Imbalanced immunity in multiple sclerosis

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CD200 and CD200R localization in the brain

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

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Abstract

CD200 is a membrane glycoprotein that suppresses immune activity via its receptor, CD200R. CD200-CD200R interactions have recently been considered to contribute to the 'immune-privileged' status of the central nervous system (CNS). The mechanisms by which these interactions take place are not well understood as detailed reports on the distribution of CD200 and CD200R are lacking. We used immunohistochemistry to characterize the distinct anatomical and cellular distribution of these molecules in multiple sclerosis (MS) lesions and controls. CD200 was robustly expressed in grey matter CNS areas including the cerebral cortex, hippocampus, striatum, cerebellum and spinal cord, where neurons appeared immunopositive. CD200 expression was also detected in oligodendrocytes, but not in astrocytes or microglia. In CNS samples from MS patients, CD200 expression was additionally observed on reactive astrocytes in chronic active lesions. This was an interesting finding as we previously showed that overall CD200 expression in MS lesions was decreased. In contrast to CD200, the localization pattern of CD200R was very distinct, showing high expression on perivascular macrophages in both grey and white matter. Using flow cytometry we show that human primary microglia express low levels of CD200R. These data suggest that CD200-mediated immune suppression may occur not only via neuron-microglia interaction, but also via glia-glia interactions, especially in inflammatory conditions in which an immune-suppressed environment needs to be restored, possibly by enhancing CD200 expression.

Introduction

A sensitive organ like the central nervous system (CNS) necessitates a shelter from peripheral immune responses to protect its cells that have low regenerative capacity. Several strategies have thus evolved to create an 'immune-privileged' environment, from which the mechanisms are only partly understood. The blood-brain barrier, which restricts the entrance of leukocytes into the CNS, is one of the best studied systems. Furthermore, communication deficiency between the CNS and the peripheral immune system is known to involve the afferent arm of the immune system and is established by the lack of competent antigen presenting cells and a conventional lymphatic system.[158] However, neurons, that hardly regenerate, not only need to be protected from the peripheral immune system, as microglia, the resident macrophages of the CNS, are present throughout the CNS and need to be continually constrained as well. Therefore, a highly important, but less well studied mechanism to protect the CNS against both peripheral and central immune responses is the presence of molecules that directly inhibit immune cells. An example of such a molecule is CD200, which is highly expressed on neurons and can down-regulate immune activity through binding with its receptor, CD200R.[121,125,159] That CD200-CD200R interaction in the brain contributes to the immune-privileged status of the CNS is supported by the observation that in other immune-suppressed organs like the placenta, CD200 expression is also remarkably high.[120]

CD200 and its receptor are homologous membrane glycoproteins belonging to the immunoglobulin superfamily. CD200 is expressed on numerous cell types in a wide variety of tissues[120] whereas in man CD200R is mainly expressed on myeloid cells like monocytes and macrophages and on a subset of T and B cells.[122,124] In rats, CD200R has been demonstrated on microglia as well.[121,160] Unlike its ligand, CD200R has signaling motifs in its intracellular domain that upon triggering deliver an inhibitory signal to the cell.[123,124] Animal studies show that CD200 is highly expressed in the CNS. When absent, mice have spontaneously activated microglia and show an enhanced susceptibility to several inflammatory disease models such as experimental autoimmune uveoretinitis (EAU) and experimental autoimmune encephalomyelitis (EAE), the animal model for multiple scherosis (MS).[125,126] Conversely, mice with inherently increased levels of CD200 due to a mutation in the *Wld*^{s} gene, have less activated and infiltrated macrophages during EAE, milder symptoms of the disease and show increased neuroprotection.[129]

Animal models thus demonstrate that loss of immune suppression via CD200 in the CNS has severe consequences for neuroinflammation and neurodegeneration. In human, activated microglia are associated with neurodegeneration in ageing, as

well as in diseases such as stroke, MS, Alzheimer's disease and Parkinson's disease. In MS, not only activated microglia but also infiltrating leukocytes cause severe inflammation leading to multiple demyelinated lesions. In a recent study, we showed that the expression of CD200, but not CD200R, was diminished in and around MS lesions and may therefore contribute to macrophage/microglia activation and inflammation in the CNS of MS patients.[111]

CD200 has been shown on neurons in the rat CNS and in the human retina,[159,160] but detailed information on CD200-CD200R expression in the human CNS is missing. With the identification of the cellular compartments that express CD200 and its receptor, we gain insight into the mechanisms by which these molecules suppress deleterious inflammatory responses in the CNS. This knowledge can help delineate the targets for therapeutic approaches to restore immune suppression when needed, for instance during MS. In this study we show in detail the broad anatomical and cellular localization of CD200 and CD200R in the human CNS, demonstrating that CD200R is expressed at low levels on human microglia and that CD200 expression is not only restricted to neurons but can also be expressed by glial cells, in particular under pathological conditions.

Methods

Tissue and donors

Snap-frozen tissue specimens of parietal cortex, hippocampus, hypothalamus, cerebellum and spinal cord of three control subjects and white matter lesions of eight MS patients obtained at autopsy were provided by the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Permission was obtained for brain autopsy and the use of the tissue and clinical information for research purposes. All MS patients were clinically diagnosed by a neurologist (Prof. C.H. Polman, VUMC, Amsterdam), and the diagnosis was confirmed post-mortem by a neuropathologist. Donor characteristics are shown in Table 1.

Immunohistochemistry

Cryostat sections (8 µm) were fixed in acetone for 10 minutes. Then, sections were incubated with monoclonal antibodies to CD200 (Clone OX104 for human and OX90 for mouse tissue, hybridomas were kindly provided by Prof. A.N. Barclay, University of Oxford, UK and has been described previously[120]), CD200R (Clone OX108[130], Serotec, Oxford, UK) or mannose receptor (Clone 15-2, Serotec), diluted in PBS containing 1% bovine serum albumine (BSA), o/n at 4°C. After washing

Table 1. Clinical and neuropathological data of donors

Patient	Sex	Age	PM time	Duration	MS type	Lesion type	Cause of death
1. Control	f	64	6:00	-	-	-	cachexia
2. Control	m	80	7:15	-	-	-	cachexia and dehydration
3. Control	f	82	11:30	-	-	-	congestive heart failure
4. MS	f	40	7:00	11	SP	chronic active	pneumonia
5. MS	f	50	7:45	17	SP	chronic active	euthanasia
6. MS	f	66	6:20	15	SP	chronic active	pneumonia
7. MS	f	69	13:20	26	RR	chronic active	probable viral infection
8. MS	f	68	7:30	39	SP	inactive	bronchitis/aspiration pneumonia
9. MS	f	71	10:15	23	SP	inactive	postsurgery respiratory problems
10. MS	f	75	8:00	42	SP	inactive	pneumonia
11. MS	m	77	4:15	26	PP-SP	inactive	cerebrovascular accident

PM time = post-mortem delay until end of autopsy expressed in hours:minutes; Duration = disease duration in years; MS type = type of MS at time of death; SP = secondary progressive; RR = relapsing remitting; PP = primary progressive.

in PBS, the sections were incubated with secondary antibody dilutions of biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), containing 10% horse serum, for 45 minutes, followed by 45 minute incubation in horseradish peroxidase-conjugated avidin-biotin-complex (ABC, VectaStain ABC Elite Kit, Vector Laboratories). To amplify the staining, CD200R-treated sections were incubated with biotinylated tyramine[161] in addition of 0.01% H_2O_2 for 10 minutes, after which the sections were again incubated in ABC for 45 minutes. Staining in all sections was visualized with DAB (Sigma Laboratories, St. Louis, MO) applied for 10 minutes. Sections were counterstained with haematoxylin for 30 seconds, dehydrated and mounted in entallan (Merck, Darmstadt, Germany). For fluorescent CD200 double stainings, sections were first incubated with a polyclonal antibody to glial fibrillary acidic protein (GFAP) (DakoCytomation, Glostrup, Denmark) and monoclonal antibodies to CNPase (Clone 11-5B, Sigma, St. Louis, MO), neurofilament (NF, clone SMI-311R, Covance, Emryville, CA) or FVIII (kindly provided by Dr. L. Bö, VU medical center, Amsterdam, The Netherlands) for 1 h at RT, followed by incubation with goat-anti-rabbit or goat-anti mouse Alexa 594-labeled antibody (Invitrogen-Molecular Probes, Eugene, OR) in the presence of 10% normal goat serum, for 1 h at RT. Next, sections were incubated with biotinylated anti-CD200 o/n at 4°C, followed by incubation with Alexa 488-labeled streptavidin-avidine (Invitrogen). Finally, sections were mounted in mounting medium (0.1 M Tris-HCl, 25% glycerol, 10% mowiol (EMD chemicals, Gibbstown, NJ). As negative controls, primary antibodies

were either omitted or substituted with an isotype control antibody (IgG1 or biotinylated IgG1, Serotec). All negative controls showed no immunoreactivity.

MS lesion inflammatory activity was characterized as described previously,[34,35,111,135] in cryostat sections (8 µm) stained for proteolipid protein (Serotec, Oxford, UK) and human leukocyte antigen (HLA)-DP, DQ, DR (Clone CR3/43, Dako). We used four chronic active lesions, determined by the presence of HLA-positive foamy macrophages in the rim surrounding the gliotic centre of the lesion, and four inactive lesions, as determined by absence of foamy macrophages.

Human microglia isolation

Corpus callosum white matter, provided by the Netherlands Brain Bank, was obtained at autopsy and stored in Hibernate A medium (Brain Bits LLC, Springfield, IL) at 4°C. Within 5 – 18 hours after autopsy, microglia were isolated using a Percoll (GE Healthcare, Diegem, Belgium) gradient, as described previously,[162] with some adaptations. In brief, tissue (per gram) was mechanically dissociated using a metal sieve in a total volume of 50 ml GKN/0.2% BSA (8 g/l NaCl, 0.4 g/l KCl, 1.77 g/l $Na_2HPO_4.2H_2O$, 0.69 g/l $NaH_2PO_4.H_2O$, 2 g/l D-(+)-glucose, 2 g/l BSA, pH 7.4). After centrifugation (400g, 7 min), the pellet was reconstituted in 5 ml dissociation buffer (4 g/l $MgCl_2$, 2.55 g/l $CaCl_2$, 3.73 g/l KCl, 8.95 g/l NaCl, pH 6-7) and enzymatically digested in 200 µg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and 150 U Collagenase Type I (Worthington, Lakewood, NJ, USA) for 1 h at 37°C. During this incubation, the tissue was mechanically disrupted by passing the mixture through a pipet tip with decreasing bore size every 5-10 minutes. After washing in GKN/0.2% BSA, the cells were resuspended in 20 ml Percoll ($\rho = 1.03$), underlain by 10 ml Percoll ($\rho = 1.095$) and overlain by 5 ml GKN/0.2%BSA and centrifuged at 1200g for 30 min with slow acceleration and no break. The myelin layer was removed from the GKN-Percoll 1.03 interface and discarded, after which the cells from the 1.03-1.095 interface were collected. The average number of recovered cells was $3x10^6$ cells/gram tissue.

Flow cytometry

The freshly isolated CNS cells were incubated with fluorescein isothiocyanate-labeled antibody against human CD45 (Clone HI30), phycoerythyrin-labeled CD11b (Clone ICRF44, Dako) and Alexa 647-labeled CD200R (Clone OX108, Serotec) or with the appropriate isotype controls in the presence of 10% human pool serum for 1 h at 4°C. Antibody dilutions and washing steps were performed in FACS buffer (PBS, 0.2% BSA, 10 mM sodium azide). After washing, the cells were analyzed using a FACSCalibur flow cytometer (BD PharMingen, San Jose, CA) and the data were

analyzed using FlowJo software version 8.7.1 (Treestar, Inc. Ashland, OR). Cells were sorted on a FACS Aria cell sorter (BD PharMingen).

Results

Anatomical and cellular CD200 expression in the human CNS

CD200 was broadly expressed throughout the human CNS, of which Figure 1 shows examples in parietal cortex, hippocampus, striatum, cerebellum and spinal cord. The most intense expression of CD200 was present in grey matter from e.g. cortex and hippocampus, where neurons appeared CD200 positive (Fig. 1A, B, insets). Although CD200 immunoreactivity seemed present on the neuronal cell membrane, this was difficult to determine as staining was diffusely present between neuronal cell bodies and, in hippocampal neurons for example, staining seemed to be present in the cytoplasm as well (Fig. 1B). CD200 staining in the striatum showed expression in white matter, but less abundant and more distinct compared to grey matter (Fig. 1C, D). In the cerebellum, CD200 was predominantly expressed in the granular and molecular layers (Fig. 1E, F). The membranes of Purkinje neurons appeared positive and the cytoplasm was unstained (Fig. 1F inset). In agreement with our earlier report, CD200 was also present on axons (Fig. 1G, H).[111] Transversal sections of the spinal cord showed that the myelin immediately surrounding the axons was unstained, although CD200 was expressed on elements surrounding the myelin sheaths (Fig. 1G inset). This peculiar expression pattern suggests that CD200 expression was not limited to neurons and their axons. We thus analyzed CD200 expression by double staining techniques. From these it was confirmed that axons expressed CD200, as seen by colocalization with neurofilament (NF) in a longitudinal section of the spinal cord (Fig. 2A). Indeed, the fluorescent images demonstrate that CD200 expression was broad and not limited to axons. Double labeling a transversal section of the spinal cord with CNPase showed that the elements surrounding the myelin sheaths as seen in Fig. 1G were oligodendrocytes expressing CD200 (Fig. 2B). In addition, CNPase and CD200 labeling in white matter clearly showed colocalization on the oligodendrocyte cell body (Fig. 2C). However, no colocalization of CD200 with GFAP was found, showing that in the non-diseased brain, astrocytes did not express CD200 (Fig. 2D). Also microglia did not express CD200, as no colocalization with CD11b was found (data not shown). Despite the fact that CD200 expression has been described on vascular endothelial cells in the rat cerebellum[159] and in the human liver[120], we could not find CD200 colocalization with the endothelial marker Factor VIII, indicating that human brain vascular en-

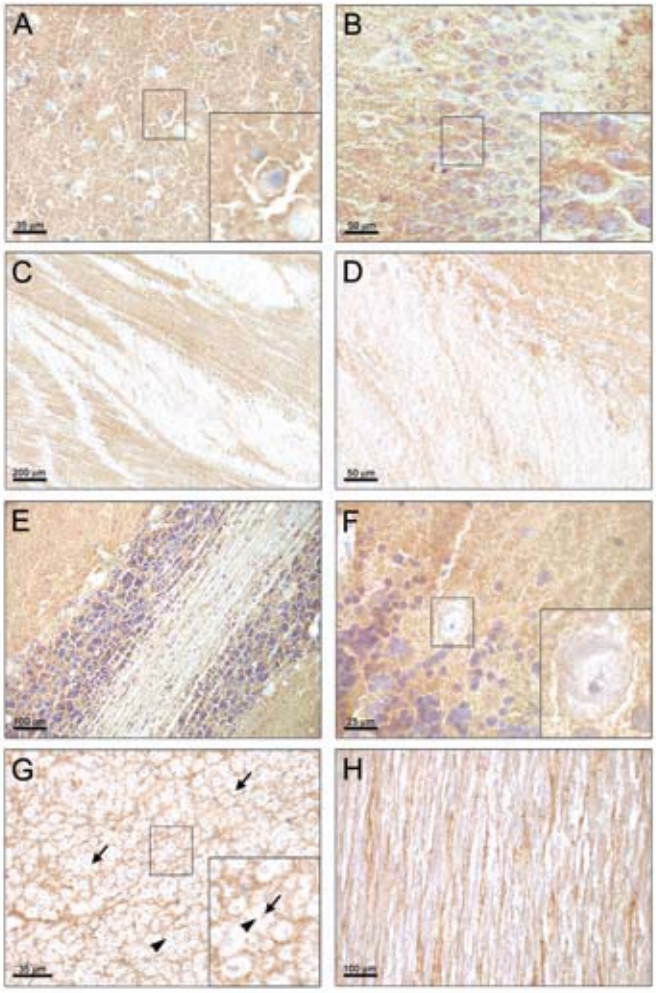

Fig. 1 CD200 expression in frozen sections (8 µm) of the human central nervous system derived from control donors. CD200 immunoreactivity is shown in parietal cortex (A), hippocampus (B), striatum (C, D), cerebellum (E, F) and in transversal and longitudinal sections of the spinal cord (G and H; respectively). CD200 is expressed on neurons (A, B, F; insets) and axons (G; arrows), but myelin is unstained (G; arrowheads). In grey matter, CD200 expression surrounding neurons is diffuse. In white matter CD200 is distinct and possibly present on non-neuronal elements.

dothelium did not express CD200 (data not shown).

CD200 expression in MS lesions

Because we previously demonstrated that CD200 expression in MS lesions was decreased, we analyzed cellular CD200 expression in MS. In and around the border of MS lesions, oligodendrocytes were CD200 positive (Fig. 3A), while staining of both

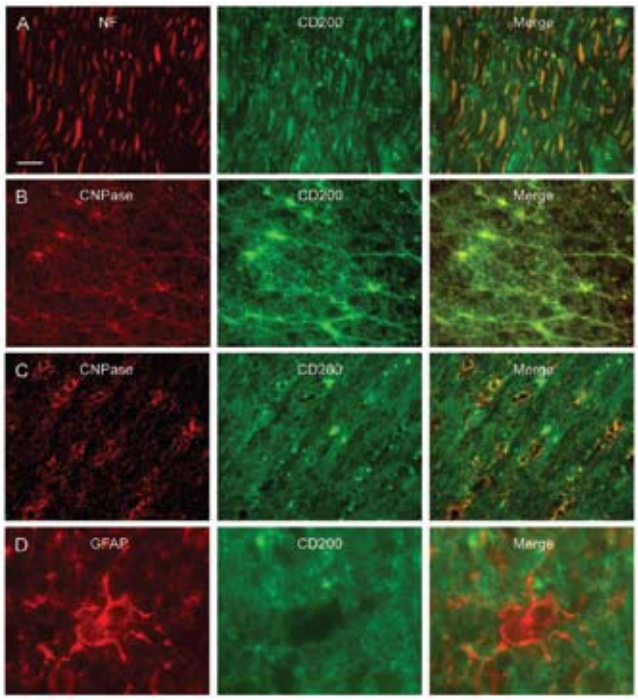

Fig. 2 Double fluorescent staining of CD200 with CNPase and GFAP in frozen human spinal cord and white matter sections (8 µm) derived from control donors. CD200 is expressed on neurofilament (NF)-positive axons shown a longitudinal section of the spinal cord (A). CD200 is expressed on CNPase-positive oligodendrocytes as shown in a transversal section of the spinal cord (B) and in white matter (C). CD200 expression is not present in GFAP positive astrocytes (D). Bar is 17.5 µm (A,B), 10 µm (C) and 5 µm (D).

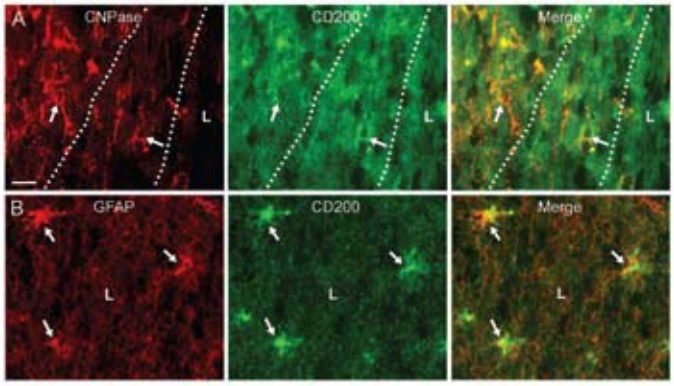

Fig. 3 Double fluorescent staining of CD200 with CNPase and GFAP in frozen sections (8 µm) of chronic active multiple sclerosis (MS) lesions. CD200 is expressed on CNPase-positive oligodendrocytes in the rim of a chronic active MS lesion (A, arrows). The area between the dotted lines is the border between the lesion (L) and the surrounding tissue. CD200 expression is present in GFAP-positive reactive astrocytes as shown in the center of a chronic active MS lesion (B, arrows). Bar is 20 µm (A) and 30 µm (B).

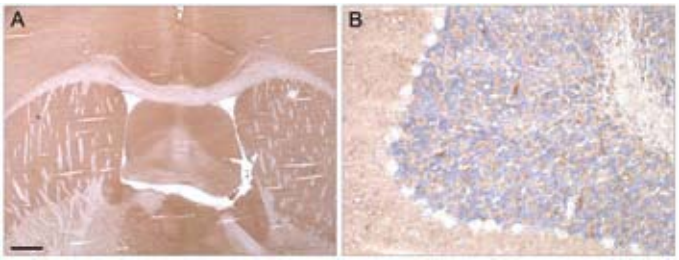

Fig. 4 CD200 expression in frozen sections (8 µm) of the mouse central nervous system. CD200 immunoreactivity is present in both grey and white matter areas, but staining intensity is low in white matter areas as corpus callosum and in the striatum compared to grey matter areas where CD200 staining is diffuse (A). CD200 staining is predominantly present in the granular and molecular layer of the cerebellum (B). Bar is 500 µm (A) and 65 µm (B).

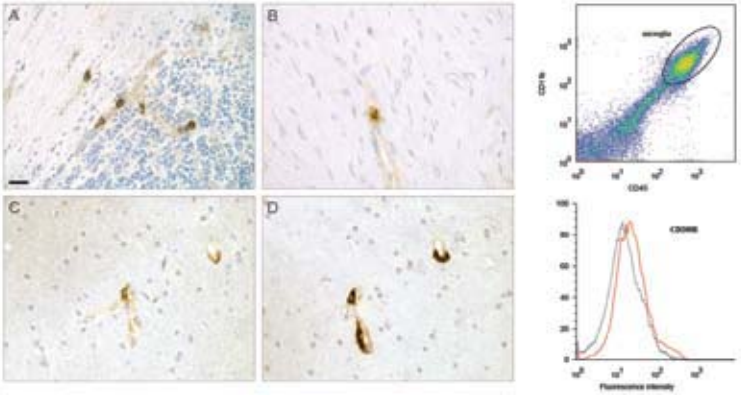

Fig. 5 CD200R staining in frozen sections (8 µm) of the human central nervous system derived from control donors. CD200R immunoreactivity is visible on perivascular cells as shown in the cerebellum (A) and in the spinal cord (B). Adjacent (8 µm) sections of white matter show CD200R expression in perivascular cells (C) and mannose receptor expression in the same cells (D). Bar is 10 µm (A-D). Human $CD45^{dim}CD11b^{+}$ microglia were isolated from corpus callosum and stained for CD200R (solid line) or an isotype-matched negative control (dotted line) (E). CD200R is present on microglia at low levels. Data are representative of 3 independent experiments.

CNPase and CD200 was diminished in the center of the lesion (data not shown, see also Koning *et al.*[111]). Unexpectedly, a subpopulation of GFAP-positive astrocytes in chronic active MS lesions also expressed CD200 (Fig. 3B). These lesion-associated astrocytes were all hypertrophic as determined by their characteristic morphology. Astrocytes situated further from the lesion did not express CD200.

CD200 expression in the normal mouse brain

Although CD200 expression has been described in the rodent brain, this has been poorly documented. In Figure 4 we show that the CD200 expression pattern in

the mouse brain was similar to that found in the human CNS. It was abundantly present throughout the brain and staining was highly intense in the grey matter. Staining was absent in CD200$^{-/-}$ mice (data not shown).[125,136,159]

Anatomical and cellular CD200R expression in the human CNS

Although CD200R immunoreactivity was present throughout the parietal cortex, hippocampus, striatum, cerebellum and spinal cord, its expression pattern was completely different from its ligand. CD200R in both grey and white matter was limited to cells associated with blood vessels (Fig. 5A, B) and staining of adjacent sections with mannose receptor (MR) demonstrated that these cells were perivascular macrophages (Fig. 5C, D).[163]

The immunohistochemical techniques we used did not show CD200R immunoreactivity on human microglia. Although studies in rodents have shown that CD200R was expressed on microglia, these were reported at low levels using flow cytometry and not supported by immunohistochemistry either.[121,136] Therefore, we analyzed CD200R expression on primary microglia, freshly isolated from human autopsy material using the highly sensitive technique of flow cytometry. We show that CD45^{dim}CD11b^{+} human microglia reproducibly expressed low amounts of CD200R (Fig. 5E), with mean fold difference of 1.6 ± 0.2 compared to isotype control. Expression of CD200R was markedly higher on choroid plexus-derived macrophages (5.3 ± 1.0) or peripheral blood monocytes (2.4 ± 0.5). In addition, gene expression analysis on these isolated microglia upon FACS sorting confirmed the presence of CD200R transcript in these cells (data not shown).[124]

In both chronic active and inactive MS lesions, CD200R expression by immunohistochemistry was clearly visible on blood vessel-associated cells that were present in all areas of the lesions and in the normal appearing white matter surrounding the lesions. In chronic active MS lesions, some perivascular cuffs contained CD200R positive cells, but others did not (Fig. 6A, B). Occasionally, CD200R expression was present on foamy macrophages, but only when located perivascularly (Fig. 6C).

Fig. 6 CD200R staining in frozen sections (8 µm) of chronic active multiple sclerosis lesions. Some perivascular infiltrates contain CD200R-positive cells (A), but others do not (B). Sometimes perivascular foamy macrophages express CD200R (C). Bar is 50 µm (A and C) and 100 µm (B).

Discussion

In this study we show that in the human CNS neurons express CD200, likely on the cell membrane and in some neurons also in the cytoplasm, confirming previous publications on the rat CNS and the human retina.[159,160] The cytoplasmic staining that was visible in some neurons may reflect transport of CD200 from the golgi to the cell membrane. It is not clear why this was not seen in the cytoplasm of all neurons, as e.g. that of Purkinje cells was negative, although this might be indicative for differential turnover rates of CD200 on these neurons. We are the first to report that human microglia express CD200R. These data indicate that neurons can suppress microglia via CD200-CD200R interaction to protect themselves from deleterious inflammatory reactions, as previously postulated for rodents. In white matter, CD200 was present on axons. Nevertheless, it is not well understood how axonal CD200 can bind its receptor since the axon is covered with myelin, except for the nodes of Ranvier. For effective immune suppression in white matter, it would therefore be reasonable if CD200 was also expressed by cells surrounding axons. Indeed, CNPase positive oligodendrocytes, but not GFAP positive astrocytes, appeared to express CD200. Hence, these results strongly suggest that not only neuron-glia but also glia-glia interactions are involved in the control of microglia activation.

The expression pattern of CD200 in grey matter is peculiar and difficult to interpret. The staining was diffuse and present on and between neuronal cell bodies. Although the majority of the immunoreactivity is ascribed to neurons, it is possible that oligodendrocytes account for the inter-neuronal staining. It is unlikely that the diffuse staining is caused by secretion of CD200 because it is a membrane-bound glycoprotein encompassing a transmembrane domain, lacking any known proteinase motifs that would be necessary for its release.[164] The diffuse staining pattern cannot be attributed to aspecificity of the antibody, as this was ruled out by specificity tests for this antibody.[120] Furthermore, immunoreactivity was absent in control stainings which included isotype-matched antibodies. Also CD200 expression in the CNS of mice showed a similar pattern as that in the human CNS, but staining was completely absent in $CD200^{-/-}$ mice, confirming specificity of our CD200 staining. Finally, such a diffuse expression pattern as that of CD200 is not uncommon in the CNS as, for example, the synaptic modulatory phosphoprotein GAP43 has been extensively described as a neuron-specific protein, but shows an expression pattern in grey matter areas resembling that of CD200.[165,166]

In contrast to the CNS of control subjects, CD200 was expressed in reactive astrocytes associated with MS lesions. This was a surprising finding as we recently showed that the overall expression levels of CD200 were decreased in the center

and rim of MS lesions.[111] This decreased expression is presumably due to axonal and oligodendrocyte damage, and may enhance macrophage and microglia activation. Since activated macrophages and microglia are thought to drive lesion formation in MS,[42] loss of CD200 will thus enhance inflammation and disease progression, as demonstrated in animal models. The implications for neuronal integrity are clearly reflected in enhanced neuronal damage in these *in vivo* models but also in macrophage-neuronal co-cultures,[125,126,128] whereas animals with increased CD200 levels display enhanced neuroprotection.[129] Our data on CD200-positive reactive astrocytes now suggest that immune activation within the CNS may lead to a protective molecular reflex by astrocytes that are otherwise not expressing CD200. Moreover, it corroborates the idea that astrocytes are protective and neurotrophic.[167] However, overall CD200 expression in MS lesions was still decreased compared to control tissues, indicating that the CNS' response to restore immune suppression in MS fails.

Expression of CD200R on microglia could not be detected using immunohistochemistry, but using flow cytometry we demonstrate that human primary microglia expressed low levels of CD200R, a finding also noted in rodents.[121,136] In contrast to microglia, CD200R was intensely stained on macrophages located in the perivascular space, in close proximity to CD200-void vascular endothelium. Hence, it is likely that in an environment where CD200 is abundantly present, the expression of CD200R is down regulated.

In conclusion, many molecules have been identified to be involved in the activation of macrophages and microglia, such as derived from cellular debris to apoptotic cells. Molecules as CD200 suppress the activation of myeloid cells in order to limit tissue damage, thereby protecting the vulnerable neurons from deleterious effects of inflammation.[168,169] Importantly, also oligodendrocytes and astrocytes can mediate neuroprotection by direct cell-cell contact through CD200-CD200R, in addition to the release of, for example, neurotrophic factors. Dysregulation of the finely tuned resident innate immunity in the CNS may cause macrophage/microglia activation as abundantly occurs in MS and also in other neurodegenerative disorders such as in stroke, Alzheimer's and Parkinson's disease.[170,171] At present, it is not known how expression of CD200 or CD200R is regulated. This will be the next step in providing tools for therapeutic use of immune suppressive mechanisms in the CNS in (inflammatory) neurodegenerative diseases.

Acknowledgments

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