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Title: Regional and functional heterogeneity of antigen presenting cells in the mouse brain and meninges

Running title: Brain and meningeal antigen presenting cells

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Abstract

The CNS is considered to be immune privileged, owing in part to the absence of major histocompatibility (MHC) class II⁺ cells in the healthy brain parenchyma. However, systemic inflammation can activate microglia to express MHC class II, suggesting that systemic inflammation may be sufficient to mature microglia into functional antigen presenting cells (APCs). We examined the effects of systemic lipopolysaccharide (LPS)-induced inflammation on the phenotype and function of putative APCs within the mouse brain parenchyma, as well as its supporting tissues – the choroid plexus and meninges. Microglia isolated from different regions of the brain demonstrated significant heterogeneity in their ability to present antigen to naïve OT-II CD4⁺ T cells following exposure to systemic LPS. Olfactory bulb microglia (but not cortical microglia) intimately interacted with T cells *in vivo* and stimulated T cell proliferation *in vitro*, albeit in the absence of co-stimulation. In contrast, myeloid cells within the choroid plexus and meninges were immunogenic and upregulated the co-stimulatory molecule CD80 following systemic inflammation. Dural APCs, which clustered around LYVE-1⁺ lymphatics, were more efficient at stimulating naïve T cell proliferation than choroid plexus APCs, suggesting that the dura may be an under-appreciated site for immune interactions. This study has highlighted the functional diversity of myeloid cells within the sub-compartments of the CNS and its supporting tissues. Furthermore, these findings demonstrate that systemic inflammation can mature selected microglia populations and choroid plexus/meningeal myeloid cells into functional APCs, which may contribute to the pathogenesis of neuroinflammation and neurodegenerative diseases.

Keywords

Central nervous system, brain parenchyma, meninges, antigen presenting cells, microglia

Main points

- Acute systemic inflammation can mature selected microglia populations and choroid plexus/meningeal myeloid cells into functional APCs
- Myeloid cells isolated from different regions of the mouse CNS and its supporting tissues are phenotypically and functionally distinct

Introduction

The brain parenchyma and its closely juxtaposed supporting tissues (the choroid plexus and meninges) contain diverse populations of resident immune cells. Microglia, the resident macrophage-like cells of the CNS parenchyma, are derived from yolk sac erythromyeloid progenitors that populate the brain during early embryonic development (Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013). Due to their location behind the blood-brain barrier and within the brain parenchyma proper, microglia exist within an ‘immune privileged’ environment, and have therefore adapted specific functions that contribute to the maintenance of CNS homeostasis (Benarroch, 2013; Gomez-Nicola *et al.*, 2015; Tremblay *et al.*, 2011). External to the brain parenchyma, the meninges, choroid plexus and perivascular spaces are also populated with myeloid cells, including macrophages and dendritic cells (DCs) (Graeber *et al.*, 1989; McMenamin, 1999)). Whilst meningeal/choroid plexus DCs are thought to be short-lived bone marrow-derived cells (Anandasabapathy *et al.*, 2011), resident macrophages within the subdural meninges, perivascular space and choroid plexus are derived from haematopoietic precursors during embryonic development and (with the exception of choroid plexus macrophages) are long-lived (Goldmann *et al.*, 2016). Notably, these cells are phenotypically and transcriptionally distinct from microglia (Anandasabapathy *et al.*, 2011; Buttgereit *et al.*, 2016; Goldmann *et al.*, 2016).

One major difference between parenchymal microglia and myeloid cells of the CNS supporting tissues is that in steady state conditions, microglia do not express MHC class II or the co-stimulatory molecules required for T cell priming and are thus unable to present antigens to CD4⁺ T cells (Dando *et al.*, 2016). Indeed, the lack of MHC class II⁺ cells within the brain parenchyma, spinal cord and neural retina is believed to contribute partly to the immune privileged status of the CNS (Louveau, Harris, *et al.*, 2015). In contrast, a large proportion of myeloid cells within the normal choroid plexus, meninges and perivascular spaces are MHC class II positive (Chinnery *et al.*, 2010; Dando *et al.*, 2016; Graeber *et al.*, 1992; McMenamin, 1999) and, due to their location between the CNS proper, CSF and peripheral circulation, are thought to be important mediators of neuroinflammation (Brendecke *et al.*, 2015; Engelhardt *et al.*, 2017). This is supported by studies demonstrating that the choroid plexus, meninges, subarachnoid space and perivascular spaces are gateways for T cell entry into the CNS during experimental autoimmune encephalitis (EAE) (Bartholomaeus *et al.*, 2009; Kivisakk *et al.*, 2009; Paterka *et al.*, 2016; Reboldi *et al.*, 2009; Schlager *et al.*, 2016), which suggests that these sites may be important for T cell licensing.

The identification of antigen presenting cells (APCs) that activate CD4⁺ T cells and initiate neuroinflammation is critical for understanding immune-mediated neurological diseases, such as multiple sclerosis. Whether T cell activation in the context of neuroinflammation occurs within the brain parenchyma, the CNS supporting tissues or the periphery remains unclear. Activation of CD4⁺ T cells is a two-step process: the first step occurs when T cell receptors (TCRs) bind to their cognate antigen peptide, which is presented in the context of MHC class II molecules on APCs. The second step occurs when the co-stimulatory molecules CD80 and CD86 (expressed by APCs) interact with CD28 on T cells, which provides an antigen nonspecific co-stimulatory signal. It is widely accepted

that steady state microglia are not APCs; however, they can upregulate MHC class II, CD80 and CD86 in some inflammatory conditions (Cazareth *et al.*, 2014; Wlodarczyk *et al.*, 2014). Furthermore, CD11c⁺ microglia isolated from mice with EAE stimulated antigen-experienced CD4⁺ T cell proliferation *in vitro* (albeit at significantly lower levels compared to spleen DCs) (Wlodarczyk *et al.*, 2014), suggesting that they can be matured into APCs under specific experimental conditions. However, two important knowledge gaps remain. Firstly, it is not known if microglia within the normal CNS parenchyma can be matured into functional APCs in response to systemic inflammation. Secondly, the antigen presentation capabilities of microglia isolated from different regions of the brain have not been investigated.

Unlike the CNS parenchyma, the mouse choroid plexus and meninges contain Flt3L-dependent classical DCs that have similar gene expression profiles to CD8⁺ spleen DCs (Anandasabapathy *et al.*, 2011). Anandasabapathy *et al.* demonstrated that Flt3L-expanded DCs isolated from pooled choroid plexus/meningeal cell suspensions presented myelin peptides to MOG 2D2 CD4⁺ T cells and induced proliferation of allogenic T cells *in vitro* (Anandasabapathy *et al.*, 2011). However, it was not clear from these studies whether APC populations within the choroid plexus and the inner (pia) and outer (dura-arachnoid) layers of the meninges were functionally equivalent, or if APCs within these tissue sub-compartments have different capacities to present antigen to naïve CD4⁺ T cells. The recent confirmation that brain interstitial fluid and CSF drain into cervical lymph nodes via dural lymphatics (Louveau, Smirnov, *et al.*, 2015) suggests that putative APCs within the dura may be strategically positioned to sample antigenic material draining from the CNS; although, to the best of our knowledge, the functional antigen presentation capacity of immune cells within the dura has not been investigated.

In this study, we examined the effects of acute, systemic inflammation on the expression of antigen presentation molecules on myeloid cell populations within the mouse brain parenchyma (including the cortex and olfactory bulbs), choroid plexus, pia and dura. Furthermore, we characterised the ability of myeloid cells within these tissues to present antigen to naïve CD4⁺ T cells. We showed that microglia isolated from different brain regions were phenotypically and functionally heterogeneous, and that olfactory bulb microglia were more efficient APCs than cortical microglia. We identified novel APC:T cell interactions within the olfactory bulbs, and demonstrated that these interactions occurred in both healthy mice and mice exposed to systemic inflammation. In addition, we showed that the choroid plexus and dura contained immunogenic APCs that stimulated higher levels of cytokine and chemokine production than spleen APCs. Overall, this study highlighted the regional heterogeneity of microglia within the brain parenchyma, and generated novel insights into the functional capacity of myeloid cells within the choroid plexus and meninges.

Methods

Mice

Adult mice (8-16 weeks of age) were used in all experiments. For this study we utilised CD11c-eYFP *Crb1*^{wt/wt} mice, which express eYFP under the control of the *Itgax* promoter and lack the *Crumbs 1* rd8 mutation. We previously demonstrated that these mice contained populations of CD11c-eYFP⁺ cells within the normal brain parenchyma, choroid plexus and meninges, and that CD11c-eYFP⁺ cells within the brain parenchyma were a subpopulation of microglia that displayed a CD45^{int} CD11b⁺ F4/80^{lo} Iba-1⁺ CD115⁺ phenotype (Dando *et al.*, 2016). In the current study, the YFP reporter allowed us to examine both CD11c-eYFP⁺ and CD11c-eYFP⁻ Iba-1⁺ microglia in the context of systemic inflammation to determine if these microglia subtypes have different phenotypic and functional responses to LPS. CD11c-eYFP *Crb1*^{wt/wt} mice were generated and genotyped as previously described (Dando *et al.*, 2016). OT-II mice (Barnden *et al.*, 1998) were purchased from the Walter and Eliza Hall Institute of Medical Research and subsequently bred at Monash Animal Research Platform. Mice were bred in specific pathogen free conditions and maintained on a 12:12 h light cycle with access to food and water *ad libitum*. All animal procedures were approved by the Monash Animal Research Platform Ethics Committee and were performed in accordance with the NHMRC Australian code for the care and use of animals for scientific purposes.

LPS injections

CD11c-eYFP *Crb1*^{wt/wt} mice received an intraperitoneal injection of *E. coli* OIII:B4 LPS (9 mg/kg, Sigma Aldrich L3024) in a 100 μ L volume; control mice were administered 100 μ L of sterile saline (vehicle). Mice were humanely killed at 2, 24 or 48 h post-injection and tissues were collected for analysis.

Tissue collection, processing and cryotomy

Mice were deeply anaesthetised with an intraperitoneal injection of sodium pentobarbital (Lethobarb 150 mg/kg) and transcardially perfused through the left ventricle with PBS followed by 4% (w/v) paraformaldehyde (PFA). Brains (with pia and choroid plexus *in situ*) and the skull cap (calvaria, with dura-arachnoid *in situ*) were collected and stored in 4% (w/v) PFA overnight, then rinsed in PBS. Brains were cryoprotected, frozen and cut into 40 μ m free-floating coronal sections as previously described (Dando *et al.*, 2016). For examination of pia, brains were obliquely sectioned so that the pia appeared multilayered and thicker in profile, allowing myeloid cells within the pia to be more easily visualised in a quasi *en face* manner.

Immunostaining

All steps were performed on a rotator at low speed. Free-floating brain sections in 96 well flat bottom plates were permeabilised in 20 mM EDTA at 37 °C for 1 h, washed in PBS (3 x 5 min washes) and then blocked in 3.0% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100 in PBS (BSA/Triton X-100/PBS) at room temperature for 1 h. Sections were then incubated in primary antibodies (rat anti-I-A/I-E [BD Biosciences 556999], rabbit anti-Iba-1 [Wako 019-19747] or rat anti-CD3 [eBioscience 14-0032-82]) diluted in BSA/Triton X-100/PBS at 4 °C overnight. After washing in PBS (3 x 5 mins), sections were then incubated in secondary antibodies (goat anti-rabbit IgG-Alexa Fluor 647 [Molecular Probes A-21244], goat anti-rat-Cy3 [Molecular Probes A-10522]) and Hoechst diluted in PBS for 2 h at room temperature, and washed again in PBS (3 x 10 mins). Sections were mounted onto SuperFrost Plus microscope slides and allowed to adhere before ProLong Diamond Antifade mountant (Molecular Probes P36961) was added and sections were coverslipped.

Whole calvaria/dura in 24 well plates underwent mild decalcification in 0.5 M EDTA at 4 °C overnight, followed by washes in PBS (3 x 5 mins) and permeabilisation in 0.5% (v/v) Triton X-100

at room temperature for 1 h. After additional washes in PBS (3 x 5 mins), calvaria/dura were blocked in BSA/Triton X-100/PBS at room temperature for 1 h. Tissues were incubated in primary antibodies (rat anti-I-A/I-E, rabbit anti-Iba-1, rat anti-CD3 or rabbit anti-LYVE-1 [Abcam ab14917]), washed in PBS and incubated in secondary antibodies and Hoechst as described above. Following final washes in PBS (3 x 10 mins), the dura was carefully dissected as a single sheet of tissue from the calvaria under a dissecting microscope using a Tooke knife and jewelers forceps (Dumont no. 5) (McMenamin, 1999). Dural whole mounts were subsequently mounted and coverslipped as described above.

Confocal microscopy, microglia Sholl analysis and 3D surface rendering

Tissues were imaged at 1024 x 1024 resolution with an inverted SP5 5-channel confocal microscope (Leica Microsystems) using a 40 x oil objective (numerical aperture 1.25). Z stacks were captured with a 2 μ m step size and maximum projection images were generated using Fiji (version 1.51r) (Schindelin *et al.*, 2012). Quantitative assessment of microglia morphology was performed in a masked fashion using Sholl analysis in Fiji. Briefly, maximum projection images were despeckled and then converted to binary via thresholding. Individual microglia for analysis (n = 6 cells per mouse, obtained from 2 fields of view) were isolated by cropping the image and using the eraser tool to remove surrounding processes from neighbouring cells. Sholl analysis was then performed using previously described parameters (Norris *et al.*, 2014). The number of primary branches originating from the cell body was manually counted. From this analysis, we quantified changes in microglia morphology by calculating the area under the curve of each Sholl profile and the Schoenen ramification index (Morrison *et al.*, 2013; Schoenen, 1982). Average cell body area was calculated for each maximum projection image using FIJI. For examination of CD11c-eYFP⁺ and CD4⁺ cellular interactions, three dimensional reconstructions were created in Imaris version 9 (Bitplane), using the surface creation wizard and absolute intensity thresholding.

Tissue collection and microdissection for flow cytometry and cell sorting

Mice were deeply anaesthetised with sodium pentobarbital (i.p.) and transcardially perfused with 40 mL of ice-cold PBS. Brains were collected and microdissected to obtain samples of cortex (free of meninges, choroid plexus and ventricle wall), choroid plexus and pia-cortex as previously described (Dando et al., 2016). Olfactory bulbs (with pia still attached), dura (dissected from the calvaria) and spleens were also collected. Due to the small size of the olfactory bulbs, no attempt was made to remove the pia via dissection; rather, contaminating leukocytes from the pia (CD45^{hi}) were excluded from the parenchymal olfactory bulb microglia (CD45^{int}) in the flow cytometry gating approach (described below). The collected tissues were pooled into separate tubes containing FACS buffer (0.1% (w/v) BSA, 100 µg/mL DNase I (Roche 10104159001) and 3mM EDTA in PBS) and held on ice for all processing steps.

Olfactory bulb, cortex and pia-cortex single cell dissociation

Dissected tissues containing myelin were passed through a 70 µm nylon cell strainer, pelleted by centrifugation at 400 x g for 5 mins at 4 °C and resuspended in 30% (v/v) Percoll (GE Healthcare 17-0891-02). Cell suspensions were then centrifuged at 700 x g for 10 mins at 4 °C without brake and the myelin layer was carefully removed with a sterile transfer pipette. Cells were washed in 10 mL of FACS buffer and centrifuged at 400 x g for 5 mins at 4 °C. Pelleted cells were then resuspended in FACS buffer and passed through a new 70 µm cell strainer into a clean tube. Cell concentration was determined by counting an aliquot of trypan blue stained cells using a haemocytometer and inverted light microscope.

Dura, choroid plexus and spleen single cell dissociation

Dura samples were initially digested in 1.5 mg/mL collagenase type IV (Worthington CLS-4) and 0.4 mg/mL DNase I at 37 °C for 60 mins on a shaker (other tissues did not require enzymatic digestion). Tissues were then processed into single cell suspensions by passing through a 70 µm nylon cell strainer. Splenocytes were treated with red cell lysing buffer (Sigma Aldrich R7757) according to the manufacturer's instructions. Cells were then pelleted by centrifugation at 400 x g for 5 mins at 4 °C and resuspended in FACS buffer. The resultant single cell suspensions were filtered through a new 70 µm cell strainer into a clean tube and quantified by trypan blue staining.

Flow cytometry immunophenotyping

Single cell suspensions were centrifuged at 500 x g for 5 mins, washed twice with ice cold PBS and stained with fixable viability dye eFluor 450 (eBioscience 65-0863) according to the manufacturer's instructions. Cells were then centrifuged at 500 x g for 5 mins, washed in FACS buffer and incubated in mouse Fc block (BD Biosciences 553141) for 15 mins on ice. Cells were stained with fluorophore-conjugated antibodies (rat anti-mouse CD45-BV605 [BioLegend 103140], rat anti-mouse I-A/I-E-PerCP-Cy5.5 [BD Biosciences 562363], Armenian hamster anti-mouse CD80-PE-CF594 [BD Biosciences 562504] and rat anti-mouse CD86-AF700 [BioLegend 105024]) for 30 mins on ice, washed twice in FACS buffer and then fixed in 2% (w/v) PFA in FACS buffer for 15 mins on ice. Samples were acquired using a LSR Fortessa X-20 flow cytometer (BD Biosciences) and a compensation matrix (generated using singly stained anti-rat and anti-hamster Ig κ/negative control compensation particles, BD Biosciences 552845) was applied post-acquisition in FlowJo (version 10, Tree Star). Fluorescence gates were set using fluorescence minus one controls.

Cell sorting

CD11c-eYFP *Crb1*^{wt/wt} mouse single cell suspensions for sorting were incubated in mouse Fc block and stained with rat anti-mouse CD45-BV605 as described above. Stained cells were then centrifuged at 500 x g for 5 mins, washed twice and resuspended in FACS buffer for sorting. Immediately prior to sorting, propidium iodide (PI, 1 µg/mL, BD Biosciences 556463) was added to tubes to enable the exclusion of dead/dying cells. Using a BD Influx cell sorter and a 100 µm nozzle, live single cells were identified by excluding doublets and debris, then gating on live cells (Supplementary figure 1a-d). Within cortical and olfactory bulb single cell suspensions, we defined microglia as CD45^{int} cells. This approach was recently validated by a fate mapping study, which confirmed that retinal microglia uniquely express low/intermediate levels of CD45 during both steady state and disease conditions (O'Koren *et al.*, 2016). We further sorted CD45^{int} microglia into CD11c-eYFP⁺ and CD11c-eYFP⁻ subpopulations, which we previously characterised as having identical CD45^{int} CD11b⁺ F4/80^{lo} Iba-1⁺ CD115⁺ I-A/I-E⁻ phenotypes (Dando *et al.*, 2016), to enable us to perform simultaneous downstream analysis of these two microglia subtypes. From non-CNS tissues, CD45⁺ CD11c-eYFP⁺ and CD45⁺ CD11c-eYFP⁻ cells were sorted from spleen, choroid plexus and dural cell suspensions. All cell populations were sorted into sterile tubes containing complete medium (RPMI 1640 [Gibco 21870-092], 10% (v/v) heat-inactivated fetal bovine serum [Gibco 10099-141], 1 x glutaMAX [Gibco 35050-061], 25 mM HEPES [Gibco 15630-080], 1 mM sodium pyruvate [Gibco 11360-070], 1 x pen-strep [Gibco 15140-122] and 55 µM beta-mercaptoethanol [Gibco 21985-023]).

CD4⁺ T cell isolation

Spleens collected from OT-II mice were processed into single cell suspensions as described above. CD4⁺ T cells were isolated from single cell suspensions by negative selection using a CD4 T cell isolation kit (Miltenyi Biotec 130-104-454) and AutoMACS Pro (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated CD4⁺ T cells was >95% as determined by

staining with rat anti-mouse CD4-APC-Cy7 (Biolegend 100413) and rat anti-mouse V α 2-TCR-PerCP-Cy5.5 (BD Biosciences 560529) antibodies and flow cytometric analysis.

Antigen presentation assay

Freshly isolated OT-II CD4⁺ T cells were diluted to 1x10⁶ cells/mL and stained with 5 μ m of CellTrace Violet Cell Proliferation Kit (Life Technologies C34571) for 20 mins at 37 °C, as per the manufacturer's instructions. Labelled cells were then diluted in complete medium containing 10 ng/mL mouse recombinant IL-7 (Miltenyi Biotec 130-094-636) and 5 x 10⁴ cells (in a 100 μ L volume) were seeded into the wells of a 96 well U bottom plate. Sorted CD11c-eYFP⁺ and CD11c-eYFP⁻ cells were added to the wells at various ratios and incubated at 37 °C for 72 h in 5% CO₂ in the presence or absence of 0.78 μ g/mL of ovalbumin 323-339 peptide (OVA, Mimotopes 51023-010). Negative controls included T cells only, and T cells + OVA; positive controls included T cells stimulated with a polyclonal cocktail (0.5 μ g concanavalin A [Sigma Aldrich C5275], 0.5 μ g LPS and 0.25 μ g CpG [Invivogen tlr-1826-1]). In addition, all antigen presentation assays included splenic CD11c-eYFP⁺ cells (known to be efficient APCs) co-cultured with T cells and OVA at various ratios. Following incubation, plates were centrifuged at 500 x g for 5 mins and cells were resuspended in FACS buffer containing anti-mouse Fc block as described above and then stained with rat anti-mouse CD3-APC (Biolegend 100235) and rat anti-mouse CD44-BV510 (Biolegend 103043) for 30 mins at 4 °C. Cells were then washed twice in FACS buffer and 1 μ g/mL PI was added to tubes prior to analysis using a LSR Fortessa X-20 flow cytometer (BD Biosciences). Cells were gated as: singlets → viable cells (PI) → non-debris → YFP⁻ → CD3⁺. T cell proliferation was measured on gated cells by dilution of CellTrace Violet and upregulation of CD44. Cytokines and chemokines were measured in the supernatants of antigen presentation assays using the Bio-Plex Pro Mouse Cytokine 23-Plex Panel (BioRad M60009RDP) as per the manufacturer's instructions.

Statistical analysis

The sample size and number of independent experiments performed are indicated in figure legends. Microglia process complexity measurements (Sholl area under the curve and Schoenen ramification index) and cell body area were analysed using one-way ANOVA with Tukey's multiple comparisons test. Data distribution was assumed to be normal; however, sample sizes were too small to be tested for normality. Statistical analysis was performed using GraphPad Prism version 7.01 (La Jolla).

Results

Microglia in the brain parenchyma respond to systemic LPS exposure in a location-dependent manner

Systemic inflammation, for example - caused by bacterial toxins (such as lipopolysaccharide [LPS]) within the bloodstream, can induce disruptive or non-disruptive changes to the blood-brain barrier (Varatharaj *et al.*, 2017) and contribute to the pathogenesis of neurodegenerative diseases such as multiple sclerosis (Dendrou *et al.*, 2015) and compromise normal cognitive function (Perry *et al.*, 2013). To determine if myeloid cells within the brain parenchyma or its supporting tissues are matured into functional APCs in response to systemic LPS, we injected adult CD11c-eYFP *Crb1*^{wt/wt} mice with LPS (9 mg/kg, i.p.) or saline and examined the phenotype of microglia populations in the cerebral cortex and olfactory bulbs, which are anatomically and functionally distinct regions of the brain parenchyma that can be precisely microdissected and are known to contain CD11c-eYFP⁺ cells.

We initially examined the morphology of microglia within the cortex and olfactory bulbs using confocal microscopy. Cortical CD11c-eYFP⁺ Iba-1⁺ microglia and CD11c-eYFP⁻ Iba-1⁺ microglia appeared to retain their characteristic ramified appearance after LPS exposure (Figure 1a); however, to more closely examine morphological changes we performed quantitative Sholl analysis, in which microglia process complexity is calculated by area under the curve analysis of the branching profile (the number of branch intersections as a function of radial distance from the centre of the cell body) and the Schoenen ramification index. Sholl analysis of cortical microglia populations demonstrated that process complexity was not different between control and LPS groups for both CD11c-eYFP⁺ microglia (Figure 1b) and CD11c-eYFP⁻ Iba-1⁺ microglia (Figure 1c). In contrast, olfactory bulb

CD11c-eYFP⁺ microglia and CD11c-eYFP⁻ Iba-1⁺ microglia were noticeably stouter and projected fewer processes 24 and 48 h post-LPS injection (Figure 1d). Sholl analysis of olfactory bulb microglia demonstrated that both CD11c-eYFP⁺ Iba-1⁺ (Figure 1e) and CD11c-eYFP⁻ Iba-1⁺ (Figure 1f) cells had a significantly reduced branching profile and Schoenen ramification index compared to controls at 24 h post-LPS, suggesting an 'activated' morphology. Olfactory bulb (but not cortical) microglia mean cell body area was also increased at 48 h compared to controls (Figure 1g).

Cortical and olfactory bulb microglia do not upregulate antigen presentation markers in response to systemic LPS

In light of the evidence, presented above, that microglia isolated from different brain regions responded differently to systemic inflammation, we next asked whether cortical and olfactory bulb microglia populations upregulated antigen presentation markers following *in vivo* LPS exposure. At 24 h post-LPS injection, we analysed the expression of MHC class II (I-A/I-E) and the co-stimulatory molecules CD80 and CD86 on microglia populations by flow cytometry. In the cortex and olfactory bulbs, microglia were identified by their unique CD45^{int} expression and were gated into CD11c-eYFP⁺ and CD11c-eYFP⁻ populations. Analysis of gated cells demonstrated that a small percentage of olfactory bulb CD11c-eYFP⁺ microglia expressed I-A/I-E (control 5.6%, LPS 4.2%), whereas no expression of CD80 or CD86 was detected (Figure 2a). The presence of small numbers of CD11c-eYFP⁺ Iba-1⁺ I-A/I-E⁺ cells and CD11c-eYFP⁻ Iba-1⁺ I-A/I-E⁺ cells within the glomerular layer and nerve fibre layer of control (Figure 2b) and LPS exposed (Figure 2c) olfactory bulbs was subsequently confirmed by immunostaining and confocal microscopy. Following LPS injection, the percentage of CD45^{int} CD11c-eYFP⁺ cells and CD45^{int} CD11c-eYFP⁻ cells within the olfactory bulbs increased; however, no overt changes in I-A/I-E, CD80 or CD86 expression were observed (Figure 2a). Within the cortex, CD11c-eYFP⁺ microglia represented a much smaller percentage of the total CD45^{int} cells

compared to the olfactory bulbs, and the percentage of cortical CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia did not increase following LPS exposure (Figure 2d). Cortical microglia populations in both control and LPS-injected mice were I-A/I-E⁻ CD80⁻ CD86⁻ (Figure 2d). In contrast to olfactory bulb and cortical microglia populations, DCs within the spleen significantly upregulated the expression of CD80 and CD86, and maintained high I-A/I-E expression following systemic LPS (Figure 2e).

The olfactory bulbs, but not cerebral cortex, contain functional APCs that interact with T lymphocytes *in vivo*

The altered morphology of olfactory bulb microglia following LPS exposure and the presence of small numbers of I-A/I-E⁺ cells within the olfactory bulbs led us to examine the functional capacity of CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia using an *in vitro* antigen presentation assay. CD45^{int} CD11c-eYFP⁺ and CD45^{int} CD11c-eYFP⁻ microglia were sorted from olfactory bulbs 24 h after i.p. LPS injection and co-cultured with CellTrace Violet-labelled naïve CD4⁺ OT-II T cells in the presence or absence of the model antigen ovalbumin (OVA). Cortical microglia (CD45^{int} CD11c-eYFP⁺ and CD45^{int} CD11c-eYFP⁻) and spleen CD45⁺ CD11c-eYFP⁺ cells (Supplementary figure 2) were also included for comparison. After 72 h of incubation, olfactory bulb CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia induced antigen-specific proliferation of naïve CD4⁺ T cells at APC: T cell ratios of both 1:5 and 1:10 (Figure 3a). Proliferation was also associated with increased expression of CD44 by CD4⁺ T cells (Supplementary figure 3a). Analysis of cytokine and chemokine production within supernatants at 72 h revealed that LPS-activated olfactory bulb CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia induced similar levels of IL-2, IL-17A, GM-CSF, IFN- γ , MIP-1 α and MIP-1 β production compared to spleen CD11c-eYFP⁺ cells within the same assay (Figure 3b). To determine if olfactory bulb APCs interacted with lymphocytes *in vivo*, we examined brain sections by confocal microscopy. Surprisingly, we found that CD3⁺ T cells were present within the olfactory bulbs of both

control and 24 h LPS-injected mice, and that these cells were often in close contact with CD11c-eYFP⁺ microglia. In control olfactory bulbs, CD3⁺ cells were frequently observed to be enwrapped by the processes of ramified CD11c-eYFP⁺ microglia (Figure 3c-d). In LPS-exposed mice, CD3⁺ T cells were also intimately associated with amoeboid CD11c-eYFP⁺ cells (Figure 3e). Combined, these data demonstrate that systemic LPS is sufficient to mature microglia within the olfactory bulbs into functional APCs, and that interactions with CD3⁺ lymphocytes may facilitate local antigen presentation *in vivo*. In addition to the interactions with CD3⁺ lymphocytes, we also observed cell:cell interactions between CD11c-eYFP⁺ cells within in the olfactory bulbs of LPS-injected mice, whereby two CD11c-eYFP⁺ cells were connected by a single nanotube-like (Chinnery *et al.*, 2008) process (Figure 3e).

In contrast to the olfactory bulbs, cortical CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia from LPS-exposed mice failed to induce *in vitro* T cell proliferation at APC:T cell ratios of both 1:5 and 1:10 (Figure 3f), and supernatants contained lower levels of key cytokines such as IL-2, IL-17A, GM-CSF and IFN- γ compared to supernatants collected from co-cultures containing spleen CD11c-eYFP⁺ cells (Figure 3g). However, high levels of MIP-1 α and MIP-1 β were detected in the supernatants of co-cultures containing cortical CD11c-eYFP⁺ microglia. CD3⁺ cells were not detected within the cortex of either control (Figure 3h) or LPS-injected mice (Figure 3i).

The expression of activation markers is altered on choroid plexus and meningeal putative APCs following *in vivo* LPS exposure

We previously demonstrated that CD11c-eYFP⁺ cells within the choroid plexus and pia mater of CD11c-eYFP *Crb1*^{wt/wt} mice expressed markers that were characteristic of DCs and macrophages, including I-A/I-E and CD86 (Dando *et al.*, 2016). In the current study, we analysed the surface

marker profile of dural CD11c-eYFP⁺ cells and identified discrete CD11c-eYFP^{lo} and CD11c-eYFP^{hi} populations. Both dural CD11c-eYFP^{lo} and CD11c-eYFP^{hi} cells were CD11b⁺ F4/80⁺, and expressed varying levels of I-A/I-E, CD11c, CD86, CD115, CD135, CD8 α and DEC205 (Supplementary figure 4). Combined with our previous findings, these data demonstrate that the dura, choroid plexus and pia contain cells with putative antigen presentation capabilities, therefore we examined their phenotype in response to systemic LPS. Confocal microscopy demonstrated that elongated, dendriform and amoeboid CD11c-eYFP⁺ cells and Iba-1⁺ cells were observed within the choroid plexus, pia and dura of control and LPS exposed mice at 2, 24 and 48 h post-injection (Figure 4a). Whilst the distribution of cells within the choroid plexus and pia was not significantly altered by LPS exposure, dural CD11c-eYFP⁺ cells and Iba-1⁺ cells accumulated in high numbers around vessels at 24 and 48 h post-LPS injection (Figure 4a).

The expression of I-A/I-E, CD80 and CD86 on leukocyte populations within the choroid plexus (Figure 4b), pia (Figure 4c) and dura (Figure 4d) was analysed by flow cytometry 24 h post-LPS injection. Following LPS challenge, the percentage of CD11c-eYFP⁺ cells in each of these tissues increased compared to controls. CD45⁺ CD11c-eYFP⁻ cells within the choroid plexus, pia and dura of control mice were largely I-A/I-E⁻ CD80⁻ CD86⁻; however, these cells upregulated CD80 in response to LPS. Similarly, the expression of CD80 was markedly increased on choroid plexus, pial and dural CD11c-eYFP⁺ cells from LPS-injected mice compared to controls (Figure 4b-d). Interestingly, CD80 upregulation was associated with a concomitant decrease in I-A/I-E expression by pial CD11c-eYFP⁺ cells and dural CD11c-eYFP^{lo} and CD11c-eYFP^{hi} cells compared to control mice. CD86 expression was also decreased on dural CD11c-eYFP^{lo} cells from LPS-injected mice. These data demonstrate that CD11c-eYFP⁺ and CD11c-eYFP⁻ leukocytes within the choroid plexus and meninges dramatically alter their expression of activation/antigen presentation markers in response to systemic LPS.

The dura and choroid plexus contain potent APCs

We next performed *in vitro* antigen presentation assays to characterise the function of LPS-activated leukocyte populations within the dura and choroid plexus. Due to variability in cell sorting yields between tissues, APC: T cell ratios tested were 1:23-1:28 for dura and 1:10-1:17 for choroid plexus. We were not able to isolate sufficient cell numbers from the pia to perform functional assays. Both CD45⁺ CD11c⁻eYFP⁺ and CD45⁺ CD11c-eYFP⁻ cells isolated from the dura of LPS-injected mice stimulated naïve CD4⁺ T cell proliferation (Figure 5a) and CD44 upregulation (Supplementary figure 3b) at an APC: T cell ratio of 1:23. Dural CD45⁺ CD11c⁻eYFP⁺ and CD45⁺ CD11c-eYFP⁻ cells continued to induce T cell proliferation at an APC:T cell ratio of 1:28; however, the percentage of proliferating T cells was reduced (Supplementary figure 5a). Co-cultures containing dural CD11c-eYFP⁺ cells produced markedly higher levels of IL-17A, G-CSF, KC, MCP-1, MIP-1 α and MIP-1 β compared to co-cultures containing either spleen CD11c-eYFP⁺ cells or dural CD45⁺ CD11c-eYFP⁻ cells assayed at the same APC: T cell ratio (Figure 5b). At an APC:T cell ratio of 1:10, choroid plexus CD45⁺ CD11c-eYFP⁺ cells also induced CD4⁺ T cell proliferation (Figure 5a) and proliferating T cells upregulated CD44 (Supplementary figure 2c). APC assays containing choroid plexus CD11c-eYFP⁺ cells uniquely produced IL-1 α , IL-1 β , IL-6, IL-12 (p70) and KC compared to assays containing spleen CD11c-eYFP⁺ cells and choroid plexus CD45⁺ CD11c-eYFP⁻ cells (Figure 5c). Both choroid plexus CD11c-eYFP⁺ cells and CD11c-eYFP⁻ cells stimulated the production of chemokines including MCP-1, MIP-1 α , MIP-1 β and RANTES in APC assays. When APC:T cell ratios of 1:17 were tested, the ability of choroid plexus CD45⁺ CD11c-eYFP⁺ cells to stimulate T cell proliferation was significantly diminished (Supplementary figure 5b).

In wholemount preparations, dural CD11c-eYFP⁺ cells were closely juxtaposed to LYVE-1⁺ lymphatic vessels, especially in LPS-injected mice (Figure 5d). CD3⁺ T cells were also observed in close proximity to dural lymphatics and CD11c-eYFP⁺ cells in both control and LPS mice (Figure 5e). 3D reconstructions demonstrated that both CD3⁺:CD11c-eYFP⁺ cell interactions and CD11c-eYFP⁺:CD11c-eYFP⁺ cell interactions occurred in the dura of LPS-injected mice (Figure 5e). CD3⁺ cells were also observed within the choroid plexus (Figure 5f) and pia (Figure 5g) of control and LPS exposed animals. Taken together, these data demonstrate that the dura and choroid plexus contain potent APCs that interact with lymphocytes *in vivo* following systemic LPS.

Discussion

Determining the identity, location and functional capacity of APCs within the CNS and its supporting tissues is crucial for understanding immune interactions within the brain in steady state conditions and during disease. As messengers between the innate and adaptive arms of the immune response, APCs can drive immune responses, activate T cells or induce immunological tolerance. To identify putative APC populations within the CNS and its supporting tissues, we characterised the phenotype and function of myeloid cells within the mouse brain parenchyma, choroid plexus and meninges following acute, systemic LPS exposure. Within the brain parenchyma, we demonstrated remarkable heterogeneity between olfactory bulb microglia and cortical microglia with respect to their phenotypic response to peripheral LPS and their functional capacity to present OVA peptide to naïve CD4⁺ T cells. We showed that the presentation of antigen by olfactory bulb microglia occurred in the absence of co-stimulation. In contrast, choroid plexus and dural APCs were immunogenic and induced higher levels of cytokine/chemokine production than spleen DCs. Moreover, we have shown that dural APCs were more potent than choroid plexus APCs, demonstrating that the dura may be an important and underappreciated site for immune interactions.

The brain parenchyma contained diverse subpopulations of microglia that were distinguished both phenotypically and functionally. Olfactory bulb CD11c-eYFP⁺ Iba-1⁺ microglia and CD11c-eYFP⁻ Iba-1⁺ microglia displayed an 'activated' morphology 24 and 48 h post-LPS injection, whereas cortical microglia populations retained their classical ramified morphology at all examined time

points according to Sholl analysis parameters. These findings contrast with previous studies in male C57Bl/6J mice, which reported that a single intraperitoneal injection of LPS (5 mg/kg) resulted in morphological 'activation' of cortical microglia at either 3 h (Qin *et al.*, 2007) or 48 h post-LPS injection (Hoogland *et al.*, 2018). Our study differed from these previous reports as we utilised: (i) a different dose of LPS; (ii) mice of both sexes and (iii) transgenic mice on a mixed genetic background. As the effects of systemic LPS on microglia activation in mice are dependent on genetic background and sex (Meneses *et al.*, 2018), these factors may explain the differences between these studies.

Acute, systemic LPS exposure was sufficient to activate olfactory bulb microglia (but not cortical microglia) into functional APCs capable of stimulating naïve CD4⁺ T cell proliferation and upregulating T cell CD44 expression. Interestingly, olfactory bulb microglia did not upregulate MHC class II, CD80 or CD86 in response to systemic LPS, which suggests that they may function as tolerogenic APCs (Bour-Jordan *et al.*, 2011; Hubo *et al.*, 2013); however additional studies are required to confirm this. Our data are consistent with the findings of Gottfried-Blackmore *et al.*, who demonstrated that acute, intracerebral IFN- γ matured CD11c-eYFP⁺ and CD11c-eYFP⁻ cells (isolated from whole brain parenchyma cell suspensions) into functional APCs, but did not induce the expression of co-stimulatory molecules (Gottfried-Blackmore *et al.*, 2009). Despite detecting high levels of IL-2 and IL-17 in olfactory bulb APC: T cell co-cultures, we did not detect cytokine profiles that suggested Th1, Th2, Th9, Th17, Th22 or Treg polarisation of proliferating T cells had occurred. The high levels of MIP-1 α and MIP-1 β within the supernatants of APC assays containing olfactory bulb and cortical microglia populations are consistent with previous studies demonstrating that microglia produce these chemotactic cytokines in response to certain inflammatory stimuli and in disease states (Kohno *et al.*, 2014; McManus *et al.*, 2000). Therefore, olfactory bulb CD11c-eYFP⁺ and CD11c-eYFP⁻ microglia may contribute to neuroinflammation by stimulating T cell proliferation

and secreting chemoattractants that facilitate recruitment of circulating leukocytes across the blood-brain barrier. In contrast, within the cortex only CD11c-eYFP⁺ microglia stimulated the production of MIP-1 α and MIP-1 β in APC assays, suggesting that CD11c-eYFP⁺ cells may be a specialised subpopulation of microglia within the cortex.

To the best of our knowledge, this is the first study to compare the functional antigen presentation capabilities of microglia isolated from different regions of the brain parenchyma. Grabert *et al.* reported that the global gene expression profile of microglia in the healthy adult mouse brain was regionally heterogeneous (Grabert *et al.*, 2016). These authors demonstrated that 2,527 genes were differentially regulated in microglia isolated from different neuroanatomical regions, and that cerebellar and hippocampal microglia were transcriptionally distinct from cortical and striatal microglia. Interestingly, analysis of genes that were highly expressed in cerebellar microglia revealed that immune-related processes (including antigen processing and presentation) were the most overrepresented biological processes (Grabert *et al.*, 2016). Based on our findings, we hypothesise that olfactory bulb microglia represent another transcriptionally distinct population of microglia that exist in a more immunocompetent state than other microglial subpopulations. This may be due to the fact that the olfactory bulbs are directly linked to the nasal cavity via the olfactory nerves (Dando *et al.*, 2014), and therefore the resident olfactory bulb microglia are more likely to encounter invading pathogens than microglia in other regions of the brain parenchyma.

In a previous study of APCs within the olfactory bulbs of mice with experimental viral encephalitis, D'Agostino *et al.* reported that distinct CD45^{hi} CD11c-eYFP⁺ populations stimulated OT-I and OT-II T cells *in vitro* (D'Agostino *et al.*, 2012). However, these APCs were most likely infiltrating cells or contaminating meningeal immune cells. One caveat of our study is that systemic LPS may have

disrupted the integrity of the blood-brain barrier at the vascular endothelium, resulting in the infiltration of blood-derived immune cells into the CNS parenchyma. Despite this possibility, a recent fate mapping study showed that CNS-infiltrating immune cells retain their CD45^{hi} phenotype in a model of light-induced retinal degeneration and thus can be distinguished from CD45^{int} microglia (O'Koren et al., 2016). As we have gated strictly on CD45^{int} cells within the neural parenchyma, it is unlikely that the sorted olfactory bulb microglia populations we tested in antigen presentation assays contained contaminating, non-parenchymal cells; however, fate mapping studies are required to definitely confirm this. Rather, we believe that the increased percentage of CD11c-eYFP⁺ microglia and CD11c-eYFP⁻ microglia within the olfactory bulbs following LPS exposure was due to local proliferation of resident immune cells.

For many years, T cells have been considered to be excluded from the naïve brain parenchyma; however, we demonstrated that CD3⁺ T cells were present within the olfactory bulbs (but not the cortex) of control mice. These data raise the possibility that the olfactory bulb blood-brain barrier may be more permeable than that in other regions of the brain. Our novel observation that olfactory bulb CD11c-eYFP⁺ microglia were intimately associated with CD3⁺ T cells in healthy control mice demonstrates that APC:T cell interactions may occur within specific regions of the naïve brain parenchyma as part of normal immune surveillance, although the nature of these interactions is unknown. Tissue-resident memory T cells have been reported to persist within the brain parenchyma following CNS infection (Korn *et al.*, 2017; Wakim *et al.*, 2010); however, the question of whether lymphocytes in the naïve brain parenchyma are recirculating or a novel resident subset is currently unclear. Intravital microscopy studies to characterise the dynamic behaviour of olfactory bulb microglial populations and lymphocytes may shed light on the nature of these immune interactions. We also demonstrated that following LPS injection, olfactory bulb CD11c-eYFP⁺ microglia were

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closely associated with CD3⁺ T cells. Taken together with our *in vitro* functional data, we conclude that olfactory bulb microglia can function as APCs and interact with T cells within the healthy and inflamed olfactory bulbs.

In naïve mice, CD11c-eYFP⁺ cells within the choroid plexus, pia and dura expressed high surface levels of I-A/I-E; however, 24 h after *in vivo* LPS exposure I-A/I-E expression was decreased. Consistent with these findings, previous studies have demonstrated that the synthesis of MHC class II is initially upregulated in activated human and mouse DCs following LPS stimulation but later (approximately 16-20 h after activation), MHC class II production is significantly reduced (Cella *et al.*, 1997; Villadangos *et al.*, 2001). Therefore, choroid plexus and meningeal CD11c-eYFP⁺ cells appeared to be in a late-activated state 24 h after systemic LPS injection. Nonetheless, at 24 h these cells upregulated CD80 and choroid plexus and dural myeloid cell populations stimulated naïve CD4⁺ T cell proliferation and CD44 upregulation *in vitro*, demonstrating that they are functional APCs. Although we were unable to obtain sufficient pial cells for antigen presentation assays, our phenotypic analysis suggested that the pia also likely contained functional, immunogenic APCs.

Dural APCs stimulated naïve CD4⁺ T cell proliferation at APC: T cell ratios of 1:23 and 1:28, whereas the functional activity of choroid plexus APCs diminished at ratios >1:10, illustrating that dural APCs were more efficient than those within the choroid plexus. In addition, both CD45⁺ CD11c-eYFP⁻ and CD45⁺ CD11c-eYFP⁺ cells within the dura were functional APCs, whilst APC activity in the choroid plexus was restricted to CD45⁺ CD11c-eYFP⁺ cells. These data demonstrate that APCs within the choroid plexus and dura have distinct functional capacities, and are consistent with a recent study, which showed that choroid plexus and meningeal macrophages differ in their origin and turnover (Goldmann *et al.*, 2016). Similar to APC co-culture experiments containing

olfactory bulb microglia, the cytokine and chemokine profiles of APC assays containing choroid plexus and dural CD11c-eYFP⁺ were not skewed towards a particular Th polarisation; however, the production of IL-1 β , IL-6 and IL-12 (p70) in choroid plexus but not dural APC assays suggests that myeloid cells at these sites induce different cytokine responses. Remarkably, both dural and choroid plexus CD45⁺ CD11c-eYFP⁺ cells induced higher levels of cytokine and chemokine production in APC assays compared to spleen CD45⁺ CD11c-eYFP⁺ cells, illustrating that APCs in the CNS supporting tissues are capable of generating more potent immune responses than APCs in a lymphoid organ such as the spleen.

To date, very few studies have investigated the role of dural myeloid cells in neuroinflammation. This lack of consideration may be partially due to the fact that the dura is the outermost layer of the meninges with its own non-cerebral blood supply and thus is not in direct contact with the CNS parenchyma and is separated from the CSF by the arachnoid barrier (Coles *et al.*, 2017). However, it was recently shown that dural lymphatic vessels absorbed CSF and brain interstitial fluid (via the ‘glymphatic’ pathway) and drained into the deep cervical lymph nodes (Aspelund *et al.*, 2015; Louveau *et al.*, 2017; Louveau, Smirnov, *et al.*, 2015), suggesting that the dura may represent an immunological interface between the CNS and the periphery. We demonstrated that dural CD11c-eYFP⁺ cells were clustered around LYVE-1⁺ lymphatics; however, the question of whether dural APCs sample CNS draining fluid within these lymphatics remains to be investigated. Classically, APCs encounter antigen in tissues and migrate within lymphatics to the draining lymph nodes, where they present antigen to T cells. However, it is not clear if dural APCs are able to migrate within meningeal lymphatics to the deep cervical lymph nodes. Our data demonstrating that dural CD11c-eYFP⁺ cells were intimately associated with CD3⁺ T cells in LPS-exposed mice raises the possibility that antigen presentation may occur locally within the dura.

In summary, this study has provided novel insights into the immunobiology of the CNS by demonstrating that immune cells isolated from different regions of the brain parenchyma, choroid plexus and meninges are functionally heterogeneous APCs. We observed marked differences between regionally distinct microglia subpopulations and choroid plexus/meningeal APCs, and demonstrated that the latter have the capacity to drive potent immune responses in the context of systemic inflammation. We demonstrated that microglia isolated from different neuroanatomical regions are phenotypically and functionally diverse, and illustrated that these cells are highly complex and adapted to their own regional microenvironments within the brain parenchyma. We propose that further investigation of APC:T cell interactions in the brain parenchyma and its supporting tissues in immune-mediated neurodegenerative diseases will identify pathologic and regulatory immune interactions that mediate a range of CNS disorders.

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Author contributions

SJD and PGMcM conceived the study. SJD, RK, HRC and PGMcM performed the experiments. SJD performed data analysis and wrote the paper. All authors reviewed and approved the manuscript prior to submission.

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Figure legends

Figure 1: Phenotypic analysis of CD11c-eYFP⁺ Iba-1⁺ microglia and CD11c-eYFP⁻ Iba-1⁺ microglia following systemic LPS exposure. **(a)** Confocal microscopy images demonstrating the morphology of CD11c-eYFP⁺ Iba-1⁺ and CD11c-eYFP⁻ Iba-1⁺ microglia within the cortex of control (saline) and LPS-injected mice. The dotted lines separate the cortex and pia. Scale bars = 100 μ m. Images are representative of data obtained from n = 3 mice/group. **(b)** Sholl analysis of cortical CD11c-eYFP⁺ Iba-1⁺ microglia (mean + SEM, n = 3 mice/group). Area under the curve (AUC, one-way ANOVA, F = 1.288, p = 0.3509) and Schoenen ramification index (one-way ANOVA, F = 0.5969, p = 0.6370) were calculated from the Sholl plot. **(c)** Sholl analysis of cortical CD11c-eYFP⁻ Iba-1⁺ microglia (mean + SEM, n = 3 mice/group; AUC: one-way ANOVA, F = 1.028, p = 0.4363; Schoenen ramification index: one-way ANOVA, F = 1.393, p = 0.3222). **(d)** Representative confocal microscopy images demonstrating the morphology of CD11c-eYFP⁺ Iba-1⁺ and CD11c-eYFP⁻ Iba-1⁺ microglia within the olfactory bulbs (OB) of control and LPS-injected mice (n = 3 mice/group). Dotted lines separate the nerve fibre layer (NFL) and glomerular layer (GL) of the olfactory bulbs. Scale bars = 100 μ m. **(e)** Sholl analysis of olfactory bulb CD11c-eYFP⁺ Iba-1⁺ microglia (mean + SEM, n = 3 mice/group; AUC: one-way ANOVA, F = 8.138, p = 0.0111; Schoenen ramification index: one-way ANOVA, F = 5.682, p = 0.0272). **(f)** Sholl analysis of olfactory bulb CD11c-eYFP⁻ Iba-1⁺ microglia (mean + SEM, n = 3 mice/group; AUC: one-way ANOVA, F = 6.241, p = 0.0217; Schoenen ramification index: one-way ANOVA, F = 4.563, p = 0.0450). **(g)** Cell body area of cortical and olfactory bulb CD11c-eYFP⁺ Iba-1⁺ and CD11c-eYFP⁻ Iba-1⁺ microglia (mean + SEM, n = 3 mice/group). Cortical CD11c-eYFP⁺ Iba-1⁺ microglia one-way ANOVA: F = 0.6326, p = 0.6171; cortical CD11c-eYFP⁻ Iba-1⁺ microglia one-way ANOVA: F = 1.13, P = 0.4002. Olfactory bulb CD11c-eYFP⁺ Iba-1⁺ microglia one-way ANOVA: F = 5.392, p = 0.0308; olfactory bulb CD11c-eYFP⁻ Iba-1⁺ microglia one-way ANOVA: F = 4.637, P = 0.0434. n.s = not significant.

Figure 2: Systemic LPS exposure does not lead to upregulation of microglial antigen presentation markers. **(a)** Left panels: gating of olfactory bulb CD45^{int} CD11c-eYFP⁻ microglia (red gate) and CD45^{int} CD11c-eYFP⁺ microglia (blue gate). Right panels: overlay histograms indicating the expression of I-A/I-E, CD80 and CD86 on gated populations. Control = shaded histogram, 24 h LPS = black line. **(b, c)** Left panel shows a low magnification image demonstrating I-A/I-E⁺ cells within the glomerular layer and nerve fibre layer of the olfactory bulbs of control **(b)** and LPS exposed **(c)** mice. Higher power images of the boxed areas are shown in the right panels and demonstrate the co-expression of I-A/I-E, CD11c-eYFP and Iba-1 in the glomerular layer. Representative data of n = 3 mice per group; scale bars = 100 μ m. **(d)** Gating of cortical CD11c-eYFP⁻ microglia (red gate) and CD11c-eYFP⁺ microglia (blue gate) and overlay histograms indicating I-A/I-E, CD80 and CD86 expression in control (shaded histogram) and LPS (black line) injected mice. **(d)** Professional APCs (CD11c⁺ I-A/I-E⁺ DCs) from CD11c-eYFP *Crb1*^{wt/wt} mouse spleens were included in each experiment as a positive control for antibody staining. Control = shaded histogram, LPS = black line. Flow cytometry data were obtained from pooled single cell suspensions from n = 8 mice/group (cortex, spleen) or n = 4 mice/group (olfactory bulbs). Numbers in flow cytometry plots indicate percentages of gated populations. Cells were pre-gated as: single cells → non-debris → live cells. Representative data of two independent experiments are shown.

Figure 3: Functional antigen presentation studies of olfactory bulb and cortical microglia populations isolated from LPS-injected mice. **(a)** Flow cytometric analysis of CellTrace Violet-labelled OT-II CD4⁺ T cell proliferation after 72 h of culture with olfactory bulb (OB) CD11c-eYFP⁻ microglia or CD11c-eYFP⁺ microglia in the presence (+ OVA) or absence (- OVA) of ovalbumin 323-339. **(b)** Heat map of cytokine and chemokine levels in the supernatants of APC assays containing either

spleen CD11c-eYFP⁺ cells, olfactory bulb CD11c-eYFP⁺ microglia (OB YFP⁺ MG) or OB CD11c-eYFP⁻ microglia (YFP⁻ MG) after 72 h of co-culture with OT-II CD4⁺ T cells + OVA (APC: T cell ratio = 1:10). Scale is in pg/mL. **(c-e)** Confocal microscopy images (left panel, scale bars = 50 μ m) of 40 μ m olfactory bulb sections and 3D reconstructions (right panel, scale bars = 10 μ m) of boxed areas showing interactions between CD11c-eYFP⁺ microglia (yellow) and CD3⁺ lymphocytes (red) in control **(c,d)** and LPS-injected mice **(e)**. Note that the 3D reconstruction shown in **(e)** is rotated to allow visualisation of CD11c-eYFP⁺:CD3⁺ interactions. **(f)** Flow cytometric analysis of CellTrace Violet-labelled OT-II CD4⁺ T cell proliferation after 72 h of culture with cortical CD11c-eYFP⁻ microglia or CD11c-eYFP⁺ microglia +/- OVA. **(g)** Heat map of cytokine and chemokine levels in APC assays containing either spleen CD11c-eYFP⁺ cells, cortical CD11c-eYFP⁺ microglia (YFP⁺ MG) or cortical CD11c-eYFP⁻ microglia (YFP⁻ MG) after 72 h of co-culture with OT-II CD4⁺ T cells + OVA (APC:T cell ratio = 1:10). Scale is in pg/mL. **(h, i)** Confocal microscopy images showing the lack of CD3⁺ lymphocytes in the cortex of control **(h)** and LPS-injected **(i)** CD11c-eYFP *Crb1*^{wt/wt} mice. Scale bars = 100 μ m. Ratios shown above flow cytometry plots indicate the APC:T cell ratio tested. Numbers in flow cytometry plots indicate the percentage of proliferating cells after 72 h. CD11c-eYFP⁺ and CD11c-eYFP⁻ cells for APC assays were sorted from pooled cell suspensions from n = 20 (olfactory bulb) or n = 24 (cortex) mice. Naïve OT-II CD4⁺ T cells were isolated from n = 3 mice for each experiment. T cell proliferation and cytokine data are representative of 2 independent experiments. Confocal microscopy images are representative of n = 3 mice/group.

Figure 4: Phenotypic analysis of CD11c-eYFP⁺ and CD11c-eYFP⁻ cells within the choroid plexus, pia and dura following systemic LPS exposure. **(a)** Representative confocal microscopy images (n = 3 mice/group) of CD11c-eYFP⁺ cells (green) and Iba-1⁺ cells (red) in 40 μ m coronal brain sections (choroid plexus), 40 μ m oblique brain sections (pia; dotted lines separate partially detached pia, which

appears as a multi-layered sheet of cells in oblique sections, from the brain parenchyma) or wholemounts (dura). Scale bars = 100 μ m. **(b)** Flow cytometry gating of CD45⁺ CD11c-eYFP⁻ (red gate) and CD45⁺ CD11c-eYFP⁺ (blue gate) populations within the choroid plexus, and overlay histograms indicating the expression of I-A/I-E, CD80 and CD86 on gated populations. **(c)** Gating of CD45^{hi} CD11c-eYFP⁺ (red gate) and CD45^{hi} CD11c-eYFP⁻ (blue gate) populations within pia-cortex suspensions (note: CD45^{int} cortical microglia, gated in black, were excluded from analysis), and overlay histograms indicating the expression of I-A/I-E, CD80 and CD86 on pial CD45^{hi} CD11c-eYFP⁺ and CD45^{hi} CD11c-eYFP⁻ cells. **(d)** Gating of dural CD45⁺ CD11c-eYFP⁻ (red gate), CD45⁺ CD11c-eYFP^{lo} (blue gate) and CD45⁺ CD11c-eYFP^{hi} (purple gate) populations, and overlay histograms indicating the expression of I-A/I-E, CD80 and CD86 on gated populations. For all overlay histograms, control = shaded histogram, 24 h LPS = black line. Flow cytometry data were obtained from pooled single cell suspensions from n = 8 mice/group. Numbers in dot plots indicate percentages of gated populations. Note that cells were pre-gated as: single cells → non-debris → live cells.

Figure 5: Functional analysis of choroid plexus and dural leukocytes isolated from LPS-injected mice. **(a)** Flow cytometric analysis of CellTrace Violet-labelled OT-II CD4⁺ T cell proliferation after 72 h of culture with dural (top panel) or choroid plexus (bottom panel) CD11c-eYFP⁻ or CD11c-eYFP⁺ cells in the presence (+ OVA) or absence (- OVA) of ovalbumin 323-339. Ratios shown above flow cytometry plots indicate the APC:T cell ratio tested. Numbers in flow cytometry plots indicate the percentage of proliferating cells after 72 h. CD11c-eYFP⁺ and CD11c-eYFP⁻ cells for APC assays were sorted from pooled cell suspensions from n = 20 mice. Naïve OT-II CD4⁺ T cells were isolated from n = 3 mice for each experiment. Representative data from 2 independent experiments are shown. **(b, c)** Heat maps of cytokine and chemokine levels in the supernatants of APC assays containing

either: **(b)** spleen CD11c-eYFP⁺ cells, dural CD11c-eYFP⁺ cells or dural CD11c-eYFP⁻ cells after 72 h of co-culture with OT-II CD4⁺ T cells + OVA (APC:T cell ratio = 1:23), or **(c)** spleen CD11c-eYFP⁺ cells, choroid plexus CD11c-eYFP⁺ cells or choroid plexus CD11c-eYFP⁻ cells after 72 h of co-culture with OT-II CD4⁺ T cells + OVA (APC:T cell ratio = 1:10). Scales are in pg/mL. Purple boxes = >600 pg/mL. **(d, e)** Representative confocal microscopy images (n = 3 mice/group) of dural wholemounts showing localisation of CD11c-eYFP⁺ cells around LVE-1⁺ lymphatic vessels **(d)** and cell:cell interactions between CD11c-eYFP⁺ cells and CD3⁺ lymphocytes, and CD11c-eYFP⁺ cells with neighbouring CD11c-eYFP⁺ cells **(e)**. **(f, g)** Representative confocal microscopy images (n = 3 mice/group) of CD3⁺ lymphocytes (arrows) within the choroid plexus **(f)** and pia **(g)**. Scale bars: d = 100 μ m; e = 100 μ m (boxed regions = 20 μ m); f-g: 100 μ m.

Supplementary figure 1: Gating strategies for the sorting of: choroid plexus CD45⁺ CD11c-eYFP⁺ cells and CD45⁺ CD11c-eYFP⁻ cells **(a)**, dural CD45⁺ CD11c-eYFP⁺ cells and CD45⁺ CD11c-eYFP⁻ cells **(b)**, olfactory bulb CD45^{int} CD11c-eYFP⁺ microglia and CD45^{int} CD11c-eYFP⁻ microglia **(c)**, and cortical CD45^{int} CD11c-eYFP⁺ microglia and CD45^{int} CD11c-eYFP⁻ microglia **(d)**.

Supplementary figure 2: Functional analysis of spleen CD11c-eYFP⁺ and CD11c-eYFP⁻ leukocytes isolated from LPS-injected mice. Flow cytometric analysis of CellTrace Violet-labelled OT-II CD4⁺ T cell proliferation after 72 h of culture with APCs in the presence (+ OVA) or absence (- OVA) of ovalbumin 323-339. Ratios shown above flow cytometry plots indicate the APC:T cell ratio tested. Numbers in flow cytometry plots indicate the percentage of proliferating cells after 72 h. CD11c-eYFP⁺ and CD11c-eYFP⁻ cells for APC assays were sorted from pooled cell suspensions from n = 20 mice. Naïve OT-II CD4⁺ T cells were isolated from n = 3 mice for each experiment. Representative data from 2 independent experiments are shown.

Supplementary figure 3: Proliferating CD4⁺ T cells in functional assays upregulate CD44 when co-cultured with ovalbumin (OVA) and APCs from the olfactory bulbs (OB) **(a)**, dura **(b)** and choroid plexus **(c)** for 72 h. Ratios shown above flow cytometry plots indicate the APC: T cell ratio tested. Numbers in flow cytometry plots indicate the percentage of CD44⁺ cells. Data are representative of two independent experiments.

Supplementary figure 4: Phenotype of CD11c-eYFP⁺ populations within the dura of control CD11c-eYFP *Crb1*^{wt/wt} mice. Dural cell suspensions (obtained from pooled tissues from n = 8 mice) were stained with: rat anti-mouse CD45-BV605 (BioLegend 103140), rat anti-mouse CD11b-APC-Cy7 (BD Biosciences 557657), rat anti-mouse F4/80-PE-Cy7 (BioLegend 123114), rat anti-mouse I-A/I-E-PerCP-Cy5.5 (BD Biosciences 562363), Armenian hamster anti-mouse CD11c-BV786 (BD Biosciences 563735), Armenian hamster anti-mouse CD80-PECF594 (BD Biosciences 562504), rat anti-mouse CD86-AF700 (BioLegend 105024), rat anti-mouse CD115-BV711 (BioLegend 135515), rat anti-mouse CD135-PE-Cy5 (BioLegend 135312), rat anti-mouse CD8α-BV650 (BD Biosciences 563234), rat anti-mouse DEC205-APC (BioLegend 138206). Cells were pre-gated as follows: single cells → non-debris → live cells.

Supplementary figure 5: Comparison of the functional capacity of dural **(a)** and choroid plexus **(b)** APCs to stimulate naïve OT-II CD4⁺ T cell proliferation at various APC: T cell ratios in the presence (+OVA) or absence (-OVA) of ovalbumin 323-339.