The role of microglia in neurodegeneration and endogenous repair during experimental autoimmune encephalomyelitis

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Faculty of Biology
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from
Master of Science in Biochemistry Sarah-Christin Starossom
Born on May 10th 1983 in Leverkusen (Germany)

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Dedicated

to my parents

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2. INTRODUCTION

2.1. The nervous System

The nervous system of vertebrate animals, including humans and mice, comprises the central nervous system (CNS) and the peripheral nervous system (PNS). The central nervous system includes the brain and spinal cord, whereas the peripheral nervous system comprises the somatic and autonomic PNS.

Sensory components of the nervous system, such as the sensory nerves of the somatic PNS or the cranial nerves of the CNS supply information about the internal and external environment to the CNS. The spinal cord can integrate sensory information directly as it is the case with simple reflexes, such as the withdrawal reflex. The majority of sensory information, however, is processed in the brain and is ultimately translated into action by the motor components of the CNS and PNS.

2.1.1. The central nervous system

The brain can be anatomically divided into forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon).

The forebrain consists of two major divisions: diencephalon and telenecephalon. The diencephalon, which contains the thalamus and hypothalamus, is responsible for processes, including the control of motor and autonomic functions, whereas the telencephalon contains the cerebral cortex, where most of the brains information processing such as decision-making, problem-solving, memory, emotion and language take place. It also harbors a part of the brains' ventricular system, the lateral ventricle (LV), where cerebrospinal fluid (CSF) circulates.

The midbrain and the hindbrain make up the brainstem. The mesencephalon is the portion of the brainstem that connects the hindbrain and the forebrain and is responsible for processes involved in the regulation of sensory functions (vision and hearing) as well as the regulation of motor functions. The inferior portion of the brainstem, the hindbrain, extends from the spinal cord and comprises of the metencephalon and the myelencephalon, which regulate functions such as breathing and heart rate as well as sleep and fine muscle movement.

Both brain and spinal cord are enveloped by meninges, a membrane system, that primarily acts as protection for the CNS.

The CNS is an organ with high metabolic rate, and thus highly vascularized, to ensure sufficient supply of oxygen and glucose. In order to shield it from potentially toxic circulating molecules and in its status as an immune privileged organ, brain and spinal cord are specifically protected by the blood-brain barrier (BBB). Tight junctions between neighboring capillary endothelial cells create a barrier that restricts “leakage” of molecules from the vascular space into the brain tissue. Thus, in order to traverse the wall of brain capillaries, substances have to either move through the endothelial cell membranes or be actively transported by specific transporters, as is the case for glucose and some ions. Furthermore, astrocytic processes surround the outside of capillary endothelial cells and may also influence the formation and maintenance of the BBB.

2.2. Cell types of the central nervous system

The main cell types of the adult central nervous system are neurons and glia cells. Cells of glial lineage can be further divided into oligodendroglia and astroglia, both derived from neural stem cells. Microglia, are CNS resident cells of hematopoetic lineage, that

populate the brain and spinal cord during development. Furthermore the CNS is populated by adult neural stem cells that inherit the capacity to differentiate into either neurons, astrocytes or oligodendrocytes.

2.2.1. Neurons

Neurons are excitable cells that function to transmit information in form of electric signals over long distances. Generally, they can be distinguished as sensory neurons, motorneurons (neurons that directly or indirectly innervate muscle in order to trigger movement) and interneurons (that connect neurons with other neurons).

Although the various types of neurons can differ in morphology, most neurons have four distinct structural regions: dentrites, cell body (soma), axon and axon terminals (synapses).

Dentrites are specialized to receive chemical signals from axon termini of other neurons to convert them into electric impulses, which are transmitted towards the cell body. Signals can also be directly received by the neuronal cell body that contains the nucleus and is the site of synthesis of almost all neuronal proteins and membranes. If the electric disturbance received by dendrites and soma passes a certain threshold, action potentials can be generated at the axon hillock and are conducted along the axon towards the axon terminals where the electric signal is either conducted to other neurons via gap junctions (electrical synapse) or where it leads to the release of neurontransmitters (chemical synapse) in order to transmit information to downstream neurons. Those action potentials, namely sudden, transient depolarization of the membrane followed by repolarization to the resting potential, are the means by which information is rapidly transmitted over long distances in order to trigger appropriate response at the target site.

Generally, neurons can be identified by their morphology, but also by their expression of neuronal marker proteins, such as the neuronal nuclear protein NeuN as well as neuron-specific cytoskeleton proteins, β-III-tubulin or microtubul-associated protein 2 (Map2).

2.2.2. Oligodendrocytes

Mature oligodendrocytes derive from oligodendrocyte precursor cells (OPCs) that originate from the ventricular and subventricular zones of the CNS[1]. As their main function, oligodendrocytes produce the myelin sheath that wraps around axons in order to support fast conduction of action potentials as well as to support axon integrity[2-5]. As precursor cells, OPCs migrate to their target site[6,7] in the future white matter in order to differentiate into mature myelinating oligodendrocytes [8]. The process of myelination is initiated after oligoderoglial processes attach to the axons and myelin-membrane components are transported to the axon-glia contact site[9].

The myelin sheath consist mainly of lipids, such as cholesterol, phospholipids, and glycosphingolipids that account for 70% of the myelin membrane's dry weight whereas the myelin proteins make up only a small proportion of the balance[2]. Proteolipid protein (PLP) and myelin basic protein (MBP) represent 80% of myelin proteins whereas the glycoproteins myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG) account only for 1% of the total myelin proteins[10]. Other myelin proteins such as 2'3'-cyclin-nucleotide-3'-phosphodiesterase (CNP)[11] or myelin-associated oligodendrocyte basic protein (MOBP)[12] make up the rest of the myelin protein composition. OPCs are commonly identified by their expression of NG2[13-16] (or its mouse homologue AN2[15]) whereas more mature oligodendrocytes can be recognized by oligodendrocyte specific

expression of the membrane bound oligodendrocyte marker O4[17] or the cytosolic enzyme 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase)[4].

2.2.3. Astrocytes

Originally associated with structural support, mature astrocytes are now known to fulfill much more diverse functions within the CNS.
Astrocytes regulate extracellular ion and neurotransmitter concentrations, BBB maintenance, provide trophic factors for other CNS cells, such as neurons, oligodendrocytes and microglia, and serve as scaffolding device for migrating neuroblasts and neurons. Along with their stellar-shape, astrocytes are characterized by expression of glial fibrillary acidic protein (GFAP)[18].

2.2.4. Neural stem cells

Neural stem cells have the following characteristics: 1) They can generate neural tissue, 2) They have the capacity for self-renewal, 3) they can undergo asymmetric cell devision[19].
The adult mammalian brain harbors neurogenic stem cells only within specialized compartments or niches[20], such as the subventricular zone (SVZ) of the lateral wall and the subgranular zone (SGZ) of the hippocampal dentate gyrus. These areas are considered repositories of stem cell activity and plasticity, engage in tissue homeostasis [21-23], and may promote repair after CNS injury [24-28].
Located close to the lateral ventricle, the SVZ consists of Type A cells (SVZ neuroblasts) that migrate along the rostral migratory stream (RMS) from the SVZ to the olfactory bulb, where they integrate and give rise to neurons. In the SVZ, type A cells are ensheathed by slowly proliferating type B cells, which express glial fibrillary acidic protein

(GFAP) and exhibit ultrastructural properties of astrocytes. Rapidly dividing type C cells are scattered along chains of type A cells. Dividing type B astrocytes give rise to type C cells, which in turn give rise to type A neuroblasts[29]. Type B cells also generate a small number of non-myelinating NG2-positive OPCs and mature myelinating oligodendrocytes[8]. Furthermore some type B cells and a small subpopulation of transit-amplifying type C cells express the oligodendrocyte lineage specific transcription factor Olig2. Olig2-positive cells that originate in the SVZ go on to migrate into the corpus callosum, striatum and fimbria fornix where they differentiate into OPCs and mature myelinating oligodendrocytes[8].

The SVZ niche area is heterogeneous and in addition to stem/progenitor cells, includes endothelial cells, that are considered bona fide niche cells that secrete products enabling stem cells to survive and maintain their identity[30] as well as microglia[31-33].

2.2.5. Microglia

Microglia constitute about 10% of the total CNS[34]. Microglia are the resident macrophage population and represent the immune system of the mammalian brain. They are distributed throughout the CNS and subtly vary in density and morphology in specific brain regions[35,36].

In the healthy mammalian brain, microglia display a ramified morphology with elongated cell bodies and spine-like processes that often branch perpendicularly[34].

These “resting” microglia were long thought to be dormant under physiological conditions becoming highly motile upon activation in pathological conditions. However, recent studies revealed that their cell bodies and main branches are stable, but their ramified processes were highly motile enabling microglia to effectively scan the brain and rapidly respond to changes in their microenvironment[37,38]. In fact, it is

estimated, that the complete brain parenchyma could be monitored every few hours, guarenteeing exhaustive screening of the brain[39]. In contrast to astrocytes, microglia carry out their maintenance works in an individualistic fashion, avoiding cell contact with other microglia cells, and covering their own surveillance territory[40]. These finding suggests a primary role for microglia in immune surveillance and has lead to the proposal to change the name of microglia from “resting” to “surveilling” as this would describe the phenotype of microglia under physiological conditions in a more appropriate fashion[39].

In addition to their function in surveillance, microglia cells are implicated in a variety of other functions under physiological conditions. In fact, microglia processes were observed to directly contact astrocytes, neuronal cell bodies and blood vessels thus suggesting communication between those cell types under physiological conditions[34]. A recent report provides evidence that microglia also contribute to the control of behavioral processes, such as grooming, and their dysregulation may lead to obsessive compulsive disorder in humans and rodents[41].

During CNS pathological process, microglia may be activated by either “stranger” signals (non-self pathogens) or danger signals, such as injured-self components [42].

Equipped with a large number and a variety of receptors such as cytokine and chemokine receptors[43], pattern recognition receptors (PRR)[44] or surface glycans[45,46], microglia are able to respond to a variety of signals and these receptors contribute to the plasticity of microglia activation.

Microglia activation leads to phagocytosis, which involves cell-to-cell contact, or the release of a variety of substances, such as pro- or anti-inflammatory cytokines, chemokines or reactive oxygen species (ROS). Both processes may in turn shape the microenvironment and

contribute to amelioration or deterioration of pathological processes[42,47-50].

Among the “stranger” signals that activate microglia, is endotoxin (lipopolysaccharid, LPS). LPS consists of a lipid portion covalently bound to a polysaccharide portion and is found in the outer membrane of Gram-negative bacteria. It induces a Toll-like receptor (TLR)4-mediated immune response in microglia, resulting in the production of pro-inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS)[44]. Danger signals, include molecules such as fibrinogen, glutamate, ATP and ADP, proinflamatory cytokines or the lack of CX3CL1[42].

Fibrinogen is a pleiotropic blood protein that usually regulates coagulation. When leaking into the CNS, after vascular damage or BBB disruption, it induces microglia activation via the CD11b/CD18 integrin receptor. Fibrinogen mediated activation then results in cytoskeleton rearrangement and enhanced phagocytosis[51].

As microglia also express the glutamate receptors mGluRs on their cell surface, they are susceptible to stimulation by extracellular glutamate. In the CNS, glutamate acts as an excitatory neurotransmitter but excess in glutamate stimulation can result in neurotoxicity[52]. Elevated levels of glutamate result from increased release into the synaptic cleft by neurons or decreased clearance, mainly by astrocytes. Either way, increased levels of glutamate leads to microglia activation and subsequent release of the pro-inflammatory cytokine TNF[52].

Furthermore, microglia activation can be triggered by extracellular ATP, which is released by damaged neurons after injury or disease. ATP signaling is generally mediated through ionotropic P2X or metabotropic P2Y receptors. Microglia express both receptor types and it has been shown, that after cortical lesions, extracellular ATP

signals through the P2Y12 receptor to mediate process extension in microglia cells[37,42].

Pro-inflammatory cytokines like TNF and IFNγ also contribute to microglia activation and have been shown to increase myelin phagocytosis during oxidative stress[53] .

Furthermore microglia express the membrane-bound chemokine receptor CX3CR1 and its ligand, the chemokine CX3CL1 is highly expressed and secreted by neurons. Signaling through the CX3CR1-CX3CL1 axis suppresses microglia activation. Thus, upon removal of CX3CL1, microglia get activated and induce neurotoxic effects in their vicinity due to the lack of inhibitory signals[42,54].

Microglia express CD200R, a receptor for the neuronal derived CD200 secretory molecule. In the absence of CD200, microglia cells show a phenotype of increased activation, including morphological changes and upregulation of MHC-II molecules as well as increased neurotoxic activity[42,55,56].

The above listed molecules induce a variety of responses in microglia, generally associated with classical activation. Even though the concept of classical and alternative activation as been described mostly in peripheral macrophages, microglia are also known to react to anti-inflammatory cytokines such as IL4 or IL13 by alternative activation. Alternative activation of microglia/macrophages is thought to be neuroprotective and to induce CNS repair.

Furthermore microglia activation has been implicated in the suppression of repair processes such as cell replacement by endogenous neural stem cells (NSCs)[32,33,57-59] or neuronal synaptic plasticity[60,61]. Reports of NSC-glial co-culture or medium transfer from microglia to NSCs provide evidence that microglia can promote self-renewal, neurogenesis and oligogenesis [33]. In fact, Butovsky et al. showed that activation of microglia by low levels of IFNγ or IL4

differentially induce neurogenesis or oligodendrogenesis respectively[58]. Consistent with this concept, it was recently demonstrated that acute or persistent activation of microglia correlates with in increased or decreased reactivity of the subventricular zone NSC population, respectively [33]

In keeping with their cellular plasticity, microglia cells have been clearly shown to play an important role during CNS pathological processes, but it is still debatable whether activated or quiescent microglia play a beneficial or detrimental role on demeylination, neurodegeneration, neuroprotection and repair.

In the human and rodent brain, these cells can be revealed by lectin cytochemistry or immunocytochemitry against selected markers such as CD11b or Iba1, which are also expressed by peripheral macrophages. As there is no unique microglia marker, the origin of microglia was under debate until recently. However, Ginhoux et al. demonstrated that adult microglia derive from primitive myeloid precursors that arrive before embryonic day 8, rather than from post-natal hematopoietic progenitors[62] or from precursors of neural lineage.

2.3. Multiple Sclerosis and experimental autoimmune encephalomyelitis (EAE)

2.3.1. Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating and degenerative disease of the CNS. To date, its cause is still unknown, but there are genetic and environmental risk factors[63-65].

The clinical disease course usually starts with reversible episodes of neurologic disability (relapsing-remitting MS; RRMS), which evolves into a progressive stage with irreversible neurologic decline (secondary progressive MS; SPMS) [65,66] in the majority of cases.

It is believed that peripheral autoreactive lymphocytes cross the BBB[65] and initiate the disease. Until recently, myelin-reactive IFNγ producing T helper cells 1 (Th1) were credited for initiating acute MS lesions in the CNS[63,65], but new evidence suggests that Interleukin 17 (IL17) secreting Th17 cells may also drive adaptive immune response in both MS and its animal model experimental autoimmune encephalomyelitis (EAE)[47,67-70].

Moreover, an increase in frequency of myelin-reactive CD8 positive cell in MS lesions was observed, suggesting that this cell population may also play a role in driving MS pathology[47].

Furthermore, the failure of regulatory T cells (Tregs) to sufficiently suppress T effector cells may also account for dysregulation of the immune response in people with MS [71-73].

IL17 also contributes to the disruption of the BBB, allowing other cells, such as B lymphocytes, plasma cells and macrophages to accumulate in the CNS where secretion of pro-inflamatory cytokines leads to the recruitment and activation of naïve microglia who then contribute to demyelination and axonal injury in such acute demyelinating lesions[47].

With the onset of the secondary progressive stage of MS comes the co-existence of demyelination with diffuse axonal and neuronal degeneration.

Remyelination leads to so-called “shadow plaques” and is most active during acute inflammation but also appears in the progressive phase of disease[47,65]. OPCs are found surrounding the lesions and presumably are the source of myelin repair, as they have to potential to myelinate naked axons[65]. Even though remyelination is insufficient, plaques are eventually remyelinated [65] in 20% of people with MS [65].

Repair processes for axonal and neuronal degeneration seem to be more difficult. Even though the adult brain harbors neural stem cells

with the capacity to replace injured neurons, axonal repair or neuronal replacement is rarely found.

As mentioned above, axonal loss occurs in both the acute and chronic phases of MS and EAE [74] and the accumulation of neuronal dysfunction correlates with the level of disability in MS patients. It is thus likely that the loss of compensatory CNS mechanisms contributes to the transition from RRMS to SPMS [66,75] where neurological decline is irreversible.

It has been believed that RRMS is mainly driven by inflammatory processes, whereas progressive MS is dominated by neuronal degeneration. However, it now becomes clear that chronic MS is also associated with chronic microglia activation[64,76,77] as well as changes of the peripheral innate immune system[78-80].

2.3.2. Experimental autoimmune encephalomyelitis (EAE)

Since the target organ in MS i.e. the brain and spinal cord, is mostly inaccessible to direct tissue examination during the patient's life, MS research highly depends on its animal model EAE.

EAE is induced by a single peripheral immunization with myelin peptides, such as protein fragments of MOG or PLP, emulsified in complete Freund's adjuvant (CFA) injected subcutaneously. CFA contains *Mycobacterium Tuberculosis* and is necessary to induce the disease, by facilitating T cell priming, and activation of macrophages. The immunization with peptide is usually accompanied by injections of pertussis toxin (PT), which leads to break-down of the BBB and allows lymphocyte trafficking to the CNS.

EAE exhibits clinical features such as limb paralysis that appear in a relapsing-remitting or progressive fashion and also pathological features, such as CNS inflammation, demyelination, and axonal degeneration.

2.4. The Role of Microglia in MS and EAE

Microglia cells contribute to the manifestation of MS and EAE as they present antigen to auto-reactive T cells in the CNS. This specific interaction is necessary to facilitate disease and specific elimination of the microglia population in mice leads to a milder severity of EAE[81]. Not only are microglia crucial for initiating the disease, but they are also found to be persistently activated during progressive MS and chronic EAE[76]. As microglia activation has been associated with processes such as facilitation of inflammation, neurondegeneration but also endogenous repair, the question whether microglia play a beneficial or detrimental role during MS and EAE is still under debate. Furthermore, it is unknown how microglia activation and functions relate to acute and chronic inflammation or specific locations, such as white matter and gray matter.

2.4.1. Role of microglia in MS neurodegeneration and neurotoxicity

Neuronal pathology is thought to be the main substrate for clinical disability in MS patients[47,64,82]. The neuronal compartment is already effected in early disease stages[82] and axonal injury is a prominent feature of acute inflammatory demyelinating lesions[83], but also appears in the absence of demyelination[47]. These findings suggesting that axonal degeneration may be partly independent from demyelination[47].
Furthermore, axonal degeneration correlates strongly with the presence of inflammatory cells such as T cells and microglia/macrophages in the lesion[84].
Axonal loss is not only a hallmark of acute RRMS lesions but can be found in primary and secondary MS. Chronic MS is characterized by

persistent microglia/macrophage activation[76], which is linked to the amount of neurodegeneration in the CNS.

The exact processes underlying neural pathology in MS and EAE are not known, but several mechanisms, such as demyelination-induced axonal degeneration or wallarian degeneration were proposed to play a role [47].

For example, mice that lack PLP show oligodendrocyte-dependent degeneration[85] and oligodendroglial support to axons seams to be important even when independent from demyelination[86,87]. Additionally, axonal degeneration is increased when remyelination is inhibited [5].

Furthermore, it has been proposed that axons that lack the protection of their myelin sheath or oligodendroglial supports are more vulnerable to degeneration when exposed to an inflammatory milieu[47].

Under normal conditions, microglia assist with tissue regeneration and maintainance by clearing apoptotic cells and debris that would otherwise have a toxic effect on its surrounding CNS tissue. In the presence of infiltrating auto-reactive lymphocytes, activated microglia exhibit a strongly pro-inflammatory phenotype, which is associated with the production of potentially neurotoxic substances as the pro-inflammatory cytokine TNF as well as NO, oxygen radicals and proteolytic enzymes [42].

Thus, activated microglia and macrophages are thought to contribute to neuro-degeneration during acute and progessive MS and EAE and quiescence or deactivation of pro-inflammatory microglia were shown benefit the clinical outcome of EAE respectively.

In fact, it was shown that myelin degeneration and subsequent cholesterol degradation products induce activation of pro-inflammatory microglia in a poly(ADP-ribose) polymerase 1 (PARP-1) dependent manner, and inhibition of this axis of activation benefitted both RR-EAE [88] as well as EAE disease progression [89].

As axonal degeneration can also occur in the absence of demyelination, its strong correlation with microglia activation may point to the possibility of neurodegeneration as an effect of direct immune attack[47]. In fact, some data suggest that wallarian degeneration is a result of inflammation-mediated neuronal injury [90-93]. Moreover it has been shown that CD200 expression is downregulated in chronic active and inactive lesions [94] and that neuronal overexpression of CD200 or treatment with CD200fc protects from microglia-induced neurotoxicity in an model of wallarian degeneration during acute and chronic EAE [55,56].

As a result, it can be concluded that activated microglia with a pro-inflammatory phenotype, so called classically activated microglia (M1), may play a crucial role in driving neurodegeneration in MS and EAE. In line with its broad receptor profile and plasticity, recent data provide evidence for the existence of microglia with an anti-inflammatory phenotype during EAE[95].

In vitro, microglia/macrophages can be activated by high levels of IFN-γ or LPS into a pro-inflammatory phenotype (M1), and when co-cultured with cortical neurons lead to neurotoxicity. However, stimulation of microglia with IL-4 or IL-13 induce a state of alternative activation (M2), which is associated with neuroprotective functions that promote repair [58,95].

Thus an important strategy to halt MS disease progression would be to understand the mechanisms underlying microglia-mediated neurodegeneration in vivo and in vitro and to develop therapeutic approaches that target this interaction.

2.4.2. Role of microglia in cell replacement by neural stem cells

Demyelination and neuronal degeneration appear during both acute RRMS and chronic progrssive MS. During RRMS the majority of attacks lead to short-term clinical disability followed by various degrees of recovery. Most patients eventually go on to develop secondary progressive MS characterized by irreversible neuronal loss and permanent disability.

The exact mechanisms that drive the transition from RRMS to SPMS and that underlie disease progression in SPMS and PPMS are still unknown. However, it was proposed that transition from RRMS to SPMS occurs when the CNS can no longer compensate for additional neuronal loss [82].

Among the possible compensatory mechanisms is the endogenous stem cell compartment SVZ, harboring cells with the potential to differentiate into neurons and oligodendrocytes in order to replace injured or dysfunctional CNS cells. During the course of EAE, NSCs in the SVZ show increased cellular activation during the acute phase [33] . However this increase in repair potential during acute inflammation is followed by significant decrease in proliferation and migration during the chronic phase of EAE [96]. Alterations of the endogenous NSC compartment are not cell-autonomous but dependent on the SVZ microenvironment [33,96] .

The SVZ niche area is heterogeneous and in addition to stem/progenitor cells, includes microglia, that populate the SVZ and proliferate during the acute phase of EAE showing marked activation, and remain activated during chronic disease [76]. These data suggest that microglia can influence the SVZ microenvironment and impact the NSC compartment during EAE. It was further shown that inactivating the microglia by minocycline, a common antibiotic and

immunomodulatory drug, results in recovery of progenitor cell proliferation and enhanced repair [33].

Furthermore, it was shown that differentially activated microglia can either limit or support neurogenesis and oligodendrogenesis in vitro and in vivo. In fact, microglia exposed to low levels of IFNγ promote neurogenesis, whereas exposure to high levels of IFNγ lead to inhibition of neurogenesis [57,58]. In addition, exposure of microglia to IL4 leads to increased oligodendrogenesis by NSCs and attenuated signs of clinical disease [57,58].

Taken together, it is clear that microglia have an important effect on the SVZ stem cell compartment and thus on repair mechanism during EAE. Furthermore, one can conclude that the effects on NSCs depend on the microenvironment that both microglia and NSCs are exposed to. However, the factors that shape the microenvironment and how such factors are linked to specific disease phases of EAE and to activation profiles of microglia remain poorly understood.

2.5. Proteins involved in this study

2.5.1. Galectin-1: protein characteristics

Recent efforts toward decoding the information encoded by the 'glycome' (the complete repertoire of glycan structures found in cells and tissues) have illuminated essential roles of glycan-binding proteins or lectins in the regulation of immune tolerance, homeostasis and inflammation [97]. Various classes of glycan-binding receptors (lectins) exist that recognize specific glycan structures presented on a protein backbone or lipid structure. These carbohydrate-binding proteins are expressed on the cell surface (e.g. C-type lectins or siglecs) or are secreted to the extracellular space (e.g. galectins) where they interact with cell surface glycoconjugates by forming multivalent

supramolecular structures-termed lattices- or by generating traditional receptor-ligand interactions [97] . Galectins are an evolutionarily conserved family of soluble lectins defined by a common structural fold and a conserved carbohydrate recognition domain (CRD) of about 130 amino acids that recognizes glycans containing the disaccharide *N*-acetyllactosamine (Gal-β(1-4)-GlcNAc) [46].

Fifteen galectins have been identified in mammals to date. Prototype galectins contain one CRD and exist as monomers (galectin-5, galectin-7, galectin-10) or dimers (galectin-1, galectin-2, galectin-11, galectin-13, galectin-14, galectin-15). In contrast, tandem-repeat galectins (galectin-4, galectin-6, galectin-8, galectin-9, galectin-12) contain two CRDs that are connected by a short linker region. Galectin-3 as the only member of chimera-type galectins that exhibit one CRD and an additional nonlectin domain, that allows oligomerisation of this protein.

Galectins are highly expressed under inflammatory conditions or in neoplastic tissue and are shown to have wide ranging effects on modulating immunity.

2.5.2. Role of Galectin-1 in multiple sclerosis and EAE

Galectin-1, a 'prototype' member of galectins, has been recently implicated in a variety of biological functions, mainly as a regulator of innate and adaptive immune responses [46,98]. In the periphery, galectin-1 promotes selective apoptosis of Th1 and Th17 cells [99], decreases the antigen presenting capacity and iNOS production of macrophages [100,101] and induces IL-27-producing tolerogenic dendritic cells (DCs) [102]. All of these cell types are implicated in both MS and EAE mechanisms and the fact that autoantibodies against galectin-1 were found in sera of MS patients [103] provides more evidence of the importance of galectin-1 in MS pathology.

Furthermore, galectin-1 was implicated in other CNS diseases, such as stroke or epilepsy. For example galectin-1 expression was markedly upregulated in astrocytes after ischemic infarct and lead to attenuated astrogliosis, reduced expression of iNOS and IL-1β, which influence the neural compartment. In this setting, galectin-1 therapy limited neuronal damage[104]. Additionally, galectin-1 was shown to induce proliferation of SVZ resident NSCs in the healthy[105] and ischemic brain[106], and of hippocampal NSCs after kainate-induced epilepsy[107].
These data suggest that galectin-1 may play a fundamental role on CNS homeostasis and pathology during MS.

2.6. Aims of this study

Activated microglia have been implicated in a variety of processes during MS and EAE. The aim of this study was to investigate about the role of microglia in inflammation-induced neurodegeneration during EAE and the factors (endogenous and exogenous) that may limit neuronal injury in EAE, such as galectin-1. We also questioned whether microglia cells have a beneficial or detrimental effect on the NSC microenviroment during the course of EAE.
Since there is currently no effective treatment for chronic progressive MS, nor any therapeutic strategies to reverse existing disability, this study may elucidate some of the underlying mechanisms of disease progression and repair and may contribute to the development of effective therapeutic strategies for MS.

3. MATERIAL & METHODS

3.1. Materials, Equipment, Buffers and Media

3.1.1. Equipment & Software

Equipment	Company
Centrifuges	
Centrifuge 5417C (Tabletop centrifuge)	Eppendorf
Centrifuge 5417R (Tabletop centrifuge, cooled)	Eppendorf
Centrifuge 5810R (centrifuge, cooled)	Eppendorf
Microscopes	
LSM 510 (confocal microscope)	Zeiss
Axioscope 2	Zeiss
StemiDV4	Zeiss
Other Equipment	
Class II A2 Biological Safety Cabinet (TC Hood)	Thermo Forma
Nuaire US Autoflow (Incubator)	Nuaire
ND1000 Spectrophotometer	NanoDrop
GeneAmp PCR System 9700	AB Applied Biosystems
7500 Fast Real-Time PCR System	AB Applied Biosystems
Luminex 100 System	Luminex
infinite F200 (Microplate reader)	TECAN
CM 3050 (Cryostat)	Leica
LSR II	BD Biosciences
Software	
Prism 4.0 for Macintosh	GraphPad Software, Inc.
Ingenuity Pathway Analysis (IPA)	Ingenuity Systems

3.1.2. Materials

Glassware was purchased from PYREX® USA, plastic ware from Corning® and BD Falcon™. Chemicals were purchased from Sigma and Fischer Scientific. The origin of special meterials and kits are indicated in the text.

3.1.2.1. Recombinant galectin-1

Recombinant mouse galectin-1 was kindly provided by Prof. Dr. GA Rabinovich.

3.1.3. Buffers and Media

Buffer	Components/Company
General Buffers	
PBS (Dublecco's Phasphate Buffered Saline)	BioWhittaker® LONZA
DMEM (Dublecco's Modified Eagle's Medium)	BioWhittaker® LONZA
Neurobasal medium	GIBCO®
HBSS (Hank's Balanced Salt Solution, Calcium Free)	GIBCO®
Protein Biochemistry	
RIPA buffer 1x (Lysis Buffer)	ThermoScientific
Immunodetection	
Fixation solution	4% (w/v) paraformaldehyde in PBS
VectaMount AQ (Mounting medium)	Vector Laboratories Inc.
Blocking Solution	8% horse serum, 3% BSA, 0.3% Triton in PBS
Flow Cytometry	
FACS Buffer	10% FBS in PBS
Cell Culture	
Laminin	Invitrogen
Poly-L-Lysine (PLL)	Sigma
Microglia medium	10% FBS, penicillin/streptomycin (50U/ml/50µg/ml), sodium pyruvate (1mM) and L-glutamine (2mM) in DMEM medium
Neurobasal medium	penicillin/streptomycin (50U/ml/50µg/ml) and 5% B27 Supplement in Neurobasal medium
Trypsin/EDTA	0.25% Trypsin-EDTA (GIBCO)
Transfection medium	Opti-MEM® + GlutaMAX™-I (GIBCO)

3.1.4. Antibodies

3.1.4.1. Primary Antibodies

Antigen	Name	Species	Dilution	Application	Source
beta-III-tubulin	Tuj1	mouse	1:100	IHC/ICC	STEMMCELL Technologies, Inc.
C3	C3	rat	1:100	IHC	Abcam
CD11b	CD11b	rat	1:50	IHC	BD Pharmingen
			1:100	FACS	
CD4	CD4	rat	1:100	IHC	BD Pharmingen
CD45	CD45	rat	1:100	FACS	BD Pharmingen
CD86	CD86	rat	1:100	FACS	BD Pharmingen
Galectin-1	Galectin-1	rabbit	1:500	IHC	GA Rabinovich
	Gal1 capture AB	goat	0.8 μg/ml	ELISA	RnD
	Gal1 detection AB	goat	100ng/ml	ELISA	RnD
Gap43	Gap43	mouse	1:100	IHC	Abcam
GFAP	GFAP	mouse	1:500	IHC	BD Pharmingen
IL18BP	IL18BP	goat	1:50	IHC	Stressgen
Insl6	Insl6	goat	1:50	IHC	Santa Cruz Biotechnology
Ionized calcium binding adaptor molecule 1	Iba1	rabbit	1:200	IHC	WAKO
Jag1	Jag1	goat	1:50	IHC	Santa Cruz Biotechnology
Map 2	Map2	mouse	1:500	IHC/ICC	Sigma
MBP	MBP	mouse	1:100	IHC	BD Pharmingen
Map 2	I-Ab	rat	1:100	FACS	BD Pharmingen
		mouse	1:100	IHC/ICC	BD Pharmingen

IHC (Immunohistochemistry); ICC (Immunocytochemistry); FACS (flow cytometry); ELISA (Enzyme linked immunoabsorbant assay)

3.1.4.2. Secondary Antibodies

Target species/ epitope	host species	Conjugation	Dilution	Application	Source
mouse	donkey	Alexa Fluor 488	1:500	IHC/ICC	Invitrogen
mouse	donkey	Alexa Fluor 594	1:500	IHC/ICC	Invitrogen
rat	donkey	Alexa Fluor 488	1:500	IHC/ICC	Invitrogen
rat	donkey	Alexa Fluor 594	1:500	IHC/ICC	Invitrogen
goat	donkey	Alexa Fluor 488	1:500	IHC/ICC	Invitrogen
goat	donkey	Alexa Fluor 594	1:500	IHC/ICC	Invitrogen
rabbit	donkey	Alexa Fluor 488	1:500	IHC/ICC	Invitrogen
rabbit	donkey	Alexa Fluor 594	1:500	IHC/ICC	Invitrogen
biotin		APC-strepdavidin	1:100	FACS	BD Biosciences
biotin		PE-strepdavidin	1:100	FACS	BD Biosciences
biotin		FITC-strepdavidin	1:100	FACS	BD Biosciences
IHC (Immunohistochemistry); ICC (Immunocytochemistry); FACS (flow cytometry)					

3.2. Methods

3.2.1. Amimals

Female C57BL/6 and SJL/J mice were purchased from The Jackson Laboratory (Ben Harbor, ME). *Lgals1*$^{-/-}$ mice (C57BL/6 background) were generated and kindly provided by Dr. Francoise Poirier (Institute Jacques Monod, Paris) and bred at the animal house of the Institute of Biology and Experimental Medicine (Buenos Aires, Argentina) and Harvard Medical School (Boston, USA). All mice were housed in conventional, pathogen-free facility at the New Research Building, Harvard Medical School (Boston, MA) according to National Institute of Health guidelines or at the Institute of Biology and Experimental

Medicine (Buenos Aires, Argentina) according to the institute's guidelines. All animal experiments were performed with the approval of the Harvard Medical Area Standing Committee on Animals.

3.2.2. Induction of EAE

3.2.2.1. Induction of EAE in C57BL/6 mice

EAE was induced in C57BL/6 wild-type and *Lgals1* knockout mice as previously described [108]. Briefly, the encephalitogenic MOG peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) corresponding to mouse sequence was synthesized by Biopolymer Laboratory (University of California, Los Angeles, CA) and purified to >99% by HPLC. Wild-type or *Lgals1*$^{-/-}$ mice (C57BL/6) were immunized subcutaneously at two sites (left and right flank) with 150 μg of MOG_{35-55} peptide that was emulsified in complete Freund's adjuvant (CFA, Sigma Aldrich, Saint Louis, MO) containing 200 μg *Mycobacterium Tuberculosis* (Difco Laboratories, Detroit, MI). Mice received 200 ng pertussis toxin (PT, List Biological Laboratories Inc., Campbell, CA) in 0.2 ml PBS (Lonza, Walkersville, MD) by intraperitoneal (i.p.) injections at the time of immunization and 48 h later. Control mice were immunized with CFA followed by PT.

3.2.2.2. Induction of EAE in SJL/J mice

For induction of RR-EAE, SJL/J mice were immunized with 150μg of PLP139-151 (H-S-L-G-K-W-L-G-H-P-D-K-F; New England Peptide LLC, Gardener, MA, USA) as described as described above.

3.2.2.3. EAE score

Mice were scored daily as follows: 0, no disease; 1, loss of tail tone; 1.5, poor righting ability; 2, hind limb weakness; 3, hind limb paralysis; 4, quadreparesis; 5, moribund.

3.2.3. Sterotactic transfer of neonatal microglia

For stereotactic placement of neonatal microglia, recipient mice (6-8 weeks old) were anesthetized with ketamine (200mg per kg body weight) and xyzlazine (10mg per kg body weight). Heads were secured in a sterotaxic head frame (Stoelting Co.). A small hole was drilled into the mouse skull, meninges were locally removed with H_2O_2 and a 10μl Hamilton syringe with a 29-gauge needle was inserted into the right lateral ventricle. Pre-treated neonatal microglia cells (4-5 X 10^5 cells in 10 μl) were in injected at a flow rate of 1 μl min per minute at the following coordinates: anteroposterior, -0.34 mm; lateral, 1.2 mm; dorsoventral, 2.4 mm. After completion of injection, the needle was left in place for aditional 5 minutes and then withdrawn at a 0.5 mm per minute. The resulting wound was sutured with surgical nylon and mice were observed daily for post-operational care.

3.2.4. Cells

3.2.4.1. Isolation of primary neonatal microglia and astrocytes

Brains from neonatal C57BL/6 mice (P0-P2) were stripped of their meninges and minced with a razor blade in Ca^{2+} free HBSS. Neural tissue was digested using the Neural Tissue Dissociation Kit (P) (Miltenyi Biotec). The cell suspension was washed and cultured in microglia culture medium (DMEM with 10% fetal bovine serum (FBS), penicillin/streptomycin (50U/ml/50μg/ml), sodium pyruvate (1mM) and L-glutamine (2mM) at 37°C and 5% CO_2. Fresh medium was added to the culture every 2 days for a total period of 10-14 days. Neonatal microglia cells were shaken off the mixed brain glia cell culture after 10 to 14 days (150 rpm, 37°C, 6 h). Microglia cells were washed in microglia culture medium and subjected to further analysis. Astroyctes were isolated as previously described [108]. At the end a of 10-day

culture period, supernatants were removed and replaced with fresh medium containing 20μM cytosine arbinoside (Sigma-Aldrich, St. Louis, Missouri, USA) for 72 hrs followed by fresh medium. The fast growing cells such as microglia were eliminated by this treatment. Oligodendrocyte precursor cells and neurons were also gradually eliminated by these culture conditions. Purity of the astroglia were verified by GFAP staining. After purification, both microglia and astrocytes were cultured in microglia culture medium.

3.2.4.2. Isolation of adult microglia from the SVZ

Mice were deeply anesthetized in a CO_2 chamber and transcardially perfused with 30ml PBS. Subventricular zone tissue was dissected from a 2mm block containing the lateral ventricle and the ventricular wall. The tissue was cut into pieces and gently digested using the papain containing Neural Tissue Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Mononuclear cells were isolated by percoll gradient (70%/37%) centrifugation and removed from the interphase, washed and resuspended in PBS containing 2% fetal bovine serum (FBS).

Mononuclear cells from HC, acute and chronic EAE SVZ tissue were labeled with FITC-conjugated anti-CD11b and APC-conjugated CD45 and sorted into a CD11b+/$CD45^{lo}$ microglia population using a FACSAria sorter (BD Bioscience).

3.2.4.3. BV 2 cells

The microglia cell line BV 2 was generated by infecting neonatal microglia with the *v-raf / v-myc* oncogene carrying retrovirus (J2) by Blasi et al. [109]. BV 2 cells are the most frequently used substitute for primary microglia [110] as they retain most phenotypical, morphological and functional properties of primary microglia [109]. These cells are

cultured in conventional microglia medium (DMEM with 10% fetal bovine serum (FBS), penicillin/streptomycin (50U/ml/50μg/ml), sodium pyruvate (1mM) and L-glutamine (2mM) at 37°C and 5% CO_2.

3.2.4.4. Isolation of neuronal cultures

Neuronal cultures were prepared from E16-16 cortices. Meninges were stripped from the brain, and cortices were minced with a razor blade in Ca^{2+} free HBSS. Neural tissue was digested using the neural tissue dissociation kit (P) (Miltenyi Biotec). The cell suspension was washed and plated at 5 x 10^4 cells per well on Laminin (Invitrogen)-precoated glass cover slips (10mm diameter, Electron Microscopy Sciences) and cultured in Neurobasal Medium (NB medium (Sigma) with B27 supplement (2%; Sigma) penicillin/streptomycin (50U/ml/50μg/ml), sodium pyruvate (1mM) and L-glutamine (2mM) at 37°C / 5%CO^2. Fresh medium was added every 2 days for a total culture time of 7-10 days.

3.2.4.5. Co-culture of microglia and neuronal cells

Freshly isolated primary microglia or BV-2 cells were cultured in microglia culture medium and pre-incubated with or without recombinant galectin-1 (10 μg/ml) for 15 min and further stimulated with our without LPS (100 ng/ml) for 24 h. Stimulated microglia cells (15 x 10^3 cells per well) were seeded with neuronal cultures and co-cultured for 48 h in microglia medium.

3.2.4.6. Isolation and polarization of CD4+ T cell subsets

Naïve CD4+ T cells were isolated from FoxP3.GFP reporter mice, using anti-CD4 magnetic beads (Miltenyi) and sorted into naïve $CD4^+CD62L^{hi}$ $FoxP3^-$ T cells and natural regulatory T cells based on their in vivo FoxP3 expression using a FACSAria sorter (BD

Biosciences). For differentiation, naïve CD4+ T cells were stimulated with plate-bound anti-CD3 (4μg/ml, PharMingen) and soluble anti-CD28 (2μg/ml, PharMingen) for 4 days in serum-free T cell media (X-VIVO-20; Lonza) supplemented with 50 μM 2-mercaptoethanol, 1mM sodium pyruvate, nonessantial amino acids, L-glutamine and 100 U/ml penicilin/100μg/ml streptomycin in the presence of the appropriate recombinant cytokines. For polarization of Th1 cells, T cells were cultured in the presence of recombinant mouse IL-12 (10ng/ml; R&D Systems) plus anti-IL4 (11B.11; 10μg/ml), whereas mouse IL-4 (10 ng/ml, R&D Systems) plus anti-IL-12 (C17.8; 10 μg/ml) were added for polarization into Th2 cells. The monoclonal antibodies agains IL-4 and IL-12 had been purified from the supernatants of hybridoma cells obtained from American Type Culture Collection (ATCC).
Human TGFβ_1 (3ng/ml) plus IL-6 (30ng/ml) was added for polarization of Th17 cells. Cells were supplemented with recombinant IL-2 (50 U/ml) after 2 days of culture.

3.2.5. Tissue preparation

In study 4.1 and study 4.2, free floating sections and glass-bound thin tissue sections were used, respectively.

3.2.5.1. Free floating sections
At the appropriate time points, mice were deeply anesthetized in a CO_2 chamber and transcardially perfused with cold PBS followed by 4% cold paraformaldehyde solution (PFA, Electron Micoscopy Sciences, Hatfield, PA) in PBS. Brains were removed and post-fixed in PFA for 24 h, placed in 30% sucrose and then frozen in cryo-protective OCT

(Electron Microscopy Sciences, Hartfield, PA) at -80°C. Tissues were cut into floating sections of 40 μm thickness in a freezing microtome.

3.2.5.2. Glass-bound thin thissue sections

At the appropriate time points, mice were deeply anesthetized in a CO_2 chamber and then transcardially perfused with cold PBS. Brains were removed, snap frozen in liquid Nitrogen and then stored in -80℃ until further use. Tissues were cut into thin tissue sections (25 μm) using a freezing microtome.

3.2.6. Histology

3.2.6.1. Hematoxylin &Eosin (H&E)

For histological assessment, 20μm thick frozen brain sections were stained with hematoxylin solution (Vector) for 5 minutes, washed with with 0.3% acid alcohol (0.3% hydrochloric acid in 70% ethanol) and then counterstained with eosin (Sigma) for 3 minutes. After staining, sections were dehydrated and mounted with coverslips.

3.2.7. Immunostaining

3.2.7.1. Immunocytochemistry

Adherent cells were plated and cultured on glass cover slips. After culture, cells were fixed with 4% PFA for 10 minutes at room temperature, rinsed with PBS, blocked with 8% horse serum in PBS for 1 hours and then incubated overnight with the appropriate primary antibodies. Sections were then rinsed and incubated for 1 h with the appropriate Alexa Flour 488 and 594 secondary antibodies (1:500, Mlecular Probes) and mounted with coverslips. Negative control sections for each animal received identical preparations for immunostaining, except that primary antibodies were omitted. Regions

of interest are analyzed with a confocal microscope (LSM 510 Laser Scanning Microscope and LSM 3D analysis software, Linux, Ogdensburg, NY).

3.2.7.2. Immunohistochemistry

Floating sections were blocked with 8% horse serum for 1 h and incubated overnight with one of the appropriate primary antibody antibodies. Sections were rinsed and incubated for 1 h at room temperature with the appropriate Alexa Fluor 488 and 594 secondary antibodies (1:500, Molecular Probes, Eugene, OR) and mounted on microscope slides and cover-slipped.

For immunostaining of thin glass-bound section, sections were fixed with 4% PFA for 10 min, washed with PBS for 15 min, blocked with 8% horse serum in PBS for 1 hours and then incubated for 48 hours with the appropriate primary antibodies. Sections were then rinsed and incubated for 1 h with the appropriate Alexa Flour 488 and 594 secondary antibodies (1:500, Mlecular Probes) and mounted with coverslips. Negative control sections for each animal received identical preparations for immunostaining, except that primary antibodies were omitted. Regions of interest were analyzed with a confocal microscope (LSM 510 Laser Scanning Microscope and LSM 3D analysis software, Linux, Ogdensburg, NY).

3.2.8. Flow cytometric anaslysis (FACS)

3.2.8.1. FACS staining of surface markers

For assessment of microglia activation, microglia cells ($1x10^5$ cells per well) were pre-incubated with or without recombinant galectin-1 (1-10 μg/ml) for 2 h followed by stimulation with LPS (10 ng/ml; Sigma), IFN-γ (10 ng/ml; R&D) or IL-4 (10 ng/ml; BD Biosciences) for a total culture

time of 24-48h. After culture, non-specific binding to microglia was blocked by incubation with anti-mouse CD16/CD32 antibody (BD Biosciences) for 5 min and then surface stained with various surface markers according to the manufacturer's instructions (BD Biosciences). After staining, all samples were aquired on a LSRII flow cytometer (BD Biosciences) and analysed using Flowjo software.

3.2.8.2. Assesment of Galectin-1 binding

Recombinant galectin-1 was pre-absorbed with biotinylated anti-galectin-1 antibody (R&D Systems) overnight at 4ºC. Microglia cells ($1x10^5$ cells per well) were stimulated with LPS (10 ng/ml; Sigma), IFN-γ (10 ng/ml; R&D) or IL-4 (10nng/ml; BD Biosciences) for a total culture time of 24h. After culture, non-specific binding to microglia was blocked by incubation with anti-mouse CD16/CD32 antibody (BD Biosciences) for 5 min and incubated with increasing concentrations of pre-absorbed galectin-1 in the absence or presence of lactose or sucrose for 1 h at 37ºC as previously described [111]. Cells were then incubated with APC conjugated strepdavidin (BD Biosciences) for 15 min at 4ºC. All samples were aquired on a LSRII flow cytometer (BD Biosciences) and analysed using Flowjo software.

3.2.8.3. Assesment of Glycoprofile

Microglia cells were stimulated with or without LPS (10ng/ml), IFNγ (10ng/ml) or IL-4 (10ng/ml) for 24h. Pre-stimulated cells were stained with biotinylated *Sambuccus nigra agglutinin* (SNA; Vector Labs, 20μg/ml), *Peanut agglutenin* (PNA; Sigma, 20μg/ml), *L-phytohemagglutenin* (PHA; Vector Labs, 2μg/ml), *Helix pomatia agglutenin* (HPA; Sigma, 20μg/ml), and *Maackia amurensis agglutinin* (MAL II; Vector Labs, 5μg/ml), followed by fluorescein isothiocyanate-

conjugated strepatavidin. Nonspecific binding was determined with fluorescein isothiocyanate-conjugated strepatavidin alone. After staining, all samples were aquired on a LSRII flow cytometer (BD Biosciences) and analysed using Flowjo software.

3.2.9. RNA analysis

3.2.9.1. RNA isolation, RT-PCR and quantitative real time PCR

Cells or tissues were lysed using stratagene lysis buffer (Agilent Technologies) and cell- or tissue lysates were then processed using the Absolutely RNA® Microprep Kit (Stratagene) according to the manufacturer's instructions. Total RNA was subject to cDNA reverse transcription using the using the Applied Biosystems high capacity cDNA Reverse Transcrition Kit according to the manufacturer's instructions. Samples were then subjected to real-time PCR analysis on an ABI 7500 system (Applied Biosystems) under fast PCR conditions. Genes analyzed were detected using commercially available TaqMan® gene expression assays (Applied Biosystems). Relative mRNA level was normalized against GAPDH.

3.2.9.2. Microarray study

A total of 120 mice were sacrificed to isolate microglia. The SVZ area of 20 mice per group were pooled to sort microglia resulting in one RNA sample per group. Two indepentendly isolated RNA samples per group were submitted for microarray analysis (Center for personalized genetic medicine at Harvard Medical School).

RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and total RNA samples where then labeled and hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix), employing standardized protocols and reagents from Affymetrix. Microarray data from biological replicates were combined and normalized using

Bioconductor R. Student's t-test was used to test for significant differences between gene expression levels of acute EAE vs. control samples, chronic EAE vs. control samples and acute EAE vs. chronic EAE samples. A correction for the false discovery rate (FDR) was not used in this pair-wise comparison. Only genes that were significantly modulated (*p* value <0.05) and passed a fold change of FC>2.0 were implemented in differential gene expression signatures and considered for further analysis.

3.2.10. Transfection of primary cells

3.2.10.1. Transfection of primary astrocytes with lgals1-siRNA

Neonatal astrocytes were exposed to TGF-β_1 (5ng/ml) or medium for 24 h in microglia culture medium before knocking down Lgals1 mRNA using Lgals1 ON-TARGET plus SMARTpool siRNA (Dharmacon and Lipofectamin 2000 RNAi MAX (Invitrogen) reagents according to the manufacturer's instructions. In brief, astrocyte were cultured up to a confluency of no more than 60-70% and antibiotics/antimycotics were withdrawn from the culture medium at least 24 hours before transfection. siRNA was resuspended to a final concentration of 2μM in siRNA dilution buffer and then further diluted to a ratio of 1:4 in OptiMEM® reduced serum medium (Invitrogen) resulting in 250 μl of Mix A. 5 μl Lipofectamin 2000 RNAi MAX (Invitrogen) were mixed with 245μl OptiMEM® reduced serum medium (Invitrogen) resulting in 250 μl of Mix B. Mix A was then added to mix B and incubated for 15 minutes at room temperature. Medium was removed from astrocytes and replaced with 0.5 ml of new microglia medium and 0.5ml Mix (A+B) resulting in a working concentration of 100nM siRNA. Transfection medium containing siRNA was replaced with normal microglia medium 4-6 hours later and galectin-1 expression was

evaluated 48 hours after transfection. *Lgals1* siRNA was omitted in control cultures.

3.2.11. Protein analysis

3.2.11.1. Cell lysis

Cells were scraped off cell culture dishes on ice in lysis buffer (RIPA buffer, Pierce) and Halt Protease inhibitor cocktail (Pierce) and Phosphatase Inhibitor cocktail 2 (Sigma) were added. Lysates were then cleared of cellular debris and nuclei by a 300xg centrifugation step for 30 minutes at 4°C and then either stored at -80C or subject to further analysis.

3.2.11.2. Signaling Luminex

BV-2 microglia cells were cultured in microglia culture medium until further analysis. BV-2 cells were pre-incubated with or without recombinant galectin-1 (25 μg/ml) for 15 min followed by stimulation with LPS (100 ng/ml) for variable time periods in microglia culture medium. Following each time point, cells were washed and lysed with lysis buffer containing protease and phophatase inhibitors. Active phosphorylated proteins were detected using a Milliplex 8-Plex Multi-Pathway Signaling Kit (Millipore). Luminex multiplex signaling assays, allow detection of phosphrylation of multiple singaling molecules within one sample-well. Assay beads detecting the following phosphoproteins were included in a premix: CREB (Ser133), Erk/MAPK 1/2 (Thr185/Tyr187), IκBα (Ser32), JNK/SAPK1 (Thr183/Tyr185), STAT3 (Ser727), STAT5A/B (Tyr694/699), p38/SAPK2A/B (Thr180/Tyr182), p70 S6 Kinase (Thr412)

Briefly, a 96-well Microtiter Filter plate is pre-weted using whasing buffer. Positive and negative controls and samples were added to the

filter plate. Unstimulated and heatschock-stimulated MILLIPLEX MAP HeLa Cell Lysate as provided by the kit served as negative and positive control, respectively. Assay buffer and assay beads were added to each well and incubated over night at 4°C.

Assay solutions were removed by vacuum and the filter was washed twice. Biotynilated detection antibody was added to each well and incubated at room temperature. After 1 hour, phycoerytrin (PE)-conjugated streptavidin was added to each well and incubated for 30 min at room temperature. Each well was washed twice and resuspended in 150ul PBS. Samples were acquired using a Luminex 100 System Instrument (Luminex Corporation). Mean Fluorescence Intensity (MFI) indicating the level of phophorylation was calculated.

3.2.12. Elisa & Cytokine luminex assay

3.2.12.1. Cytokine detection by Luminex

Cell culture supernatants were collected and the secreted cytokines were determined by fluorescent bead-based Luminex technology (Luminex) for the indicated cytokines, according to the manufacturer's instructions.

Luminex multiplex assays, allow detection of multiple cytokines and chemokines within one sample-well. Assay beads detecting the following cytokines ans chemokines were included in a premix: G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2 IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, RANTES, TNFα.

Briefly, a 96-well Microtiter Filter plate is pre-weted using washing buffer. Standards, ranging from 10.000 pg/ml to 3.2 pg/ml, negative control and samples were added to the filter plate. Assay buffer and assay beads were added to each well and incubated overnight at 4°C.

Assay solutions were removed by vacuum and the filter was washed twice. Biotinylated detection antibody was adde to each well and incubated at room temperature. After 1 hour, phycoerytrin (PE)-conjugated streptavidin was added to each well and incubated for 30 min at room temperature. Each well was washed twice and resuspended in 150ul PBS. Samples were acquired using a Luminex 100 System Instrument (Luminex Corporation) and the concentration was calculated.

3.2.12.2. Galectin-1 ELISA

Supernatants were subject to enzyme-linked immunoabsorbent assay (ELISA) and processed according to the manufacturers intructions (RnD Mouse Galectin-1 Duo Set).

In brief, a high-binding surface microplate (Costar EIA Plate, Corning) is incubated with diluted capture antibody overnight at room temperature. Each well is aspirated, washed with washing buffer containing 0.05% Tween-20 in PBS and dried blotting it against clean paper towels. Each well is incuabted with reagent diluent containing 1% BSA in PBS for 1 hour at room temperature in order to avoid non-specific binding. Each well is again aspirated and washed as described above. Standards, ranging from 8000 pg/ml to 62.5 pg/ml, negative control and samples are added to the wells and incubated for 2 hours at room temperature. After the wells are aspirated and washed, biotinylated detection antibody is added to each well and incubated 2 hours at room temperature. Wells are again aspirated and washed and horseradish peroxydase (HRP) conjugated strepdavidin is added to each well and incubated for 20 minutes at room temperatre. After aspiration and washing, subtrate solution containing H_2O_2 and Tetramethylbenzidine is added to each well and incubated for another 20 minutes. Optical density was measured at a wavelength of 450nm

using a microplate reader (Tecan), and the resulting galectin-1 concentration was calculated.

3.2.13. Statistical Ansalysis & Software

3.2.13.1. Ingenuity Pathway Analysis

Gene Ontology, canonical pathway and functional network analysis were executed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mointain View, CA). IPA is a curated database of previously published findings on mammalian biology from the public literature. Reports on individual studies of genes in human, mouse or rat were first identified from peer-reviewed publications, and findings were then encoded into an ontology by content and modeling experts.

Each clone identifier was mapped to its corresponding gene in the Ingenuity pathway knowledge base. This knowledge base of pathway interactions is scientifically accurate, semantically consistent, contextually rich, broad in coverage, and up-to-date[1].

Location mapping and functional analysis were then applied to the so-called focus genes, to identify cellular location and biological functions and/or diseases that were algorithmically significant (Fischer's exact test) to the data set. For generation of functional networks, focus genes were used as a starting point. A score was computed for each network, that reflects the negative logarithm of the P that indicated the likelihood of the focus genes in a network being found together due to random chance, where only networks of a score >10 were considered as biologically relevant.

[1] http://wwwingenuity.com/products/PathwayKnowledge.pdf.

3.2.13.2. Statistical Analysis

Prism software or Microsoft Excell were used for statistical analysis. For comparison of 2 groups, the two-tailed unpaired student's *t*-test or analysis of variance (ANOVA) were used. Significant differences were assumed at the 5% level and represented as *P*-values ($P < 0.05$).

4. RESULTS

4.1. Galectin-1 deactivates classically-activated microglia and protects from inflammation-induced neurodegeneration in a mouse model of Multiple Sclerosis

Inflammation-mediated neurodegeneration occurs in the acute and the chronic/progressive phases of MS and its animal model experimental autoimmune encephalomyelitis. Classically-activated microglia are key players mediating this process through secretion of soluble factors including NO and TNF [112,113].

4.1.1. Dynamic regulation of endogenous galectin-1 in the CNS during the course of EAE

There is emerging evidence indicating an essential role for galectin-1-glycan lattices in regulating innate and adaptive immune responses within peripheral compartments [46,99], but the immunoregulatory role of galectin-1 within the CNS has not yet been explored. To investigate the relevance of endogenous galectin-1 during neuroinflammation, the expression of galectin-1 in the CNS during the course of MOG-induced EAE in C57BL/6 mice was examined. Galectin-1 expression in the spinal cord was analyzed in naïve mice, and in preclinical (d10pi), acute EAE (d15-16) and chronic EAE (d30 to d40pi). Protein expression was assesed by immunoreactivity against galectin-1 in the white matter of spinal cord (**Fig. 4.1.1-1 b)**, and regulation of its corresponding gene (*Lgals1*) was assesed by quantitative real time PCR (**Fig. 4.1.1-1 a)**. In naive mice there was very little expression of galectin-1 in the CNS, but its expression was dynamically regulated during the course of the disease **(Fig. 4.1.1-1 a,b).** During preclinical EAE, *Lgals1* gene expression remained unchanged but galectin-1

protein expression was marginally elevated. However, *Lgals1* gene expression and galectin-1 protein expression were highest during the peak of the disease and persisted at lower levels during the chronic phase of EAE **(Fig. 4.1.1-1a,b)**.

Thus, expression of galectin-1 is locally regulated within the inflamed CNS consistent with a homeostatic function for this glycan-binding protein at the peak of the demyelinating neurodegenerative disease.

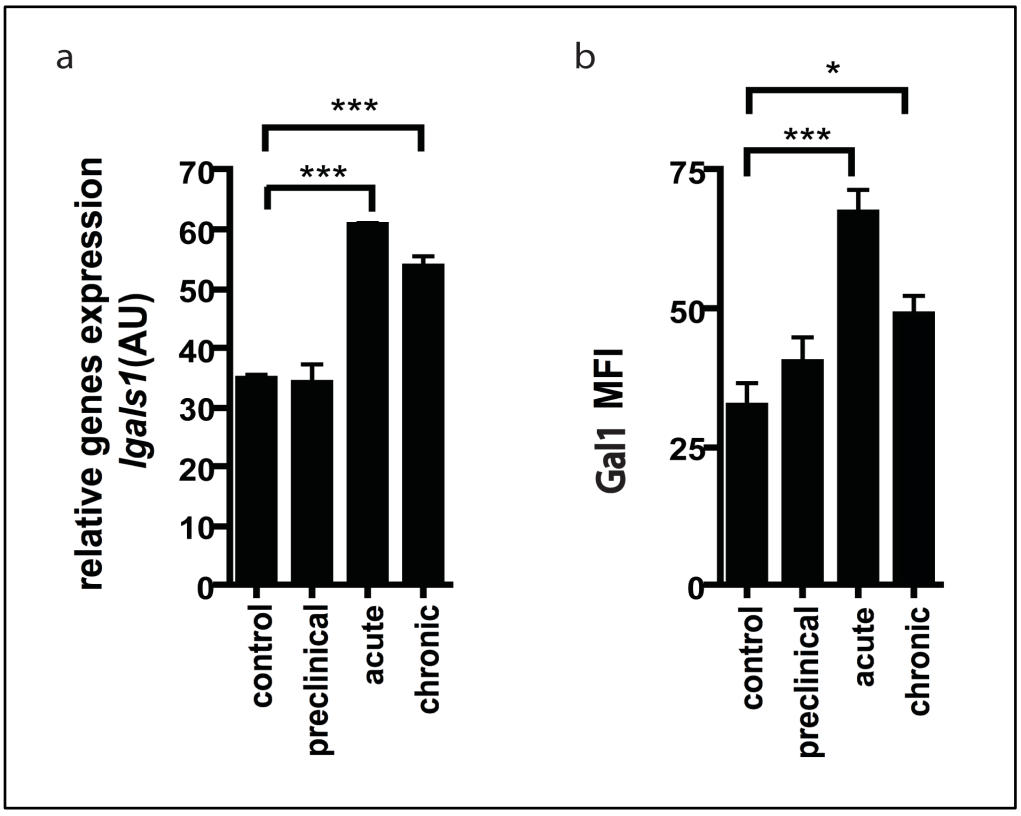


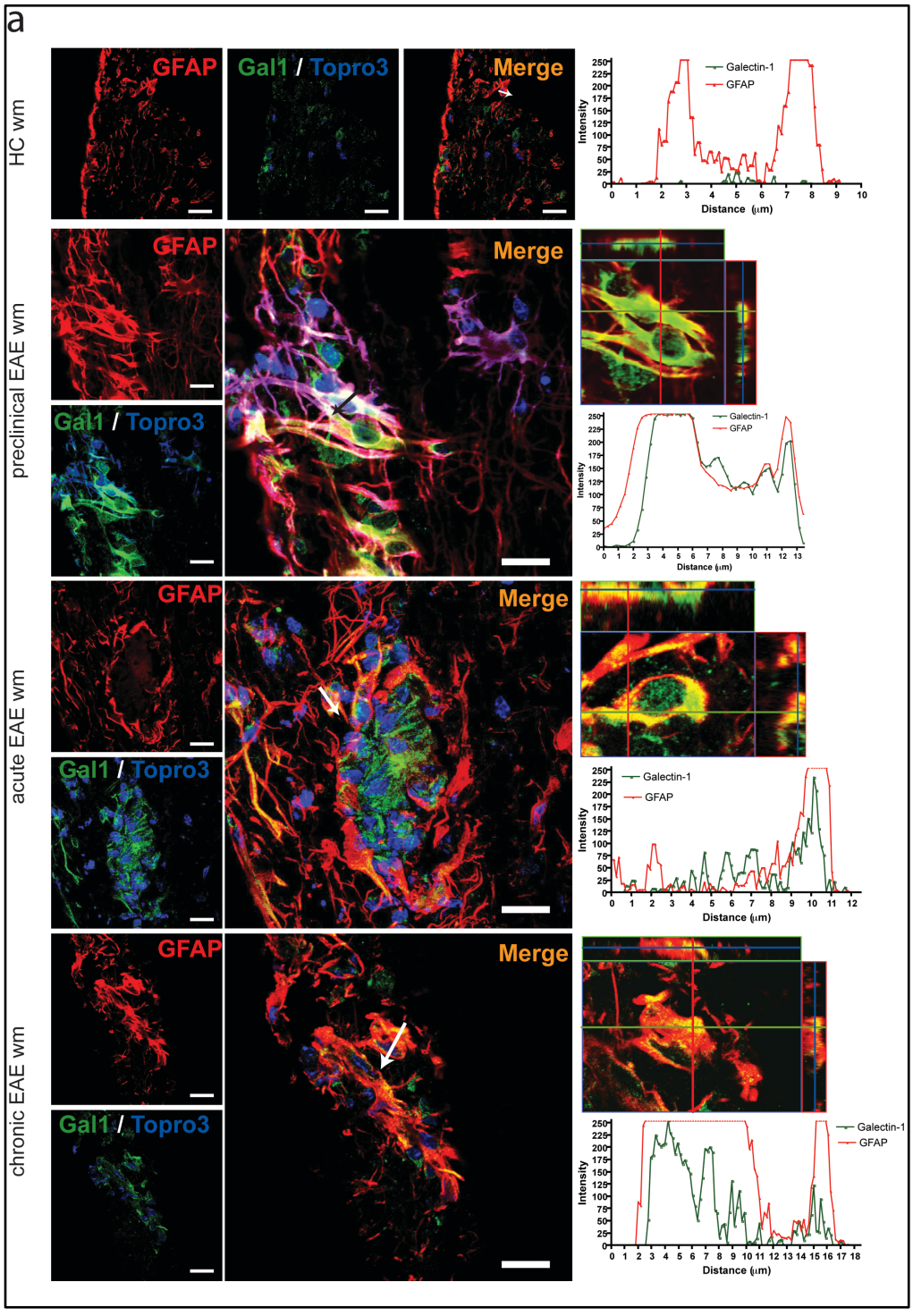
Figure 4.1.1-1: *Lgals1* gene and galetin-1 protein expression in CNS during EAE.

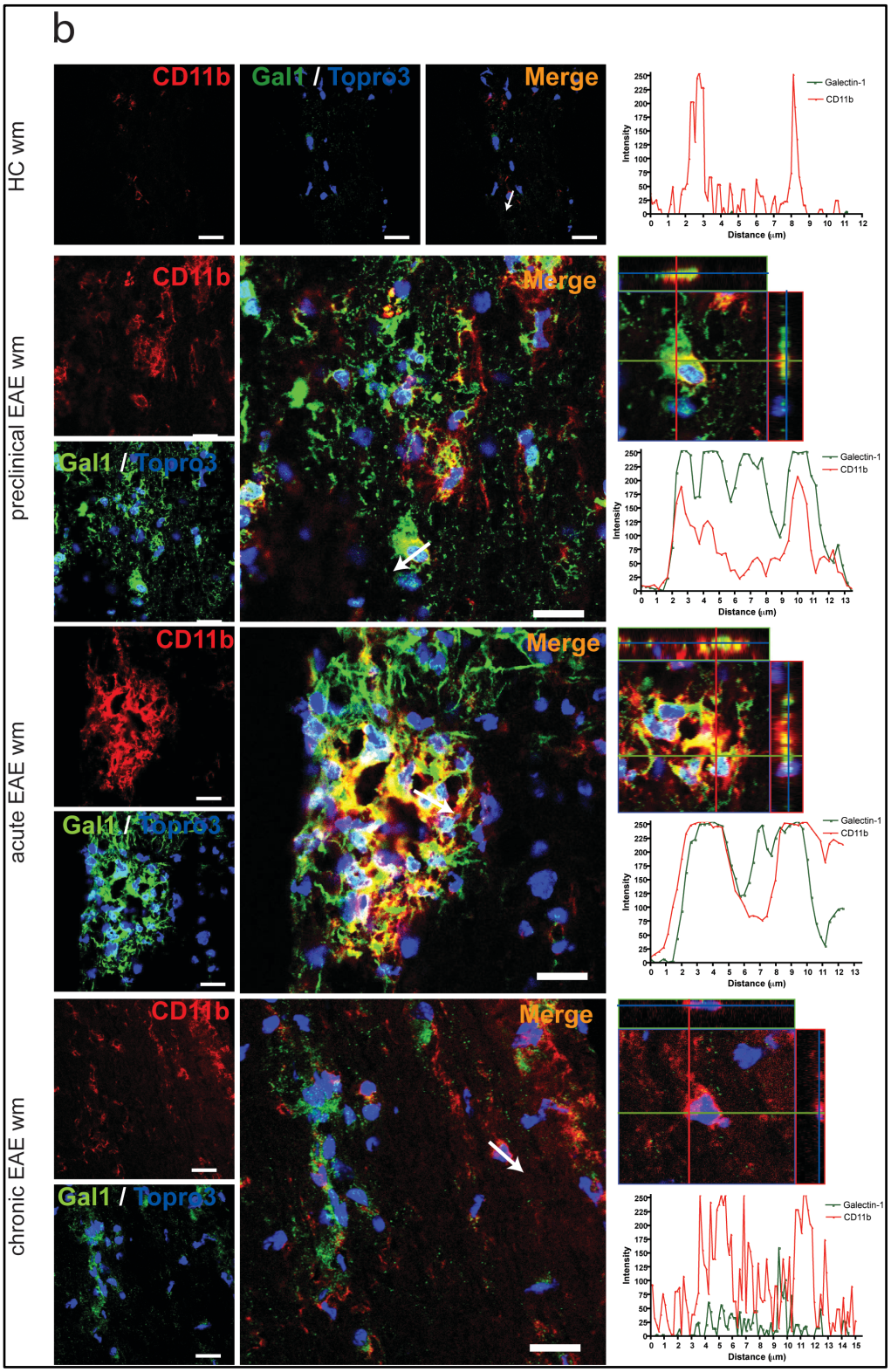
Lgals1 gene expression and galectin-1 protein expression in the spinal cord of CFA immunized (control), preclinical (10 dpi), acute EAE (acute, 20 dpi) and chronic EAE (chronic, 40 dpi) mice.

(a) Displayed are the levels of *Lgals1* gene expression in the spinal cord during EAE as measure by quantitative RT-PCR (**b**) Mean Fluorescence Intensity (MFI) of immunoreactivity against galectin-1 after confocal microscopy of the spinal cord white matter. $P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$ (Student's *t* test)

In order to investigate the cellular source of galectin-1 during the course of EAE, the spinal cord white matter regions of mice from preclinical, acute, and chronic EAE were immuno-stained for galectin-1 and cellular markers for astrocytes (GFAP), CD4+ T cells (CD4) and microglia/macrophages (CD11b). Co-localization was displayed in ortho-view of high-magnification light micrographs (Z stacks) of galectin-1 expressing cells and quantified by line-profile analysis.

Of note, galectin-1 expression was present in astrocytes, some CD4+ T cells and CD11b+ cells during preclinical EAE **(Fig. 4.1.1-2 a-c)**, but increased in $GFAP^+$ astrocytes bordering the lesion area **(Fig. 4.1.1-2 a)** and subsets of $CD11b^+$ cells and $CD4^+$ T cells **(Fig. 4.1.1-2 b,c)** in acute EAE**.** Interestingly, during the chronic phase of EAE only astrocytes maintained significant expression of this lectin **(Fig. 4.1.1-2 a)**. Thus, astrocytes and subsets of CD11b+ cells and CD4+ T cells accounte for a high abundance of galectin-1 in the white matter spinal cord during acute EAE but only astrocytes consistanly contribute to galectin-1 expression over the course of EAE.


a
HC wm
preclinical EAE wm
acute EAE wm
chronic EAE wm
GFAP
Gal1 / Topro3
Merge
Galectin-1
GFAP
Intensity
Distance (µm)


b
HC wm
preclinical EAE wm
acute EAE wm
chronic EAE wm
CD11b
Gal1 / Topro3
Merge
Galectin-1
CD11b
Intensity
Distance (µm)

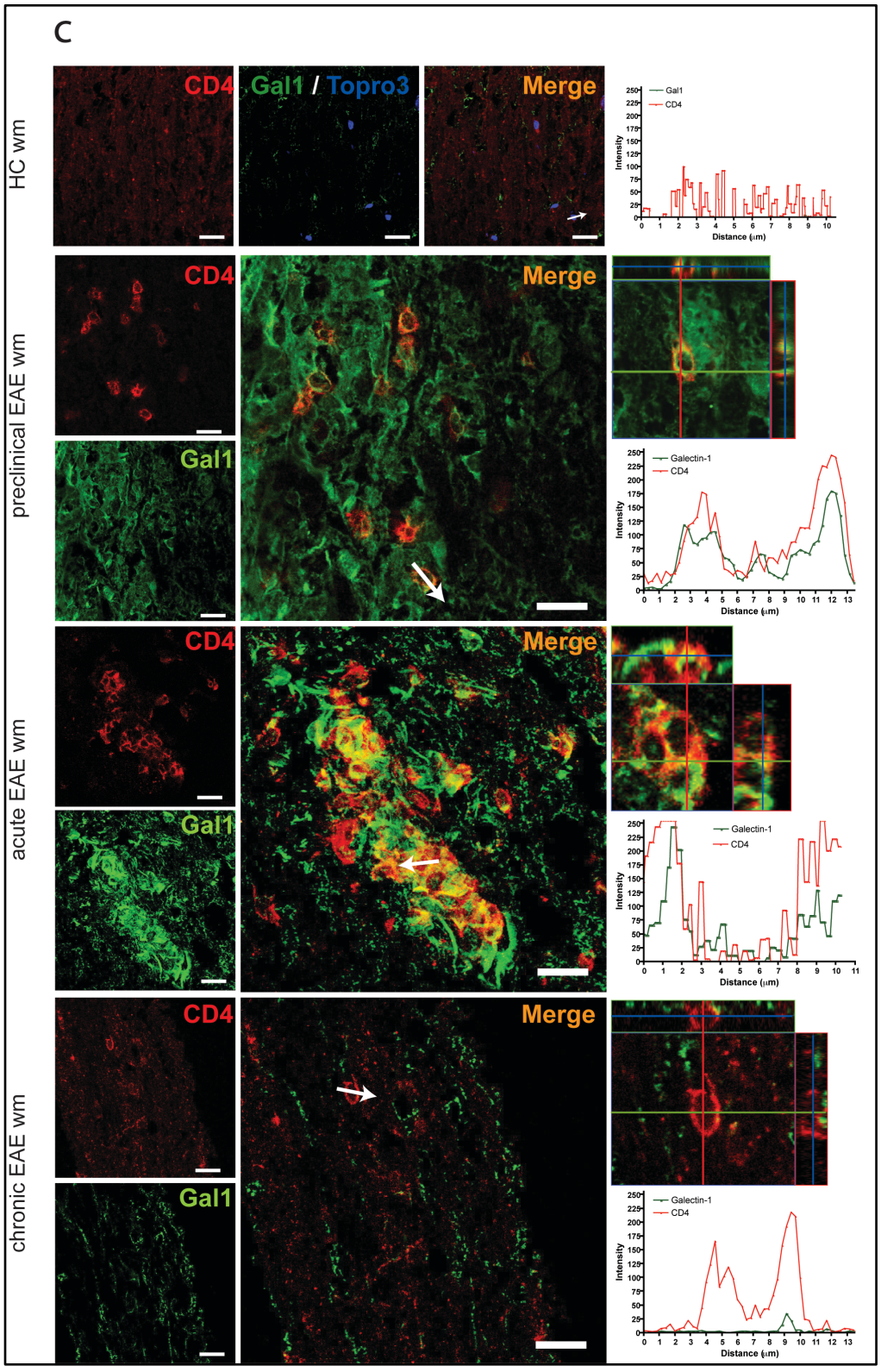

C
HC wm
preclinical EAE wm
acute EAE wm
chronic EAE wm
CD4
Gal1 / Topro3
Merge
Gal1
Galectin-1
Intensity
Distance (µm)

Figure 4.1.1-2 CNS expression of galectin-1 is dynamically regulated during EAE.
Confocal microscopy of spinal cord white matter sections from CFA immunized (control), preclinical EAE (preclinical EAE wm, 10dpi), acute EAE (acute EAE wm, 15-16 dpi) and chronic EAE (chronic EAE wm, 30-40 dpi) mice. Spinal cord sections were stained with anti-galectin-1 (green), the nuclear marker Topro3 (blue) and anti-GFAP (**a**, red), anti-CD11B (**b**, red) or anti-CD4 (**c**, red).
Representative cells were displayed in high magnification ortho-view.
Scale bars= 20μm. The chart shows pixel intensity of galectin-1 (green) and GFAP **(a,** red**),** CD11b (**b,** red) and CD4 (**c,** red) for correlating cells (as indicated by white arrow).

The CNS microenvironment may include various pro- and anti-inflammatory stimuli during EAE. Since only some subsets of CD11b+ cells and CD4+ T cells expressed galectin-1 during preclinical and acute EAE, specific stimulatory or polarization conditions must induce galectin-1 expression in those subsets.

Astrocytic regulation of galectin-1 was assessed by exposing neonatal astrocyte cultures to LPS, IFNγ, IL4, TGFβ$_1$ and recombinant galectin-1 and measuring *Lgals1* gene expression and galectin-1 protein secretion by quantitative RT-PCR and ELISA, respectively.

A strong downregulation of *Lgals1* mRNA expression following stimulation with LPS, a slight increase after IFNγ treatment and a strong upregulation after exposure to tolerogenic stimuli such as IL-4, TGFβ$_1$ and galectin-1 itself (**Fig. 4.1.1-3 a**) were observed. However only stimulation with IL-4 or TGFβ$_1$ led to increases in secreted galectin-1, whereas no changes were detected in the levels of soluble galectin-1 in astrocytes stimulated with pro-inflammatory factors (**Fig. 4.1.1-3 b**).

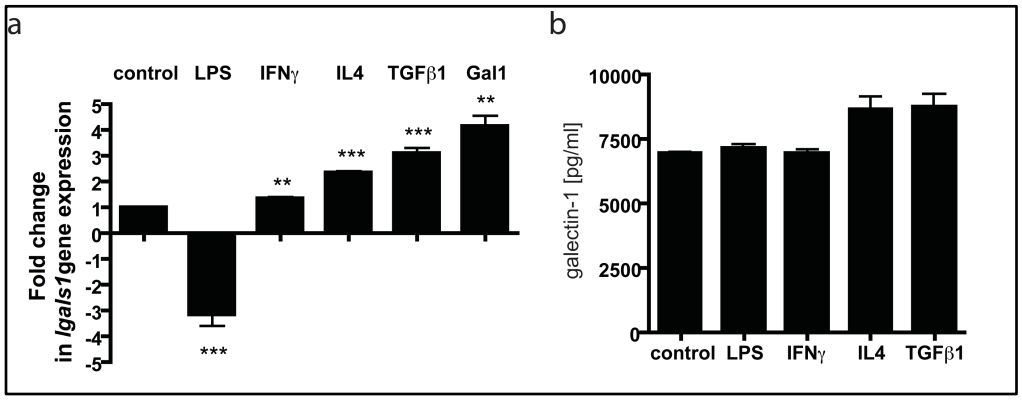


Figure 4.1.1-3. *Lgals1* gene expression and galectin-1 protein secretion in cultured astrocytes.
Neonatal astroyctes were stimulated with various pro- and anti-inflammatory cytokines for 48 hours. **(a)** Displayed is the fold change in relative *Lgals1* espression compared to control microglia as determined by quantitative RT- PCR. **(b)** Displayed are the levels of secreted galectin-1 as determined by ELISA. Significance * $P<0.05$; ** $P<0.01$; *** $P<0.005$ (Student's *t* test)

In microglia, *Lgals1* gene expression and galectin-1 protein secretion were tested by activating neonatal microglia with various pro- and antiinflammatory stimuli for 48 hours *in vitro*. **Fig 4.1.1-4** shows that both gene expression (**Fig 4.1.1-4 a)** and protein secretion (**Fig 4.1.1-4 b)** were highly increased after stimulation with the anti-inflammatory cytokines IL4 and IL13, whereas LPS and the pro-inflammatory cytokine IFNγ significantly suppressed galectin-1 expression. Furthermore stimulation of microglia with recombinant galectin-1 itself slightly inceased *lgals1* gene expression **Fig 4.1.1-4 a)**.

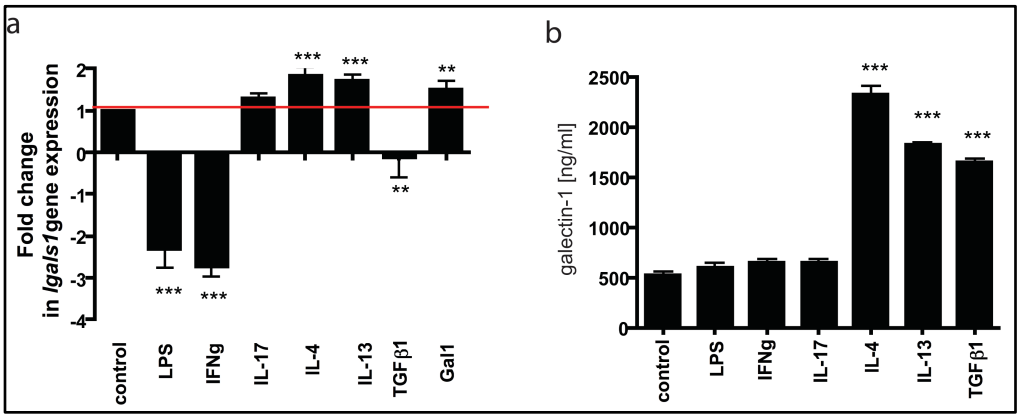


Figure 4.1.1-4. *Lgals1* gene expression and galectin-1 protein secretion in cultured microglia.
Neonatal microglia were stimulated with various pro- and anti-inflammatory cytokines for 48 hours. **(a)** Displayed is the fold change in relative *Lgals1* espression compared to control microglia as determined by quantitative RT- PCR. **(b)** Displayed are the levels of secreted galectin-1 as determined by ELISA. Significance * $P<0.05$; ** $P<0.01$; *** $P<0.005$ (Student's *t* test)

As for galectin-1 expression in T cells, FACS sorted naïve CD4+ $CD62L^{hi}$ $FoxP3^{-}$ T cells were isolated from FoxP3.GFP knock in (FoxP3.GFP.KI) reporter mice and differentiated into Th1, Th2, Th17 according to established protocols [114] or left undifferentiatied (Th0). Furthermore, natural occurring regulatory T cells (nTregs) were sorted from naïve FoxP3.GFP.KI mice based on their in vivo FoxP3 expression [114]. Using quantitative RT-PCR, *lgals1* expression was identified to be highly upregulated in Th1 cells and Tregs whereas other polarization conditions induced only moderate galectin-1 gene expression **(Fig. 4.1.1-5)**.

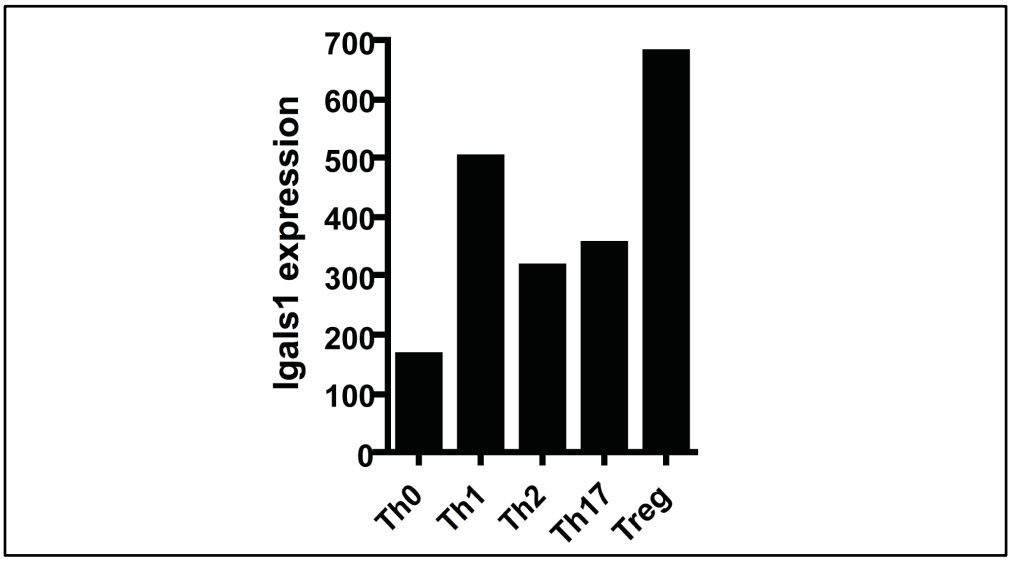


Figure 4.1.1-5. *Lgals1* gene expression in T cell subsets.
$CD4^+$ $CD62L^{hi}$ $FoxP3/GFP^-$ T cells from FoxP3.GFP.KI mice were stimulated with anti-CD3 & anti-CD28 in the presence of the coresponding cytokines for 4 days. Displayed are the levels of *Lgals1* relative espression as determined by quantitative RT- PCR.

Thus, with the exception of proinflammatory Th1 cells, galectin-1 is mainly produced in response to a tolerogenic microenvironment *in vitro.* Therefore it is likely that Th1 and Tregs as well as alternatively activated CD11b cells account for the galectin-1 production in the CNS during EAE. Moreover, tolerogenic stimuli may also induce galectin-1 production by astrocytes during EAE.

4.1.2. Galectin-1 negatively regulates M1 microglia activation through modulation of p38MAPK and pCREB pathways

Activation with LPS or IFN-γ induces a M1 phenotype in microglial cells characterized by high MHC II, CD86, and *iNOS* expression, and production of pro-inflammatory cytokines and chemokines such as TNF and CCL2 [115], whereas stimulation with IL-4 induces a M2 phenotype [95] characterized by *Arginase1* (*Arg1*) expression. Gal-1 differentially binds to selected cellular populations bearing a restricted set of glycans resulting from the regulated expression of glycosyltransferases [99]. Thus, the binding of this glycan-binding protein to either M1 or M2 microglia was investigated and the 'glycosylation signature' of these cells was assesed by flow cytometry. For this, neonatal microglia were stimulated with LPS or IFNγ (M1), IL-4 (M2) or left unstimulated (resting microglia) and exposed to increasing concentrations of galectin-1, and its binding was measured by flow cytometry. Galectin-1 bound to all primary microglia subsets in a dose-dependent manner (**Fig. 4.1.2-1).**

Interestingly, galectin-1 binding was markedly increased when microglia were polarized toward an M1 phenotype by exposure to either LPS or IFN-γ for 24h (**Fig. 4.1.2-1)**. In contrast, galectin-1 binding to M2-polarized microglia was significantly decreased compared to unstimulated microglia **(Fig. 4.1.2-1)**.

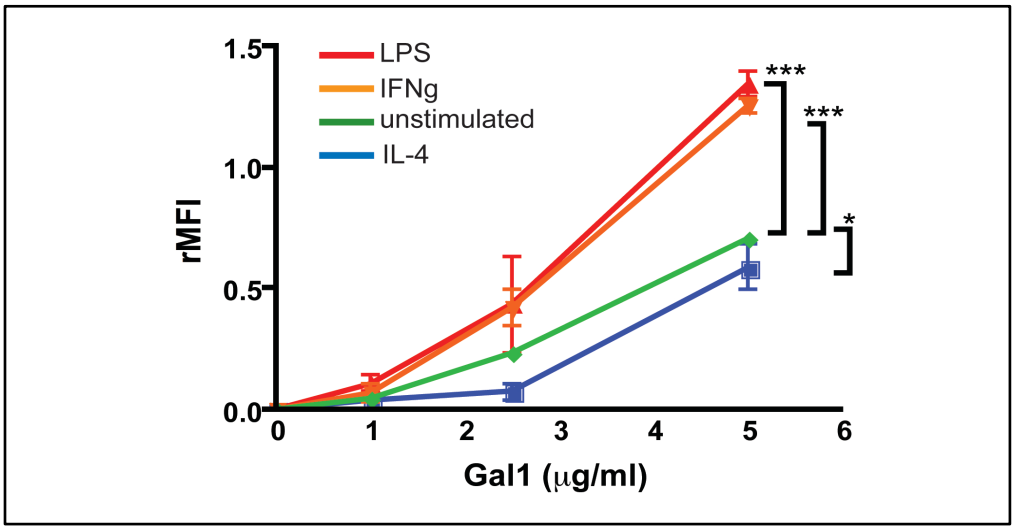


Figure 4.1.2-1: Galectin-1 binding to polarized microglia.
Flow cytometry of resting and M1 (IFNg, LPS) or M2 (IL-4) polarized microglia subsets, incubated with increasing concentrations of antibody-pre-absorbed recombinant mouse galectin-1 (Gal1) and analyzed with APC-conjugated streptavidin.
*P<0.05; **P<0.01; ***P<0.005 (2-way ANOVA)

To determine whether the observed binding was carbohydrate dependent, galectin-1 was added to polarized microglia in the presence or absence of lactose and sucrose. While the addition of lactose, which is a gelactin-1 specific sugar, fully abrogated galectin-1 binding to all microglia subsets, addition of the disaccharide sucrose, which has a very low binding affinity to galectin-1, was not able to prevent its binding to microglia (**Fig. 4.1.2-2)**. Thus, galectin-1 binds all microglia subsets in a saccharide-dependent fashion.

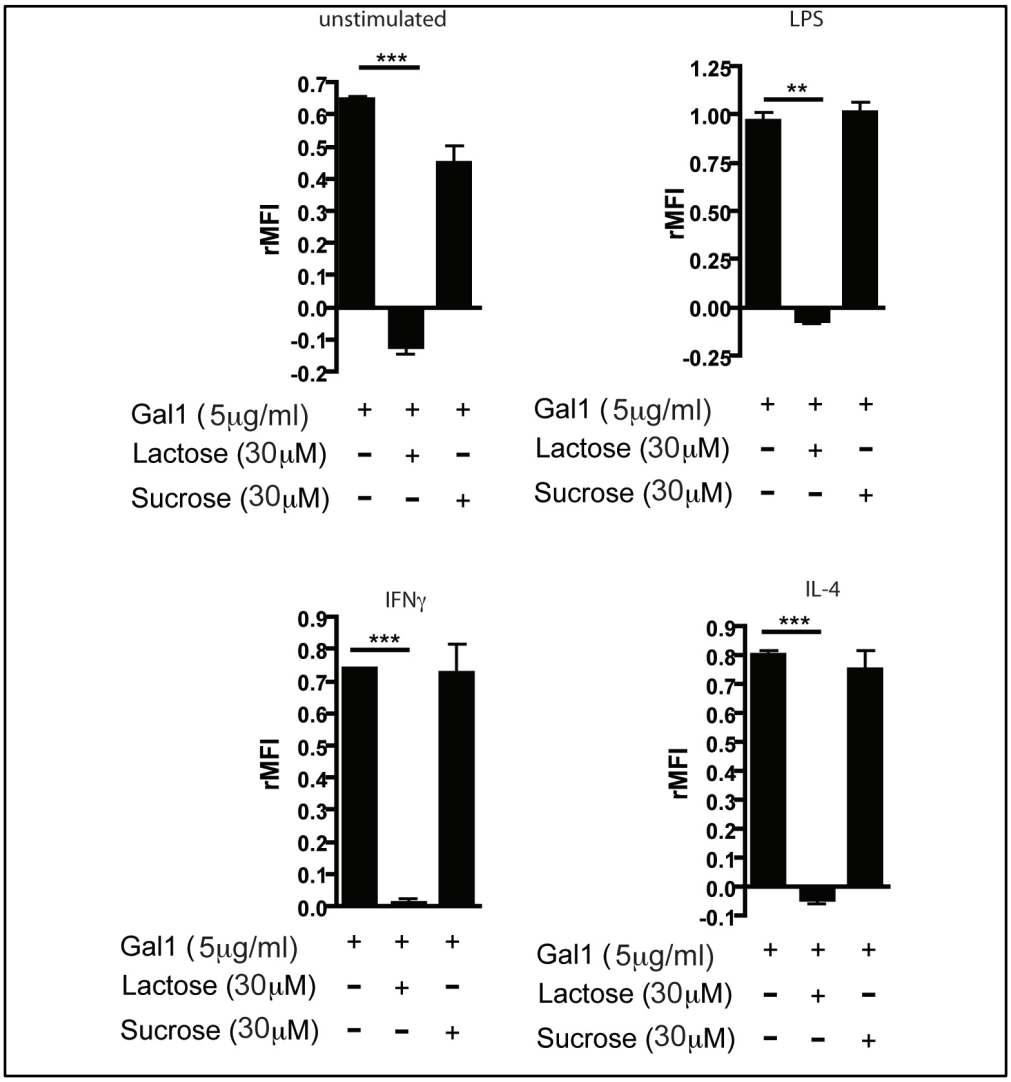


Figure 4.1.2-2: Galectin-1 binds microglia in a carbohydrate-dependent manner.
Resting (unstimulated), LPS, IFN-γ or IL-4 polarized microglia subsets were incubated with antibody-absorbed recombinant galectin-1 (5 □g/ml) in the presence or absence of specific (lactose; 30mM) or non-specific (sucrose; 30mM) disaccharides and further stained with APC-conjugated streptavidin. Bar graph represents the relative mean fluorescence intensity (rMFI): mean fluorescence intensity of galectin-1 - mean fluorescence intensity without galectin-1). *P<0.05; **P<0.01; ***P<0.005 (Student's t test)

Next, the differential binding capacity was correlated with the distinct glycosylation signatures of these cells.

To directly compare these 'glycophenotypes', a panel of plant lectins that selectively recognize specific oligosaccharide sequences as previously described [99] was made use of: *Sambucus nigra agglutinin* (SNA), a plant lectin that recognizes the α2-6-linked sialic acid and interferes with Gal-1 binding, *Maackia amurensis* agglutinin (MAL II) binds to α2-3 sialic acid linkages, L-phytohemagglutinin (PHA) recognizes β1-6 branching on complex *N*-glycans and *Helix pomatia* (HPA) binds specifically to terminal α-N-acetylgalactosamine residues.

The binding of biotynilated plant lectins was assessed by flow cytometry and displayed as histograms, comparing each binding to control-streptavidin binding. Furhermore, the relative binding was quantified by calculating the relative median fluorescence (rMFI) displayed in red (**Fig. 4.1.2-3).**

As compared to M1 polarized cells, M2 polarized microglia bound with higher avidity to SNA consistent with the observed decreased binding of galectin-1 to the surface of M2 polarized cells and the well-recognized ability of sialic acid-α2-6 sequences to interfere with Gal-1 recognition. In addition, M1, but not M2 polarized microglia showed higher binding capacity to PHA, suggesting increased β1,6 branching of complex N-glycans which are the preferred ligands of galectin-1. Furthermore, the results showed decreased sialylated core 1-*O*-glycans in M1-polarized compared to M2-polarized microglia as shown by abundant reactivity of these cells for peanut agglutinin (PNA) that recognizes asialo-galactose β1-3-N-acetylgalactosamine (core-1) O-glycans. Augmented PNA reactivity indicates increased availability of glycan structures required for elongation of core-2 O-glycans through the action of core-2-N-acetylglucosaminyltransferases (C2GnTs),

which favors LacNAc incorporation and galectin-1 binding. This lectin-binding pattern was accompanied by higher HPA reactivity in classically versus alternatively-activated microglia. Finally, both LPS-stimulated M1- and M2-polarized microglial cells had similar binding profiles for MAL II, although IFN-γ-stimulated cells had lower MAL II binding (**Fig. 4.1.2-3**). As α2,3-linked sialic acid may allow binding of galectin-1 with different affinities than asialo-LacNAc structures [116] these results suggest that subtle differences may exist in the glycoprofile of different subpopulations of classically-activated microglia (LPS or IFN-γ).

Collectively, these findings suggest that M1 but not M2 microglia show the complete repertoire of cell surface glycans that are critical for galectin-1 binding and function, suggesting that this endogenous lectin may preferentially modulate the physiology of M1-polarized microglia.

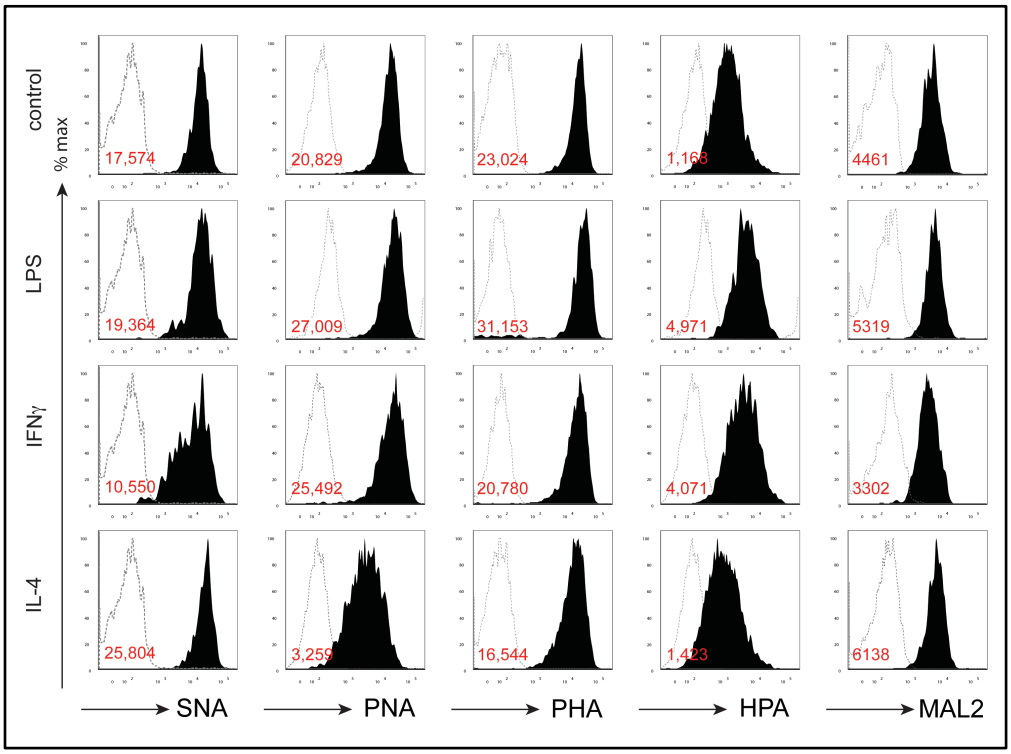


Figure 4.1.2-3: Differential glycosylation of M1- and M2-polarized microglia selectively regulates susceptibility to Gal1 binding.
Expression of cell surface oligosaccharides on M1 (LPS, IFN-g), M2 (IL-4) and resting (unstimulated) microglia, detected with biotinylated *Sambucus nigra* agglutinin (SNA), *Arachis hypogaea* peanut lectin (PNA), L-phytohemagglutinin (L-PHA), *Helix pomatia* (HPA) and *Maackia amurensis* agglutinin (MAL II) (black filled histograms) or with fluorescein isothiocyanate-conjugated streptavidin alone (dashed open histogram). Red numbers represent the relative median of intensity (median of intensity (Lectin) – median of intensity (streptavidin control)) Data are representative of three independent experiments.

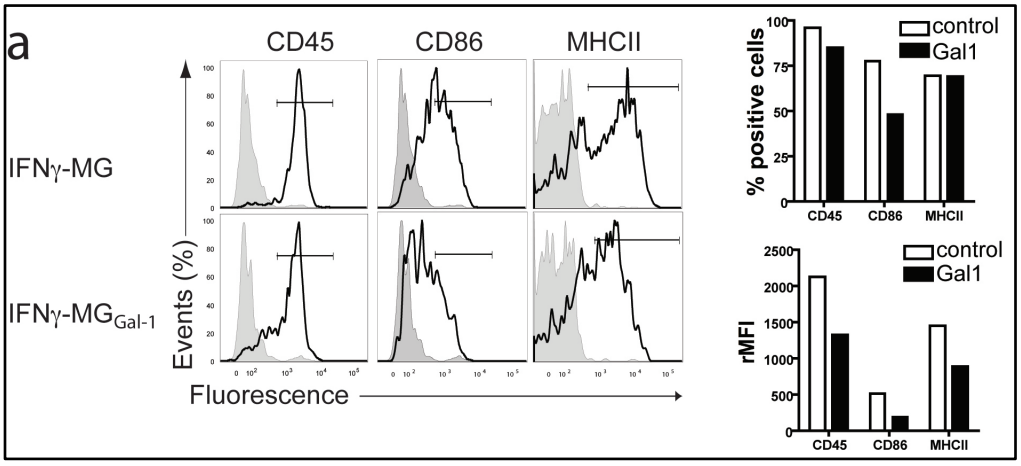
In order to investigate whether binding of galectin-1 to microglia results in phenotypic or functional changes, neonatal microglia were stimulated with IFNγ and cultured in the presence or absence of Galectin-1. As an increase in CD45, MHC II and CD86 in microglia and macrophages indicates an increase in classical activation [122], the

expression of these cell-surface activation was measured by flow cytometry.

Interestingly, surface expression of CD45, MHC II, and CD86 in IFNγ-polarized M1 microglia was substantially decreased following exposure to recombinant galectin-1 (**Fig. 4.1.2-4 a)**.

Furthermore, the change in expression of the activation markers *iNOS* (M1) and *Arg1* (M2) as well as changes in the secretion of TNF (M1) and CCL2 (M1), were measured by quantitative RT-PCR and luminex technology, respectively.

iNOS mRNA expression, as well as the production of TNF were significantly decreased by galectin-1exposure (**Fig. 4.1.2-4 b-e**). While the decrease in CCL2 production did not reach statistical significance in the galectin-1-stimulated microglia polarized with IFN-γ (**Fig. 4.1.2-4 c**), it was significantly decreased in LPS-polarized M1 microglia (**Fig. 4.1.2-4 e**). On the other hand, M2 polarized microglia experienced a relative increase in *arginase1* and *iNOS* mRNA compared to unstimuated microglia; while exposure to galectin-1 (at the highest dose) enhanced arginase expression but decreased iNOS expression (**Fig. 4.1.2-4 f**). Thus, galectin-1 deactivates M1 microglia and may induce a shift towards a M2 activation phenotype.



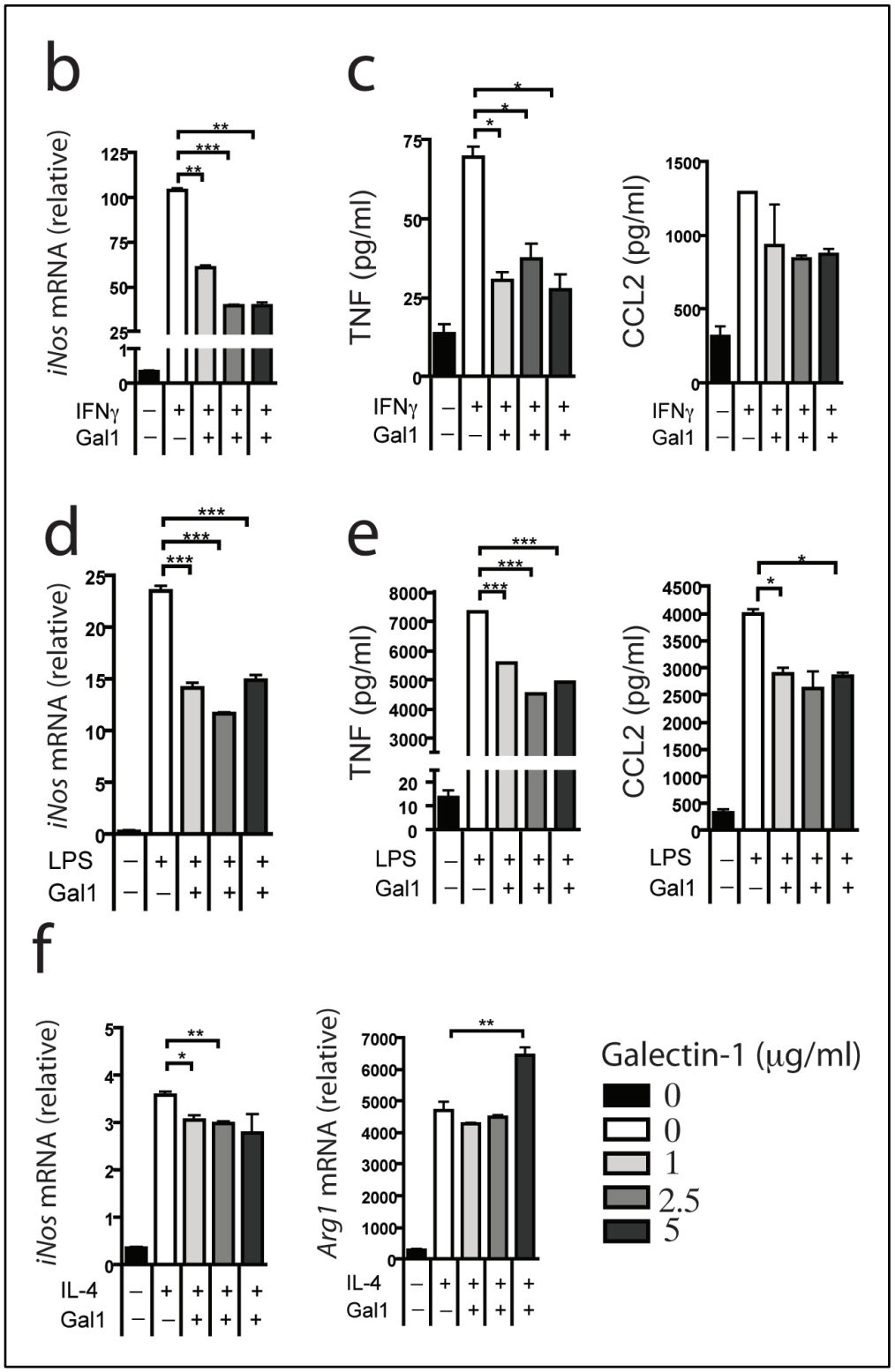

b
iNos mRNA (relative)
IFNγ
Gal1
c
TNF (pg/ml)
CCL2 (pg/ml)
d
e
LPS
f
IL-4
Arg1 mRNA (relative)
Galectin-1 (μg/ml)
0
0
1
2.5
5

Figure 4.1.2-4: Galectin-1 differentially modulates microglia activation *in vitro*
(a) Flow cytometry of M1 microglia activated by IFN-γ (24 h). Control and galectin-1-treated microglia were stained with antibodies against the surface markers CD45, CD86 and MHC II. Black lines represent specific antibody binding whereas tinted lines represent unspecific fluorescence signal. Below, percentage of positive cells and relative median fluorescence (rMFI): (median fluorescence intensity of specific marker signal – median fluorescence intensity of unspecific signal). **(b-f)** Effect of galectin-1 on the expression of specific activation markers of IFNγ- (b,c), LPS- (d,e) or IL-4 (f) stimulated microglia as determined by quantitative RT-PCR (b,d,f) for *iNos* and *Arg1* (d,f) or by bead-based Luminex assay for TNF and CCL2 (c,e).
* $P<0.05$; ** $P<0.01$; *** $P<0.005$ (Student's *t* test)

As galectin-1 selectively deletes Th1 and Th17 cells [99] we examined whether exposure to this lectin may also induce apoptosis in microglia; Therefore, resting (unstimulated) and activated microglia (LPS) neonatal microglia were cultured in the presence of increasing concentrations of recombinant galectin-1 (ranging from 0.1 to 10μg/ml) for 24 hours followed by staining with the cell death marker 7AAD and the apoptosis specific marker Anexin-V. Intensity of staining was analyzed by flow cytometry and resulting percentages of doube positive cells are diplayed below.

Remarkably, galectin-1 had no effect on the survival of microglial cells, as no significant changes in double positive could be detected upon exposure of both resting and activated microglia to increased concentrations of galectin-1. However, a slight increase in cell death was observed after activation with LPS **(Fig. 4.1.2-5**).

Thus, galectin-1 actively inhibits the expression of markers of classical activation and promotes a phenotype of alternative activation in microglia without affecting microglia survival.

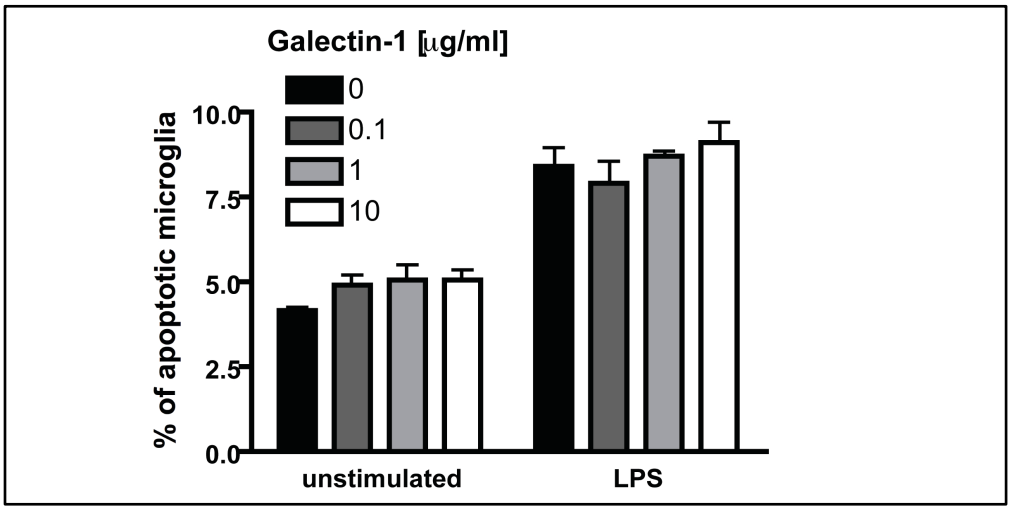


Figure 4.1.2-5: Galectin-1 does not effect microglia survival.

Percentage of 7AAD and AnexinV double positive cells. Resting (unstimulated) or LPS-stimulated neonatal microglia were cultured in the presence or absence of increasing concentrations of galectin-1 (ranging from 0.1 to 10 μg/ml) for 24 hours. 7AAD and AnnexinV staining was assed by flow cytometry.

The production of NO and TNF is controlled by pro-inflammatory signaling pathways involving nuclear factor-kappa B (NF-κB) [102,117,118], extracellular signal-regulated kinase (ERK) [119], p38 mitogen-activated protein kinase (p38MAPK) [120] and cAMP response element binding (CREB) [121]. The effect of galectin-1 on the phosphorylation of these signaling molecules was investigated by stimulating BV-2 microglia cells with LPS in the presence or absence of recombinant galectin-1. Cells were lysed after 5 min and 15 min and resulting cell lysates containing total protein were subjected to phosphorylation analysis using Luminex technology. LPS induced phosphorylation of the inhibitor of kappa B-alpha (IκB-α), a negative regulator of NF-κB pathway after 5 min, and p38-MAPK, pERK and pCREB after 15 min of incubation **(Fig. 4.1.2-6).** However, activation of BV-2 microglia with

LPS in the presence of galectin-1 promoted a striking decrease in the phosphorylation of p38-MAPK (58.09%) and CREB (63.15%), but only slight inhibition of phosphorylation of ERK (26.03%) and IκB-α (28.8%). These data suggest that galectin-1 may limit microglia activation through modulation of p38MAPK and pCREB dependent pathways.

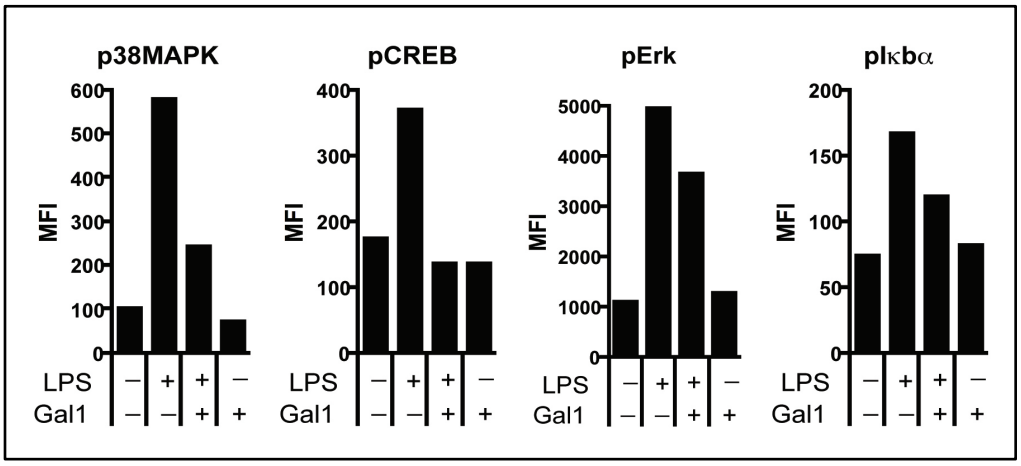
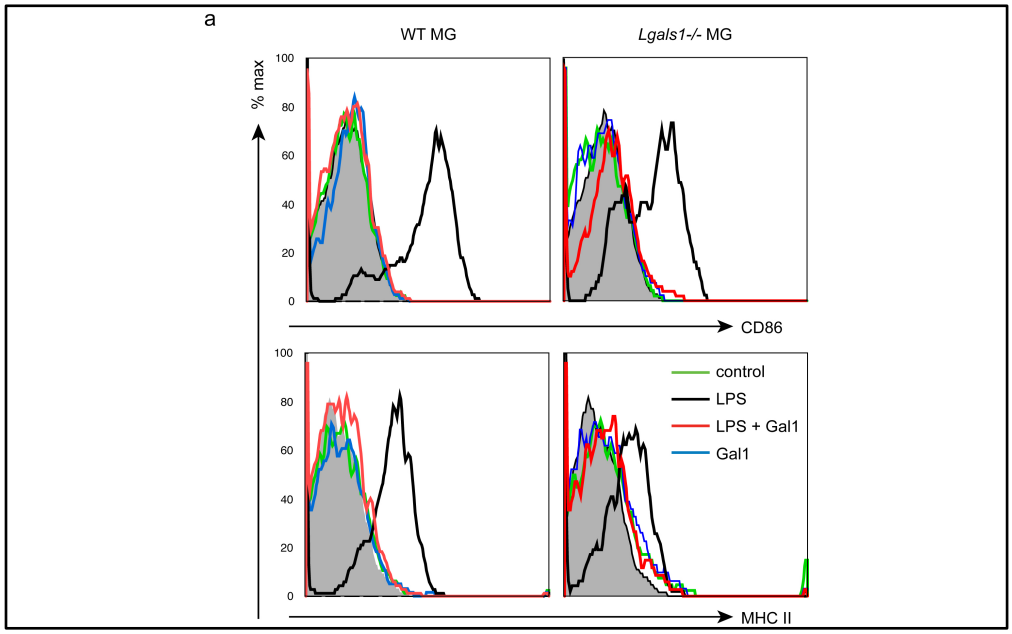


Figure 4.1.2-6: Galectin-1 differentially modulates microglial phsophrylation pathways *in vitro*
Effect of galectin-1 on LPS-induced signaling pathways in BV-2 microglia. Phosphorylation of p38-MAPK, CREB, ERK1,2 and IκB-α was analyzed by bead-based luminex assay as described in *Materials & Methods*.

Since microglia expresse and secrete galectin-1 under certain conditions, it was hence questioned whether intracellular galectin-1 expression impacts the modulatory effect of exogenous galectin-1 on microglia. To investigate this possibility, neonatal microglia were isolated from WT and *Lgals1-/-* mice and stimulated with PBS (control) LPS, LPS + Gal1 and Gal-1. After 24 hours, microglia cells were stained for the classical activation markers CD86 and MHC II and analyzed by flow cytometry **(Fig. 4.1.2-7 a)**. Changes in activation

were quantified as percentage of positive cells and median fluorescence intensity (MFI) **(Fig. 4.1.2-7 b)**.

In both, wild type and *Lgals1*-/- microglia, LPS induced a strong phenotype of classical activation, whereas stimulation with galectin-1 did not induce any changes in microglia activation. However, when stimulated with LPS and galectin-1 both cell types showed a down-regulation of CD86 and MHC II surface expression. In Lgals1-/- microglia the down-regulation of CD86 and MHC II was similar to that observed in WT microglia, suggesting that endogenously produced galectin-1 does not participate in the negative regulation of classical microglia activation by exogenous galectin-1. Interestingly, Lgals1-/- microglia showed a slightly reduced expression of CD86 and MHCII after LPS activation compared to WT cells.



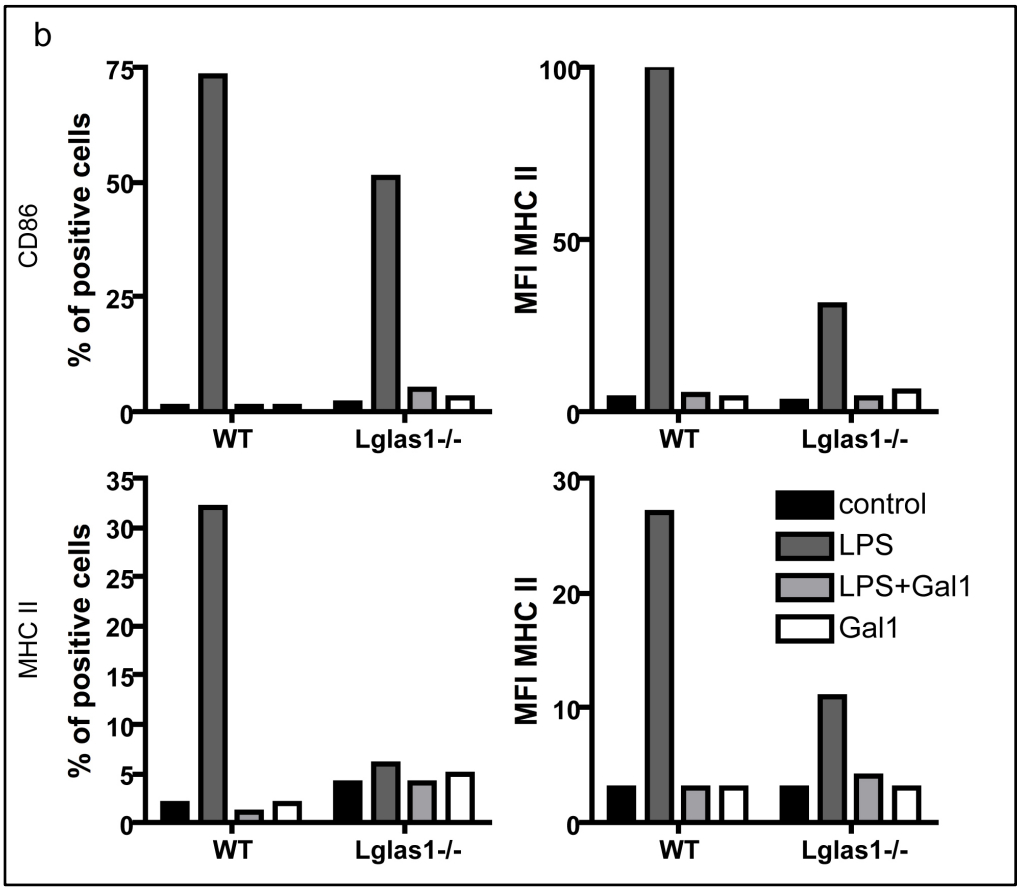


Figure 4.1.2-7: Effect of endogenous galectin-1 on microglia activation.
(a) Flow cytometry of M1 wild type (left) and Lgals1-/- (right) microglia, activated by PBS (control, green line), LPS (black line), LPS+Gal1(red line) and Gal1 (blue line) for 24h. Treated microglia were stained with antibodies against the surface markers CD86 and MHC II. Tinted lines represent unspecific fluorescence signal. (b) Percentage of positive cells and median fluorescence (MFI) of CD86 and MHC II surface expression.

4.1.3. Lack of endogenous galectin-1 enhances classical microglia activation and worsens axonal damage during EAE

In the healthy CNS, microglia cells are in a resting state, monitoring the CNS microenvironment, but get activated under inflammatory conditions [76,122] or after CNS injury [37]. Once activated, microglia may contribute to CNS pathology or repair depending on the prevalent microenvironment and their mode of activation [123-125]. Whereas classically activated M1 microglia are involved in inflammation-mediated neurotoxicity, alternatively-activated M2 microglia are thought to have neuroprotective functions.

EAE was induced in galectin-1-deficient (*Lgals1*$^{-/-}$*; KO*) and wild-type (WT) C57BL/6 mice, in order to investigate the role of galectin-1 on microglia activation *in vivo*. Spinal cord section obtained on 30 post immunization were co-stained for the microglia marker Iba1 and the activation marker MHC II in order to assess micorglia activation.

Remarkably, Iba^{+}MHC II^{+} microglia were considerably more abundant in *Lgals1*$^{-/-}$ mice, when compared to WT EAE mice **(Fig. 4.1.3-1 a)**, while healthy control mice had no Iba1^{+}/MHC II^{+} cells in the CNS (data not shown).

Furthermore spinal cord sections were stained for the neuronal and axonal marker β-III-tubulin (Tuj1) **(Fig. 4.1.3-1 b)**, Gap43 (a marker for axonal growth cones) **(Fig. 4.1.3-1 c)**, myelin basic protein (MBP; a marker of demyelination) **Fig. 4.1.3-1 d)** and GFAP (a astrocyte marker) **Fig. 4.1.3-1 e).**

Lgals1$^{-/-}$ mice had decreased immunoreactivity against Tuj1 and Gap43 compared to WT mice, indicating a higher degree of axonal and neuronal loss as well as impairement in neuronal repair, respectively. Immunoreactivity against MBP was also decreased, suggesting a

higher degree of demyelination as compared to WT EAE mice. However, immunoreactivity against GFAP was increased in Lgals1-/- EAE mice.

Thus, targeted deletion of endogenous galectin-1 significantly increases axonal loss, demyelination and astrogliosis and decreases axonal outgrowth during ongoing autoimmune neuroinflammation.

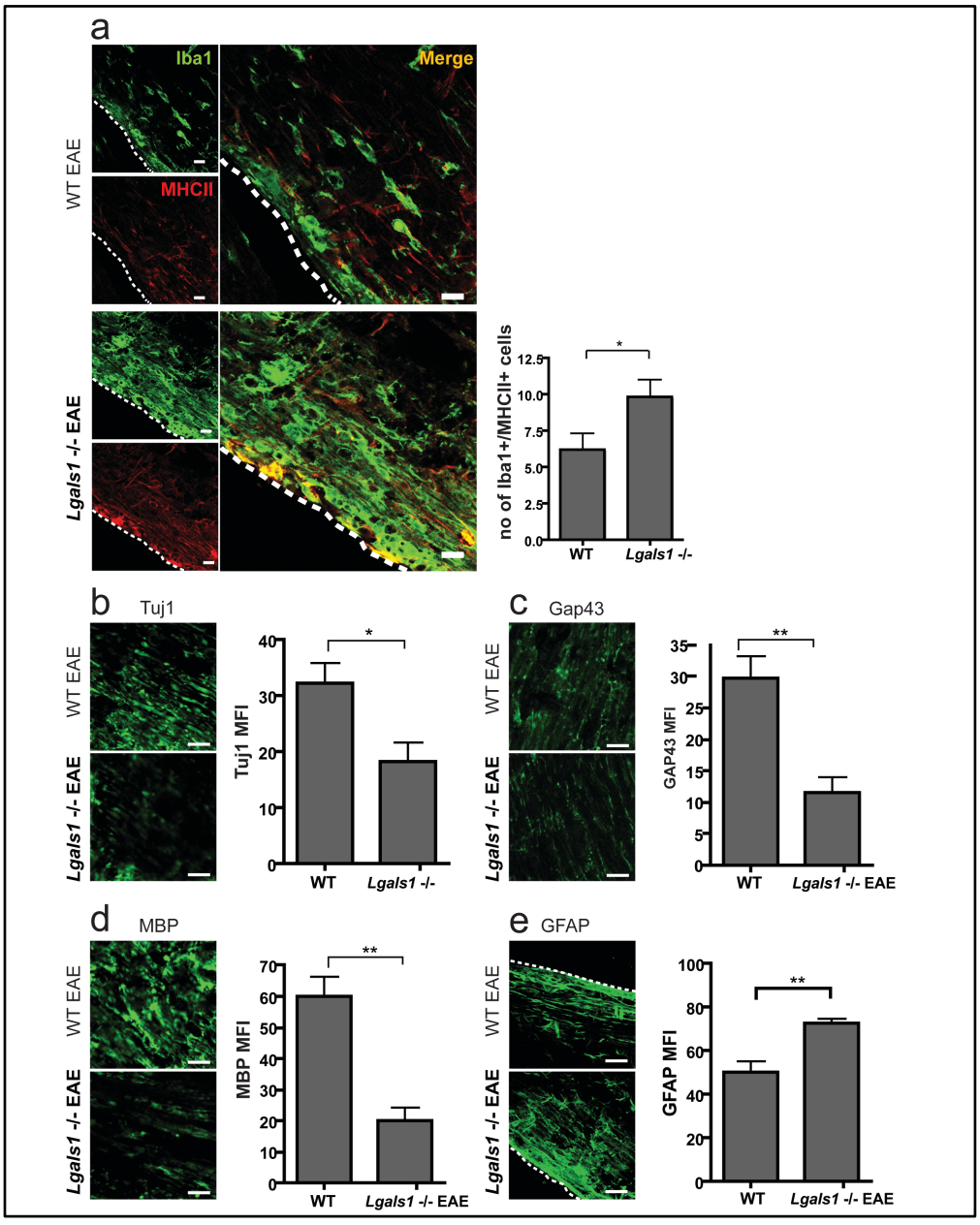


Figure 4.1.3-1: Endogenous galectin-1 modulates classical microglia activation *in vivo* and limits EAE neuropathology
Confocal microscopy of spinal cord white matter of wild-type (WT EAE) and *Lgals1*$^{-/-}$ mice (Gal-1 KO EAE) 35 days after immunization with MOGp35-55. (**a**) Left. Spinal cord sections were stained for Iba1 (green) and MHC II (red). Right. Quantification of MHC II/Iba1 double positive microglia galectin-1 and vehicle-treated mouse spinal cords sections. Quantification data results from 20 independently analyzed areas (n=20) (**b**) Left. Spinal cord sections were stained for Tuj1 (green). Right mean fluorescence intensity (MFI) of immunoreactivity against Tuj1. (**c**) Left. Spinal cord sections were stained for Gap43 (green). Right mean fluorescence intensity (MFI) of immunoreactivity against Gap43. (**d**) Left. Spinal cord sections were stained for MBP (green). Right mean fluorescence intensity (MFI) of immunoreactivity against MBP. (**e**) Left. Spinal cord sections were stained for GFAP (green). Right mean fluorescence intensity (MFI) of immunoreactivity against GFAP. Scale bars= 20 μm;
* P<0.05; ** P<0.01; *** P<0.005 (Student's t test)

4.1.4. Galectin-1 controls microglia-mediated neurotoxicity

To gain insight into the functional consequences of galectin-1-induced regulation of microglia activation, an *in vitro* model of microglia-mediated neurotoxicity [126] was established. Neurons were co-cultured with resting microglia, or microglia pre-activated by LPS in the absence or presence of recombinant galectin-1 or galectin-1 alone. Since neurotoxicity is closely associated with a collapse of cytoskeleton proteins [127], the intensity of immunoreactivity against microtubule-associated protein 2 (Map2) as well as the density of Map2-positive cells per condition served as measure for the severity of neurotoxicity.
Co-culture with LPS-activated microglia resulted in increased neuronal cell body destruction with decreased Map2 immunoreactivity **(Fig. 4.1.4-1 a-b)** indicating that LPS activated microglia induce a collapse

of cytoskeleton proteins and subsequent cell death in cultured neurons.

Interestingly, co-culture with microglia activated in the presence of LPS and galectin-1, showed significantly better preservation of neurons **(Fig. 4.1.4-1 a-b)**. Moreover, galectin-1-treated resting microglia behaved like resting microglia. Thus, galectin-1 provides neuroprotection through deactivation of M1 activated microglia cells *in vitro.* This mechanism may also explain the increase in neurodegeneration observed *in vivo* in galectin-1-/- mice (Section 4.2.3).

A recent report suggested that galectin-1 may directly influence neuronal integrity and survival [128]. In order to investigate this possibility, we added recombinant galectin-1 to cortical neuronal cultures for 48 hours. Unlike the observation reported by Plachta et al, we found no significant changes in neuronal density **(Fig. 4.1.4-2 c)** or cytoskeleton integrity **(Fig. 4.1.4-2 c)** by direct addition of galectin-1.

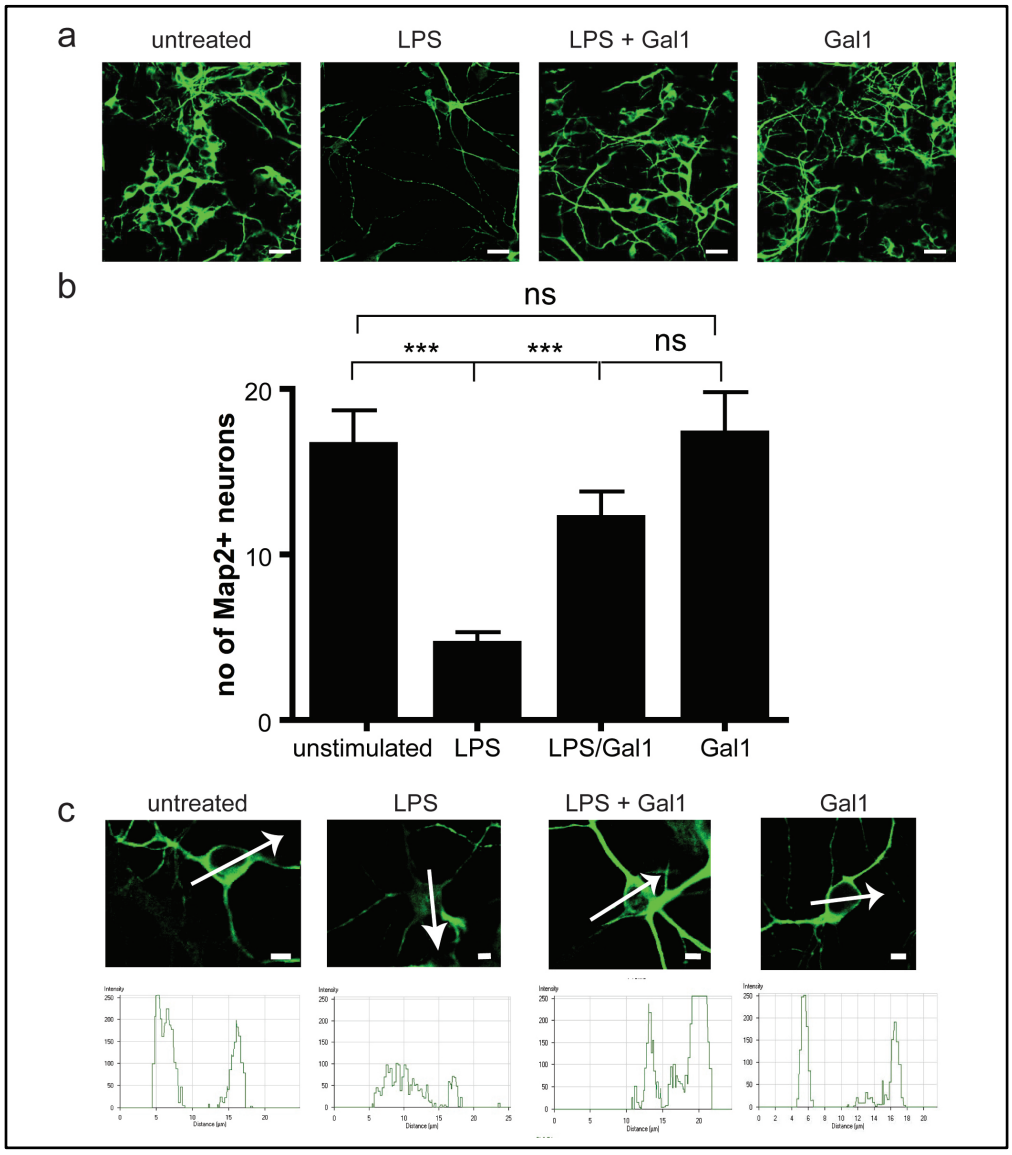


Figure 4.1.4-1 Galectin-1 negatively regulates microglia-induced neurotoxicity

24 h co-culture of pre-treated microglia (untreated MG, LPS treated MG, LPS+Gal1 treated MG and Gal1-treated MG) with high-density cortical neuronal cultures. (a) Representative fluorescence photomicrograph of Map-2$^+$ (green) neurons. (b) Quantification of neuronal density measured as Map2$^+$ cell bodies per culture (n=20) (c) High magnification photomicrograph of single Map2$^+$ neurons and pixel intensity analysis (below). (a) Scale bar= 50 μm (b) Scale bar=5 μm;

* *P*<0.05; ***P*<0.01; ****P*<0.005 (Student's *t* test)

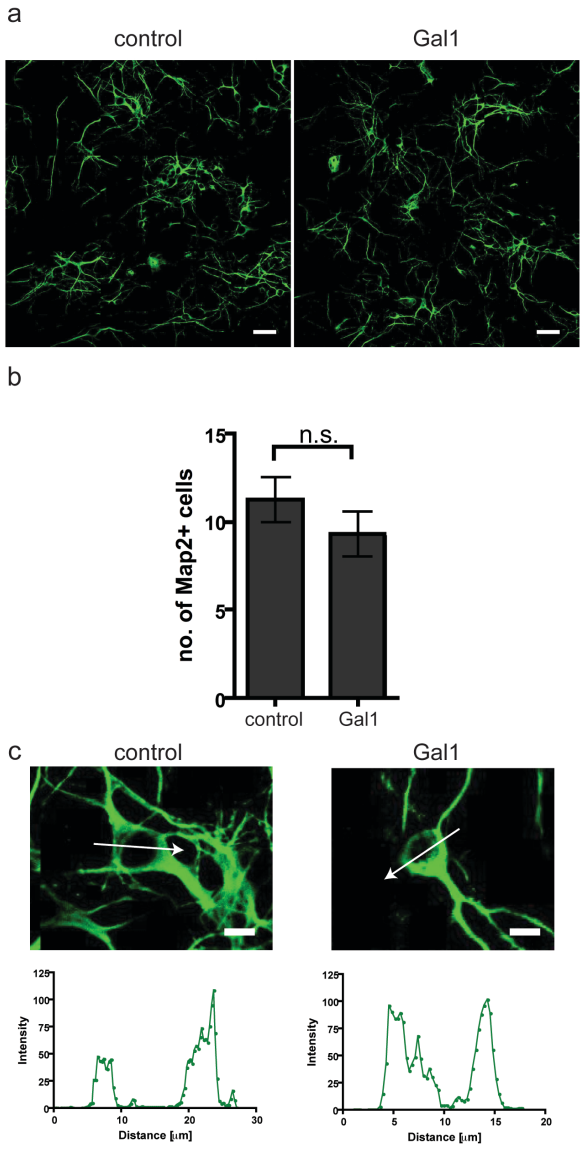


Figure 4.1.4-2 Galectin-1 does not directly effect neurotoicity
High-density cortical neuronal cultures were incubated with galectin-1 (5μg/ml) for 48 hours and then stained for Map2 (a) Representative fluorescence photomicrograph of Map-2^{+} (green) neurons. (b) (b) Quantification of neuronal density measured as Map2^{+} cell bodies per culture (n=20) (c) High magnification photomicrograph of single Map2^{+} neurons and pixel intensity analysis (below). (a) Scale bar= 200 μm (b) Scale bar=5 μm;
* *P*<0.05; ***P*<0.01; ****P*<0.005 (Student's *t* test)

4.1.5. Impact of galectin-1 modulated microglia on acute EAE

Neonatal microglia were purified and stimulated with LPS, galectin-1 or both, LPS and galectin-1 *in vitro*. Control microglia were left untreated. After 24 hours of stimulation, cultured neonatal microglia (5x 10^5 / mouse) were transferred into the right lateral ventricle (RLV) *of Lgals1 -/-* mice on day 9 post immunization. The clinical score was followed until day 20 pi.

Transfer of LPS treated microglia resulted in a significantly worse clinical score of EAE compared to untreated microglia **(Fig. 4.1.5-1**). This result is not suprizing, since adoptive transfer of LPS-treated microglia lead to neurotoxicity *in vivo* [54]. Interestingly, transfer of LPS+galectin-1 treated microglia significantly ameliorated clinical disease as compared to LPS treated microglia **(Fig. 4.1.5-1**). Furthermore, transfer of galectin-1 treated resting microglia resulted in an even lower clinical disease course as compared to untreated resting microglia **(Fig. 4.1.5-1**), suggesting that galectin-1 may inhibit *in vivo* activation of microglia after intracranial transfer.

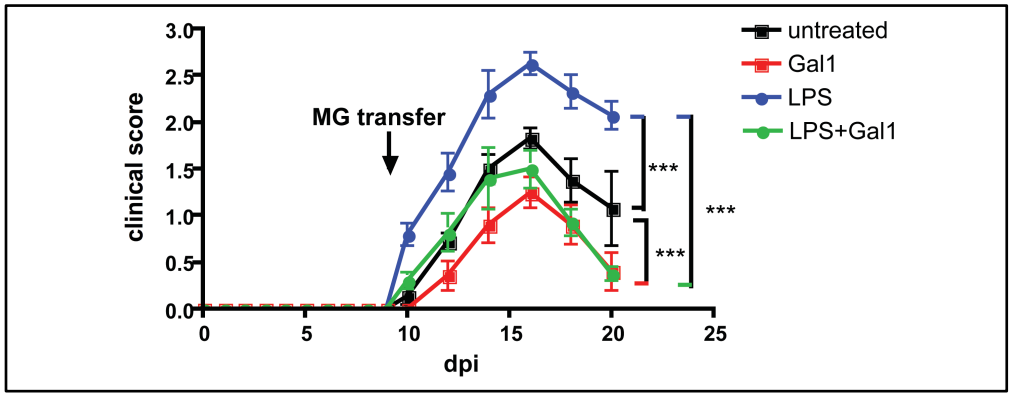


Figure 4.1.5-1 Intraventricular transfer of galectin-1 modulated microglia improves clinical symptoms of EAE.
Neonatal microglia were pre-treated with PBS (untreated), LPS, galectin-1 or LPS and galectin-1 for 24 hours before transfert to the right lateral ventricle of Lgals1-/- EAE mice (day 9 post immunization, n=6 per group). Displayed is the mean clinical score ±SEM.
* $P<0.05$; ** $P<0.01$; *** $P<0.005$ (2-way ANOVA)

4.1.6. Astrocytes control microglia activation via galectin-1

Since astrocytes express high levels of galectin-1 during both the acute and chronic phases of EAE, it was investigated whether this contributes to astrocyte-mediated regulation of microglia activation. *Lgals1* mRNA was transiently knocked down in PBS (control) or TGFβ_1 treated astrocytes. IFN-γ-stimulated microglia where then exposed to astrocyte-conditioned media for 24 hours, after which microglia were stained for CD11b and MHC II and analyzed by flow cytometry.

Stimulation of astrocytes with TGFβ_1 led to an increase in *Lgals1* mRNA and secreted galectin-1, while *Lgals1*-siRNA led to a significant decrease in *Lgals1*- mRNA **(Fig. 4.1.6-1 a)** and secreted galectin-1 **(Fig. 4.1.6-1 b)** in both control and TGFβ_1-stimulated astrocytes.

There was no difference in MHC II expression on classically-activated microglia compared to microglia that were exposed to conditioned media from unstimulated control- or *Lgals1*-siRNA treated astrocytes. However, M1 microglia that had been exposed to conditioned media from TGFβ_1-treated astrocytes showed increased activation (increased MHCII expression) following galectin-1 silencing as compared to lgals1 expressing cells. **(Fig. 4.1.6-1 c)**. Taken together these results indicate that, upon exposure to anti-inflammatory or tolerogenic stimuli,

astrocytes can downregulate classical microglia activation through mechanisms involving galectin-1.

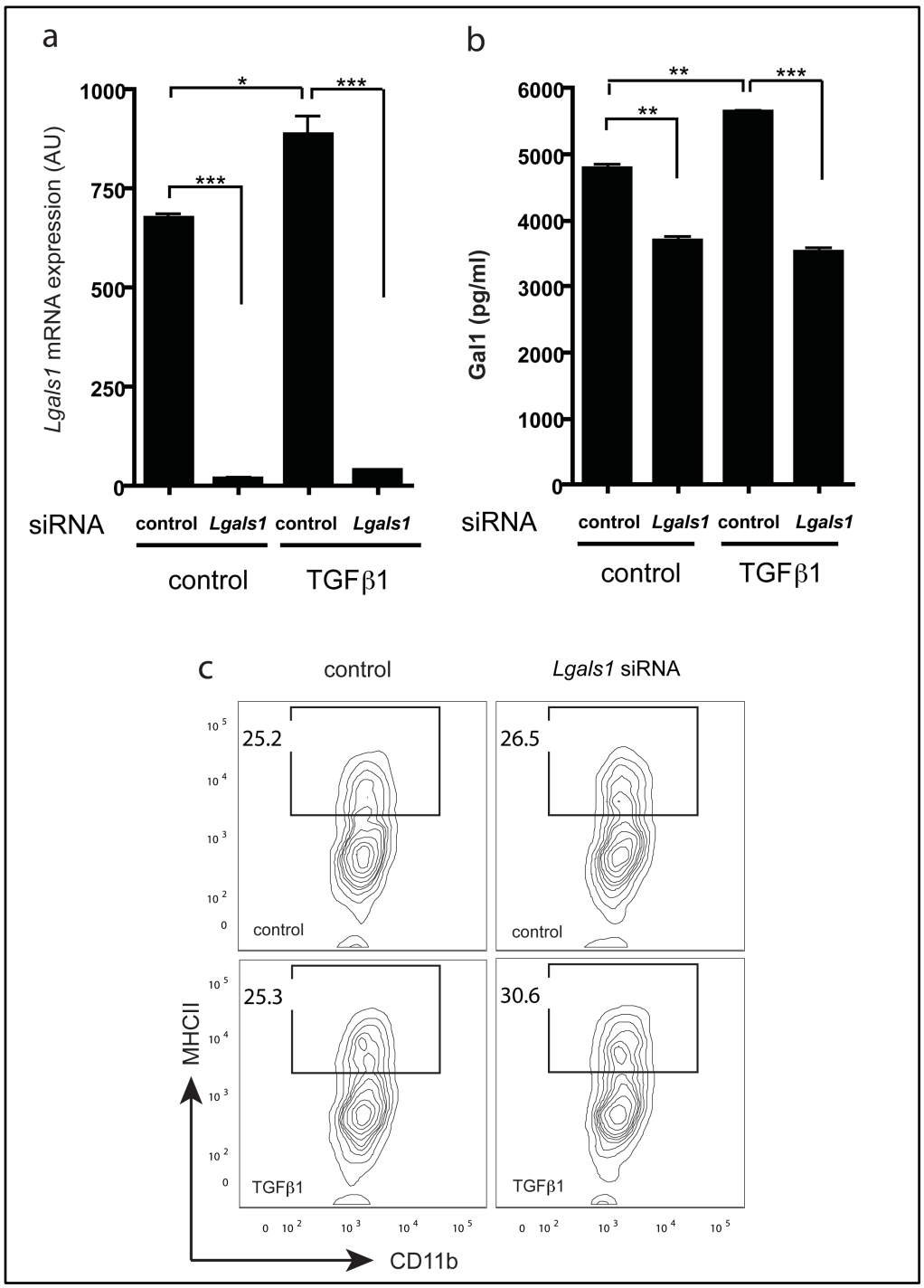


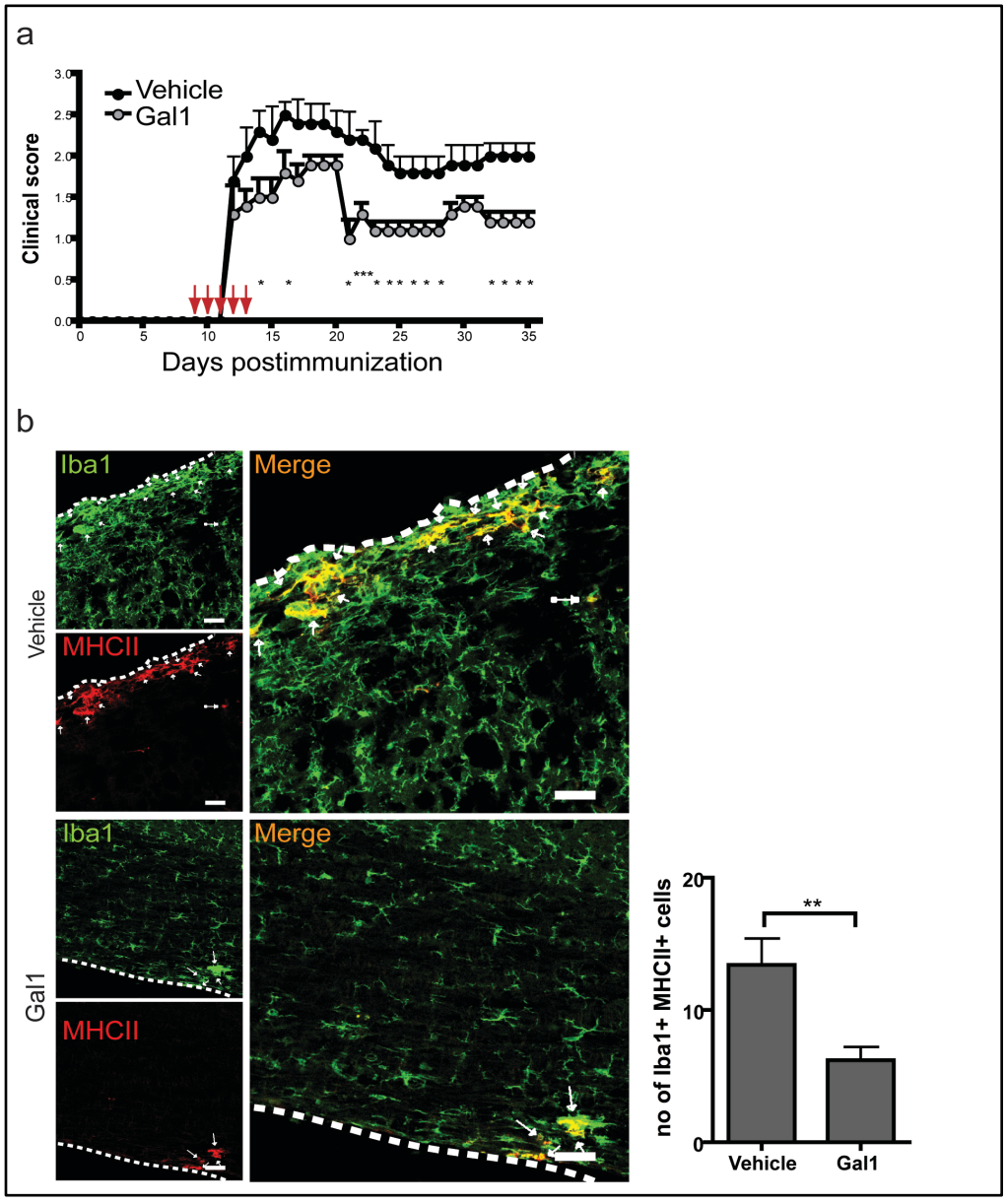
Figure 4.1.6-1: Astrocytes control microglia-activation via galectin-1.
Transient knock-down of the *Lgals1* gene by *Lgals1* siRNA in unstimulated (control) and $TGFb_1$ stimulated astrocytes as determined by quantitative RT-PCR(a) and ELISA(b). (c) Flow cytometry of MHCII activation marker of cultured CD11b+ microgli. IFNγ treated microglia were exposed to conditioned media from control and $TGFb_1$ stimulated astrocytes where the *Lgals1* gene had or had not been knocked-down by *Lgals1* siRNA. Numbers represent percentage of MHCII positive gated microglia. * $P<0.05$; ** $P<0.01$; *** $P<0.005$ (Student's *t* test)

4.1.7. Therapeutic administration of galectin-1 decreases microglia activation and prevents neurodegeneration and demyelination

In order to investigate whether galectin-1 therapy influences the microglia compartment and neuropathology during EAE, mice were immunized and treated with recobinant galectin-1 or PBS (vehicle) around the onset of clinical disease (day 9 to 13 pi), a time when peripheral inflammatory cells will have already entered the CNS. Spinal cord sections were collected on day 35 post immunization and stained for the microglia marker Iba1 and the microglia activation marker MHC II, and for β-III-tubulin and Gap43.

It could be observed that galectin-1 administration significantly attenuated EAE disease severity **(Fig. 4.1.7-1 a)**. and decreased microglia activation in the spinal cord **(Fig. 4.1.7-1 b)**. Consistent with our *in vitro* data, axonal damage and neuronal degeneration were substantially reduced in galectin-1-treated mice as reflected by increased staining of β-III-tubulin, Gap43 **(Fig. 4.1.7-1 c,d).** Furthermore, immunoreactivity against MBP was increased and GFAP

staining was decreased, suggesting a decrease in demyelinaton **(Fig. 4.1.7-1 e)** and astrogliosis (**Fig. 4.1.7-1 f)**.



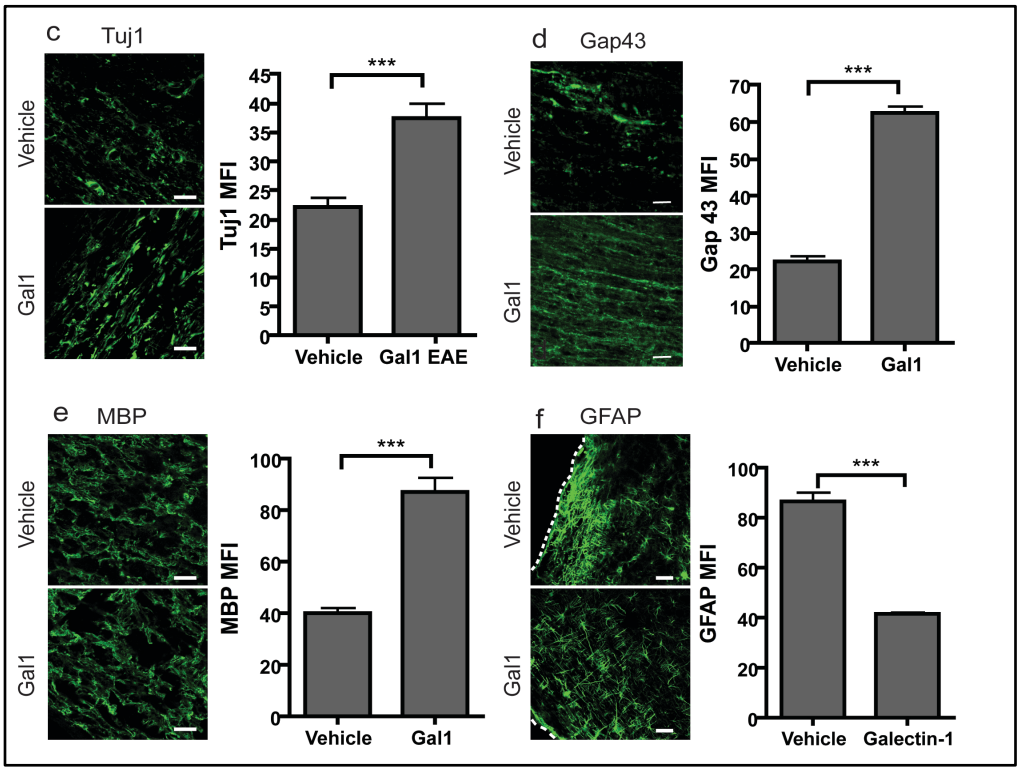


Figure 4.1.7-1: Galectin-1 therapy attenuates clinical signs of EAE, limits microglia activation, axonal loss and demyelination and promotes synaptic repair.

(a) Disease score of vehicle-treated and galectin-1-treated (100 μg/day) C57BL/6 mice, immunized with 200 μg MOG_{35-55}. (b-e) Confocal microscopy of spinal cord white matter after 35 days of EAE in galectin-1 treated and untreated EAE mice. (b) Sections were stained for Iba1 (green) and MHC II (red). Right Graph represents a quantification of Iba1/MHCII double positive cells in different groups.

(**c**) Left. Spinal cord sections were stained for b-III-tubulin (Tuj1 ,green). Right mean fluorescence intensity (MFI) of immunoreactivity against Tuj1. (**d**) Left. Spinal cord sections were stained for Gap43 (green). Right mean fluorescence intensity (MFI) of immunoreactivity against Gap43. (**e**) Left. Spinal cord sections were stained for MBP (green). Right mean fluorescence intensity (MFI) of immunoreactivity against MBP. (**f**) Left. Spinal cord sections were stained for GFAP (green). Right mean fluorescence intensity (MFI) of immunoreactivity against GFAP.

Scale bars= 20 μm; * P<0.05; **P<0.01; ***P<0.005 (Student's t test)

4.2. Subventricular Zone Microglia Transcriptional Networks.

4.2.1. Systems biology strategy for the analysis of microglia in the acute and chronically inflamed SVZ

SJL/J mice immunized with PLP 130-151 develop relapsing-remitting EAE with a peak of disease at around day 14 post immunization (dpi) followed by a remission and then a relapse [76]. Mice enter a phase of chronic inflammation at around 50-55 days post immunization, characterized by minor changes in clinical disease severity. Microglia activation persists through the chronic phase of EAE [76], and microglia activation in the SVZ correlates with alterations in endogenous stem cell repair mechanism and niche activity [33].

In order to investigate how microglia influence the SVZ niche during the course of EAE, the subventricular zone was dissected from the brain of mice with acute (n=40) and chronic EAE (n=40) as well as from healthy control mice (HC; n=40), that were immunized with Freud's Complete Adjuvant (CFA). Microglia, defined as $CD11b^{+}/CD45^{lo}$ expressing cells, were isolated by flow cytometric sorting, total RNA was isolated and microarray profiling was then performed to investigate patterns of gene expression **(Fig. 4.2.1-1).**

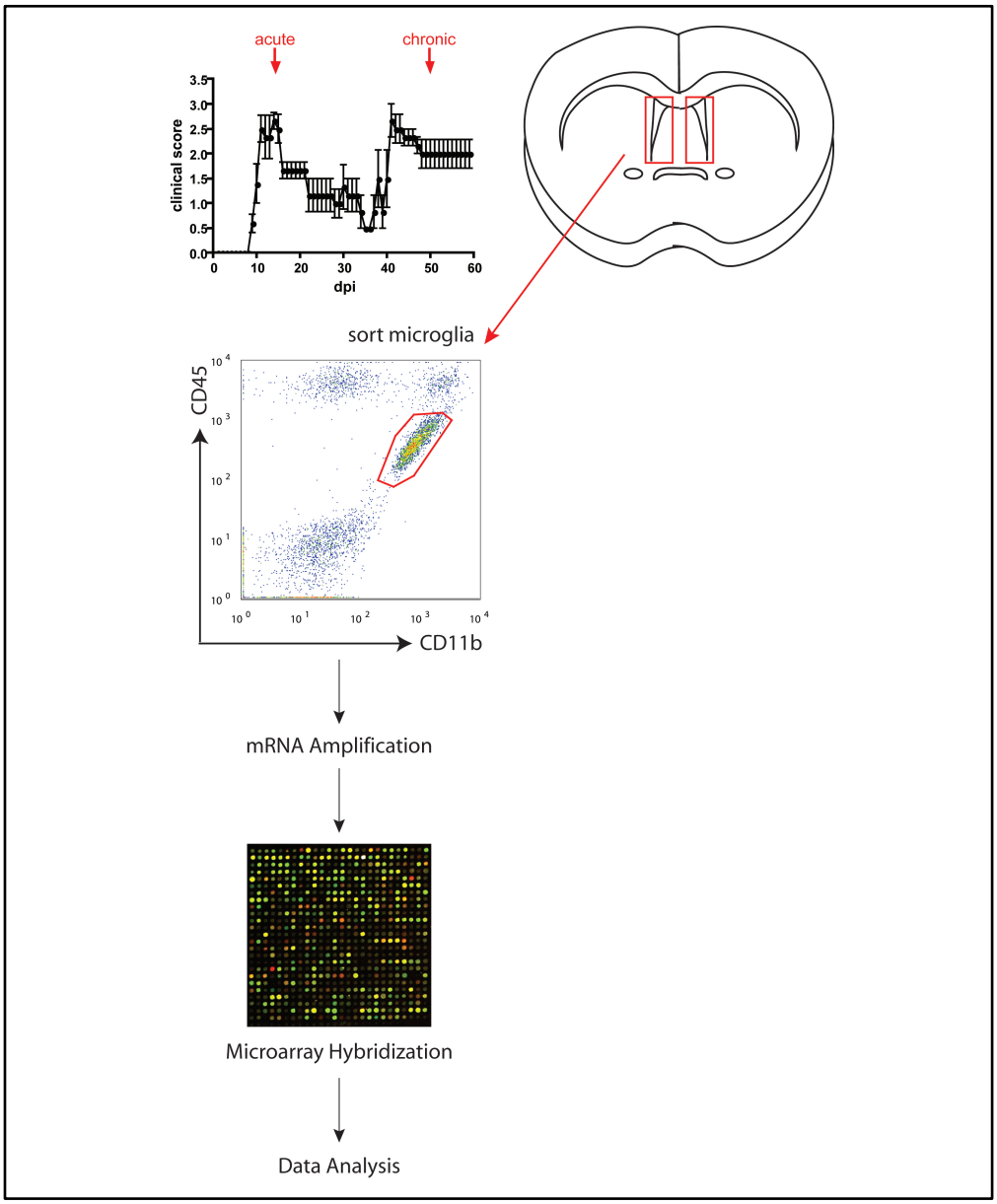


Figure 4.2.1-1: Systematic Strategy of SVZ Microglia isolation and Microarray analysis.

Schematic representation microglia isolation from the SVZ and microarray strategy. Microglia were isolated from RR EAE during the acute and chonic disease phase (red arrows) and from CFA injected healthy control mice, followed by dissection of the SVZ with flow cytometry sorting of CD11b/CD45^{lo} microglia. Subsequent microarray and data analysis was performed after 2 rounds of RNA amplification.

The known microglia genes HLA-DQ A1, CD40, Vcam1, IL1b, Cx3cr1, CCL2 and P2x7 were highly abundant, whereas neuronal, astroglial, oligodendroglial and precursor genes were not expressed in the sorted microglia population in HC or EAE, confirming the high purity of the $CD11b^{+}/CD45^{lo}$ microglia population **(Fig. 4.2.1-2)**. Furthermore the SVZ did not contain foci of inflammation **(Fig. 4.2.1-3)**, making contamination of peripheral immune cells unlikely [76].

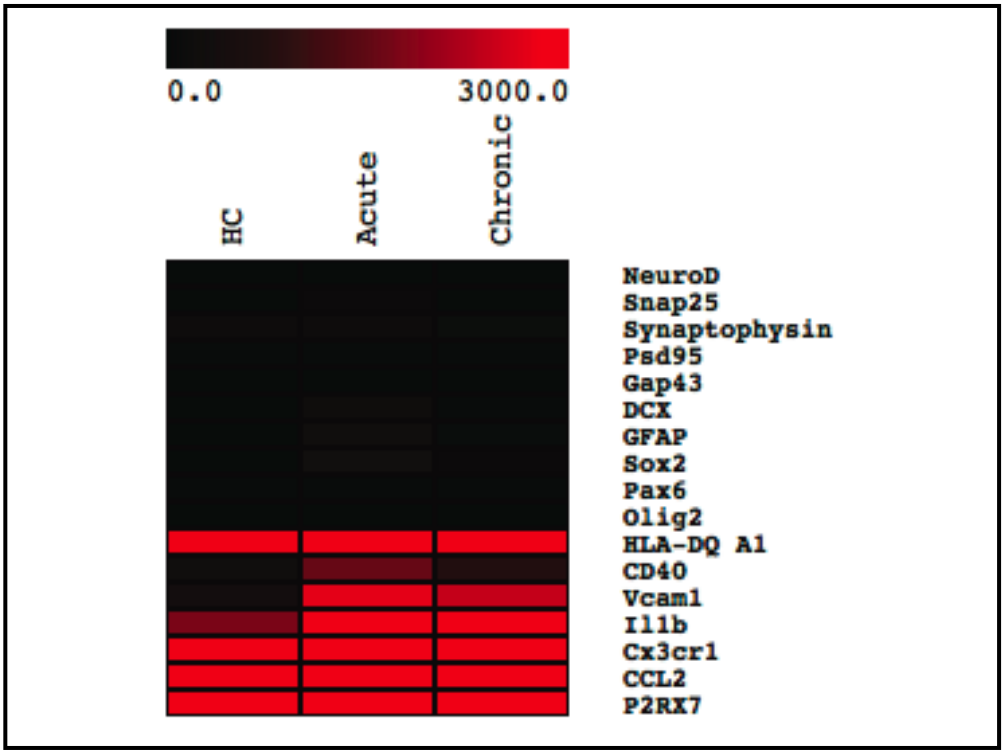


Figure 4.2.1-2 Transcription profile of CNS celltype specific genes.

Expression profile of neuronal, astroglial, progenitor, oligodendroglia and microglial genes in HC, Acute and Chronic EAE. Color denotes expression intensity with a single gradient (black represents absance of gene expression; red represents high gene expression). Expression data shows a high enrichment of micorglia genes as compared to genes specific to other cell types.

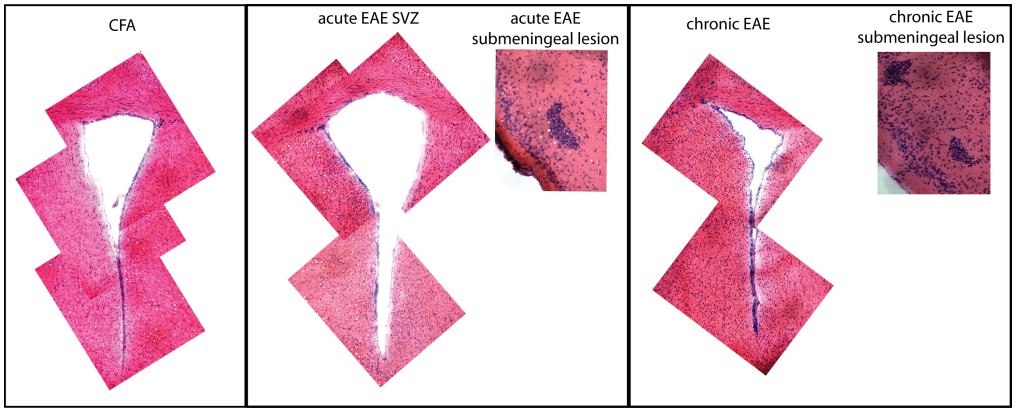


Figure 4.2.1-3: Histology of SVZ.

H&E histological staining of the SVZ. No inflammarory infiltrates were detected in close proximity to the subventricular zone in control, acute and chronic EAE.

4.2.2. Genomic signature of microglia in the acute and chronically inflamed SVZ

In the primary analysis, gene expression between $CD11b^{+}/CD45^{lo}$ microglia isolated from HC mice was compared to that of microglia from acute or chronic EAE. There were 1414 transcripts that were differentially regulated in EAE SVZ microglia. Among these, several genes were shared between acute and chronic EAE but were not expressed among control SVZ microglia (fold change (FC) of acute vs. HC >2 or chronic vs. HC >2). Despite being expressed both in acute and chronic EAE several genes were significantly enriched in one disease phase more than the other (FC acute vs. chronic >2 or FC chronic vs. acute >2). Genes sharing similar expression pattern between acute and chronic EAE, but different than HC are likely important for microglia functions throughout the whole disease course, whereas genes that are enriched in either acute or chronic EAE are more likely to reflect functions that are disease phase specific. Thus

the differentially regulated transcripts converged into a pattern of 1171 transcripts with significant enrichment in acute EAE microglia, 41 transcripts were upregulated in chronic microglia and 201 transcripts were co-expressed and co-regulated in both acute and chronic EAE (common EAE) **(Venn diagram, Fig. 4.2.2-1 a,b)**.

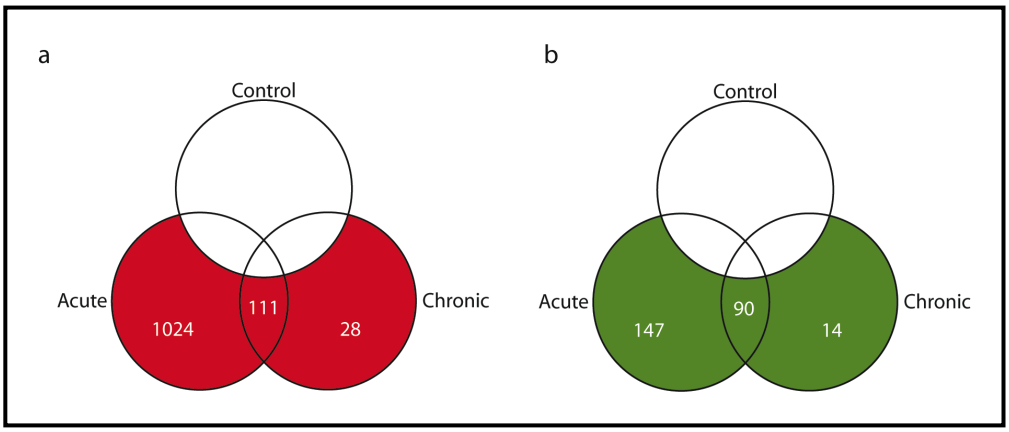


Figure 4.2.2-1: Venn Diagramm.

Comparison of the significant genes identified as differentially expressed in acute EAE, chronic EAE and acute and chronic EAE. (a) Identification of 1024, 28 and 111 upregulated genes for acute, chronic and common EAE respectively. (b) Identification of 147, 14 and 90 downregulated genes for acute, chronic and common EAE respectively.

4.2.3. Gene Ontology analysis

Bioinformatic analysis was performed by uploading the previously identified differentially expressed gene patterns **(Venn Diagramm, Fig. 4.2.2-1)** into the Ingenuity pathway knowledge base (IPA). Transcripts were clustered according to the reported subcellular location of the transcripts' gene products **(Fig. 4.2.3-1 a).** A large number of transcripts of secreted molecules (86 transcripts in acute EAE, and 11 shared by acute and chronic EAE), and plasma membrane expressed

molecules (202 transcripts in acute EAE, and 29 shared between acute and chronic EAE) were upregulated, whereas only a small number of such transcripts were downregulated: for secreted molecules 6 transcripts in acute EAE, and 4 shared by acute and chronic EAE were downregulated and for surface expressed molecules 14 transcripts in acute EAE, and 16 shared by acute and chronic EAE were downregulated. In chronic EAE only 3 up-regulated and no down-regulated transcripts for secreted gene products, and for membrane expressed products 2 up-regulated transcripts and 2 down-regulated transcripts were found. Furthermore, a large number of differentially regulated genes whose products have a nuclear (134 up-regulated molecules, 29 down-regulated molecules) and cytoplasmic location (209 up-regulated molecules, 33 down-regulated molecules) were found to be specific for the acute phase of EAE. The dynamic regulation of gene expression during EAE suggests that microglia respond to the inflammatory microenvironment especially during acute disease, but many of these genes revert to normal expression during the chronic phase **(Fig. 4.2.3-1)**.

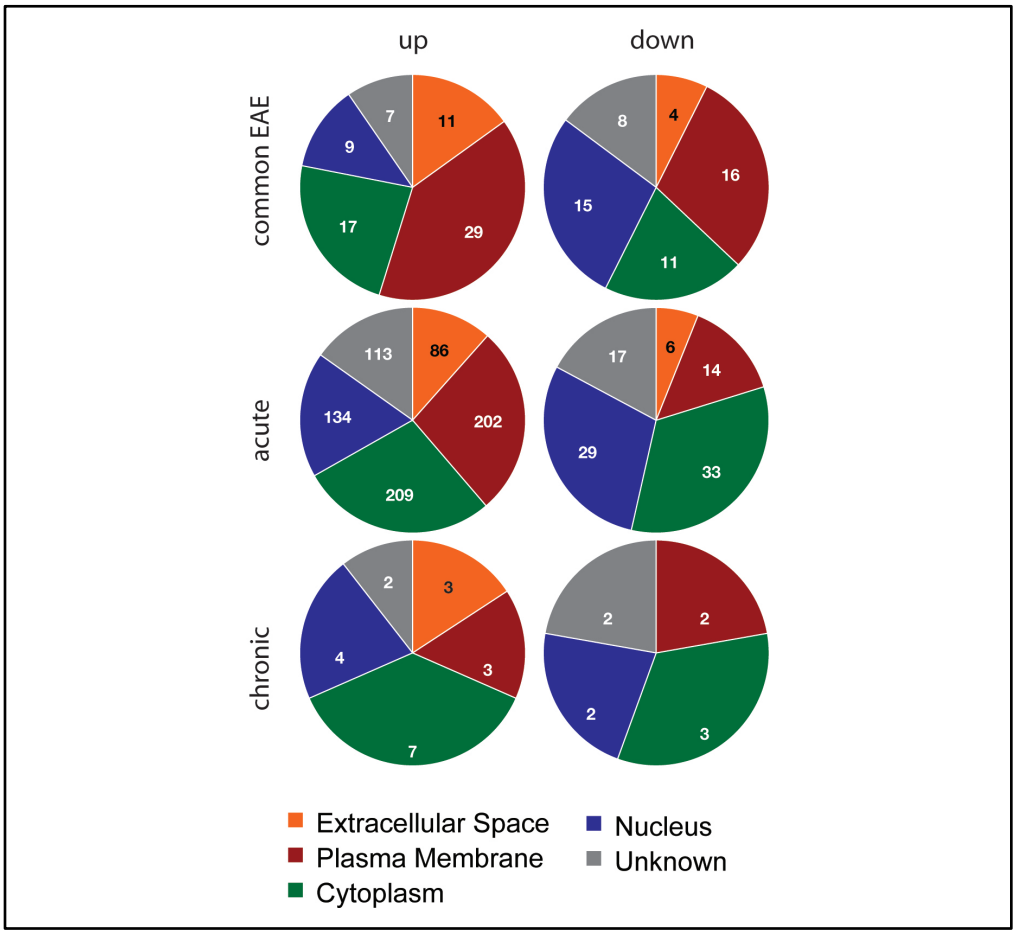


Figure 4.2.3-1 Gene Ontology analysis: subcellular location clustering
Pie-diagram of the cellular location of genes from each up- and downregulated signiture set for acute, chronic and common EAE, with each number representing the number of genes in a given cellular location.

In order to investigate how microglia influence the stem cell niche, only transcripts of molecules that are secreted or expressed on the surface of microglia were subjected to subsquent analysis, implying that all of those molecules have a possible effect on other cells within the niche. To understand the functional significance of the gene expression data, gene ontology (GO) analysis was applied on data sets of transcripts

with subcellular location in plasma membrane and extracellular space in acute, chronic and common EAE signatures using IPA[129]**.** Biological functions were assigned to each data set by using the Ingenuity knowledge base as a reference set. Using the web-based entry tool IPA, biological functions are assigned to each data set and Fisher's exact tests were then performed to calculate a p-value determining the probability that genes of interest participate in a given biological function, relative to the total number of occurrences of these genes in all functions stored in IPA.

IPA organizes GO terms into three categories ("Molecular Functions", "Physiological Functions" and "Diseases and Disorders") and ranks them according to their p value. The functional profiles of acute EAE, chronic EAE and common microglia gene expression are shown in pie charts in **Fig. 4.2.3-2.** Under the category of "Molecular and cellular functions" it can be clearly seen that genes related to "cellular growth and proliferation", "cellular movement" and "cell-to-cell signaling" are predominantly regulated during acute EAE with a fraction of these being regulated during both phases of disease, but very few genes with these functions are differentially regulated during the chronic phase **(Appendix Suppl. Table 2).**

This is consistent with the hypothesis that microglia proliferation, migration and interactions occur predominantly during acute disease and with our observation that the largest increase in number of microglia in the SVZ occurs during acute EAE [76]. This data may also point to the fact that acutely activated microglia may also support the growth and proliferation of other niche resident cells, such as neural stem cells.

More intriguing, under the categories of "physiological system development" and "diseases and disorders", genes related to tissue

development and cancer appeared to be predominantly regulated in acute EAE **(Appendix Suppl. Table 1)**.

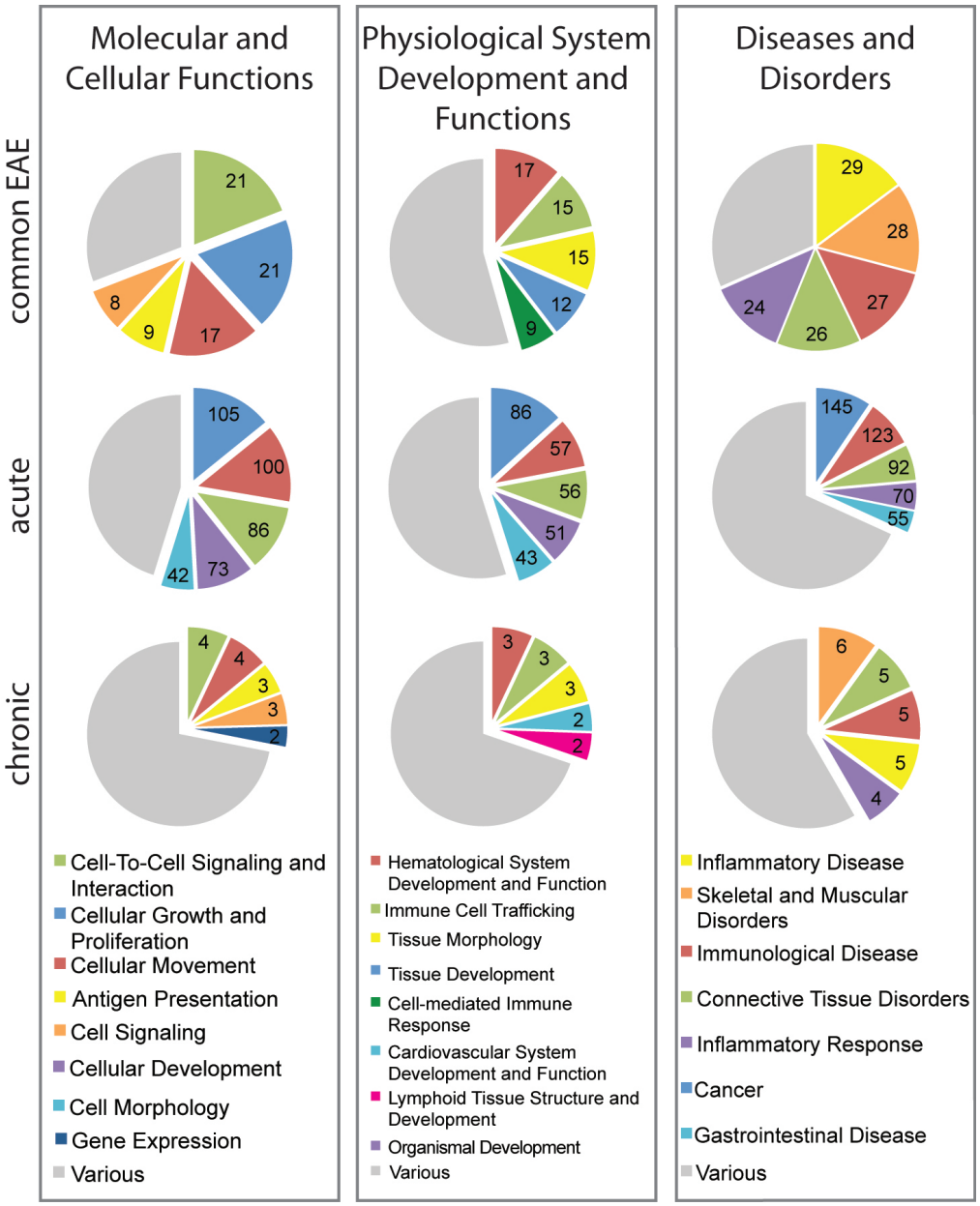


Figure 4.2.3-2: GO analysis of differential gene expression in with subcellular lovation in extracellular space and plasma membrane
Gene Ontology functions of up-regulated genes with location in extracellular space and plasma membrane for acute, chronic and common EAE. Top 5 significant GO functions categorized into "Molecular and Cellular Functions", Physiological System Development and Functions" and "Diseases and Disorders" are displayed as pie-diagrams with each number representing the number of molecules associated with the given function.

Brain tumors and neural stem cells share several genes [130,131] and recently a hypothesis that tumor-derived stem cells originate from a population of endogenous neural stem cells has been proposed [132]. So the fact that regulation of genes in these categories are observed only during the acute but not the chronic phase of EAE, supports the hypothesis that microglia produce niche promoting molecules only during acute disease. Some of the genes listed in **Appendix Suppl. Table 1** are associated with processes such as neurite outgrowth, tubulation of endothelial cells, adhesion of tumor cells, assembly of extracellular matrix, and development of blood vessels and were unique to the acute EAE signature. This suggests an active role of acute microglia in supporting the SVZ niche during acute EAE.

On the other hand, genes related to inflammation such as "immunological disease", "connective tissue disease" and "inflammatory response", are regulated during both phases of EAE **(Appendix Suppl. Table 3)** consistent with the observation that microglia remain activated throughout the disease course [76].

Interestingly, signature genes associated with canonical pathways such as antigen presentation pathway, allograft rejection pathway and graft-versus-host disease signaling were upregulated in acute and chronic EAE; leukocyte extravasation signaling, atherosclerosis

signaling and complement system for genes were upregulated in acute EAE, and reelin signaling, ephrin receptor signaling and phospholipase C signaling in chronic EAE **(Fig. 4.2.3-3)**. The latter three pathways are associated with cytoskeleton reorganization. Taken together these results predict that microglia cells display specific canonical pathway phenotypes in EAE with additional distinct phenotypes in acute versus chronic EAE.

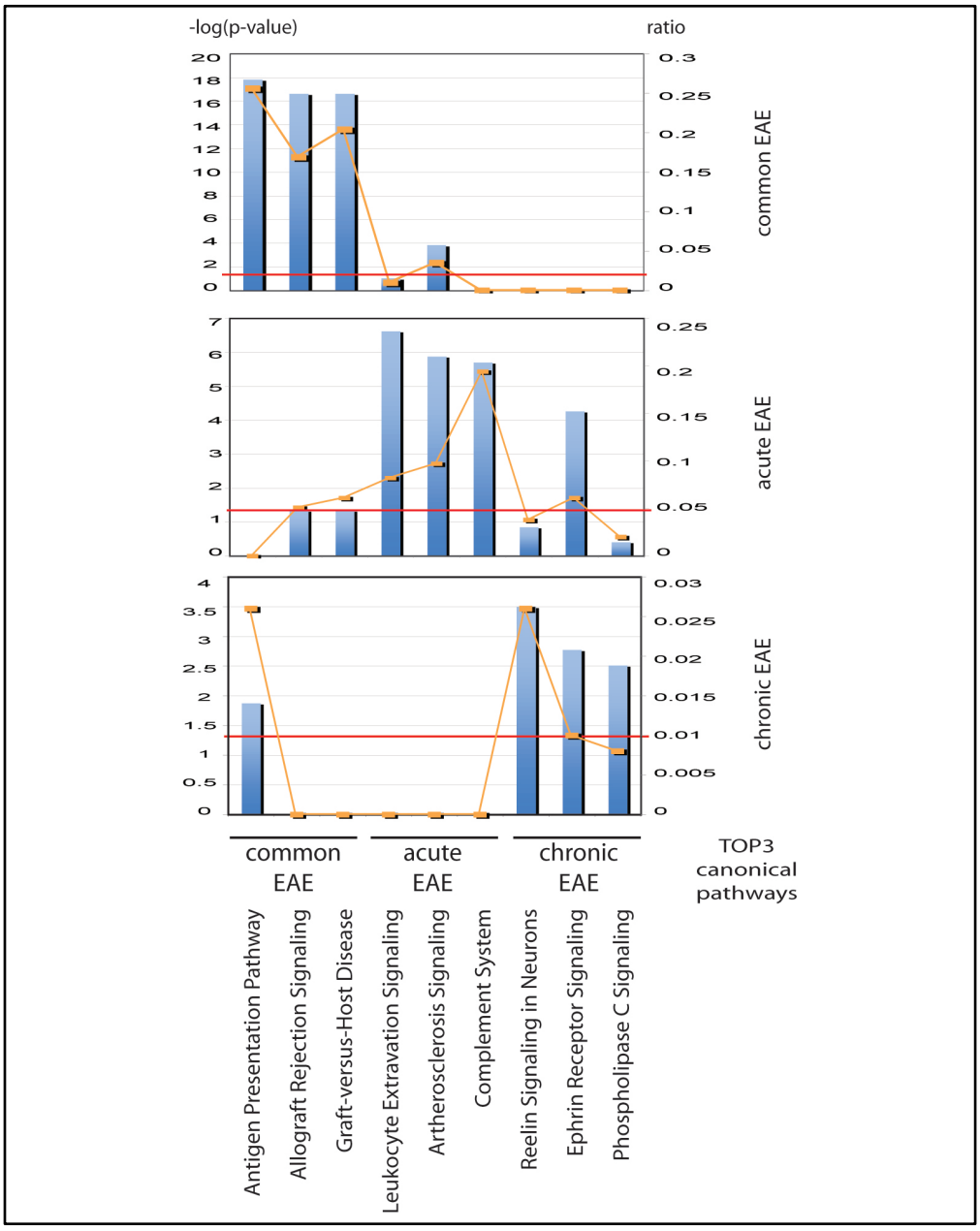


Figure 4.2.3-3: GO analysis of canonical pathways
Top 3 canonical pathways associated with upregulated genes of secreted and plasma membrane expressed molecules for the signature sets in common EAE, acute EAE, and chronic EAE and their dynamics in each disease phase. Displayed are the significance (–log(p-value); blue bars; left y-axis) and the ratio (number of signature genes present in the canonical pathway / total number of genes associated with the canonical pathway; orange line; right y-axis). The red lines show the threshold for significance (-log (p=0.05))

4.2.4. Functional Network analysis

Network analysis was performed to evaluate how genes associated with different EAE signatures are related, using Ingenuity Pathway Analysis (IPA) (ingenuity.com). IPA generated networks of 35 genes are stored in the ingenuity knowledge base as having protein-protein interactions with the given set of focus genes. Furthermore IPA computes a score, reflecting the relevance of the network to the empirical data sets, which allows identification of networks with highest importance to the data set. Based on the computed score (score above 10, with a maximum score of 50 and a minmal score of 0), 16 netwoks in the signature set of acute EAE, 1 in chronic and 2 networks in common EAE were identified to be significant. Additionally, biological functions were calculated and assigned to each network, based on their significance and relevance to the network ($p<0.05$).

Given its overrepresentation of up- and down-regulated genes and its unique GO functional profile, we focused on acute EAE signature genes. As before this signature set only included genes resulting in gene products with subcellular location in extracellular matrix and plasma membrane.

Two networks appeared to be most interesting, according to their gene content and assigned GO functions **(Fig. 4.2.4-1)**. The top-scoring network **(Fig. 4.2.4-1 a,** score=45**)** was associated with the GO functions of “cell-to-cell signaling and interaction”, “tissue development” and “tissue morphology”. Furthermore, it included genes that are known to be associated with the proliferation of oligodendroglial precursors (vitronectin; VTN, [133]), neurogenesis (jagged 1; Jag1 [134]), increase of neurite outgrowth (discoidin domain receptor tyrosine kinase 1 (DDR1) [135]; syndecan 1 (SDC1) [136]; tissue plasminogen activator (PLAT) [137]; gap junction protein alpha 1 (GJA1) [138])

neuritogenesis (tissue plasminogen activator (PLAT) [139]), axonal growth (SPACR-like 1 (SPARCL1) [140]) neuroprotection (tissue plasminogen activator (PLAT) [141]) and tubulugenesis of endothelial cells (thrombospondin 1 (THBS1) [142]). Another network was enriched in complement genes that were highly over-expressed in acute EAE, such as complement component 3 (C3) (FC=48.85), CD40 (FC=7.66), and vascular endothelial growth factor α (VEGFA) (FC=6.199) and **(Fig. 4.2.4-1 b)**. The complement component C3 was among the highly interconnected nodes within this pathway. This finding supports an important role for microglia in the SVZ niche in acute EAE, since C3a is reported to increase neurogenesis in addition to its role in inflammation [143]. These results suggest that microglia play a neuroprotective and neuroregenerative role during the acute phase by up-regulating non-classical niche supporting factors.

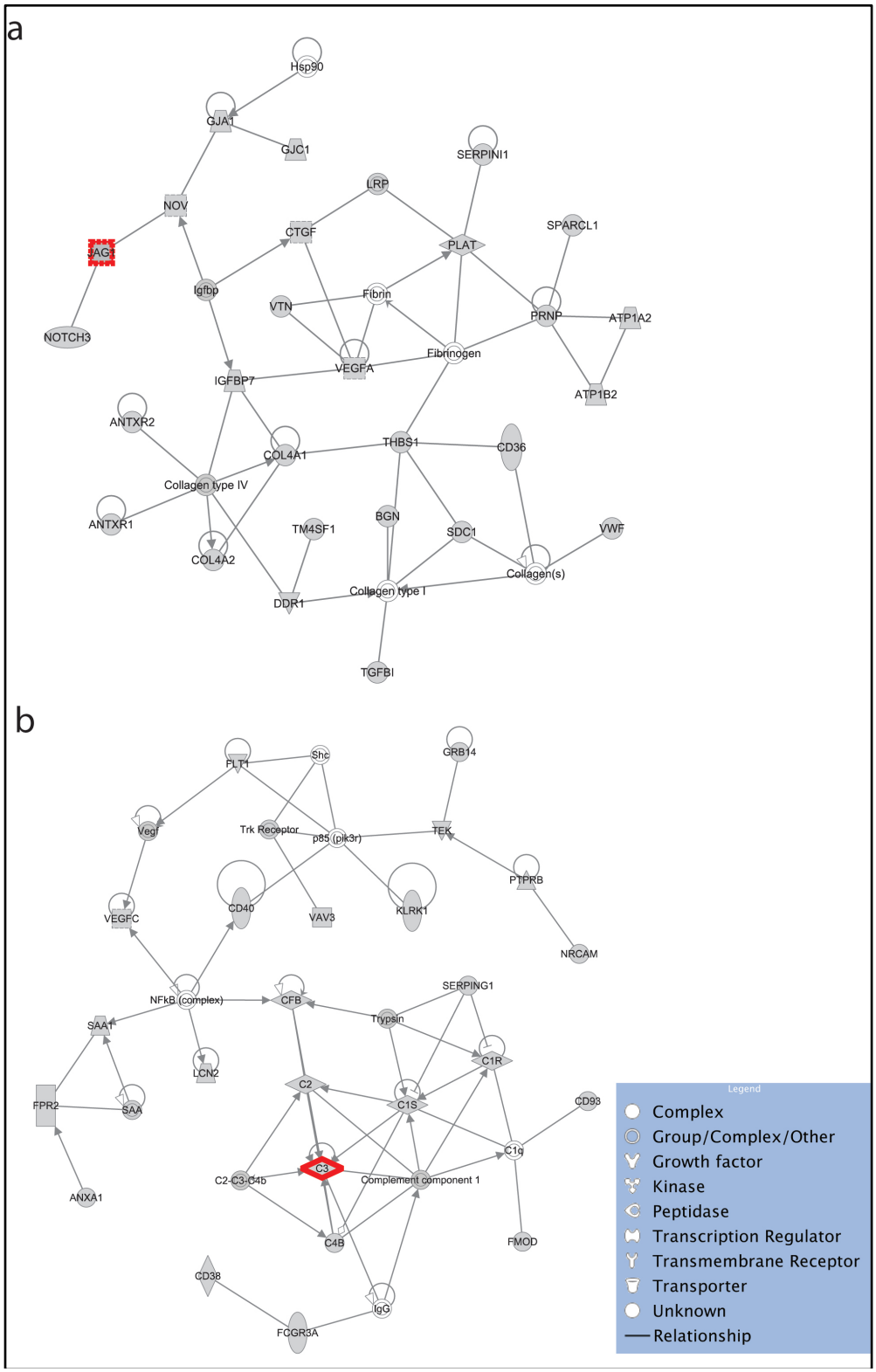

a
Hsp90
GJA1
GJC1
SERPINI1
LRP
NOV
CTGF
PLAT
SPARCL1
AGT
Igfbp
Fibrin
VTN
PRNP
ATP1A2
NOTCH3
VEGFA
Fibrinogen
IGFBP7
ATP1B2
ANTXR2
COL4A1
THBS1
CD36
Collagen type IV
ANTXR1
BGN
TM4SF1
SDC1
VWF
COL4A2
Collagen(s)
DDR1
Collagen type I
TGFBI
b
PLT1
Shc
GRB14
Vegf
Trk Receptor
p85 (pik3r)
TEK
PTPRB
VEGFC
CD40
VAV3
KLRK1
NRCAM
NFkB (complex)
CFB
SERPING1
Trypsin
SAA1
C1R
LCN2
C2
C1S
CD93
FPR2
SAA
C1q
C3
C2-C3-C4b
Complement component 1
ANXA1
C4B
FMOD
CD38
IgG
FCGR3A
Legend
Complex
Group/Complex/Other
Growth factor
Kinase
Peptidase
Transcription Regulator
Transmembrane Receptor
Transporter
Unknown
Relationship

Figure 4.2.4-1: Functional network analysis
(a) Top scoring network for signature genes of secreted and plasma membrane expressed molecules in acute EAE, many of which are involved in neural- or oligodendroglial precursor function, neuroprotection, neurite outgrowth or angiongenesis. (b) High scoring network with an enrichment of complement factors. Nodes represent genes, with their shape representing the functional class of the gene product and edges indicate the biological relationship between the nodes. Genes that were selected as target genes for microarray validation are highlighted with red borders.

4.2.5. Target gene validation by real-time reverse transcription PCR

Several target genes seemed biologically interesting for their differential expression in acute and chronic EAE as well as their ontological association with cancer or stem cell functions. Furthermore, the products of the identified target genes are secreted in the extracellular space or located in the plasma membrane; *Jag1* (jagged1*)* promotes proliferation of cancer cell lines [144] and tumor cells [145], the complement component 3 (*c3*), which is primarily known for its immune function, has been shown to be a potent inducer of neurogenesis [143]. The gene for IL18bp *(*Interleukin 18 binding protein*)* is of interest since IL18bp neutralizes IL18 [146] that acts as an inhibitor of neuronal differentiation[147]. *Igals1 (Galectin-1)* was shown to promote neural progenitor proliferation in the dentate gyrus [107], whereas *htra1 (*HtrA serine peptidase 1*) and insl6 (*insulin-like 6*)* are members of the insulin-like growth factor 2 pathway (IGF-2) that plays a role in stem cell recruitment to the site of injury [148] and in differentiation potential of stem cells [149].

In order to confirm the results of the cDNA microarray, quantitative RT-PCR analysis was utilized for validation at the gene level, and immunostaining of brain sections for validation at the protein level.

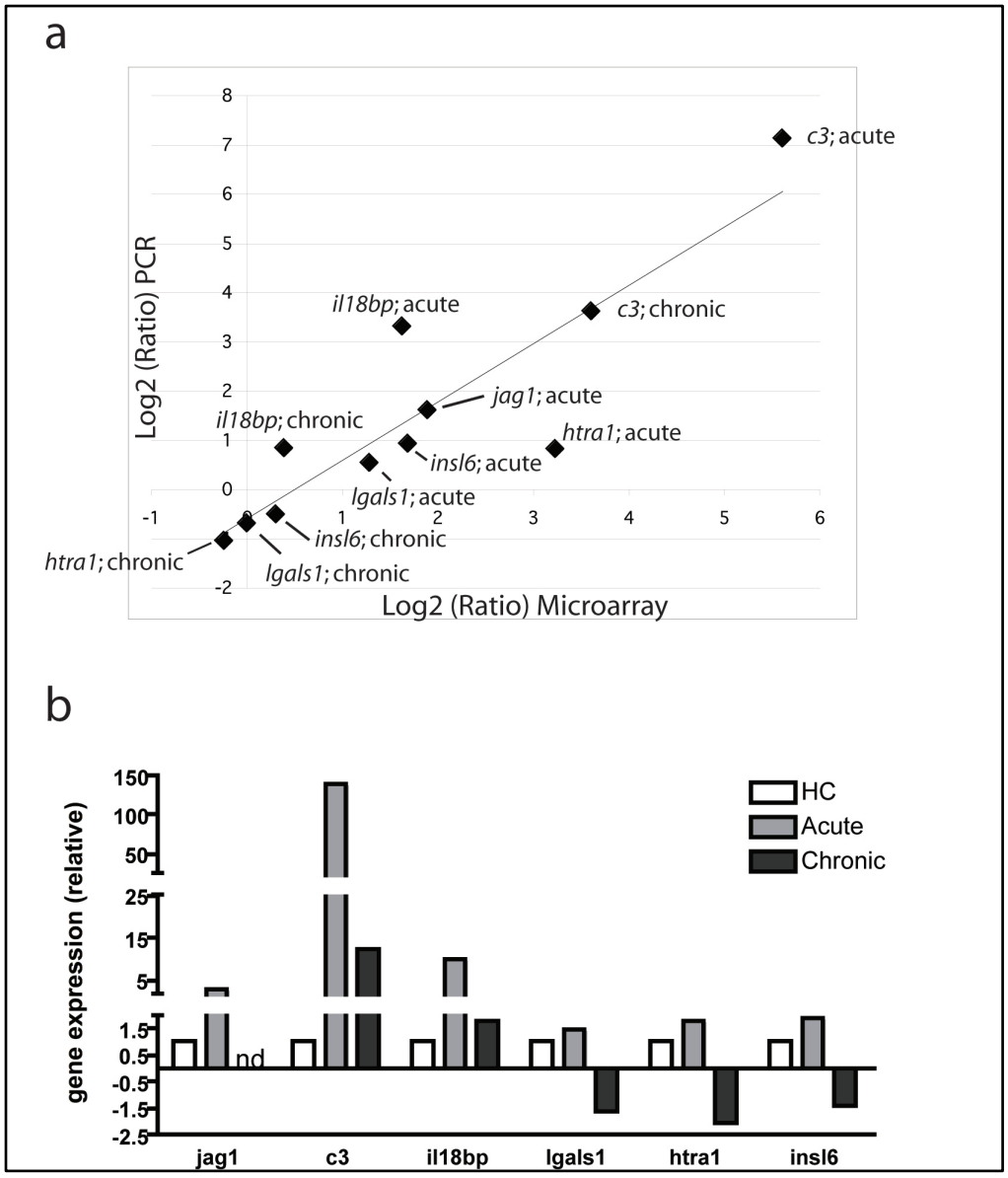
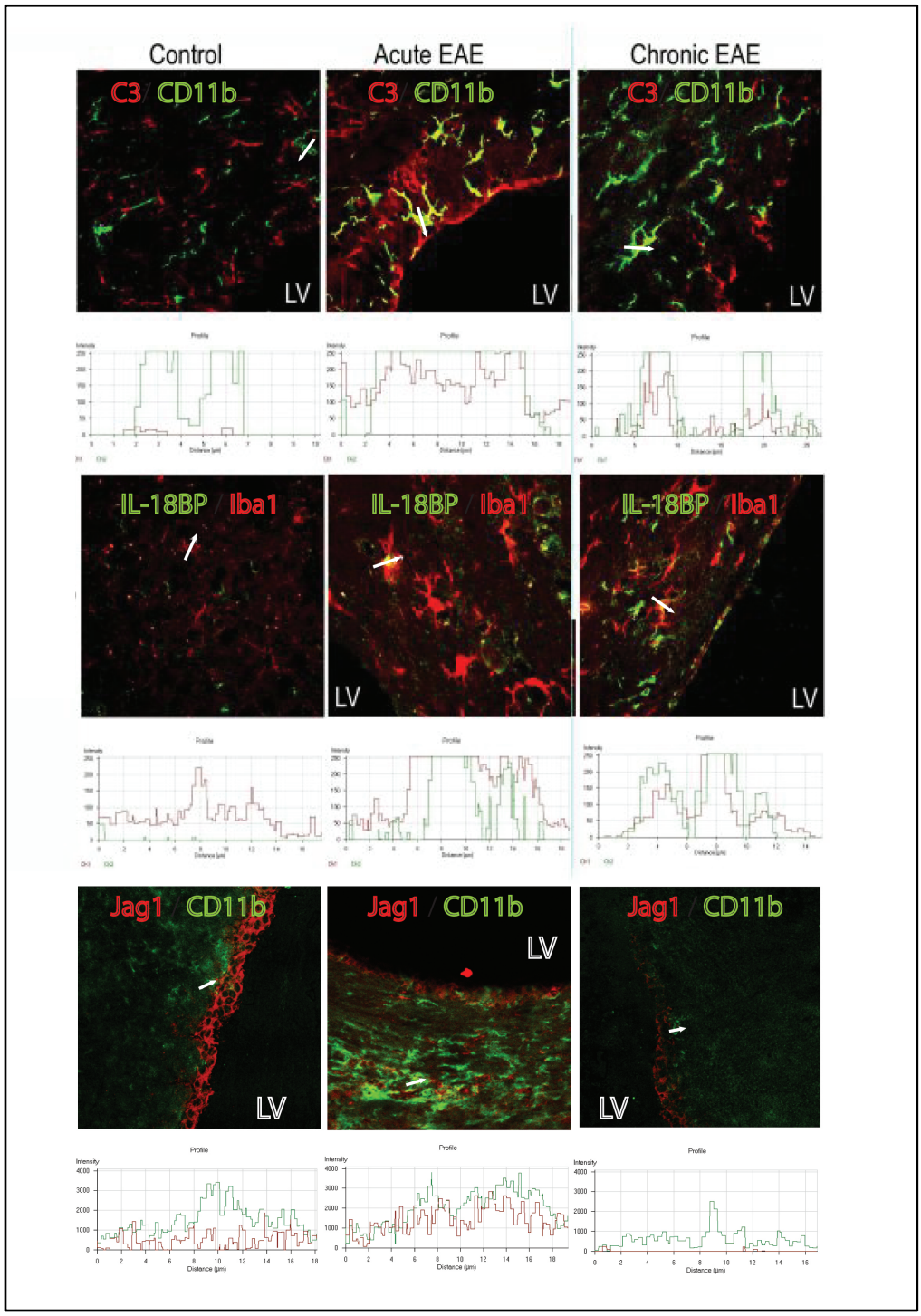


Figure 4.2.5-1: Correlation of microarray with RT-PCR.
The microarray data was validated by RT-PCR, correlating the Log2 ratio of the microarray genes with the Log2 ratio of given genes as measured by quantitative RT-PCR. The data show a good linear fit with a correlation coefficient of 0.78 and a slope of 1.18.

Figure 4.2.5-1 demonstrates the correlation between expression ratios (Log^2) of PCR samples and microarray data (acute EAE vs HC; chronic EAE vs HC) with a correlation coefficient of 0.78 and a slope of 1.18. At the protein level, frozen brain sections of CFA control, acute EAE and chronic EAE mice were immunostained with CD11b as a microglia marker and the proteins of interest **(Figure 4.2.5-2)**. As expected, either CD11b or Iba1 as a microglia marker colocalized with Jag1, C3, IL18BP, galectin-1, and INSL6 in the SVZ microglia during the acute phase of EAE. Furthermore Iba1 co-localization with IL18BP or C3 in chronic EAE, but to a lesser extent than in acute EAE microglia. Also, Iba1 or CD11b did not co-localize with IL18BP or C3 in CFA control microglia nor with galectin-1 or Insl6 in chronic or control microglia. Furthermore no Htra1 positive cells were found in the SVZ area of healthy or EAE mice. Overall these results confirm the validity of the microarray study on the gene and protein level.


Control
Acute EAE
Chronic EAE
C3 CD11b
LV
IL-18BP Iba1
Jag1 CD11b
Profile
Intensity
Distance (µm)

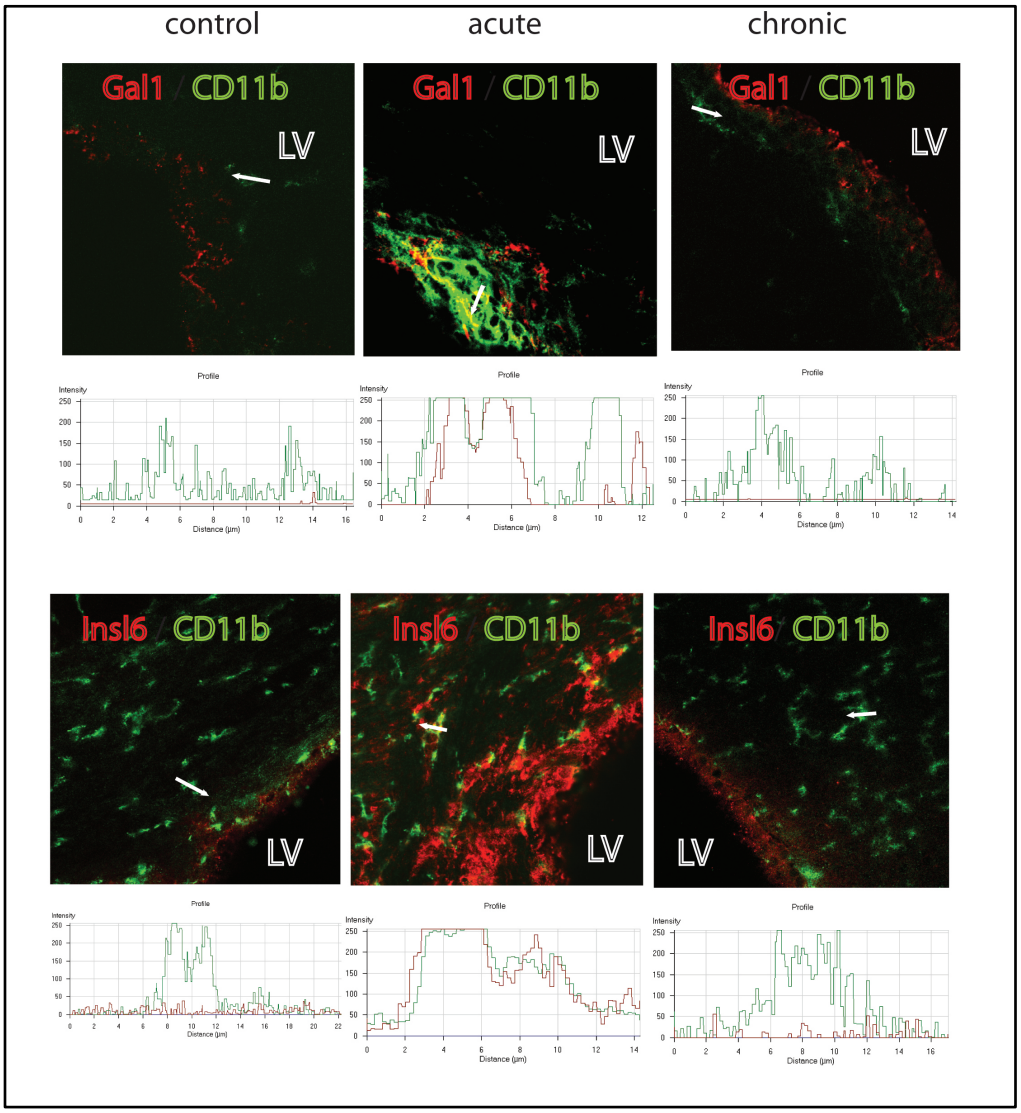


Figure 4.2.5-2: Validation of the microarray data at the protein level.
The microarray data was validated at the protein level by co-immunostaining of the microglia/macrophage marker CD11b and the protein of interest (Jag1, C3, IL18BP, Galectin-1, INSL6). Light micrographs and profile analysis showed that proteins of interest were not detectable in SVZ microglia of the control and chronic EAE, but highly expressed in acute SVZ microglia.

5. Discussion

5.1. Galectin-1 deactivates classically-activated microglia and protects from inflammation-induced neurodegeneration in a mouse model of Multiple Sclerosis

Research over the past few years, focused on deciphering the biological information encoded by the 'glycome', revealed dramatic remodeling of cell surface glycans during immune cell activation, differentiation and homeostasis. [97]. Galectins, an evolutionarily conserved family of glycan-binding proteins play pleiotropic roles in innate and adaptive immune responses through non-classical mechanisms, possibly involving the establishment of multivalent lectin-glycan lattices on the surface of immune cells [150]. Galectins do not appear to have specific receptors, but can mediate communication between immune cells through the recognition of a preferred set of cell surface glycoconjugates such as CD45, CD43, and CD71 [151-153]. In particular, galectin-1 has been associated with the induction of T-cell apoptosis [151-153], regulation of the fate of T helper cell subsets [99], although other studies supported a role for this glycan-binding protein in promoting IL-10 synthesis and blocking IFN-γ and IL-17 secretion by T cells [154-158]. Furthermore, exposure to galectin-1 or galectin-9 promoted the differentiation of $CD4^+CD25^+$ T_{reg} cells [159] [155,160], while galectin-1 signaling on dendritic cells contributes to amplifying a tolerogenic circuit mediated by IL-27, pSTAT3 and IL-10 [102]. In keeping with its anti-inflammatory functions, galectin-1 inhibits nitric oxide synthesis [161], increases arginase activity [161] and impairs the ability of T cells to stimulate macrophages [100]. However, the regulatory functions of galectins within the CNS compartment still need to be determined in both physiological and pathological settings.

This study provides insight on the role of endogenous and exogenous galectin-1 on CNS resident innate immune cells.

5.1.1. Galectin-1 is expressed in the CNS during EAE

Galectin-1 has been shown to exhibit tolerogenic functions and thus contributing to tempering inflammation and driving the resolution of autoimmune pathology by acting on the peripheral immune compartment during EAE. In this context, it was previously shown that galectin-1 expression in spleen and lymph nodes incerased during the peak and recovery phases of EAE [102]. Additionally it was shown that galectin-1 is expressed in neuronal subsets in the forbrain and in GFAP+ cells in both SVZ and dendate gyrus in naïve mice [105]. Furthermore, galectin-1 expression was reported to be increased after CNS pathological processes such as spinal cord injury [162] and stroke, with GFAP+ astrocytes as the main cellular source [104,106]. However it is not know how galectin-1 expression is regulated in the CNS during EAE.

Here, it was demonstrated that endogenous galectin-1 expression is slighly increased in the spinal cord of preclinical EAE mice and is highly up-regulated in acute EAE whereas its expression remains high during the chronic phase though to a lesser extent.

Focusing on the cellular source during EAE, data provided here suggest that galectin-1 is expressed by the majority of astrocytes and a subset of CD4+ T cells and CD11b+ microglia/macrophages. However during chronic EAE its expression is mainly restricted to astrocytes.

It was shown that tolerogenic stimuli, including galectin-1 itself increase galectin-1 expression in astrocytes. Furthermore, unstimulated astrocytes produce high levels of galectin-1 protein *in vitro*, which is probably related to the presence of TGF-β_1 or other

stimuli in the serum that is included in the culture media. Additionally, the pro-inflammatory molecule LPS supressed astrocytic expression of galectin-1 mRNA, but not galectin-1 protein secretion. These data suggest that galectin-1 gene expression and protein secretion may be independently regulated in astrocytes, or that downregulation of galectin-1 protein expression or secretion may take more than 24 hours to respond to changes in the environment.

Furthermore, tolerogenic stimuli such as IL-4, IL-13 and galectin-1 itself also induced galectin-1 expression and realease in cultured micorglia. Similar to what was observed in astrocytes, pro-inflammatory stimuli like LPS or IFNγ lead to downregulation of *Lgals1* mRNA expression but not protein secretion. Since both IL-4 and IL-13 are thought to insuce a state of alternative activation in CD11b+ microglia and macrophages, these results indicate that galectin-1 may act as a novel marker for M2 microglia/macrophges, along with Arginase1, and Chi3l3 (Ym1). Furthermore, these data suggests that alternatively activated micoglia/macrophages account for the galectin-1 expressing CD11b+ cells in the CNS during EAE. However, further in vivo evidence needs to be provided to support this conclusion.

The data provided in section 4.1.1 show that natural regulatory T cells (nTregs) and Th1 cells express high levels of Lgals1 mRNA. High *Lgals1* expression in nTregs is in line with previous reports, showing not only that CD4+ CD25+ T regs express high levels of *Llgals1* mRNA, but also that this expression is FoxP3 independent and that galectin-1 expression in those cells may provide a mechanism for effector T cell suppression [163,164] However, Motran et al. report that galectin-1 is equally expressed in cultured Th1 and Th2 cells 48 hours after activation with anti-CD3/anti-CD28 [165] . The data provided in section 4.1.1, however, suggest that 4 days after polarization/activation only Th1 cells but not Th2 or Th17 cells expressed relatively high

levels of the *Lgals*1 gene. Even though no data on protein secretion by T cell subsets was provided here, this observation suggests that both nTregs and Th1 cells express galectin-1 and may account for the galectin-1 expressing T cell subsets in vivo. Galectin-1 expression in Tregs provides a possible mechanism for resolving neuroinflammation and is in line with the general finding of galectin-1 as a tolerogenic molecule, whereas expression of galectin-1 by Th1 cell may be an Th1 autocrine mechanism to limit inflammatory processes.

Taken together, the results provided in section 4.1.1 suggest a role for galectin-1 during EAE, not only in the peripheral immune compartment, but also within the CNS.

5.1.2. Galectin-1 downregulates activation of M1 microglia in vitro

The dichotomy of classical activation versus alternative activation is well studied in peripheral macrophages, where pro-inflammatory stimuli, such as high levels of IFNγ, TNF or LPS leads to classical activation (M1) while anti-inflammatory cytokines such as IL-4 or IL-13 induce alternatively activated macrophages (M2) [166]. Classical activation in macrophages is closely associated with increased iNos and TNF expression as well as functional processes, such as neurodegeneration, whereas M2 macrophages are characterized by increased expression of Arginase1 and Chi3l3 (Ym1) and associated with anti-inflammatory processes. Ponomarev et al. have recently demonstrated that such dichotomy also exists in microglia, where CNS endogenous IL-4 is responsible for alternative activation in microglia. they also provide evidence of the high abundance of M2 microglia in the CNS of acute EAE mice.

In agreement with its anti-inflammatory role, data provided here suggests that galectin-1 binds to all microglia subtypes but has a significantly higher affinity to classically activated microglia. Galectin-1 binding to microglia is dose- and carbohydrate dependent and this increase in binding afiinity to M1 microglia is consistent with the differential glycosylation signatures of these cells as compared to M2 microglia. Strikingly, cell surface binding of galectin-1 as well as the glyco-profile of classically-activated microglia recapitulate the pattern observed on Th1, Th17 cells and immature dendritic cells, while the pattern observed on alternatively activated microglia is similar to that displayed by Th2 cells [99,102]

Data in section 4.1.2 also suggest a functional role of galectin-1 in negatively regulating microglia activation. In fact, classically-activated microglia respond to galectin-1 by down-regulating activation markers, pro-inflammatory cytokine production, and expression of iNOS. Alternatively activated microglia, on the other hand respond by further up-regulating the M2 marker arginase1.

As a proof of concept, transfer of conditioned media from stimulated astrocytes after silencing of *Lgals1-/-* (as in section 4.1.6) also increased classical activation in microglia as compared to mock silencing. This observation also point to a role of galectin-1 in modulating microglia activation.

The dampening of classical activation was not associated with a decrease in cell survival, but correlated with a strong downregulation of pro-inflammatory signaling pathway involving p38MAPK and CREB and Erk and IκB-α to a lower extent.

Signaling pathways involving p38MAPK and CREB are widely implemented in classical activation of microglia and macrophages, suggesting that the galectin-1 deactivates M1 microglia by targeting p38MAPK and CREB (Ref: Xing, B et al (123) & Mirzoeva et al (124)).

However, it is possible that galectin-1 also targets other signaling pathways not investigated in this study.

Furthermore, section 4.1.1 shows that activation of microglia by pro-inflammatory stimuli down-regulates endogenous galectin-1 expression by microglia and the data shown in Figure 4.1.2-7 provide evidence that microglia-endognous galectin-1 is not necessary to down-regulate M1 microglia activation by exogenous galectin-1. Nevertheless, *Lgal1*-/- microglia showed a slightly decreased response to activation by LPS, which may indicate a dual role of galectin-1 in microglia activation rather than deactivation.

5.1.3. Galectin-1 reduces classical microglia activation and promotes axonal damage during EAE

It was previously reported that mice lacking endogenous galectin-1 develop severe EAE when compared to wild type EAE mice concurrent with an increase in Th1 and Th17 effector cells in the periphery and CNS [99]. Furthermore endogenous galectin-1 drives the generation of IL-27-producing tolerogenic dendritic cells (DCs) in the periphery, and transplantation of galectin-1 expressing DCs to *Lgals1*-/- EAE mice ameliorates clinical severity compared to transplantation of *Lgals1-/-* DCs [102].

Section 4.1.3 shows that in the absence of endogenous galectin-1, classical microglia activation is favored, concurrent with an increase in demyelination, axonal loss, and astrogliosis and a reduction in endogenous synaptic repair. Furthermore, section 4.1.7 shows that therapy with galectin-1 during EAE reduces classical microglia activation along with a reduction of neurodegeneration, demyelination, astrogliosis and an increase in synaptic repair.

Within the CNS inflamed tissues, Iba1$^+$ cells may include microglia and peripherally-derived macrophages. Galectin-1 is known to suppress macrophage activation [100,161] in the periphery, thus it is likely that both microglia and macrophages in the CNS may respond to galectin-1 with a decrease in classical activation. This in vivo finding is consistent with the in vitro finding in section 4.1.2 and the previously reported in vitro findings on macrophages, suggesting that galectin-1 induces a state of aternative activation or deactivation of M1 in microglia and macrophages [101].

Other members of the galectin family have also been associated with the control of the activity of CNS macrophages and microglia. This is the case for galectin-3, which is up-regulated in microglia in a model of traumatic brain injury and induces the release of nerve growth factor [167,168] and galectin-9 which signals through Tim-3 in CD11b$^+$ CNS cells to stimulate innate immunity [36].

Furthermore, the observation that lack of endogenous galectin-1 leads to increased neurodegeneration correlates well with increase classical microglia activation, as those cells are known to mediate such pathological processes. However, the data provided in section 4.1.3 suggest but does not provide solid proof of the involvement of endogenous galectin-1 on damplening neuropathological processes via microglia in vivo.

Moreover, an increase in GFAP immunoreactivity in the inflamed CNS of *Lgals1*$^{-/-}$ mice was observed here. In this regard, previous studies indicated that galectin-1 promotes astrocyte maturation and inhibits astrocyte proliferation *in vitro* [169]. Thus, it is likely that in addition to microglial cells, astrocytes also respond to galectin-1 and contribute to disease modulation, by promoting a neuroprotective microenvironment.

5.1.4. Galectin-1 controls microglia-mediated neurotoxicity

There is ample evidence that microglia may mediate neuronal damage for instance through release of reactive oxygen species.

In vitro results provided in section 4.1.4 demonstrate that galectin-1 decreases neuronal loss and axonal degeneration, possibly by limiting LPS-induced p38-MAPK and CREB phosphorylation, the signaling pathways that are activated upstream of the neurotoxic molecules NO and TNF in the microglia.

In the experiments outlined in section 4.1.4, no direct effect of galectin-1 on cultured neurons was observed, in contrast to a recent report [128]. Plachta et al. observed a direct neurodegenerative effect of galectin-1, based on in vitro experiments similar to the one used here. The divergence in findings may be related to the source and biochemical properties of the galectin-1 used, including the prevalence of monomeric versus dimeric forms, the endotoxin content of the preparation and/or the oxidizing versus reducing microenvironment. Of note, occasional contamination of endotoxin were found in different batches of carrier containing- and carrier free recombinant galetin-1 preparations provided by RND (and used by Plachta et al. [128]), raising questions on the purity of this product.

Furthermore, the degeneration of peripheral neuronal processes in *Lgals1*$^{-/-}$ mice (as observed by Plachta et al.[128]) may be due to the loss of endogenous neuron-derived galectin-1 or to differences between the peripheral nervous system and the CNS.

However, the results presented here are in line with the general understanding of galectin-1 as a mediator of protection in EAE [99,170] and demonstrate, for the first time, that the mechanisms of neuroprotection are mediated by inactivation of M1 microglia, suggesting that the establishment of galectin-1-glycan lattices within

different glial cells may provide an endogenous mechanism to limit CNS inflammation and neuropathology.

The data provided here highly suggest that galectin-1 dampens neurodegeneration by acting on microglia in vivo. This hypothesis is further strengthened by the finding (section 4.1.5) that transfer of galectin-1 ex vivo-treated microglia into EAE mice down-regulates clinical disease.

5.1.5. Galectin-1 as a potential therapeutic target indiseases involving inflammation mediated neurodegeneration

The results in section 4.1 provide evidence that galectin-1 reduces neurodegeneration by deactivating classically activated microglia, thus leading to a decrease in clinical severity of EAE.

It is likely that during acute EAE galectin-1 is highly expressed in inflammatory lesions, through secretion by astrocytes, M2 microglia/macrophages and Th1 and nTreg cells. The increase in ambient galectin-1 may upregulate galectin-1 in microglia through autocrine mechanisms leading to a decrease in classical microglia activation and subsequent microglia-mediated neurotoxicity. This process is likely mediated by the downregulation of the proinflammatory signaling pathways in microglia, involving p38MAPK and CREB and downstream mediators, such as TNF and NO. Furthermore, galectin-1 acts as a powerful therapeutic agent, leading to amelioration of clinical signs in EAE. Thus galectin-1 may provide a new therapeutic target for diseases involving inflammation-associated neurodegeneration, such as multiple sclerosis as well as Alzheimer's and Parkinson's disease.

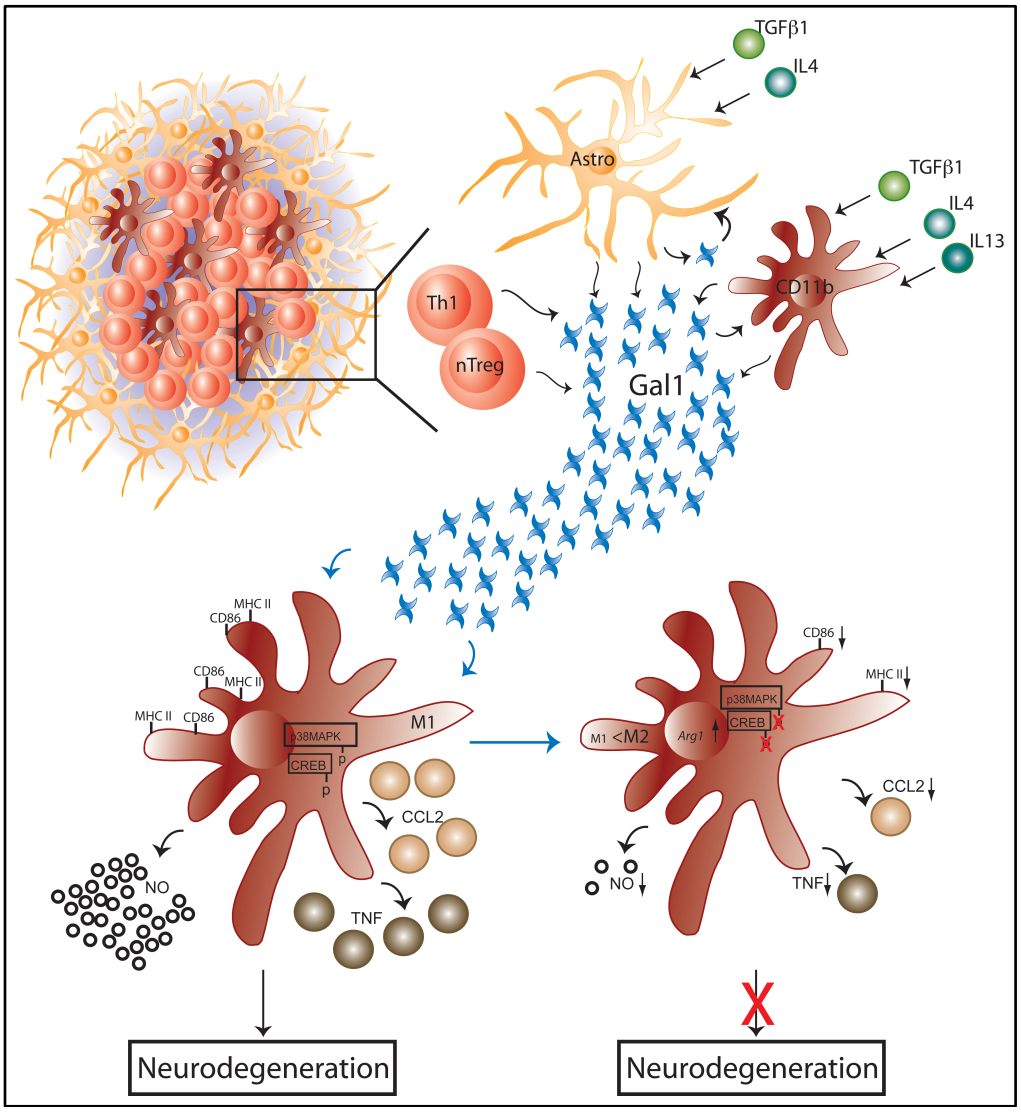


Figure 5.1.5-1: Model illustrating a novel role for galectin-1 in microglia-mediated neurodegeneration
See text for details

5.2. Subventricular Zone Microglia Transcriptional Networks.

Adult stem cells are viewed as repositories for the tissues repair potential. Endogenous neural stem cells have the potential of participating in regeneration in neurological diseases. In MS, an inflammatory neurodegenerative disease of the CNS, the clinical disease course usually starts with reversible episodes of neurologic disability (Relapsing Remitting Multiple Sclerosis, RRMS), that become progressive with irreversible neurological decline [66]. In the acute phase of MS and EAE the CNS may compensate for tissue damage through local repair mechanisms [171], whereas endogenous repair mechanisms are lost in the chronic phase [33] resulting in progression of clinical disability [75,82,171].

Microglia have been closely associated with the SVZ niche during EAE and appear to remain activated during acute and chronic disease. There are conflicting reports on the effect of microglia on NSC function with some reports suggesting a deleterious role [172] while others suggest a beneficial role [58,173]. Previous data suggest that microglia have both beneficial and deleterious roles at different phases of disease, and that manipulation of microglia activation may impact the repair potential in the CNS [33]. However the underlying molecular mechanisms of such dichotomy have not been investigated.

5.2.1. Systems biology approach to study $CD11b^{+}/CD45^{lo}$ microglia in the acute and chronically inflamed SVZ ex vivo

Investigation of cells in a complex microenvironment calls for an un-biased approach with the least possible degree of experimental manipulation.

This study provides a detailed analysis of the microglia transcriptome by direct isolation of $CD11b^{+}/CD45^{lo}$ microglia from the subventricular zone.

Section 4.2.1 demonstrates that the transcriptional profile of microglia showed no contamination with neurons, astroglia, oligodendroglia or progenitors cells as shown by enrichment of CD11b+ microglia specific transcripts and absence of non-microglia cell-type specific transcripts **(Fig. 4.2.1-2).** However, it remains possible that the population of $CD11b^{+}/CD45^{lo}$ expressing cells used in the analysis, contains microglia as well as peripheral macrophages. $CD11b^{+}/CD45^{lo}$ cells were selected for this study as they are thought to represents microglia cells, as opposed to $CD11b^{+}/CD45^{high}$ cells that are thought to represent peripheral macrophages. In fact, two distinct populations of CD11b+ cells were detected during EAE: a CD11b+ CD45hi and a CD11b+ CD45lo population **(Fig. 4.2.1-1)**.

Furthermore, a previous study on SVZ microglia in EAE found no inflammatory lesions that were located in close proximity to the subventricular zone area[76]. The data provided in section 4.2.1 confirms the absence of inflammatory lesions in close proximity to the subventricular zone area by hematoxylin & eosin (H&E) staining of the subventricular zone area in control, acute EAE and chronic EAE (Figure 4.2.1-3). Thus, this data suggests that the selected population represents microglia and is of high purity.

5.2.2. Genomic signature reveals persistant microglia activation through acute and chronic RR-EAE.

Previous data by Rasmussen et al. showed persistent microglia activation in the SVZ area during acute and chronic EAE with overexpression of the pro-inflammatory activation marker p38MAPK [33].

The results, provided in section 4.2.2 show that during the acute phase of EAE there are a larger number of up-regulated transcripts than during the chronic phase. This finding confirms that microglia get activated during the acute phase and remain activated during chronic EAE.

5.2.3. Acutely activated microglia support NSC functions by secretion of niche supporting molecules.

Recent studies have revealed that the NSC compartment of the SVZ is highly activated during acute EAE, and loses its capacity for endogenous repair during the chronic phase[33]. Data by Pluchino et al. suggest that the loss of stem cell activity during the chronic phase is reversible when the stem cells are cultured ex-vivo [96], suggesting that the niche microenvironment is responsible for the stem cell dysfunction. Ultrastructural and confocal microscopic data revealed that microglia are closely associated with the SVZ stem cells in the niche, supporting the hypothesis that microglia have the ability to influence the SVZ microenvironment.

Focusing on molecular signatures, section 4.2.3 shows that microglia may actively influence the SVZ microenvironment by secreting molecules in the extracellular space and expressing molecules on the plasma-membrane.

Moreover this data provides evidence that during acute EAE, microglia secrete molecules that may lead to the recruitment, adhesion and development of other immune cells, such as monocytes and T cells suggesting that microglia may react and orchestrate the inflammatory processes. Furthermore, microglia may also affect the neural stem cell niche through molecules that are known to influence adhesion, development and growth of tumor cells and tumor cell lines, that are

known to share many unique functions with stem cell [132,174-176]. Upregulation of genes that support cardiovascular functions such as the development of blood vessels and angiogenesis solely during acute EAE suggests a structural impact on the stem cell niche. Since functions related to cancer and angiogenesis are thought to influence the self-renewal, differentiation and migration capacity of stem cells [132,175,176], a functional upregulation of those pahtways through microglia secreted molecules may reinforce the notion of a strong niche supporting role of microglia during this disease phase.

Thus, it can be concluded that during the acute phase of EAE, microglia exhibits a dual phenotype: on the one hand microglia support and orchestrate the inflammatory process, while on the other hand they secrete molecules that actively support neural stem cell functions such as self-renewal, differentiation and recruitment to the site of injury.

An interesting question is whether microglia are a homogeneous population, or whether there are subpopulations with different activation profiles.

In contrast to the acute phase of EAE, chronically activated microglia seem to have predominantly a detrimental role as the surface expressed and secreted molecules promote only inflammatory functions but no niche supporting functions as shown by the GO analysis provided in section 4.2.2. This finding correlates very well with the observation that the repair potential of the SVZ niche, as measured by increased self-renewal, neurogenesis and oligodendrogenesis, is enhanced only during the acute phase of RR-EAE, but does not persist into the chronic phase [33].

5.2.4. Functional Network analysis reveals VEGFa and C3 as targets to enhance SVZ niche activity

Functional Networks provide insight into the molecular mechanisms underlying a predicted GO function. As functional networks consist of molecules with reported physical interaction and a common association for distinct biological functions, they may reveal interesting drug targets for dampening or enhancing a particular biological function in the given biological context.

Within a given protein-interaction network there are proteins with high connectivity, also called hubs that are suggested to have high importance for the associated biological function. Targeting a hub, or a gene that translates into a hub protein may impact a given function more than regulating a gene or protein with lesser connectivity[177] .

The functional network, identified in section 4.2.4 contains genes such as Jag1, C3 or vascular endothelial growth factor alpha (VEGFα) that affect stem cell functions. The transmembrane type notch ligand, Jag1 induces self-renewal in neural progenitors through the notch signaling pathway [134,178-180]. In addition to its immunological functions, the complement component C3 is known to induce neurogenesis and migration of neural progenitors in vivo and in vitro [143,181], while VEGFa induces proliferation of neural [182-185] and glial progenitors [186] as well as neurogenesis [187]. In particular, the VEGFa and C3 genes show a high degree of interconnectivity within their network, suggesting hat targeting those genes may result in an alteration of their related function.

These findings are of great interest since they open the door for indentifying new therapeutic targets for chronically activated microglia, which are associated with neuronal and progenitor dysfunction [33,76,188]. Enhancing C3 and VEGFa functions during the chronic phase of MS

and EAE in particular may increase endogenous repair functions and thus provide a mechanism to ameliorate MS and EAE.

5.2.5. Validation of microarray study

Results from this microarray study were validated by direct correlation of expression levels of genes of interest with those observed by the microarray platform. Here, dynamic changes of gene expression during EAE were taking into account and are shown to follow the same pattern when analyzed by qPCR and microarray. Furthermore, the findings were also validated at the protein level, also taking into acount dynamic changes during the disease phases of EAE.

GO and functional network analysis of gene expression profiling data assumes that changes in gene expression levels directly translate into changes in protein level. While this premise may be true for the majority of regulated genes/proteins (section 4.2.5), post-translational events and protein regulation may significantly affect other genes and impact biological functions. Thus, proof of invovlement of particular networks will require further testing at the protein level to directly test their involvement in the predicted functions.

Our data highlights the importance of microglia in MS and EAE and provides insight into new avenues of investigation that may lead to halting MS progression and facilitating CNS repair.

5.2.6. Micorglia support of NSC functions during EAE

Taken together, these results demonstrate for the first time that during inflammatory CNS disease, microglia display distinct genomic signatures in different disease phases, and provide support to the stem cell niche during the acute inflammation.

5.3. Outlook

The work described in this thesis provides insight into the role of microglia during EAE and the regulatory function of galectin-1 in the CNS.

Further studies are needed to address whether endogenous galectin-1 plays a role during chronic-progessive EAE, and whether therapy with recombinant galectin-1 may ameliorate clinical disease when administered at a later disease stage.

More importantly, further investigations need to be carried out to determine if our findings can be translated to human disease. Expression of galectin-1 in acute and chronic-active MS lesions as well as in shadow-plaques during RR-MS, PP-MS and SP-MS would be informative, especially if correlated with the presence of activated microglia and neurodegeneration in those areas.

The second part of this thesis proves insight into the functional relation of differential microglia activation durign acute and chronic EAE. The data point out that functional outcome of microglia activation is highly dependent on the environement. The structure of the human SVZ differs from the murine SVZ, so the results of this study may not be directly translated to humans. Thus, it would be interesting, to study functional changes in acute and chronically activated microglia isolated from the human SVZ, as well as in acute and chronic white matter leasons in multiple sclerosis. Such studies rely on human-post mortem tissues, where no living cells can be isolated, but laser-capture microdissection technology may allow isolation of RNA from microglia/macrophages from the areas of interest.

This work and the potential subsequent investigations provide hope that modulation of micorglia may ultimatly result in amelioration of chronic MS enhance repair.

6. SUMMARY

Inflammation-mediated neurodegeneration occurs in the acute and the chronic/progressive phases of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Classically-activated microglia (M1) are key players mediating this process through secretion of soluble factors including nitric oxide (NO) and tumor necrosis factor (TNF). Here, galectin-1, an endogenous glycan-binding protein, was identified as a pivotal regulatory mechanism that limits M1 microglia activation and neurodegeneration, by targeting the activation of p38MAPK- and CREB-dependent pathways and hierarchically controlling downstream pro-inflammatory mediators such as iNOS, TNF and CCL2. Galectin-1 is highly expressed in the acute phase of EAE and its targeted deletion results in pronounced inflammation-induced neurodegeneration. These findings identify an essential role of galectin-1-glycan lattices in tempering microglia activation, brain inflammation and neurodegeneration with critical therapeutic implications in relapsing-remitting and secondary progressive MS.

Microglia with distinct phenotypes are implicated in neurotoxicity, neuroprotection, and in modulation of endogenous repair by NSCs. However the precise molecular mechanisms underlying this diversity in fuction are still unknown.

Using a model of EAE, transcriptional profiling of isolated SVZ microglia from the acute and chronic disease phases of EAE was performed. The results from this study suggest that microglia exhibit disease phase specific gene expression signatures, that correspond to unique GO functions and genomic networks. These data demonstrate for the first time, distinct transcriptional networks of microglia activation in vivo, that support their role as mediators of injury or repair.

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8. Appendix

8.1. Appendix Supplementary Table 1

This table can be found on the supplementary data CD

8.2. Appendix Supplementary Table 2

This table can be found on the supplementary data CD

8.3. Appendix Supplementary Table 3

This table can be found on the supplementary data CD

9. List of abbreviations

7AAD	**7**-**A**mino**a**ctinomycin **D**
A	**a**mpere
ADP	**A**denosine 5' (trihydrogen **d**iphos**p**hate)
Arg1	**Arg**inase **1**
ATP	**A**denosine 5' **t**riphos**p**hate
BBB	**B**lood **b**rain **b**arrier
BSA	**B**ovine **S**erum **A**lbumin
C	**C**elsius
C2GnTs	**c**ore-**2**-N-acetyl**g**lucosamin**y**l**t**ransferases
C3	**C**omplement component **3**
cAMP	3'-5'-cyclic adenosine monophasphate
CD	**C**luster of **d**ifferentiation
CFA	**c**omplete **F**reund's **A**djuvant
Chi3l3	**Chi**tinase **3** **l**ike **3**
CNP	2'3'-**c**yclic-**n**ucleotide-3'-**p**hosphodiesterase
CNPase	2'3'-**c**yclic-**n**ucleotide-3'-**p**hosphodiester**ase**
CNS	**C**entral **n**ervous **s**ystem
CRD	**C**arbohydrate **r**ecognition **d**omain
CREB	**c**AMP **r**esponse **e**lement **b**inding
CSF	**C**erebro**s**pinal **f**luid
DC	**D**endritic **C**ell
DDR1	**d**iscoidin **d**omain **r**eceptor tyrosine kinase **1**
DMEM	**D**ublecco **m**odified **e**agle's **m**edium
DNA	**D**eoxyribo**n**ucleic **a**cid
dpi	**d**ay **p**ost **i**mmunization
EAE	**E**xperimental **A**utoimmune **E**ncephalomyelitis
ECL	**E**nhanced **c**hemo**l**uminescence
ELISA	**E**nzyme-**L**inked **I**mmunoab**s**orbent **A**ssay
ERK	**e**xtracellular signal-**r**egulated **k**inase
FBS	**F**etal **b**ovine **s**erum
FC	**F**old **c**hange
FDR	**F**alse **D**iscovery **R**ate
FoxP3	**F**o**r**khead bo**x** **P3**

g	relative centrifuge force
g	gram
G-CSF	Granulocyte colony-stimulating factor
Gal1	Galectin-1
GAP43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GJA1	Gap junction protein alpha 1
GO	Gene Ontology
h	hour
HBSS	Hank's balanced salt solution
HC	Healthy Control
HPA	Heliz pomatia agglutenin
htra1	Htra serine peptidase 1
IFNγ	Interferon gamma
IGF2	Insulin-like growth factor 2
IL	Interleukin
insl6	insulin-like 6
IPA	Ingenuity pathway analysis
Jag1	Jagged 1
l	liter
LPS	Lipopolysaccharide
LV	Lateral ventricle
m	meter
M	Molar
M1	classically activated microglia/macrophages
M2	alternatively activated microglia/macrophages
MAG	Myelin-associated glycoprotein
MAL II	Maackia amurensis agglutinin
Map2	Microtubul-associated protein 2
MBP	Myelin basic protein
MFI	Mean flourescence intesity or Median flourescence intensity
µg	microgram
MHC-II	Major histocompatibility complex-II

min	minute
ml	Milliliter
µl	Microliter
MOBP	Myelin-associated oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
mRNA	messanger Ribonucleic acid
MS	Multiple Sclerosis
NB	Neurobasal medium
NF-κB	Nuclear factor-kappa B
ng	nanogram
NSC	Neural stem cell
OPC	Oligodendrocyte precursor cell
p38MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PHA	L-phytohemagglutenin
PLAT	tissue plasminogen activator
PLP	Proteolipid protein
PNA	Peanut agglutenin
PNS	Peripheral nervous system
PP-MS	Primary Progressive- Multiple Sclerosis
PT	Pertussis toxin
RMS	Rostral migratory stream
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
RR-MS	Relapsing Remitting-Multiple Sclerosis
RT	room temperature
RT	reverse transcription
RT-PCR	Real time-Polymerase Chain Reaction
SDC1	syndecan 1
SEM	standard error of the mean
SGZ	Subgranular zone
siRNA	small interference Ribonucleic acid

SNA	**S**ambuccus **n**igra **a**gglutinin
SP-MS	**S**econdary **P**rogressive-**M**ultiple **S**clerosis
SPARCL1	**SPARC**-like **1**
SVZ	**S**ub**v**entricular **z**one
TBST	**T**ris **b**uffered **s**aline with **T**ween20
TGFβ1	**T**ransforming **g**rowth **f**actor beta **1**
Th1	**T** **h**elper cell **1**
Th17	**T** **h**elper cell **17**
Th2	**T** **h**elper cell **2**
THBS1	**Th**rom**bo**s**p**ondin **1**
TLR	**T**oll-**l**ike **r**eceptor
TNF	**T**umor **n**ecrosis **f**actor
Treg	**reg**ulatory **T** cell
U	**U**nit
V	**V**olt
VEGFA	**V**ascular **e**ndothelial **g**rowth **f**actor **a**lpha
VTN	**Vit**ro**n**ectin
WM	**W**hite **m**atter
WT	**W**ild **t**ype

10. Eidesstattliche Erklaerung

Hiermit erklaere ich, Sarah-Christin Starossom, geboren am 10.05.1983 in Leverkusen, dass ich meine Dissertation selbststaendig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.
Ich habe keinen anderen Promotionsversuch unternommen.

Mainz, 02/2011

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Sarah-Christin Starossom