

**CANNABINOID RECEPTOR 2 AGONIST REDUCES IMMUNE CELL
MIGRATION IN NEUROINFLAMMATION VIA INHIBITION OF MATRIX
METALLOPROTEINASE-9**

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ABSTRACT

Several studies have reported that administration of cannabinoid receptor agonists in inflammatory/autoimmune and CNS injury models resulted in significant attenuation of clinical disease. The beneficial effects correlated with the observed reduction of inflammatory mediators and peripheral immune cell infiltration into the site of inflammation. Previous studies from our laboratories demonstrated that administration of cannabinoid type 2 receptor agonist attenuated disease score and improved recovery in two murine models of neuroinflammation; spinal cord injury (SCI) and experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis. The goal of the current investigation was to evaluate the mechanisms through which administration of selective cannabinoid-2 receptor (CB2R) agonists modify inflammatory responses and help to improve function in SCI and EAE.

In SCI, an acute neuroinflammatory disorder, administration of CB2R agonist at 1 h and 24 h following contusion injury to the cord resulted in improved recovery of motor function and bladder function (the ability to spontaneously void) compared to control animals. Evaluation of inflammatory mediators at 48h demonstrated a dramatic reduction in the expression of the chemokines CXCL9, 10, 11 and cytokines IL-23 and its receptor in CB2R agonist-treated cords. There was also a reduction in the expression of toll-like receptors (TLR1, TLR4, TLR6, and TLR7), which correlated with a decreased number of immunoreactive microglia. Interestingly, at seven days post injury, CB2R agonist-treated injured cords showed a significant reduction in both hematopoietic and myeloid cell infiltration.

In EAE, a chronic neuroinflammatory disorder, our laboratories demonstrated previously that administration of a CB2R agonist led to lower disease scores and improved recovery. In this study, we observed reduced numbers of infiltrating hematopoietic and myeloid cells into the spinal cord and brain of CB2 agonist-treated mice. This reduction was observed at the peak of disease (day 17) and the effect was maintained at the chronic stage of disease (day 30). Evaluation of molecules associated with cell migration showed decreased levels of the adhesion molecule VCAM-1 and matrix metalloproteinases MMP-2 and 9 at peak of EAE in treated mice. The decrease in VCAM-1 correlates with our previous observation of decreased leukocyte rolling and adhesion to brain microvasculature. However, the reduction in MMP-2/9 expression suggests that CB2R agonists may also affect leukocyte transmigration into the perivascular space and further infiltration into the CNS parenchyma. This process requires both chemokine cues and the gelatinases MMP2/9. Animals deficient in these MMPs show leukocyte accumulation in the perivascular space and are resistant to EAE. There are no reports in the literature on possible CB2R agonist effects on gelatinases in myeloid cells. Although both MMP-2 and -9 are produced by antigen-presenting cells and act on similar substrates, MMP-9 appears to play a crucial role in EAE. Therefore, we decided to examine the effects of CB2 signaling on MMP-9 expression in myeloid cells, focusing on myeloid bone marrow-derived dendritic cells (BMDC).

Activation of bone marrow-derived macrophages, dendritic cells, and primary microglia with the cytokine cocktail TNF α , IL-1 β , IL-6, containing PGE₂, which mimicked an inflammatory milieu, resulted in expression of high levels of MMP-9. Treatment with

CB2R agonists reduced MMP-9 in all three cell types. Since migration of DC to various sites is required for their activation and for the initiation of adaptive immune responses, we evaluated the effects of CB2R agonists on migration. The reduced levels of MMP-9 correlated with reduced migration of DC to the draining lymph nodes in vivo, as well as reduced migration vitro in the matrigel migration assay. The effect on MMP-9 expression was mediated through CB2R, resulting in reduction in cAMP levels, subsequent decrease in ERK activation, and reduced binding of c-Fos and c-Jun to the AP-1 site in the MMP-9 promoter. We postulate that, by dampening production of MMP-9 and subsequent MMP-9-dependent DC migration, cannabinoids contribute to resolve acute inflammation and to reestablish homeostasis. Selective CB2R agonists might be valuable future therapeutic agents for the treatment of chronic inflammatory conditions by targeting activated immune cells including DC.

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CHAPTERS

CHAPTER 1

BACKGROUND

1.1. Neuroinflammation

CNS was considered to be an immune privileged site due to observations in early 20th century that rat sarcoma cells implanted within the brain parenchyma evaded systemic immune response and grew well, in contrast to implantation subcutaneously or intramuscularly in the periphery. However, when these tumor cells were implanted into the CNS along with spleen cells, tumor growth was inhibited, suggesting that the CNS was not capable of mounting an immune response [1]. This idea of “immune privilege” was further enforced when researchers reported that the CNS lacked lymphatic drainage, showed very low levels of MHCII staining, and most importantly that it was separated from the vasculature by the presence of blood brain barrier [1, 2]. However, this concept is misleading. In recent years, with mounting research in the area of various neurodisorders, the concept of neuroinflammation is evolving. Neuroinflammation was first described in context of Alzheimer’s Disease [3]. It encompassed the idea that immune responses of innate glial cells particularly the microglia in the central nervous system (CNS) regulate the pathogenesis and progression of various neurological disorders. The importance of peripheral immune cells in exacerbation of neuroinflammation was demonstrated in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Mice deficient in the chemokine receptor

CCR2 were resistant to EAE. Further analysis showed that in contrast to wild type mice, the CCR2-deficient mice failed to develop mononuclear cell inflammatory infiltrates in the CNS [3]. Neuroinflammation as we understand today involves both CNS resident cells and peripheral immune cells.

1.1.1. The Architecture of CNS

The CNS is organized into different compartments: the parenchyma, the meninges that ensheath the parenchyma, and the ventricles that contain the choroid plexus and cerebrospinal fluid (CSF) (**Figure 1**) [1]. In steady state conditions the CNS parenchyma does show the characteristics of an “immune priveleged” organ. It lacks lymphatic drainage, expresses low level of MHCII and it is sealed off from the surrounding meninges as well as the blood vessels that penetrate into it, by a structure called the glia limitans which is part of the blood brain barrier (BBB).

To go into detail, the meninges underlying either skull bone or the vertebral column are composed of three membranes; the outer dura, the inner arachnoid and the inner most pia which covers the parenchyma. The space between the two inner meninges, known as subarachnoid space, is bathed in cerebrospinal fluid and traversed by blood vessels that supply the CNS. These vessels are lined with highly-specialized endothelial cells that lack fenestrations and have complex tight junctions between them which restrict the paracellular route and force transcellular passage of various molecules and cells into the CNS [4]. Underlying these cells is the endothelial basement membrane composed of extracellular proteins such as laminins, collagen type IV, nidogens, and

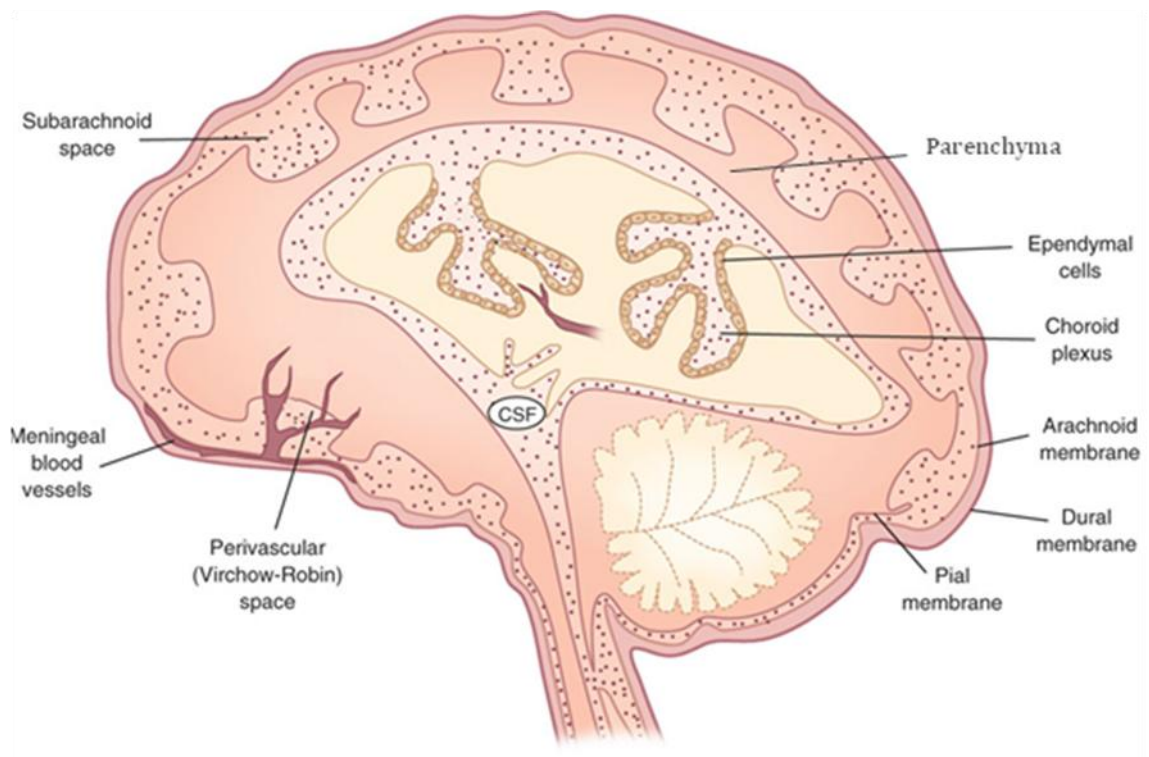


Figure 1. The architecture of the CNS.

The CNS is organized into different compartments: parenchyma, meninges and ventricles that contain the choroid plexus. Meninges underlying the skull ensheath the brain parenchyma. The cerebrospinal fluid (CSF) produced by the choroid plexus is continuous with CSF in the subarachnoid space between the pial and the arachnoid membrane. Adapted from Ousman & Kubes [2]

heparan sulfate proteoglycan perlecan [5]. Of particular note here are the isoforms of laminins present in the endothelial basement membrane, i.e. $\alpha 4$ and $\alpha 5$ isoforms [6]. The $\alpha 4$ isoforms are permissive for T cell diapedesis while $\alpha 5$ isoforms are inhibitory, providing further selectivity [7].

Some of these meningeal vessels penetrate the CNS parenchyma and are lined by the arachnoid and pia membranes for several millimeters from the site of parenchymal entry (**Figure 2**). They form the inner parenchymal basement membrane and are composed of

laminin isoform $\alpha 1$. The space created between the endothelial basement membrane and the inner parenchymal membrane is known as the perivascular space (also called Virchow–Robin space) and is directly continuous to the subarachnoid space. The inner membrane is surrounded by the outer parenchymal basement membrane which is composed of laminins $\alpha 2$ and heparan sulfate proteoglycan agrin [6, 8]. Beyond the parenchymal basement membrane lie the processes of astrocytes termed astrocyte endfeet which are tightly tethered to the parenchymal basement membrane forming an impenetrable wall collectively known as the glia limitans perivascularis. The meningeal vessels that end in the subarachnoid space don't have a defined perivascular space. Rather their perivascular space spills into the subarachnoid space and the entire subarachnoid space is sealed with glia limitans superficialis (the outer parenchymal basement membrane and the astrocytes endfeet) separating it from the parenchyma. The glia limitans covers the entire surface of the brain and spinal cord delineating the CNS parenchyma and forming part of the blood brain barrier. Therefore leukocytes entering from the vasculature have to cross through two barriers to reach the CNS parenchyma; one posed by the endothelial cells and the endothelial basement membrane and the second one posed by glia limitans.

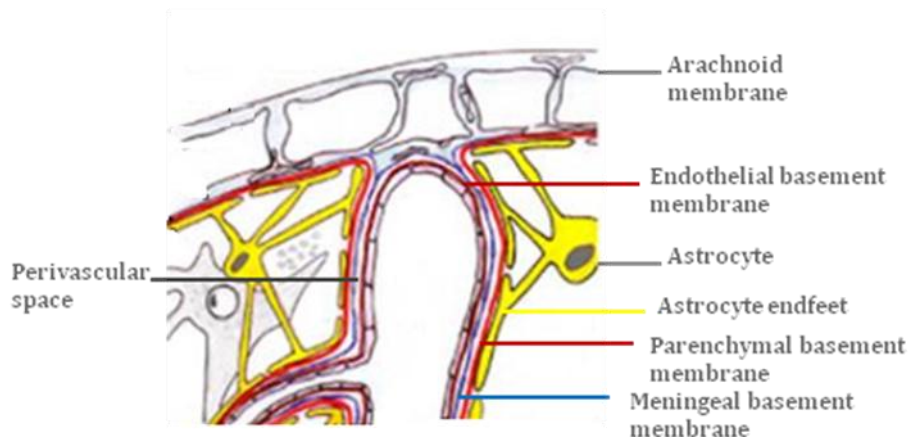


Figure 2. The ultrastructure of the CNS blood vessels.

The meningeal vessels that penetrate the CNS parenchyma are lined by the arachnoid and pia membranes for several millimeters from the site of parenchymal entry forming the inner parenchymal basement membrane. The space created between the endothelial basement membrane and the inner parenchymal membrane is known as the perivascular space and is directly continuous to the subarachnoid space. The inner membrane is surrounded by the outer parenchymal basement membrane. Beyond the parenchymal basement membrane lie the processes of astrocytes termed astrocyte endfeet which are tightly tethered to the parenchymal basement membrane forming an impenetrable wall collectively known as the glia limitans. Adapted from Engelhardt & Sorokin [8].

An easier site of entry for peripheral immune cells into the CNS (but not necessarily the parenchyma) is through the choroid plexus. The choroid plexus, a structure in the ventricles of the brain, is composed of a single layer of cuboidal epithelial cells (ependymal cells) and is the site of cerebrospinal fluid (CSF) production (**Figure 3**). It interacts with capillaries within the choroid plexus parenchyma which differ from those of the brain. The endothelial cells lining these capillaries have fenestrations and intercellular gaps and allow free movement of molecules and cells across them. Though the choroid plexus epithelial cells have been demonstrated to have tight junctions, they constitutively express the adhesion molecules ICAM-1 and VCAM-1 [9]. Also the fact that the CSF bathes the entire CNS (meninges surrounding the brain and the spinal cord)

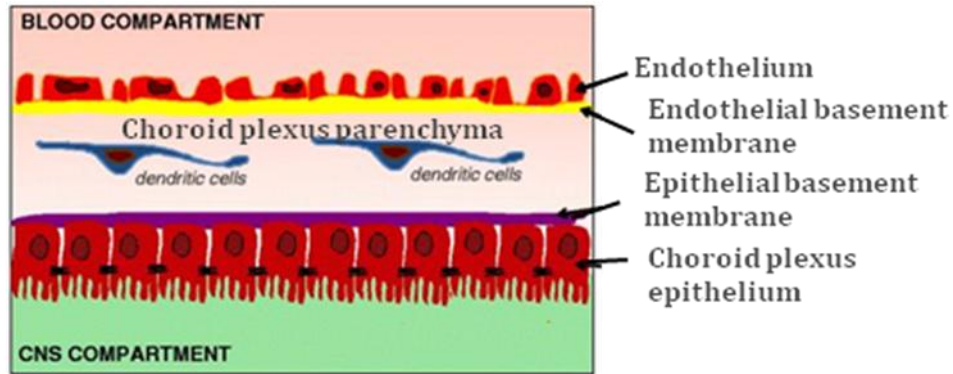


Figure 3. The detailed structure of the choroid plexus.

The fenestrations in the endothelium are more permissible to cell entry into the choroid plexus parenchyma. The epithelial cells that constitute the choroid plexus have tight junctions to limit cell entry. However, they also express adhesion molecules ICAM-1 and VCAM-1 that facilitate cell entry into the ventricles. Once cells enter the ventricles and the CSF compartment they are able to circulate throughout the brain. Adapted from Engelhardt & Sorokin [8].

allows leukocytes that enter into the choroid plexus to enter the meninges and circulate throughout the brain. All these factors make the choroid plexus more accessible to cells in the circulation.

1.1.2 Immune cells in the steady state CNS

T cells are present in the CNS in steady state condition. The CSF contains 1,000–3,000 cells per ml, of which 90% are T cells, very few of them are naïve. The predominant population has been shown to be $CD4^+/CD45RA^-/CD45RO^+/CD27^+/CD69^+$, recently activated central memory T cells expressing high levels of CCR7, CXCR3, and L-selectin in normal human post mortem brains [10]. This phenotype is consistent with surveillance, rather than effector function. They are comparable with the corresponding $CD4^+$ memory T cell populations in the blood, except for the expression of the recent-activation antigen

CD69. Very few T cells in blood express this marker. It is not known whether recent activation aids in T cell entry into the CSF of healthy individuals.

Though a healthy CNS parenchyma is devoid of peripheral mononuclear phagocytes, macrophages and dendritic cells (DC) are found in the CNS associated with either the meninges or the perivascular spaces located next to the parenchyma [11] [12]. Both meningeal and perivascular macrophages have a surface phenotype (CD11b+, CD45hi, F4/80+) that is typical of tissue macrophages. Perivascular macrophages can be closely associated with the endothelial basement membrane and express MHC II molecules. Similarly, dendritic cells have been detected in the choroid plexus and meninges. The meningeal/ choroid plexus DC were shown to resemble conventional splenic DC in terms of surface markers (CD11c+, MHCII+, CD115-), transcription factors (ID2, BATF3), and their functions of antigen presentation [13]. Similar to splenic DC, they originate from pre-DC and their development is fms-like tyrosine kinase 3 ligand (Flt3L) dependent [13]. Both macrophages and DC are believed to be involved in immune surveillance.

The only mononuclear phagocytes present in the CNS parenchyma behind the blood brain barrier are the microglia. They are characterized by the expression of high levels of integrin CD11b and intermediate expression of CD45, a member of tyrosine phosphatase family found on all leukocytes. They can range from 5 -12% of CNS cells and are evenly distributed throughout the parenchyma [14]. In mice, they arise from a yolk sac progenitor before hematopoiesis (embryonic day 7 (E7)), enter the brain after blood vessel formation at around E10.5 and thereafter are maintained by local proliferation [11]. They exhibit many processes that are rapidly moving and sampling the CNS

environment and express low levels of MHCII. They support neurogenesis, maintain synaptic plasticity and provide trophic support to neurons via various neurotrophins they secrete [2].

Microglia are capable of mounting an immune response but are quiescent in the healthy CNS. The CNS parenchyma houses the neurons and glial cells, oligodendrocytes, astrocytes, and microglia. Oligodendrocytes synthesize myelin that surrounds the axons and facilitates neurotransmission while the astrocytes situated around neurons maintain normal brain function by adjusting the CNS environment as required [15]. With their processes interacting with the blood vessels, they regulate energy reserves, maintain ionic homeostasis, clear excess neurotransmitters from synaptic zones. Neurons can keep microglia in a quiescent state by cell-cell contact via CD200–CD200 receptor (CD200R) and CD172A (also known as SIRP- α)–CD47 [11]. Similarly, neurons are prevented from aberrant sprouting via myelination by oligodendrocytes and release of inhibitory extracellular matrix molecules known as chondroitin sulphate proteoglycans by astrocytes [15]. Therefore, these inhibitory signals help to preserve the complex neural networks formed during development of CNS and keep the CNS quiescent.

1.1.3. Immune cells in inflamed CNS

During inflammation, this quiescent state is perturbed and can manifest as either acute or chronic neuroinflammation [16]. Acute inflammation is characterized by the immediate response to an injury and is mainly glial in nature. Resident microglia can get activated via engagement of various receptors which include 1) purine receptors when ATP is

dysregulated, 2) glutamate receptors, when there is excitotoxic damage, 3) Toll-like receptors (TLRs) that generally recognize pathogen associated pattern molecules during infection, and bind endogenous ligands like heat shock protein or high-mobility group protein B1 released from injured tissue, and 4) chemokine and cytokine receptors [2]. Chronic inflammation is a prolonged response to stimuli that are persistent and consist of resident glial cells as well as infiltrating peripheral cells. Activated microglia upregulate various receptors associated with innate immunity which include TLRs, scavenger receptors, and phagocytic complement receptors to clear damaged apoptotic neurons. They also upregulate MHCII and other costimulatory molecules associated with antigen presentation (CD80, CD86, CD40), proliferate, and secrete inflammatory cytokines, chemokines, and inflammatory mediators that can lead to a compromised blood brain barrier and subsequent recruitment as well as activation of peripheral immune cells.

Astrocytes also can contribute to neuroinflammation. Similar to microglia they also express many chemokine and cytokine receptors and they can upregulate a variety of pattern recognition receptors which include TLRs, scavenger receptors, and mannose receptors in response to activation [17]. Astrocytes proliferate and secrete various cytokines and chemokines. In response to neural injury, astrocytes migrate to the site of insult, produce high levels of chondroitin sulfate proteoglycans, and form a glial scar to wall off damaged neurons from healthy tissue. Neurons also have certain receptors for cytokines and chemokines and respond to these stimuli by secreting more of these inflammatory mediators.

Peripheral immune cell infiltration relies on expression of chemokines, adhesion molecules, and matrix metalloproteinases [5]. Chemokines are short peptides (small cytokines) that serve as chemoattractants for immune cells as well as stimuli for glial cells. They are classified on the basis of their structural properties, regarding the number and position of the conserved cysteine residues at the amino-terminal, into two major (CXC and CC) and two minor (C and CX3C) subfamilies [18]. They interact with specific G protein coupled receptors, chemokine receptors, which are differentially expressed on leukocytes. Therefore, the type of chemokine expressed in the CNS can determine the type of infiltrating peripheral leukocyte. The inflammatory response in the CNS relies on expression of chemokines which serve as chemoattractants for immune cells as well as stimuli for glial cells. Upregulation of adhesion molecules on the endothelial cells allow peripheral immune cells to adhere to endothelium and chemokine gradients direct them into the perivascular space. Once in the perivascular space, they require matrix metalloproteinases that help breach the glia limitans to facilitate their infiltration into the parenchyma.

Compromise of the blood brain barrier starts with the activation of the endothelial cells that line the blood vessels [19]. In response to inflammatory stimuli, they upregulate expression of various adhesion molecules which include selectins as well as ICAM-1 and VCAM-1. Activated leukocytes express integrins which interact with these adhesion molecules. For example, PSGL-1 interacts with selectins on endothelial cells allowing leukocytes to roll along the surface of the blood vessels. LFA-1 and VLA-4 interact with ICAM-1 and VCAM-1 allowing the leukocytes to tether firmly to the vessel. Endothelial

cells and perivascular cells provide a chemokine gradient directing the leukocytes to the CNS. Inflammatory mediators also cause changes in the tight junction proteins allowing the tethered leukocytes to extravasate through the endothelial layer. T cells were observed to crawl on the endothelium to find permissive sites with high levels of $\alpha 4$ laminin isoforms in the endothelial basement membrane for diapedesis [7]. Once the leukocytes cross this barrier, they enter the perivascular space and can remain there unless they acquire the ability to cross the glia limitans perivascularis. As described before, the composition of the parenchymal basement membrane is completely different from the endothelial basement membrane; it mainly consists of laminins $\alpha 1$ and $\alpha 2$ and heparan sulfate proteoglycan agrin, which form a tight interaction with the proteoglycan dystroglycan expressed in the astrocytes endfeet and requires matrix metalloproteinases to breach this barrier [6, 8].

With the compromise of the blood brain barrier and increased expression of various chemokines, there is an increase in the number of peripheral immune cells in the CNS. High levels of the chemokine CCL2 expressed in the CNS were reported to attract monocytes from the periphery. Ly6C^{hi}CD11b⁺ monocytes have been demonstrated to exit the bone marrow in a chemokine receptor 2 (CCR2)-dependent manner [20, 21]. In the CNS, these cells were observed to produce the cytokine GM-CSF and to differentiate into cells that resemble DC and mature macrophages [22]. They produced proinflammatory mediators such as TNF α and iNOS, and increased IL-2 and interferon- γ responses in T cells. Deletion of either monocyte specific CCR2 or systemic expression

of GM-CSF resulted in reduced number of Ly6C^{hi}CD11b⁺ in circulation as well as in the CNS and decreased neuroinflammation [20].

1.1.4 Mononuclear phagocytes and their generation

Both macrophages and DC belong to the mononuclear phagocyte system that includes monocytes and they originate from common monocyte/DC progenitors or MDP in bone marrow (**Figure 4**). Though they are both phagocytes, DC are specialized in antigen capture, processing, and presentation to T lymphocytes to initiate adaptive immunity and therefore are termed professional antigen presenting cells. Macrophages are better equipped to clear up apoptotic cells and tissue debris during development or inflammation and contribute to tissue remodeling [23].

Monocyte/ DC progenitors (MDP) in the bone marrow express receptors for M-CSF (CD115) and Flt3L (CD135) but are negative for integrins CD11b and CD11c [24]. They give rise to either 1) monocytes which acquire CD11b and lymphocyte antigen 6C (Ly6C^{hi}), maintain CD115, but lose the CD135 expression or 2) common DC progenitors which maintain both CD115 and CD135 expression and are negative for CD11b. Ly6C^{hi} monocytes enter into circulation, where they can reach up to 5% of blood cells and seed various tissues. They can give rise to macrophage in the presence of M-CSF. In its absence, they are thought to return to the bone marrow and give rise to Ly6C^{lo} monocytes. Common DC progenitors give rise to pre-DC that enter circulation and express CD11c and CD11b while maintaining expression of CD135 and CD115. These cells populate most tissues where they terminally differentiate into either conventional

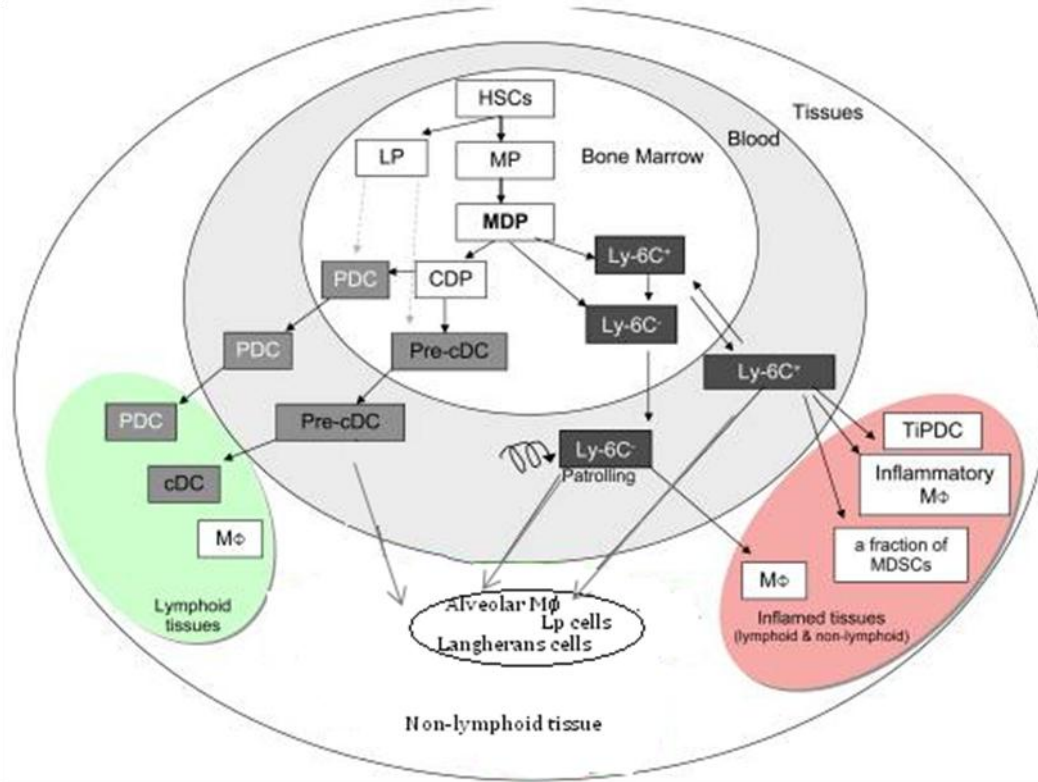


Figure 4. Differentiation of DC and macrophages in mice.

In the bone marrow, hematopoietic stem cells (HSC) give rise to either lymphoid (LP) or myeloid (MP) committed precursors. MP in turn give rise to monocyte/macrophages or DC precursors (MDP). MDP can become either Ly6C^{+/−} monocytes or common DC precursor (CDP). Two monocyte subsets, Ly-6C⁺ and Ly-6C[−] leave the bone marrow to enter the blood. CDP give rise to pre-classical dendritic cells (pre-cDC) and plasmacytoid dendritic cells (PDC). Pre-cDC circulate in blood and give rise to conventional DC in lymphoid tissue, and other DC in non-lymphoid tissues. Under homeostatic conditions, Ly-6C⁺ monocytes can become macrophages and Ly-6C[−] monocytes may contribute to alveolar macrophages (MΦ). During inflammation, Ly-6C⁺ monocytes give rise to monocyte-derived DCs, e.g. TNF and iNOS-producing dendritic cells (TipDC), and inflammatory macrophages. Langerhans cells can renew independently from the bone marrow. HSC can also leave their bone marrow niche and enter peripheral tissues, where they differentiate to myeloid cells during inflammation. It is unclear at this time if LP contribute significantly to PDC and cDCs (dashed arrow). Adapted from Geissmann et al [25].

DC (cDC), or plasmacytoid DC (pDC). Classical DC are CD11c⁺CD11b⁺CD135⁺ CD115[−]MHCII^{hi} and are highly efficient at antigen presentation, while plasmacytoid DC

are CD11c^{int}CD11b⁺CD135⁺MHCII^{lo} and B220⁺ (a marker for B cells) and are less efficient at antigen presentation, but function as a major source of type I interferons after activation. However, under inflammatory conditions, various studies have demonstrated that monocytes travel to inflammatory site, undergo diapedesis and translocate into the inflamed tissue to differentiate into monocyte-derived DC or macrophages depending on the local cytokine milieu.

For in vitro experiments, macrophages and DC are generated either from peripheral blood or bone marrow cells. In mice, since only 5% of blood cells are monocytes, and there are even fewer DC precursors, bone marrow cells are used to generate both types of cells. In the 1990s, a protocol for generating large quantities of pure DC was established [26, 27]. It consists of culturing of fresh whole bone marrow cells in the presence of GM-CSF with 10% heat-inactivated fetal bovine serum (FBS) supplemented medium. After 7-8 days of continuous culture, non-adherent cells develop into immature DC [27]. If the culture period is extended to 12 days, the purity and yield of DC is further increased, but with various degrees of maturation [26]. GM-CSF containing medium has to be supplemented every 3-4 days to obtain high numbers of cells. On the other hand, macrophage differentiation relies on M-CSF. The same protocol as DC differentiation is used with 10 ng/mL of M-CSF substituted in place of GM-CSF. In GM-CSF culture conditions non-adherent cells give rise to DC that express the classic DC marker CD11c⁺, while in M-CSF culture conditions adherent cells (which are collected by trypsinization) give rise to macrophages that express the classic macrophage markers CD11b⁺F4/80⁺. Though these cells are not derived from monocytes, they resemble monocyte derived macrophages and

DC in inflammatory conditions. Study of macrophages observed at injury site in spinal cord injury revealed these cells resembled bone marrow derived macrophages and not other tissue macrophages [28]. Therefore, for in vitro studies, bone marrow derived DC (BMDC) and bone marrow derived macrophages (BMMΦ) serve as a great tool to study the inflammatory response.

1.2 Spinal cord injury

1.2.1. Pathophysiology

Spinal cord injury is a response to mechanical force directed to the cord that results in compression or contusion of the cord. In the early stage, defined as primary injury, there is rupturing of the vasculature and cell death at the impact site. Dysregulation of the surrounding cells and tissue causes tissue damage to expand both in rostral and caudal directions from the injury “epicenter” resulting in secondary injury. Factors that contribute to secondary injury are ionic imbalance, necrotic and apoptotic cell death, generation of free radicals, lipid peroxidation products and alterations in microvascular permeability [29]. Secondary injury starts out as acute neuroinflammation. However, it is further exacerbated by activation of immune cells and their recruitment and can become chronic.

The earliest cell populations to get activated are the CNS resident cells which include neurons, microglia and astrocytes (**Figure 5**). Activation occurs as early as 5 minutes

post injury as observed by expression of proinflammatory cytokines $\text{TNF}\alpha$ and $\text{IL-1}\beta$ [30]. The primary injury results in release of various inflammatory mediators from necrotic cells, which include activators of transcription factor nuclear Factor- κB (NF κB) such as reactive oxygen species and excitotoxic glutamate as well as endogenous toll-like receptor (TLR) activators such as heat shock proteins and high-mobility group box 1 (HMGB1) which activate microglia as well as astrocytes via TLR engagement ([31-33]). Activated microglia change shape, retract their processes, and appear more like macrophages. They upregulate surface antigens and secrete cytokines and chemokines to recruit peripheral cells. Activated microglia are observed by the first day of injury, increase in numbers by 7 days, and plateau between 2-4 weeks [34]. Similar to microglia, activated astrocytes proliferate and migrate to the site of injury. They secrete various cytokines and chemokines as well as components of extracellular matrix which contribute to the formation of a glial scar. The glial scar secludes the injury site from healthy tissue to prevent further tissue damage [35].

Neutrophils are the first peripheral cells to be recruited to the injury site. They accumulate within a few hours after spinal cord injury, reaching a peak at 1-3 days post-injury followed by a second peak several weeks later [34]. They respond to the chemokines CXCL1, 2, and 3 via receptors CXCR1 and CXCR2.

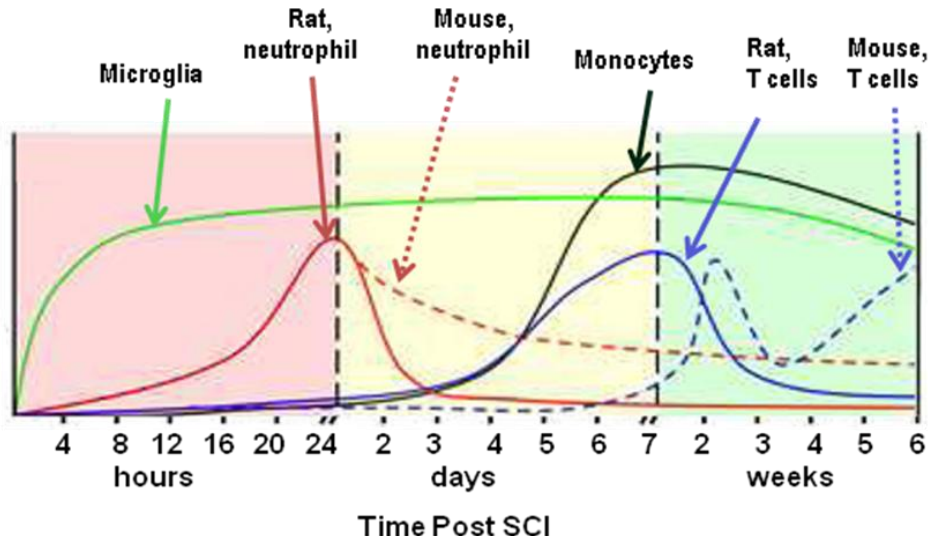


Figure 5. Temporal correlation of immune cell activation and infiltration into injured spinal cord after SCI.

Temporal correlation between inflammatory cascades, secondary neurodegenerative events and functional recovery in SCI rodents. A) Anatomical and functional outcomes, including de- and remyelination, axonal sprouting/plasticity and locomotor recovery. B) Resident CNS microglia are activated immediately post SCI in both mice and rat. The first wave of peripheral cells that infiltrate into the cord are neutrophils, followed by monocytes/ macrophages, and finally T cells. Dashed lines are used to depict data from SCI mice in cases where they differ from SCI rats which are depicted by solid lines. Adapted from Donnelly and Popovich [36].

Neutrophils release reactive oxygen species (ROS), inflammatory cytokines, chemokines, and a variety of proteases. Though neutrophils normally function to sterilize and rid the wounded area of bacterial infection, in spinal cord injury they can exacerbate secondary tissue damage by enhancing apoptosis of neuronal and glial cells and by breaking down the blood spinal cord barrier. Inhibition of the neutrophil derived protease called elastase was shown to reduce neurological damage in spinal cord injury [37].

The next wave of cells to infiltrate the injured spinal cord are the monocytes/ macrophages. Monocytes that express chemokine receptor CCR2 start to infiltrate in

small numbers within 24 hours, in response to CCL-2 expressed in the CNS, and develop into macrophages upon entering the injured tissue [21]. They reach maximal levels at 7 days, followed by a subtle decrease over the next 2–5 weeks [36, 38]. The role of macrophages in spinal cord injury is a subject of great debate [39]. Macrophages are needed for the removal of apoptotic and necrotic cells as well as myelin debris that may block axonal regeneration. However, activated macrophages (and microglia) can also contribute to secondary injury by production of toxic molecules such as nitric oxide and ROS and numerous proinflammatory cytokines (i.e. TNF α , IL-1 β) which exacerbate inflammation. They are also a source of various proteases including metalloproteinases that help break down the blood-spinal cord barrier and facilitate infiltration of peripheral immune cells [21].

T-lymphocytes start to increase, in parallel with the activation of microglia and macrophages. They are evident at 2 weeks post injury and continue to increase in numbers for weeks and months after injury [36, 38]. Larger proportions of these cells have been characterized to be CD4 T cells. Though the role of T cells is highly controversial in spinal cord injury, there is a potential for an autoimmune response through their ability to recognize specific auto antigens, such as myelin basic protein, and their proliferation in response to those antigens [40, 41]. Also activated T cells can target and kill cells and they produce cytokines such as IFN- γ that activate macrophages and microglia, sustaining inflammation.

In spinal cord injury, the initial neuronal and glial cell death due to the primary insult is irreversible. However, the secondary injury resulting from exacerbated inflammatory

response leads to more apoptotic cell death which can occur for weeks after injury and at a great distance from the point of mechanical impact. Astroglial scar, though formed by astrocytes as a neuroprotective response, ends up by being detrimental. The extracellular components of the scar form a physical barrier to axon regeneration at the injury site [35]. Apoptosis of oligodendrocytes results in impairment of remyelination as well as ultimate loss of neurological functions. Thus, spinal cord injury which starts out as acute neuroinflammation turns into chronic neuroinflammation.

1.2.2 Treatment for SCI

Spinal cord injury has no effective treatment for long term recovery. Corticosteroids have been used for neurotrauma in general. A soluble form of corticosteroid Methylprednisolone Sodium Succinate is standard treatment, though its benefits are highly controversial. North American Spinal Cord Injury Study II (NASCIS II) showed that treatment within 8 hours of injury benefited patients neurologically but NASCIS III showed that treatment within 3-8 hours only resulted in improved neurological function at 6 weeks and 6 months but not at 1 year [42]. In addition to its poor efficacy, this treatment led to significant increase in wound infection as well as increased rates of sepsis, pulmonary embolism, delayed wound healing, and death [43]. Therefore, an effective treatment is badly needed to treat spinal cord injury. Since modulation of cellular responses immediately after injury can have neuroprotective effect and improve the outcome, there is ongoing research targeting some of the factors involved in secondary damage, such as excitotoxicity, inflammation, and cell death (apoptosis). A treatment that would attenuate the early exacerbation of inflammatory response and

prevent the spread of post-injury damage while preserving surrounding tissue would be ideal.

1.2.3. Animal model

Rodent models of spinal cord injury, mainly mice and rats, are used extensively. Three types of injury models are commonly used in rodents: transection, compression, and contusion [44]. Transection involves opening the dura and cutting some or all of the spinal cord with a sharp instrument to cause axotomy. Compression injuries are induced by squeezing the spinal cord with a modified aneurysm clip or forceps. Contusion injuries are induced by hitting the exposed spinal cord with a selected force delivered using an electromagnetically controlled impactor device. The device can be programmed for speed of the impactor and displacement of the cord. A contusion injury model is viewed as the most appropriate to mimic human spinal cord injury which is rapid and nonpenetrating and results in vertebral dislocation or burst fracture injuries. Mice are a popular model since many transgenic and gene knockout mice are available for studying mechanisms of secondary injury. Of all mice strains, C57BL/6 mice show a robust inflammatory response to spinal cord injury as observed by induction of inflammatory cytokines and chemokines and the magnitude of spinal cord inflammation correlates with lesion size and impaired functional recovery [45].

However, the pathologic response to SCI in mice is different from other mammals. In rats, as in humans, there is a formation of a fluid-filled cystic cavity at the site of contusion which is surrounded by a rim of anatomically preserved white matter. In contrast, the mouse spinal cord becomes filled with dense fibrous connective tissue

matrix [38]. The contribution of inflammatory cells in these two species is relatively similar. The reactions of microglia and macrophages are comparable, with a peak of invasion and activation occurring at approximately 7 days. There is a difference in the timing of T-cell invasion between these two species. In rats the peak of T-cell invasion occurs between 3 and 7 days, whereas in mice T-cell infiltration starts much later (14 days), but continues over the next several weeks similar to humans. In human post mortem spinal cord injury tissue there were very few T cells at early timepoints but increased from weeks to months, and T cells were found in perivascular spaces, in regions of tissue damage, including areas at the margins of cystic cavities, usually were randomly distributed among macrophages [46]. Despite these differences, the murine model of spinal cord injury is quite relevant in studying the mechanisms and perhaps therapeutic targets in secondary injury.

1.3. Experimental Autoimmune Encephalomyelitis

1.3.1. Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the human central nervous system (CNS). It is characterized by the presence of focal areas of inflammatory-mediated demyelination of the brain and spinal cord white matter. Brain-imaging studies have correlated breakdown of the blood brain barrier and the presence of focal areas of demyelination with the neurological disability in MS patients. Focal inflammatory demyelinating lesions are characterized by perivascular infiltrates that predominantly

contain clonally expanded T cells, monocytes, rare B cells as well as large numbers of macrophages containing myelin debris [47]. Demyelination leads to axonal degeneration which is manifested in MS patients as various neurological symptoms including optic neuritis, sensory dysfunction and muscle weakness.

Though the etiology is unknown, involvement of the immune system in MS pathology is indisputable. Inflammatory cytokines that are present in MS lesions include innate factors such as interleukin (IL)-1 β and IL-6, as well as more specialized T cell specific cytokines such as interferon (IFN)- γ , IL-23 and IL-17 among others [47]. Chemokines including CCL2, CCL3, CCL4, CCL5, CXCL10, CXCL12 and CXCL13 are also found [18]. Other inflammatory mediators like reactive oxygen and nitrogen species are also present [48]. Increased susceptibility to MS has been found to be genetically linked to the major histocompatibility complex (MHC) class II [47]. Interestingly, the latest study on susceptibility to MS identified 52 MS risk loci with genomewide significance and the vast majority of these loci were found to contain genes encoding immune system-related molecules, particularly those involved in T cell activation [49]. This finding strongly supports the hypothesis that MS is primarily an immune-mediated disease.

Therapeutics targeting inflammation have been more successful in long term care but are by no means a cure. Targets have focused on pathways involved in either T cell activation or T cell trafficking. The most widely used therapeutics are Interferon beta (IFN β) and glatiramer acetate (GA) which appear to act on immune cell activation [50]. IFN β , a cytokine, has been demonstrated to reduce antigen presentation and pro-inflammatory cytokine production in antigen presenting cells and inhibit T cell

proliferation and differentiation. GA, composed of four amino acids found in myelin basic protein, is thought to also act as a sort of decoy, diverting an autoimmune response against myelin [50]. In the murine model of MS, GA shifted T cells from pro-inflammatory Th1 cells to anti-inflammatory Th2 cells that suppress the inflammatory response. Other therapeutics that are used in treatment are agents that target migration of immune cells. Natalizumab is a monoclonal antibody against the adhesion molecule VLA4 expressed on activated T cells which interacts with the adhesion molecule VCAM1 expressed on endothelial cells lining the cerebral blood vessels. Fingolimod is a modulator of a G protein called sphingosine-1-phosphate receptor 1 (S1P1R1). It blocks the interaction between sphingosine-1 phosphate (S1P) with its receptor (S1P1R1) expressed on lymphocytes required for their egress from lymphoid organs (such as lymph nodes and spleen) into the lymphatic vessels. Thus it interferes with the trafficking of activated T cells from the periphery to the CNS.

1.3.2. Animal model – experimental autoimmune encephalomyelitis (EAE)

Animal models of MS, known as experimental autoimmune encephalomyelitis (EAE), have been a useful tool in studying the mechanism as well as testing various therapeutics. MS and EAE share many similarities. Both are characterized by a massive infiltration of circulating immune cells into the CNS, resulting in neuroinflammation. The resulting demyelination correlates with the onset of the clinical manifestations in both diseases and they share histopathological similarities.

EAE can be induced by direct immunization with myelin peptides such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP) in susceptible strains of mice [51]. As seen in MS, the MHCII molecule dictates the susceptibility due to its varied binding affinities to different peptides. For example, SJL (H-2^s) display a relapsing-remitting course of paralysis in response to PLP peptide representing residues 139-151, while C57BL/6 (H-2^b) react to MOG peptide 35-55 and show a chronic progressive clinical course. Most studies are presently done using C57BL/6 mice, which are immunized subcutaneously with MOG peptide, emulsified in Freund's adjuvant supplemented with additional *Mycobacterium tuberculosis* extract to activate the immune system. Mice are also injected with pertussis toxin on the day of immunization and 2 days thereafter to compromise the blood brain barrier via upregulation of various matrix metalloproteinases [52].

Typically, classic symptoms are observed within two weeks of disease induction and manifest in mice as ascending paralysis, starting at the distal end of the tail and developing rostrally to the whole tail, the hindlimbs and the forelimbs [53-55]. Histopathological analyses showed the inflammatory cells to be located in the meninges of the brain and the parenchyma of spinal cord. However, some mice develop atypical EAE characterized by forelimb paralysis in the absence of hind limb or tail paralysis. In this form of disease, the inflammatory cells were found to infiltrate into the parenchyma of the brain. Strommes et al showed that the type of activated CD4 T cells determined the outcome. If the ratio of IL-17 producing Th17 cells to IFN- γ producing Th1 cells was greater than 1, then inflammatory cells infiltrated into the brain parenchyma. If the ratio

was less than 1, then the cells stayed in the meninges in the brain and only infiltrated into the parenchyma of the spinal cord [56]. Both types of symptoms are observed in MS though the atypical type of symptoms is more prevalent.

1.3.3. Pathophysiology of experimental autoimmune encephalomyelitis (EAE)

The sequence of events that have been demonstrated to occur upon immunization with MOG is as follows [57]: antigen presenting cells (APC) such as dendritic cells are activated in the periphery and migrate to secondary lymphoid organs where they present MOG antigen to T cells, leading to activation and clonal expansion of MOG specific CD4⁺T cells. Activated T cells migrate to the circulation. Due to the inclusion of adjuvant and pertussis toxin, the endothelium lining the vasculature in the CNS is activated and expresses adhesion molecules as well as chemokines, which provide cues and facilitate the entry of circulating CD4⁺T cells to the meninges or the perivascular space of the CNS. Here they are reactivated by local or infiltrating APCs, which results not only in the release of pro-inflammatory and cytotoxic mediators but also of proteases that digest the glia limitans components leading to entry of the immune cells into the CNS parenchyma resulting in tissue damage and demyelination. Finally, the inflammation induces apoptosis of oligodendrocytes which prevents remyelination and manifests as a chronic paralysis.

CD4 T cells garnered most of the attention when Pettinelli et al demonstrated that adoptive transfer of myelin antigen specific CD4⁺ T cells led to induction of EAE [58]. However, myeloid cells also play a crucial role. In active MS lesion, macrophages

outnumber T cells by 10:1 [59]. Their importance in disease initiation has been demonstrated in various experiments. When encephalitogenic T cells were transferred into macrophage-depleted mice, they failed to induce EAE. Although T cells trafficked to the CNS, they remained in the perivascular space [60]. CD11c⁺ DC associated with the meninges and CNS blood vessels have been demonstrated to be crucial in the reactivation of T cells. When encephalitogenic T cells were transferred into naïve mice lacking MHCII, there was no EAE development. When MHCII was present on CNS parenchymal cells, mainly microglia, but not on peripheral APC, there still was no induction of EAE. However restoration of MHCII expression on just CD11c⁺ DC allowed development of the disease. These cells colocalized with T cells in the meninges and perivascular space, indicating that peripheral DC are required for the reactivation process in the CNS to induce clinical symptoms [61]. Interestingly, clinical signs of EAE only start following the penetration of inflammatory cells across the glia limitans and entry into CNS parenchyma [62]. These results indicate that peripherally derived antigen presenting cells, especially CD11c⁺ DC, are required to reactivate T cells in the CNS, and possibly to provide matrix metalloproteinases to breach the glia limitans facilitating T cell entry into the parenchyma.

1.4. Matrix Metalloproteinase-9

1.4.1. Structure of MMP-9

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that are secreted by various cell types and are involved in remodeling extracellular matrix and cell-matrix interactions. Matrix metalloproteinase-9 (MMP-9) belongs to the gelatinase subfamily of the MMPs along with MMP-2 and its main substrate is gelatin (a denatured collagen) [63]. It also cleaves type IV collagen, a major component of the basement membranes lining blood vessels, which allows leukocytes to enter and leave the blood circulations.

MMP-9 is composed of a 1) N terminal Signal peptide domain that directs it to the endoplasmic reticulum, 2) pro-domain that maintains it in an inactive form, 3) zinc containing catalytic domain that contains the catalytic machinery, 4) C terminal hemopexin-like domain that is involved in interactions with substrate as well inhibitors known as TIMPs, and a 5) hinge region that connects the catalytic domain to the hemopexin like domain. MMPs in general are kept inactive by the interaction of a cysteine in the pro-domain with the zinc in the catalytic domain. Removal of this interaction, called the “cystein switch” results in their activation. The catalytic domains in gelatinases MMP-2 and 9 contain a fibronectin-like gelatin binding domain and are responsible for binding to gelatin, laminin and collagens type IV and V [64].

1.4.2. Regulation of MMP-9

MMP-9 is produced by many cell types, including peripheral blood mononuclear cells [65]. It was first identified in neutrophils and later, in rabbit alveolar macrophages activated with complete Freund's adjuvant [63, 66]. Its production is regulated at both transcriptional and post translational level. MMP-9 expression is induced by diverse signals such as cytokines, chemokines, eicosanoids and peptidoglycans in various immune cells resulting in increased expression during inflammation [63]. MMP-9 is highly upregulated in monocytes in response to proinflammatory cytokines such as TNF α , IL-1 β , and GM-CSF and chemokines CCL-2 and CCL-5 [67-69]. Besides cytokines and chemokines, arachidonic acid metabolite prostaglandin E2 also contributes to increase MMP-9 in these cells [70]. Interestingly, differentiation of monocytes into macrophages results in upregulation of MMP-9 expression which can be further increased by inflammatory cytokines, TNF α and IL-1 β and PGE2 [71-73]. Similar observations have been made in dendritic cells which upregulate MMP-9 in response to TNF α /IL-1 β , IL-6, CCL5 and PGE2 [74, 75]. Neutrophils, in contrast to other cell types, express MMP-9 during their maturation and store it within granules. Therefore, stimulation of mature neutrophils does not result in up-regulation of MMP-9 at gene level as seen in myeloid cells, but induces release of the enzyme by degranulation [64].

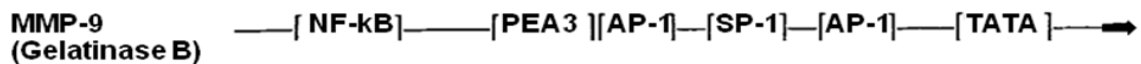


Figure 6. MMP-9 promoter.

The minimal MMP-9 promoter contains two AP-1 binding sites along with an Sp-1, an NF κ B, and an Ets (PEA3) binding site. Adapted from Chun He [76].

Analysis of MMP-9 promoter demonstrates a presence of a TATA-like consensus, Ap-1 binding sites, an Sp-1 binding site, an NFκB-like sequence and PEA3-like sequence (Ets binding site) (**Figure 6**) [76]. AP-1 complexes are known to play a critical role in the regulation of MMP-9, but not in that for MMP-2 [77]. Elimination of either of the two sites results in complete abolishment of MMP-9 expression [78]. Cytokines like TNFα have been demonstrated to act mainly through increased transcriptional activity at the NFκB site while PGE2 acts through AP-1 sites and complement C5a acts through both [79-81]. Inhibitors of MMP-9 can act through inhibition at these sites. Interferons type I and type II have been reported to inhibit MMP-9 induction in macrophage and DC in a STAT-1 dependent manner [82-84] and this is one of the methods by which IFN-β therapy appears to work in MS [85, 86].

Activity of MMP-9 can also be regulated in a post translational manner. MMP-9 is secreted in an inactive pro-form. The “cystein switch” catalyzed by other proteases results in active MMP-9. Active MMP-9 can be inhibited by tissue inhibitor of metalloproteinase 1 (TIMP-1), which binds to the catalytic site of MMP-9 forming tight 1:1 stoichiometric noncovalent complexes [63, 87, 88].

1.4.3. MMP-9 in neuroinflammation

Though MMP-9 has physiological roles, such as remodeling of endometrial tissue during female reproductive cycle, endochondral ossification, migration of immune cells, and tissue regeneration [89, 90], it has been reported to be mostly detrimental in neuroinflammation. Detrimental effects are attributed to its ability along with MMP-2

and MMP-3 to degrade various extracellular proteins in the blood brain barrier, which includes the tight-junction proteins, occludin and claudin-5, which form the endothelial barrier, and the basement membrane components fibronectin, laminin, and heparan sulfate [91]. Also its ability to cleave various cytokines like IL-1 β , IL8 to their active form, leads to promotion of inflammation [89]. Most of these data were obtained from in vitro incubation of potential substrate with proteases and may not hold true in vivo. Also, the fact that MMPs can share substrates makes it hard to study the role of each MMP. Therefore, MMP-9 deficient mice have provided a better tool to elucidate the role of this enzyme in CNS diseases such as spinal cord injury and EAE.

In spinal cord injury, even though there is rupture of vasculature due to mechanical injury, the blood-spinal cord barrier is disrupted rostral and caudal to the epicenter. Barrier leakage to luciferase protein at the epicenter of injury was observed to reach peak within 24 hours of injury, while the cord area rostral and caudal showed an increase in luciferase protein 3-7 days post injury [92]. The peak staining observed within 24 hours correlated to an increased MMP-9 expression around the epicenter and the source of this enzyme was shown to be microglia/macrophages, neutrophils and astrocytes [93]. Induction of spinal cord injury in mice that were either deficient for MMP-9 or were treated with MMP-9 inhibitor (within 3 days), resulted in decreased numbers of neutrophils and long term improvement in recovery of locomotor function [93]. After 3 days, the source of MMP-9 at the site of injury was F4/80+ macrophages derived from bone marrow monocytes and resembled bone marrow derived macrophages [21]. MMP-9 deficient mice showed reduced numbers of macrophage infiltration in SCI. Treatment with MMP-9 inhibitor 4-6 days post injury resulted in F4/80+ cells that were mostly

clustered near the site of impact whereas in untreated cords these cells were distributed at as well as away from the site of impact [21]. In astrocytes, MMP-9 facilitated the migration of these cells to the injury site. MMP-9 deficient mice had decreased migration and reduced astroglia scarring. In contrast, MMP-2 deficient mice displayed more extensive astroglial scarring and poorer recovery due to a compensatory increase in MMP-9 expression [94].

MMP-9 has also been associated with multiple sclerosis. Cerebrospinal fluid levels of MMP-9 increased in patients going through relapses while they decreased during remission [95]. Similarly, in EAE MMP-9 has been observed to be upregulated in the CNS along with a few other MMPs [96] and myeloid cells have been shown to be source of MMP-9 in EAE CNS tissue [62]. MMP inhibitors have been shown to reverse the EAE symptoms [97]. However, similar to the observation in spinal cord injury, MMP-2 deficient mice exhibited an early onset and more severe EAE due to a compensatory increase in MMP-9 expression [98]. In contrast, double MMP-9/2 and young MMP-9 deficient mice were resistant to EAE [62, 99]. Interestingly, though there was no difference in the migration of leukocytes into the CNS, the cells were retained in the perivascular space in the absence of MMP-9/2. In EAE mice, it was shown that both MMP-9/2 cleave β -dystroglycan, a cell surface receptor that anchors the astrocyte endfeet to the laminins of the parenchymal basement membrane [62]. T lymphocyte penetration of the parenchymal basement membrane correlated with sites of focal matrix metalloproteinase (MMP) activity [8]. MMP-9, along with other MMPs, has also been shown to cleave myelin compounds such as myelin basic protein (MBP) to generate immunogenic peptides that can induce EAE [100], though the implications of this effect

are not known. Although both MMP-2 and -9 are produced by antigen-presenting cells and act on similar substrates, knock out studies indicate that MMP-9 appears to play the crucial role in neuroinflammation. Targeting MMP-9 in neuroinflammatory disorders has therapeutic potential.

1.5. Cannabinoids

1.5.1. Cannabinoids and Receptors

Cannabis plant has been used in controlling pain and inflammation in central and south Asia since ancient times. However, its therapeutic value was only reported to the western world in 1830s by William O'Shaughnessy, a British physician working in India [101]. A century later, the major psychoactive ingredient of cannabis plant, delta-9-tetrahydrocannabinol (THC), was identified. Interest in this system was vastly increased with the identification of the cannabinoid type 1 (CB1) receptor in rat brain as the binding site of THC [102] and its subsequent cloning [103]. The second cannabinoid (CB2) receptor was identified and cloned in immune cells [104]. With the identification and characterization of two endocannabinoid ligands arachidonylethanolamide (anandamide) [105] and 2-arachidonoylglycerol (2-AG) [106, 107], there was increased interest in modulation of endocannabinoid system in inflammation.

The endocannabinoid system consists of cannabinoid receptors and their ligands, the endocannabinoids. The two classical cannabinoid receptors, CB1R and CB2R, have different distribution and function [101, 108, 109]. CB1R is abundantly expressed on

CNS and peripheral neurons and involved in various neural functions including analgesia, feeding behavior, modulation of synaptic transmission, regulation of neuronal excitotoxicity and glia-neuron interactions [110]. In contrast, CB2R are mostly expressed on immune cells and involved in immunoregulation [101].

1.5.2. Cannabinoids as anti-inflammatory agents

Recently, there has been an increased interest in CB2R as an anti-inflammatory agent. CB2R is highly expressed in myeloid derived cells, which include monocytes, macrophages, dendritic cells as well as T and B lymphocytes [101]. It acts via inhibition of adenylate cyclase and cyclic AMP [106, 111-113]. Administration of CB2R selective agonists in models of inflammatory and autoimmune diseases such as systemic sclerosis, experimental autoimmune uveoretinitis, inflammatory bowel diseases, and experimental autoimmune encephalomyelitis (EAE) resulted in significant attenuation of clinical disease [114-119]. In addition, CB2R agonists have been reported to have a beneficial effect in models of CNS injury such as cerebral infarction and spinal cord injury [120-124].

In addition to attenuation of clinical disease, treatment with selective CB2R agonists reduced the levels of inflammatory mediators in various experimental models [114, 124, 125]. A possible mechanism is the direct action of CB2R agonists on the immune cells responsible for the induction and maintenance of the inflammatory environment. Indeed, *in vitro* studies indicated that CB2R signaling inhibited the production of proinflammatory cytokines such as TNF α , IL-6, IL-2, and IFN γ by activated microglia

and T cells, and reduced the capacity of macrophages and dendritic cells to stimulate CD4+ T cells [126-129].

1.5.3. Effect of Cannabinoids in immune cell migration

The anti-inflammatory role of CB2R signaling may also be due to a reduction in immune cell migration. Maresz et al showed that the number of encephalitogenic T cells in the CNS was significantly increased in CB2R-deficient EAE mice, suggesting that endocannabinoids play a role in controlling T cell migration [129]. Administration of exogenous CB2R agonists has been also reported to reduce migration of inflammatory cells in various disease models. Decreased rolling and adhesion of leukocytes to brain microvessels have been reported in models of EAE [118], middle cerebral artery occlusion and reperfusion (MCAO/R) [121], and in LPS-induced encephalitis [130]. In the EAE model, CB2R activation was shown to reduce the total number of peripheral CD34+ hematopoietic cells in the CNS [131], while in the MCAO/R model of stroke, CB2R agonists reduced the number of myeloperoxidase positive neutrophils in the ischemic brain [123]. There are also several in vitro reports showing the ability of CB2R agonists to modulate migration of various types of immune cells. CB2R activation reduced chemotaxis of human T cells to CXCL12 [132], human monocytes to CCL2/CCL3 [133], murine macrophages to CCL5 [134], and LPS activated murine microglial cells to ADP [135].

1.5.4. Cannabinoids and MMP-9

There have been some reports of regulation of MMP-9 and MMP-2 by cannabinoids and both transcriptional and post translational mechanisms have been involved. In a model of atherosclerosis, administration of a CB2R agonist was shown to reduce MMP-9 levels in neutrophils via inhibition of TNF α induced ERK phosphorylation [136]. A few studies have also reported the inhibitory effects of CB2R agonists on MMP-2 in various tumors [137-139]. Interestingly, a recent study has also shown that cannabinoids inhibit cancer cell invasion via upregulation of TIMP-1 in a CB1 and CB2 receptor dependent manner [140]. However, there are no reports on effects of CB2R agonist on MMP-9 induction in macrophages or DC during inflammation.

As mentioned before, myeloid cells are major producers of MMP-9 in inflammatory condition. Among immune cells, DC play a two-pronged role in EAE. Their initial activity requires migration to the nearest lymph node where they act as antigen-presenting cells and activate cognate naïve T cells. The second activity requires migration to the CNS to reactivate encephalitogenic T cells and to facilitate immune cell infiltration into the CNS parenchyma [141]. Both activities require CCR7 and MMP-9 expression by DC. In this study we evaluated the effects of CB2R agonist treatment in a murine model of acute inflammation, i.e. spinal cord injury, and in a model of chronic inflammation, i.e. EAE and investigated the effects of CB2R signaling on MMP-9 production in innate immune cells, i.e. bone-marrow derived DC (BMDC), bone marrow derived macrophages (BMM Φ), and primary neonatal microglia, as outlined in the specific aims below.

1.6 Specific Aims

Previous studies from our laboratories demonstrated that administration of cannabinoid type 2 receptor agonist attenuated disease score and improved recovery in three murine models of neuroinflammation: spinal cord injury (SCI), experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, and middle cerebral artery occlusion-reperfusion (MCAO/R) injury, a model of stroke [119, 122]. In the EAE and MCAO/R models, administration of CB2R agonists resulted in decreased numbers of leukocytes rolling and adhering to meningeal vessels as observed through a cranial window [119]. The goal of the current investigation is to evaluate the molecular mechanisms through which cannabinoid-2 receptor (CB2R) selective agonists result in beneficial effects in SCI (acute neuroinflammation) and EAE (chronic neuroinflammation) focusing on various factors involved in the migration of peripheral cells. The proposed studies will be conducted at both molecular/cellular and whole animal level. We propose that CB2 receptor activation by selective CB2R agonists results in: 1. changes in the expression of chemokine/chemokine receptors, adhesion molecules and matrix metalloproteinases in vivo SCI and EAE models; 2. subsequent reduction of peripheral immune cells infiltration into the central nervous system (CNS); and 3. the effects are mediated through inhibition of cyclic AMP induction in vitro. The following specific aims are addressed:

1. To evaluate the effects of CB2R agonist treatment on inflammation in SCI. Infiltration of peripheral cells into the injured cord and expression of chemotactic signaling molecules (chemokines and cytokines) will be evaluated via flow cytometric analysis and real-time PCR and the effects of CB2R agonist treatment will be determined. We expect that the treatment with CB2R agonist will prevent chemokine upregulation resulting in a decrease in glial cell activation as well as peripheral immune cell infiltration.
2. To evaluate how the CB2R agonist treatment in EAE may result in decreased leukocyte rolling and adhesion to brain microvasculature and lower disease scores. The effects of CB2R agonist treatment on peripheral cell infiltration into the central nervous system and expression of molecules that facilitate leukocyte migration which includes adhesion molecules and matrix metalloproteinases 2 and 9 (MMP-2/9) will be evaluated in the EAE model via flow cytometric analysis and real-time PCR. We expect that CB2R agonist treatment will result in reduced expression of MMP-9 and VCAM-1 and lower number of infiltrating immune cells into the CNS.
3. To determine whether CB2R agonists have direct effects on leukocyte migration and the molecular mechanisms involved.
 - a. The effects of CB2R agonists on MMP-9 production by myeloid cells and the subsequent MMP-9 dependent migration will be evaluated via in vitro and in vivo migration assays. We expect that CB2R agonist treatment will result in decreased level of MMP-9 and affect migration of treated cells in matrigel migration as well as in vivo migration assays.

- b. The signaling pathway(s) involved in the effects of CB2R agonists on MMP-9 induction in myeloid dendritic cells will be determined. Various targets which include cyclic AMP, kinases such as extracellular signal regulated kinase (ERK) and transcription factors cFos and cJun will be analyzed by ELISA, western blot, intra cellular flow cytometry, and chromatin immunoprecipitation assays. We expect that CB2R agonist acts via inhibition of cAMP induction and subsequent downstream activators.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals and Reagents

Animals

For a murine SCI contusion model, 7-9 week old female C57BL/6 mice weighing approximately 16-21 g (Taconic, Hudson, NY) were used. For EAE model, 7-9 week old male C57BL/6 mice (Jackson Laboratory (Bar Harbor, ME) were used. All procedures, interventions, and animal care were done in accordance with protocol approved by the Temple University Institutional Animal Care and Use Committee following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in the Temple University School of Medicine (TUSM) animal facility under pathogen-free conditions for one week prior to surgical intervention for acclimation and observation. A light/dark cycle of 12 hours was maintained, and mice were allowed free access to food and water including hydrogel at all times.

For MMP-9 studies, 6-8 week-old B10.A mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the TUSM animal facility. CB2R^{+/+} and CB2R^{-/-} mice on C57BL/6 background were bred at TUSM by mating CB2R^{+/-} originally obtained from NIH. CB2R deficiency was confirmed by polymerase chain reaction (PCR) as described [126]. For the in vivo studies 8 week-old C57BL/6 were purchased from The Jackson Laboratory.

Reagents

PGE2 was purchased from Sigma (St. Louis, MO). M-CSF, GM-CSF, TNF α , IL-1 β , IL-6, and CCL19 were purchased from Peprotech, Inc. (Rocky Hill, NJ). CB2R agonist GP1a (Ki: CB1 363 nM, CB2 0.037 nM), antibodies for pro-MMP-9 ELISA and MMP-9 standards were purchased from R&D Systems (Minneapolis, MN). CB2R agonist O-1966 (Ki: CB1 5055 nM, CB2 23 nM) was a generous gift from Anu Mahadevan (Organix Inc., Woburn, MA). Dibutyryl-cAMP (dbcAMP) and MMP-9 inhibitor I were purchased from Calbiochem (La Jolla, CA). The cAMP kit was purchased from Applied Biosystems (now Life Technologies, Grand Island, NY). For flow cytometric analyses conjugated antibodies CD45-APC, CD11b-PE, Ly6G and Ly6C (Gr-1)-FITC, CD3-PeCy7 and CD3-FITC and corresponding isotypes were purchased from BD Bioscience (Mountain View, CA). Antibodies to phospho-ERK, ERK, I κ B α , phospho-p65, phospho-c-Jun, c-Jun, and c-Fos were obtained from Cell Signaling Technology, Inc. (Danvers, MA) and anti-GST from Abcam (Cambridge, MA).

2.2. In vivo models

Spinal Cord Injury - Surgical Procedures

For contusion SCI, mice were weighed and then anesthetized using an IP injection of a 1:1 combination of Ketamine (100 mg/mL) and Xylazine (20 mg/mL) at a dose of 1 mL/kg. Once under anesthesia, back hair was shaved, protective eye gel applied and the surgical site was prepped with povidone-iodine solution. Body temperature was maintained at $37^{\circ} \pm 0.5^{\circ}$ C during the procedure and recovery period with a heating pad and lamp. The paraspinal musculature was dissected free from the lamina from T8-T10. Mice were then held by the lateral aspects of the T7 vertebra using Adson forceps. Using

the operative microscope, laminectomies were performed at the T8 and T9 levels using fine microscissors and laminectomy forceps. The ligamentum flavum was gently dissected free using a cotton swab taking care not to injure the spinal cord and to ensure adequate width of laminectomy.

Mice were then transferred to the Infinite Horizons (IH) impactor device (PSI Inc., Lexington, KY, USA). They were suspended via modified Adson forceps clamped to the vertebra above and below the level of the laminectomies. The impactor tip was positioned directly above exposed dura and raised to a 3mm height. The device was set to deliver a 60kdyne force to the spinal cord. The actual force, displacement, velocity and injury time was recorded as well as injury characteristics such as bruise severity visible to the naked eye. Spinal musculature was re-sutured and the dorsal fat pad placed back in its normal position. The skin was closed with clips.

A selective CB2 agonist (O-1966) was dissolved with 1:1:18 ratio of anhydrous ethanol (Sigma), emulphor and 0.9% saline. The mice were randomly divided into experimental (drug treated), control (vehicle treated) and sham groups. Treated animals received 0.2ml vehicle or O-1966 (5mg/kg) by IP injection. Mice were treated at two timepoints; 1 hour and 24 hours post contusion. Sham mice had lamina removed without contusion.

SCI - Post-operative Care

At the conclusion of surgery the mice were given subcutaneous (SC) injections of fluid (0.9% NSS; 0.5 mL) and placed in a recovery cage under a heating lamp. All mice cages were kept on a heating pad on the first night post contusion. The mice were also given SC injections of fluid (0.9% NSS; 0.5 mL) and buprenorphine (0.03 mg/kg) twice daily and

antibiotics Bytril (2.5 mg/kg) once for the first three post-operative days. The mice had their bladders emptied via the Credé maneuver twice a day until recovery of autonomic function.

SCI - Motor Function Evaluation

The mice were evaluated for motor function recovery using two scales for open field assessment of locomotion, the 9 point Basso Mouse Scale (BMS) and the 17 point Basso, Beattie, Bresnahan (BBB) locomotor scale modified for mice by Dergham [142-145]. Scoring in each of these scales relies on movement of lower limb, the ability to plantar place the hind-paw, stepping with weight support, coordination in movement, and trunk stability. Each mouse was evaluated on post-operative day 1, 3, 7, and 14 by investigators that were blinded to treatment.

SCI - Autonomic Function Evaluation

All mice had autonomic impairment with urine retention following SCI. To relieve their bladders and to assess for autonomic function recovery, bladders were emptied via the Credé maneuver and urine mass determined. Mice were considered to have recovered autonomic function once the total urine mass collected via Credé maneuver was less than 500 mg/day for three consecutive days indicating that mice had recovered the ability to void spontaneously.

EAE induction

Mice were injected with 200 µg MOG33-55 peptide emulsified in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37 RA (final concentration 2 mg/ml)

subcutaneously on day 0 and 100 ng pertussis toxin (PTX) intraperitoneally on day 0 and day 2. Clinical scores were as follows: 0, normal mouse, no overt signs of disease; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state. At stage 5, animals were euthanized and removed from the calculation for the clinical score. Both clinical scores and weight were followed for 60 days.

Treatment regimen was started at day 7 post immunization induction and mice were treated with either vehicle of CB2R agonist biweekly (every 3-4 days) via intraperitoneal route. Treatment dose was either O-1966 at 1 mg/kg or GP1a at 5 mg/kg.

2.3. Evaluation of Inflammatory Cell Invasion

Cell extraction from CNS and spleen

Anaesthetized mice were perfused with 30 ml PBS administered intracardiacally. Organs were dissected and in SCI model, the area of contusion on the spinal cord (1cm around the impact site) was separated. In EAE the entire spinal cord and brain were harvested. CNS tissue and spleen were passed 5x through 18G needle and digested in HBSS with Ca^{2+} or Mg^{2+} (Invitrogen) based enzyme solution of DNaseI (0.1 mg/ml) (Roche) and Liberase TL (0.03 mg/ml) (Roche) for 45 min at 37°C with shaking. Ten milliliters and 5 ml of enzyme solution were used for spleen and CNS tissue, respectively. Enzyme digestion was followed by adding ice-cold blocking solution (10% FBS, 10 mM EDTA in HBSS) for 5 min with 40 and 20 ml per spleen and cord, respectively.

CNS samples were subjected to centrifugation at 4°C at 1,100 rpm (200 g) for 10 min (low brake). The tissue was pelleted and resuspended in 10 ml of 30% isotonic Percoll (GE Healthcare) (diluted with 10x HBSS w/o Ca²⁺ and Mg²⁺ (Invitrogen) and distilled water). The tissue was underlaid with 5 ml of 70% isotonic Percoll. Mononuclear cells were isolated from the 30/70 interphase after gradient centrifugation (2,000 rpm (1000 g) for 20 min, slow brake at RT). Collected cells were washed three times in 7x volume of FACS buffer (1 mM EDTA, 2% FCS in PBS), pelleted, and counted.

Spleen samples for SCI were subjected to centrifugation at 4°C at 1,100 rpm (200 g) for 10 min (low brake). Red blood cell lysis was done with RBC lysis buffer (eBioscience) by following the manufacturer's instructions. Collected cells were pelleted and counted.

Flow cytometric analyses

All cells were resuspended in Flow cytometry or Fluorescence activated cell sorter (FACS) buffer and stained with anti-mouse CD16/CD32 (BD Biosciences) for 5 min and afterward with combination of anti-mouse monoclonal conjugated Abs: CD45-APC, CD11b-PE, Ly6G and Ly6C (Gr-1)-FITC, and CD3-PeCy7 or CD3-FITC (for cells extracted from EAE tissue) for 30 min at 4°C. For staining control purposes samples were also stained with appropriate isotypes.

All samples were then immediately analyzed using BD FACSCalibur and /or BD FACSCanto (BD Biosciences). SCI data were analyzed on FlowJo 7.2.5 (Treestar, OR) while EAE data were analyzed on Cell Quest (BD Biosciences).

2.4. Evaluation of mRNA expression via Real Time PCR

Tissue harvesting for RNA extraction

For both SCI and EAE models, anaesthetized mice were perfused with 30 ml PBS administered intracardiacally. Both control and experimental groups consisted of 3 mice for each timepoint in both SCI and EAE. Naïve and sham group (used only for SCI) consisted of 2 mice. In SCI model, spinal cords were removed by dissection, and 1 cm of cord around the site of injury was homogenized in Trizol. In EAE model, the entire spinal cord and brain were harvested and homogenized in Trizol.

RNA extraction

RNA was extracted according to the manufacturer's protocol from either CNS tissue or cultured myeloid cells. RNA pellet obtained was resuspended in DEPC water. RNA concentration was determined by measuring optical density at 260 nm using a nanodrop spectrophotometer.

Reverse transcription

1 µg equivalent of RNA was diluted in 16 µl volume of DEPC water and heated at 65°C for 5 min to denature the secondary structures. Tube was cooled immediately on ice to prevent the formation of secondary structures. 14 µL of mastermix consisting of 300 U of Moloney murine leukemia virus reverse transcriptase, 40 U of RNasin, Moloney murine leukemia virus buffer, 500 ng of random primers, 30 µg of BSA and 15 mM of dNTP mix was added to make up a total volume of 30 µL (Promega). Reverse transcription was

carried out at 42°C for 90 min to obtain cDNA which was then used in Real-time PCR analysis.

Real-time PCR

cDNA was diluted 10 fold in DEPC water. 4 µL of the diluted cDNA was added to 16 µL of mastermix consisting of SYBR green-containing PCR master mixture (2X) (ABI) and 250 nM of forward and reverse primers. Real-time RT-PCR was performed using the Stratagene Mx3005P. Cycling conditions were 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 75°C for 30 sec, 57°C for 30 sec, and a melting point determination or dissociation curve. Each sample was amplified with primers for target and housekeeping gene β -actin. Amount of cDNA for each target was calculated by the number of cycles required for the fluorescence emission to reach threshold level (cT value) and was normalized to the housekeeping gene. Data was expressed in fold difference to housekeeping gene.

Primers used:

Table 1: Real-time PCR primer sequences

CXCL1	(F) 5'-TCGCGAGGCTTGCCTTGACC-3' (R) 5'-GCGTTCACCAGACGGTGCCA-3';
CCL2	(F) 5'-CACAGTTGCCGGCTGGAGCA-3' (R) 5'-CAGCAGGTGAGTGGGGCGTT-3';
CXCL9	(F) 5'-CAAAATTCATCACGCCCTT-3' (R) 5'-CCAGACAGCTGTTGTGCATT-3';
CXCL10	(F) 5'-ATTCTTTAAGGGCTGGTCTGA-3' (R) 5'-CACCTCCACATAGCTTACAGT-3';

Table 1, continued

CXCL10	(F) 5'-ATTCTTTAAGGGCTGGTCTGA-3' (R) 5'-CACCTCCACATAGCTTACAGT-3';
CXCL11	(F) 5'-GGGCGCTGTCTTTGCATC-3' (R) 5'-AAGCTTTCTCGATCTCTGCCAT-3'
IL-23p19	(F) 5'-TGCTGGATTGCAGAGCAGTAA-3' (R) 5'-ATGCAGAGATTCCGAGAGA-3';
IL-23R	(F) 5'-ACATTGGACTTTTGTCTGGGAA-3' (R) 5'-AAAATCGGCAACATG-3';
MMP-9	(F) 5'-AAAACCTCC-AACCTCACGGA-3' (R) 5'-GCGGTACAAGTATGCCTCTGC-3';
CCR7	(F) 5'-TTCCAGCTGCCCTACAATGG-3' (R) 5'-GAAGTTGGCCACCGTCTGAG-3';
MMP-2	(F) 5'-CGCTCAGATCCGTGGTGA-3' (R) 5'-CGCAAATAAACCGGTCCTT-3';
TIMP-1	(F) 5'-TATCCGGTACGCCTACACCC-3' (R) 5'-TGGGCATATCCACAGAGGCT-3';
CB2R	(F) 5'-GTGATCTTCGCCTGCAACTTT -3' (R) 5'-GGAGTCGACCCCGTGGA -3'.
β -actin	(F) 5'-AGCTTCTTTGCAGCTCCTTCGTTGC-3' (R) 5'-ACCAGCGCAGCGATATCGTCA-3'.
β -actin (used in SCI)	(F) 5'-TCCACCACCACAGCTGAGAGG-3' (R) 5'-CAGCTTCTC TTTGATGTCACG-3'

RT² Profiler PCR array

The expression of proinflammatory chemokines and chemokine receptors and mediators of the TLR-signaling pathway was evaluated by using the RT² Profiler PCR array based on SYBR green-containing PCR technique. 20 μ L of cDNA was diluted with double-distilled H₂O to a total of 100 μ L. The experimental mixture was prepared by mixing the

following components: 1225 μ L of 2x SYBR-green containing PCR master mix, 98 μ L of diluted cDNA, and 1127 μ L of double-distilled H₂O. A total of 25 μ L of the mixture was loaded in each well of a 96-well plate precoated with primers for different genes (SABiosciences). The PCR array was performed using the Stratagene Mx3005P detector. PCR cycling conditions were 15 s at 95°C, 1 min at 60°C for 40 cycles followed by a melting point determination of dissociation curves. Cycle threshold values were determined by automated threshold analysis and results were normalized to 5 housekeeping genes which included glyceraldehyde-3 phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (Hprt1), glucuronidase beta (GusB), heat shock protein 90 alpha (hsp90sb1), and β -actin.

2.5. Cell cultures

Bone marrow macrophages (BMM Φ) and DC (BMDC) were generated in vitro from bone marrow. Briefly, bone marrow cells were flushed from femurs and tibiae. Approximately 2×10^6 cells were cultured in 100 mm Petri dishes containing 10 ml RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine. Medium was supplemented with either M-CSF (10 ng/mL) or GM-CSF (20 ng/mL) for BMM Φ or BMDC cultures, respectively. On day 3, another 10 mL of fresh medium containing either M-CSF or GM-CSF was added to each dish. For BMM Φ cultures, adherent cells were collected by trypsinizing at day 8 and the purity was determined to be 85% or greater by FACS analysis of CD11b and F480 markers. For BMDC cultures, the nonadherent cells were harvested at day 7 and purified by immunomagnetic sorting with anti-CD11c coated magnetic beads using the

autoMACS system (Miltenyi Biotech, Bergish-Gladbach, Germany) and the purity was determined to be 95% or greater by FACS analysis of CD11c marker.

Microglial cells were generated from neonatal mice. Briefly, cerebral cortical cells from 1-2-day-old mice were dissociated and plated in 75 cm² Falcon culture flasks in Dulbecco's modified Eagle's medium (DMEM) -F12 (Hyclone, Logan, Utah) supplemented with 10% heat-inactivated FBS, containing 2 mM glutamine and 1x antibiotic/antimycotic (complete medium). The medium was removed and replenished with complete medium containing 10 ng/ml of GM-CSF at day 5 and 10 after plating. On day 15, microglia were harvested by shaking the flasks at 350 rpm, 37°C for 25 min. The harvested cells were centrifuged (1100 rpm, 5 min) and plated at 1×10⁶ cells/mL in complete medium containing 10 ng/mL of GM-CSF (CD11b+F4/80+ cells >90% by FACS analysis).

All three cell types, BMMΦ, BMDC, and microglia were cultured at 1×10⁶ cells/mL in GM-CSF supplemented complete medium and matured with TNFα (20 ng/mL), IL-1β (10 ng/mL), IL-6 (10 ng/mL), and PGE2 (10⁻⁷M) for either 24 or 48h in the presence or absence of GP1a.

2.6. Migration assays

Matrigel migration assay

Matrigel migration was performed in Transwell inserts (6.5 mm) fitted with polycarbonate filters (8-μm pore size; Corning, Acton, MA). The upper sides of the transwells were coated with Matrigel (BD Biosciences, Mountain View, CA) diluted in

PBS (100 µg/filter). CD11c⁺ DC cultured with TNF α , IL-1 β , IL-6, and PGE2 with or without GP1a (5 or 1 µM) for 48h were tested for migration to CCL19 (100 ng/mL). In some experiments cells treated with the MMP-9 inhibitor I (10⁻⁶M) were also included. Briefly, the lower chambers of the plate were filled with 500 µL serum-free medium with or without CCL19 (100 ng/mL). DC (1 \times 10⁵ cells in 0.1 mL) were deposited in the upper Transwell chambers and allowed to migrate for 3h at 37°C in 5% CO₂. Migrated DC harvested from the lower chambers were counted by FACS (60-second counts).

Chemotaxis assay

Chemotaxis assay was performed in Transwell inserts (6.5 mm) fitted with polycarbonate filters (8-µm pore size; Corning, Acton, MA). CD11c⁺ DCs cultured with TNF α , IL-1 β , IL-6, and PGE2 with or without GP1a (5 or 1 µM) for indicated timepoints were tested for migration to either 500 µL serum-free medium or CCL19 (100 ng/mL) placed in the lower chambers. DC (1 \times 10⁵ cells in 0.1 mL) were deposited in the upper Transwell chambers and allowed to migrate for 2 h at 37°C in 5% CO₂. Migrated DCs harvested from the lower chambers were counted by FACS (60-second counts).

In vivo migration assay

Bone marrow-derived DC from CB2R^{+/+} and CB2^{-/-} mice were treated with TNF α , IL-1 β , IL-6, and PGE2 in the presence or absence of GP1a. 48h later DC were labeled with PKH 26 red fluorescent dye (Sigma) according to the manufacturer's instructions, and 10⁶ labeled DC were inoculated subcutaneously in the footpads of mice pre-injected 24h earlier with 40 ng TNF α (s.c. in the footpads). At 48h the numbers of labeled DC

collected from the draining popliteal lymph nodes were determined by FACS (50,000 event counts).

2.7. Biochemical assays for cyclic AMP and protein analysis

cAMP assay

Purified CD11c⁺ DC (1×10^6 cells/ml) were seeded in 96-well plates in 100 μ L of complete medium and rested overnight. Next day, cells were washed with PBS and the medium was exchanged for cAMP assay medium (serum free XViVO-15 medium with 0.5 mM 3-isobutyl-1-methylxanthine). After incubation for 1h at 37°C, the cells were treated with PGE₂ in the presence or absence of the CB₂R agonist. After treatment, the cAMP amounts were determined using a cAMP screening kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

MMP-9 ELISA

Purified CD11c⁺ DC (1×10^6 cells/ml) were seeded in 12-well plates and treated as described in Results. The amounts of pro-MMP-9 released in the medium were measured by sandwich ELISA with antibodies and standards obtained from R&D Systems. The absorbance was determined using a Polarstar Optima plate reader at a wavelength of 450 nm.

FACS Analysis for Phospho-ERK, Phospho-p65, and c-Fos

Cells treated as indicated were fixed, permeabilized, and incubated with rabbit anti-mouse phospho-ERK, rabbit anti-mouse phospho-p65 or rabbit anti-mouse c-Fos for 40

min at room temperature followed by Alexa Fluor-conjugated goat anti-rabbit IgG (Invitrogen / Life Technologies, Carlsbad, CA) for 30 min. Data were collected for 10,000 cells by FACS analysis.

Western Blot Analysis

$3-6 \times 10^6$ DC were serum-starved for 3h before treatment. Samples were lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) plus a protease inhibitor mixture (5 mM phenylmethyl-sulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.2 μ M okadaic acid (Sigma). 20–30 μ g of whole protein lysate were mixed with 6 \times sample buffer and heated at 90°C for 5 min, followed by loading on 10% SDS-PAGE gels. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies against phospho-p44/p42 MAP Kinase (Thr-202, Tyr-204), total p44/p42 MAP Kinase (L34F12), phospho-cJun (Ser-63) II, total cJun (L70B11), and total I κ B α (L35A5) (Cell Signaling Technology, Inc.) at 1:1000 dilution in 50:50 Odyssey blocking buffer:PBS (LiCor Biosciences, Lincoln, NE). Antibody against GAPDH was diluted 1:5000. Goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680CW antibodies (LiCor Biosciences) were used as secondary antibodies. Transferred proteins were visualized by using the Odyssey infrared imaging system (LiCor Biosciences).

ChIP Assay

DC were prepared for ChIP analysis as described in Yen et al. [79]. Following various treatments, DC were fixed with 1% formaldehyde (final concentration) for 30 min,

followed by 125 mM glycine (final concentration) for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitors, collected, resuspended in SDS lysis buffer containing protease inhibitors, incubated for 30 min on ice, and sonicated to shear DNA. After sonication, the lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatants were diluted in ChIP dilution buffer (0.01% SDS, 0.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, plus protease inhibitors). The chromatin was immunoprecipitated with anti-c-Fos, anti-c-Jun, or anti-GST as a negative control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After overnight incubation at 4 °C, protein A/G-agarose was added for 1h, and the immune complexes were washed sequentially with low-salt wash buffer once (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl), high-salt wash buffer once (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl), LiCl wash buffer once (0.25 M LiCl, 1% octyl phenyl-polyethylene glycol, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)), and Tris/EDTA buffer twice (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). Antibodies were eluted from the immune complexes with elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0)) and cross-linking was reversed by heating at 65 °C overnight. After Proteinase K digestion, input DNA and precipitated DNA were purified and real-time PCR-amplified with primers encompassing the MMP-9 promoter region containing the distal AP-1 site (sense, GACCCTGGGAACCGGGTCCA and antisense, CAGGGACCGGCCGTGGAAAC) which has been shown to be essential in MMP-9 expression [78].

2.8. Statistical analysis

All results obtained from in vitro assays are expressed as mean \pm SD. Animal scores in spinal cord injury and cell infiltration data from EAE are expressed as mean \pm SEM. Comparisons between multiple groups were performed by ANOVA followed by Bonferroni t-test. Statistical significance was determined with p values <0.05 (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). For in vivo migration, data were analyzed using ratio paired t test in which the average of the logarithm of the ratio of treated/control is taken and tested for null hypothesis. Data were analyzed using Graphpad Prism 5 software. FACS data were analyzed using CellQuest software from BD Biosciences.

CHAPTER 3

RESULTS

Previously, our laboratories as well as a few other groups have demonstrated that CB2R agonist administration had beneficial effects on clinical outcome in both spinal cord injury and experimental autoimmune encephalomyelitis (EAE) [119, 122, 131, 146]. The two neurodisorders are very different in their etiology. Spinal cord injury is an acute response to traumatic injury to the cord and is antigen independent while EAE is a chronic inflammatory response to myelin antigens. However, both neurodisorders are exacerbated by the infiltration of inflammatory immune cells from the periphery into the CNS. In the present study we examined the effect of CB2R agonist on expression of various molecular markers involved in peripheral immune cell recruitment into the CNS in the two disease models.

3.1. Effects of CB2R agonists on recovery in spinal cord injury

3.1.1. CB2R agonist treatment improves autonomic and locomotor function recovery after SCI

Previous studies had demonstrated that pretreatment of animals with CB2R agonist 1 hour prior to spinal cord injury improved locomotor functions and bladder functions [122]. In this study we investigated if intervention after injury, i.e. administration of CB2R agonist at 1h and 24h post injury, would have a beneficial effect.

CB2R agonist treated mice showed a better recovery of locomotor function in both Basso Mouse Scale (BMS) and modified Basso, Beattie, Bresnahan (BBB) scores for open-field assessment of locomotion at 3, 7, and 14 days post-injury (**Figure 7A**).

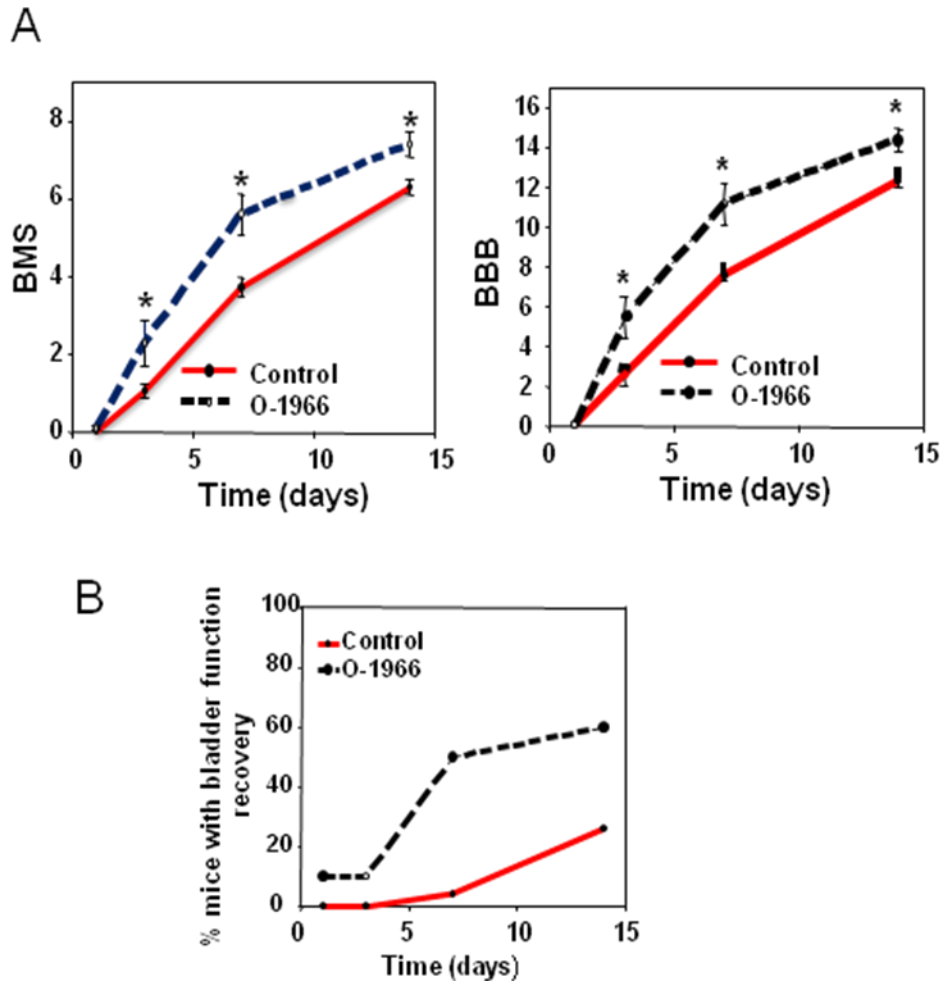


Figure 7. CB2 agonist-treated mice show statistically significant improvement in the BMS and BBB open field assessment for motor function.

Mice ($n=10$) were injected *i.p.* with O-1966 (5 mg/kg) or vehicle ($n=14$), 1h and 24h post-injury and evaluated for recovery of (A) locomotor functions by an open field assessment of Basso mouse scale (BMS) and Basso, Beattie, Bresnahan (BBB) and (B) autonomic function as the ability to spontaneously empty their bladder. Recovery of autonomic recovery is expressed as percent mice with bladder function recovery ($*p<0.05$). Adapted from Adhikary et al [124].

Spinal cord injury results in bladder dysfunction. Injured mice are not able to spontaneously relieve their bladder. Animals treated with the selective CB2 agonist had significantly improved ability to spontaneously void compared to controls (**Figure 7B**). Three days post-injury 10% of the CB2-agonist-treated animals were able to spontaneously void. By day 7 approximately 50% of the treated animals had recovered their bladder function, while less than 5% of the vehicle-treated animals were able to empty their bladder. By day 14 60% of the CB2-agonist-treated animals could spontaneously void, compared to only 25% of vehicle-treated animals.

3.1.2. CB2R agonist modulates inflammatory chemokines and cytokines in SCI

To investigate if CB2R agonist administration alters expression of early chemokines and cytokines at the site of injury, RNA was extracted from injured cords and PCR array analysis was performed at 48h post spinal cord injury. PCR array analysis showed a significant increase in the expression of CXCL9, CXCL11, IL-23 p19 subunit, IL-23 receptor, CCR1, CCR2, CCR3 and CXCR2 in injured cords compared to sham operated animals which underwent only laminectomy but not the injury. (**Figure 8A**). Animals treated with O-1966 1h and 24h post injury showed a significant decrease in the expression of CXCL9, CXCL10, CXCL11, IL-23 p19, and IL-23 receptor compared to animals treated with vehicle. These findings were confirmed by Real-time PCR as shown in **Figure 8B**. There was also a trend for a decrease in the expression of CCR1, CCR2, CCR3 and CXCR2 in the treated cords though the data did not reach statistical significance. CCL2, along with chemokines CCL3, and CCL4 are expressed early during spinal cord injury and we also observed very high expression of these chemokines at 6h

post injury. While expression of CCL3 and CCL4 returned to basal levels at 48h, CCL2 remained elevated and there was no difference between treated and untreated cords.

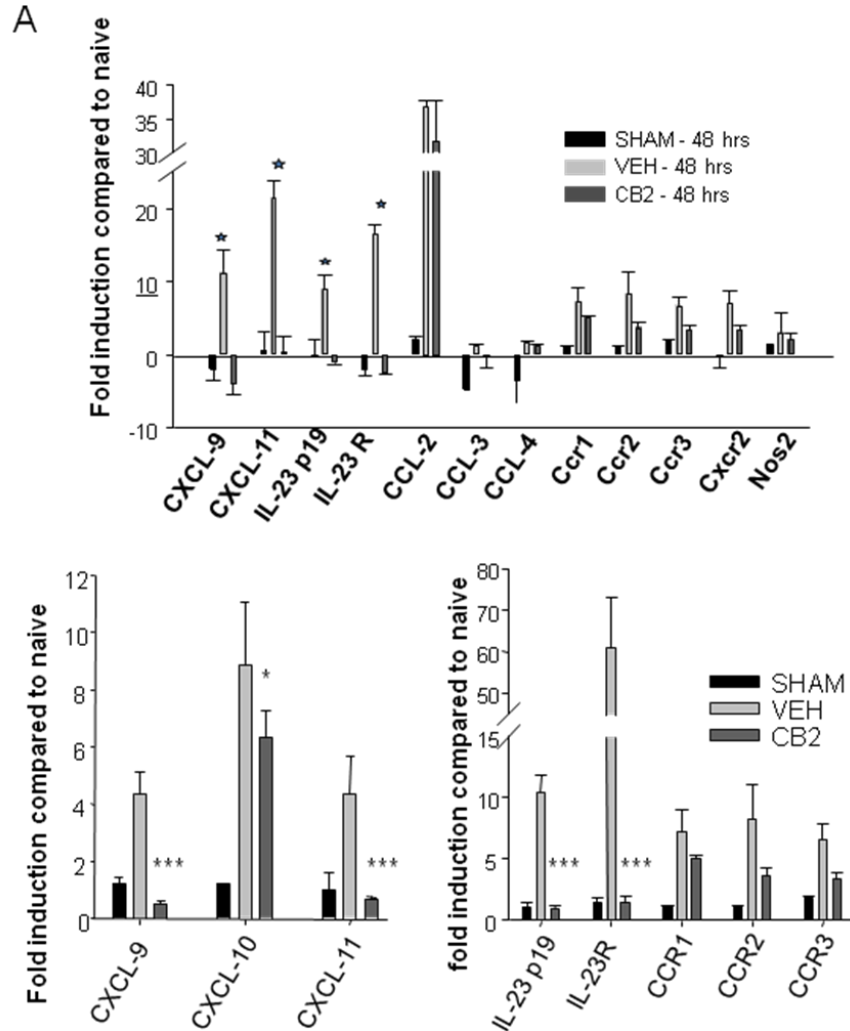


Figure 8. CB2R agonist treatment reduces expression of cytokines and chemokines in spinal cord at 48h post injury.

*Analysis of mRNA expression of cytokines and chemokines via (A) PCR-array and (B) Quantitative real-time PCR. Data are represented as fold changes compared to naïve cords. (n=2 for sham and n=3 for SCI vehicle-treated and O-1966-treated groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). Adapted from Adhikary et al [124].*

3.1.3. CB2R agonist modulates expression of TLRs

Spinal cord injury also led to the upregulation of various Toll-like receptors. TLR-1 and 2 were upregulated 6h post injury (**Figure 9A**, vehicle versus sham) while TLR-1, 2, 4, 6, and 7 were upregulated 48h post injury compared to sham operated animals (**Figure 9B**, vehicle versus sham). Treatment with O-1966 had no effect on the expression of TLR-2 at either time points. However, it significantly attenuated the expression of TLRs-1, 4, 6 and 7 at 48h post injury.

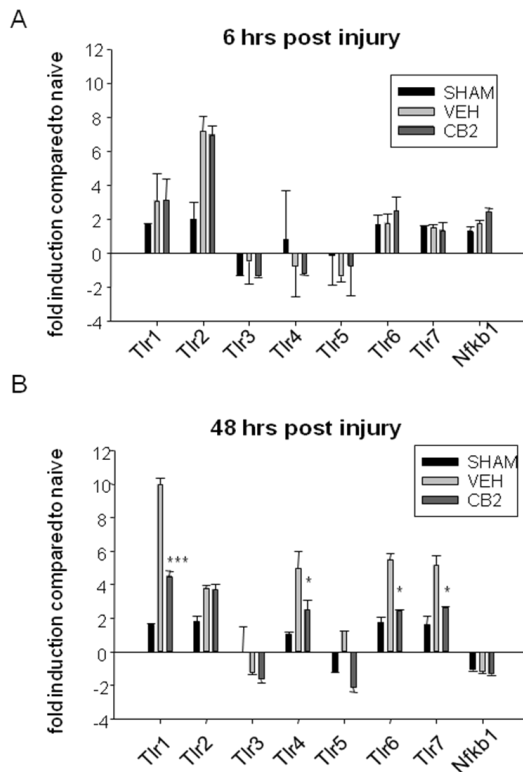


Figure 9. CB2R agonist treatment inhibits upregulation of Toll-like receptor expression in spinal cord at 48h post injury.

*Analysis of mRNA expression of Toll-like receptors via PCR-array at (A) 6h and (B) 48h post SCI. Data are represented as fold changes compared to naïve cords. (n=2 for sham and n=3 for SCI vehicle-treated and O-1966-treated groups for each timepoint; *p<0.05, **p<0.01, ***p<0.005). Adapted from Adhikary et al [124].*

3.1.4. CB2R agonist treatment leads to reduced numbers of peripheral immune cell infiltration into the injured

To assess the effects of the altered chemokine and cytokine expression on peripheral immune cell infiltration to the injury site, cells were extracted from injured cords and analyzed by flow cytometry. To determine the various infiltrating population, we immunostained for the following cell surface markers; hematopoietic cells (CD45⁺), myeloid cells (CD45^{hi}CD11b^{hi}), monocytes (CD45^{hi}CD11b^{hi}GR-1^{lo}), neutrophils (CD45^{hi}CD11b^{hi}GR-1^{hi}), microglia (CD45^{lo}CD11b^{hi}), and T cells (CD45^{hi}CD11b⁻CD3⁺). First, we compared the cell infiltration pattern into the injury site to the distal cord. Most of the immune cells, i.e. neutrophils (CD45^cCD11b^{hi}GR-1^{hi}) and monocytes (CD45^{hi}CD11b^{hi}GR-1^{lo}), infiltrated to the injury site. The only cell population observed in the distal cord was the microglia (CD45^{lo}CD11b^{hi}GR-1^{lo}) (**Figure 10A**).

At 7 days post injury, there was a significant decrease in the number of hematopoietic cells as well as myeloid cells in the CB2R agonist treated cords. There was a trend towards a decrease in the number of monocytes, neutrophils, and microglia (**Figure 10B**). To rule out that the treatment altered cell counts in the periphery we analyzed the spleen for total cells, monocytes, neutrophils, and T cells. There was no difference between CB2R agonist treated and untreated mice (**Figure 9C**).

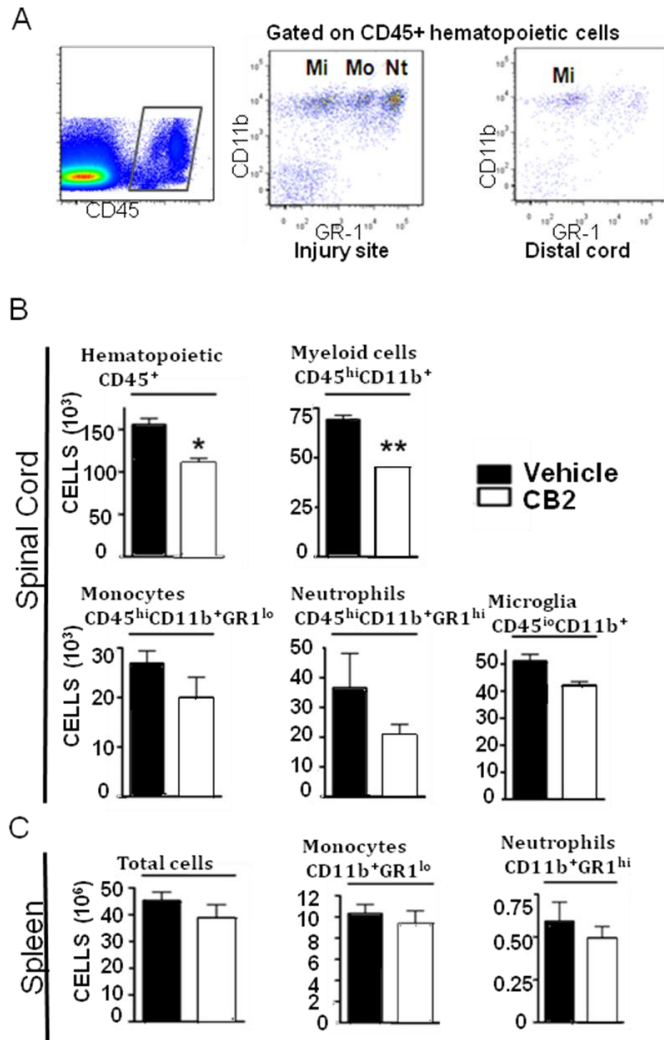


Figure 10. CB2R agonist treatment results in lower numbers of CNS infiltrating hematopoietic cells in the injured spinal cord area post injury (7 days).

(A) Cells extracted from spinal cord were gated for CD45 expression and analyzed for expression of CD11b and GR-1. Cell populations extracted from injured area were compared to the ones obtained from distal cord. (B) Cellular composition and cell numbers obtained from the injured spinal cord 7 days area post injury were analyzed. Data are represented as number of cells extracted from either vehicle or CB2R agonist treated cords. (3-4 mice pooled, $n = 2$). C. As in B, number of spleen cells obtained at 7d post injury from the two groups of mice were compared. ($n = 7$); * $p < 0.05$, ** $p < 0.01$; Adapted from Adhikary et al [124].

3.1.5. CB2R agonist treatment attenuates activation of microglia

Though we didn't find a significant decrease in the number of total microglia between CB2R treated and untreated mice, we proceeded to determine whether there was a difference in microglial activation. Upon activation, microglia change shape, retract their processes, and upregulate expression of Iba-1 (ionized calcium binding adaptor molecule 1) also known as allograft inflammatory factor-1 [147].

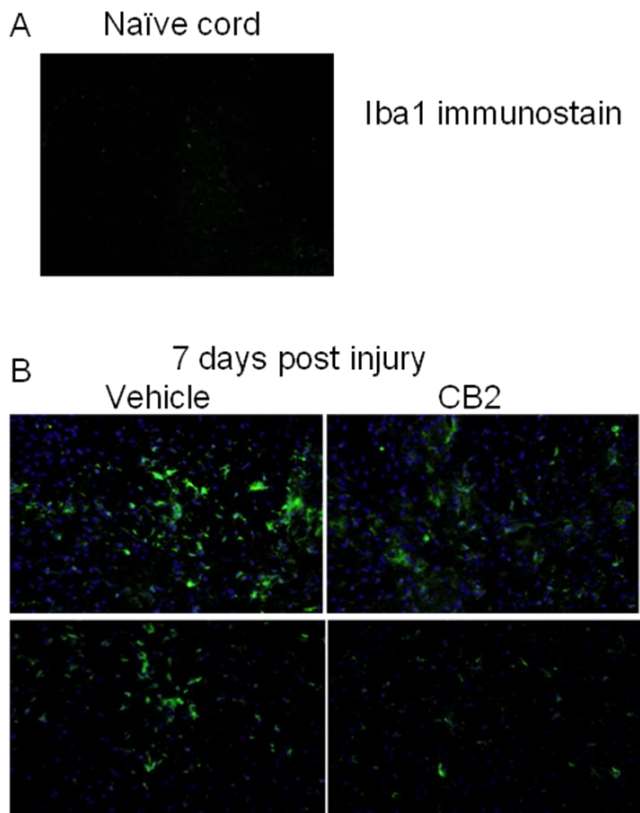


Figure 11. Analysis of Iba-1 upregulation via immunohistochemical staining at 7 days post SCI.

(A) naïve cord (B) Left panels are vehicle treated cords either 1.5 mm rostral (top) or caudal (bottom) and right panels are CB2R agonist treated cords. ($n = 2$ for naïve and $n=3$ for vehicle treated and O-1966 treated groups).

Immunostaining for Iba-1 showed decreased fluorescence in both rostral and caudal regions of cord from mice treated with CB2R agonist (**Figure 11B**). For reference uninjured (naïve) cord showing almost no Iba-1⁺ was also included (**Figure 11A**).

In summary, treatment with CB2R agonist in spinal cord injury resulted in improved locomotor and bladder functions, which correlated with reduced infiltration of immune cells into the injured cord. There was also a decreased expression of chemokines (CXCL10 family) and cytokines (IL-23 and its receptor). Lastly, CB2R agonist treated cords showed a reduction in Iba-1⁺ activated microglia/macrophages that correlated with the decreased expression of toll-like receptors (TLR-1,4,6, and 7).

3.2. Effects of CB2R agonist in Experimental Autoimmune Encephalomyelitis (EAE)

In our previous publications we had reported beneficial effects of CB2R agonist in an active EAE model. This effect correlated with the observed reduction in rolling and adhesion of leukocytes to pia microvessels at the peak of EAE [118, 119]. In a mouse model of ischemia/ reperfusion injury, CB2R agonist treatment resulted in decreased levels of ICAM-1 in brain tissue. To determine if the beneficial effects of CB2R agonist treatment in EAE is due to decreased immune cell recruitment into the CNS, we evaluated the number of peripheral immune cells in the brains and spinal cords of EAE mice at peak of the disease (day 17). Animals treated with the CB2R agonist had decreased numbers of CD45⁺ hematopoietic cells including myeloid cells (CD45^{hi}CD11b⁺), and T cells (CD45^{hi}CD11b⁻CD3⁺) infiltrating into both spinal cord and

brain (Figure 12 and 13). Interestingly, this effect was maintained on day 30, the chronic EAE stage (Figure 13).

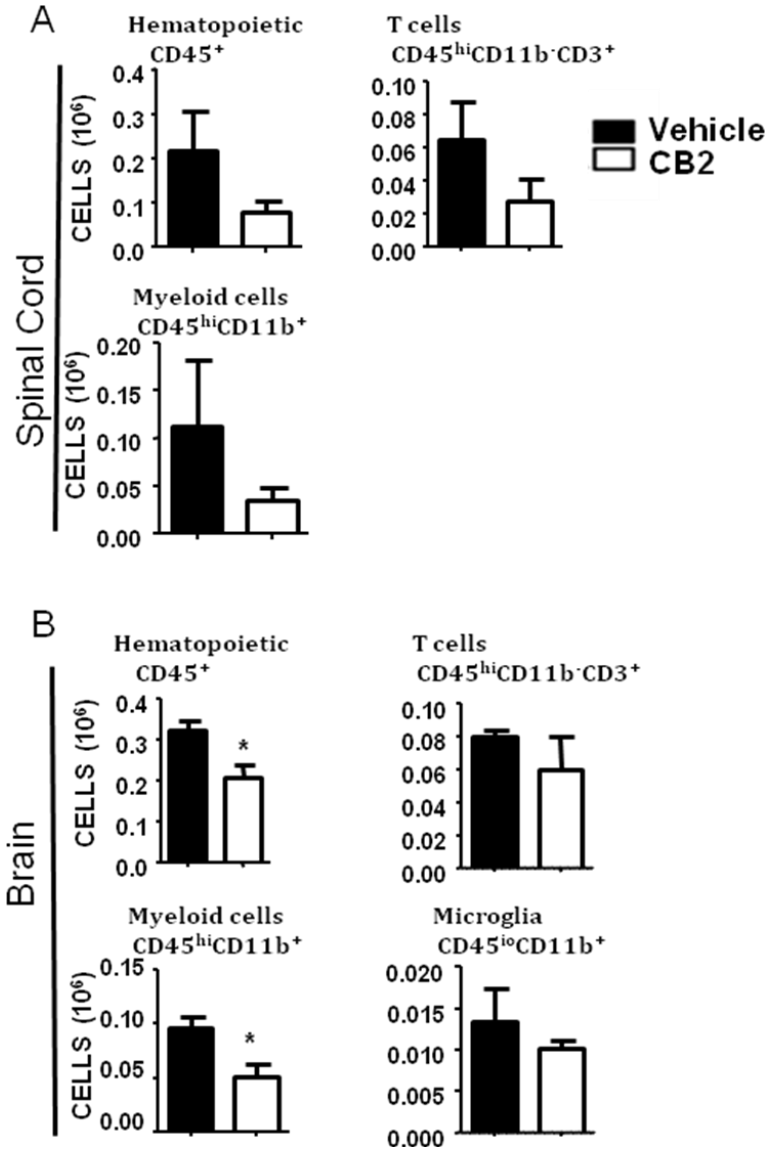


Figure 12. CB2R agonist treatment results in reduced numbers of hematopoietic cell infiltration into CNS at peak of EAE.

Cellular composition and cell numbers obtained from EAE (A) spinal cord and (B) brain at day 17 or peak of disease were analyzed. Data are represented as number of cells extracted from either vehicle or CB2R agonist treated cords and brains. (* $p < 0.05$), ($n = 3$ for EAE vehicle treated and CB2R agonist treated groups).

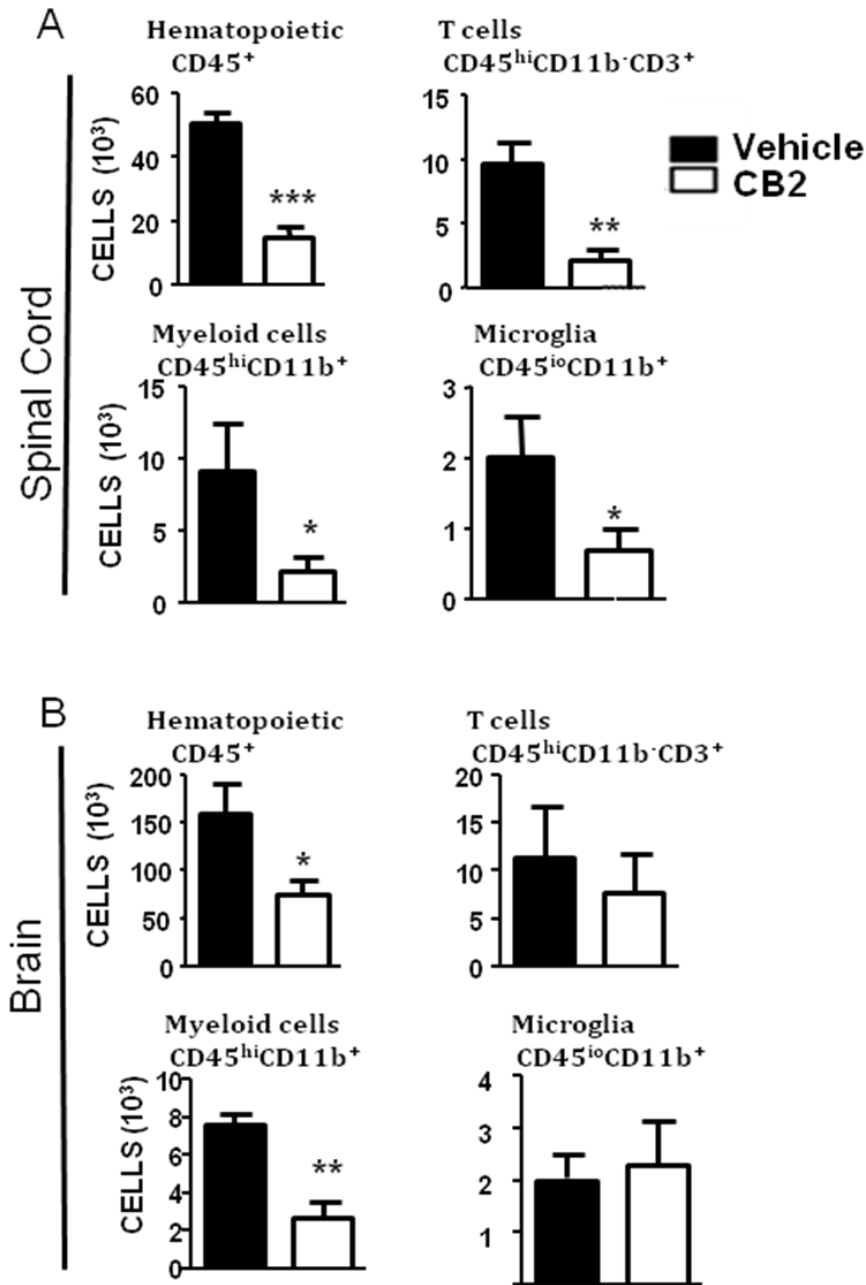


Figure 13. CB2R agonist treatment results in lower numbers of CNS infiltrating hematopoietic cells in chronic stage of EAE.

Cellular composition and cell numbers obtained from EAE (A) spinal cord and (B) brain at day 30 or the chronic stage of disease were analyzed. Data are represented as number of cells extracted from either vehicle or CB2R agonist treated cords and brains. (* $p < 0.05$, ** $p < 0.02$, *** $p < 0.005$), ($n = 4$ for EAE vehicle treated and CB2R agonist treated groups).

3.2.1. CB2R agonist administration reduces TLR4 expression in EAE

Since TLR4 expression was greatly attenuated in SCI upon CB2R agonist treatment we analyzed its expression in EAE. RNA was extracted from EAE mice (brain and spinal cord) at two different timepoints; 10 days post immunization i.e. prior to disease manifestation, and 16 days post immunization i.e. peak of the disease. The experiment included EAE mice treated with either vehicle or CB2R agonist. TLR4 was upregulated in both brain and especially spinal cord of EAE mice. There was a significant reduction in TLR4 expression in spinal cord following CB2R agonist treatment both on day 10 and 17 (Figures 14A and B).

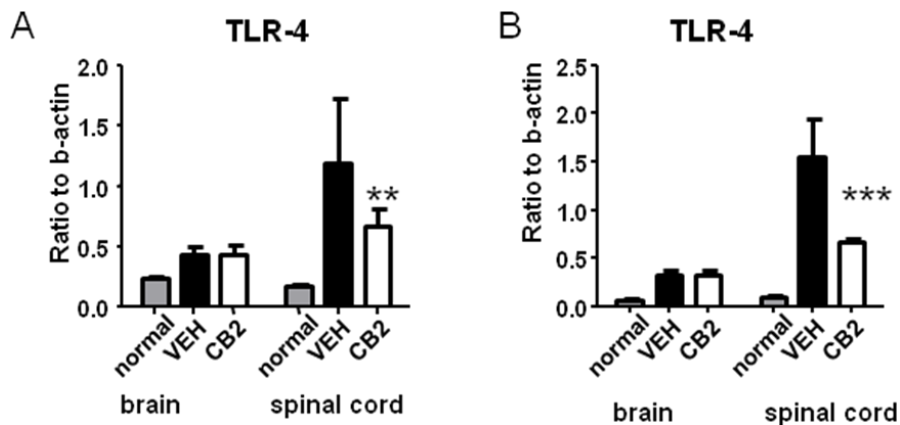


Figure 14. CB2R agonist treatment reduces TLR-4 expression in spinal cord.

*Analysis of mRNA expression of TLR-4 via qRT-PCR (A) on 10 days and (B) on 16 days post immunization (peak of disease). (** $p < 0.02$, *** $p < 0.005$). ($n = 2$ for naïve and $n = 3$ for EAE vehicle treated and O-1966 treated groups).*

Our observed decrease in TLR-4 expression in the RNA prepared from spinal cord of EAE mice treated with CB2R agonists could be the result of a direct effect on TLR-4 expression in both microglia and infiltrating myeloid cells or the consequence of fewer

infiltrating peripheral myeloid cells. This issue could be resolved by determining TLR-4 expression in mononuclear cells separated from brain and spinal cord of EAE mice treated with CB2R agonists or by using laser capture technology to analyze TLR-4 expression separately in microglia and infiltrating monocytes/macrophages.

3.2.2. CB2R agonist administration reduces VCAM-1 and MMP expression at the peak of EAE disease

Infiltration into the CNS is a two step process. The first step, in which peripheral immune cells need to traverse through the endothelium to reach the perivascular space, depends on adhesion molecules expressed by endothelial cells. The second step requires matrix metalloproteases to breach the glia limitans to facilitate their infiltration into the parenchyma.

To investigate if the CB2R agonist had any effects on these molecules, RNA was extracted from the brain and spinal cord of EAE mice and analyzed for expression of adhesion molecule VCAM-1 and gelatinases MMP-2 and MMP-9, via real time PCR. Though VCAM-1 was not upregulated 10d post immunization, it was highly upregulated on day 16 and treatment with the CB2R agonist induced a significant decrease in VCAM-1 expression both in brain and spinal cord (**Figures 15A and B**).

Similarly, MMP-9 was not upregulated on day 10 post immunization (**Figure 15C**). However, on day 16, both members of the gelatinase family MMP-9 and MMP-2, as well as the inhibitor TIMP-1 were upregulated in EAE mice (**Figures 14D-F**). CB2R agonist treated mice expressed lower levels of both MMP-9 and MMP-2 in the spinal cord

(Figures 15D and E). Interestingly, the levels of TIMP-1 expression were the same in treated and untreated mice (Figure 15F).

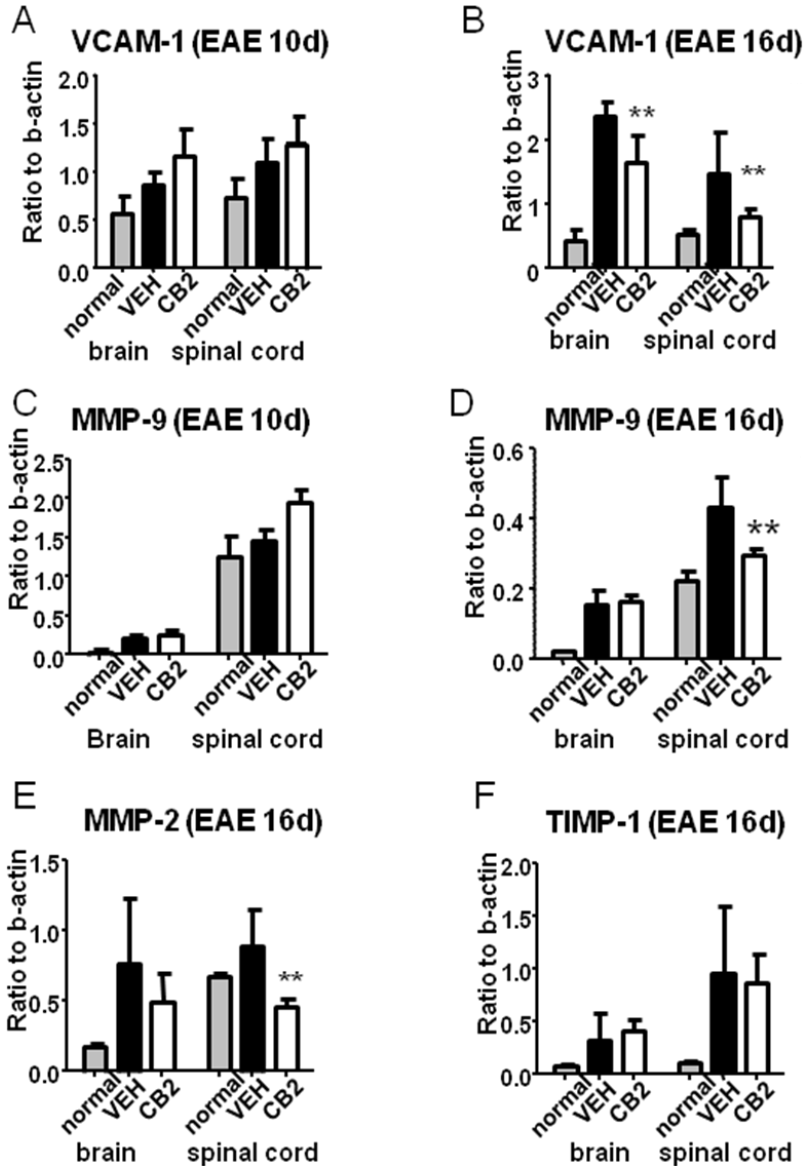


Figure 15. CB2R agonist treatment reduces expression of VCAM-1 and MMP 2 and 9 at peak of disease.

*Analysis of mRNA expression of VCAM-1, MMP-9, MMP-2 and TIMP-1 via qRT-PCR (A and C) on day 10 and (B and D-F) on day 16 post immunization (peak of disease). (** $p < 0.02$, *** $p < 0.002$). ($n = 2$ for naïve and $n = 3$ for EAE vehicle treated and O-1966 treated groups).*

The decrease in VCAM-1 and MMP-2/9 expression suggests that CB2R agonists may play a bigger role than the previously reported inhibition of leukocyte rolling and adhesion to brain microvasculature [119], and that it might also affect leukocyte transmigration into the perivascular space and further infiltration into the CNS parenchyma. This process requires both chemokine cues and the gelatinases MMP-2/9. There are no reports in the literature on possible CB2R agonist effects on gelatinases in myeloid cells. Although both MMP-2 and -9 are produced by antigen-presenting cells and act on similar substrates, MMP-9 appears to play the crucial role in EAE. Therefore, we decided to study the effects of CB2 signaling on MMP-9 expression in myeloid cells, focusing on myeloid bone marrow-derived dendritic cells (BMDC).

3.3. CB2R agonists affect migration of activated DC by inhibiting MMP-9

3.3.1. GPIa inhibits MMP-9 expression in myeloid derived immune cells including DC

Myeloid derived cells are major producers of MMP-9 in EAE. To study the molecular mechanisms involved in the CB2R effects on MMP-9, we generated bone marrow derived macrophages (BMM Φ), dendritic cells (BMDC), and neonatal primary microglia. We have previously reported that treatment of BMDC with a cytokine cocktail consisting of TNF α , IL-1 β , IL-6, with PGE2 (CCP) induced high levels of MMP-9. Other studies have reported similar results in macrophages [73]. We matured all three types of myeloid cells with CCP for 24h in the presence or absence of CB2R agonists. CB2R activation resulted in decreased expression of MMP-9 in all three cell types (**Figure 16A-C**). In

contrast to MMP-9, the other member of the gelatinase family, MMP-2, is constitutively expressed in immature DC and downregulated upon maturation. However, even in these circumstances, the presence of the CB2R agonist resulted in further reduction of MMP-2 expression (**Figure 16D**).

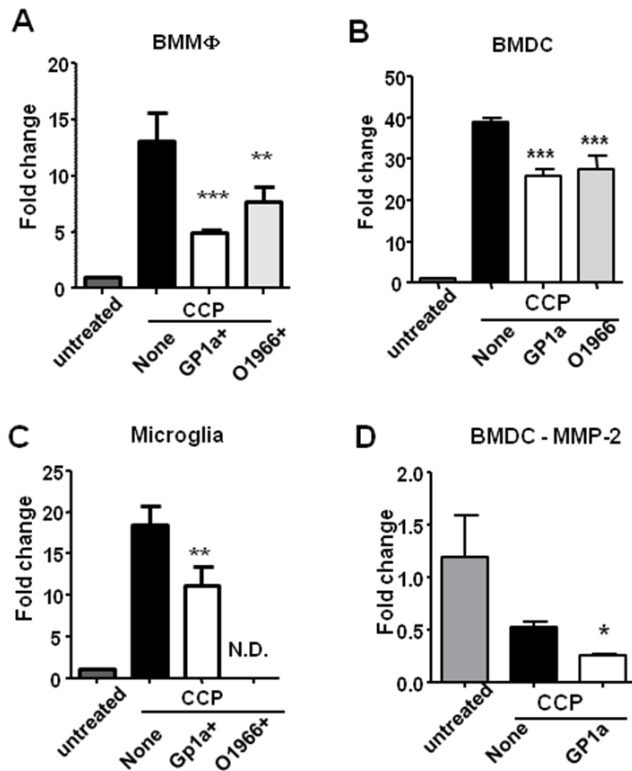


Figure 16. GP1a inhibits MMP-9 expression in myeloid cells.

*GP1a inhibits MMP-9 induced by cytokine cocktail and PGE2 (CCP) in bone marrow-derived macrophages (BMM ϕ), dendritic cells (BMDC) and microglia. (A-C) BMM ϕ , BMDC and primary microglia were treated with CCP with or without CB2R agonists GP1a (5 μ M) or O-1966 (5 μ M) for 24h. RNA was extracted and subjected to qRT-PCR for MMP-9. (D) BMDC were treated with CCP with or without GP1a (5 μ M), RNA was extracted and subjected to qRT-PCR for MMP-2. Data were normalized to housekeeping gene β -actin expression and represented as fold change compared to untreated samples. * p <0.05, ** p <0.01, *** p <0.001 compared with CCP (no CB2R agonist). Data are representative of two independent experiments.*

Since migration of DC to secondary lymphoid organs is essential to initiate the adaptive immune response, we focused on the effects of the CB2R agonist GP1a on MMP-9 expression in BMDC. CCP induced high levels of MMP-9 mRNA expression at 24h and MMP-9 protein secretion at 48h (**Figures 17A and B**); GP1a inhibited MMP-9 expression and secretion in a dose-dependent manner (**Figure 17C**).

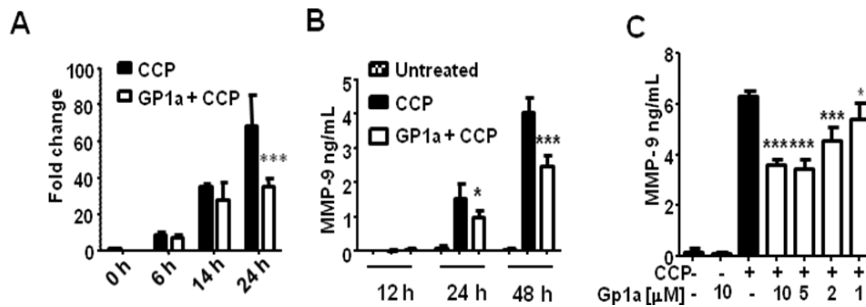


Figure 17. GP1a inhibits MMP-9 expression in BMDC in a concentration dependent manner.

(A) BMDC were treated CCP with or without GP1a (5 μ M), and RNA was extracted at different timepoints and subjected to qRT-PCR for MMP-9. (B) BMDC were treated as in A, supernatants were collected at various timepoints and subjected to MMP-9 ELISA. (C) BMDC were treated with different concentrations of GP1a for 48h and supernatants were subjected to MMP-9 ELISA. Data were normalized to housekeeping gene β -actin expression and represented as fold change compared to untreated samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. compared with CCP (no CB2R agonist). Data are representative of two (A) and four (B and C) independent experiments.

3.3.2. Inhibition of MMP-9 production by GP1a is mediated through CB2R

To confirm that wild type BMDC express CB2R we carried out qRT-PCR for CB2R in BMDC. Since the murine CB2R gene lacks introns, we included a no reverse transcriptase (no RT) control to rule out amplification of genomic DNA. BMDC obtained

from CB2R^{-/-} mice were analyzed to ensure the lack of CB2R expression. In contrast to CB2R^{-/-} BMDC, wild type DC express CB2R (**Figure 18A**).

To test if the effect of GP1a on MMP-9 expression was dependent on CB2R signaling, we generated BMDC from CB2R^{+/+} and CB2R^{-/-} littermates and matured them with CCP for 24 or 48h in the presence of varying concentrations of GP1a. GP1a inhibited MMP-9 production in a concentration dependent manner at both 24 and 48h in BMDC generated from CB2R^{+/+} mice (**Figure 18B**). In contrast, GP1a had no significant effect on MMP-9 production in CB2R^{-/-} mice. Interestingly, lack of this receptor resulted in significantly higher levels of MMP-9 production which was not due to increased BMDC proliferation (**Figure 18C**). This observation is suggestive of modulation of MMP-9 by endogenous cannabinoids in CB2R^{+/+} BMDC.

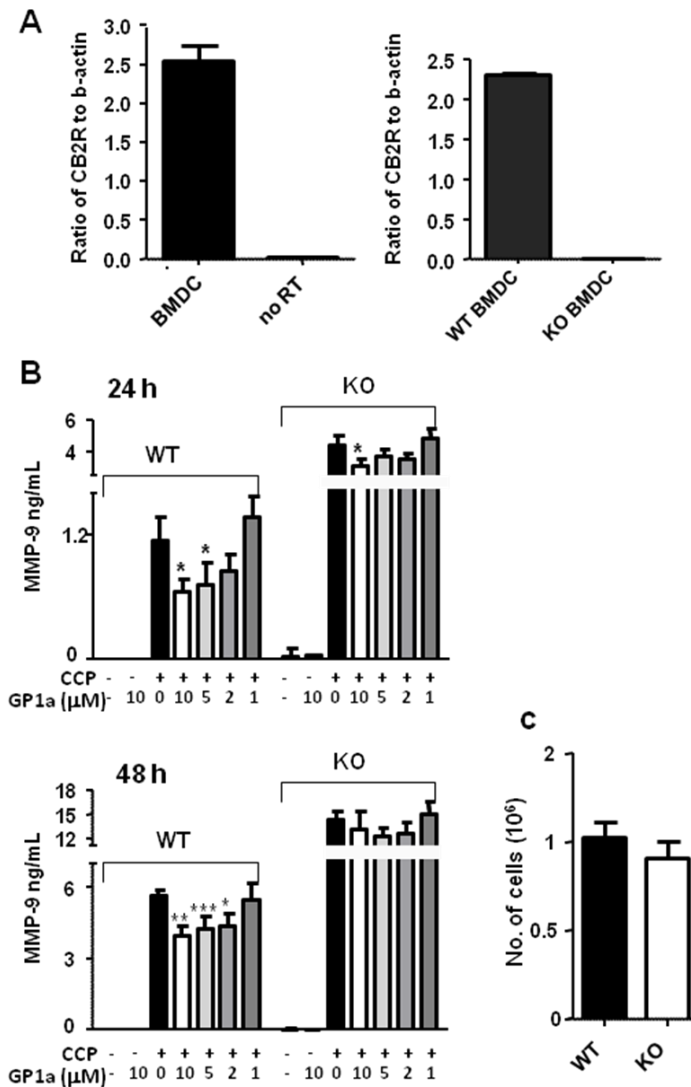


Figure 18. Inhibition of MMP-9 production by GP1a is mediated through CB2 receptor.

(A) RNA was extracted from purified $CD11c^+$ BM-DCs from $CB2R^{+/+}$ or $CB2R^{-/-}$ mice and subjected to RT-qPCR for CB2 receptor. A sample with no reverse transcription was included for the wt BM-DCs. Data are represented as ratio of CB2R to β -actin. (B) $CD11c^+$ BM-DCs from $CB2R^{+/+}$ or $CB2R^{-/-}$ mice were treated with CCP with or without various concentrations of GP1a for 24 and 48h timepoints and the culture supernatant was subjected to total MMP-9 ELISA. (C) Cells cultured with CCP were collected after 48h of culture and counted via hemocytometer. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with CCP. Data are representative of two (A) and three (B) independent experiments.

3.3.3. GP1a prevents DC migration through matrigel

MMP-9 facilitates DC migration through digestion of the extracellular matrix and of basement membranes underlying the blood vessels. To test the functional relevance of the GP1a-induced decrease in MMP-9 levels, we subjected DC to migration assays through transwells coated with matrigel which contains extracellular matrix components. DC matured for 24 or 48h with CCP in the presence or absence of GP1a were allowed to transmigrate in response to CCL19 through matrigel coated transwells. DC treated with GP1a migrated in lower numbers compared to control cells (**Figure 19A**). Migration through matrigel was dependent on active MMP-9 since the inclusion in the assay of an MMP-9 inhibitor abrogated the migratory capacity of control cells (**Figure 19B**). In agreement with the matrigel migration results, DC treated with 5 μ M GP1a secreted significantly lower amounts of MMP-9 at both 24 and 48h (**Figure 19C**).

Metalloproteinases are modulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Through its interactions with pro-MMP-9, the inducible TIMP-1 inhibits cell migration. Therefore, the inhibitory effect of GP1a on DC migration could be the result of TIMP-1 upregulation. We tested the effects of GP1a on TIMP-1 expression in DC and although TIMP-1 was upregulated following CCP activation, GP1a did not further increase TIMP-1 expression (**Figure 19D**).

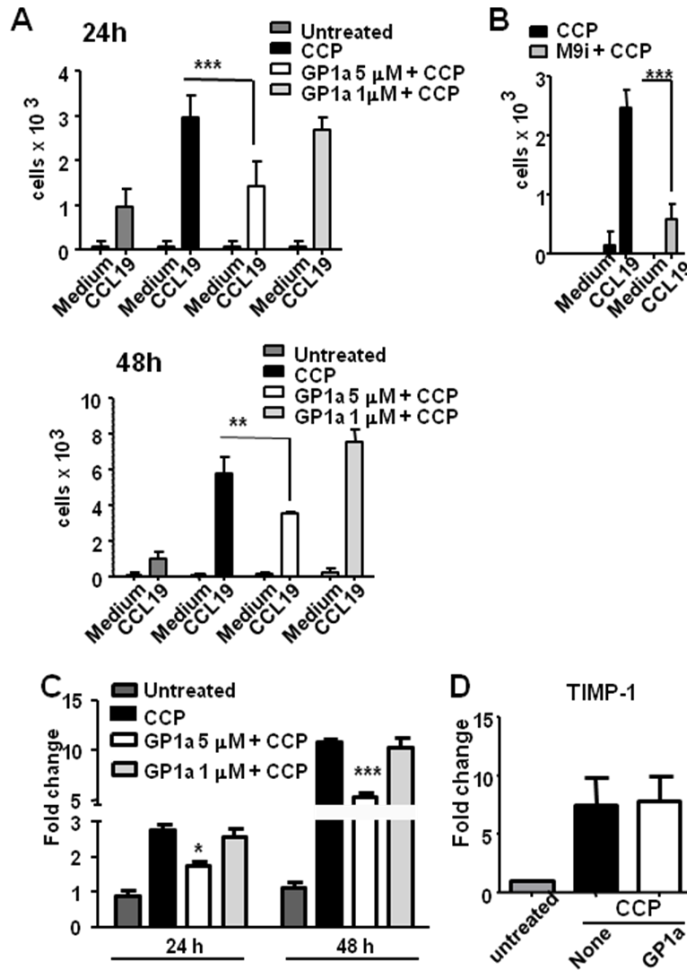


Figure 19. GP1a prevents matrigel migration of CCP treated DC.

(A-C) DC (1×10^5 cells) were placed in upper chambers coated with Matrigel. The bottom chambers were filled with serum free medium with or without CCL19. After 3h incubation at 37°C , the migrated cells were collected from the lower chambers and counted by FACS. (A) DC were treated with CCP with or without GP1a (1 μM and 5 μM) for 24 or 48h. (B) DC were treated with CCP with or without the MMP-9 inhibitor (M9i) for 24h. Data are representative of 3 independent experiments. (C) DC culture supernatants collected prior to migration assay were analyzed for MMP-9 production by ELISA. (D) BMDC were treated with CCP with or without CB2R agonists GP1a (5 μM) for 24h. RNA was extracted and subjected to qRT-PCR for MMP-9 inhibitor TIMP-1.

3.3.4. GP1a neither modulates DC CCR7 expression nor affects CCL19 induced chemotaxis.

Since chemokines and chemokines receptors play a major role in DC migration we wanted to see if decreased matrigel migration was partly due to decreased expression of chemokine receptor CCR7. The chemokines CCL19 and CCL21 constitutively expressed in lymph nodes act as ligands for the chemokine receptor CCR7 expressed on mature DC. To determine if CB2R signaling prevents CCR7 expression, we matured BMDC with the inflammatory cytokine cocktail consisting of TNF α +IL-6+IL-1 β and PGE2 (CCP), in the presence or absence of different concentrations of GP1a. CCP upregulated CCR7 mRNA expression as expected, whereas the GP1a treatment did not affect this increase (**Figure 19A**).

Since cannabinoids have been previously reported to inhibit macrophage migration to CCL5 through the trans-deactivation of CCR1/CCR5 without changes in chemokine receptor expression, we investigated the effects of GP1a on DC chemotaxis. Mature DC migrated in response to CCL19 and this migration was not affected by GP1a 5 μ M at either 24h or 48h (**Figure 19B**).

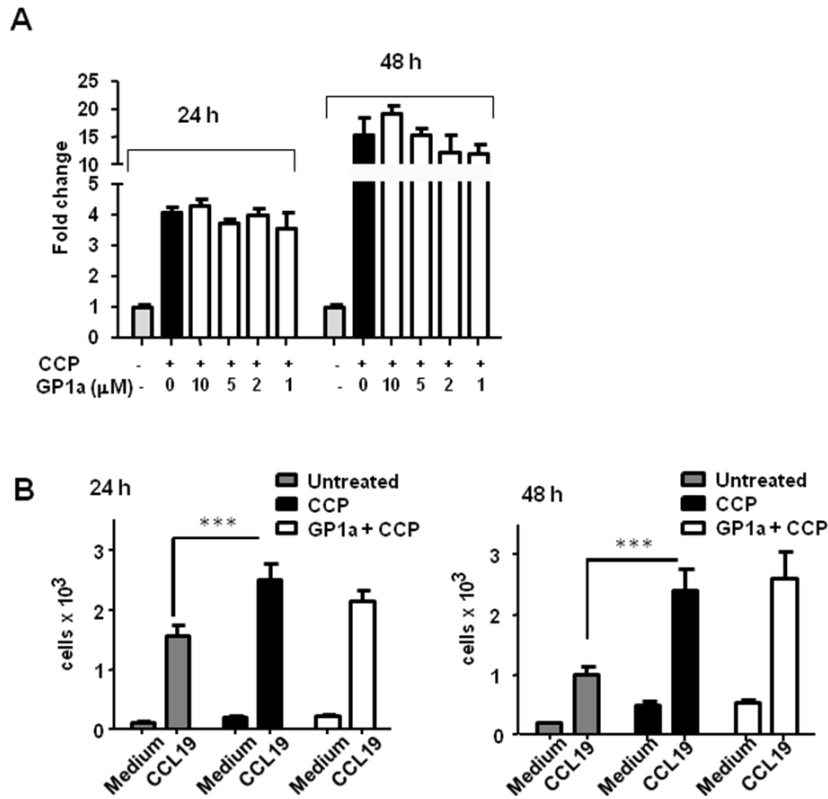


Figure 20. GP1a neither modulates expression of CCR-7 nor affects chemotaxis of mature DC to CCL19.

DC were treated with CCP with or without different concentrations of GP1a for 24 and 48h. Controls consisted of DC cultured in medium. (A) RNA was extracted and subjected to qRT-PCR for CCR7. (B) DC (1×10^5 cells) were placed in the upper chambers of a Transwell plate and the bottom chambers were filled with serum free medium with or without CCL19. 2h later the migrating cells were collected from the lower chambers and counted by FACS. *** $p < 0.001$ compared with medium. Data are representative of 2 independent experiments.

3.3.5. GP1a treated DC exhibit lower migratory capacity in vivo

Besides chemokine-chemokine receptors and MMPs, adhesion molecules also affect in vivo migration. CB2R agonist has been demonstrated to reduce LFA-1 on T cells in a model of experimental autoimmune ureovitis (EAU) [114]. To see if GP1a had a similar

effect on mature DC, we looked at expression of LFA-1 and L-selectin. L-selectin was upregulated upon maturation with CCP and GP1a treatment did not have any effect on it (**Figure 21A**). LFA-1 was expressed in untreated DC but was not upregulated upon maturation with CCP. There was no difference between GP1a treated and untreated cells (**Figure 21B**).

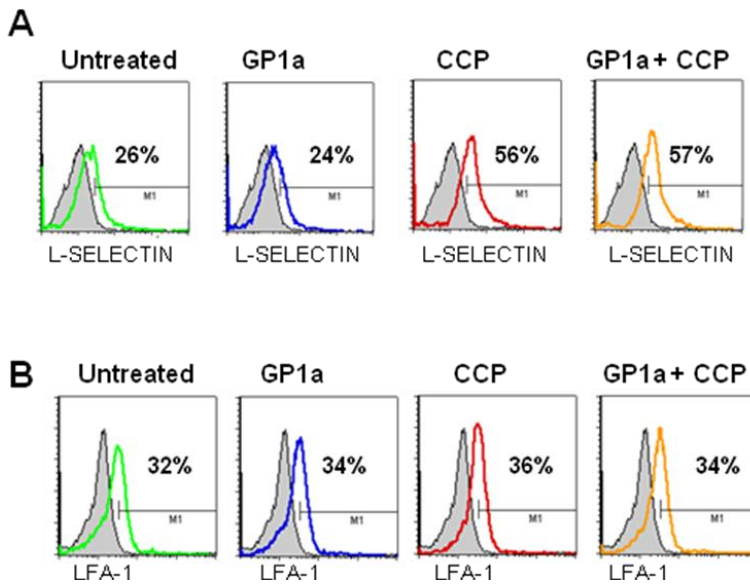


Figure 21. GP1a does not modulate expression of L-selectin or LFA-1.

(A-B) DC were treated with CCP with or without GP1a (5 μ M) for 48h. Cells were harvested and stained with antibodies against L-selectin and LFA-1 for 30 min and analyzed via FACS. Data are representative of 2 independent experiments.

Our in vitro results suggest that GP1a treated mature DC migrate less in the matrigel assay due to the inhibition of MMP-9 production. To investigate whether GP1a-treated DC exhibit reduced migration in vivo, we differentiated DC from CB2R^{+/+} and CB2R^{-/-} littermates and treated them with CCP in the presence or absence of GP1a. The cells were collected 48h later, labeled with PKH-26, and injected into the footpads of wt mice. Each mouse was injected with control DC in the right footpad and GP1a-treated DC in

the left footpad. Draining popliteal lymph nodes were harvested 48h later, and the numbers of labeled DC were determined by flow cytometry. Data from different experiments were normalized by plotting the number of fluorescent cells from control legs as 100%. GP1a treatment resulted in the recovery of lower numbers of CB2R^{+/+} DC. No reduction was observed for CB2R^{-/-} DC (**Figure 22A**). In agreement with these results, CB2R^{+/+} DC, but not CB2R^{-/-} DC treated with GP1a, secreted lower amounts of MMP-9 prior to inoculation into footpads (**Figure 22B**).

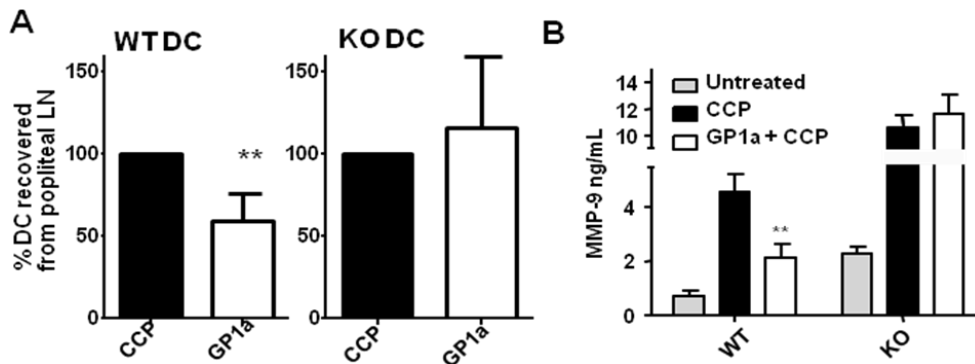


Figure 22. GP1a treated DC exhibit lower in vivo migratory capacity.

(A) DC generated from CB2R^{+/+} or CB2R^{-/-} littermates were treated with CCP with or without GP1a (5 μ M) for 48h, followed by labeling with the fluorescent dye PKH. 1×10^6 labeled DC were injected s.c in the footpads of wild-type mice pre-injected 24h earlier with 40 ng TNF α s.c in the each of the hind footpads. All recipient mice received CCP-treated DC (control) in the right footpad and CCP+Gp1a-treated DC in the left footpad. 48h later, cells were collected from popliteal lymph nodes and PHK-labeled cells were counted by FACS. Data from 3 different experiments were normalized by considering the numbers of labeled cells from the control (right leg) as 100%. (B) DC culture supernatants collected prior to injection were analyzed for MMP-9 production by ELISA.

3.4. CB2R agonist inhibits PGE2 induced MMP-9 by reducing cAMP levels, thus reducing cAMP induced ERK phosphorylation and subsequent c-Fos and c-Jun binding to the AP1 sites in the MMP-9 promoter

3.4.1. PGE2 is the main contributor to MMP-9 induction by cytokine cocktail treatment of DCs and GP1a treatment reduces MMP-9 levels

Although CCP is an excellent inducer of MMP-9 in DC, the signaling pathways leading to MMP-9 expression are complicated by the presence of different cytokines and PGE2. In previous studies we reported that PGE2 is the major MMP-9 inducer. This was confirmed by comparisons between the effects of the cytokine cocktail without PGE2 (CC) and with PGE2 (CCP) (**Figure 23A**). Therefore, we tested the effect of GP1a on PGE2-induced MMP-9 production, and determined that, similar to the effects on CCP-treated DC, GP1a reduced MMP-9 production in PGE2-treated DC (**Figure 23B**).

3.4.2. GP1a reduces PGE2-induced MMP-9 in DC via inhibition of cAMP induction

The major PGE2 receptors expressed in BMDC are EP2 and EP4 [148] which signal through activation of adenylate cyclase resulting in increased cAMP levels [149]. Cannabinoid signaling through CB2R was reported to inhibit early cAMP induction in macrophages and T cells [112]. To investigate whether CB2R activation in our experimental system affects cAMP levels, we activated DC with PGE2 in the presence or absence of GP1a for various time periods and measured cAMP levels via an ELISA assay. GP1a treatment resulted in lower levels of cAMP, reaching statistical significance at 30 and 60 min post treatment (**Figure 23C**). These results led us to hypothesize that

the effects of CB2R signaling on MMP-9 expression might be mediated through a reduction in cAMP. To test this hypothesis, we assessed the effect of GP1a in the presence of dibutyryl cAMP (dbcAMP), a stable exogenous cAMP analog. Although GP1a reduced PGE2-induced MMP-9, it did not affect MMP-9 production induced by dbcAMP (Figure 23D).

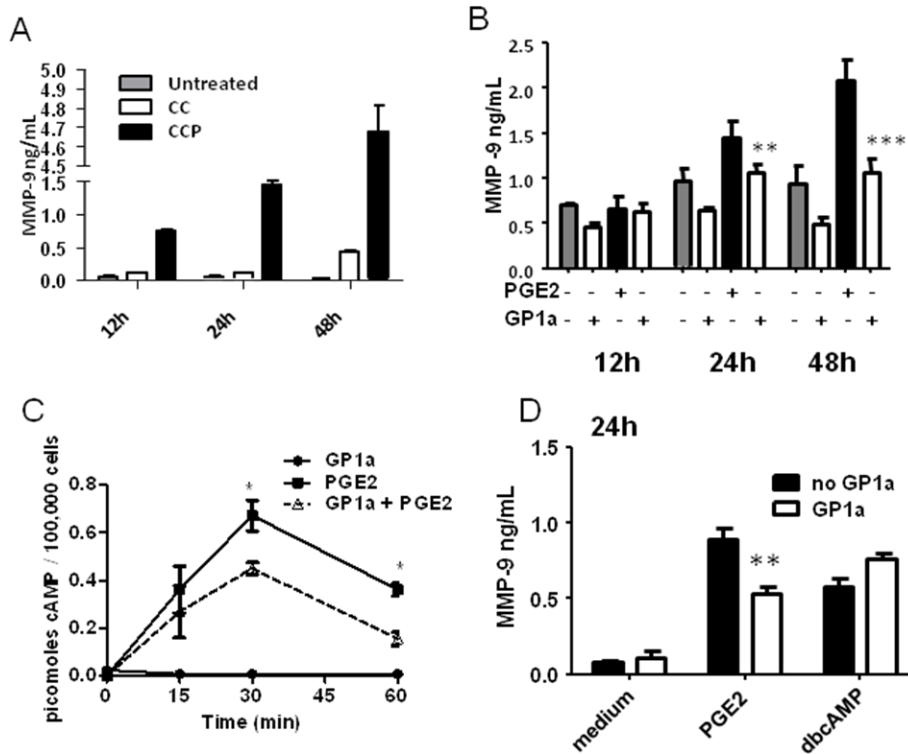


Figure 23. GP1a reduces PGE2-induced MMP-9 in DC through inhibition of cAMP induction.

(A) DC were treated with cytokine cocktail without PGE2 (CC) or with cytokine cocktail plus PGE2 (CCP). Culture supernatants were collected at different timepoints and analyzed for MMP-9 by ELISA. (B) DC were treated with PGE2 (0.1 μ M) with or without GP1a (5 μ M). Culture supernatants were collected at different timepoints and analyzed for MMP-9 by ELISA. (C) DC (1x10⁵ cells) were treated with PGE2 with or without GP1a (5 μ M). Cells lysates were collected at different timepoints and analyzed for cAMP by ELISA. (D) DC were treated with PGE2 or dibutyryl cAMP (10 μ M) with or without GP1a (5 μ M). After 24h, culture supernatants were collected and analyzed for MMP-9 via ELISA. Data are representative of 3 independent experiments.

This confirms the previously described role of cAMP in MMP-9 expression in DC [75], and suggests that the inhibitory effect of CB2R signaling is mediated through a reduction in cAMP levels.

3.4.3. GP1a reduces PGE2-induced ERK phosphorylation but not NFκB p65

We showed previously that MMP-9 expression induced by PGE2 is mediated through the activation of protein kinase A (PKA), a well-known cAMP target, followed by subsequent ERK1/2 phosphorylation [79]. GP1a reduced PGE2-induced ERK phosphorylation as determined by flow cytometry (20 min post-treatment) and Western blot (**Figures 24A and B**).

Since NFκB is a major transcription factor involved in MMP-9 expression, we also evaluated the effect of GP1a on CCP induced phosphorylation of p65 subunit of NFκB by flow cytometry and IκBα degradation via western blot. IκB binds to NF-κB proteins (including p65) and keeps them sequestered in an inactive state in the cytoplasm. Phosphorylation marks IκB for ubiquitination, thus releasing NF-κB proteins from the complex, allowing their (p65) phosphorylation and subsequent translocation into the nucleus. We did not observe any difference in p65 phosphorylation or IκB degradation between CB2R treated or untreated cells (**Figures 25A and B**). CB2R agonist does not modulate CCP induced NFκB activation.

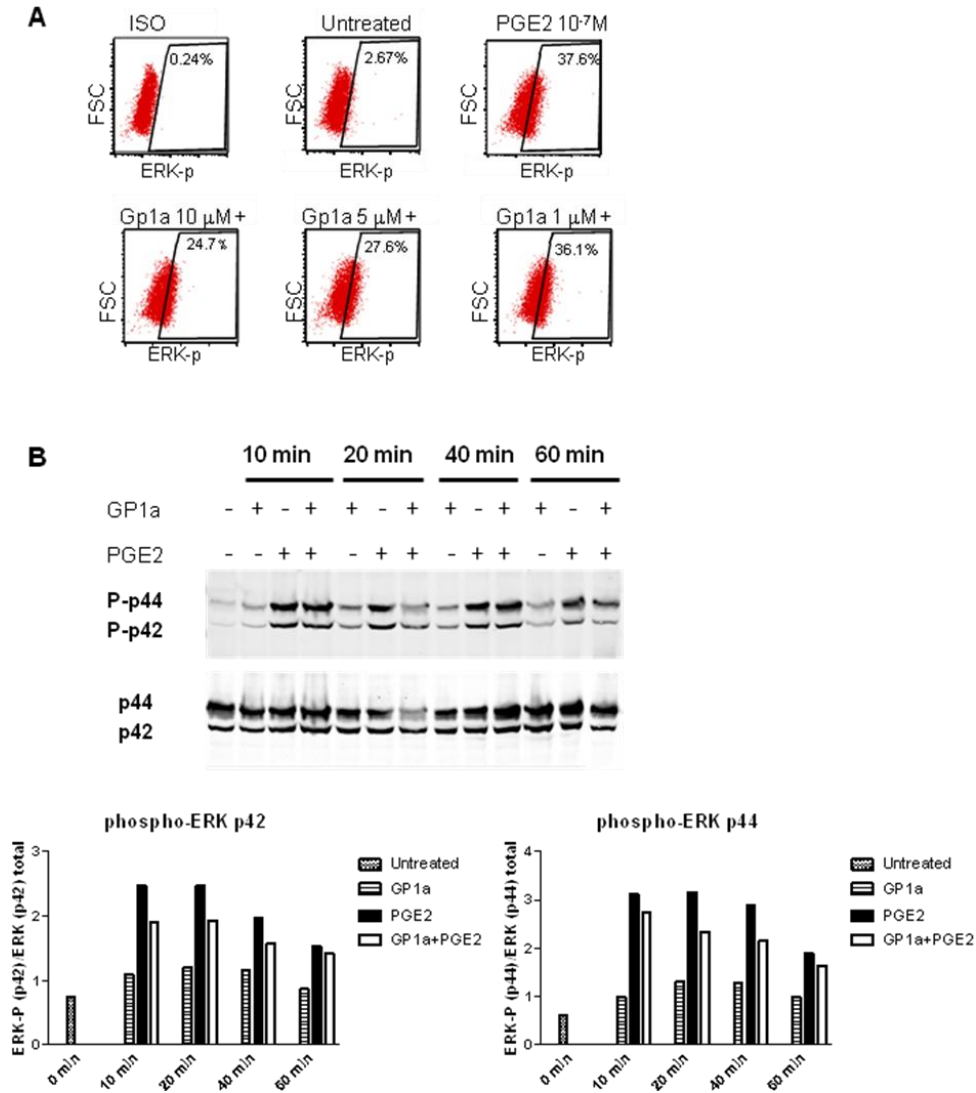


Figure 24. GP1a treatment reduces PGE2 induced ERK phosphorylation.

(A) DC were treated with PGE2 with or without different concentrations of GP1a for 20 min. Cells were fixed, permeabilized and analyzed by intracellular staining with phospho-ERK antibody via FACS. (B) DC were treated with PGE2 with or without GP1a (5 μ M) for various timeperiods. Cells were lysed and analyzed for phosphorylation of ERK by western blot. Densitometric analyses are plotted in graphs normalizing phospho-ERK to total ERK. Data are representative of 2 (B) and 3 (A) independent experiments.

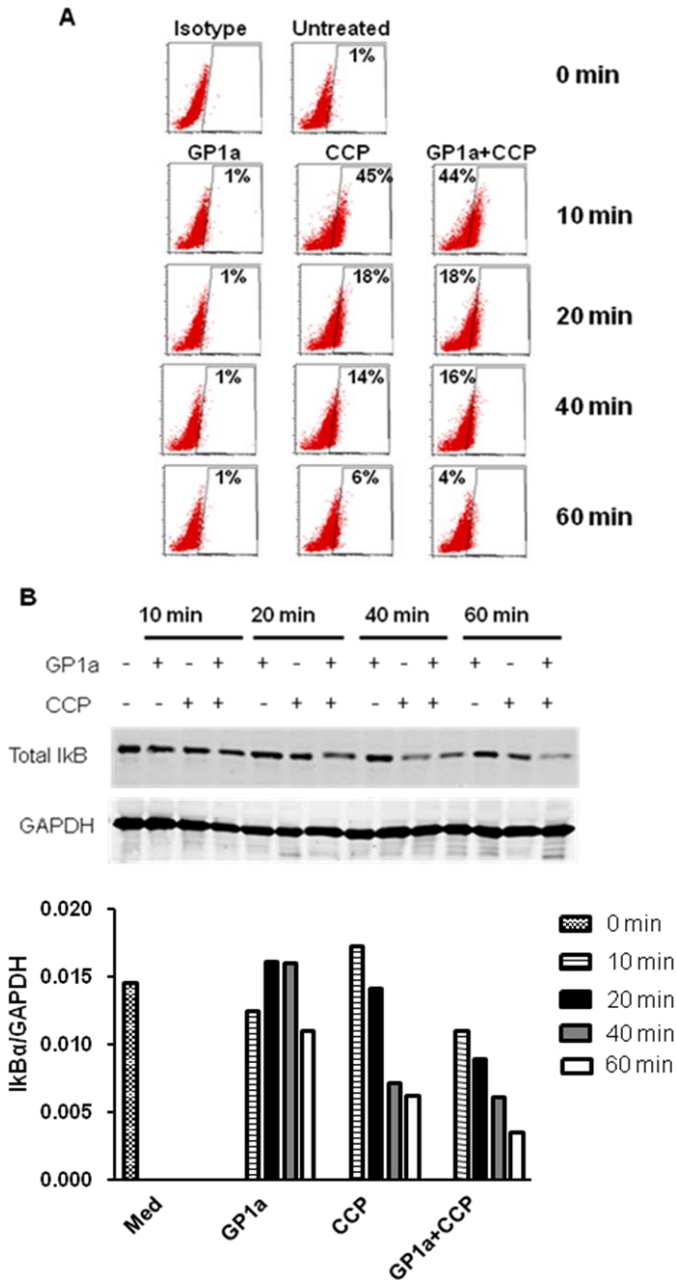
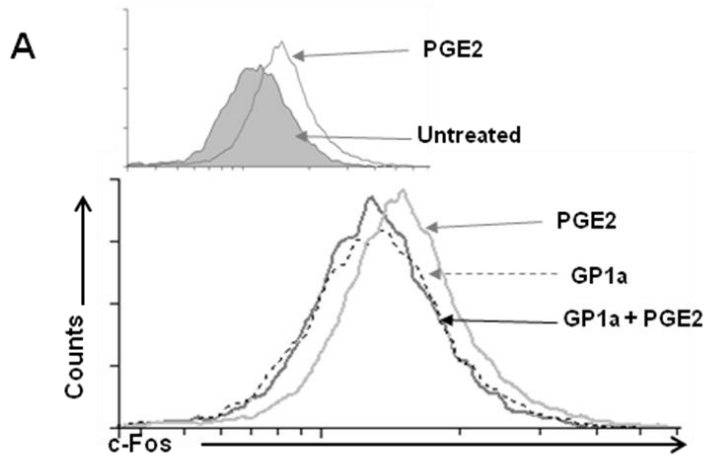


Figure 25. GP1a treatment affects neither CCP induced p65 phosphorylation or IκBα degradation.

(A) DC were treated with CCP with or without GP1a (5 μ M) for various periods of time. Cells were fixed, permeabilized and analyzed by intracellular staining with phospho-p65 antibody via FACS. (B) DC were treated as described in A. Cells were lysed and analyzed for IκB by western blot. Densitometric analyses are plotted in graphs normalizing IκB to GAPDH. Data are representative of 2 (B) and 1 (A) independent experiments.

3.4.4. GP1a decreases total c-Fos and phospho c-Jun and subsequent binding of c-Fos and c-Jun to the AP-1 site on the MMP-9 promoter

We have previously shown that PGE2 induces MMP-9 via a cAMP-dependent pathway, leading to ERK phosphorylation and subsequent activation and binding of c-Fos and c-Jun to AP-1 binding sites on the MMP-9 promoter [79]. Here, we treated DC with PGE2 in the presence or absence of GP1a and measured the amounts of c-Fos via flow cytometry and of phosphorylated c-Jun via Western blot. GP1a decreased both the amount of c-Fos and the level of phospho-c-Jun (**Figures 26A and B**). The MMP-9 promoter has two AP-1 binding sites, and we have shown previously that PGE2 induces binding of c-Fos/c-Jun to the AP-1 sites on the MMP-9 promoter. We used ChIP assays to determine whether GP1a affects c-Fos/c-Jun binding to the MMP-9 promoter. DC were treated with PGE2 in the presence or absence of GP1a for 1h and cell lysates were immunoprecipitated with anti-c-Fos, anti-c-Jun, or anti-GST antibody as negative control, followed by real-time PCR amplification using primers encompassing the distal AP-1 site in the murine MMP-9 promoter. As expected, PGE2 increased c-Fos and c-Jun binding to the AP-1 site. In contrast, treatment with GP1a led to a significant decrease in binding of both c-Fos and c-Jun (**Figures 27A and B**).



B

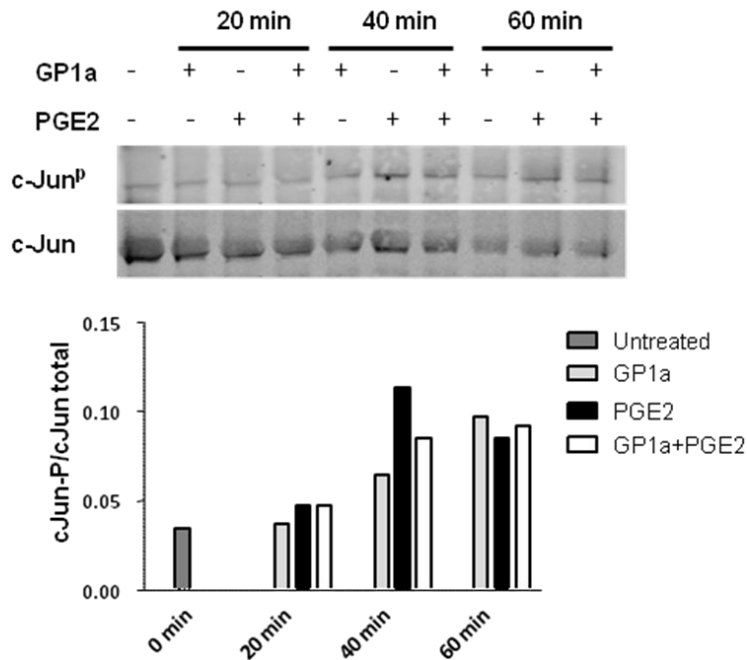


Figure 26. GP1a treatment reduces both c-Fos induction and c-Jun phosphorylation.

(A) DC were treated with PGE2 with or without GP1a (5 μ M) for 40 min. Cells were fixed, permeabilized and analyzed for c-Fos by intracellular FACS staining. (B) DC were treated with PGE2 with or without GP1a (5 μ M) for various time periods and analyzed for phosphorylation of c-Jun by western blot. Densitometric analyses are plotted in graphs normalizing phospho-c-Jun to total c-Jun. Data are representative of 2 (B) and 3 (A) independent experiments.

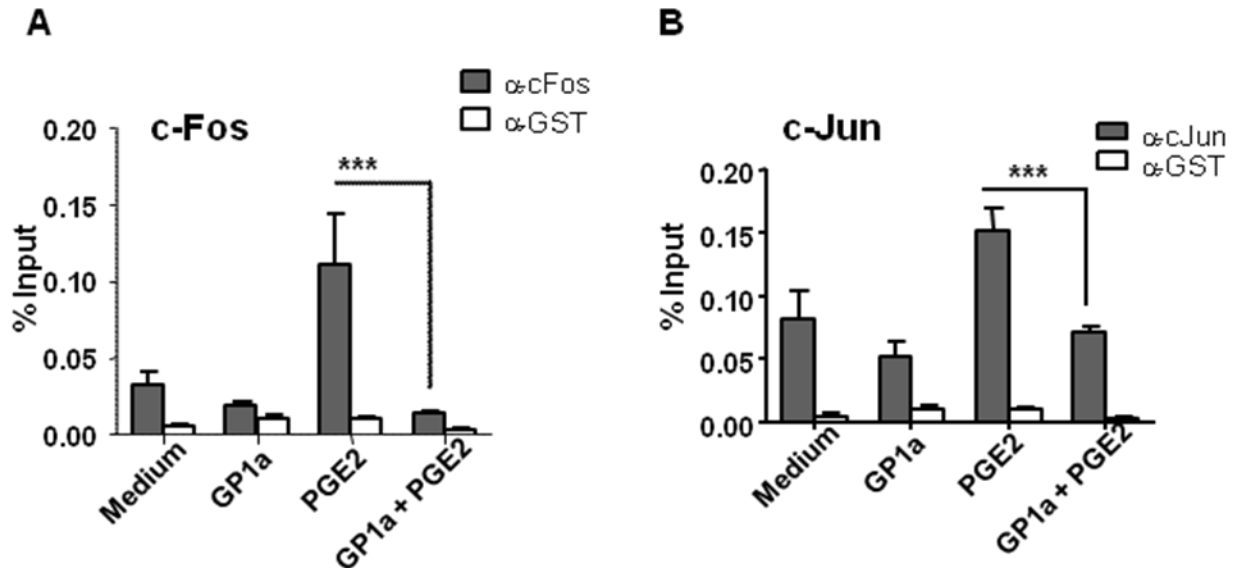


Figure 27. GP1a leads to decreased binding of c-Fos and c-Jun to the AP-1 site on MMP-9 promoter.

*DC were treated with PGE2 with or without GP1a (5 μ M) for 1h. Cells were fixed, sonicated and subjected to ChIP analysis using antibodies to c-Fos (A), c-Jun (B) or control IgG. Precipitated DNA was isolated and evaluated by PCR using specific primers for the distal AP-1 site in the MMP-9 promoter. *** $p < 0.001$ compared with PGE2. Data are representative of 2 independent experiments.*

CHAPTER 4

DISCUSSION

In spinal cord damage, the secondary injury that follows the physical damage to the cord is a major contributor to long term damage and spinal cord dysfunction. Secondary injury consists of prolonged neuroinflammatory responses and a dysregulated blood spinal cord barrier (BSCB), which prevent repair of the cord and lead to neuronal and glial apoptosis [36, 150, 151]. In recent years considerable efforts have been made towards modulation of inflammation in secondary injury. However, generalized broad based suppression of inflammation, as achieved by corticosteroids, has not been beneficial [43]. Although there is significant evidence that the inflammation contributes to secondary damage, components of the inflammatory response have also been shown to play an important role in enhancing recovery and promoting neuroregeneration [152-155]. Therefore, an appropriate therapeutic strategy following spinal cord injury would be selectively inhibiting the detrimental components of inflammation while sparing or enhancing the beneficial components. However, since the role played by the various inflammatory elements is not known it has been hard to tailor an appropriate therapy.

Manipulation of the endocannabinoid system has been one of the areas of study in recent years. Previously Baty et al showed that administration of a selective CB2R agonist 1 hour prior to spinal cord injury resulted in improved motor and bladder function over the 14 day period of observation. In this study, we show that even treatment 1 h post injury results in similar results. Autonomic dysfunction causing a loss of bladder control

following spinal cord injury is a major source of morbidity in spinal cord injured patients. Therefore, the return of spontaneous bladder emptying in the animals treated with the CB2R agonist was impressive. It is not known if the CB2R agonist leads to bladder function recovery by reducing secondary injury and preserving more axons, or by directly acting on the bladder.

Since increased production of proinflammatory cytokines and chemokines have been reported to be associated with enhanced injury, the effect of treatment with the CB2R agonist on cytokine/chemokine upregulation was investigated through real-time PCR. Six hours post spinal cord injury a number of chemokines and cytokines were upregulated in the injured cords compared to cords from sham operated animals. Several of these were reported in various studies [156, 157]. However, the CB2R agonist treatment did not have any effect on most of these molecules.

At 48 hours post-injury, most of the mediators upregulated at the earlier timepoint, had been reduced to baseline levels and a different set of chemokines and cytokines were upregulated. The effects of the CB2R agonist treatment at 48h were more striking. At this later timepoint, the CXCL10 family of chemokines, which includes CXCL9, 10, and 11, were upregulated in injured cords compared to sham. CB2 agonist treated cords showed a significantly lower expression of all three chemokines, which function as chemoattractants for activated T cells, especially the IFN γ producing T helper type 1. CXCL10 upregulation has been shown to correlate with increased immune cell infiltration into the damaged cord [158]. Treatment with neutralizing antibody against CXCL10 resulted in reduced neuroinflammation as well as significant reduction in

neuronal loss and apoptosis, and better recovery of locomotor functions [159-161]. CB2 treatment also resulted in a dramatic reduction of IL-23p19 expression and of its receptor IL-23R. These results indicate a potential for CB2 treatment to influence CD4 T-cell differentiation, with a reduction in the number of IL-17 producing T-cells (Th17) which depend on IL-23. Recent reports have demonstrated that Th17 cells establish immune-neuronal synapses and induce neuronal dysfunction via sustained calcium oscillations [162]. Beside CD4 T cells, IL-23 together with IL-1 β can directly activate $\gamma\delta$ T cells to produce IL-17 in the absence of antigen presentation or T cell receptor stimulation. Though their role in SCI is not known, $\gamma\delta$ T cells are reported to play a pivotal role in the delayed phase of brain ischemia [163]. The role of different subtypes of T cells in SCI is not known. However T-cells have been reported as important contributors to the exacerbation of spinal cord injury [158]. Intervention into signals that recruit or activate T cells may provide another strategy in treating SCI.

In a previous study, CB2R agonist treatment 1h prior to spinal cord injury resulted in decreased TNF α expression [122]. In our experimental system, where we administer CB2R agonists 1h post injury, we did see high expression of TNF α as well as IL-6 and IL-1 β 6h post injury but the CB2R treatment made no difference. All three cytokines TNF α , IL-6, and IL-1 β are already expressed as early as 1h post injury in murine models of SCI [30]. Therefore, the post SCI treatment may not modulate these early molecules, but have an effect on molecules expressed later on in SCI.

The most interesting result was the effect of CB2R agonist on the expression of Toll-like receptors. Toll-like receptors, originally identified as reacting to pathogen-associated

molecular patterns from invading organisms, have recently been shown to also be activated by endogenous molecules called danger-associated molecular patterns (DAMPs) [164-168]. A number of DAMPs, including heat shock proteins, necrotic cells, degeneration products of extracellular matrix molecules, mRNA and high mobility group box 1 can be formed following injury to the spinal cord and can activate TLR-2 and TLR-4 [33]. In the injured CNS, the majority of Toll-like receptors are located on monocytes/macrophage and microglia [169, 170]. In this study, at 6 hours post injury we saw upregulation of TLR-2 and treatment with CB2R agonist had no effect. However at 48 hours post injury, we saw upregulation of TLR-1, TLR-4, TLR-6, and TLR-7 and the CB2R agonist significantly reduced their expression. In a mouse model of spinal cord injury, it was demonstrated that both astrocytes and microglia/monocytes contribute to TLR2 expression while TLR4 was primarily restricted to microglia/macrophage [33]. Activation of Toll-like receptors results in NF κ B signaling and the production of pro-inflammatory cytokines [171-174]. However, animals deficient in TLR-2 and TLR-4 show worse recovery after spinal cord injury and increased monocyte/macrophage infiltration. In our study, the CB2R agonist treatment resulted in reduced numbers of monocytes/macrophage infiltration into the injured cord at 7 days post injury. Unlike the TLR4 deficient animals, CB2R agonist treatment only attenuated injury induced increases in TLR-4 receptor expression. The reduction observed in TLR expression correlated with decrease in immunoreactive microglia, suggesting that the CB2R agonist is partially exerting its effect through inhibition of microglia activation.

Unlike spinal cord injury, experimental autoimmune encephalomyelitis (EAE) is a chronic neuroinflammatory disease which shares many characteristics with multiple

sclerosis. Similar to MS, EAE is characterized by demyelination caused by inflammatory infiltrates in the CNS and demyelination correlates with the onset of the clinical manifestations. Since EAE is induced through active immunization, it can be attenuated at two steps: activation of peripheral immune cells and their migration into the CNS. In these experiments, mice were treated with CB2R agonists 7 days after immunization, and the treatment resulted in reduced numbers of peripheral hematopoietic cell infiltrates into the CNS. There was a reduction in the number of monocytes/macrophage and T cells in both brain and the spinal cord on day 17, which corresponds to the peak of disease. Interestingly, this effect was maintained for a longer period of time, as observed on day 30 post immunization. Treated mice showed reduced levels of VCAM-1, TLR-4, and MMP-2 and 9 expression in the CNS prior to peak of the disease. These findings correlated with a lower number of infiltrating cells and suggested that CB2R agonists may affect the migration of immune cells by modulating adhesion molecules on endothelial cells, activation of innate immune cells in the CNS, and expression of gelatinases required for the breakdown of glia limitans. This finding agrees with a study that found CB2R agonist O-1966 to inhibit the upregulation of VCAM-1 in LPS induced encephalitis [130]. Since myeloid cell derived gelatinases are essential for EAE induction [62], we investigated the effects of CB2R agonists on expression of the MMP-2 and MMP-9 on myeloid cells.

Among the two gelatinases, MMP-2 is constitutively expressed by myeloid cells whereas MMP-9 is induced in inflammatory conditions [89]. We mimicked the inflammatory environment by treating myeloid cells with a cytokine cocktail consisting of TNF α , IL-6, and IL-1 β plus prostaglandin E2 (PGE2) [175]. This treatment has been shown to mature

DC and induce high levels of MMP-9 in macrophages and DC [73, 82]. In agreement with previous reports, we saw an upregulation of MMP-9 expression in bone marrow derived macrophages (BMM Φ), and bone marrow derived DC (BMDC) matured with the cytokine cocktail, and treatment with various CB2R agonists inhibited this upregulation. MMP-2 expression was not upregulated upon activation of these cells. However, in neonatal microglial cultures expression of both MMP-9 and MMP-2 were upregulated upon activation and treatment with CB2R agonist GP1a had an inhibitory effect on both MMPs.

There are few reports on cannabinoid effects on gelatinases, especially MMP-2. CB2R agonists were shown to inhibit MMP-2 expression in gliomas, and MMP-9 expression in activated T cells [132, 137]. The inhibitory effect of cannabidiol, a non-CB1R, non-CB2R ligand, on tumor cell invasion has been attributed in some cases to the induction of TIMP-1, and subsequent inhibition on MMPs [176]. However, other reports show downregulation of both MMP-2 and TIMP-1 in human glioma cells by CB2R agonists [137, 177]. We compared expression of TIMP-1 between DC matured with cytokine cocktail and PGE2 in the presence of absence of CB2R agonist and found no difference and therefore, ruled out the involvement of TIMP-1 as a factor in MMP-9 modulation by CB2R agonist.

MMP-9, secreted primarily by activated DC and macrophages, plays an essential role in immune cell migration by degrading the extracellular matrix and basement membranes through cleavage of collagen IV. MMP-9 can play a critical role in DC function especially in EAE. Their initial activity requires migration to the nearest lymph node

where they act as antigen-presenting cells and activate cognate naïve T cells. The second activity requires migration to the CNS to reactivate encephalitogenic T cells and to facilitate immune cell infiltration into the CNS parenchyma [141]. The role of MMP-9 in DC migration to inflammatory sites, draining lymph nodes, and out of brain capillaries has been demonstrated recently [74, 178, 179]. MMP-9 deficient mice were demonstrated to have impaired migration of Langerhans cells to the lymph nodes [180].

Given the importance of the capacity of dendritic cells to migrate, we examined the effects of the CB2R agonist GP1a on DC migration. In an in vitro matrigel migration assay, GP1a reduced migration of mature DC to CCL19 but it had no effect on chemotaxis in the absence of matrigel. When DC were subjected to an in vivo migration assay, GP1a treated cells migrated less to the draining lymph node, though there was no difference in expression of CCR7, the chemokines receptor for CCL19. In contrast, reduced in vitro matrigel and in vivo migration correlated with reduced levels of MMP-9 production. Besides chemokines and MMPs, adhesion molecules also affect leukocyte migration. Administration of CB2R agonists in EAE and ischemia/reperfusion injury has been shown to reduce leukocyte rolling and adhesion to brain microvessels [118, 121, 181] and shown to correlate with a reduction in the expression of the adhesion molecules P-selectin glycoprotein ligand 1 and of LFA-1 on T cells in a model of experimental autoimmune uveoretinitis [114]. In contrast, we did not observe any differences in DC expression of L-selectin and LFA-1 following treatment with GP1a.

Previously, we reported that the stimulatory effect of PGE2 on MMP-9 expression in DC was mediated through the EP2/EP4→cAMP→PKA/PI3K→ERK signaling pathway,

leading to AP-1 activation and binding to the MMP-9 promoter [79, 82]. Here we show that CB2R signaling interferes with the stimulatory pathway at an early point, by reducing cAMP levels and subsequent ERK and AP-1 activation (**Figure 28**). This is in agreement with the fact that CB2R are $G\alpha_{i/o}$ -protein coupled receptors whose primary function is the inhibition of adenylate cyclase [182].

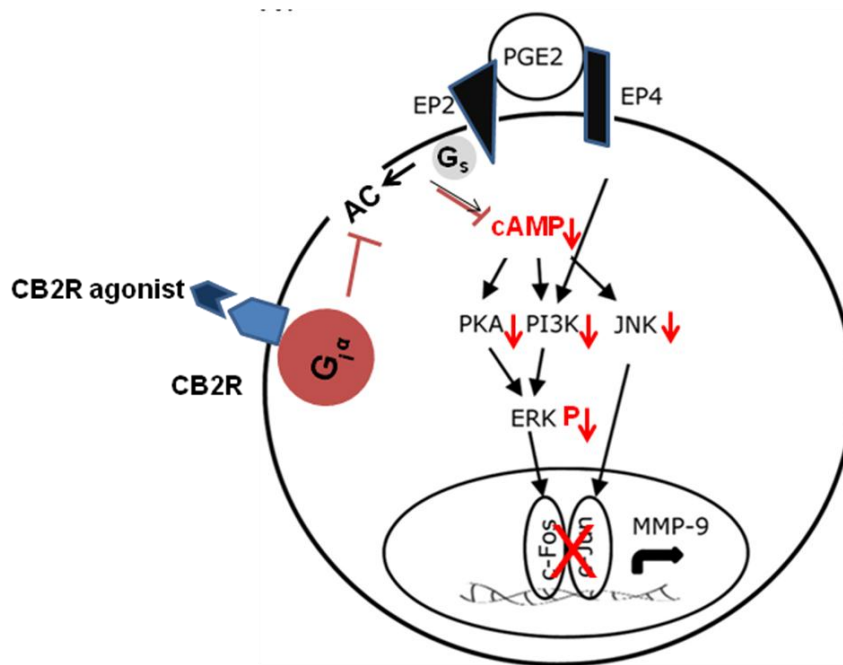


Figure 28. Proposed model of CB2R agonist's mode of action in inhibition of MMP-9.

PGE2 binds to EP2/EP4 receptor and activates adenylate cyclase via $G_{\alpha s}$ and results in increased levels of cAMP. cAMP induced protein kinase A and PI3K activation leads to increased ERK phosphorylation, and cFos induction. cAMP also leads to Jun kinase and subsequent cJun phosphorylation. cFos and cJun dimerize and colocalize to the nucleus and bind to AP-1 sites on the MMP-9 promoter. CB2 receptor activation inhibits PGE2 induced adenylate cyclase activation via $G_{\alpha i}$, which leads to subsequent inhibition of cAMP induction and PKA, PI3K, and JNK activation resulting in decreased c-Fos/c-Jun binding to the AP-1 site on MMP-9 promoter and suppression of MMP-9 expression. Adapted from Yen et al. [79].

Though NF κ B plays a role in inducing MMP-9, CB2R agonist did not have an effect on this pathway. Interestingly, in bone marrow derived DC AP-1 sites were found to play a major role with minor contributions by NF κ B [79]. As we showed in this study the cytokine cocktail alone induced very low levels of MMP-9 which was vastly increased upon the addition of PGE2. There are reports of MMP-9 induction in DC via other pathways [74, 183], but the question whether endogenous PGE2 contributed to MMP-9 induction has not been addressed in these studies. During inflammation high levels of PGE2 are induced and in such conditions, we expect that CB2R agonists would exert a beneficial effect by inhibiting MMP-9 expression.

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APPENDIX

Abbreviations

2-AG, 2-arachidonoylglycerol;

AP-1 activator protein-1;

APC, antigen presenting cells;

ATP, adenosine triphosphate;

BBB, blood-brain barrier;

BMDC, bone marrow derived dendritic cells;

BMM Φ , bone marrow derived macrophages;

cAMP, Cyclic adenosine monophosphate;

CB, cannabinoid;

CB1R, cannabinoid type 1 receptor;

CB2R, cannabinoid type 2 receptor;

CC, cytokine cocktail consisting of TNF α , IL-1 β , IL-6, without PGE2;

CCL, chemokine (C-C motif) ligand; CCP, cytokine cocktail consisting of TNF α , IL-1 β , IL-6, with PGE2;

CCR, Chemokine (C-C motif) receptor;

CSF, cerebrospinal fluid;

CXCL, chemokine (C-X-C motif) ligand;

CXCR, Chemokine (C-X-C motif) receptor;

CX3CL, chemokine (C-X3-C motif) ligand;

CX3CR, Chemokine (C-X3-C motif) receptor;

CD, cluster of differentiation;

cDC, conventional DC;

CLP, common lymphoid progenitor;

CLR, C-type lectin receptor;

CMP, common myeloid progenitors;

CNS, central nervous system;

DC, dendritic cell;

EAE, experimental autoimmune encephalomyelitis;

ERK, Extracellular signal-regulated kinase;

FACS, fluorescence-activated cell sorting;

FITC; fluorescein isothiocyanate;

FBS, Fetal Bovine Serum;

Flt3, fms-like tyrosine kinase 3;

Flt3L, fms-like tyrosine kinase 3 ligand;

GA, glatiramer acetate;

GM-CSF, granulocyte-macrophage colony-stimulating factor;

Hi, high;

HMGb1, High-mobility group protein B1;

hMo-DC, human monocyte-derived DC;

HRP, horseradish peroxidase;

HSC, hematopoietic stem cells;

ICAM -intercellular adhesion molecules;

IFN, interferon;

I κ B, inhibitor of kappa B;

IL, interleukin;

iNOS, inducible nitric oxide;

ISO, isotype;

JNK, c-Jun N-terminal kinases;

LFA-1, Lymphocyte function-associated antigen 1;

Lo, low;

LP, lymphoid committed precursors;

LPS, lipopolysaccharide;

Ly6C, lymphocyte antigen 6 complex, locus C;

KO, knock out;

MBP, myelin basic protein;

M-CSF, macrophage colony-stimulating factor;

MDP, Monocyte/ DC progenitors;

MED, medium;

MHC, major histocompatibility complex;

MMP, matrix metalloproteinase;

MOG, myelin oligodendrocyte glycoprotein;

MP, myeloid committed precursors

MS, multiple sclerosis;

NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells;

PAMP, pathogen associated molecular patterns;

pDC, plasmacytoid DC;

PGE2, prostaglandin E2;
PLP, proteolipid protein;
ROS, reactive oxygen species;
SCI, spinal cord injury;
S1P1R, sphingosine-1-phosphate receptor;
STAT, signal transducer activator of transcription;
TCR, T cell receptor;
Th, T helper cells;
THC, delta-9-tetrahydrocannabinol;
TIMP, tissue inhibitor of metalloproteinase;
TLR, toll-like receptor;
TMB, tetramethylbenzidine;
TNF, tumor necrosis factor;
VCAM, vascular cell adhesion molecule;
VLA-4, Very Late Antigen-4;
WT, wild type;