



Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Classical activation of microglia in CD200-deficient mice is a consequence of blood brain barrier permeability and infiltration of peripheral cells

Stephanie Denieffe¹, Ronan J. Kelly¹, Claire McDonald, Anthony Lyons, Marina A. Lynch^{*}

Trinity College Institute for Neuroscience, Trinity College, Dublin 2, Ireland

ARTICLE INFO

Article history:

Received 29 May 2013

Received in revised form 22 July 2013

Accepted 29 July 2013

Available online xxxx

Keywords:

CD200

Blood brain barrier permeability

Inflammation

Microglial activation

Lipopolysaccharide

Claudin

ABSTRACT

The interaction between CD200, expressed on several cell types, and its receptor CD200R, expressed on cells of the myeloid lineage, has been shown to be an important factor in modulating inflammation in macrophage function in several conditions including colitis and arthritis. More recently its modulatory effect on microglial activation has been identified and CD200-deficiency has been associated with increased microglial activation accompanied by increased production of inflammatory cytokines. The response of glia prepared from CD200-deficient mice to stimuli like lipopolysaccharide (LPS) is markedly greater than the response of cells prepared from wildtype mice and, consistent with this, is the recent observation that expression of Toll-like receptor (TLR)4 and signalling through NF κ B are increased in microglia prepared from CD200-deficient mice. Here we show that glia from CD200-deficient mice are also more responsive to interferon- γ (IFN γ) which triggers classical activation of microglia. We investigated the effects of CD200-deficiency *in vivo* and report that there is an increase in expression of several markers of microglial activation including tumor necrosis factor (TNF)- α , which is a hallmark of classically-activated microglia. These changes are accompanied by increased IFN γ , and the evidence suggests that this is produced by infiltrating cells including T cells and macrophages. We propose that these cells enter the brain as a consequence of increased blood brain barrier (BBB) permeability in CD200-deficient mice and that infiltration is assisted by increased expression of the chemokines, monocyte chemoattractant protein-1 (MCP-1), IFN γ -induced protein-10 (IP-10) and RANTES. This may have implications in neurodegenerative diseases where BBB permeability is compromised.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Microglia, like macrophages, are plastic cells, responding to different stimuli by adopting different morphological and functional phenotypes indicative of their broad array of functions (Lynch, 2009). These cells are the immune cells of the brain and therefore respond to pathogen-associated, and danger-associated, molecular patterns which trigger the responses that result from insult. An acute stimulus generally induces release of inflammatory mediators, but this relatively brief inflammatory state is followed by restoration of microglia to their resting state. In contrast, exposure of cells to chronic activation is undesirable and can result in damage to neighboring cells.

A number of endogenous mechanisms ensure that microglial activation is kept in check and these include the interaction with other cells through ligand–receptor binding. Among the

ligand–receptor pairs is CD200–CD200R which has complementary cell expression patterns characteristic of this method of controlling microglial activation. Thus CD200 is expressed on several cell types including neurons (Lyons et al., 2007a; Webb and Barclay, 1984) and astrocytes (Costello et al., 2011) whereas CD200R is expressed primarily on cells of the myeloid lineage and on microglia in the brain (Barclay et al., 2002; Koning et al., 2009). The evidence has indicated that decreased expression of CD200, which occurs with age (Cox et al., 2012; Frank et al., 2006), A β treatment (Lyons et al., 2007a) or in the brain of AD patients (Walker et al., 2009), is associated with the type of microglial activation that is characterized by inflammatory changes (Colton and Wilcock, 2010). At least in some cases, these inflammatory changes are attenuated by a CD200 fusion protein (CD200Fc) which stimulates CD200R activation (Cox et al., 2012; Lyons et al., 2012), although the evidence indicates that the reversal is incomplete.

We have recently shown that glial cells prepared from CD200^{−/−} mice respond more profoundly to Toll-like receptor (TLR) agonists, specifically lipopolysaccharide (LPS) which interacts with TLR4, and Pam₃CSK₄ which interacts with TLR2 (Costello et al., 2011);

* Corresponding author. Tel.: +353 1 896 8531; fax: +353 1 896 3545.

E-mail address: lynchma@tcd.ie (M.A. Lynch).

¹ These authors contributed equally to the work.

expression of both receptors was increased on microglia prepared from CD200^{-/-} mice, compared with wildtype mice, and this was associated with a genotype-related increase in signaling through NFκB. Thus the loss of CD200 in CD200-deficient mice is accompanied by a number of changes that play a role in modulating microglial activation.

In the present study, we set out to evaluate the changes that accompany the loss of CD200 *in vivo* with the objective of increasing our understanding of the mechanisms that modulate microglial function. First, we showed that interferon (IFN)-γ, which induces a classically-activated microglial phenotype exerted a greater effect in cultured cells from CD200^{-/-}, compared with wildtype mice. *In vivo*, CD200 deficiency was associated with increased expression of markers of microglial activation including tumor necrosis factor (TNF)-α, which is a hallmark of classically-activated microglia, in the absence of any stimulus. Increased expression of IFNγ was observed in hippocampus of CD200^{-/-} mice, although it is generally considered that IFNγ is not produced by resident cells in the brain (Lynch, 2010; Renno et al., 1995). The probable explanation for this is that there was a significant infiltration of T cells and macrophages into the brain of CD200^{-/-} mice, and the data suggest that cell infiltration is a consequence of the increase in blood brain barrier (BBB) permeability and increased expression of the chemokines, monocyte chemoattractant protein-1 (MCP-1), IFNγ-induced protein-10 (IP-10) and RANTES (also known as CCL5).

The data highlight the consequences of infiltration of peripheral immune cells on microglial function and suggest that the inflammatory changes which occur in neurodegenerative diseases might be modulated by targeting the BBB.

2. Methods

2.1. Animals

C57BL/6 mice (wildtype) and CD200-deficient mice were used in this study. Mice were bred in an SPF housing facility in the Bio-Resources Unit, Trinity College, Dublin and were maintained under veterinary supervision in a controlled environment (12-hour light-dark cycle; 22–23 °C) for the duration of the experiment. In one series of experiments, wildtype mice were anaesthetized with urethane (1.5 g/kg; Sigma Aldrich; IRE) and injected with IFNγ (5 μl @ 1 μl/min; 50 ng/ml; R&D Systems, UK; co-ordinates: 0.34 mm caudal and 1 mm lateral to bregma, at a depth of 2.1 mm. After 3 h, animals were perfused intracardially with ice-cold PBS (20 ml). In all experiments, the brain was rapidly removed and placed on ice, the cerebellum, brain stem, olfactory lobes and hippocampus were removed and the remaining tissue was taken to prepare a single cell suspension for flow cytometric analysis as described below. Animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act 1876 and the European Community Directive, 86/609/EEC, and in accordance with local ethical guidelines.

2.2. MRI analysis to assess BBB permeability and blood flow

MRI analysis, to assess extravasation of the gadolinium-based contrast agent, gadopentate dimeglumine (Magnevist; Clissmann, Ireland) as an indicator of BBB permeability, was carried out on a small rodent Bruker Biospec (Bruker Biospin, Germany) system with a 7 Tesla magnet and a 30 cm core. Anaesthesia was induced and maintained using isoflurane (5% in 100% O₂ and 1.5–2% at 1 l/min of 100% O₂, respectively). Respiration rate was monitored and body temperature was controlled throughout.

Mice were placed into an MR-compatible cradle and positioned in a stereotaxic frame. The right lateral tail vein was cannulated

using a 30 gauge in-dwelling, paediatric intravenous cannula needle (Introcan, Ireland) to which an 80 cm long polyethylene tubing cannula extension, connected to a 3-way tap, was attached; this allowed delivery of contrast agent (200 μl/mouse) without disturbing the animals and enabled pre- and post-contrast measurements to be determined within the same contrast scan. Contrast imaging was carried out using a 10 repetition T₁-weighted fast low angle shot (FLASH) sequence; the repetition duration was 2 min, 11 s and the 15-slice protocol had dimensions of 128 × 128 voxels per slice. Pre-contrast measurements were acquired on the first repetition of the scan to provide a contrast-free baseline after which the contrast agent was injected; the following 9 scan repetitions recorded the passage of the bolus throughout the brain. Gadolinium-enhanced contrast images acquired from the BBB permeability studies were analysed in the MIPAV software package. The average signal intensity changes in each treatment group were plotted against repetition. Anatomically-distinct ROIs, viewed in the first repetition, were overlaid with a 2 × 2 voxel square and the average pre-contrast intensity was measured. The ROI squares were copied into the same locations in the remaining 9 repetitions for each mouse brain, measuring the contrast change following injection of the gadolinium-based contrast agent at the commencement of the second repetition. Values for each data set were normalised to the pre-contrast measurement and expressed as a proportion of the pre-contrast value.

Quantitative blood flow and volume measurements were investigated using a bolus-ASL methodology (Kelly et al., 2010). This method uses a bolus-tracking ASL sequence to provide groups of 11 one slice images on a single time curve following the passage of a 3 s bolus through the imaging slice. Control and labelled images were created for wildtype and CD200-deficient mice and corresponding pairs were subtracted to provide perfusion-weighted maps. Mean values for cortical and hippocampal ROIs, manually drawn on perfusion-weighted images, were plotted against the acquisition time point in the bolus tracking ASL sequence. The curve was fitted to the solution of the non-compartmental cerebral perfusion ASL model (Kelly et al., 2009), and the curve-fitting utility in Mathematica was used to find the least squares fit to the experimental curves. The mean transit time (MTT) and capillary transit time (CTT) were calculated from the first and second statistical moments of the signal-time curves, respectively.

Following MRI scanning, animals were anaesthetised with urethane (1.5 g/kg; Sigma Aldrich; IRE) and perfused intracardially with ice-cold PBS (20 ml). The brain was rapidly removed and placed on ice, the cerebellum and olfactory bulbs were removed and the brain was bisected along the midline. The hippocampus was dissected free and snap-frozen in liquid nitrogen, for later analysis by PCR and/or ELISA, and the remaining brain tissue was used to prepare a single cell suspension for analysis by flow cytometry.

2.3. Preparation of cultured glia

Mixed glial cells were prepared from 1 day-old wildtype and CD200^{-/-} mice (BioResources Unit, Trinity College, Dublin, Ireland) as described (Costello et al., 2011). To prepare mixed glia, whole brain minus cerebellum was roughly chopped, added to pre-warmed Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Ireland) containing 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml), triturated and filtered through a sterile mesh filter (70 μm). Samples were centrifuged (2000g, 3 min, 20 °C), the pellet was resuspended in warmed DMEM, cells were seeded in 6 ml flasks (1 × 10⁶ cells/flask) and incubated for 2 h before addition of warmed DMEM. Cells were grown for 10–12 days at 37 °C in a humidified 5% CO₂:95% air environment and

media was changed every 3 days. Mixed glia were approximately 70% astrocytes and 30% microglia (Costello et al., 2011). Purified microglia were harvested by shaking off (110 rpm, 2 h, room temperature) the non-adherent cells from the mixed cultures which had been cultured for 10–12 days. Samples were centrifuged (2000 rpm, 5 min), the pellet was resuspended in cDMEM, the microglia were plated onto 6-well plates (2×10^5 cells/ml) and cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere for up to 3 days prior to treatment. Cells, which were 97% CD11b⁺ as assessed by FACS, were incubated for 24 h in the presence or absence of IFN γ (50 ng/ml; R&D Systems, UK).

2.4. Analysis of mRNA by PCR

RNA was isolated from hippocampal tissue and from cultured cells using Nucleospin[®] RNAII KIT (Macherey–Nagel, Duren, Germany) and cDNA was prepared using High-Capacity cDNA RT kit according to the manufacturer's instructions (Applied Biosystems, UK). Real-time PCR was performed with predesigned Taqman gene expression assays (Applied Biosystems, UK). The assay IDs were as follows: CD11b (Mm01271263_m1), MHCII (Mm00439221_m1), CD40 (Mm0041895_m1), MCP-1 (Mm00441242_m1), IP-10 (Mm00445235_m1), RANTES (CCL5; Mm01302428_m1), MIP-1 α (CCL3; Mm00441258_m1), claudin 5 (Mm00727012_m1), TNF α (Mm0043258_m1), iNOS (Mm00440502_m1), IL-6 (Mm00446191_m1), mannose receptor (MR; Mm00485148_m1) and arginase-1 (Arg-1; Mm00475988_m1). Samples were assayed on an Applied Biosystems 7500 Fast Real-Time PCR machine and gene expression was calculated relative to the endogenous control samples (β -actin) to give a relative quantification (RQ) value ($2^{-\text{DDCT}}$, where CT is threshold cycle).

2.5. Flow cytometry

Flow cytometry was carried out on cells prepared from brain using a 5-layer Percoll gradient to obtain mononuclear cells, as described (Costello et al., 2011). Cells were washed 3 times with FACS buffer (2% FBS, 0.1% NaN₃ in PBS), blocked for 15 min at room temperature with mouse CD16/CD32 Fc γ RIII block (1:100 in FACS buffer; BD Biosciences, UK) and incubated with APC-Cy[™]7 rat anti-mouse CD11b, PE-Cy[™]7 rat anti-mouse CD45, PerCP rat anti-mouse CD8a, PeCy[™]7 rat anti-mouse CD4, FITC hamster anti-mouse CD40, PE rat anti-mouse MHCII (all BD Biosciences, UK), Alexa Fluor 488 rat anti-mouse CD68 (AbD Serotec, UK), PE hamster anti-mouse CD119 (IFN gamma Receptor 1 (IFNGR1; eBioscience, UK), APC rat anti-mouse CD3 and PerCP/Cy5.5 rat anti-mouse CD86 (Biolegend, UK), diluted (1:200) in FACS buffer. Immunofluorescence analysis was performed on a DAKO CyAn-ADP 7-colour flow cytometer (DAKO Cytomation, UK) with Summit version 4.3 software and data analysis was carried out using FlowJo v7.6.5 software. Microglia and macrophages were identified as CD11b⁺CD45^{low} and CD11b⁺CD45^{high} cells, respectively. CD4⁺ and CD8⁺ T cells were initially gated on CD45 and subsequently on CD3, and CD4 or CD8.

2.6. Western immunoblotting

Hippocampal lysate or lysate prepared from cultured cells was assessed for the expression of IFN γ , IFNGR1 or pSTAT1 using standard Western immunoblotting methods; the membranes were incubated overnight in the presence of the primary antibodies, IFN γ (R&D, UK; 1:1000 in TBS containing 0.05% Tween and 2.5% dried non-fat milk), IFN γ R α (IFNGR1; Santa Cruz, US; 1:200 in

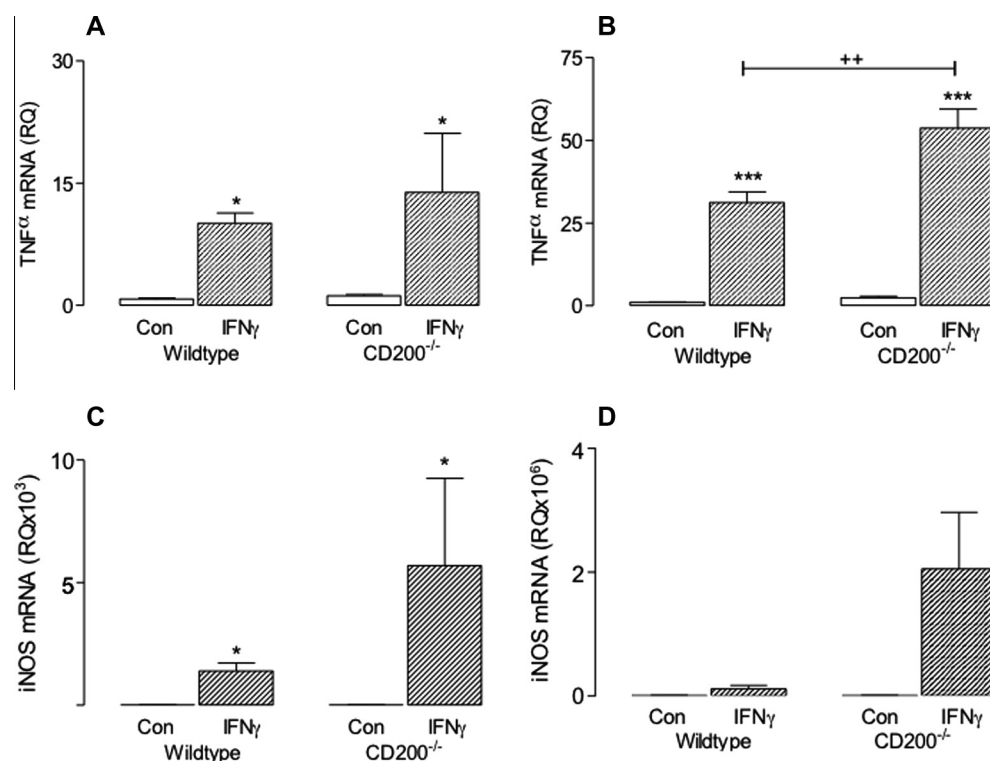


Fig. 1. IFN γ induced markers of classical activation in microglia prepared from wildtype and CD200^{-/-} mice. Mixed glial cells, prepared from 1 day-old wildtype and CD200^{-/-} mice, were grown for 10–12 days at 37 °C in a humidified 5% CO₂:95% air environment and media was changed every 3 days. Purified microglia were obtained by shaking off the non-adherent cells from the mixed cultures (110 rpm, 2 h, room temperature), centrifuging (2000 rpm, 5 min), resuspending in cDMEM and plating onto 6-well plates (2×10^5 cells/ml) and cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere for 1–3 days. Cells were incubated for 24 h in the presence or absence of IFN γ (50 ng/ml; R&D Systems, UK). IFN γ increased TNF α mRNA in mixed glia (A) and purified microglia (B) prepared from wildtype and CD200^{-/-} mice; a significant treatment effect was observed in the case of mixed glia ($F(1,9) = 10.41$; $*p < 0.05$) and a significant interaction between treatment and genotype was observed in the case of purified microglia ($F(1,20) = 10.13$; $**p = 0.01$). iNOS mRNA was increased in response to IFN γ in mixed glia (C) and purified microglia (D) prepared from wildtype and CD200^{-/-} mice; a significant treatment effect was observed for mixed glia ($F(1,8) = 10.55$; $*p < 0.05$). Values are presented as means \pm SEM.

TBS containing 0.05% Tween and 2.5% dried non-fat milk) and pSTAT1 (Cell Signaling, Ireland; 1:1000 in TBS containing 0.05% Tween and 2.5% dried non-fat milk) and subsequently in peroxidase-conjugated secondary antibody for 2 h. Membranes were washed and protein complexes were visualized using enhanced chemiluminescence. Membranes were stripped (Re-blot plus; Chemicon, US) and reprobbed for β -actin. Images were captured using a Fijifilm LAS-4000 imager, and densitometric analysis was used to quantify expression of the proteins.

2.7. Analysis of cytokine expression by ELISA

The concentrations of IFN γ , TNF α and IL-6 were assessed by ELISA in hippocampal tissue or supernatant from glial cultures as previously described (Lyons et al., 2007a). In brief, 96-well plates were coated overnight with capture antibody (rat anti-mouse IL-6 (2 μ g/ml in PBS; BD Biosciences, UK), goat anti-mouse TNF α antibody (0.8 μ g/ml in PBS; BD Biosciences, UK), or rat anti-mouse IFN γ (4 μ g/ml in PBS; R&D Systems). Triplicate samples and standards (50 μ l) were added, plates were incubated (2 h, room temperature), washed and detection antibody (100 ng/ml biotinylated anti-mouse TNF- α or biotinylated anti-mouse IL-6 in PBS containing 10% FBS or biotinylated goat anti-mouse IFN γ in TBS containing 0.1% bovine serum albumin and 0.05% Tween-20; R&D Systems, UK) was added. Samples were incubated with streptavidin-horse radish peroxidase conjugate (100 μ l; 1:200; 20 min, room temperature) and washed before addition of substrate solution (100 μ l; 1:1 H $_2$ O $_2$:tetramethylbenzidine; R&D Systems, US). After colour development, the reaction was stopped by adding 50 μ l 1 M H $_2$ SO $_4$ and plates were read at 450 nm (Labsystem Multiskan RC,

UK). The limits of detection for IFN γ , TNF α and IL-6 were 0.38 pg/ml, 4.3 pg/ml and 10 pg/ml, respectively.

2.8. Statistical analysis

Data are expressed as means \pm standard error of mean (SEM). Two-way analysis of variance (ANOVA) was performed to determine whether significant differences existed between conditions. Two-tailed unpaired Students *t*-tests were also performed, where indicated, to compare treatment groups; significance was set as $p < 0.05$. All statistical analyses were carried out using GraphPad Prism.

3. Results

Classical activation of macrophages is identified by increased expression of TNF α and iNOS and their upregulation is designed to reinforce the initial innate immune response (Colton, 2009). The data presented here show that IFN γ (50 ng/ml) increased TNF α mRNA in mixed glia (Fig. 1A) and purified microglia (Fig. 1B) prepared from wildtype and CD200 $^{-/-}$ mice ($F(1,9) = 10.41$; $*p < 0.05$; $F(1,20) = 150.3$, $***p < 0.001$; 2-way ANOVA). In purified microglia, a significant interaction between IFN γ treatment and CD200-deficiency was observed ($**p < 0.01$). IFN γ also increased iNOS mRNA expression in mixed glia (Fig. 1C) prepared from wildtype and CD200 $^{-/-}$ mice ($F(1,8) = 10.55$, $*p < 0.05$) and although the effect was greater in purified microglia prepared from CD200 $^{-/-}$, compared with wildtype, mice, this did not reach statistical significance ($F(1,17) = 14.37$, $p = 0.058$). IFN γ increased release of TNF α and IL-6 from mixed glia (Fig. 2A and C) and purified microglia (Fig. 2B and D) prepared from wildtype and CD200 $^{-/-}$ mice; a sig-

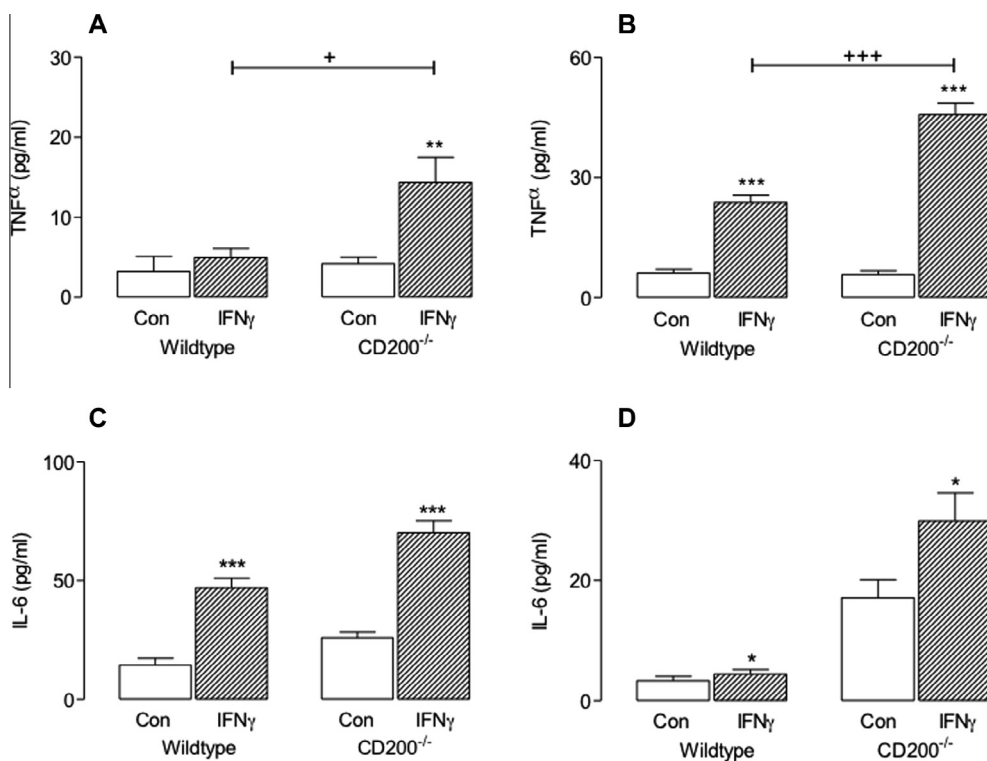


Fig. 2. IFN γ -induced TNF α and IL-6 release was enhanced in mixed glia and purified microglia prepared from CD200 $^{-/-}$ mice. Cells (mixed glia and purified microglia) were prepared as described in the Methods and in the legend to Fig. 1. IFN γ increased supernatant concentrations of TNF α (A and B) and IL-6 (C and D) in mixed glia (A and C) and purified microglia (B and D) prepared from wildtype and CD200 $^{-/-}$ mice. A significant interaction between treatment and genotype was observed in the case of TNF α ($F(1,13) = 5.086$; $p < 0.05$ for mixed glia and $F(1,20) = 31.81$; $***p < 0.001$ for purified microglia). In the case of IL-6, significant treatment ($F(1,12) = 119.0$; $***p < 0.001$ and $F(1,14) = 4.804$; $*p < 0.05$) and genotype ($F(1,12) = 24.21$; $***p < 0.001$ and $F(1,14) = 38.13$; $***p < 0.001$) effects were observed for mixed glia and microglia, respectively. Values are presented as means \pm SEM.

nificant interaction between genotype and treatment was observed in the case of TNF α (A: $F(1,13)=5.086$; $*p < 0.05$; B: $F(1,20)=31.81$; $***p < 0.001$) and significant treatment (C: $F(1,12)=119.0$; $***p < 0.001$; D: $F(1,14)=4.804$; $*p < 0.05$) and genotype (C: $F(1,12)=24.21$; $p < 0.001$; D: $F(1,14)=38.13$; $p < 0.001$) effects were observed in the case of IL-6. Overall, CD200 $^{-/-}$ mice display an enhanced response to IFN γ and suggest a role for CD200 in modulating the classical activation pathway. Interestingly, when unstimulated IL-6 concentration was extracted and compared in cells from wildtype and CD200 $^{-/-}$ mice, a significantly greater concentration was observed in cells prepared from CD200 $^{-/-}$, compared with wildtype, mice ($p < 0.05$ and $p < 0.01$ for isolated microglia and mixed glia, respectively; student's t -test for independent means). We also examined the effect of IFN γ on >95% purified astrocytes from wildtype and CD200 $^{-/-}$ mice and no genotype-related changes were observed (data not shown).

To explore the possible mechanism underlying the increased responsiveness of glia prepared from CD200 $^{-/-}$ mice to IFN γ , the expression of IFNGR1 was assessed. Flow cytometric analysis indicated that the number of IFNGR1 $^{+}$ CD11b $^{+}$ cells was increased in cell preparations obtained from CD200 $^{-/-}$, compared with wildtype, mice ($F(1,12)=12.27$; $**p < 0.01$; 2-way ANOVA; Fig. 3A and B). In addition, IFN γ significantly increased STAT1 phosphorylation in glia prepared from CD200 $^{-/-}$ mice; a significant interaction between genotype and treatment was observed ($F(1,9)=63.34$, $***p < 0.001$; Fig. 3C).

In contrast to the differential genotype-related effect of IFN γ , IL-4, which induces alternative activation of macrophages and microglia (Colton et al., 2006; Gordon and Martinez, 2010), significantly increased expression of 2 hallmarks of alternative activation, arginase-1 mRNA and mannose receptor mRNA albeit to the same extent in mixed glia prepared from wildtype and CD200 $^{-/-}$ mice (data not shown). These results demonstrate the specific nature of the modulatory effect of CD200 on classically-induced activation of microglia.

To examine the effect of CD200 deficiency on microglial activation *in vivo*, hippocampal tissue was assessed for expression of 3 markers of activation, CD11b mRNA, CD40 mRNA and MHCII mRNA, and cells were prepared from brain tissue to investigate expression of CD40 on CD11b $^{+}$ cells. The data indicate that mRNA expression of CD11b, CD40 and MHCII was significantly increased in hippocampal tissue prepared from CD200 $^{-/-}$, compared with wildtype, mice ($*p < 0.05$; $**p < 0.01$; Fig. 4A–C). However since there was an increase in macrophage infiltration, these changes could also be indicative of increased macrophage activation. The finding that the percentage of CD11b $^{+}$ CD40 $^{+}$ CD45 low cells was doubled in tissue prepared from CD200 $^{-/-}$, compared with wildtype, mice is indicative of microglial activation rather than macrophage activation (Fig. 4D and E). The evidence suggested that microglia adopted the classical phenotype since mRNA expression of TNF α , iNOS and IL-6 was increased in the hippocampus of CD200 $^{-/-}$ mice compared with wildtype mice ($*p < 0.05$; $**p < 0.01$; Student's t -test for independent means; Fig. 4F–H). Hippocampal concentrations of TNF α in WT and CD200-deficient mice were 2.55 pg/mg (± 0.01 , SEM) and 4.69 (± 1.67), respectively and IL-6 in WT and CD200-deficient mice were 4.32 (± 1.56 SEM) and 17.93 (± 3.57), respectively ($p < 0.05$; student's t -test for independent means).

Since IFN γ triggers the classical activation state, it was important to assess hippocampal concentration of the cytokine. We demonstrate that IFN γ concentration was significantly increased in hippocampus prepared from CD200 $^{-/-}$, compared with wildtype, mice ($*p < 0.05$; $**p < 0.01$; student's t -test for independent means; Fig. 5A) and this was confirmed by showing increased IFN γ expression in hippocampal tissue using Western immunoblotting ($*p < 0.05$; student's t -test for independent means; Fig. 5B). Additionally, there was a significant increase in expression of IFNGR1

as revealed by Western immunoblotting ($*p < 0.05$; student's t -test for independent means; Fig. 5C); whereas the cells expressing this receptor was not assessed in this study, the data in Fig. 3 indicate increased expression on microglia from CD200 $^{-/-}$, compared with wildtype, mice. The cell source of IFN γ which is responsible for the increase in brain tissue remains to be established, but we have been unable to detect its presence in cultured glia or neurons

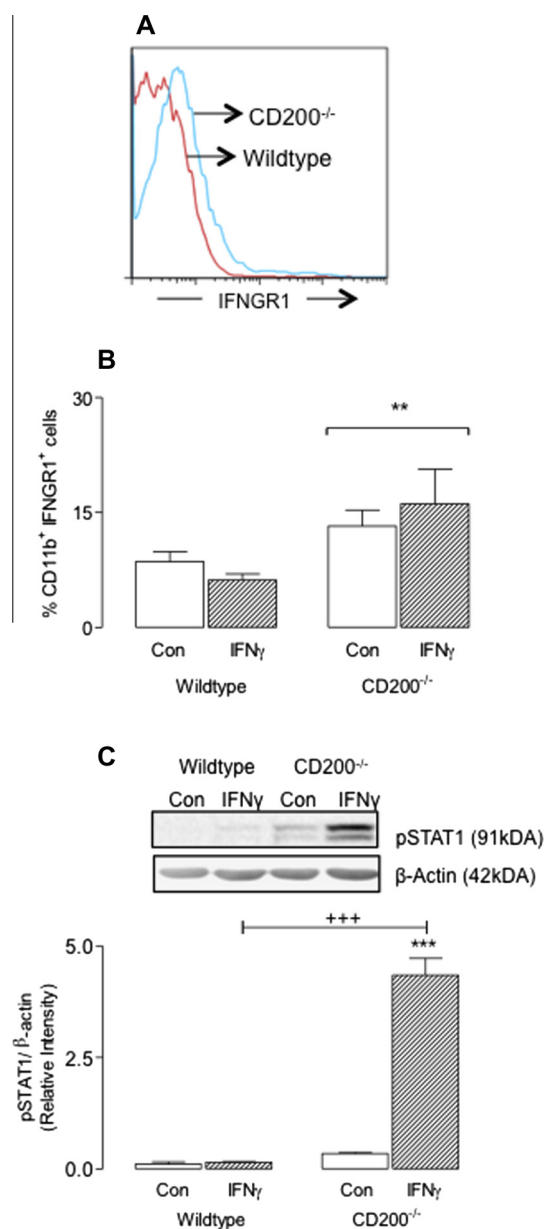


Fig. 3. CD200 deficiency is associated with upregulation of IFN γ receptor 1 and signalling. Purified microglia were prepared as described in the Methods and in legend to Fig. 1. Cells were washed and blocked and incubated with antibodies including APC-Cy7TM rat anti-mouse CD11b and PE hamster anti-mouse CD119 (IFN gamma Receptor 1; eBioscience, UK; see Methods for details). (A and B) Flow cytometric analysis revealed that the percentage of CD11b $^{+}$ IFNGR1 $^{+}$ cells was increased in cells prepared from CD200 $^{-/-}$, compared with wildtype, mice; a significant genotype effect was observed ($F(1,12)=12.27$, $**p < 0.01$). (C) Microglia were incubated for 24 h in the presence or absence of IFN γ (50 ng/ml), cells were harvested and lysate was assessed by Western immunoblotting for analysis of STAT1 phosphorylation. A sample immunoblot reveals that IFN γ increased STAT1 phosphorylation in cells prepared from CD200 $^{-/-}$, compared with wildtype, mice and analysis of the densitometric data indicated a significant interaction between treatment and genotype ($F(1,9)=63.34$; $***p < 0.001$). Values are presented as means \pm SEM.

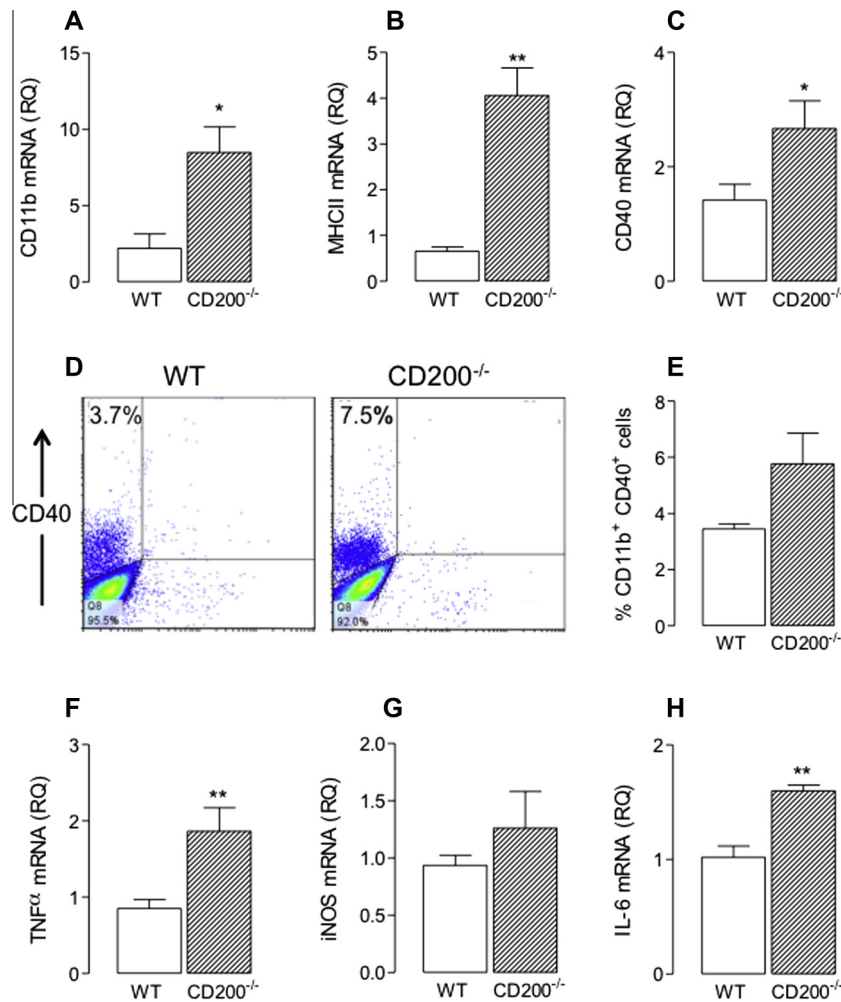


Fig. 4. The expression of CD11b, MHC class II and CD40 was increased in the hippocampus of CD200^{-/-} mice. Real-time PCR was performed on cDNA prepared from hippocampal tissue of wildtype and CD200-deficient mice. Details of the predesigned Taqman gene expression assays are given in the Methods. Gene expression was calculated relative to the endogenous control samples (β -actin) to give a relative quantification (RQ) value ($2^{-\Delta\Delta CT}$, where CT is threshold cycle). Expression of CD11b (A), MHC II (B) and CD40 (C) was significantly increased in the hippocampus of CD200^{-/-} mice compared with wildtype mice ($*p < 0.05$; $**p < 0.01$; Student's *t* test for independent means). (D and E) Cells, prepared for flow cytometry as described in the Methods, were incubated with antibodies including APC-CyTM7 rat anti-mouse CD11b, PE-CyTM7 rat anti-mouse CD45 and FITC hamster anti-mouse CD40. Representative FACS plots for CD11b⁺ cells expressing CD40 from wildtype and CD200^{-/-} mice demonstrate there was an apparent increase in CD40 expression on CD11b⁺ cells prepared from CD200^{-/-}, compared with wildtype, mice, however this failed to reach statistical significance. mRNA expression of TNF α (F), iNOS (G) and IL-6 (H) was significantly increased in the hippocampus of CD200^{-/-} mice compared with wildtype mice ($*p < 0.05$; $**p < 0.01$; Student's *t* test for independent means). Values are presented as means \pm SEM ($n = 4-6$).

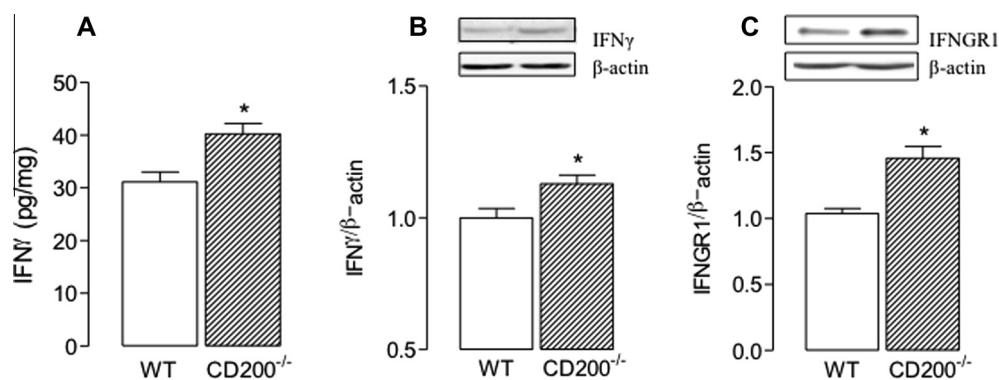


Fig. 5. IFN γ and IFN γ receptor 1 expression was increased in the hippocampus of CD200^{-/-} mice. (A) IFN γ concentration was assessed by ELISA in samples of hippocampal homogenate prepared from CD200^{-/-} and wildtype mice. IFN γ concentration was increased in samples prepared from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; Student's *t*-test for independent means). (B) IFN γ was also assessed in hippocampal lysate prepared from these animals and the sample immunoblot and mean data from densitometric analysis revealed a significant genotype-related difference ($*p < 0.05$; Student's *t*-test for independent means). (C) Similarly, the sample immunoblots and the mean data from densitometric analysis revealed that IFN γ receptor 1 was significantly increased in tissue from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; Student's *t*-test for independent means). Values are presented as means \pm SEM ($n = 4-6$).

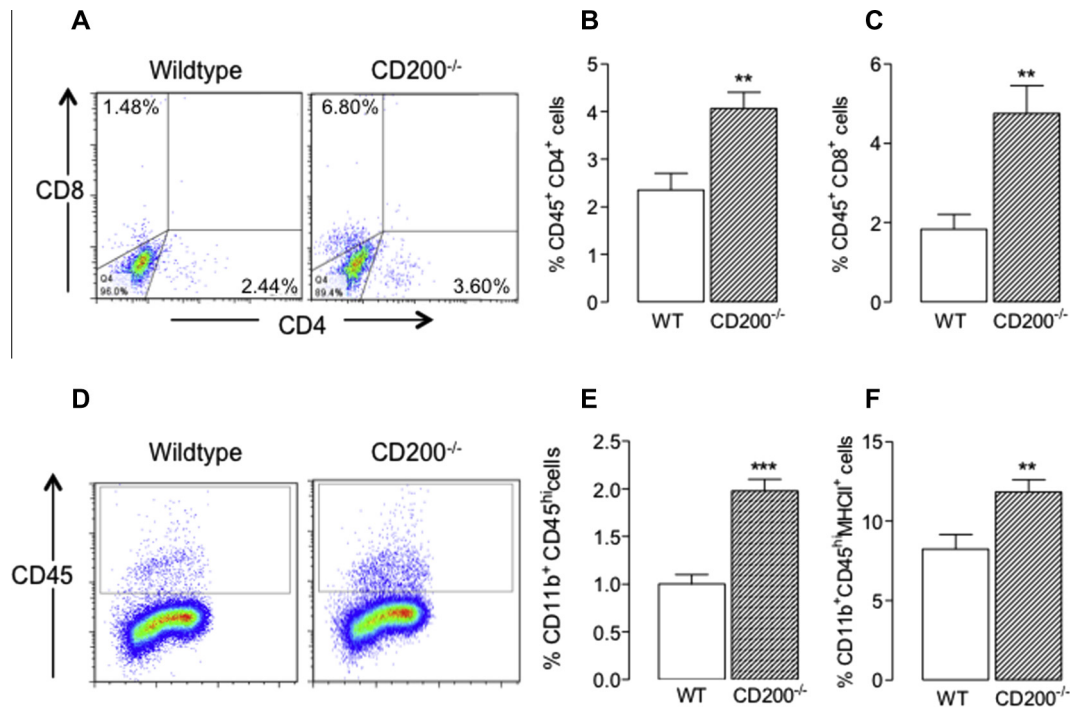


Fig. 6. Deficiency of CD200 is associated with infiltration of immune cells. A single cell suspension, prepared from perfused brain, was added to a 5-layer Percoll gradient to obtain mononuclear cells, as described previously (Costello et al., 2011) and in the Methods. Cells, prepared for flow cytometry as described in the Methods, were incubated with antibodies including APC-Cy7TM rat anti-mouse CD11b, PE-Cy7TM rat anti-mouse CD45, PerCP rat anti-mouse CD8a, PE rat anti-mouse CD4 and PE rat anti-mouse MHCII. Flow cytometric analysis indicated that there was a significant increase in the number of CD45⁺ CD3⁺ CD4⁺ cells and CD45⁺ CD8⁺ cells in preparations obtained from CD200^{-/-} mice compared with wildtype mice ((A–C); ***p* < 0.01; student's *t*-test for independent means). There was also a significant increase in the number of CD11b⁺ CD3⁺ CD45^{high} cells (i.e., macrophages), as well as CD11b⁺ CD45^{high} MHCII⁺ cells, in single cell suspensions prepared from the brains of CD200^{-/-}, compared with wildtype, mice ((D–F); ***p* < 0.01; ****p* < 0.001; student's *t*-test for independent means). Values are presented as means ± SEM (*n* = 4–6).

suggesting that infiltrating cells may be the source. IFN γ is primarily produced in, and released from, immune cells and we have previously reported that infiltrating CD3⁺ CD4⁺ and CD3⁺ CD8⁺ cells in mice that overexpress amyloid precursor protein and presenilin 1 were IFN γ ⁺ (Browne et al., 2013). Here we considered that the observed increase in IFN γ in the brain of CD200-deficient mice may be a consequence of infiltrating IFN γ -producing peripheral cells which may, in turn, contribute to the observed microglial activation. Therefore a single cell suspension was prepared from perfused brain tissue of wildtype and CD200^{-/-} mice and assessed by flow cytometry for evidence of infiltrating cells. The data indicate that there was a significant increase in CD4⁺ and CD8⁺ cells in tissue prepared from CD200^{-/-}, compared with wildtype, mice (***p* < 0.01; student's *t*-test for independent means; Fig. 6A–C). There was also a significant genotype-associated increase in the number of CD11b⁺CD45^{high} macrophages (****p* < 0.001; student's *t*-test for independent means; Fig. 6D and E) and, interestingly, the proportion of these cells which stained positively for MHCII was also increased (***p* < 0.01; student's *t*-test for independent means; Fig. 6F).

Infiltration of cells may be facilitated if BBB permeability increases and we examined this possibility in wildtype and CD200^{-/-} mice by assessing time-related changes in signal intensity following intravenous injection of the gadolinium-based contrast agent, gadopentate dimeglumine. Signal intensity, indicative of extravasation of the contrast agent (Blamire et al., 2000), was significantly increased in several areas of the brain of CD200^{-/-} mice compared with wildtype mice (Fig. 7A); significant differences were observed in the dentate gyrus and area CA1 (Fig. 7B and C) and also in the motor cortex and entorhinal cortex (Fig. 7D and E). Changes were also observed in several other regions including area CA3 of the hippocampus, the somatosensory cortex, frontal associational cortex, thalamus and cerebellum. We assessed CTT and MTT in

hippocampal and cortical tissue in wildtype and CD200^{-/-} mice and no genotype-related changes were observed (Fig. 7F–I). Analysis in whole brain similarly showed no genotype-related changes. We examined expression of the tight junction proteins claudin 5, claudin 12 and occludin mRNA and the data indicate that claudin 5 mRNA was significantly decreased in hippocampus (***p* < 0.01; student's *t*-test for independent means; Fig. 7J) and cortex (***p* < 0.01; student's *t*-test for independent means; Fig. 7K) of CD200^{-/-} mice compared with wildtype mice. Analysis of claudin 5 in hippocampus by Western immunoblotting indicated that there was a genotype-related decrease (1.29 ± 0.11 vs 1.01 ± 0.09; (mean ± SEM; *n* = 5) wildtype vs CD200^{-/-}; *p* < 0.05; student's *t*-test for independent means; densitometric data expressed as a ratio, claudin 5:β-actin). However there was no significant genotype-related difference in either claudin 12 mRNA or occludin mRNA (data not shown). Interestingly, there was a significant inverse relationship between the signal intensity and claudin 5 mRNA in hippocampus and cortex (*p* < 0.01; Fig. 7L). While these data are consistent with the idea that increased BBB permeability enables infiltration of peripheral cells into the brain, it is interesting that expression of two chemokines, MCP-1 and IP-10, which act as chemotactic factors for recruitment of cells including macrophages and T cells, were significantly increased in hippocampal tissue prepared from CD200^{-/-} mice compared with wildtype mice (**p* < 0.05; student's *t*-test for independent means; Fig. 8A and B). The data also show that there was a genotype-related increase in CCL5 mRNA (**p* < 0.05; student's *t*-test for independent means; Fig. 8C) but not CCL3 mRNA (Fig. 8D).

If IFN γ is the key to the increased microglial activation in CD200^{-/-} mice, then it must be predicted that injection of IFN γ will mimic the effects of CD200 deficiency. To assess this, a single cell suspension was prepared from the perfused brain of control-

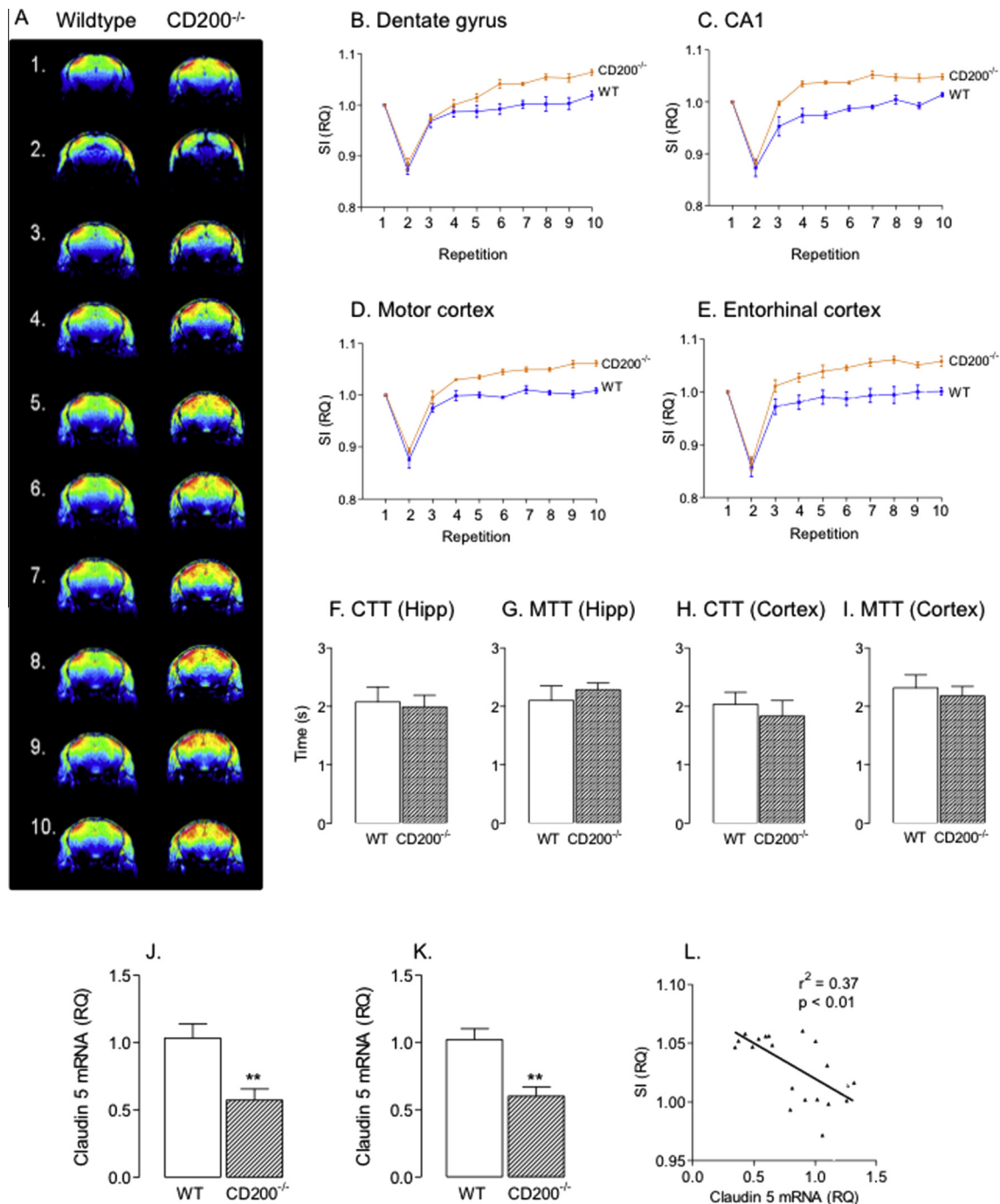


Fig. 7. BBB permeability is increased in hippocampus and cortex of CD200^{-/-} mice. Signal intensity (SI) generated by extravasation of the gadolinium-based contrast agent, gadopentate dimeglumine, was used to assess BBB permeability using MRI. Mice were anaesthetized using isoflurane (5% in 100% O₂) and maintained under anaesthesia using 1.5–2% isoflurane in 100% O₂. Mice were placed in a cradle, positioned in a stereotaxic frame and the right lateral tail vein was cannulated to allow delivery of gadopentate dimeglumine. Contrast imaging was carried out using a T₁-weighted fast low angle shot (FLASH) sequence (10 repetitions; duration 2 min 11 s/repetition; 15-slice @ 128 × 128 voxels per slice). The first of 10 repetitions provided the pre-contrast measurement and contrast intensity was assessed in anatomically-distinct ROIs as described in the Methods. (A) Mean SI was assessed in the last 4 repetitions and the data indicated that it was significantly greater in dentate gyrus (B) and CA1 (C) of CD200^{-/-}, compared with wildtype, mice ($F(1, 9) = 8.91$; $p < 0.01$ and $F(1, 9) = 10.72$; $p < 0.01$, respectively; 2-way ANOVA). (D) and entorhinal (E) cortices ($F(1, 9) = 32.4$; $p < 0.001$ and $F(1, 9) = 17.57$; $p < 0.001$, respectively; 2-way ANOVA). (F–I) Capillary transit time (CTT) and mean transit time (MTT) was similar in hippocampus and cortex of wildtype and CD200^{-/-} mice. Expression of claudin 5 mRNA was significantly decreased in hippocampal (J) and cortical (K) tissue obtained from CD200^{-/-} mice compared with wildtype mice (** $p < 0.01$; student's *t*-test for independent means). (L) Signal intensity (SI) derived from extravasation of gadopentate dimeglumine, which is an indicator of BBB permeability, was significantly correlated with claudin 5 mRNA ($r^2 = 0.37$, $p < 0.01$).

treated and IFN γ -injected mice and subjected to flow cytometric analysis. The data indicate that the expression of CD11b per cell, as indicated by the mean fluorescence intensity, was increased in IFN γ -injected, compared with control-treated, mice ($*p < 0.05$; student's *t*-test for independent means; Fig. 9A) and, in addition, that

there was a greater number of CD11b⁺ CD86⁺, as well as CD11b⁺ CD68⁺, cells in samples prepared from IFN γ -injected mice (** $p < 0.01$; *** $p < 0.001$; student's *t*-test for independent means; Fig. 9B–D). Injection of IFN γ also increased TNF α mRNA ($*p < 0.05$; student's *t*-test for independent means; Fig. 9E).

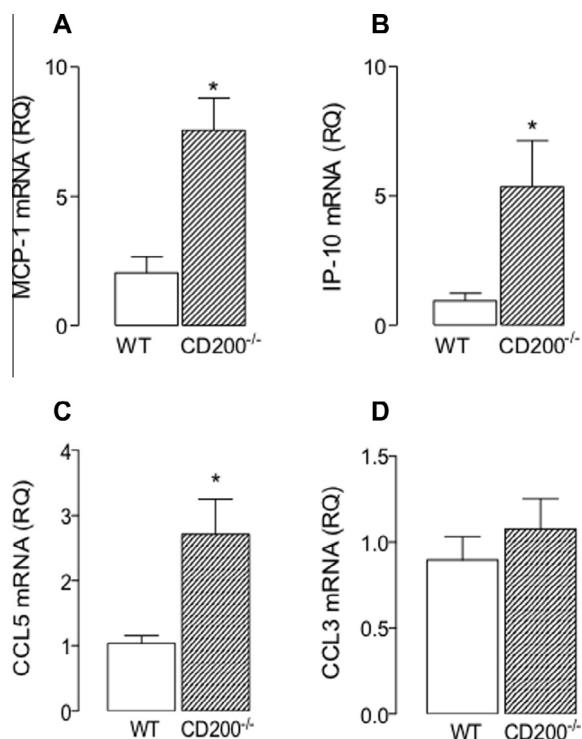


Fig. 8. Expression of MCP-1, IP-10 and RANTES mRNA was increased in hippocampal tissue prepared from CD200-deficient mice. Real-time PCR was performed on cDNA prepared from hippocampal tissue of wildtype and CD200-deficient mice. Details of the predesigned Taqman gene expression assays are given in the Methods. Gene expression was calculated relative to the endogenous control samples (β -actin) to give a relative quantification (RQ) value ($2^{-\Delta\Delta CT}$, where CT is threshold cycle). MCP-1 mRNA (A), IP-10 mRNA (B) and CCL5 mRNA (C), but not CCL3 mRNA (D), were significantly increased in hippocampal tissue prepared from CD200-deficient, compared with wildtype, mice ($*p < 0.05$; Students *t*-test for independent means). Values are presented as mean \pm SEM ($n = 6$).

4. Discussion

The significant finding reported here is that microglia in CD200-deficient mice adopt the inflammatory classical activation phenotype because IFN γ -producing T cells and macrophages enter the brain as a consequence of increased BBB permeability, perhaps assisted by increased expression of chemotactic factors in the brain. The proposal is that the infiltrating T cells interact with the microglia which have antigen presenting capability, and that the released IFN γ exerts a more profound effect since receptor expression and receptor-induced signalling are enhanced in cells from CD200-deficient mice.

It is established that the interaction between CD200 and its receptor is an important factor in modulating microglial activation and that loss of CD200 is coupled with increased microglial activation (Lyons et al., 2007a, 2009) and CD200 deficiency is associated with increased responsiveness of microglia to TLR agonists, LPS and Pam₃CSK₄ (Costello et al., 2011). Here we show that the response to IFN γ is greater in cells prepared from CD200-deficient, compared with wildtype, mice and that this is associated with increased expression of IFNGR1 and STAT1 phosphorylation. This genotype-linked propensity to adopt the classical microglial activation state was not mirrored by a genotype-related sensitivity to IL-4.

In vivo, even under resting conditions, the loss of CD200 is associated with upregulation of inflammatory markers, including IL-6, CD11b, MHCII and CD40 mRNA, as well as TNF α and iNOS mRNA which are hallmarks of classical activation of microglia. Increased expression of CD40 on CD11b⁺ cells was also observed. IFN γ induces microglia to adopt the classical activation state (Colton,

2009) and has been shown to increase expression of several inflammatory molecules *in vitro* and *in vivo* (Clarke et al., 2008; Downer et al., 2009; Lyons et al., 2007b; Maher et al., 2006); here the increased microglial activation in CD200^{-/-} mice is accompanied by increased IFN γ concentration. A similar parallel between IFN γ concentration and increased markers of microglial activation has been shown in the brain of aged rats and LPS/A β -treated rats (Clarke et al., 2008; Maher et al., 2006).

The evidence indicates that there was a genotype-related increase in hippocampal concentration of IFN γ as assessed by ELISA and also by western immunoblotting. Interestingly an increase in expression of IFNGR1 was also observed in hippocampus of CD200^{-/-} mice identifying a mechanism by which microglia might be more responsive to IFN γ . However the source of the IFN γ in the brain of CD200^{-/-} mice is unclear since it is generally considered that resident cells in the brain do not produce it; we have consistently failed to induce release from microglia, or indeed astrocytes, using a range of stimuli. We have also been unable to show any meaningful IFN γ mRNA expression in primary microglial cells, although there have been sporadic reports in the literature suggesting that glia produce IFN γ under specific circumstances (Kawanokuchi et al., 2006; Suzuki et al., 2005; Xiao and Link, 1998). In this study we considered that peripheral cells might infiltrate the brain and explain the presence of IFN γ .

An intact BBB provides one of the most important protective strategies for the brain and the evidence has indicated that increased BBB permeability is increased in conditions which are associated with inflammation including AD (Farrall and Wardlaw, 2009; Starr et al., 2009), multiple sclerosis (Kirk et al., 2003) and Parkinson's disease (Kortekaas et al., 2005). An age-related increase in BBB permeability has also been reported in rats (Blau et al., 2012). Here we demonstrate that gadolinium extravasation is increased in several areas of the brain of CD200^{-/-}, compared with wildtype, mice and that this is associated with decreased expression of claudin 5. It is well established that maintenance of BBB integrity is dependent on the interaction between the transmembrane proteins, the claudins, occludin and junctional adhesion molecule (Ballabh et al., 2004; Capaldo and Nusrat, 2009; Hawkins and Davis, 2005) and, consistent with this, is the observation that expression of claudin 5 is inversely correlated with the signal intensity derived from extravasation of gadolinium. Interestingly we have observed a similar decrease in expression of claudin 5 and claudin 12 following LPS injection and these changes are also associated with BBB permeability (Kelly et al., unpublished). Expression of tight junction proteins, including claudin 5, is decreased by inflammatory cytokines like TNF- α and IL-1 β which are released from activated microglia (Kebir et al., 2007; Walsh et al., 2000). Although the BBB is considered by some to be primarily involved in preventing entry of high molecular weight molecules into the brain, it also plays a role in preventing unrestricted infiltration of cells, as suggested by the presence of large numbers of peripheral cells in the brain in conditions where BBB permeability is compromised (Kroenke et al., 2008; Murphy et al., 2010; Togo et al., 2002). Consistent with this, we show that the increase in BBB permeability in CD200^{-/-} mice is associated with the presence of CD4⁺ and CD8⁺ T cells and macrophages in the brain. While the data demonstrate marked infiltration of peripheral cells, the next step is to assess, using immunohistochemical analysis, the anatomical distribution of the cells; these analyses, which form part of a major new study, are underway. Interestingly, a role for another neuroimmune regulatory molecule, fractalkine, in cell infiltration into the brain has been described; specifically adenosine-induced fractalkine upregulation in the choroid plexus has been shown to trigger lymphocyte infiltration in experimental autoimmune encephalomyelitis (Mills et al., 2012), whereas fractalkine deficiency has been associated with decreased leukocyte infiltration

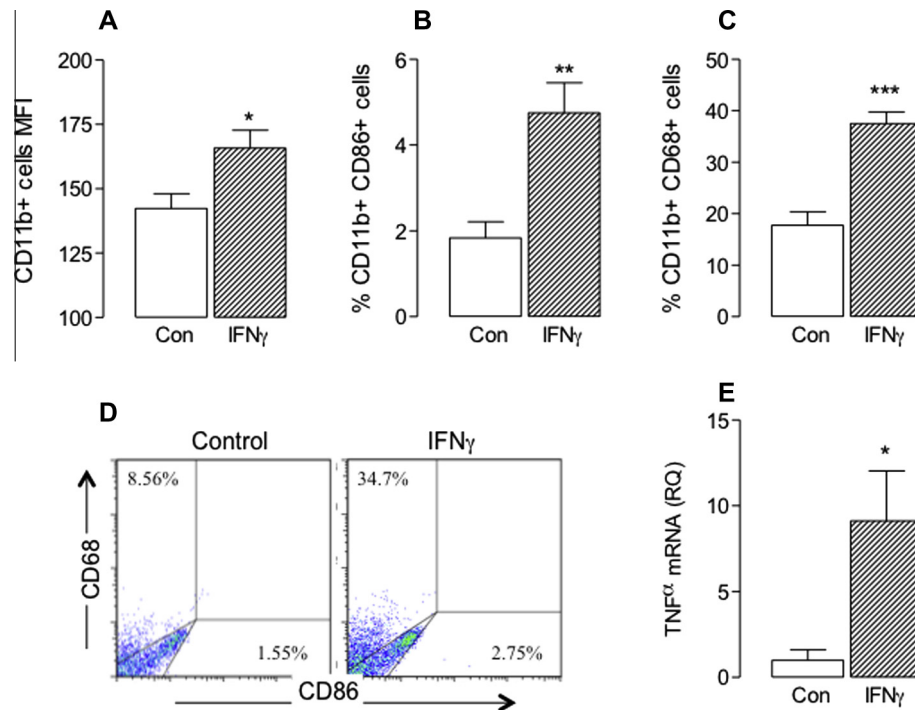


Fig. 9. IFN γ increased CD11b, CD86 and CD68 on cells prepared from the cortex of wildtype mice. Mice were anaesthetized with urethane (1.5 g/kg), injected intracerebroventricularly with saline or IFN γ (50 ng/ml) and transcardially perfused 3 h after injection. The cerebellum, olfactory lobes and hippocampus were removed and cells, prepared for flow cytometry as described in the Methods from the remaining tissue, were incubated with antibodies including APC-Cy7TM rat anti-mouse CD11b, Alexa Fluor 488 rat anti-mouse CD68 and PerCP/Cy5.5 rat anti-mouse CD86. (A) Mean fluorescence intensity of CD11b was increased in cells prepared from IFN γ treated animals compared with saline controls (**p* < 0.05; Student's *t*-test for independent means). The expression of CD86 (B) and CD68 (C) was increased on CD11b⁺ cells prepared from IFN γ treated animals compared with saline controls (**p* < 0.05; ****p* < 0.001; Student's *t*-test for independent means). Representative FACS plots are shown (D). IFN γ also increased TNF α mRNA expression (**p* < 0.05; student's *t*-test for independent means). Values are presented as mean \pm SEM (*n* = 6).

and smaller lesions in a model of cerebral ischaemia (Denes et al., 2008).

Interestingly CD200 deficiency has also been associated with increased infiltration of iNOS-producing macrophages into the retina in experimental autoimmune uveoretinitis. In this case, it was argued that the infiltration was a consequence of the loss of the down-regulatory signal, which arises from CD200–CD200R interaction, at the retinal vascular endothelium (Copland et al., 2007). Whether a similar mechanism contributes to BBB permeability remains to be assessed. However the CD200–CD200R interaction between endothelial cells and microglia (Miller et al., 2011), like neurons and astrocytes, increases production of inflammatory cytokines including IL-1 β from microglia (Cox et al., 2013; Lyons et al., 2007a) creating an inflammatory microenvironment which has been shown to increase BBB permeability (Argaw et al., 2006; McCandless et al., 2009; Zhao et al., 2007). Similarly the age-related increase in neuroinflammatory changes, which have previously been coupled with increased IFN γ expression (Lyons et al., 2011) and decreased CD200 expression (Lyons et al., 2007a), are associated with increased gadolinium extravasation and infiltration of macrophages (Blau et al., 2012), while recent findings have revealed similar age-related changes in APP/PS1 mice (Minogue et al., unpublished).

The clinical relevance of CD200 has been recognized recently with the observation that expression of the ligand and receptor are decreased in post mortem brain tissue prepared from individuals with Alzheimer's disease (Walker et al., 2009), whereas a decrease in CD200 has been reported around inactive and chronic active lesions in multiple sclerosis (Koning et al., 2007). It has also been reported that macrophage expression of CD200 receptor is dysregulated in patients with Parkinson's disease (Luo et al., 2010). These data suggest that targeting CD200 may be a potential

therapeutic option in neurodegenerative diseases which are characterized by neuroinflammatory changes.

Recruitment of immune cells into the brain is also influenced by chemokines and therefore the present finding that MCP-1, IP-10 and RANTES are increased in tissue prepared from CD200^{-/-} mice is significant. Interestingly, increased expression of MCP-1 and IP-10 has been reported in brain in animal models of neurodegenerative diseases including EAE (Boztug et al., 2002; Duan et al., 2008) in which infiltration of inflammatory CD4⁺ T cells is considered to be a primary factor in triggering demyelination and degeneration of axons (Prendergast and Anderton, 2009). Especially significant in the context of CD200-deficiency is the observation that CD200–CD200R interaction plays a role in T cell adhesion and migration into tissues (Ko et al., 2009).

IFN γ is the signature cytokine of Th1 cells, but there is a wealth of evidence indicating that CD8⁺ cells and macrophages also produce IFN γ (Fenton et al., 1997; Ngai et al., 2007; Szabo et al., 2002; Wang et al., 1999). Thus it is possible that the genotype-related increase in IFN γ described here is derived from the infiltrating cells and that it triggers microglial activation. To further explore the role of IFN γ in microglial activation, we evaluated the effect of intracerebroventricular injection of IFN γ on microglial activation in the hippocampus. The data show that IFN γ markedly increased the number of CD11b⁺ cells which stained positively for both CD86 and CD68, and this concurs with earlier data indicating that IFN γ increased NO and TNF α in murine macrophages (Mosser, 2003). This observation, and the finding that IFN γ was increased in brain tissue prepared from CD200^{-/-} mice, is clearly consistent with the possibility that IFN γ provides the trigger for microglial activation. This proposal is supported by recent data from this laboratory which showed that infiltrating IFN γ -producing cells in a mouse model of Alzheimer's disease was associated with marked

microglial activation and, importantly, that administration of an anti-IFN γ antibody blocked this effect (Browne et al., 2013).

The data demonstrate that perturbation of the BBB, such as occurs in certain neurodegenerative diseases which are characterized by neuroinflammatory changes, may be a significant factor in driving further microglial activation and may therefore contribute to the development of a persistent and damaging inflammatory cascade. Targetting the BBB may therefore provide a useful approach in breaking this cycle of events and limiting the consequent neuronal damage.

Conflict of interest

All authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants obtained from Science Foundation Ireland and the Health Research Board (Ireland). RK was the recipient of funding from the Dublin Region Higher Education Alliance funded by the Strategic Innovation Fund (Cycle 2; grant code) from the Higher Education Authority.

References

- Argaw, A.T., Zhang, Y., Snyder, B.J., Zhao, M.L., Kopp, N., Lee, S.C., Raine, C.S., Brosnan, C.F., John, G.R., 2006. IL-1 β regulates blood–brain barrier permeability via reactivation of the hypoxia–angiogenesis program. *J. Immunol.* 177, 5574–5584.
- Ballabh, P., Braun, A., Nedergaard, M., 2004. The blood–brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiol. Dis.* 16, 1–13.
- Barclay, A.N., Wright, G.J., Brooke, G., Brown, M.H., 2002. CD200 and membrane protein interactions in the control of myeloid cells. *Trends Immunol.* 23, 285–290.
- Blamire, A.M., Anthony, D.C., Rajagopalan, B., Sibson, N.R., Perry, V.H., Styles, P., 2000. Interleukin-1 β -induced changes in blood–brain barrier permeability, apparent diffusion coefficient, and cerebral blood volume in the rat brain: a magnetic resonance study. *J. Neurosci.* 20, 8153–8159.
- Blau, C.W., Cowley, T.R., O'Sullivan, J., Grehan, B., Browne, T.C., Kelly, L., Birch, A., Murphy, N., Kelly, M.E., Kerskens, C.M., Lynch, M.A., 2012. The age-related deficit in LTP is associated with changes in perfusion and blood–brain barrier permeability. *Neurobiol. Aging* 33 (1005), e1023–e1035.
- Boztug, K., Carson, M.J., Pham-Mitchell, N., Asensio, V.C., DeMartino, J., Campbell, I.L., 2002. Leukocyte infiltration, but not neurodegeneration, in the CNS of transgenic mice with astrocyte production of the CXCL chemokine ligand 10. *J. Immunol.* 169, 1505–1515.
- Browne, T.C., McQuillan, K., McManus, R.M., O'Reilly, J.A., Mills, K.H., Lynch, M.A., 2013. IFN- γ Production by amyloid beta-specific Th1 cells promotes microglial activation and increases plaque burden in a mouse model of Alzheimer's disease. *J. Immunol.* 190, 2241–2251.
- Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. *Biochim. Biophys. Acta* 1788, 864–871.
- Clarke, R.M., Lyons, A., O'Connell, F., Deighan, B.F., Barry, C.E., Anyakoha, N.G., Nicolau, A., Lynch, M.A., 2008. A pivotal role for interleukin-4 in atorvastatin-associated neuroprotection in rat brain. *J. Biol. Chem.* 283, 1808–1817.
- Colton, C.A., 2009. Heterogeneity of microglial activation in the innate immune response in the brain. *J. Neuroimmune Pharmacol.* 4, 399–418.
- Colton, C.A., Wilcock, D.M., 2010. Assessing activation states in microglia. *CNS Neurol. Disord.: Drug Targets* 9, 174–191.
- Colton, C.A., Mott, R.T., Sharpe, H., Xu, Q., Van Nostrand, W.E., Vitek, M.P., 2006. Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. *J. Neuroinflammation* 3, 27.
- Copland, D.A., Calder, C.J., Raveney, B.J., Nicholson, L.B., Phillips, J., Cherwinski, H., Jenmalm, M., Sedgwick, J.D., Dick, A.D., 2007. Monoclonal antibody-mediated CD200 receptor signaling suppresses macrophage activation and tissue damage in experimental autoimmune uveoretinitis. *Am. J. Pathol.* 171, 580–588.
- Costello, D.A., Lyons, A., Denieffe, S., Browne, T.C., Cox, F.F., Lynch, M.A., 2011. Long term potentiation is impaired in membrane glycoprotein CD200-deficient mice: a role for Toll-like receptor activation. *J. Biol. Chem.* 286, 34722–34732.
- Cox, F.F., Carney, D., Miller, A.M., Lynch, M.A., 2012. CD200 fusion protein decreases microglial activation in the hippocampus of aged rats. *Brain Behav. Immun.* 26, 789–796.
- Cox, F.F., Berezin, V., Bock, E., Lynch, M.A., 2013. The neural cell adhesion molecule-derived peptide, FGL, attenuates lipopolysaccharide-induced changes in glia in a CD200-dependent manner. *Neuroscience* 235, 141–148.
- Denes, A., Ferenczi, S., Halasz, J., Kornyei, Z., Kovacs, K.J., 2008. Role of CX3CR1 (fractalkine receptor) in brain damage and inflammation induced by focal cerebral ischemia in mouse. *J. Cereb. Blood Flow Metab.* 28, 1707–1721.
- Downer, E.J., Cowley, T.R., Cox, F., Maher, F.O., Berezin, V., Bock, E., Lynch, M.A., 2009. A synthetic NCAM-derived mimetic peptide, FGL, exerts anti-inflammatory properties via IGF-1 and interferon-gamma modulation. *J. Neurochem.* 109, 1516–1525.
- Duan, R.S., Yang, X., Chen, Z.G., Lu, M.O., Morris, C., Winblad, B., Zhu, J., 2008. Decreased fractalkine and increased IP-10 expression in aged brain of APP(swe) transgenic mice. *Neurochem. Res.* 33, 1085–1089.
- Farrall, A.J., Wardlaw, J.M., 2009. Blood–brain barrier: ageing and microvascular disease—systematic review and meta-analysis. *Neurobiol. Aging* 30, 337–352.
- Fenton, M.J., Vermeulen, M.W., Kim, S., Burdick, M., Strieter, R.M., Kornfeld, H., 1997. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. *Infect. Immun.* 65, 5149–5156.
- Frank, M.G., Barrientos, R.M., Biedenkapp, J.C., Rudy, J.W., Watkins, L.R., Maier, S.F., 2006. MRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiol. Aging* 27, 717–722.
- Gordon, S., Martinez, F.O., 2010. Alternative activation of macrophages: mechanism and functions. *Immunity* 32, 593–604.
- Hawkins, B.T., Davis, T.P., 2005. The blood–brain barrier/neurovascular unit in health and disease. *Pharmacol. Rev.* 57, 173–185.
- Kawanokuchi, J., Mizuno, T., Takeuchi, H., Kato, H., Wang, J., Mitsuma, N., Suzumura, A., 2006. Production of interferon-gamma by microglia. *Mult. Scler.* 12, 558–564.
- Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., Prat, A., 2007. Human TH17 lymphocytes promote blood–brain barrier disruption and central nervous system inflammation. *Nat. Med.* 13, 1173–1175.
- Kelly, M.E., Blau, C.W., Kerskens, C.M., 2009. Bolus-tracking arterial spin labelling: theoretical and experimental results. *Phys. Med. Biol.* 54, 1235–1251.
- Kelly, M.E., Blau, C.W., Griffin, K.M., Gobbo, O.L., Jones, J.F., Kerskens, C.M., 2010. Quantitative functional magnetic resonance imaging of brain activity using bolus-tracking arterial spin labeling. *J. Cereb. Blood Flow Metab.* 30, 913–922.
- Kirk, J., Plumb, J., Mirakhor, M., McQuaid, S., 2003. Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood–brain barrier leakage and active demyelination. *J. Pathol.* 201, 319–327.
- Ko, Y.C., Chien, H.F., Jiang-Shieh, Y.F., Chang, C.Y., Pai, M.H., Huang, J.P., Chen, H.M., Wu, C.H., 2009. Endothelial CD200 is heterogeneously distributed, regulated and involved in immune cell–endothelium interactions. *J. Anat.* 214, 183–195.
- Koning, N., Bo, L., Hoek, R.M., Huitinga, I., 2007. Downregulation of macrophage inhibitory molecules in multiple sclerosis lesions. *Ann. Neurol.* 62, 504–514.
- Koning, N., Swaab, D.F., Hoek, R.M., Huitinga, I., 2009. Distribution of the immune inhibitory molecules CD200 and CD200R in the normal central nervous system and multiple sclerosis lesions suggests neuron–glia and glia–glia interactions. *J. Neuropathol. Exp. Neurol.* 68, 159–167.
- Kortekaas, R., Leenders, K.L., van Oostrom, J.C., Vaalburg, W., Bart, J., Willemsen, A.T., Hendrikse, N.H., 2005. Blood–brain barrier dysfunction in parkinsonian midbrain *in vivo*. *Ann. Neurol.* 57, 176–179.
- Kroenke, M.A., Carlson, T.J., Andjelkovic, A.V., Segal, B.M., 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* 205, 1535–1541.
- Luo, X.G., Zhang, J.J., Zhang, C.D., Liu, R., Zheng, L., Wang, X.J., Chen, S.D., Ding, J.Q., 2010. Altered regulation of CD200 receptor in monocyte-derived macrophages from individuals with Parkinson's disease. *Neurochem. Res.* 35, 540–547.
- Lynch, M.A., 2009. The multifaceted profile of activated microglia. *Mol. Neurobiol.* 40, 139–156.
- Lynch, M.A., 2010. Age-related neuroinflammatory changes negatively impact on neuronal function. *Front. Aging Neurosci.* 1, 6.
- Lyons, A., Downer, E.J., Crotty, S., Nolan, Y.M., Mills, K.H., Lynch, M.A., 2007a. CD200 ligand–receptor interaction modulates microglial activation *in vivo* and *in vitro*: a role for IL-4. *J. Neurosci.* 27, 8309–8313.
- Lyons, A., Griffin, R.J., Costelloe, C.E., Clarke, R.M., Lynch, M.A., 2007b. IL-4 attenuates the neuroinflammation induced by amyloid-beta *in vivo* and *in vitro*. *J. Neurochem.* 101, 771–781.
- Lyons, A., McQuillan, K., Deighan, B.F., O'Reilly, J.A., Downer, E.J., Murphy, A.C., Watson, M., Piazza, A., O'Connell, F., Griffin, R., Mills, K.H., Lynch, M.A., 2009. Decreased neuronal CD200 expression in IL-4-deficient mice results in increased neuroinflammation in response to lipopolysaccharide. *Brain Behav. Immun.* 23, 1020–1027.
- Lyons, A., Murphy, K.J., Clarke, R., Lynch, M.A., 2011. Atorvastatin prevents age-related and amyloid-beta-induced microglial activation by blocking interferon-gamma release from natural killer cells in the brain. *J. Neuroinflammation* 8, 27.
- Lyons, A., Downer, E.J., Costello, D.A., Murphy, N., Lynch, M.A., 2012. Dok2 mediates the CD200Fc attenuation of Abeta-induced changes in glia. *J. Neuroinflammation* 9, 107.
- Maher, F.O., Clarke, R.M., Kelly, A., Nally, R.E., Lynch, M.A., 2006. Interaction between interferon gamma and insulin-like growth factor-1 in hippocampus impacts on the ability of rats to sustain long-term potentiation. *J. Neurochem.* 96, 1560–1571.
- McCandless, E.E., Budde, M., Lees, J.R., Dorsey, D., Lyng, E., Klein, R.S., 2009. IL-1R signaling within the central nervous system regulates CXCL12 expression at the blood–brain barrier and disease severity during experimental autoimmune encephalomyelitis. *J. Immunol.* 183, 613–620.
- Miller, A.M., Deighan, B.F., Downer, E., Lyons, A., Henrich-Noack, P., Nolan, T., Lynch, M.A., 2011. In: Raymond Chuen-Chung Chang (Ed.), *Analysis of the Impact of CD200 on Neurodegenerative Diseases, Neurodegenerative Diseases* –

- Processes, Prevention, Protection and Monitoring. ISBN: 978-953-307-485-6. InTech, Available from: <<http://www.intechopen.com/books/neurodegenerative-diseases-processes-prevention-protection-and-monitoring-analysis-of-the-impact-of-cd200-on-neurodegenerative-diseases>>.
- Mills, J.H., Alabanza, L.M., Mahamed, D.A., Bynoe, M.S., 2012. Extracellular adenosine signaling induces CX3CL1 expression in the brain to promote experimental autoimmune encephalomyelitis. *J. Neuroinflammation* 9, 193.
- Mosser, D.M., 2003. The many faces of macrophage activation. *J. Leukoc. Biol.* 73, 209–212.
- Murphy, A.C., Lalor, S.J., Lynch, M.A., Mills, K.H., 2010. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav. Immun.* 24, 641–651.
- Ngai, P., McCormick, S., Small, C., Zhang, X., Zganiacz, A., Aoki, N., Xing, Z., 2007. Gamma interferon responses of CD4 and CD8 T-cell subsets are quantitatively different and independent of each other during pulmonary *Mycobacterium bovis* BCG infection. *Infect. Immun.* 75, 2244–2252.
- Prendergast, C.T., Anderton, S.M., 2009. Immune cell entry to central nervous system – current understanding and prospective therapeutic targets. *Endocr. Metab. Immune Disord. Drug Targets* 9, 315–327.
- Renno, T., Krakowski, M., Piccirillo, C., Lin, J.Y., Owens, T., 1995. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J. Immunol.* 154, 944–953.
- Starr, J.M., Farrall, A.J., Armitage, P., McGurn, B., Wardlaw, J., 2009. Blood–brain barrier permeability in Alzheimer's disease: a case-control MRI study. *Psychiatry Res.* 171, 232–241.
- Suzuki, Y., Claflin, J., Wang, X., Lengi, A., Kikuchi, T., 2005. Microglia and macrophages as innate producers of interferon-gamma in the brain following infection with *Toxoplasma gondii*. *Int. J. Parasitol.* 35, 83–90.
- Szabo, S.J., Sullivan, B.M., Stemmann, C., Satoskar, A.R., Sleckman, B.P., Glimcher, L.H., 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295, 338–342.
- Togo, T., Akiyama, H., Iseki, E., Kondo, H., Ikeda, K., Kato, M., Oda, T., Tsuchiya, K., Kosaka, K., 2002. Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. *J. Neuroimmunol.* 124, 83–92.
- Walker, D.G., Dalsing-Hernandez, J.E., Campbell, N.A., Lue, L.F., 2009. Decreased expression of CD200 and CD200 receptor in Alzheimer's disease: a potential mechanism leading to chronic inflammation. *Exp. Neurol.* 215, 5–19.
- Walsh, S.V., Hopkins, A.M., Nusrat, A., 2000. Modulation of tight junction structure and function by cytokines. *Adv. Drug Deliv. Rev.* 41, 303–313.
- Wang, J., Wakeham, J., Harkness, R., Xing, Z., 1999. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J. Clin. Invest.* 103, 1023–1029.
- Webb, M., Barclay, A.N., 1984. Localisation of the MRC OX-2 glycoprotein on the surfaces of neurones. *J. Neurochem.* 43, 1061–1067.
- Xiao, B.G., Link, H., 1998. IFN-gamma production of adult rat astrocytes triggered by TNF-alpha. *NeuroReport* 9, 1487–1490.
- Zhao, C., Ling, Z., Newman, M.B., Bhatia, A., Carvey, P.M., 2007. TNF-alpha knockout and minocycline treatment attenuates blood–brain barrier leakage in MPTP-treated mice. *Neurobiol. Dis.* 26, 36–46.