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RESOURCE ARTICLE

A simple method for in vivo labelling of infiltrating leukocytes in the mouse retina using indocyanine green dye

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ABSTRACT

We have developed a method to label and image myeloid cells infiltrating the mouse retina and choroid in vivo, using a single depot injection of indocyanine green dye (ICG). This was demonstrated using the following ocular models of inflammation and angiogenesis: endotoxin-induced uveitis, experimental autoimmune uveoretinitis and laser-induced choroidal neovascularization model. A near-infrared scanning ophthalmoscope was used for in vivo imaging of the eye, and flow cytometry was used on blood and spleen to assess the number and phenotype of labelled cells. ICG was administered 72 h before the induction of inflammation to ensure clearance from the systemic circulation. We found that in vivo intravenous administration failed to label any leukocytes, whereas depot injection, either intraperitoneal or subcutaneous, was successful in labelling leukocytes infiltrating into the retina. Progression of inflammation in the retina could be traced over a period of 14 days following a single depot injection of ICG. Additionally, bright-field microscopy, spectrophotometry and flow cytometric analysis suggest that the predominant population of cells stained by ICG are circulating myeloid cells. The translation of this approach into clinical practice would enable visualization of immune cells in situ. This will not only provide a greater understanding of pathogenesis, monitoring and assessment of therapy in many human ocular diseases but might also open the ability to image immunity live for neurodegenerative disorders, cardiovascular disease and systemic immune-mediated disorders.

KEY WORDS: Indocyanine green, Inflammation, In vivo imaging

INTRODUCTION

Resident and infiltrating leukocytes have an important role in sight-threatening diseases of the eye. Substantial evidence exists to confirm not only that activation and altered immune response happen in uveitic syndromes (Lee et al., 2014) but that they may also occur in the form of parainflammation (Xu et al., 2009) and initiate or exacerbate age-related macular degeneration and diabetic retinopathy (Whitcup et al., 2013). Here, the cellular participants of the immune system act as drivers for pathological angiogenesis, forming the two main causes of blindness in Western industrialized countries, namely, age-related macular degeneration and diabetic retinopathy (Penfold et al., 1987; Lopez et al., 1991; Lutty et al., 1997; Joussen et al., 2001). A better understanding of the role of inflammation in the development of pathological angiogenesis is essential for the development of disease-modifying strategies not only for monitoring disease progression but also to assess the efficacy of treatments.

The development of clinically viable imaging tools for in vivo visualization of inflammation is a crucial step in this process. To date, live imaging of inflammation has been restricted to experimental animal models, with examples such as magnetic particles (iron oxide and gadolinium chelates) for magnetic resonance imaging, fluorescent nanoparticles which can be tagged with aptamers or peptides targeted against cell surface markers, circulating factors or nucleic acid structures, or dyes such as acridine orange, which is a known human carcinogen (Joussen et al., 2001; Rausch et al., 2001; Montet-Abou et al., 2010; Roivainen et al., 2012; Cibiel et al., 2012; Nahrendorf et al., 2007; Hossain et al., 1998). The only application that is currently used to image inflammatory cells in humans is based on in vitro radionucleotide labelling of leukocytes from the patient’s own blood. Although this allows direct visualization of cell migration patterns, current imaging techniques do not allow sufficient resolution to track single cells (van Hemert et al., 2007).

Here, we present the use of indocyanine green (ICG) for in vivo visualization of myeloid cells in the mouse. ICG is a near-infrared (NIR) fluorescence tricarbocyanine dye with a peak spectral absorption of 800-810 nm (in blood) that is approved by the US Food and Drug Administration for clinical use. The introduction of ICG in ophthalmic angiography in the late 1960s was largely because of its minimal toxicity and favourable optical and biophysical properties (Kogure et al., 1970). NIR light could penetrate the ocular pigments of the eye, such as melanin and xanthophyll, thereby allowing visualization of the deep choroidal vasculature (not possible with conventional fluorescein angiography). In addition, its tendency for conjugation to plasma proteins meant that the dye did not readily leak from the fenestrated choriocapillaris (Cherrick et al., 1960).

Despite the wide use of intravenous (i.v.) ICG, US Food and Drug Administration approval and a good safety profile, there is limited evidence for its use in cellular imaging. This is probably because of its pharmacokinetic properties; ICG is rapidly removed from the circulation via hepatic clearance and it has a half-life of approximately 3-4 min, which precludes in vivo labelling of cells. However, we observed that by administering ICG as a depot injection, we could obtain reproducible labelling of peripheral CD11b+ circulating myeloid cells, establishing a novel method for in vivo tracking of these cells at near single-cell resolution as they
invade the eye in response to inflammation and injury. Furthermore, we found in vitro evidence that human myeloid cells stain in a similar manner, which strongly supports the translation of this promising technique into clinical practice.

RESULTS

**In vitro labelling of peripheral blood mononuclear cells (PBMCs) and splenocytes with ICG**

To establish whether ICG can label circulating leukocytes, we incubated whole blood with the dye in vitro for 30 min at room temperature. Visual inspection of a blood smear from human blood by fluorescent microscopy revealed a small population of fluorescent cells in the NIR channel (Fig. 1A,B). Next, we exposed PBMCs isolated from human and mouse blood, and mouse splenocytes to ICG (30 min at room temperature) and then analysed dye uptake and cell identity by flow cytometry. In human PBMCs, whilst all cells stained slightly with the dye, around 2-5% of all cells were strongly stained with ICG (Fig. 1C,D). Conversely, ICG did not activate BMDMs, as assessed by interleukin-6 (IL-6) production (Fig. 2E). In order to assess whether ICG was internalized by the cells or whether it only stained the cell membrane, we measured ICG release of stained cells after lysis by spectrophotometry. Increasing absorption at 790 nm suggested the release of internalized dye (Fig. 2F).

**Assessment of the efficiency of ICG cell labelling and its effects on macrophages and lymphocytes**

To assess the efficiency of ICG uptake of different populations of cells, we used cultured mouse bone-marrow-derived macrophages (BMDMs) and magnetic-activated cell sorted CD4+ mouse lymphocytes from the spleen. We observed, using flow cytometry, that whilst both populations were stained with ICG, BMDMs were more strongly stained than CD4+ cells (Fig. 2A). A 2 h exposure of BMDMs to ICG (200 ng/ml) led to a green stain of BMDMs that was visible in bright-field microscopy (Fig. 2B). The uptake of ICG in BMDMs was not affected by activation of the cells with lipopolysaccharide (LPS; Fig. 2C,D). Conversely, ICG did not activate BMDMs, as assessed by interleukin-6 (IL-6) production (Fig. 2E). In order to assess whether ICG was internalized by the cells or whether it only stained the cell membrane, we measured ICG release of stained cells after lysis by spectrophotometry. Increasing absorption at 790 nm suggested the release of internalized dye (Fig. 2F).
The third model was the laser-induced choroidal neovascularization (CNV) model, a model widely used to study pathological angiogenesis in the retina (Montezuma et al., 2009). The vascular response in this model is accompanied by an accumulation of infiltrating leukocytes in the laser lesion from the circulation and from within the retina (Horie et al., 2013; Liu et al., 2013). We hypothesized that the infiltrating leukocytes can be visualized by labelling cells with ICG in the periphery. Given that laser injury can induce autofluorescence, we first imaged animals without ICG. In the NIR channel, no fluorescence was detected in the lesion (Fig. 4A). Next, we tested whether administration (i.p.) of ICG immediately after the laser would result in ICG leakage into the retina. Although ICG could be visualized in the retinal vasculature and laser lesions, overt leakage of ICG into the retina was not apparent (Fig. 4B). As before, in order to ensure that there was sufficient time for ICG labelling of cells in the circulation and the clearance of ICG from the circulation, we injected ICG 3 days before laser induction of CNV. Given that ICG is rapidly cleared from the circulation, we reasoned that a 3-day delay would be sufficient to minimize any ICG in the circulation that might leak directly into the retina from the bloodstream and label resident retinal macrophages. Consistent with this, ICG could no longer be detected in the circulation by NIR imaging at this stage (not shown). Seven days after induction of the laser lesion (10 days after ICG), we observed a marked aggregation of clearly labelled cells in and around the laser lesions (Fig. 4C, C'). After a further 3 days (10 days after laser), the signal started to subside (Fig. 4D, D'). ICG+ cells were observed in laser lesions that developed CNV and also in those that had no fluorescein angiographic evidence of CNV after 7 days. This is illustrated by an example shown in Fig. 4E. Here, six laser...
lesions were applied. Although all of them accumulated ICG+ cells (Fig. 4E), only two lesions showed signs of neovascularization based on fluorescein angiography (Fig. 4F,G).

Furthermore, dissection of the mice 7 days after administration of ICG i.p. revealed green staining of lymphatic tissue in the thoracic cavity (Fig. S1A,B), mediastinal lymph nodes (Fig. S1C), thymus (not shown) and the greater omentum in the abdominal cavity (Fig. S1D-F). This suggests that inflammatory cells in the circulation, most probably both monocytes and lymphocytes that continuously circulate between the bloodstream and lymphoid organs, are labelled by the ICG. To check whether ICG might be toxic to the cells that take it up, we used flow cytometry to measure cell death in inflammatory cells that had been stained in vitro (after 30 min) and in vivo (10 days after ICG injection) in the CNV model. This showed no noticeable differences in cell counts, indicating that ICG has no gross effects on inflammatory cell populations in the spleen, the retina and the choroid (Fig. S2) within the time frame of our experiments (10 days).

We next tested whether cellular infiltration around the laser-induced CNV lesion could be monitored over time. As before, ICG was administered i.p. 3 days before the laser, and animals were imaged sequentially 2, 5 and 8 days after laser CNV induction (Fig. 5). The number of ICG-labelled cells in and surrounding the CNV lesion could be observed qualitatively to increase over time (Fig. 5C-E). Next, we assessed the feasibility with which this could be quantified and monitored over time (Fig. 5B,C’-E’).

Characterization of in vivo ICG-labelled cells
To test the identity of ICG+ cells invading the retina in the laser-induced CNV model, we used an in vivo staining protocol. After ICG imaging (Fig. 6A-C), a fluorescently labelled (fluorescein isothiocyanate) antibody against CD11b was injected. Over the course of 30 min, this labelled a population of cells in the choroid and subretinal space, with diffuse leakage of ICG into the laser-induced CNV lesion, and individual cells could not be detected (not shown). Oral administration (by gavage or in drinking water) did not lead to any labelling (Fig. S5).
ICG-labelled cells spatially (Fig. 6D-F). The CD11b signal was weaker than the ICG signal and there was not a perfect overlap. Nevertheless, all CD11b-positive cells were also ICG positive. The}

same approach was also taken with an anti-CD45 antibody, with the same outcome (data not shown). This suggests the identity of most of the ICG-labelled cells in the retina to be infiltrating myeloid cells.

**DISCUSSION**

We describe here a novel technique for in vivo labelling of a subpopulation of circulating cells with ICG dye. We found that in order to label these cells reproducibly in vivo, ICG must be administered as a depot preparation. Using this approach, we showed in three disease models of retinal inflammation that infiltrating leukocytes can be detected, tracked over time and quantified in the murine retina.

We found that the commonly used i.v. route of administration of ICG failed to label infiltrating leukocytes. However, by administering ICG as a depot, we have shown that infiltrating cells were clearly labelled in our disease models. A possible explanation for this is the increase in the period of ICG exposure that circulating cells have, in order to be labelled. In keeping with this, we observed in vivo that an increased time of exposure to ICG resulted in increased cell labelling. Furthermore, we observed both in vivo and in vitro that the majority of labelled cells were of myeloid origin.

Our technique will be useful in the above-mentioned mouse models. In particular, the laser-induced neovascularization model is widely used to study pathological vessel growth in the retina. This model also contains an important inflammatory component, which has so far been assessed mainly by immunohistochemistry on postmortem tissue. With our ICG technique, it will be possible to monitor inflammatory cell invasion longitudinally in these animals.

More generally, in the field of inflammation, the ability to monitor a sequence of events over time is particularly important in order to establish cause-and-effect relationships. As such, recent developments have been focused on the ability to visualize inflammation in vivo using molecular imaging techniques, such as intravitral microscopy using fluorescent antibodies, nanoparticles and transgenic animals that express fluorescent proteins (Miyahara et al., 2004; Shen et al., 2007). One study has used intravitreal ICG in murine CNV models (Paques et al., 2010). The vitreous cavity acts as a reservoir, thereby allowing prolonged exposure of cells to ICG (similar to that of a depot application). Intravitreal ICG was observed to label microglial cells for up to several weeks, allowing time-lapse observation of migratory behaviour after injury. In a similar fashion, our ICG method labels peripheral circulating cells but avoids potential neurotoxic effects of prolonged exposure of ICG to the retina, as observed by vitreoretinal surgeons who use ICG as a vital stain to visualize the internal limiting membrane during macular hole surgery (Stanescu-Segall and Jackson, 2009). Although depot ICG does not target specific cell types or epitopes like transgenic animals or antibodies, nor have we identified the identities or proportion of infiltrating cells stained, it has the advantage of having a simple application technique (depot injection), does not depend on antibodies, nor have we identified the identities or proportion of infiltrating cells stained, it has the advantage of having a simple application technique (depot injection), does not depend on specific mouse strains and, most importantly, has the potential for rapid translation to human use.

Current strategies for in vivo molecular imaging in humans include conjugation of NIR dyes, such as ICG, with ligands such as small molecules, antibodies, peptides, DNA and nanoparticles. Nevertheless, none of these probes has been approved for human use because of their so far insufficiently characterized safety profiles. In particular, nanoparticles suffer from poorly characterized distribution, accumulation and clearance from the
human body, and potential cytotoxicity of heavy metal ingredients (Nel et al., 2006; Mancini et al., 2008). In contrast, ICG is an inert, water-soluble organic dye that is rapidly bound to plasma proteins and solely removed from the circulation through the liver via a specific carrier-mediated transport system (Donald and Yipintsoi, 1973). Furthermore, ICG is known not to provoke inflammation even when injected directly into tissues.

The ICG dye was first developed in the mid-1950s to determine cardiac output and hepatic function. The dye is rapidly cleared, low in toxicity and well tolerated by subjects, even at doses that exceed what is now routinely given by tenfold (Miller et al., 1962; Wood, 1962). This has led to its application to a variety of clinical uses, with examples including sentinel node biopsy, tumour demarcation surgery and lymphatic vessel assessment. The main advantage of ICG is its track record of safety and tolerance, more than 60 years of application in clinical practice, and the ability of an NIR dye to be visualized without the need for tissue windows, i.e. for non-invasive imaging.

Techniques for in vivo cellular imaging require a biocompatible, non-toxic agent that allows reproducible quantification of these infiltrating leukocytes. Alternative methods that have been described for in vivo imaging are dyes which are conjugated to antibodies, allowing more specificity of binding or ex-vivo labelling (labelling cells outside the circulation and reintroduction into the circulation). Our results clearly demonstrate that this simple method of using ICG as a depot injection to label cells in vivo can be achieved in mouse models. Translation of this insight into the clinic will involve the development of a formulation suitable for depot ICG application in humans. This might be useful not only for monitoring ocular inflammation but might also have applications further afield, for example, the detection of an immune response to new treatment strategies, such as stem cell therapies or retinal implants, or serve as an imaging biomarker for predicting the onset of angiogenesis in age-related macular degeneration or diabetic retinopathy.

MATERIALS AND METHODS

In vitro labelling of peripheral blood mononuclear cells and splenocytes with ICG

Mouse blood was drawn via cardiac puncture with a 0.5 M EDTA-coated 23-gauge needle, before either red cell lysis or Ficoll-gradient separation using Histopaque-1077 (Sigma-Aldrich, UK) according to the manufacturer’s guidelines. Cells were stained with ICG, then incubated with Fc-block (BD Biosciences, UK) before primary antibody staining at the manufacturer’s recommended concentrations at 4°C for 20 min. All antibodies were from BD Biosciences.
Human PBMCs were isolated from 20 ml of whole blood using a Ficoll gradient. PBMCs were isolated from murine whole blood using Percoll-gradient separation, and splenocytes were mechanically dissociated before being incubated in 6.25 µg/ml ICG for 30 min at room temperature. PBMCs and splenocytes were washed and co-stained with CD45, CD11b and CD3 fluorescent antibodies (Miltenyi Biotech, Bisley, UK).

Flow cytometric analysis of ICG-labelled cells
ICG-stained PBMCs, whole blood and splenocytes were analysed using a BD Bioscience LSRII flow cytometer because no commercial machine was available with a near-infrared laser for ideal excitation of ICG. Suboptimal excitation by the 633 nm red laser nonetheless resulted in a reliable signal using a 780/60 bandpass filter. A minimum of 10,000 events was collected for each sample, and fluorescence-minus-one controls were used to determine the placement of gates. Data were processed using FlowJo v10.1 (TreeStar, Ashton, OR, USA). For the toxicity assessment, gating on the following populations was performed (after exclusion of debris and cellular aggregates): lymphocytes (CD4+/CD8+/B220+); total myeloid populations (CD11b+); neutrophils (Ly6G+); natural killer cells (NK1.1+); dendritic cells (CD11chigh); and monocytes/macrophages (CD11b+/CD11clow/Ly6G–/NK1.1+). Cell death within each population was then analysed using a live:dead stain.

Generation of bone-marrow-derived macrophages
Generation of bone-marrow-derived macrophages (BMDMs) followed the protocol from the original report (Munder et al., 1999). In brief, mouse femurs and tibias were collected and bone marrow cells were flushed out, followed by 8 days of maturation in Teflon bags with Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 5% normal horse serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 50 mM 2-mercaptoethanol (all from Life Technologies) and 100 pg/ml colony-stimulating factor 1 generated from L929 fibroblast-conditioned media.

Mouse interleukin-6 ELISA
BMDMs were plated at a density of 5×10^5 cells/well in a 96-well flat-bottomed plate. Four hours later, the medium was exchanged for 0.2 mg/ml ICG dye, with either medium alone or 5 ng/ml LPS. After the indicated times, supernatants were collected and frozen at −80°C. Mouse IL-6 sandwich ELISA was performed in technical triplicate according to the manufacturer’s instructions (BD Biosciences, UK) using rat anti-mouse IL-6, 554400 and biotin rat anti-mouse IL-6, 554402.

Spectrophotometry of ICG
BMDMs were plated in flat-bottomed 96-well plates for 4 h in 10% fetal calf serum and Dulbecco’s modified Eagle’s medium, before the addition of ICG to a concentration of 0.2 mg/ml. Cells were incubated for the specified amount of time before washing five times with PBS. After this, 200 µl of PBS was added to the well for 5 min and then removed for spectrophotometry. Triton (2%) in 200 µl PBS was then added to each well and the supernatant also removed after 5 min. Spectrophotometry was performed using a SpectraMax 190 (Molecular Devices, Wokingham, UK), with SoftMax Pro (v6.0) software, set to an absorbance of 790 nm.

Bright-field microscopy
BMDMs were imaged in flat-bottomed 96-well plates with a Leica DMIRB microscope using Leica QFluoro software v3.1. No filters were used, but light intensity was adjusted.
facility using this protocol.

26 days later at the time point determined to be the disease peak in our peritoneal space (Tocris Bioscience, Bristol, UK). Imaging was performed

Pertussis toxin (1.5 μg) was simultaneously administered into the

Mycobacterium tuberculosis H37RA (Difco Laboratories, BD, Oxford, UK). Freund human RBP-1-20 peptide subcutaneously, emulsified in complete

Biotech, UK) according to the manufacturer instructions.

Female C57BL/6J mice (Harlan, UK) at 7 weeks of age received 500 μg of human RBP-1-20 peptide subcutaneously, emulsified in complete Freund’s adjuvant (Sigma-Aldrich, UK) supplemented with 1.5 mg/ml Mycobacterium tuberculosis H37RA (Difco Laboratories, BD, Oxford, UK). Pertussis toxin (1.5 μg) was simultaneously administered into the peritoneal space (Tocris Bioscience, Bristol, UK). Imaging was performed 26 days later at the time point determined to be the disease peak in our facility using this protocol.

Magnetic-activated cell sorted separation of CD4+ cells

Single-cell spleen suspensions from C57BL/6J mice were erythrocyte lysed by ammonium-calcium-potassium (ACK) buffer. Splenic CD4 cells were prepared by enriching CD4+ cells using anti-CD4 microbeads (Miltenyi Biotech, UK) according to the manufacturer’s instructions.

Animals

All animals were handled in accordance with the UK Animals (Scientific Procedures) Act 1986. Female C57BL/6J mice (Harlan, UK) at 7-8 weeks of age were used. For in vivo procedures, the mice were anaesthetized with an i.p. injection of medetomidine hydrochloride (1 mg/kg body weight; Domitor; Pfizer Animal Health, New York, NY, USA) and ketamine (60 mg/kg body weight) in water. Pupillary dilatation was achieved with one drop of 1% Tropicamide (Bausch and Lomb, Kingston-upon-Thames, UK).

Induction of endotoxin-induced uveitis

Female C57BL/6J mice (Harlan, UK) at 7 weeks of age received a single i.p. injection of 0.2 mg of LPS from E. coli (Sigma-Aldrich, St Louis, MO, USA) in PBS. A 1 mg i.p. bolus injection of ICG was given 24 h before the LPS treatment. Imaging was performed 48 h later, at a previously determined time point when there is peak infiltration by myeloid cells.

Induction of experimental autoimmune uveoretinitis

In vivo imaging

Ocular imaging was performed using a scanning laser ophthalmoscope (Spectralis™ HRA; Heidelberg Engineering, Heidelberg, Germany). A lens with a 55° field of view was used, and a mean of 100 consecutive frames was taken for each image. In order to visualize ICG-labelled cells, a near-infrared filter (790 nm diode excitation laser and 800 nm long-pass filter) was used. Cell labelling was achieved with various doses (from 1 to 0.0125 mg; Fig. S4) of 5 mg ICG (Pulsion Medical Systems AG) dissolved in 5 ml of water and administered 3 days before laser induction of CNV, i.p. injection of LPS or imaging of the experimental autoimmune uveoretinitis model. The different routes of administration are summarized in Fig. S5. To assist the identification of laser CNV lesions, infrared-reflectance imaging was performed with an 820 diode excitation laser and no barrier filter. Autofluorescence imaging and fluorescein angiography, a blue-light filter (488 nm solid-state excitation laser and 500 nm long-pass filter) was used. Cell labelling was achieved with various doses (from 1 to 0.0125 mg; Fig. S4) of 5 mg ICG (Pulsion Medical Systems AG) dissolved in 5 ml of water and administered 3 days before laser induction of CNV, i.p. injection of LPS or imaging of the experimental autoimmune uveoretinitis model. The different routes of administration are summarized in Fig. S5. To assist the identification of laser CNV lesions, infrared-reflectance imaging was performed with an 820 diode excitation laser and no barrier filter. Autofluorescence imaging and fluorescein angiography, a blue-light filter (488 nm solid-state excitation laser and 500 nm long-pass filter) was used. Fluorescein angiography was performed 1 week after laser CNV induction with an i.p. injection of 0.2 ml fluorescein sodium (2%). Images were acquired at 90 s and 7 min after injection.

Quantification of ICG+ cells in the retina

Images were exported from the Heidelberg eye explorer version 1.7.1.0 and processed in Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA). Details of image processing and quantification can be found in the Results section and Fig. S5.
Competing interests

The authors declare no competing or financial interests.

Author contributions

D.A.S., C.J.C., S.L., D.A.C., J.W.B.B. and M.F. designed the research. D.A.S., C.J.C., S.S., M.B.P. and S.L. generated and collected data. D.A.S., C.J.C., S.S., M.B.P., S.L., D.A.C., P.A.K., A.T., C.A.E., J.W.B.B., R.W.L., A.D.D. and M.F. analysed and/or interpreted data. D.A.S., C.J.C., S.L., D.A.C., P.A.K., A.T., C.A.E., J.W.B.B., R.W.L., A.D.D. and M.F. wrote and/or revised the manuscript.

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Supplementary information

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