Neuronal CD200 Signaling is Protective in the Acute Phase of Ischemic Stroke

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Abstract

Background and Purpose—CD200, a highly glycosylated protein primarily expressed on neurons in the central nervous system (CNS), binds with its receptor CD200R to form an endogenous inhibitory signal against immune responses. However, little is known about the effect of neuronal CD200 signaling in cerebral ischemia. The aim of this study was to investigate how neuronal CD200 signaling impacts post-stroke inflammation and the ischemic injury.

Methods—CD200 tma1[lf/fl]:Thy1CreER mice were treated with tamoxifen (TMX) to induce conditional gene knockout (ICKO) of neuronal CD200. The mice were subjected to a 60-min transient middle cerebral artery occlusion (MCAO). Stroke outcomes, apoptotic cell death, immune cell infiltration, microglia activation and other inflammatory profiles were evaluated at 3 and 7 days after stroke.

Results—Infarct volumes were significantly larger, and behavioral deficits more severe in ICKO vs. control mice at 3 d after MCAO. TUNEL assay also revealed a significant increase in apoptotic neuronal death in CD200 ICKO mice. An enhancement in lymphocytic infiltration and microglial pro-inflammatory responses were revealed by flow cytometry at 3 and 7 days after stroke in ICKO mice, accompanied by an increased microglial phagocytosis activity. Plasma pro-inflammatory cytokine (TNFα and IL-1β) levels significantly increased at 3d, and IL-1β/IL-6 levels increased at 7d in ICKO vs. control animals. ICKO led to significantly lower baseline level of CD200 both in brain and plasma.

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Disclosures

None of the authors have a conflict of interest relevant to this work.

Supplemental Materials:

- Expanded Material & Methods

- Online Supplemental Figure I, II, and III.

- References 61–62.

Conclusion—Neuronal CD200 inhibits pro-inflammatory responses and is protective against stroke injury.

Keywords

CD200; Neuroprotection; ischemic stroke; Neuroinflammation

Introduction

The inflammatory response to ischemic stroke is a fundamental pathophysiological process that significantly contributes to ischemic brain injury.[1, 2] Under normal conditions, neurons cross talk with both resident and circulating immune cells through either membrane-bound signaling molecules or soluble factors to maintain cerebral homeostasis.[3, 4] After ischemia, vast amounts of neurons die and trigger post-stroke inflammation. The post-stroke immune response causes secondary ischemic damage, and peaks within days of the stroke onset and lasts for months, potentially providing a longer time window for intervention compared to energy failure, oxidative stress[5, 6] or calcium excitotoxicity.[7, 8] Holding pro-inflammatory responses in check can mitigate stroke injury and has significant translational potential.

CD200 (OX-2) is a highly glycosylated membrane protein mainly expressed on the cell surface of neurons and endothelia in CNS.[9] CD200 was shown to exert its immunosuppressive and anti-inflammatory activities through interaction with CD200 receptor (CD200R), which belongs to the immunoglobulin superfamily and is expressed by myeloid cells and lymphocytes.[10–12] The CD200-CD200R signaling maintains immune cells in a quiescent state, and disruption of this interaction leads to pro-inflammatory activation of myeloid cells/lymphocytes and consequently pathological outcomes.[10] The contribution of neuronal CD200 signaling to stroke recovery has not been well studied. In the present study, we hypothesized that the neuronal CD200 signaling is protective in acute stroke and investigated the role of neuronal CD200 by using an inducible conditional knock out mouse model. We demonstrate that the neuronal CD200 signaling functions as a critical regulator of the immune response to ischemic brain injury.

Methods

The data that support the findings of this study are available from the authors upon reasonable request.

Animals

To generate neuronal CD200 inducible conditional knockout (CD200 ICKO) mice, we crossed CD200 tma1 floxed mice with Thy1-Cre (thy1Cre^{ER}) mice (Jackson Laboratory) in the C57BL/6 background. All the breeding pairs were housed in pathogen-free rooms and had access to food and water ad libitum. All mice were kept and generated under normal light-dark-cycles. As no sex difference in neuronal CD200 was noted, male (6–8 weeks old) CD200tma1$^{fl/fl}$/Thy1creER mice received injection (ip) of tamoxifen (TMX) (75mg/Kg BW) to induce Cre/LoxP mediated CD200 conditional gene deletion, or received corn oil (Sigma) injection as vehicle (VEH) controls. The injection was given a single dose every day

for five consecutive days. All the mice were subjected to stroke/sham surgery 2 weeks after the last injection. All procedures were performed in accordance with NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal care and use committee of the University of Texas Health Science Center Houston.

Ischemic stroke model

Cerebral ischemia was induced by 60-minute reversible middle cerebral artery occlusion (MCAO) under isoflurane anesthesia as previously described.[2, 10, 13] Details of the model are in the online-only supplementary material.

Behavior testing

Neurological deficit scores (NDS), corner test, and spontaneous alternation in Y-maze were assessed as previously reported[1, 2]; for details, see the online-only supplementary material.

Immunohistochemistry, Cresyl Violet (CV), TUNEL staining

Brain sections were stained with mouse anti-NeuN (1:500, R&D Systems, MN, USA), rat anti-CD200 (1:300, abcam, USA), mouse anti-von Willebrand Factor (VWF, sc-365712, Santa Cruz biotechnology Inc, TX, USA), and rabbit anti-glial fibrillary acidic protein (GFAP) (1:500, Cell Signaling Technology # 80788). Brain images were obtained on Leica DMi8 confocal microscope. At day 3, infarct was evaluated by performing CV staining as described previously.[14, 15] TUNEL assay was performed according to manufacture instruction (In Situ Cell Death Detection Kit, Fluorescein, Millipore Sigma). For details, see the online-only supplementary material.

Flow cytometry (FC) and Phagocytosis assay

Leukocytes from the ipsilateral brain tissue were prepared as previously described.[16–18] Isolated leukocytes were stained with antibodies for cell membrane and intracellular markers. Microglial phagocytosis was evaluated in isolated microglia with bio-particles. Details of these assays were provided in the online-only supplementary material.

Determination of CD200 and cytokine Levels

The levels of CD200 in mouse brain, plasma and in the supernatants of primary neuronal culture were measured by a sandwich ELISA kit according to manufacture instruction ((# EK1185, Mouse CD200 PicoKine ELISA Kit, Boster Biological, Pleasanton, CA, USA.). Other plasma cytokine levels (IL-1β, TNF-α, IL-6, IL-10, IL-4) were measured by commercially available specific multiplex quantitative Bio-Plex Pro™ Mouse Cytokine 23-plex assay according to manufacturer instructions (# M60009RDPD, Bio-Rad Laboratories, Hercules, CA).

Primary neuronal culture and Oxygen-Glucose Deprivation (OGD)

Culture of primary neurons was performed as described previously.[19, 20] Oxygen-glucose deprivation (OGD) was slightly modified from a previously described method.[21] After OGD, CD200 levels in cell culture supernatant was evaluated as described in.[19] For details please see the online-only supplementary material.

Splenic CD3⁺ isolation, co-culture with primary neurons, and CD200-CD200R binding assay

CD3$^+$ T cells were isolated from the spleen of a 7-week old naïve male C57BL/6 mouse using the MojoSort™ Mouse CD3 T Cell Isolation Kit according to manufacture instruction (# 480024 BioLegend). The purity of isolated CD3$^+$ cells was confirmed by flow cytometry. The isolated cells were plated at 1×10^6 cell per T-75 flask, and rested for 5–7 days before co-culture with cortical neurons, and followed by ICC. CD200-CD200R binding assay was performed by using recombinant mouse CD200R1 Fc chimera peptide_2554-CD (R&D Systems) and biotinylated recombinant mouse CD200-Fc Chimera (carrier-free)_786004 (BioLegend). For details please see the online-only supplementary material.

Statistical Analysis

Data from individual experiments were presented as mean ± SD and assessed by Student t test or one-/two-way ANOVA with Tukey post-hoc test for multiple comparisons and Hold-Sidak test for paired comparisons (GraphPad Prism Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant. The ordinal data of the NDS was analyzed with Mann-Whitney U test. Investigators were blinded to mouse strains for stroke surgery, behavioral testing, infarct, and inflammation analysis.

Results

Stroke outcomes in CD200 ICKO mice.

We first confirmed CD200 deletion from neurons by performing NeuN and CD200 immunofluorescence staining in brain slices from CD200 ICKO mice. We found that almost none of the NeuN positive cells express CD200 in Tamoxifen treated mice (TMX); however, NeuN and CD200 were colocalized in vehicle treated mice (Sup. Fig. IA). We confirmed that TMX injection had minimal effect on the expression of CD200 level in endothelial or astrocytic cells as we found there was no difference in CD200 immunofluorescence intensity in VWF or GFAP positive cells between TMX and VEH treated mice (Sup. Fig. IB–D), demonstrating that the ICKO only affects neuronal but not endothelial or astrocytic CD200.

Next, we examined brain infarct at 3 days after stroke in both VEH and TMX treated mice. Quantitative data showed the TMX treated (CD200 ICKO) mice had significantly larger infarct in the cortex ($p = 0.0172$), striatum ($p = 0.0161$), and ipsilateral hemisphere ($p = 0.0191$) than the VEH treated (CD200 intact) mice (Fig. 1A). We also performed functional behavior tests after 3d of stroke. The NDS significantly increased in TMX group compared to the VEH control ($p = 0.025$, Fig. 1A lower left). In the corner test, the proportion of right turns was significantly higher in TMX vs. VEH stroke group ($p = 0.0011$, Fig. 1A lower right). There was no difference in Y-maze test between TMX and VEH groups (data not shown). We also evaluated the effect of CD200 deletion on neuronal apoptosis near the peri-infarct area. DNA damage is one of the main features of apoptosis and can be visualized with TUNEL staining.[22] The nuclear morphology of apoptotic neuronal cells was evaluated with TUNEL counterstained with DAPI. There was no difference in TUNEL positive cells between sham VEH and TMX treated mice; however, in the MCAO groups, TMX mice showed significantly more TUNEL positive cells compared to the VEH control ($p = 0.001$,

Fig. 1B). These data indicated that neuronal CD200 signaling is protective against ischemic injury.

CD200 ICKO mice had more infiltration of leukocytes into the ischemic brain.

To evaluate the effect of neuronal CD200 on peripheral immune cell infiltration in the ischemic brain, we quantified infiltrating leukocytes in sham and stroke mice brains with flow cytometry (FC). Total peripheral myeloid cells were gated as $CD45^{high}CD11b^+$, and lymphocytes as $CD45^{high}CD11b^-$ (Fig. 2A). Among the peripheral myeloid cells, monocytes were further gated as $CD45^{high}CD11b^+Ly6C^+Ly6G^-$, and neutrophils as $CD45^{high}CD11b^+Ly6G^+$. Quantitative data showed that there was significantly more lymphocyte infiltration in the TMX vs. VEH treated mice brains at 3 days (p = 0.003, Fig. 2B) and 7 days (p = 0.0182, Fig. 2C) after MCAO. More neutrophil infiltration was also seen in TMX vs. VEH brains at 7 days (Fig. 2C).

Microglia are more activated in ischemic brains of TMX vs. VEH treated mice.

We next examined microglial activation after stroke with FC, as microglial activation is a key element in initiating and perpetuating inflammatory responses to ischemia. CD68 is a well-established marker of phagocytic pro-inflammatory activation.[22, 23] We gated microglia as $CD45^{intermediate}CD11b^+$, and quantified the median fluorescence intensity (MFI) of CD68 and CD206 in microglia. Gating strategy of microglia is in Fig. 2A. Our data revealed that the MFI of CD68 significantly increased in TMX vs. VEH treated microglia 3 (p = 0.023, Fig.3A) and 7 days (p = 0.003, Fig.3B) after stroke. In contrast, there was no difference in CD206 (an anti-inflammatory marker)[1, 2] MFI between the two groups (Fig.3 A, B). *Ex vivo* assays was performed on freshly isolated microglia by FC from the ischemic brains at 7 days after MCAO to evaluate microglial phagocytosis activity. The gating strategy of the isolated microglia can be found in Sup. Fig. II. Our data demonstrated a significant increase in the phagocytic activity of microglia (MFI of phagocytic beads) in TMX treated mice compared to VEH controls (p < 0.0001, Fig. 3C). These data suggest that CD200 signaling dampens microglial pro-inflammatory response to ischemic stroke.

Serum cytokine levels after stroke in CD200 ICKO vs. CD200 intact mice.

Serum cytokine levels are reflective of the degree of immune responses to ischemic injury.[1, 24] To investigate how CD200 deletion affects serum cytokine levels, we measured pro-inflammatory (IL-1β, TNF-α, and IL-6) and anti-inflammatory (IL-10, and IL-4) cytokine levels in the serum of sham and stroke mice with MultiPlex at 3 and 7 days after MCAO. As expected, in both the TMX and VEH cohorts, levels of all cytokines significantly increased after stroke. When the comparison was made between TMX vs. VEH groups, we observed no difference in the levels of these cytokines (Fig. 4A&B) between the two sham groups, both of which were equivalently low. However, in the 3d MCAO group (Fig. 4A), TNFα (p=0.0001) and IL-1β (p= 0.0002) levels were significantly higher in TMX vs. VEH mice; IL-6 level showed an increase trend in TMX vs. VEH stroke mice, and there was no difference in IL-10 and IL-4 cytokine levels between the two groups. Similar results were seen in the 7d MCAO group (Fig. 4B): IL-1β and IL-6 levels were significantly increased in TMX vs. VEH stroke mice. However, we observed an increased IL-4 level in VEH vs. TMX mice at 7d, suggesting the ICKO suppressed the anti-inflammatory cytokine

which begins to increase at the sub-acute phase of stroke. The data further suggested that the CD200 signaling mainly impacts the pro-inflammatory responses in the acute stroke.

Since neurons are the primary cells that express CD200 in the CNS, we wondered if neurons can secret CD200 into the blood circulation to bind to CD200R expressed on circulating leukocytes. We first examined the base line level of CD200 in both TMX and VEH mice brains. Our data demonstrate a significant decrease in CD200 baseline level in the TMX treated naïve brains compared to their VEH treated counterparts (p = 0.027, Fig. 4C). Next, we examined the plasma level of CD200 in these mice 3d after MCAO with ELISA. Interestingly, the quantitative analysis showed the plasma level of CD200 protein was significantly decreased in TMX injected compared to VEH sham mice, indicating that deletion of neuronal CD200 alters circulating CD200 levels. We also observed a significant decrease in serum CD200 after stroke in Vehicle treated mice. This suggests that neurons are a major source of circulating CD200 as the stroke injury causes death of vast amount of neurons, leading to a significant loss of CD200. The level of CD200 did not significantly change in TMX mice after stroke, probably due to a floor effect of CD200 ICKO from neurons (Fig. 4C). To confirm this finding, we performed primary neuronal culture, used OGD as an *in vitro* ischemia model, and examined CD200 levels in the culture medium. Consistent with our *in vivo* results, we also observed a significant decrease of soluble CD200 levels in the culture supernatant after OGD (Fig. 4C).

Administration of CD200Fc during the acute phase of stroke improves outcomes.

To confirm the protective effect of CD200 during the acute phase of stroke, CD200Fc or mouse IgG2a (control) was administered in C57BL/6 mice before and after stroke (the method was provided in the online-only supplementary material). Stroke outcomes were evaluated at 3d of MCAO, and we found that CD200Fc-treated mice had a smaller infarct in the ipsilateral cortex (p = 0.026) and hemisphere (p = 0.001) than the IgG treated mice (Fig. 5A). Consistent with the infarct data, CD200Fc-treated mice showed improved NDS compared to control mice (p = 0.0214, Fig. 5A), although there was no significant difference in corner test scores between the two groups. We also performed flow cytometry in CD200 Fc treated mice to examine microglial expression of cell membrane and intracellular markers. As shown in Fig. 5B, the anti-inflammatory marker CD206 was significantly higher expressed in ischemic microglia in CD200 Fc treated vs. control mice. However, CD200 Fc ischemic microglia expressed significantly less intracellular pro-inflammatory markers (IL-1β, TNFα) compared to controls after stroke (Fig. 5C). The intracellular anti-inflammatory markers (IL-10, IL-4) did not show differences between the two groups (Sup. Fig. III). These data confirmed that the CD200 signaling is protective against ischemic injury by quenching the inflammatory response.

Neuronal CD200 interaction with lymphocytic CD200R

Neuronal CD200 functions through interactions with CD200R expressed on leukocytes. To confirm the interaction, we performed neuron and T cell co-culture to examine the actual ligand-receptor binding with confocal microscoping. Fig. 6A shows the purity of T cells (CD45^{+}CD3^{+}) separated from a C57BL/6 mouse spleen by flow cytometry. These T cells express CD200R1(the primary receptor that binds to CD200 in CD200R family)[25, 26], and

the cultured primary neurons express CD200 (Fig. 6B). After a co-culture of T cell and neurons, ICC was performed. Fig. 6C showed CD3 and MAP2 (a neuronal marker) were colocalized (upper panel), and CD200R1 from T cells bound to CD200 on neurons (lower panel). To further confirm the interaction, we additionally performed ligand-receptor binding assay, and found CD200 binds to CD200R in a concentration dependent manner (Fig. 6D).

Discussion

The present study demonstrated several key findings which support our hypothesis that neuronal CD200 signaling is essential to limit post-stroke inflammation and ischemic injury. Firstly, neuronal CD200 signaling mainly targets the pro-inflammatory response to acute stroke, evidenced by the data of microglial activation and circulating cytokines (Fig. 3&4). Secondly, neuronal CD200 is protective against ischemic injury and promotes functional recovery after stroke. This is consistent with previous studies showing CD200 signaling is beneficial in neurodegenerative diseases.[27, 28] Thirdly, neuronal CD200 preferably targets the circulating lymphocytes and inhibits their infiltration in the ischemic brain. This is consistent with our previous report that lymphocytes robustly express the ligand receptor CD200R.[10] Lastly, neurons secrete soluble CD200 into the blood circulation and are the primary source of circulating CD200. Of note, these findings were attributed to the neuronal CD200 signaling, as our ICKO model based on Thy1-CreER recombination only affects the CD200 expression in neurons but not endothelia or astrocytes (Sup.Fig.I), although it was reported these cells are also Thy1 positive. To our knowledge, the present study is the first report that has mechanistically investigated the involvement of neuronal CD200 signaling in post-stroke inflammation.

The CD200-CD200R signaling axis has been described as an "inflammation brake" in experimental allergic encephalomyelitis (EAE) and other neurodegenerative diseases.[28–32] In stroke, the ischemic injury not only causes neuronal death but also induces inflammatory responses characterized by the activation of immune cells and release of cytokines/ chemokines.[33] Post-stroke inflammation plays a fundamental role in the progress of ischemic infarction and causes secondary neuronal damage.[2, 34] In the CNS CD200 was primarily expressed by neurons,[35] and the loss of neurons in the ischemic brain may lift the "brake" of CD200-CD200R inhibitory signaling and enable a more robust inflammatory response. It is controversial as to which immune cell population the CD200 binds to as a brake. Studies with other CNS disease models have suggested neuronal CD200 interacts with CD200R expressed on microglia to dampen inflammatory responses.[36, 37] However, our study[10] and others have found adult microglia express near null levels of CD200R.[35, 38] The present study did show an enhanced inflammatory response in microglia demonstrated by an increased level of CD68 and phagocytic activity in CD200 ICKO vs. VEH stroke mice (Fig. 3). We hypothesize that the enhanced pro-inflammatory phenotype of microglia is secondary to the loss of CD200-CD200R brake between neurons and other immune cells. Our previous study used a different transgenic animal model, i.e. CD200R global knockout mice, and found the similar neurobehavior deficits and inflammatory responses after stroke as in the present study, indicating the protective role of CD200-CD200R signaling. This is consistent with a previous study[39] which showed injection of exogenous recombinant CD200 protein into the cerebral ventricle reduced the expressions of Iba-1, IL-1β, and

TNF-α at 48 h after permanent MCAO. Interestingly, the authors also reported a negative correlation between brain CD200 levels and neuronal death.

Our previous study demonstrated that lymphocytes were the predominant cell type expressing CD200R in the ischemic brain, with moderate expression in infiltrating monocytes.[10] The present study found the deletion of CD200 signal from neurons exacerbates lymphocytic cell infiltration, but has no effect on the infiltration of monocytes (Fig. 2), suggesting the neuronal CD200 signaling has cell specific affinity and mainly binds to CD200R on lymphocytes. Although the mobilization of lymphocytes has long been considered a chronic inflammatory response,[40, 41] the infiltration of lymphocytes in the ischemic brain has been extensively reported at the acute stage of stroke.[42, 43] Lymphocytes can be divided into many subtypes, including $CD4^+/CD8^+$ T cells, B cells, natural killer cells, etc.[44, 45] Our latest study has found the intracellular levels of TNFα and IFNγ in $CD8^+$ T cells (but not in $CD4^+$ cells or microglia) were increased after the disruption of CD200-CD200R signaling, and these changes mirrored the levels of these cytokines in whole brain. This is consistent with the findings in the present study that showed robust infiltration of lymphocytes and increased levels of circulating pro-inflammatory cytokines (TNFα, IL-1β, IL-6) (Fig. 5) in CD200 ICKO vs. control mice. Our *in vitro* data further confirmed the interaction of neuronal CD200 ligand with lymphocytic CD200R (Fig.6). Of note, the CD200-CD200R signaling functions on both central (brain) and peripheral inflammatory responses. Our previous study has found that CD200R global knockout mice developed more severe spontaneous bacterial infections of the lung after stroke compared to WT mice.[10] Further studies are underway to investigate how CD200-CD200R signaling affects T cell activation in both the central (brain) and peripheral immune response to stroke.

To date, the relationship of neuronal CD200 signaling with peripheral CD200R expressing leukocytes has not been studied, as most studies have focused on neuron-microglial interactions. The neuroimmune cross-talk between neurons and peripheral leukocytes happens via two avenues: soluble factors[4] and direct contact with infiltrating leukocytes. The involvement of neuronal soluble factors in immune cell activation has been increasingly reported; these include anti-inflammatory cytokines,[46] chemokines and neurotrophins,[47] neuropeptides,[48] and neurotransmitters.[49] It was found that soluble CD200 (sCD200) plays a critical role in the pathophysiology of many diseases.[50–53] Consistently in the present study, we administered soluble CD200 Fc to WT mice and also induced protective effects (Fig. 5). Furthermore, our data showed that neurons are the primary source of circulating sCD200 as the neuronal CD200 ICKO mice had significantly lower levels of sCD200 compared to the CD200 intact mice; the ischemic insult significantly decreased sCD200 as seen in both *in vitro* and *in vivo* assays (Fig. 4C). The sCD200 secreted from neurons may bind to CD200R expressed on lymphocytes to quench lymphocytic activation and prevent them from infiltrating into the ischemic brain. Neuronal CD200 may also directly bind to adjacent infiltrating lymphocytes from the disrupted BBB after stroke and lower the lymphocytic activation level. The interaction between neurons and lymphocytes through CD200-CD200R signaling warrants further investigation.

Thy-1 is a widely accepted neuronal biomarker,[54–56] and Thy1-CreER mice has been extensively used to generate inducible conditional knockout of neuronal genes.[57, 58]

However, it has also been reported that Thy1 was expressed not only by neurons, but also by endothelia, thymocytes, peripheral T cells, myoblasts, epidermal cells, and keratinocytes.[56, 59, 60] By using the Thy1-CreER mice in the present study, we found the TMX induction affects the neuronal CD200 expression but not the endothelial or astrocytic levels; nevertheless, we cannot rule out the effect of CD200 deletion in other Thy1 positive cells. Ongoing studies in this lab are using an additional neuronal conditional knockout animal model, enolase-2 cre mouse, to study the impact of neuronal CD200 signaling on post-stroke inflammation. Another caveat of this study is that we only examined the immune response and stroke outcomes at the acute stages of stroke (3d and 7d) in young mice. It is important to also evaluate the effect of the neuronal CD200 signaling at the chronic phase of stroke and in older animals, which is underway in the lab.

In conclusion, our data demonstrated that neuronal CD200 signaling is critical for the control of post-stroke inflammation and limits stroke injury. The effect is unlikely achieved through a direct interaction between neurons and microglia as microglia express near null CD200R,[10] but rather through the dampening of lymphocytic activation after stroke. The role of CD200 signaling is unique in post-stroke inflammation, as it primarily inhibits pro-inflammatory responses but not the anti-inflammatory response. Our observations provide new insights into the effect of the CD200 signaling on neuroinflammation not only in stroke, but also in other CNS disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Nonstandard Abbreviations and Acronyms

CNS	central nervous system
CV	cresyl violet
FC	flow cytometry
ICKO	inducible conditional knock out
MCAO	middle cerebral artery occlusion
MFI	mean fluorescence intensity

| NDS | neurological deficit score |
OGD	oxygen-glucose deprivation
TMX	tamoxifen
VEH	vehicle

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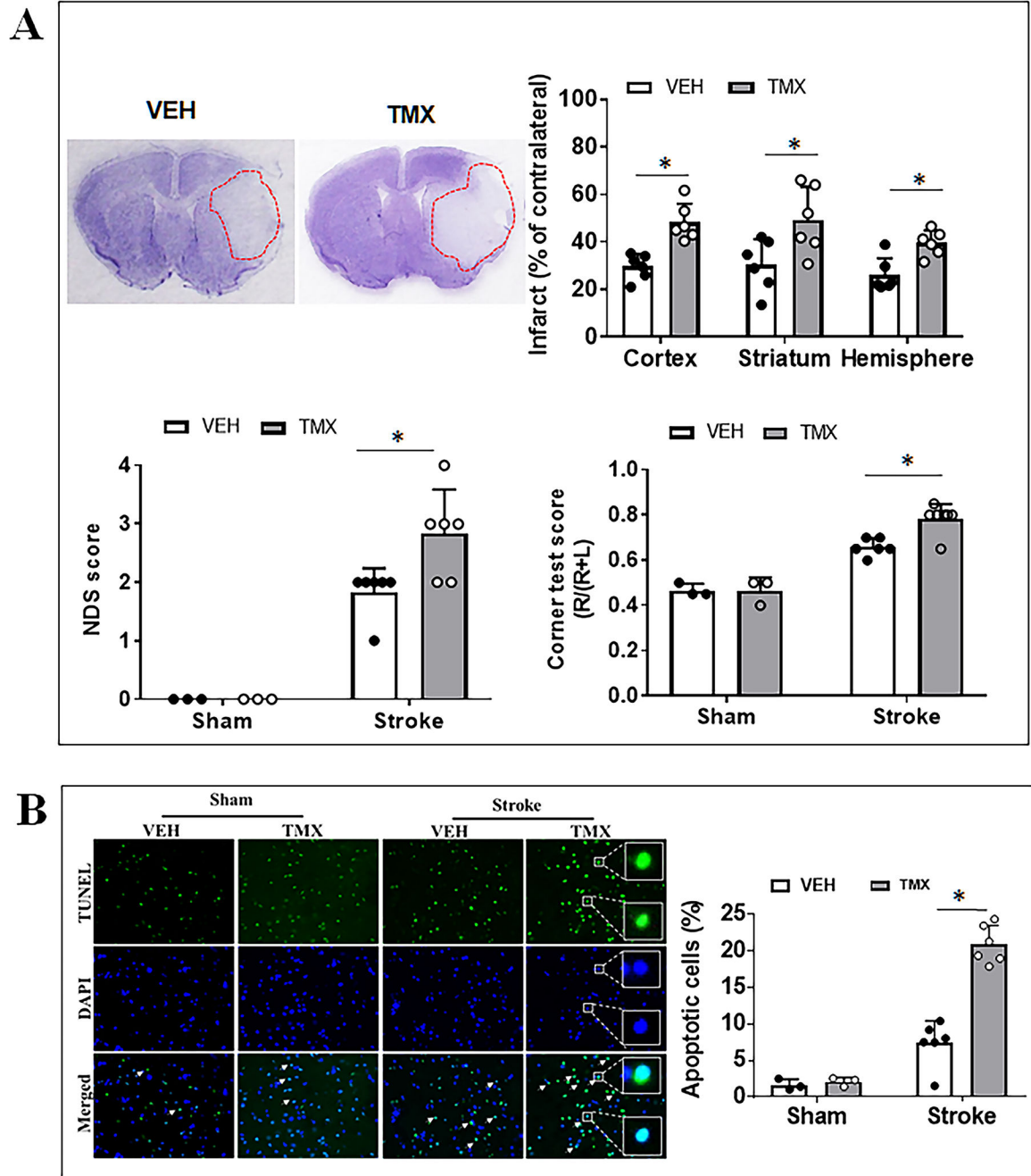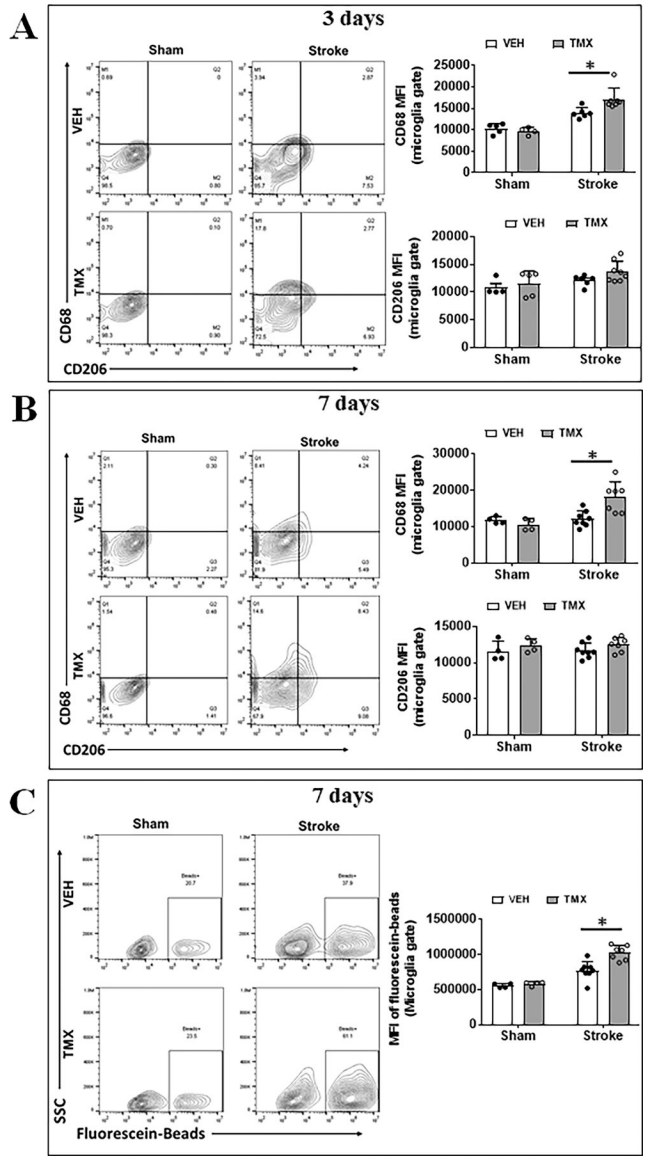
Figure 1.
Effects of neuronal CD200 signaling on stroke outcomes and neuronal apoptosis at 3 days after stroke. **(A)** Representative images of brain slices stained with cresyl violet (CV) and quantification of brain infarct volumes, NDS, and corner test scores in TMX vs. VEH mice. **(B)** TUNEL labelled cell apoptosis in peri-infarct area of TMX and VEH treated mice and the quantification of percent of TUNEL positive cells in ipsilateral hemispheres Each dot represents the average of % apoptotic cells from eight $20\times$ fields/animal in the peri-infarct area. N=3 for sham and 6–7 for stroke per group; $*P < 0.05$.
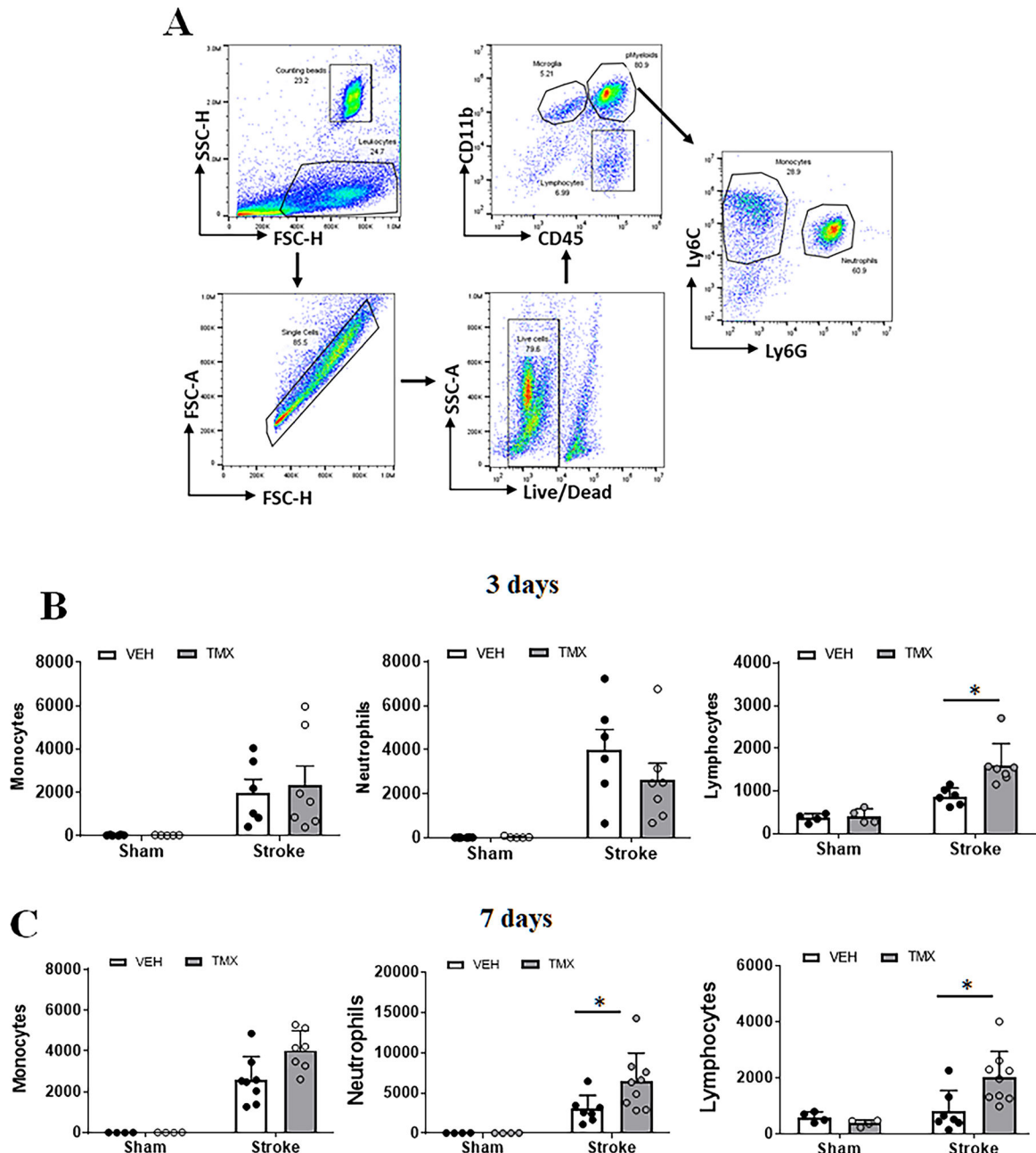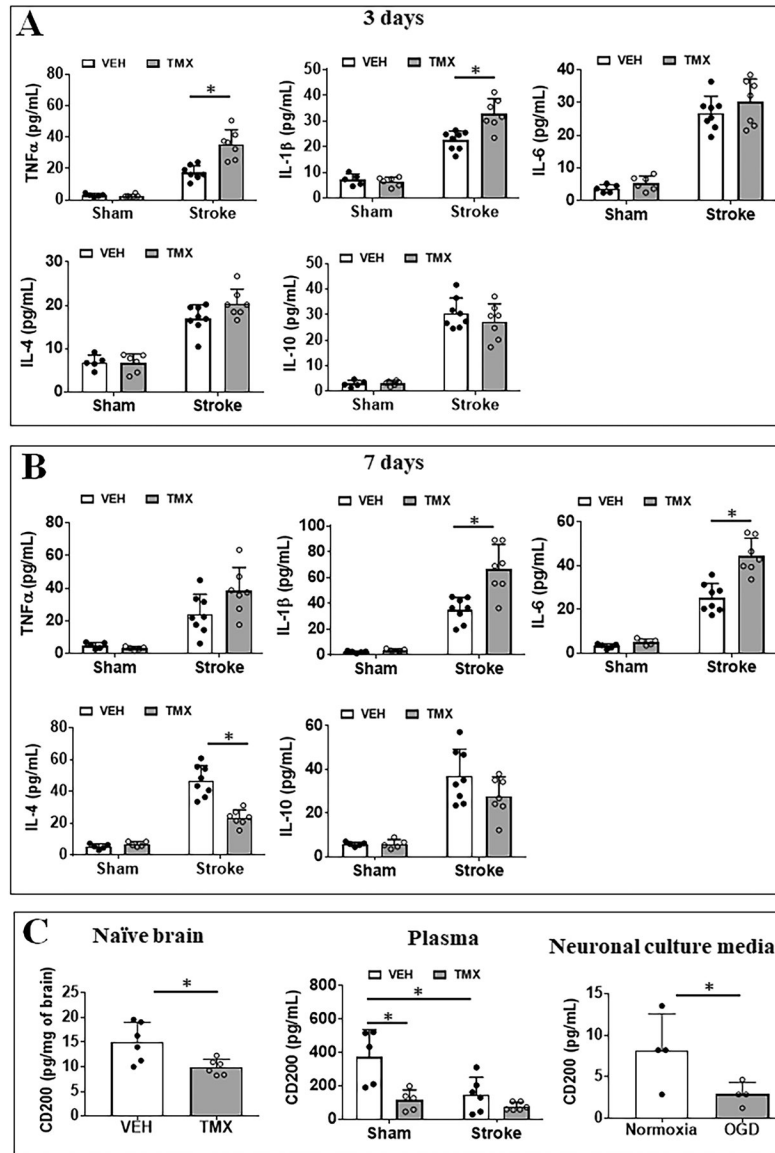
Figure 2.
Peripheral immune cell infiltration in ischemic brains at 3 and 7 days after stroke. **(A)** Representative dot plots depict the gating strategy used to differentiate the microglia (CD45^{intermediate}CD11b$^+$), peripheral myeloid cells (CD45^{high}CD11b$^+$) (pMyeloid), and lymphocytes (CD45^{high}CD11b$^-$). In pMyeloid cells, monocytes are further gated as CD45^{high}CD11b$^+$Ly6C$^+$Ly6G$^-$, and neutrophils as CD45^{high}CD11b$^+$Ly6G$^+$ Ly6C$^-$. Absolute counts of infiltrating monocytes, neutrophils, and lymphocytes at 3d **(B)** and 7d **(C)** after stroke were shown. N=4–5 sham and 6–8 stroke animals per group; $*P < 0.05$.
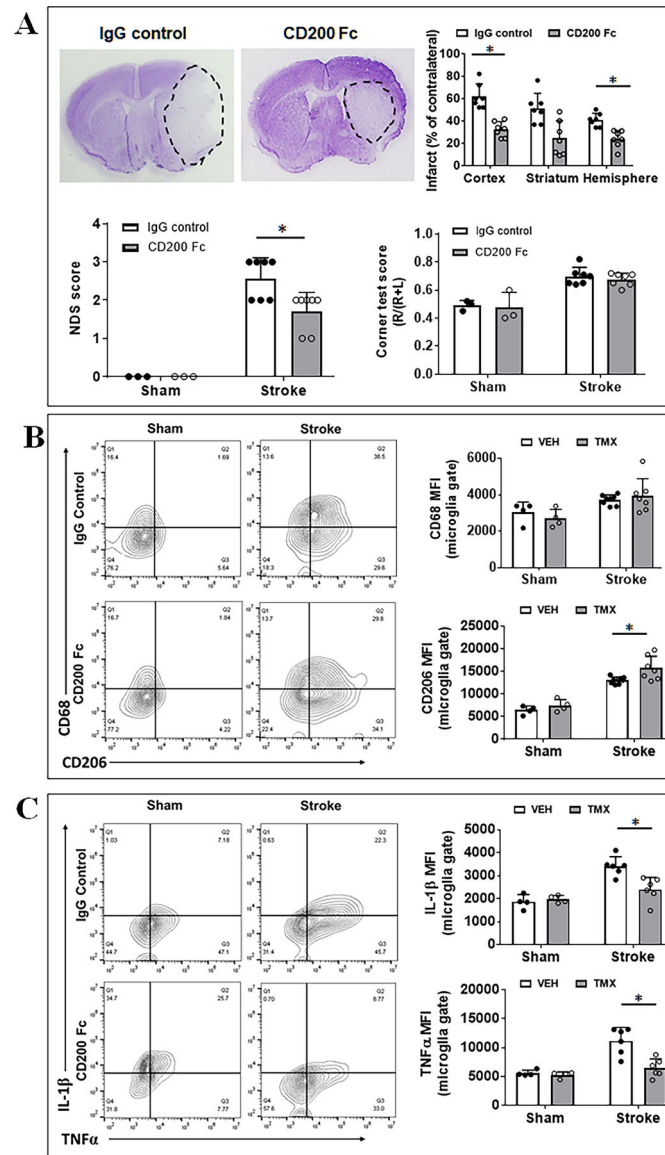
Figure 3.
Microglial activation after stroke in TMX and VEH treated mice. Representative FC plots of CD68/CD206 expressed on microglia at 3d (**A**) and 7d (**B**) after MCAO. Quantitative expression of CD68 and CD206 was measured as mean fluorescence intensity (MFI). (**C**) Representative plots illustrate the phagocytic activity of microglia at 7d after MCAO, which was quantified by MFI of bio-particles in microglia. N=6–8 stroke/4–5 sham animals per group; *$P < 0.05$.
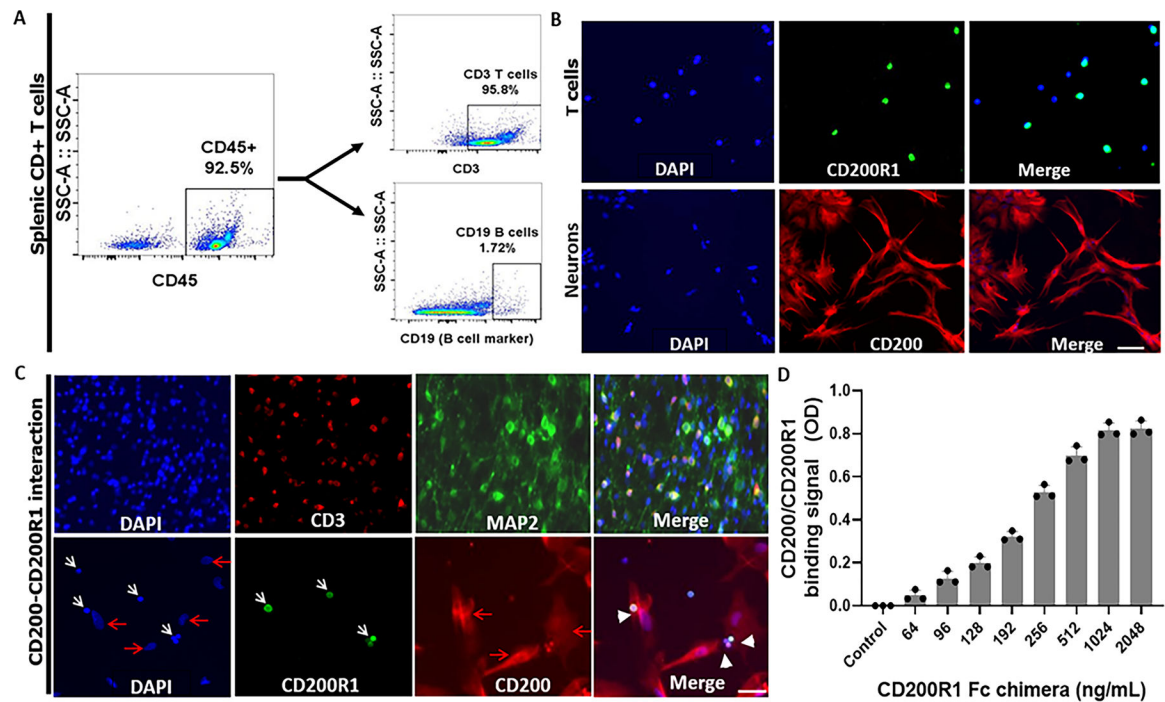
Figure 4.
Plasma cytokine levels after ischemic injury. Pro-inflammatory (TNFα, IL-1β and IL-6) and anti-inflammatory (IL-10 and IL-4) cytokine levels (pg/mL) were measured in the plasma of VEH and TMX treated sham/stroke mice at 3d (**A**) and 7d (**B**). N=5–6 sham and 6–7 stroke animals per group; *$P < 0.05$. (**C**) CD200 levels in naïve brains (left), in the plasma (middle) of TMX and VEH treated mice, and in neuronal culture medium after OGD (right). N=5–6 sham and 6–8 stroke animals per group; *$P < 0.05$. For neuronal culture medium, N=4 independent OGD experiments; *$P < 0.05$.

Figure 5.
Stroke outcomes and flow cytometry in CD200 Fc treated mice. **(A)** Representative images of brain slices stained with cresyl violet (CV) and quantification of brain infarct volumes, NDS, and corner test scores in CD200Fc vs. IgG treated mice. N=3 for sham and 6–7 for stroke per group; *$P < 0.05$. **(B)** Microglial expression of cell membrane markers CD68/CD206 measured as mean fluorescence intensity (MFI). **(C)** Microglial expression of intracellular markers IL-1β/TNFα. For (B) and (C), n=4/sham group and n=6/stroke group; *$P < 0.05$.

Figure 6.
Interaction of neuronal CD200 with lymphocytic CD200R1. **(A)** The purity of CD45+CD3+
T cells from spleens of C57BL/6 mice were confirmed by flow cytometry. **(B)**
Representative ICC images showed splenic CD3+ T cells express CD200R1 (upper panels;
green) and cultured primary neurons express CD200 (lower panels; red). **(C)** Neurons
interact with CD3+ T cells. Upper panels, colocalization of CD3 (marker for T cells) and
MAP2 (marker for neurons). Lower panels, colocalization of CD200 ligand on neurons
and CD200R1 on T cells. White arrows indicate T cells; red arrows indicate neurons;
white arrow heads indicate the interaction of CD200 with CD200R. Scale bar=100 μm.
(D) ELISA binding assay of mouse biotinylated CD200Fc chimera protein with mouse
CD200R1 chimera protein (captured by anti-CD200R1 antibody) immobilized on a Nunc™
MaxiSorp™ ELISA plate and in a concentration dependent manner (n = 4 assays per group).
Control ELISA wells were without CD200R1 Fc chimera protein.