F,I,L,O) taken over five time points illustrated experimental autoimmune uveoretinitis disease progression and GFP+ myeloid cell perivascular infiltrates during disease progression. d, Day; p.i. post-immunization. GFP, green fluorescent protein; IRBP, interphotoreceptor retinoid binding protein.
fundoscopy (Figure 3C) and a large influx of perivascular CD11c-eYFP+ cells along retinal veins was observed in the fluorescent mode (Figure 3D) as well as in the brightfield due to the strong eYFP signal. Clinical examination on d21 p.i. revealed that vasculitis and the large influx of CD11c-eYFP+ cells was partly resolved but multiple retinal lesions were still present, and CD11c-eYFP+ cells were more evenly distributed in the retina (Figure 3E,F). FA examination revealed absence of fluorescein leakage (data not shown). All IRBP1-20 immunized mice developed EAU while adjuvant treated control mice appeared normal at all time points.

**Visualization of LysM-eGFP+ myelomonocytic cell infiltration during experimental autoimmune uveoretinitis in C57Bl/6 J LysM-eGFP mice**

In C57Bl/6 J LysM-eGFP mice, eGFP is cloned into the LysM locus which is expressed in myelomonocytic cells (macrophages-eGFPlow and neutrophils-eGFPbigh) [26] and have been used for *in vivo* imaging of neutrophils in healthy...
and diseased states [37]. In vivo imaging of the naïve C57Bl/6 J LysM-eGFP mouse fundus (n = 5) revealed a normal appearance (Figure 4A) and genotyping of these mice demonstrated that they did not carry the rd8 mutation (data not shown). In the naïve retina of these mice there were 16.6 ± 1.18 (mean ± SEM) LysM-eGFP+ cells present in the entire normal fundus (Figure 4B). However, upon examination of the clinical fundus video, transient LysM-eGFP+ cells were observed travelling at high velocity through the lumina of the retinal vessels (Additional file 4: Supplementary video 2). In vivo imaging of IRBP1-20 immunized C57Bl/6 J LysM-eGFP mouse fundus on d14 p.i. illustrated mild EAU with swelling of the optic nerve head, mild vasculitis (Figure 4C) and accumulations of the LysM-eGFP+ cells around the optic nerve head and along the retinal vessels (Figure 4D). Clinical fundus examination on d21 p.i. revealed multiple large retinal lesions and moderate vasculitis (Figure 4E) and the LysM-eGFP+ cells were found along the retinal vessels and in retinal lesions (Figure 4F). FA examination revealed absence of fluorescein leakage (data not shown). All IRBP1-20 immunized mice developed EAU while adjuvant treated control mice appeared normal at all time points.

Discussion

Intravital imaging using genetically modified reporter mice in which leukocyte subtypes are endogenously labelled with a fluorescent reporter gene transcript has greatly enhanced our understanding of cellular and immunological mechanisms during inflammation of several tissues [38-40]. Many of these experimental approaches are partly hindered or complicated by the potential effects of surgical intervention needed to exteriorise or surgically alter the tissue under investigation such as mesentery [41,42], cremaster muscle [42-44], liver [45], lung [37], kidney [40,43], and skin [46]. In the case of intravital imaging of the brain a craniotomy window is required [47,48]. The eye has several advantages over most organs because by
its very nature it provides a clear transparent window on both neural tissue (retina) and connective tissues (cornea, iris) [33,49], thus avoiding surgical intervention. We sought to exploit the recent development of multi-modal imaging techniques which allow high-quality examination of the mouse fundus [16,22] to investigate the behaviour of cells of myeloid origin during the course of EAU, a widely used model of ocular autoimmune disease.

Disease severity of EAU is routinely determined using histopathological grading methods [28,29,50]. To circumvent the issue of single time point post-mortem grading, several research groups have developed non-invasive clinical grading methods including topical endoscopic fundus imaging [51], otoscope imaging [6], scanning laser ophthalmoscopy [52] and optical coherence tomography [53-55]. In this present study, multi-modal fundus ophthalmoscopy was chosen to grade the disease severity as it has the advantages of being relatively inexpensive and has the capability of capturing both video and still frame images in brightfield, together with green and red fluorescence wavelengths.

Many previous phenotypic analyses of the inflammatory cell infiltrate during EAU have used multi-parameter flow cytometry to show that the majority of infiltrating cells are myeloid-derived with a peak in T cell infiltration around d14 p.i. [11,56,57]. Alternative approaches to visualize leukocyte trafficking in vivo in the eye during EAU have included use of acridine orange, a non-specific nuclear dye which can be visualized by fluorography. This method revealed leukocytes rolling along the retinal veins as early as d14 p.i. [58,59]; however, their specific phenotype was obviously not determined. More recently, in vivo imaging of the leukocyte subtypes with more specificity has become easier with the availability of genetically modified mouse models in which genes regulating leukocyte subtypes are used as promoters to express fluorescent reporter proteins. Although these transgenic mouse models are useful for providing insights to myeloid lineage cell

Figure 4 Clinical fundus examination of C57Bl/6 J LysM-eGFP mice in normal and diseased state. Fundus images of naïve C57Bl/6 J LysM-eGFP mouse fundus in brightfield (BF) (A) and fluorescent mode (Fluor) (B) appear normal (n=5). Fundus images of C57Bl/6 J LysM-eGFP mouse (n=4) immunized with IRBP₁₋₂₀ on day 14 post-immunization (d14 p.i.) (C-D) and d21 p.i. (E-F) illustrated the peripapillary infiltrates of vasculitis. eGFP, enhanced green fluorescent protein.
types in normal and diseased state, some authors have warned of cautious interpretation of these mice as the sole means of identifying and distinguishing macrophages and DCs [24]. Such limitations are also true of the three transgenic mouse lines chosen for the present study.

There are several subpopulations of Cx3cr1-GFP+ myeloid-derived cells in the normal retina including the hyalocytes on the retinal surface [60], subretinal macrophages on the other aspect of the neural retina [61-63], and the extensive network of microglial populations in the retinal parenchyma [13,64]. C57Bl/6 J Cx3cr1GFP/+ mice [12] have been widely used to investigate the role of microglia in numerous ocular conditions that may have an inflammatory element in their pathogenesis including potential models of retinal degeneration [65], retinopathy of prematurity [66,67] and diabetic retinopathy [16]. In the present study we demonstrate highly distinctive perivascular infiltrates of Cx3cr1-GFP+ cells at d14 p.i. to d35 p.i. which is in agreement with previous studies [8,68]. The perivascular infiltrate could theoretically represent haematogenous Cx3cr1-GFP+ (GFPlow) myeloid cells recently extravasated into the retina or the chemotactic migration of resident Cx3cr1-GFP+ (GFPhigh) microglia towards the vasculature. We believe the former is the case as the Cx3cr1-GFP+ (GFPhigh) microglia network seemed largely undisturbed, something that was subsequently confirmed by retinal whole mount analysis (data not shown).

The difficulty in distinguishing subpopulations of macrophages from cells of DC lineage was the motivation for the creation of CD11c transgenic reporter mice, specifically the CD11c-eYFP [25] and CD11c-DTR/GFP mice [69]. In these transgenic mice the promoter for the Itgax (CD11c) gene is used to drive eYFP expression (CD11c-eYFP mice) or GFP and diphtheria toxin receptor (DTR) expression (CD11c-DTR/GFP mice). CD11c is a leukocyte integrin comprised of an alpha X subunit that along with CD18, a leukocyte beta 2 integrin polypeptide, forms the CD11c/CD18 heterodimer which is important in leukocyte adhesion, migration and cell to cell interaction during immune responses. CD11c is expressed heterogenously by different populations of DCs [25] and is important in T cell priming [69]. However, it is also expressed to at least one log lower than DCs on other immune cells such as natural killer cells, subpopulations of macrophages and activated T cells [25]. As such immune cells are not normally a feature of the resting central nervous system, we, like other previous investigators [31,32,70], thought is reasonably safe to assume that in the resting and disease state CD11c-eYFP+ cells may represent predominantly DCs. The view that CD11c-eYFP mice are valuable for examining the distribution of DCs has recently been strongly challenged by Hume [24] who points out that CD11c has no function in antigen presentation; not all DCs are CD11c+ and that not all CD11c+ cells are antigen-presenting cells. It was thus with caution that we chose to take advantage of the transgenic C57Bl/6 N CD11c-eYFP mice to examine the dynamic of DCs in EAU. Indeed at the commencement of this study we had the further complication of discovering that these mice had a pre-existing retinal dystrophy due to the presence of the rd8 mutation in the crb1 gene [71] and that the CD11c-eYFP+ cells in the retina represented activated microglia [22]. In the present study, CD11c-eYFP+ cells were recruited into the eye at d14 and d21 p.i. in a similar pattern to that observed in C57Bl/6 J Cx3cr1GFP/+ mice leading us to conclude that these CD11c-eYFP+ cells are likely a mixture of myeloid-derived cells.

Interestingly, despite the C57Bl/6 N CD11c-eYFP mice carrying the rd8 mutation and the pre-existing disrupted retinal architecture prior to immunization, they did not develop a more severe form of EAU as may have been predicted if one were to assume that this dystrophic condition compromised the immune status of the retina as we have previously concluded [22], although we have not specifically proven that the blood-ocular barrier was compromised.

In the C57Bl/6 J LysM-eGFP mouse line generated by Faust and colleagues [26], homologous recombination was used to insert the eGFP gene into the LysM locus. This was chosen because LysM is expressed specifically in the myelomonocytic cell lineage (macrophages and neutrophil granulocytes). Characterization of LysM-eGFP+ cells in the blood revealed that the eGFPhigh polymorphonuclear granulocytes outnumbered LysM-eGFP+ monocytes by 50:1. In the present study, we observed exceedingly small numbers (16.6 ± 1.18) of largely static LysM-eGFP+ myelomonocytic cells around the optic nerve head in the normal C57Bl/6 J LysM-eGFP fundus. We propose that these are likely of monocyte lineage as it is highly unusual to detect extravasated neutrophils in the normal retina. However, video analysis (not shown) revealed many LysM-eGFP+ travelling at high velocity in retinal vessel lumina, which we conclude are likely to be circulating neutrophils. In these mice we demonstrated increased numbers of LysM-eGFP+ cells in the peripapillary retinal vessels at d14 to d21 of EAU. Closer examination of the high power images (see inset, Figure 4) suggests that many of these are marginating in vessel lumina, a pattern which differs from the perivascular infiltrates observed in the C57Bl/6 J Cx3cr1GFP/+ and C57Bl/6 N CD11c-eYFP mice. Subsequent flow cytometry of retinal tissue during EAU in C57Bl/6 J LysM-eGFP mice revealed these cells to be largely a neutrophilic infiltrate (Goldberg and colleagues, unpublished data).

Conclusions

In conclusion, in vivo fundus imaging of C57Bl/6 J Cx3cr1GFP/+ and C57Bl/6 N CD11c-eYFP mice revealed
the dynamics of the myeloid cell infiltrates in EAU, particularly the perivenular accumulations. In contrast, the C57Bl/6 J LysM-eGFP mice revealed intravascular margination of LysM-eGFP+ neutrophils as well as less distinctive perivascular infiltrates. The grading of disease using in vivo imaging of these genetically modified reporter mice correlated strongly with the histopathological changes.

Additional files

Additional file 1: Figure S1. Clinical appearance of C57Bl/6 J LysM-eGFP mouse fundus with different severities of EAU. Fundus images of adjuvant control C57Bl/6 J LysM-eGFP mouse in brightfield (A), fluorescent mode (B), and fluorescent angiography (C) revealed normal retinal vasculature with no fluorescent leakage and normal retinal microglia network. Multi-modal fundus images from C57Bl/6 J LysM-eGFP mice immunized with IRBP+20 displaying mild (D-F), moderate EAU (G-I), and severe (J-L) EAU. The classical features of EAU include perivenular vasoconstriction and dilated retinal vessels that is associated with GFP+ peripheral infiltrates (E and K, arrows) and multiple focal retinal lesions (G, arrows), venular dilation (L, arrows), and fluorescent leakage (L, arrowhead).

Additional file 2: Supplementary Video 1. Video of real time in vivo visualization of rolling leukocytes in retinal veins of C57Bl/6 J LysM-eGFP mouse during EAU. Duration: 5 seconds (20 frames per second).

Additional file 3: Figure S2. Clinical appearance of immunized with IRBP+20 WT C57Bl/6 J mouse fundus on d21 p.i. These fundus images of WT controls in brightfield (A), fluorescent mode (B), and fluorescent angiography (C) revealed that the inflammatory infiltrated did not autofluoresce.

Additional file 4: Supplementary Video 2. Video of real time in vivo visualization of naive C57Bl/6 J LysM-eGFP mouse fundus revealed static and circulating LysM-eGFP+ myelomonocytic cells. Duration: 5 seconds (20 frames per second).

Abbreviations
CFA: complete Freund's adjuvant; C6b1: crumbs 1; d: day; DC: dendritic cell; DTR: diphtheria toxin receptor; EAU: experimental autoimmune uveoretinitis; eGFP: enhanced green fluorescent protein; eYFP: enhanced yellow fluorescent protein; FA: fluorescent angiography; i.p.: intraperitoneal; IRBP: interphotoreceptor retinoid binding protein; LysM: lysosome M; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PFA: paraformaldehyde; p.i.: post-immunization; rd8: retinal degeneration 8; s.c.: subcutaneous.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
XC performed the immunization, in vivo analysis, tissue collection and subsequent histology, and drafted the manuscript. JMK was part supervisor of XC during her PhD studies and helped plan the study. JVF and CCB were also part supervisors of XC and advised on the experimental approach and reviewed the manuscript. GLG and IPW provided data from the C57Bl/6 J LysM-eGFP mice. PGM is the principle chief investigator in whose laboratory the studies were performed, and was primarily responsible with XC for the clinical grading, data analysis, data interpretation and writing of the manuscript. All authors read and approved the final manuscript.

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References
1Durrani OM, Meads CA, Murray PI. Uveitis: a potentially blinding disease. Ophthalmologica. 2004;221:223–6.
2Suttrop-Schulten MS, Rothova A. The possible impact of uveitis in blindness: a literature survey. Br J Ophthalmol. 1996;80:894–8.
3Rothova A, Suttrop-van Schulten MS, Frits Tewers W, Klijastra A. Causes and frequency of blindness in patients with intraocular inflammatory disease. Br J Ophthalmol. 1996;80:332–6.
4Gapo RR. A look at autoimmunity and inflammation in the eye. J Clin Invest. 2010;120:3073–83.
5Forrest JE. Endogenous posterior uveitis. Br J Ophthalmol. 1990;74:620–3.
6Xu H, Kuch P, Chen M, Lau A, Reid DM, Forrest JE. A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. Exp Eye Res. 2008;87:319–26.
7Rao NA, Kimoto T, Zamar E, Giri R, Wana R, Ito R, et al. Pathogenic role of retinal microglia in experimental uveoretinitis. Invest Ophthalmol Vis Sci. 2003;44:22–31.
8Kezic J, McMenamin PG. The monocyte chemokine receptor CX3CR1 does not play a significant role in the pathogenesis of experimental autoimmune uveoretinitis. Invest Ophthalmol Vis Sci. 2010;51:1521–7.
9Chan C-C, Li Q. Immunopathology of uveitis. Br J Ophthalmol. 1998;82:91–6.
10Jiang H-R, Lumsdon L, Forrest JV. Macrophages and dendritic cells in IRBP-induced experimental autoimmune uveoretinitis in B10 RIIm mice. Invest Ophthalmol Vis Sci. 1999;40:3177–85.
11Kerr EC, Raveney BJ, Copland DA, Dick AD, Nicholson LB. Analysis of retinal cellular infiltrate in experimental autoimmune uveoretinitis reveals multiple regulatory cell populations. J Autoimmun. 2008;31:354–61.
12Jung S, Aliberti J, Greemel P, Sunshine MI, Keutzberg GW, Sher A, et al. Analysis of fractalkine receptor CX3CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol. 2000;20:4106–14.
13Langmann T. Microglia activation in retinal degeneration. J Leukoc Biol. 2007;81:1345–51.
14Lee JE, Liang KL, Fariss RN, Wong WT. Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. Invest Ophthalmol Vis Sci. 2008;49:1696–76.
15Liang KL, Lee JE, Want YD, Ma W, Fontainhas AM, Fariss RN, et al. Regulation of dynamic behavior of retinal microglia by CX3CR1 signaling. Invest Ophthalmol Vis Sci. 2009;50:4444–51.
16Kezic JM, Chen X, Rakoczy EP, McMenamin PG. The effects of age and Cx3cr1 deficiency on retinal microglia in the Ins2Akita diabetic mouse. Invest Ophthalmol Vis Sci. 2013;54:8543–62.
17Kohteister M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: insights from novel mouse models. Immunobiology. 2010;215:685–91.
18Liu Z, Cordello C, Schan A, Harb R, Gruzenjler J. CX3CR1 in microglia regulates brain amyloid deposition through selective profamily amyloid β-phagocytosis. J Neurosci. 2010;30:10101–101.
19Michaud JP, Bellavance M-A, Préfontaine P, Rivest S. Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. Cell Reports. 2013;5:65–63.
20Nimmesjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillance of brain parenchyma in vivo. Science. 2003;308:1314–8.
21. Alt C, Runnels JM, Teo GSL, Lim PC. In vivo tracking of hematopoietic cells in the retina of chimeric mice with a scanning laser ophthalmoscope. Intravital. 2012;1:32–40.

22. Chen X, Kezic J, Bernard C, McMenamin PG. RGD mutation in the CD1 gene of CD1c-eftFP transgenic reporter mice results in abnormal numbers of CD1c-positive cells in the retina. J Neuroophthal Exp Neurol. 2013;72:82–90.

23. Spencer D, Lee E, Kawaguchi T, Rosenbaum J. In vivo imaging of the immune response in the eye. Semin Immunopathol. 2008;30:79–90.

24. Hume DA. Applications of myeloid-specific promoters in transgenic mice. Bio imaging. 2011;8:525–38.

25. Lindquist RL, Shakhar G, Dudaiko D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks in vivo. Nat Immunol. 2004;5:1243–50.

26. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of enhanced green fluorescent protein into the lysosome gene creates mice with green fluorescent granulocytes and macrophages. Blood. 2000;96:719–26.

27. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. Biophotonics Int. 2004;11:36–42.

28. Dick AD, Cheng YF, Liveridge J, Forrester JI. Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens. Eye. 1994;8:52–9.

29. Chan CC, Caspi RR, NJ M, Leake WC, Wiggert B, Chader GJ, et al. Pathology of experimental autoimmune uveoretinitis in mice. J Autoimmun. 1990;3:247–55.

30. Cortes LM, Mattappalli MJ, Silver PB, Donoso LA, Liou GI, Zhu W, et al. Repertoire analysis and new pathogenic epitopes of IRBP in C57BL/6 (H-2b) and B10.HII (H-2d) mice. Invest Ophthalmol Vis Sci. 2008;49:1946–56.

31. Anandasabapathy N, Victoria GD, Meredith M, Feder R, Dong B, Kluger C, et al. FLT3 controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain. J Exp Med. 2011;210:1695–705.

32. Kauzner UW, Miller MM, Gottfried-Blackmore A, Gal-Toth J, Felger JC, McEwen BS, et al. Accumulation of resident and peripheral dendritic cells in the aging CNS. Neurobiol Aging. 2012;33:681–93. e681.

33. Lee EJ, Rosenbaum JT, Planck SR. Epifluorescence intravital microscopy of the live kidney in health and disease. J Biomed Opt. 2014;19:020901.

34. Lee EJ, Rosenbaum JT, Planck SR. Epifluorescence intravital microscopy of the live kidney in health and disease. J Biomed Opt. 2014;19:020901.

35. Ng LG, Hsu A, Mandell MA, Roediger B, Hoeller C, Mrass P, et al. Migratory lymphoid organs. J Immunol. 2009;182:3969–75.

36. Laliotou B, Dick AD. Modulating phenotype and cytokine production of leucocytic retinal infiltrate in experimental autoimmune uveoretinitis following intranasal tolerance induction with retinal antigens. Br J Ophthalmol. 1999;83:478–85.

37. Thurai SR, Mempel TR, Flügel A, Diedrichs-Möhling M, Kornbach F, Kawakami N, et al. The fate of autoreactive, GFP+ T cells in rat models of uveitis analyzed by intravital fluorescence microscopy and FACS. Int Immunol. 2004;16:1573–82.

38. Hamada M, Ogun Y, Miyamoto K, Nishiwaki H, Hiroshiba N, Honda Y. Retinal leukocyte behavior in experimental autoimmune uveoretinitis of rats. Eye Exp Res. 1997;65:445–9.

39. Parnaby-Price A, Stanley MR, Biggestaff J, Howe L, Whiston RA, Marshall J, et al. Leukocyte trafficking in experimental autoimmune uveitis in vivo. J Leukoc Biol. 1996;64:434–40.

40. Vagaja NN, Chinnery HP, Binni Z, Kezic JM, Rakozy EP, McMenamin PG. Changes in murine hyalocytes are valuable early indicators of ocular disease. Invest Ophthalmol Vis Sci. 2012;53:1445–51.

41. Chinnery HR, McNelchan S, Humphries T, Kezic JM, Chen X, Ruitenbeek MJ, et al. Accumulation of murine subretinal macrophages: effects of age, pigmentation and CX3CR1. Neurobiol Aging. 2011;33:1769–76.

42. Raoul W, Auwynyt C, Camello S, Gulloneou X, Feurini C, Combadere C, et al. CCLe2/CCL4 and CXCCLI1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration. J Neuroinflammation. 2010;7:87.

43. Luhmann UFC, Robbie S, Munro PMG, Barker SE, Duran Y, Luong V, et al. The Drusenlike phenotype in aging C2d2-knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages. Invest Ophthalmol Vis Sci. 2009;50:5934–43.

44. Dick AD. Influence of microglia on retinal progenitor cell turnover and cell replacement. Eye. 2008;22:1939–45.

45. Combadere C, Feurini C, Raoul W, Keller N Rod, Ro M, et al. CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. J Clin Invest. 2007;117:2920–9.

46. Zhao L, Ma W, Faris RN, Wong WT. Retinal vascular repair and neovascularization are not dependent on CX3CR1 signaling in a model of ischemic retinopathy. Exp Eye Res. 2009;88:1004–13.

47. Fischer F, Martin G, Agostini H. Activation of retinal microglia rather than microglial cell density correlates with retinal neovascularization in the mouse model of oxygen-induced retinopathy. J Neuroinflammation. 2011;8:120.
68. Dagkalis A, Wallace C, Hing B, Liversidge J, Crane UJ. CX3CR1-deficiency is associated with increased severity of disease in experimental autoimmune uveitis. Immunology. 2009;128:25–33.

69. Jung S, Unutmaz D, Wong P, Sano GI, Delos Santos K, Spanvasser T, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity. 2002;17:211–20.

70. Bulloch K, Miller MM, Gal-Tooth J, Milner TA, Gottfried-Blackmore A, Waters EM, et al. CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain. J Comp Neurol. 2008;508:687–710.

71. Mattapallil MJ, Wawrousek EF, Chan C-C, Zhao H, Roychoudhury J, Ferguson TA, et al. The R8β mutation of the Crb1 gene is present in vendor lines of C57BL/6 N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. Invest Ophthalmol Vis Sci. 2012;53:2021–7.

72. Medina-Contreras O, Geem D, Laur Q, Williams IR, Li SA, Nusrat A, et al. CX3CR1 regulates intestinal macrophage homeostasis, bacterial translocation, and colitogenic Th17 responses in mice. J Clin Invest. 2011;121:4787–95.

73. Kim K-W, Vallon-Eberhard A, Zigmond E, Farache J, Shezen E, Shalhar G, et al. In vivo structure/function and expression analysis of the CX3C chemokine fractalkine. Blood. 2011;118:e156–e167.

74. Hochheiser K, Heuser C, Krause TA, Teteris S, Ilias A, Weisheit C, et al. Exclusive CX3CR1 dependence of kidney DCs impacts glomerulonephritis progression. J Clin Invest. 2013;123:4242–54.

75. Lionakis MS, Swanzydas M, Fischer BG, Plantinga TS, Johnson MD, Jaeger M, et al. CX3CR1-dependent renal macrophage survival promotes Candida control and host survival. J Clin Invest. 2013;123:5035–51.

76. Huang D, Shi F-D, Jung S, Pien GC, Wang J, Salazar-Mather TP, et al. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. FASEB J. 2006;20:896–905.

77. Ueno M, Fujita Y, Tanaka T, Nakamura Y, Kikuta J, Ishii M, et al. Layer V cortical neurons require microglial support for survival during postnatal development. Nat Neurosci. 2013;16:543–51.

78. Kezic J, Xu H, Chinnery HR, Murphy CC, McMenamin PG. Retinal microglia and uveal tract dendritic cells and macrophages are not CX3CR1 dependent in their recruitment and distribution in the young mouse eye. Invest Ophthalmol Vis Sci. 2008;49:1599–608.

79. Singh-Jasuja H, Thiolat A, Ribon M, Boissier M-C, Bessis N, Rammensee H-G, et al. The mouse dendritic cell marker CD11c is down-regulated upon cell activation through Toll-like receptor triggering. Immunobiology. 2013;128:28–39.

80. Felger JC, Abe T, Kurunzer UW, Gottfried-Blackmore A, Gal-Tooth J, McEwen BS, et al. Brain dendritic cells in ischemic stroke time course, activation state, and origin. Brain Behav Immun. 2010;24:724–37.

81. Kin A, Loughman JA, Zinselmeyer BH, Miller MJ, Caparon MG. Streptolysin S inhibits neutrophil recruitment during the early stages of streptococcosis pyogenes infection. Infect Immun. 2009;77:190–201.

82. Howe C, LaFrance-Corey R, Sundsbrak R, LaFrance S. Inflammatory monocytes damage the hippocampus during acute picornavirus infection of the brain. J Neuroinflammation. 2012;9:50.

83. Byrne R, Rath E, Hladik A, Niedereiter B, Bonelli M, Frantal S, et al. A dynamic real time in vivo and static ex vivo analysis of granulomonocytic cell migration in the collagen-induced arthritis model. PLoS ONE. 2012;7:e35194.
