A case of mistaken identity: CD11c-eYFP⁺ cells in the normal mouse brain parenchyma and neural retina display the phenotype of microglia, not dendritic cells.

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Main points:

- CD11c-eYFP⁺ cells in the normal mouse CNS do not phenotypically resemble dendritic cells or their immature precursors
- CD11c-eYFP⁺ cells are present in the neural retina and brain parenchyma of mice with and without the *Crb1*^{rd8} mutation
- CD11c-eYFP⁺ cells are a subpopulation of microglia

Keywords: microglia, central nervous system, *Crumb1*, retinal dystrophic lesions, antigen presenting cells

Abstract:

Under steady-state conditions the central nervous system (CNS) is traditionally thought to be devoid of antigen presenting cells; however, putative dendritic cells (DCs) expressing enhanced yellow fluorescent protein (eYFP) are present in the retina and brain parenchyma of CD11c-eYFP mice. We previously showed that these mice carry the *Crb1*^{rd8} mutation, which causes retinal dystrophic lesions, therefore we hypothesized that the presence of CD11c-eYFP⁺ cells within the CNS may be due to pathology associated with the *Crb1*^{rd8} mutation. We generated CD11c-eYFP *Crb1*^{wt/wt} mice and compared the distribution and immunophenotype of CD11c-eYFP⁺ cells in CD11c-eYFP mice with and without the *Crb1*^{rd8} mutation. The number and distribution of CD11c-eYFP⁺ cells in the CNS was similar between CD11c-eYFP *Crb1*^{wt/wt} and CD11c-eYFP *Crb1*^{rd8/rd8} mice. CD11c-eYFP⁺ cells were distributed throughout the inner retina, and clustered in brain regions that receive input from the external environment or lack a blood-brain barrier. CD11c-eYFP⁺ cells within the retina and cerebral cortex of CD11c-eYFP *Crb1*^{wt/wt} mice expressed CD11b, F4/80, CD115 and Iba-1, but not DC or antigen presentation markers, whereas CD11c-eYFP⁺ cells within the choroid plexus and pia mater expressed CD11c, I-A/I-E, CD80, CD86, CD103, DEC205, CD8α and CD135. The immunophenotype of CD11c-eYFP⁺ cells and microglia within the CNS was similar between CD11c-eYFP *Crb1*^{wt/wt} and CD11c-eYFP *Crb1*^{rd8/rd8} mice; however, CD11c and I-A/I-E expression was significantly increased in CD11c-eYFP *Crb1*^{rd8/rd8} mice. This study demonstrates that the overwhelming majority of CNS CD11c-eYFP⁺ cells do not display the phenotype of DCs or their precursors and are most likely a subpopulation of microglia.

Introduction:

Most peripheral tissues contain resident populations of dendritic cells (DCs), which present antigens to major histocompatibility complex (MHC) class II restricted naive T cells in concert with co-stimulatory molecules CD80 and CD86 (Banchereau and Steinman 1998). In contrast to peripheral tissues, the central nervous system (CNS), which comprises the brain parenchyma proper, spinal cord and neural retina, is considered to have a degree of immune privilege. The brain parenchyma and neural retina lack lymphatic vessels and (to a certain extent) are immunologically sequestered from the systemic circulation by the blood-brain and blood-retina barriers respectively (Galea et al. 2007; Perez and Caspi 2015; Ransohoff and Engelhardt 2012). CNS tissues do contain rich populations of resident microglia; although the question of whether DCs or functional antigen presenting cells are present in the normal CNS remains unanswered.

Early immunohistochemical studies reported that the rat steady-state brain parenchyma and neural retina do not contain MHC class II⁺ cells or OX-62⁺ cells and thus the CNS was traditionally considered to be devoid of antigen presenting cells (Forrester et al. 1994; Matyszak and Perry 1996). Other studies have suggested that the resident microglia of the CNS may be uncommitted myeloid progenitors of immature DCs (Santambrogio et al. 2001). Microglia are highly specialized macrophages that are maintained through *in situ* self-renewal without reconstitution from the bone marrow (Ajami et al. 2007; Ginhoux et al. 2010) and can be driven to a DC-like phenotype *in vitro* (Butovsky et al. 2006; Santambrogio et al. 2001). In the normal mouse CNS, microglia express ionized calcium-binding adapter molecule-1 (Iba-1), CD11b, F4/80 and are CD45 intermediate (CD45^{int}), but very few express MHC class II, CD80 or CD86. In response to injury or inflammation, microglia can upregulate antigen presentation and activation markers; however, they retain poor antigen presentation capabilities (Gregerson et al. 2004; Włodarczyk et al. 2014).

In recent years transgenic reporter mice, in which expression of a fluorescent reporter is driven by the CD11c promoter (*Itgax*), have been used to identify potential DC populations within the CNS. CD11c is

highly expressed by conventional DC subsets and has been widely used as a pan DC marker; however, it can also be expressed by other cell types including macrophages and microglia (Hume 2008; Immig et al. 2015). Using CD11c-eYFP mice, Bulloch et al. identified an extensive network of CD11c-eYFP⁺ cells within the normal mouse brain parenchyma, which were referred to as 'brain DCs' (Bulloch et al. 2008). These data challenged the accepted paradigm that the normal mammalian CNS was devoid of DCs. We extended these findings and identified a population of CD11c-eYFP⁺ cells within the quiescent neural retina of CD11c-eYFP mice (Chen et al. 2013). CD11c-eYFP⁺ cells within the brain parenchyma (Bulloch et al. 2008) and retina (Chen et al. 2013) expressed macrophage markers such as Iba-1, F4/80 and CD68, but were MHC class II negative. It was therefore suggested that CD11c-eYFP⁺ cells within the normal CNS may represent either a macrophage-derived immature DC lineage (Bulloch et al. 2008), or a subset of microglia (Chen et al. 2013).

We previously found that CD11c-eYFP mice display abnormal retinal pathology, due to the presence of the *rd8* mutation in the *Crumb1* (*Crb1*) gene (Chen et al. 2013). *Crb1* encodes a transmembrane protein that is involved in cell-cell contact and is required for formation of the zonula adherens (Klebes and Knust 2000; Tepass 1996). The *rd8* mutation is an autosomal recessive single nucleotide deletion, which results in a premature stop codon and truncation of the transmembrane and cytoplasmic domain of *Crb1* (Mehalow et al. 2003). In humans and mice *Crb1* is expressed exclusively in the eye and brain (den Hollander et al. 2002) and mutations in this gene can result in a range of neurological defects. Screening of a wide range of commercially available mouse colonies from major vendors demonstrated that all C57Bl/6N substrains and embryonic stem cells of C57Bl/6N origin carry the *Crb1*^{rd8} mutation in the homozygous (*Crb1*^{rd8/rd8}) form, whereas C57Bl/6J mice possess the wild type (wt) allele (*Crb1*^{wt/wt}) (Mattapallil et al. 2012).

Our previous work further showed a close spatial relationship between the observed retinal degenerative lesions and CD11c-eYFP⁺ cells in naïve C57Bl/6N CD11c-eYFP *Crb1*^{rd8/rd8} mice (Chen et al. 2013). In the same study, we found that CD11c-GFP⁺ cells were virtually absent from the retinæ of C57Bl/6J

CD11c-DTR-GFP transgenic mice, which do not carry the *Crb1*^{rd8} mutation. On the basis of these findings, we hypothesised that the accumulation of CD11c-eYFP⁺ cells within the retina and brain of CD11c-eYFP *Crb1*^{rd8/rd8} mice may be due to pathology associated with the *Crb1*^{rd8} mutation. To address this hypothesis, we bred out the *Crb1*^{rd8} mutation and compared the distribution of CD11c-eYFP⁺ cells within the brain and retina between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. Furthermore, we performed detailed immunophenotyping of CD11c-eYFP⁺ cells within the normal CNS to determine if these cells are a subpopulation of microglia, or DCs.

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Materials and Methods:

Mice

Naïve CD11c-eYFP *Crb1*^{rd8/rd8}, CD11c-eYFP *Crb1*^{wt/wt}, CD11c-DTR-GFP and C57Bl/6J mice were used at 8-10 weeks of age. CD11c-eYFP *Crb1*^{rd8/rd8} mice (Lindquist et al. 2004) were obtained from Professor Michael Hickey (Monash Medical Centre, Monash University, Clayton, VIC) with permission from Professor Michael Nussenzweig (Rockefeller University, New York, NY). To breed out the *Crb1*^{rd8} mutation, we crossed CD11c-eYFP *Crb1*^{rd8/rd8} mice with C57Bl/6J mice and then intercrossed the heterozygous F1 generation to obtain CD11c-eYFP *Crb1*^{wt/wt} mice, which were maintained on a mixed C57Bl/6N/C57Bl/6J background. CD11c-DTR-GFP mice (Jung et al. 2002) were provided by Dr Frank Carbone (University of Melbourne, Parkville, VIC), who initially obtained the strain from Jackson Laboratories (Sacramento, CA). C57Bl/6J mice were obtained from Monash Animal Services (Clayton, VIC). All experimental procedures were approved by the Monash Animal Research Platform Animal Ethics Committee (approval MARP/2014/074) and were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse genotyping

Mice were screened for the *Crb1*^{rd8} mutation by allele-specific PCR using previously described primers (*Crb1* mF1: GTGAAGACAGCTACAGTTCTGATC, *Crb1* mF2: GCCCCTGTTTGCATGGAGGAACTTGGAAGACAGCTACAGTTCTTCTG and *Crb1* mR: GCCCATTTGCACACTGATGAC; (Mehalow et al. 2003)). Separate reactions were prepared for detection of the wt allele (220 bp, amplified by the *Crb1* mF1 and *Crb1* mR primers) and *rd8* allele (244 bp, amplified by the *Crb1* mF2 and *Crb1* mR primers). Reactions contained 100 µM dNTPs, 3.0 mM MgCl₂, 0.5 U Go *Taq* polymerase (Promega, Madison, WI), 0.5 µM of forward and reverse primer (Geneworks, Perth, WA) and 20 ng of DNA. The wt allele was amplified using previously described

cycling conditions (Chen et al. 2013). A three-step touchdown PCR protocol was used for amplification of the *rd8* allele. Step 1 involved initial denaturation at 95 °C for 5 mins, followed by 15 cycles of : 95 °C for 30 sec, 80-65 °C (temperature decreased by 1 ° per cycle) for 30 sec and 72 °C for 20 sec. Step 2 involved : 95 °C for 30 sec, 65 °C for 30 sec and 72 °C for 20 sec. A final extension step at 72 °C for 5 mins was then performed. Amplicons were separated on a 3.0% (w/v) agarose gel. PCR results were confirmed by sequencing of the *Crb1* gene (supplementary figure 1), using previously described primers (Mattapallil et al. 2012).

CD11c-eYFP *Crb1^{rd8/rd8}* and CD11c-eYFP *Crb1^{wt/wt}* mice were also genotyped for the presence of the transgenic reporter, using primers oIMR9715: 5' TGCTGGTTGTTGTGCTGTCTCATC 3' and oIMR9716: 5' GGGGGTGTCTGCTGGTAGTGGTC 3' (Jackson Laboratories https://www2.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:6851,009422). CD11c-DTR-GFP mice were genotyped using primers DTR-1: 5' GCCACCATGAAGCTGCTGCCG 3' and DTR-2: 5' TCAGTGGGAATTAGTCATGCC 3' (Jung et al. 2002).

In vivo imaging of the retina

CD11c-eYFP *Crb1^{rd8/rd8}* (n = 55), CD11c-eYFP *Crb1^{wt/wt}* (n = 121), CD11c-DTR-GFP (n = 61) and C57Bl/6J (n = 6) mice underwent clinical fundus imaging using a Micron III camera (Phoenix Research Labs, Pleasanton, CA) and StreamPix 5 software (NorPix, Montreal, QC) as previously described (Chen et al. 2013).

Tissue collection and processing

Mice were humanely killed by an intraperitoneal injection of sodium pentobarbital and were then perfused through the left ventricle with 25 mL of phosphate buffered saline (PBS) containing 1% (v/v) heparin followed by 25 mL of 4% paraformaldehyde (PFA). The eyes and brains were collected and stored in 4% PFA at 4 °C overnight. Retinae were dissected from the posterior eye cup as previously

described (McMenamin 2000) and radial incisions were made to flatten the retina prior to whole mount immunostaining. Brains were incubated in 10% (w/v) sucrose in PBS for 48 hours at 4 °C, then 30% (w/v) sucrose in PBS for 48 hours at 4 °C before being embedded in Tissue-Tek optimal cutting temperature medium (Sakura Finetek, Torrance, CA) and frozen in chilled isopentane (Sigma Aldrich, Castle Hill, NSW). Free-floating, 40 µm brain sections were cut on a cryostat (Leica Microsystems, Mannheim, Germany) and stored in cryoprotectant solution (Watson et al. 1986) at -20 °C.

Immunofluorescence staining and confocal microscopy

Retinae and free-floating brain sections were permeabilised in 20 mM EDTA at 37 °C for 1 hour. Tissues were blocked in 3.0% (w/v) bovine serum albumin (Sigma) and 0.3% (v/v) Triton X-100 (ProSciTec, Kirwan, QLD) in PBS for 1 hour at room temperature prior to being incubated with primary antibodies (rat anti-mouse MHC class II [I-A/I-E] and rabbit anti-Iba-1; Table 1) overnight at 4 °C. Tissues were washed in PBS, and subsequently incubated with fluorophore-labelled secondary antibodies (Table 1) and Hoechst (1:1000) for 2 hours at room temperature. Tissues were again washed and then mounted onto microscope slides and coverslipped. Samples were imaged with an inverted SP5 5-channel confocal microscope (Leica Microsystems) using a x20 objective (numerical aperture 0.7) or x63 objective (numerical aperture 1.3). Z stacks were captured every 2 µm and maximum projection images were created using FIJI (Schindelin et al. 2012). Brightness and contrast adjustments were performed using Adobe Photoshop CC.

Quantitative analysis of CD11c-eYFP⁺ cells in the inner retina

To quantify CD11c-eYFP⁺ cells in the inner retina, retinae from CD11c-eYFP *Crb1*^{rd8/rd8} (n = 4) and CD11c-eYFP *Crb1*^{wt/wt} (n = 6) mice were mounted vitreous side up and imaged with a SP5 5-channel confocal microscope (Leica Microsystems) using a x40 objective (numerical aperture 1.25). Three random fields of view, each representing 0.1502 mm², were captured for each mouse. The number of CD11c-eYFP⁺ cells in maximum projection images were manually counted using FIJI (Schindelin et al.

2012). The total number of CD11c-eYFP⁺ cells across the three fields of view was divided by the total area analysed; hence data are represented as cells/mm².

Quantitative analysis of CD11c-eYFP⁺ cells in the subretinal space

To quantify CD11c-YFP⁺ cells in the subretinal space, retinal and choroidal whole mounts (n = 6 mice per group) were analysed by epifluorescence microscopy (Provis AX70, Olympus, Tokyo, Japan) using a x20 objective and GFP filter as previously described (Chinnery et al. 2015).

Immunoperoxidase staining and anatomical mapping of CD11c-eYFP⁺ cells in the brain

To determine the distribution of CD11c-eYFP⁺ cells in the brain, immunoperoxidase staining was performed on free-floating, coronal sections using an anti-GFP antibody, which also strongly binds to YFP. Brain sections from CD11c-eYFP *Crb1*^{rd8/rd8} mice (n = 5), CD11c-eYFP *Crb1*^{wt/wt} mice (n = 4), CD11c-DTR-GFP mice (n = 4) and C57Bl/6J mice (n = 2) were examined. Sections were initially incubated in 10% (v/v) methanol (Sigma Aldrich) and 0.6% (v/v) hydrogen peroxide (Merck Millipore, Darmstadt, Hesse) in PBS for 30 mins. Sections were then washed in PBS and incubated in blocking solution (as described above) for 30 mins. Sections were incubated in polyclonal rabbit anti-GFP (2.5 µg/mL; Merck Millipore) in blocking solution overnight at 4 °C, and then washed. Biotinylated goat anti-rabbit secondary antibody (1:400; Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature and avidin-biotin complexes were formed using Vectastain Elite ABC Kit (Vector Laboratories; 1:400). Anti-GFP labelling was detected using Sigmafast 3,3'-diaminobenzidine tablets (Sigma Aldrich). Stained sections were mounted onto Super Frost Plus microscope slides and allowed to dry overnight. Slides were then rehydrated in water and counterstained with 0.1% (w/v) cresyl violet, before being dehydrated in 100% ethanol and xylene and coverslipped with DPX mounting medium. Slides were scanned at x40 magnification using an Aperio ScanScope (Leica Biosystems)

The anatomical distribution of labelled cells was determined by mapping CD11c-eYFP⁺ cells onto coronal section figures from 'The Mouse Brain in Stereotactic Coordinates' (Franklin and Paxinos 2007)

in Adobe Illustrator CC. A digital image of each stained section was overlaid onto the corresponding coronal section figure from the atlas. Labelled cells were mapped as dots onto the brain atlas image, which enabled their anatomical distribution to be determined. Often CD11c-eYFP⁺ cells were observed in clusters, thus each dot may represent between 1 and 4 CD11c-eYFP⁺ cells.

Flow cytometry immunophenotyping of CD11c-eYFP⁺ cells

Pooled spleens, retinæ and brains were collected from mice perfused with PBS containing 1% (v/v) heparin (CD11c-eYFP *Crbl*^{rd8/rd8} mice: n = 7; CD11c-eYFP *Crbl*^{wt/wt} mice: n = 7). Brains were microdissected to isolate samples of choroid plexus, cortex and pia mater. Briefly, the choroid plexi were removed from the lateral and fourth ventricles of the brain using fine forceps under a dissecting microscope. A 2 mm tissue biopsy punch was then used to collect multiple samples of brain parenchyma from the cerebral cortex; the superior surface of the core samples was then trimmed off using a flat-edged razor blade to obtain samples predominantly containing pia mater. Any inner part of the core sample that contained ventricle or ependyma was also trimmed and the remaining sample containing cerebral cortex was collected. Dissected cortex and pia mater samples were then pooled into separate tubes; the remainder of the brain was also pooled. Tissues were processed into single cell suspensions in FACS buffer (PBS containing 0.1% (v/v) bovine serum albumin and 1 mM EDTA) by passing through a 70 µm nylon cell strainer (Falcon, Corning, NY). Spleen cell suspensions were treated with red cell lysing buffer (Sigma Aldrich) as per the manufacturer's instructions. To enrich for brain leukocytes, cortex and pia samples were resuspended in 30% (v/v) Percoll (GE Healthcare, Little Chalfont, Buckinghamshire) and centrifuged at 700 x g for 10 mins at 4 °C without brake. The myelin layer was removed; FACS buffer was then added and the cells were pelleted at 400 x g for 5 mins at 4 °C.

Cell suspensions were stained with fixable viability dye eFluor 450 (eBioscience, San Diego, CA) according to the manufacturer's instructions and then incubated in mouse Fc block (Table 1) for 15 mins on ice. Cells were then incubated with fluorophore-conjugated antibodies (Table 1) for 30 mins on ice, washed in FACS buffer and subsequently fixed in 2% PFA in FACS buffer for 15 mins. For intracellular

staining, fixed cells were permeabilised in 0.1% Triton X-100 in PBS for 45 mins and then stained with antibody for 45 mins on ice. Samples were acquired using a LSR Fortessa X-20 flow cytometer (BD Biosciences) and a compensation matrix was applied post-acquisition in FlowJo (version 10.1, Tree Star, Ashland, OR). CD11c-eYFP⁺ cells were gated as follows: single cells → viable cells → non-debris → CD45⁺ YFP⁺. Microglia within the retina and cortex cell suspensions were defined as: single cells → viable cells → non-debris → CD11b⁺ CD45^{int}. Due to the limited number of cells available for analysis in some tissues, we calculated the coefficient of variation (CV) for each sample according to published formulae for the analysis of rare events by flow cytometry (Hoy 2001). The coefficient of variation for each sample (supplementary table 1) was within the acceptable limits for rare event analysis (Donnenberg and Donnenberg 2007). Gates for fluorescent parameters were set using fluorescence minus one and unstained controls.

Statistical analysis

Unpaired, two-tailed t tests were used to compare the density of CD11c-eYFP⁺ cells in the retina and subretinal space between mouse strains. The percentage of cells expressing cell surface markers was compared between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice using chi-square tests. Statistical analyses were performed in Graph Pad Prism (version 6, La Jolla, CA) and significance was accepted as $p < 0.05$.

Results:*Retinal degenerative lesions are absent in CD11c-eYFP $Crb1^{wt/wt}$ mice*

In vivo fundus imaging of naïve CD11c-eYFP $Crb1^{rd8/rd8}$ mice revealed the presence of retinal lesions and a network of CD11c-eYFP⁺ cells (figure 1). To address our hypothesis that the accumulation of CD11c-eYFP⁺ cells within the retina of CD11c-eYFP $Crb1^{rd8/rd8}$ mice may be due to pathology associated with the $Crb1^{rd8}$ mutation, we generated CD11c-eYFP $Crb1^{wt/wt}$ mice. Brightfield imaging revealed normal appearance of the fundus of CD11c-eYFP $Crb1^{wt/wt}$ mice, with an obvious lack of retinal degenerative lesions, similar to C57Bl/6J mice. Unexpectedly, fluorescent imaging demonstrated the continued presence of a network of CD11c-eYFP⁺ cells throughout the retina (figure 1). These cells were similar in appearance and distribution to those observed in CD11c-eYFP $Crb1^{rd8/rd8}$ mice. Retinal lesions were not observed in the fundus of CD11c-DTR-GFP mice (supplementary figure 2A), nor were any fluorescent cells detected in the retinæ of these mice (supplementary figure 2B).

Distribution and quantitation of CD11c-eYFP⁺ cells in retinal whole mounts

CD11c-eYFP⁺ cells were uniformly distributed throughout the retina in both CD11c-eYFP $Crb1^{rd8/rd8}$ and CD11c-eYFP $Crb1^{wt/wt}$ mice (figure 2A). CD11c-eYFP⁺ cells were observed surrounding the optic nerve head and throughout the central and peripheral margins of the retina. CD11c-eYFP⁺ cells were predominantly located within the ganglion cell layer, inner plexiform layer and outer plexiform layer of the retina (figure 2B). CD11c-eYFP⁺ cells were also occasionally seen in the subretinal space (figure 2C). Disruption of the outer nuclear layer was observed in CD11c-eYFP $Crb1^{rd8/rd8}$ mice, but not CD11c-eYFP $Crb1^{wt/wt}$ mice (Figure 2C). The density of CD11c-eYFP⁺ cells/mm² in the retina (figure 2D) and subretinal space (figure 2E) did not differ between CD11c-eYFP $Crb1^{rd8/rd8}$ and CD11c-eYFP $Crb1^{wt/wt}$ mice ($p > 0.05$). Consistent with *in vivo* imaging, GFP⁺ cells were not detected in whole mount retinæ from CD11c-DTR-GFP mice (supplementary figure 2C-D).

CD11c-eYFP⁺ cells within the mouse retina display the phenotype of microglia

We next sought to define the immunophenotype of retinal CD11c-eYFP⁺ cells to: (i) characterise their expression of macrophage/microglia, DC and antigen presentation cell surface markers; and (ii) determine if the *Crbl*^{rd8} mutation affected the phenotype of these cells. Using confocal microscopy, we demonstrated that retinal CD11c-eYFP⁺ cells from both CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice expressed Iba-1. A small proportion of CD11c-eYFP⁺ cells located at the peripheral margins of the retina (figure 3A) and surrounding the optic nerve head (figure 3B) also expressed MHC class II (I-A/I-E⁺); however, all CD11c-eYFP⁺ cells located within the central retina (figure 3C) and subretinal space (supplementary figure 3) were I-A/I-E⁻. Retinal CD11c-eYFP⁺ cells resembled typical Iba-1⁺ microglia, with a small cell body extending multiple, branching processes.

We next analysed retinal microglia and CD11c-eYFP⁺ cells by flow cytometry, and compared the expression of surface markers between CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice (figure 4A). Retinal CD11c-eYFP⁺ cells from both strains of mice were CD45^{int} and expressed the myeloid cell marker CD11b, the mouse macrophage marker F4/80 and the macrophage colony-stimulating factor receptor CD115, which is consistent with the phenotype of microglia in the quiescent retina. In contrast, very few retinal CD11c-eYFP⁺ cells expressed DC subset markers (CD103, CD8α, DEC205 and 33D1), fms-like tyrosine kinase-3 (Flt3) receptor (CD135), co-stimulatory molecules (CD80 and CD86) and/or the conventional DC-specific transcription factor Zbtb46. Intriguingly, although YFP expression is driven by the CD11c promoter, only 6.2% of retinal CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{wt/wt} mice expressed CD11c. CD11c expression was significantly increased in retinal CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} mice (19%, *p* = 0.005). In addition, a significantly higher percentage of retinal CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} mice were I-A/I-E⁺, compared to CD11c-eYFP *Crbl*^{wt/wt} mice (34.6% versus 4.8%, *p* = 0.0001). Fewer CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} mice expressed F4/80 (*p* = 0.003), associated with a decrease in F4/80 median fluorescence intensity (MFI, figure 4B).

As mentioned above, the phenotype of CD11c-eYFP⁺ cells was remarkably similar to that of retinal microglia (CD45^{int} CD11b⁺), which expressed F4/80 and CD115, but did not express DC or antigen presentation markers (figure 4A). CD11c was expressed by 3.5% and 7.8% of microglia from CD11c-eYFP *Crbl*^{wt/wt} and CD11c-eYFP *Crbl*^{rd8/rd8} mice respectively. Microglia were typically I-A/I-E⁻; however, a significantly higher percentage of CD11c-eYFP *Crbl*^{rd8/rd8} microglia were I-A/I-E⁺ compared to CD11c-eYFP *Crbl*^{wt/wt} microglia (6.2% versus 0.4%, $p = 0.013$). Interestingly, 25.1% of CD11c-eYFP *Crbl*^{wt/wt} microglia and 39.7% of CD11c-eYFP *Crbl*^{rd8/rd8} microglia also expressed YFP (supplementary figure 4).

To determine if the phenotypic differences observed between CD11c-eYFP *Crbl*^{wt/wt} and CD11c-eYFP *Crbl*^{rd8/rd8} mice were specific to CNS tissues, we also analysed the surface phenotype of CD11c-eYFP⁺ cells from the spleen (supplementary figure 5A). There were no differences in the percentage of positive cells between mice with or without the *Crbl*^{rd8} mutation ($p > 0.05$), but a decrease in the intensity of F4/80 expression was again observed in CD11c-eYFP⁺ cells from the spleen of CD11c-eYFP *Crbl*^{rd8/rd8} mice (supplementary figure 5B). Taken together, these data demonstrate that retinal CD11c-eYFP⁺ cells cannot be readily distinguished from microglia based on their phenotype, and that the observed phenotypic differences between resident leukocytes of CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice are specific to neural tissues.

*CD11c-eYFP⁺ cells cluster around specific neuroanatomical landmarks within the brains of CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice*

Crumbs 1 is expressed in both the brain and retina, therefore we next sought to map the anatomical location of CD11c-eYFP⁺ cells within brain sections of CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice. CD11c-eYFP⁺ cells were distributed bilaterally throughout the brains of CD11c-eYFP mice with and without the *Crbl*^{rd8} mutation (supplementary figure 6A-C). Three distinct morphologies of CD11c-eYFP⁺ cells were observed: (i) amoeboid cells with few, thick process were found in the choroid plexus and pia mater (figure 5A); (ii) elongated CD11c-eYFP⁺ cells extending thin bipolar processes were

seen in white matter tracts (figure 5B); and (iii) highly ramified/dendriform cells, the most common morphotype, were observed in several regions, including the piriform cortex (figure 5C).

The anatomical location of CD11c-eYFP⁺ cells in adult CD11c-eYFP *Crb1*^{rd8/rd8} mice was extensively described by Bulloch and colleagues (Bulloch et al. 2008). Similar to this previous study, we found that there were distinct clusters of CD11c-eYFP⁺ cells throughout the glomerular layer of the olfactory bulbs and the olfactory nerve (figure 5D). CD11c-eYFP⁺ cells persistently surrounded the olfactory ventricle, and were highly concentrated around the lateral and third ventricles (figure 5E-G), the choroid plexus and pia mater. CD11c-eYFP⁺ cells were also located within the rostral migratory stream and subventricular zone of the lateral ventricles (supplementary figure 7). Interestingly, the majority of CD11c-eYFP⁺ cells within the cerebrum were located ventrally, rather than in dorsal regions. Although labelled cells were observed randomly throughout the cerebral cortex (in regions such as the primary and secondary motor cortex, agranular insular cortex, medial orbital cortex and somatosensory cortex), distinct clusters of CD11c-eYFP⁺ cells were consistently found within ventral regions including the piriform cortex, olfactory tubercle, ventral pallidum and ventral regions of the striatum (figure 5F). CD11c-eYFP⁺ cells were consistently distributed in white matter tracts including the corpus callosum, anterior commissure, internal capsule, medial amygdaloid nucleus and optic tract (figure 5F-G). Circumventricular organs such as the subfornical organ, vascular organ of the lamina terminalis and area postrema also contained clusters of CD11c-eYFP⁺ cells.

Within the hindbrain, labelled cells were prominent in regions associated with the trigeminal nerve, including the sensory and motor root of the trigeminal nerve (figure 5H), the interpolar part of the spinal trigeminal nucleus, the spinal trigeminal tract and the dorsomedial spinal trigeminal nucleus (figure 5I). Within the cerebellum, CD11c-eYFP⁺ cells were located primarily within white matter tracts, but were also scattered throughout the granule layer. The distribution of CD11c-eYFP⁺ cells within the brain was very similar between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice, with the exception of the spinal trigeminal regions, in which there appeared to be fewer labelled cells in CD11c-eYFP *Crb1*^{rd8/rd8}

mice (figure 5I). Examination of CD11c-DTR-GFP brain sections revealed that there were occasional GFP⁺ cells present within the ependymal cells lining the lateral ventricles (supplementary figure 2E), but no GFP⁺ cells were detected within the brain parenchyma (supplementary figure 2F).

The brain contains distinct CD45^{int} and CD45^{hi} CD11c-eYFP⁺ populations

Flow cytometric analysis of CD11c-eYFP⁺ cells from whole brain preparations demonstrated the presence of CD45^{int} and CD45^{hi} populations in both CD11c-eYFP *Crb1^{rd8/rd8}* (figure 6A) and CD11c-eYFP *Crb1^{wt/wt}* mice (figure 6B). CD11c, I-A/I-E and Zbtb46 expression was notably increased in CD45^{hi} cells compared to CD45^{int} cells. We hypothesized that the CD11c-eYFP⁺ CD45^{hi} population represented contaminating cells from the choroid plexus and/or pia mater, which are not part of the brain parenchyma. Therefore, in subsequent experiments we performed brain microdissections and separately analysed the immunophenotype of CD11c-eYFP⁺ cells from the choroid plexus, pia mater and cerebral cortex.

CD11c-eYFP⁺ cells within the choroid plexus and pia mater express macrophage, DC and antigen presentation markers

CD11c-eYFP⁺ cells within the choroid plexus and pia mater co-localised with Iba-1 staining in brain sections (figure 7A). The majority of CD11c-eYFP⁺ cells within the choroid plexus and pia mater were also I-A/I-E⁺. As expected, CD11c-eYFP⁺ cells from the choroid plexus and pia mater contained a mixture of CD45^{int} and CD45^{hi} cells (figure 7B). Choroid plexus and pia mater CD11c-eYFP⁺ cells displayed a heterogeneous immunophenotype, with multiple peaks seen in flow cytometry histograms (figure 7B). Similar to spleen and retinal CD11c-eYFP⁺ cells, choroid plexus and pia mater CD11c-eYFP⁺ cells expressed CD11b, F4/80 and CD115. Populations of CD11c⁺, CD103⁺, CD80⁺, CD86⁺, CD8α⁺, CD135⁺ and DEC205⁺ cells were also seen in the choroid plexus and pia mater, which is consistent with the fact that rich populations of DCs and macrophages are known to reside in these tissues.

CD11c-eYFP⁺ cells within the brain parenchyma display the phenotype of microglia

The majority of CD11c-eYFP⁺ cells within the brain parenchyma resembled microglia. Confocal microscopy demonstrated that both the ramified (figure 8A) and elongated (figure 8B) CD11c-eYFP⁺ cells co-localised with Iba-1 staining, but not I-A/I-E. CD11c-eYFP⁺ I-A/I-E⁺ cells were occasionally found scattered throughout the spinal trigeminal regions (figure 8C); these cells were similar in appearance to the stout CD11c-eYFP⁺ cells that were located within the choroid plexus and pia mater.

Flow cytometric analysis demonstrated that cerebral cortex CD11c-eYFP⁺ cells were CD11b⁺, CD45^{int}, F4/80⁺, CD115⁺, and largely negative for CD103, CD80, CD86, CD8α, CD135, DEC205 and 33D1 expression (figure 9A). Similar to retinal CD11c-eYFP⁺ cells, the phenotype of these brain parenchymal CD11c-eYFP⁺ cells was indistinguishable from that of microglia. The percentage of CD11c-eYFP⁺ cells expressing CD11c was significantly higher in CD11c-eYFP *Crbl*^{rd8/rd8} mice compared to CD11c-eYFP *Crbl*^{wt/wt} mice (23.1% versus 11.3%, *p* = 0.02). Furthermore, the percentage of CD11c-eYFP⁺ cells expressing I-A/I-E was increased in CD11c-eYFP *Crbl*^{rd8/rd8} mice (32% versus 7.4%, *p* = 0.0001). An increase in the MFI of CD11c and I-A/I-E was also observed in CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} mice (figure 9B). Cortical microglia from CD11c-eYFP⁺ *Crbl*^{wt/wt} mice did not express CD11c (0.9%); however, 7.1% of microglia from CD11c-eYFP *Crbl*^{rd8/rd8} mice were CD11c⁺ (*p* = 0.03). Similarly, the percentage of microglia expressing I-A/I-E was increased in CD11c-eYFP *Crbl*^{rd8/rd8} mice compared to CD11c-eYFP *Crbl*^{wt/wt} mice (8.8% versus 0.1%, *p* = 0.002).

The brain flow cytometry immunophenotyping data is summarised in figure 9C. This heat map highlights: (i) stark differences in the expression of surface markers between CD11c-eYFP⁺ cells from the choroid plexus and pia compared to those from the cortex, particularly with respect to DC and antigen presentation markers; (ii) that the phenotype of cortical CD11c-eYFP⁺ cells resembles with that of microglia; and (iii) subtle differences in the phenotype of cortical CD11c-eYFP⁺ cells and microglia between CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice.

Discussion:

The recent identification of large numbers of putative DCs within the brain parenchyma (Bulloch et al. 2008) and neural retina (Chen et al. 2013) of CD11c-eYFP mice potentially has significant implications for our understanding of immune mechanisms within the CNS; however, these findings may be confounded by our discovery that CD11c-eYFP mice carry the *Crbl^{rd8}* mutation and that CD11c-eYFP⁺ cells co-localise with retinal degenerative lesions in CD11c-eYFP *Crbl^{rd8/rd8}* mice (Chen et al. 2013). This led us to suspect that their accumulation within the retina, and possibly the brain, may be due to pathology associated with the *Crbl^{rd8}* mutation. This would have implied that the retina and brain of mice carrying the *Crbl^{rd8}* mutation are unlikely to be representative of normal steady-state tissue, and thus further scrutiny of CNS CD11c-eYFP⁺ cells was warranted. Therefore, in the present study we sought to compare the distribution and immunophenotype of CNS CD11c-eYFP⁺ cells between CD11c-eYFP *Crbl^{rd8/rd8}* and CD11c-eYFP *Crbl^{wt/wt}* mice.

DCs are broadly grouped into conventional and plasmacytoid subtypes, which are derived from common myeloid progenitors. DC development is dependent on Flt3 signalling and thus both conventional and plasmacytoid DC precursors express the Flt3 receptor, CD135 (Karsunky et al. 2003). Immature conventional DCs and their progenitors also express the transcription factor Zbtb46 (Meredith et al. 2012). In contrast, common monocyte progenitors lack CD135 and Zbtb46 expression, but express the macrophage colony stimulating factor receptor CD115 (Schraml and Reis e Sousa 2015). Mature conventional DCs express high levels of CD11c and can be further divided into migratory or lymphoid DCs based on CD11b, CD4, CD8 α , CD103 and DEC205 expression (Shortman and Liu 2002). Upon activation DCs express high levels of MHC class II, CD80 and CD86 (Banchereau and Steinman 1998). In the present study, we analysed the immunophenotype of CD11c-eYFP⁺ cells from the retina and cerebral cortex of CD11c-eYFP *Crbl^{wt/wt}* mice and found that very few of these cells expressed DC subset markers or antigen presentation/activation markers, indicating that they are not mature DCs. Given that the overwhelming majority of CNS CD11c-eYFP⁺ cells also did not express CD135 or Zbtb46 our data

suggest that these cells are unlikely to be immature DCs or their progenitors. Rather, CD11c-eYFP⁺ cells were CD45^{int}, Iba-1⁺, CD11b⁺, F4/80⁺, CD115⁺ and thus could not be phenotypically distinguished from microglia.

Microglia are now recognised as a collection of diverse subtypes that can be distinguished based on phenotype and function (Gertig and Hanisch 2014; Hanisch 2013). This diversity is particularly evident during inflammation and it has been suggested that subsets rather than a uniform population of microglia may be responsible for responding to danger signals and coordinating tissue responses (Scheffell et al. 2012). Specifically, it has been shown that only subpopulations of murine microglia upregulate MHC class II following IFN- γ challenge. As another example of functional diversity, MHC class II⁺ microglia selectively internalise exosomes from oligodendrocytes, suggesting overall that distinct microglial subpopulations may be responsible for the functions of micropinocytosis and antigen presentation (Fitzner et al. 2011). Heterogeneous microglia responses have also been observed following lipopolysaccharide challenge, with the detection of functionally distinct populations of microglia on the basis of TNF- α , CCL3 and COX2 expression (Scheffell et al. 2012). Microglia diversity is also observed under physiological conditions, with only a subpopulation of microglia from the neonatal and adult mouse brain having the ability to phagocytose myelin (Scheffell et al. 2012). Our data posit that the overwhelming majority of CD11c-eYFP⁺ cells within the neural retina and brain parenchyma are a subset of microglia that cannot be distinguished from microglia by immunophenotype in the normal CNS. Whether these cells have a specific or divergent function that distinguishes them from other microglia populations remains a question for future research.

Consistent with previous reports, we found that fluorescent reporter levels did not correlate with CD11c expression (Anandasabapathy et al. 2011; Bulloch et al. 2008; Lehmann et al. 2010), as only a small percentage of YFP⁺ cells expressed CD11c. Although the reasons for this are unclear, brain CD11c-eYFP⁺ cells can upregulate CD11c expression following GM-CSF stimulation *in vitro* (Bulloch et al. 2008), indicating that its expression is inducible in these cells. It has also been suggested that the 5.5 kb

Itgax promoter fragment used in these transgenic mice does not contain all of the control elements required to precisely recapitulate CD11c expression (Bar-On and Jung 2010; Hume 2011), which may partially explain the discordance between fluorescent reporter and CD11c expression. Alternatively, expression of CD11c but not YFP may be regulated at the post-transcriptional level.

Regardless of the reasons behind this discrepancy, our analysis revealed that an extensive network of CD11c-eYFP⁺ cells is present in the retina of CD11c-eYFP *Crb1*^{wt/wt} mice, suggesting that this is not wholly a consequence of the *Crb1*^{rd8} mutation. CD11c-eYFP⁺ cells were distributed predominantly within the ganglion cell layer, inner plexiform layer and outer plexiform layer of the retina, which corresponds with the normal distribution of retinal microglia (Hume et al. 1983). There were no differences in the density of CD11c-eYFP⁺ cells/mm² in the retina and subretinal space between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. The density of Iba-1⁺ microglia/mm² in the retina and subretinal space also did not differ between these mouse strains (data not shown).

Our study does not support the generic classification of retinal CD11c-eYFP⁺ cells as DCs; however, others have identified putative DC populations within the quiescent mouse retina. Xu et al. (2007) observed low numbers of MHC class II⁺ cells, restricted in distribution to the juxtapapillary area and the peripheral margins of the normal mouse retina, which are sites of early retinal inflammation in experimental autoimmune uveitis (EAU). Unlike the CD11c-eYFP⁺ cells characterised in the present study, the MHC class II⁺ cells identified by Xu et al. did not express F4/80 and were weakly positive for CD11b. The function of these cells is yet to be determined; however, the presence of retinal MHC class II⁺ cells promoted resistance to EAU (Xu et al. 2007). Using the CD11c-DTR-GFP transgenic mouse line, Lehmann et al. identified 95 ± 42 GFP⁺ cells in the quiescent retina. These cells were CD45^{int}, CD11b⁺, but only 4.4% were MHC class II⁺ (Lehmann et al. 2010). Following optic nerve crush injury, these cells upregulated MHC class II expression (49.6%), whereas retinal microglia did not. Functional assays demonstrated that CD11c-DTR-GFP⁺ cells from the normal mouse retina promoted the generation of

Foxp3⁺ T cells and inhibited splenic DCs from activating naïve CD4⁺ T cells (Heuss et al. 2012), suggesting a role in immune regulation.

In contrast to Lehmann et al., our analysis of CD11c-DTR-GFP mice did not identify GFP⁺ cells within the quiescent retina. It is well known that CD11c-DTR-GFP mice exhibit weak GFP expression, but in our study sensitive anti-GFP immunostaining failed to detect significant numbers of retinal GFP⁺ cells. We also detected only low numbers of GFP⁺ cells within the brains of CD11c-DTR-GFP mice. GFP⁺ cells were largely restricted to the ependymal lining of the lateral ventricles, but were absent in the brain parenchyma. In contrast, Prodinger et al. described CD11c-DTR-GFP⁺ cells within the corpus callosum, fornix and white matter tracts of the olfactory bulbs, brain stem, cerebellum and spinal cord. The majority of GFP⁺ cells were located in the juxtavascular parenchyma and extended processes into the glia limitans, suggesting that they are uniquely positioned to present intraparenchymal antigens to T cells within the perivascular spaces (Prodinger et al. 2011). The differences in the number and distribution of GFP⁺ cells between these studies may be attributed to variations between mouse colonies worldwide, including differences in environmental factors and microbiota that may alter the immune system (Wu and Wu 2012).

As observed in the retina, the distribution of CD11c-eYFP⁺ cells within the brain also did not significantly differ between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. In agreement with Bulloch et al. (2008), we found that CD11c-eYFP⁺ cells clustered in regions of the brain that receive input from the external environment or lack a blood-brain barrier. In particular, the olfactory nerve, the glomerular layer of the olfactory bulbs and the trigeminal nerve were 'hot spots' for CD11c-eYFP⁺ cells. Within the olfactory pathway, olfactory sensory neurons within the olfactory epithelium project cilia into the nasal cavity mucosa. From the olfactory epithelium, olfactory axons course through the lamina propria, penetrate the cribriform plate and enter the olfactory bulbs (Dando et al. 2014). Similarly, the V1 and V2 branches of the trigeminal nerve innervate the olfactory and respiratory epithelia of the nasal cavity and then project to the olfactory bulb (V1), or brainstem (V1, V2 and V3 branches) (Schaefer et al.

2002). These pathways provide potential routes of entry to the brain for airborne chemicals, allergens and a wide range of pathogens (Dando et al. 2014). CD11c-eYFP⁺ cells were also seen within the choroid plexus which, although not part of the brain parenchyma, is an important site of entry for macromolecules, trafficking immune cells and pathogens into the cerebrospinal fluid and sub-arachnoid space (Schwerk et al. 2015). In addition, CD11c-eYFP⁺ cells were found within the circumventricular organs, which possess a fenestrated vascular network and thus lack a blood-brain barrier (Morita et al. 2015). Taken together, our data suggest that CD11c-eYFP⁺ cells are located in the regions of the brain that are the most susceptible to insult from the 'external' milieu.

Consistent with Bulloch et al. (2008), CD11c-eYFP⁺ cells were also distributed in regions of neurogenesis, such as the subventricular zones of the lateral ventricles, and throughout the rostral migratory stream. Microglia within the subgranular zone of the hippocampus and subventricular zone of the lateral ventricles regulate neurogenesis via the secretion of neurotrophic factors, fractalkine/CX3CR1 signalling and phagocytosis of apoptotic cells (Sato 2015). Therefore, it is possible that CD11c-eYFP⁺ cells within the subventricular zone and rostral migratory stream may also play a role in the regulation of neurogenesis; a matter that remains an area for future research. Recently, Mohammad et al. (2014) identified a significant number of myeloid-derived CD11c⁺ cells within the rostral migratory stream of normal adult mice. Following intracerebroventricular infusion of CFSE, labelled CD11c⁺ cells were observed within the cervical lymph nodes, but not other secondary peripheral immune organs, via a mechanism that was pharmacologically inhibited by the immune-sequestering drug fingolimod. The authors proposed a model of immunosurveillance in which CD11c⁺ cells: (i) are recruited to the subventricular zone and rostral migratory stream; (ii) migrate along the rostral migratory stream towards the olfactory bulb and encounter CNS antigens; (iii) exit the brain at the central terminus of the rostral migratory stream in the olfactory bulb via the cribriform plate and (iv) access the cervical lymph nodes where they promote Treg function and suppress naïve and memory T cell responses (Mohammad et al. 2014). Although we found that CD11c-eYFP⁺ cells were highly concentrated along the rostral migratory

stream and the olfactory bulb, it is currently unclear how the cells identified in our study relate to the CD11c⁺ cells identified by Mohammad et al. as this area of the brain cannot be easily identified by microdissection and was thus not further scrutinised by flow cytometry.

Flow cytometric analysis of enriched leukocytes from whole brain cell suspensions revealed the presence of CD45^{int} and CD45^{hi} CD11c-eYFP⁺ populations. Consistent with previous studies, we demonstrated by using a tissue microdissection approach to separate the choroid plexus, pia mater and cerebral cortex, that the CD45^{hi} cells were mostly contaminants from the choroid plexus and pia mater (Anandasabapathy et al. 2011). CD11c-eYFP⁺ cells from the choroid plexus and pia mater expressed mature DC and macrophage markers, and were phenotypically distinct from those within the parenchyma of the cerebral cortex. It has been well established that the choroid plexus and meninges are a rich source of macrophages and DCs (Chinnery et al. 2010; McMenamin 1999; Quintana et al. 2015). Meningeal/choroid plexus DCs significantly expand in numbers following Flt3 treatment and have a similar transcriptional profile to CD8⁺ spleen DCs. In contrast to microglia, meningeal/choroid plexus DCs can stimulate allogenic T cells and present peptides to naïve myelin oligodendrocyte glycoprotein-specific CD4⁺ T cells *in vitro* (Anandasabapathy et al. 2011). At the present time, there have been few functional studies of CNS CD11c-eYFP⁺ cells. Gottfried-Blackmore et al. showed that intracerebral injection of IFN- γ into the CA1 region of the hippocampus induced MHC class II expression on brain CD11c-eYFP⁺ cells, but did not affect CD80, CD86 or CD40 expression. Following IFN- γ treatment, brain CD11c-eYFP⁺ cells and microglia stimulated the proliferation of naïve OVA-specific T cells, but only CD11c-eYFP⁺ cells stimulated cytokine production (Gottfried-Blackmore et al. 2009). The antigen presenting capability and thus function of CD11c-eYFP⁺ cells in the normal brain and retina are yet to be determined, but investigations are underway in our laboratory to answer this question.

Lastly, our analysis of CD11c-eYFP⁺ cells and microglia within the retina and cerebral cortex of CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice revealed that the expression of the majority of markers that we examined was not different between these strains of mice. These findings suggest that the *Crb1*^{rd8}

mutation does not overly affect the immunophenotype of resident immune cells within the CNS parenchyma. We did, however, observe a statistically significant increase in the percentage of CD11c-eYFP⁺ cells within the retina and cerebral cortex of CD11c-eYFP *Crb1*^{rd8/rd8} mice that expressed CD11c and I-A/I-E compared to CD11c-eYFP *Crb1*^{wt/wt} mice. Whilst it is tempting to attribute these findings solely to the *Crb1*^{rd8} mutation, the two CD11c-eYFP mouse lines used in this study were maintained on different backgrounds and, in addition to the *Crb1*^{rd8} mutation, C57Bl/6N and C57Bl/6J mice are distinguished by 34 single nucleotide polymorphisms and 1 indel (Simon et al. 2013). Therefore additional genetic differences between CD11c-eYFP *Crb1*^{rd8/rd8} mice (C57Bl/6N background) and CD11c-eYFP *Crb1*^{wt/wt} mice (mixed C57Bl/6N/C57Bl/6J background) may have contributed to the increased expression of CD11c and I-A/I-E within the CNS parenchyma of CD11c-eYFP *Crb1*^{rd8/rd8} mice. These issues may be ameliorated by crossing C57Bl/6N lines to the *Crb1*^{rd8} corrected C57Bl/6NJ strain (C57Bl/6NJ-*Crb1*^{rd8+em1Mvw}/MvwJ), which was generated by TALEN-mediated homology-directed repair (Low et al. 2014), but were only made commercially available after the commencement of our experiments.

In summary, we have demonstrated that the CD11c-YFP⁺ cells are present in the normal CNS of CD11c-eYFP transgenic mice with and without the *Crb1*^{rd8} mutation. Unlike choroid plexus and pia mater CD11c-eYFP⁺ cells, CD11c-eYFP⁺ cells within the retina and brain parenchyma of CD11c-eYFP *Crb1*^{wt/wt} mice could not be phenotypically distinguished from microglia, thus we challenge the notion that these cells are indeed 'brain DCs' or their immature precursors. Rather, our data suggest that the vast majority of CD11c-eYFP⁺ cells within the naïve brain parenchyma and retina are a subpopulation of microglia.

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Figure legends

Figure 1: Representative *in vivo* fundus images of CD11c-eYFP *Crb1*^{rd8/rd8}, CD11c-eYFP *Crb1*^{wt/wt} and C57Bl6/J mice. Retinal degenerative lesions (arrows) are seen in brightfield images (top panel) of CD11c-eYFP *Crb1*^{rd8/rd8} mice, but not CD11c-eYFP *Crb1*^{wt/wt} or C57Bl6/J mice. Fluorescent imaging revealed that CD11c-eYFP⁺ cells are distributed throughout the retina of both CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice (bottom panel).

Figure 2: Confocal microscopy was used to compare the distribution of CD11c-eYFP⁺ cells in the retinae of CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. (A) Confocal tiling of flat mounted retinae; scale bar = 500 μ m. Orthogonal views of Z stacks demonstrate the localization of CD11c-eYFP⁺ cells in the GCL, IPL and OPL (B); few cells were observed in the subretinal space (C, arrows). Arrowheads illustrate disruptions to the architecture of the ONL in CD11c-eYFP *Crb1*^{rd8/rd8} mice. The density of CD11c-eYFP⁺ cells in the retina (D) and subretinal space (E) was similar between mouse strains (mean + standard error of the mean; $p > 0.05$). GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer.

Figure 3: A subpopulation of CD11c-eYFP⁺ cells (yellow) located at the periphery of the retina (A) and adjacent to the optic nerve head (B) express I-A/I-E (red), whereas the majority of retinal CD11c-eYFP⁺ cells are I-A/I-E⁻ (C). All CD11c-eYFP⁺ cells are Iba-1⁺ (magenta). Nuclei in merged images (blue) were stained with Hoechst. Left panels = CD11c-eYFP *Crb1*^{wt/wt} mice, right panels = CD11c-eYFP *Crb1*^{rd8/rd8} mice. Images are maximum projection images generated from Z stacks taken through the entire thickness of the retina (A and B) or the outer plexiform layer (C). Scale bars = 50 μ m.

Figure 4: Immunophenotype of retinal microglia and CD11c-eYFP⁺ cells from naïve CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. (A) Retinal CD11c-eYFP⁺ cells are CD45^{int}, express CD11b, F4/80 and CD115. Light grey histograms: microglia CD11c-eYFP *Crb1*^{wt/wt}; dark grey histograms: microglia CD11c-eYFP *Crb1*^{rd8/rd8}; blue histograms: CD11c-eYFP⁺ CD11c-eYFP *Crb1*^{wt/wt}; red

histograms: CD11c-eYFP⁺ CD11c-eYFP *Crb1*^{rd8/rd8}. Numbers indicate the percentage of positive cells, determined by gating using fluorescence minus one and unstained controls (shown by vertical line). * $p < 0.05$. (B) Comparison of median fluorescence intensity (MFI) between retinal microglia and CD11c-eYFP⁺ cells from CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. Representative data from one of three experiments is shown.

Figure 5: Three morphologies of CD11c-eYFP⁺ cells within the mouse brain were observed: (A) stout cells with thick processes (choroid plexus shown); (B) elongated cells with thin, bipolar processes (corpus callosum shown); (C) ramified/dendriform cells extending multiple, branched processes (piriform cortex shown). Images in (A-C) are representative of CD11c-eYFP⁺ cells observed in both CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. (D-I): selected brain regions demonstrating the anatomical distribution of CD11c-eYFP⁺ cells in 40 μ m brain sections from CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice. Dots are representative of 1-4 CD11c-eYFP⁺ cells. Relative to bregma, the brain maps shown are: 4.28 mm (D), 2.22 mm (E), 0.14 mm (F), -1.22 mm (G), -4.72 mm (H) and -6.72 mm (I).

Figure 6: Brain CD11c-eYFP⁺ cells from CD11c-eYFP *Crb1*^{rd8/rd8} (A) and CD11c-eYFP *Crb1*^{wt/wt} (B) mice contain CD45^{int} and CD45^{hi} populations. Histograms demonstrate significant differences in the CD11c, I-A/I-E and Zbtb46 expression between gated CD45^{int} cells (light grey, dotted line) and CD45^{hi} cells (dark grey, solid line).

Figure 7: (A) CD11c-eYFP⁺ cells in the choroid plexus of both CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice co-localise with Iba-1 and I-A/I-E staining. A small percentage of CD11c-eYFP⁺ cells were I-A/I-E⁻. Left panels = CD11c-eYFP *Crb1*^{wt/wt} mice, right panels = CD11c-eYFP *Crb1*^{rd8/rd8} mice. Scale bars = 50 μ m. (B) CD11c-eYFP⁺ cells within the choroid plexus and pia mater express macrophage, DC and antigen presentation markers. Numbers indicate the percentage of positive cells, determined by gating using fluorescence minus one and unstained controls (shown by vertical line). * $p < 0.05$ between choroid plexus CD11c-eYFP⁺ cells from CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP

Crbl^{wt/wt} mice; # $p < 0.05$ between pia mater CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice. Representative data from one of two experiments is shown.

Figure 8: Confocal microscopy of brain sections demonstrates that ramified (A) and elongated (B) CD11c-eYFP⁺ cell types within the brain parenchyma express Iba-1, but not I-A/I-E. Stout CD11c-eYFP⁺ I-A/I-E⁺ cells were occasionally observed within brain sections, such as the in the spinal trigeminal tract as shown in (C). Left panels = CD11c-eYFP *Crbl*^{wt/wt} mice, right panels = CD11c-eYFP *Crbl*^{rd8/rd8} mice. Scale bars in (A-B) = 50 μ m; scale bars in (C) = 100 μ m.

Figure 9: CD11c-eYFP⁺ cells from the cerebral cortex display a similar immunophenotype to brain microglia (A). Light grey histograms: microglia CD11c-eYFP *Crbl*^{wt/wt}; dark grey histograms: microglia CD11c-eYFP *Crbl*^{rd8/rd8}; blue histograms: CD11c-eYFP⁺ CD11c-eYFP *Crbl*^{wt/wt}; red histograms: CD11c-eYFP⁺ CD11c-eYFP *Crbl*^{rd8/rd8}. Numbers indicate the percentage of positive cells, determined by gating using fluorescence minus one and unstained controls (shown by vertical line). * $p < 0.05$. (B) Comparison of median fluorescence intensity (MFI) between brain microglia and CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice. (C) The percentage of positive cells for each surface marker represented as a heat map. Heat map compares microglia, cortical CD11c-eYFP⁺ cells, choroid plexus CD11c-eYFP⁺ cells and pia CD11c-eYFP⁺ cells from CD11c-eYFP *Crbl*^{rd8/rd8} and CD11c-eYFP *Crbl*^{wt/wt} mice. Representative data from one of two experiments is shown.

Supplementary figure 1: The *Crbl*^{rd8} mutation is a single nucleotide deletion within the *Crumbs 1* gene. Sequencing of the *Crumbs 1* gene was performed to confirm that CD11c-eYFP *Crbl*^{wt/wt} mice possess the wild type allele.

Supplementary figure 2: GFP⁺ cells are not detected in the retina or brain of CD11c-DTR-GFP mice. Representative *in vivo* fundus imaging demonstrates normal appearance of the retina (A) and a lack of GFP⁺ cells (B). Confocal tiling (C) and analysis of orthogonal views of Z stacks (D) of flat mounted retinæ also did not identify GFP⁺ cells. Examination of brain sections demonstrated that GFP⁺ cells were

found only in the ependymal cells lining the lateral ventricles (E), but were not found within the brain parenchyma (F, image shows the piriform cortex). Scale bar in (C) = 500 μm . GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer.

Supplementary figure 3: Subretinal CD11c-eYFP⁺ cells (green) from CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice express Iba-1 (red), but not I-A/I-E. Confocal micrographs were obtained using a x63 oil objective and represent maximum projection images of Z stacks taken through the subretinal space. Scale bars = 50 μm .

Supplementary figure 4: Subpopulations of CD11b⁺ CD45^{int} microglia within the retina (left) and brain cortex (right) express YFP. Numbers indicate the percentage of YFP⁺ microglia from CD11c-eYFP *Crb1*^{rd8/rd8} (red) and CD11c-eYFP *Crb1*^{wt/wt} mice (blue). * $p < 0.05$.

Supplementary figure 5: Spleen CD11c-eYFP⁺ cells display the immunophenotype of red pulp macrophages (CD45^{hi}, F4/80⁺, I-A/I-E⁺, CD11b^{lo}, CD11c^{lo}). (A) Histograms comparing CD11c-eYFP⁺ cells from CD11c-eYFP *Crb1*^{rd8/rd8} mice (red) and CD11c-eYFP *Crb1*^{wt/wt} mice (blue) demonstrate that there are no differences between the two mouse strains ($p < 0.05$). Numbers indicate the percentage of positive cells. (B) Comparison of median fluorescence intensity (MFI) of CD11c-eYFP⁺ cells between CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice.

Supplementary figure 6: (A-C) Complete comparative anatomical mapping of CD11c-eYFP⁺ cells in coronal brain sections of CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice.

Supplementary figure 7: CD11c-eYFP⁺ cells are distributed throughout the rostral migratory stream (RMS), beginning at the subventricular zone surrounding the lateral ventricles (A) and continuing to the olfactory bulb (B). CD11c-eYFP⁺ cells within the RMS displayed an amoeboid morphology (C, D) similar to that of choroid plexus and pia CD11c-eYFP⁺ cells. The RMS was visualized in 40 μm sagittal

sections (shown in inset); images are representative of both CD11c-eYFP *Crb1*^{rd8/rd8} and CD11c-eYFP *Crb1*^{wt/wt} mice.

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Antibody	Clone	Manufacturer and catalogue number	Amount used per 10 ⁶ cells or working dilution
Purified rat anti-mouse I-A/I-E	M5/114.15.2	BD Biosciences, 556999	1:300
Rabbit anti-Iba-1	-	Wako, 019-19747	1:300
Rabbit anti-GFP	-	Merck Millipore AB3080	2.5 µg/mL
Goat anti-rabbit IgG-biotin	-	Vector Laboratories BA1000	1:400
Goat anti-rat IgG (H+L)-AF549	-	Molecular Probes, A-11007	1:300
Goat anti-rabbit IgG (H+L)-AF647	-	Molecular Probes, A-21244	1:300
Purified rat anti-mouse CD16/CD32 (mouse Fc block)	2.4G2	BD Biosciences, 553141	0.50 µg
Rat anti-mouse CD103-BV510	M290	BD Biosciences, 563087	0.25 µg
Rat anti-mouse CD45-BV605	30-F11	BioLegend, 103140	2.5 µL
Rat anti-mouse CD8α-BV650	53-6.7	BD Biosciences, 563234	0.25 µg
Rat anti-mouse CD115-BV711	AFS98	BioLegend, 135515	0.50 µg
Armenian hamster anti-mouse CD11c-BV786	HL3	BD Biosciences, 563735	0.125 µg
Rat anti-mouse I-A/I-E-PerCP-Cy5.5	M5/114.15.2	BD Biosciences, 562363	0.03 µg
Rat anti-mouse 33D1-PE	33D1	BioLegend, 124905	0.60 µg
Rat anti-mouse Zbtb46-PE	U4-1374	BD Biosciences, 565832	0.25 µg
Armenian hamster anti-mouse CD80-PE-CF594	16-10A1	BD Biosciences, 562504	0.06 µg
Rat anti-mouse CD135-PE-Cy5	A2F10	BioLegend, 135312	0.60 µg
Rat anti-mouse F4/80-PE-Cy7	BM8	BioLegend, 123114	0.25 µg
Rat anti-mouse DEC205-APC	NLDC-145	BioLegend, 138206	0.06 µg
Rat anti-mouse CD86-AF700	GL-1	BioLegend, 105024	1.00 µg
Rat anti-mouse CD11b-APC-Cy7	M1/70	BD Biosciences, 557657	0.25 µg

Table 1: Antibodies used for confocal microscopy and flow cytometry immunophenotyping.

Figure 1
70x38mm (300 x 300 DPI)

Accepted

Figure 2
180x200mm (300 x 300 DPI)

A

Figure 3
199x301mm (300 x 300 DPI)

A

Figure 4
194x208mm (300 x 300 DPI)

AC

Figure 5
205x234mm (300 x 300 DPI)

A

Figure 6
115x103mm (300 x 300 DPI)

Acce

Figure 7
202x248mm (300 x 300 DPI)

Figure 8
188x282mm (300 x 300 DPI)

A

Figure 9
173x162mm (300 x 300 DPI)

Acc

Supplementary Figure 1
84x18mm (300 x 300 DPI)

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Supplementary Figure 2
132x134mm (300 x 300 DPI)

Acc

Supplementary Figure 3
56x26mm (300 x 300 DPI)

Accepted

Supplementary Figure 4
68x56mm (300 x 300 DPI)

Accel

Supplementary Figure 5
200x437mm (300 x 300 DPI)

A

Supplementary Figure 6A
271x368mm (300 x 300 DPI)

A

Supplementary Figure 6B
274x368mm (300 x 300 DPI)

A

Supplementary Figure 6C
217x475mm (300 x 300 DPI)

A

Supplementary Figure 7
191x302mm (300 x 300 DPI)

A

		Total number of events acquired	Number of YFP ⁺ cells analysed	Coefficient of variation (%)
CD11c-eYFP <i>Crbl^{rd8/rd8}</i>	Spleen	1.21 x 10 ⁵	8480	1.05
	Retina	4.24 x 10 ⁶	321	5.74
	Bulk brain	6.11 x 10 ⁶	1373	2.67
	Choroid plexus	8.59 x 10 ⁵	686	3.82
	Pia mater	1.72 x 10 ⁶	289	5.91
	Cortex	4.27 x 10 ⁶	7404	1.16
CD11c-eYFP <i>Crbl^{wt/wt}</i>	Spleen	2.59 x 10 ⁵	8991	1.04
	Retina	3.69 x 10 ⁶	273	6.05
	Bulk brain	6.71 x 10 ⁶	1892	2.29
	Choroid plexus	8.01 x 10 ⁵	250	6.32
	Pia mater	4.40 x 10 ⁶	247	6.57
	Cortex	5.44 x 10 ⁶	6072	1.28

Supplementary table 1: the number of YFP⁺ cells analysed in flow cytometry experiments and the associated coefficient of variation.

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Title:

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