Role of the cell adhesion molecule L1 during neuroinflammation in the central nervous system

Dissertation

Zur Erlangung des Grades

Doktor der Naturwissenschaften

(Dr. rer. nat.)

Am Fachbereich Biologie

Der Johannes Gutenberg-Universität Mainz

Lutz Menzel

geboren am 02.10.1985 in Berlin

Mainz, 2016

Dekan: [redacted]

Berichterstatter: 1. [redacted]

2. [redacted]

Tag der mündlichen Prüfung:

Table of Contents

Abbreviations

ADAM	a disintegrin and metalloproteinase domain-containing protein
APC	antigen presenting cell
APP	amyloid precursor protein
ara-c	cytosine-arabinoside
ATP	adenosine triphosphate
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BMDC	bone marrow derived dendritic cell
BMDM	bone marrow derived macrophages
BS	brain stem
BSA	bovine serum albumin
CAM	cell adhesion molecule
CB	cerebellum
CFA	Freud's complete adjuvance
CFSE	carboxyfluorescein succinimidyl ester
CHL1	cell adhesion molecule L1-like
CNS	central nervous system
CSF	Cerebrospinal fluid
ctl	control
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTX	cortex
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
eGFP	enhanced green fluorescence protein
ER	endoplasmic reticulum
ERK	extracellular-signal regulated kinase
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FCS	fetal calve serum

FN	fibronectin
FoxP3	forkhead box protein 3
GFAP	glial fibrillary acidic protein
GM	grey matter
GM-CSF	granulocyte-monocyte colony stimulating factor
HIP	hippocampus
iDC	immature dendritic cell
IFNγ	interferon gamma
IgG	immunoglobulin G
IgSF	immunoglobulin superfamily
IL	interleukin
IMDM	Iscove's modified Dulbeco's medium
IRDye	infrared fluorescence dyes
L1	L1 cell adhesion molecule
LFA-1	lymphocyte function-associated antigen-1
LFB	luxol fast blue
LPS	lipopolysaccharide
LysM	lysozyme M
M-CSF	macrophage colony stimulating factor
MAC-1	macrophage-1 antigen
MAC3	macrophage-3 antigen
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
mDC	mature dendritic cell
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NeuN	neuronal nuclei
NF	neurofilament
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NrCAM	neuronal glial-related cell adhesion molecule

Nrp-1	neuropilin-1
PAS	periodicacidSchiff
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDL	poly-D-lysine
PI	propidium iodide
PLP	proteolipid protein
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
REST	repressor element-1 silencing transcription factor
RGMa	repulsive guidance molecule A
RPMI	Roswell Park Memorial Institute
RRMS	relapse remitting multiple sclerosis
RT	room temperature
SC	spinal cord
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Sema3A	semaphorin3A
SPMS	secondary progressive multiple sclerosis
Syn	synapsin
TCR	T cell receptor
tg	transgene
TGFβ	transforming growth factor beta
Th	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
WM	white matter
WT	wild type

1 Introduction

1.1 The central nervous system

The nervous system of vertebrates is composed of the peripheral nervous system (PNS) and the central nervous system (CNS). The CNS comprises the brain and the spinal cord, whereas the PNS consists of nerves that connect all body parts of the periphery with the CNS. The mammalian brain is a highly complex organ that is organized in specialized structures, including the distinct areas of cortex, hippocampus, thalamus, cerebellum and brain stem. The CNS is basically composed of two kinds of specialized cells, neurons as major cell type responsible for signal transmission and processing and glia cells, responsible to support and modulate neuronal function, provide metabolic factors, set up CNS structure, assist in repair processes and maintain CNS integrity. Glia cells comprise astrocytes, oligodendrocytes, NG2-glia and microglia, which maintain an undisturbed and healthy neuron environment and support neuronal function in the CNS. Astrocytes are involved in almost all aspects of brain structure, function and homeostasis. Oligodendrocytes are the myelin sheath forming cell population and therefore enable an appropriate neuronal signal transmission and maintain long-term axonal integrity [1]. NG2-glia modulate synaptic activity, and often ensheath synapses [2]. Microglia are the resident innate immune cells of the CNS, albeit their immunological activity is tightly restricted and continuously modulated by the immunosuppressive microenvironment of the CNS. Neurons differentiate from neuronal precursor cells during the CNS development in the processes of neurogenesis. Subsequently to their generation, neurons migrate to their designated CNS structure to form neuroanatomically defined brain and spinal cord structures [3, 4]. Upon arrival at their destination site, axon branching and presynaptic differentiation results in the formation of highly stereotypic and topographically specific neuronal circuits [5]. As the primary signal conduit in neurons, axon fibers are in average 20,000 times longer than the cell body and therefore constitute extended neurites of the neuronal cell membrane [6]. An axon protrudes from the axon hillock at the cell body and extends towards its target cell. The interior structure of the axon maintains its shape and caliber and permits a rapid internal transport. The axoplasm is basically resembled of loosely packed cytoskeleton proteins and a main fiber axis. The axonal cytoskeleton consists of

microtubules, which stiffen the axon and provide transport scaffolding; neurofilaments filling the axoplasm, and actin microfilaments that form the axon cortex together with spectrins. There are unmyelinated and myelinated axons in the CNS. Therefore the most significant extracellular interaction of myelinated axons is with processes of oligodendrocytes, which form the myelin sheath around the axon [7].

1.2 The immune system

The immune system is a complex composition of different cell types, immunologically active factors, and signaling molecules. Myeloid cells (including macrophages), dendritic cells (DC), and mast cells are the most abundant cells of the innate immune system which conducts pathogen recognition and executes an immediate and direct immune response. In contrast, the adaptive immune system needs days to initiate an appropriate immunological response. It is composed of populations of lymphocytes - specialized immune cells, which include B cells, T cells, and natural killer cells (NKC). Lymphocytes express antigen-specific cell surface receptors that enable a specific recognition and differentiation of pathogenic and self-antigens. The adaptive immune system reacts specifically against foreign pathogens and is tolerant to all endogenous antigens [8]. T lymphocytes, commonly known as T cells, derive from hematopoietic stem cells in a process of thymocyte development into mature T cells in the thymus before puberty of every individual. T cells bear antigen-specific T cell receptors (TCR) that recognize antigens presented by antigen-presenting cells (APC) via major histocompatibility complexes (MHC). Therefore, APCs process phagocytized material, including potential pathogens, to presentable fragments, which can be recognized by T cells that bear the specific TCR for the presented antigen [9]. There are two major T cell populations, cytotoxic T cells, referred as CD8 T cells and T helper cells (Th cells), referred as CD4 T cells. CD8 T cells are activated through recognition of viral particles and short foreign or mutated peptides presented by infected cells via MHC class I (MHC I). The cytotoxic capacities of CD8 T cells mediate the killing of the affected cells, which prevents further replication of the virus and expansion to neighboring tissue. CD4 T cells recognize antigens of pathogenic organisms such as bacteria, which are phagocytosed, processed by APCs and presented via MHC class II (MHC II) [10]. The activation of CD4 T cells results in their differentiation into specific T helper cell subsets with characteristic cytokine production patterns. Th1 cells are characterized by Interferon-γ (IFNγ) and

interleukin (IL)-2 production and primarily activate macrophages, monocytes, and under specific circumstances also microglia cells [11]. Th2 cells secrete IL-4, IL-5 and IL-13 which influence the immunoglobulin class switch in B cells and the release of antibodies from B cells at sites of inflammation [12]. Another CD4 T cell subset is known as Th17 cells due to the characteristic IL-17 production. Th17 cells amplify the inflammatory process through cytokine secretion, recruitment of neutrophil granulocytes, and repression of regulatory T cells [13]. Regulatory T cells (Treg) are in turn known for their role in the modulation of inflammatory processes by secreting immunosuppressive cytokines IL-10 and transforming growth factor-beta (TGF-β) [14] and by cell-cell contact via CTLA-4 [15].

1.3 Immunological processes within the CNS

The CNS has been regarded as an immune-privileged site for a long time, delimited from the immune system by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier. This hypothesis is supported by only minimal MHC II expression levels on parenchymal cells, low cytokine and chemokine levels, low numbers of lymphocytes and the inability of cells from the healthy, un-inflamed brain to prime naïve T cells [16, 17]. Recent studies challenge the rigid concept of an absolute separation of the immune system from the CNS. It has become clear that immune cells are present within the CNS not only during acute and chronic inflammation, but also under non-pathological conditions as an ongoing process of CNS immune surveillance [18, 19]. It has been shown that T cells also survey the brain during daily-life infections and specific subpopulations even remain as memory T cells after the infection has cleared [20]. A healthy CNS contains a negligible level of immunologically relevant molecules and in contrast to the historical view of the immunological incompetence, there are CNS-resident cells like microglia and astrocytes, which actively maintain the CNS environment in order to keep immunological processes at a minimum level. The BBB is composed of CNS specific endothelia cells (EC), which are characterized by the formation of tight junctions and the deficiency in *trans*-cellular transport systems. ECs together with basal lamina, pericytes, and astrocytes form a functional neurovascular unit. The highly selective permeability of the BBB stringently restricts the diffusion of pathogens, blood cells, and large hydrophilic molecules from the peripheral circulating blood into the cerebrospinal fluid (CSF), but enables the passage of small hydrophobic

molecules like O_2, CO_2, and small signal proteins [21]. The integrity of BBB can be disrupted by several diseases, which lead to the infiltration of immune cells into the CNS. BBB dysfunction and breakdown and the disruption of tight junctions have been implicated, for example, in Alzheimer's and Parkinson's disease, multiple sclerosis, stroke, and brain trauma and is intimately associated with the entry of immune cells into the brain parenchyme [22].

1.4 Multiple sclerosis

The neurodegenerative disease multiple sclerosis (MS) results from escalated inflammatory processes within the CNS. MS is the most frequent neurological disease in young adults, which can lead to severe disabilities and progress into invalidity with very limited treatment options and no actual cure currently available. In Germany, the MS incidence is estimated to be up to a number of 150,000 affected people [23] with a clear predominance of female patients [24]. The first symptom of the disease is episodic neurological dysfunction, which is followed by the remission and relapse phases. These phases are characterized by the formation of disseminated focal cell infiltrates which is accompanied by the damage of the myelin sheath around axons and a glial scar formation within the affected area [25]. More recent observations indicate an inflammatory nature of the MS disease that was assumed to occur in individuals with a genetic predisposition for auto-reactive pro-inflammatory T cells with specificity for CNS antigens. Due to the failure of immunological control mechanisms, these T cells are not depleted in the periphery and cause damage to the CNS tissue when passing the BBB [26, 27]. MS is a complex disease that is not only characterized by a variety of factors triggering its onset and progression, but also by different disease symptoms and clinical courses [28]. The relapsing-remitting MS (RRMS) is the most common disease course, which displays a pattern of acute exacerbation and periods of remission and stability. The RRMS disease course most often develops into the secondary progressive MS (SPMS), which leads to progressive deterioration of relapses and worse remission over time. Between 10-15 % of MS patients exhibit the primary progressive MS (PPMS). These patients display progressive deterioration right from the onset of the disease and are unlikely to experience a significant recovery phase or relapse-remission episodes [29]. The benign MS is characterized by a milder course of disease progression with relapse-remission episodes but without progressive deterioration over time and

relatively extended episodes of stability [30]. Even though MS has been intensely studied over the last decades, the actual etiology of the disease is still unclear. There is evidence of geographical variations of the MS incidence, implications of environmental factors, and genetic predisposition to MS [31-33]. A variety of exogenous events have been identified as potential triggering factors for the exacerbation of MS, e.g. stress, trauma, infection, immunization, climate changes, and physical exertion [34]. Due to the fact that infectious agents have long been cleared from the body before the first signs of immunopathology or even neuropathology occur, most of the epidemiologic attempts to reveal a direct connection between the infection and MS etiology have failed so far. However, some clinical and epidemiologic studies support the hypothesis of a link between infections and the onset and exacerbation of autoimmune diseases like MS [28, 35]. The disease itself is characterized by heterogeneous autoimmune processes including CNS inflammation, demyelination and axon loss [36]. The pathologic hallmarks of MS are multiple focal areas of myelin loss in the CNS tissue, also called plaques or lesions. The event of demyelination is induced and accompanied by inflammation and the formation of reactive gliosis. MS lesions can be found throughout the CNS but most often they affect the optic nerve, the subpial spinal cord, brainstem, cerebellum, and juxtacortical and periventricular white matter regions [25, 37]. Although MS has long been considered as disease that primarily affects white matter tissue, more recent studies have demonstrated demyelinated lesions also in the cortical gray matter [38, 39].

Active plaques most frequently occur during acute and relapse-remitting MS and are characterized by hypercellular-demyelinated areas that are massively infiltrated by macrophages containing phagocytosed myelin debris. The occurrence of specific myelin degradation products (e.g. CNPase, myelin-associated glycoprotein), major myelin proteins like proteolipid protein (PLP), myelin basic protein (MBP) or the myelin oligodendrocyte glycoprotein (MOG) phagocytosed by macrophages indicate demyelination activity in active lesions [40]. Besides activated macrophages, inflammatory infiltrates are predominantly composed of auto-reactive CD8 T cells [41] and to a lesser extent of CD4 T cells [42], γδ T cells [43], B cells, and plasma cells [44]. The MS pathology is thought to be induced by infiltrated T cells bearing an auto-reactive specification against myelin-associated antigens. After infiltrating the CNS, T cells are confronted with antigen-presenting cells such as microglia and/or perivascular

macrophages, dendritic cells and astrocytes that contain and present myelin debris [45], which re-activate T cells and initiate an inflammatory cascade [46]. Re-activated T cells produce characteristic signatures of cytokines, which activate neighboring immune cells and astrocytes that in turn enhance the inflammatory reaction and attract further immune cell infiltration into the CNS [47]. Astrocytes present in active lesions were activated and form centers of proliferation and reactive astrogliosis [40]. The ongoing inflammation disturbs the integrity of the BBB, which in turn facilitates infiltration of attracted peripheral immune cells. These processes form a detrimental cycle of infiltration and inflammation [48].

Chronic plaques are most frequently seen in patients with progressive MS. Chronic active plaques are sharply demarcated demyelinated lesions with numerous macrophages. These plaques are "loaded" with myelin debris that is concentrically disposed at the expanding edges of the lesion, referred to as the "active rim". Perivascular inflammatory infiltrates are often found in chronic lesions, even though the BBB remains mostly intact. Therefore during chronic progressive MS inflammation is restricted to the CNS by a closed and/or repaired BBB [40, 49]. Axonal degeneration and loss are also observed in chronic MS plaques and the reduction of the axonal density down to 20 % can be observed. As in the active lesions, neurodegeneration in all demyelinated plaques is invariably associated with inflammatory processes [49, 50]. During the progression of the MS, plaques develop from acute active over chronic active to chronic inactive, which are characterized by edema resolution, decrease of inflammation, and a gradual disappearance of macrophages. These processes are accompanied by glial scar formation, during which reactive astrocytes fill the demyelinated plaque site [38]. The cortex is a site of MS pathology that is either characterized by neuronal loss and atrophy or classical demyelinated areas. However, demyelination occurs spatially and has been generally disregarded for investigation due to the insufficient visualization of lesion demyelination when using classical staining strategies. Though, inflammatory infiltrates are present during early cortical lesions and are mainly composed of macrophages and T cells. These inflammatory cells are observed in close association to neurons and their neurites and induce detrimental inflammatory processes and thus cause axonal and neuronal damage [40].

1.5 Experimental autoimmune encephalomyelitis (EAE)

EAE is the most common mouse model for CNS neuroinflammation which mimics several aspects of MS [51]. However, some major differences between the EAE model and MS compromise the ability to study all aspects of the human disease. A major difference between EAE and MS is that the former involves artificial immunization for myelin-specific antigens, the activation of the innate immune system, and the disruption of the BBB using bacterial component-containing adjuvant. In the case of MS, no explicit self-antigens are identified and the induction of the disease is a rather complex undirected process [52]. Nevertheless, EAE is an approved model of autoimmune inflammation in the CNS that resembles many features of MS and facilitates the study of inflammatory MS mechanisms [51, 53]. For the experiments carried out in this thesis, mice of the most commonly used mouse strain C57BL/6 and the SJL strain were used. In C57BL/6 mice, immunization with the MOG immunodominant epitope MOG_{35-55} induces a severe CNS attack by CD4 T cells followed by an incomplete recovery, which allows to study peak and the chronic progression of the disease. In contrast, SJL mice immunized with the PLP immunodominant epitope $PLP_{139-151}$ develop EAE progression, which displays relapsing and remitting disease stages similar to the situation in human RRMS [47]. The EAE disease is induced by a peripheral injection of adjuvant-containing specific CNS peptides as auto-antigens in combination with pro-inflammatory adjuvants, e.g., complete Freud's adjuvant (CFA) containing mineral oil and inactivated particles of mycobacterium tuberculosis. The auto-reactive T cells activated after immunization within lymph nodes and spleen infiltrate the CNS, where they recognize their cognate antigen presented by the local APC and initiate the inflammatory cascade. This in turn results in a massive immune cell infiltration and CNS tissue damage [54]. The cascade is further enforced by APCs which endocytose CNS debris and drain into the cervical and lumbar lymph nodes and into spleen by means of interstitial and cerebrospinal fluids. These APCs trigger the T cell response within these compartments and lead to an exacerbated immune reaction and an increasing number of CNS-infiltrating auto-reactive T cells [55-57]. The pathology of EAE is not entirely understood yet, but demyelination, axonal degeneration and loss, and neuronal death have been recognized as the major events of the T cell-mediated CNS tissue damage in acute EAE plaques [48].
In MS as well as in the EAE mouse model, primarily DCs and macrophages but also CNS-residents microglia and astrocytes are APCs which take up, process and present

antigens, co-stimulate T cells and produce cytokines. Together these processes drive the proliferation and differentiation of auto-reactive T cell subsets [58]. Auto-reactive Th1 and Th17 cells of the CD4 T cell lineage are thought to be the main players in EAE induction and progression as they promote the accumulation of inflammatory immune cells in typical lesion sites and mediate oligodendrocyte and neuron damage. CD4 T cell activation and survival requires two specific signals from APCs: TCR-antigen recognition via MHC II of APCs and secondary signaling which occurs via the interaction between co-stimulatory molecules like CD80/CD86 in APCs and CD28 in T cells [59]. Additionally, APCs determine the direction of naïve CD4 T cell development through cytokine secretion patterns, which induce specific subset differentiation (mainly Th1, Th2, Th17, Treg). The APCs that encounter myelin antigens mature and migrate to lymph nodes and spleen to present respective antigens to naïve T cells [14, 60]. Once activated, T cells cross the BBB, invade the CNS, and are re-activated by microglia, perivascular macrophages, and DCs, all of which present the respective self-antigens [61-63].

Different types of cells can present antigens. B cells, DCs, macrophages, microglia, and astrocytes express MHC II and co-stimulatory molecules in a constitutive and/or conditional fashion and therefore are capable to present antigens to CD4 T cells. However, these cells massively differ in their efficiency to prime, activate and differentiate T cells [64].

Macrophages are phagocytic and antigen-presenting effector cells, which execute, assist, and control innate and adaptive immune processes. The activation of macrophages is one of the factors mediated by the Th1-cytokines IFNγ and the tumor necrosis factor α (TNFα), which stimulate the up-regulation of the surface molecules MHC II, CD80, CD86, and CD40 during inflammation and likewise in MS and EAE [65]. The role of macrophages in the processes of CNS inflammation and demyelination has been demonstrated in studies involving clodronate-mediated macrophage *in situ* depletion: while the clinical signs of EAE diminished, the number of infiltrated CD4 T cells into the CNS was unaffected [66, 67].

Microglia are bone marrow-derived CNS-resident macrophages, which can be distinguished from macrophages by lower levels of CD45 and Ly6c [68, 69]. Under resting conditions, “quiescent” microglia express minimal level of the inflammation-related surface molecules MHC I, MHC II, CD80, CD86 and CD40 [70]. Quiescent microglia maintain their resting state in an autocrine manner by secreting the tumor

growth factor beta (TGFβ), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) [71], and are supported by neurons via cell-cell contacts (CD47-CD172, CD200-CD200R, CD22-CD45) [72], secreted chemokines such as CX3CL1 [73], and immunosuppressive cytokines IL-4, IL-10, and TGF-β, which are produced by astrocytes and neurons [74]. Under resting conditions, microglia are the major cell subset that maintains CNS homeostasis, display a ramified morphology, and constantly survey the CNS microenvironment [75]. Even though quiescent microglia display minimal inflammatory features, inflammatory stimuli rapidly cause microglia to switch to an active state, which is characterized by enhanced phagocytic properties, the up-regulation of MHC II, CD80, CD86, and CD40 expression and thereby the capacity to activate CD4 effector T cells [68, 70, 76]. Similar to mentioned studies with macrophages, the depletion of microglia attenuates EAE severity [77]. Altogether, macrophages and microglia display a wide range of effector and regulatory functions including secretion of inflammatory factors and control of T cell responses that contribute to induction and progression of the MS disease and the EAE mouse model.

Dendritic cells are bone marrow-derived cells, which develop from myeloid progenitors. DCs together with B cells and macrophages are considered to constitute the cell population of professional APCs. They are also considered to be the most potent antigen-presenting cell type that stimulates and controls T cell activation, proliferation, and differentiation [78]. Classically, T cell activation occurs in lymph nodes and spleen, where mature DCs migrate to after encountering antigens in the peripheral tissue. Mature DCs have been also found in the inflamed brain, although there is still an ongoing discussion concerning the relevance of CNS-infiltrating DCs and brain's own DCs during MS or EAE. Even though earlier studies suggested infiltrating DCs to be poor T cell stimulators [79, 80], there is evidence that antigen presentation of DCs in mice, that lack for MHC II expression in all other APCs populations, is sufficient to initiate EAE disease [81]. Moreover, the number of CNS-infiltrating DCs correlates with EAE disease progression and severity [82]. Studies with MS patients and EAE model have indicated myelin and neuron antigen-containing DCs in cervical lymph nodes, which are considered to be the draining lymph nodes of the CNS [83]. Since APC-containing myelin debris has also been found in the meninges and perivascular spaces of MS patients, these DCs are assumed to be en route from the CNS towards the draining lymph nodes, where they perform peripheral priming of naïve auto-reactive T cells [84-87]. Besides

Th1 cells, Th17 cells are considered to be the most critical CD4 T cell subset in EAE pathology. It has been shown that CNS myeloid DCs are not only able to activate naïve CD4 T cells, but can also preferentially promote T cell differentiation into Th17 effector cells upon co-stimulation with CD40 and secretion of TGF-β, IL-6, and IL-23 [60, 88]. Taken together, accumulated evidence point to a critical role of DCs in MS and EAE inflammatory and demyelination disease.

Astrocytes are the most abundant cell type in the CNS with a great variety of functions, including BBB maintenance, glutamate metabolism, and the production of neurotropic factors. Astroglia cells are not commonly associated with immunological process, but they clearly respond to inflammation and are considered to have a regulatory role, which involves cytokine production and glial scar formation [89, 90]. The expression of MHC II and their co-stimulatory molecules is required for APC function and T cell priming, activation, and differentiation induction. Astrocytes express very low levels of MHC and co-stimulatory molecules, but are known to up-regulate MHC II expression in human MS lesions [91] and after IFNγ stimulation *in vitro* [92]. Although it has been shown that astrocytes are capable to prime T cells, induce Th1 cell differentiation, and maintain Th1 and Th17 effector cell activation [93, 94], it is still unclear whether the specific environment which induces astrocyte APC capabilities occurs during MS or EAE disease. Astrocytes are capable of modifying inflammatory responses of all other cell types in the CNS, which makes them powerful regulators of inflammation and repair processes. Since astrocytes secrete GM-CSF and the macrophage colony-stimulating factor (M-CSF) that is required for microglial antigen presentation, they have a direct influence on microglia/macrophages. Astrocytes have also been reported to produce immunosuppressive TGFβ and IL-10, both of which suppress microglia activation and MHC II expression [95-97]. The constitutive expression of high levels of Fas and FasL marks another astrocyte inflammatory process regulating mechanism in the CNS. Being resistant to Fas/FasL [98-100], they regulate the survival of Fas-expressing cells, including activated T cells and microglia/macrophages, for which Fas-FasL signaling is cytotoxic. Thus, astrocytes do not perform the “traditional” function of antigen presentation. However, they respond and regulate inflammatory processes and are capable to either boost the inflammation or to respond with suppression and protection. They can even influence the survival of CNS-infiltrating immune cells and microglia and hence attenuate the inflammation [90].

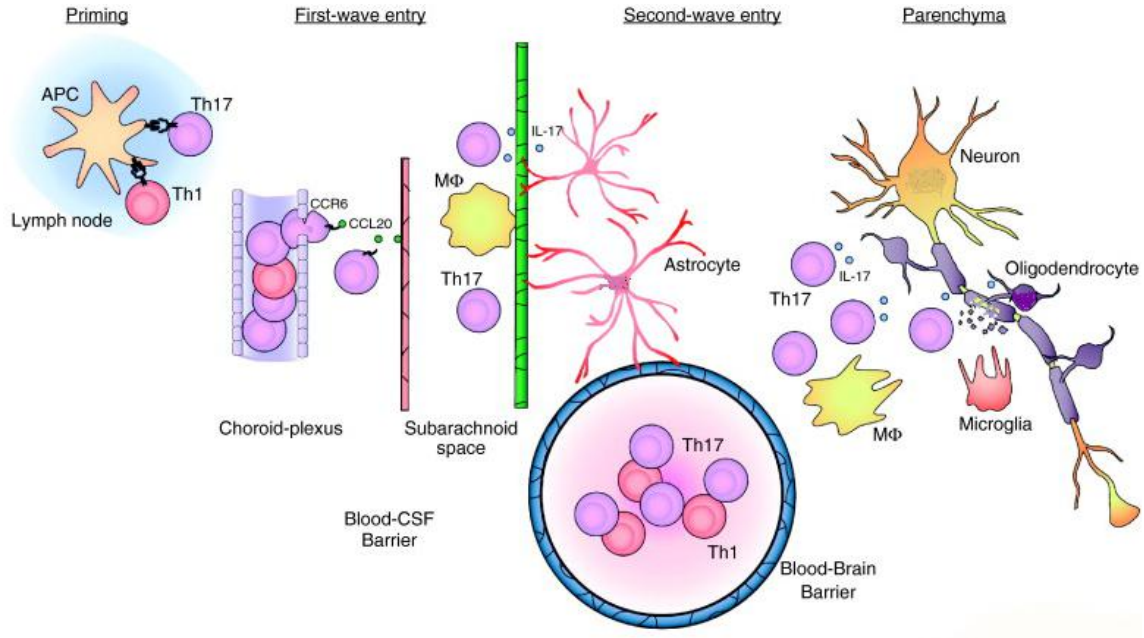

Fig. 1.1 T cell priming, CNS-infiltration and effector activity during EAE

T cells were primed and activated by APCs in the secondary lymphatic organs, cross the BBB and were restimulated by microglia/macrophages and/or DCs. Th1, Th17 and other inflammatory cells migrate within the CNS parenchyma, resulting in tissue destruction including demyelination and axonal degeneration. Adapted from Zepp et al. [101]

1.6 Immune-mediated axonal degeneration

Demyelinating diseases such as MS are considered to cause axonal degeneration which is responsible for neurological impairment and crucial for the neuropathology of MS and in the EAE mouse model [102, 103]. The details of the axonal degeneration mechanism during inflammatory processes are still poorly understood. When the normal function of axons is compromised by an injury, blockade of axonal transport, or chemical toxicity, distinct morphological and molecular changes result in cytoskeletal disassembly and granular degeneration of the axon at the injured site [104]. Inflammation-mediated axonal injury in the EAE model occurs even before the signs of demyelination and is characterized by destabilization of microtubules [105], axonal transport deficit [106], focal axonal swelling followed by fragmentation [107] and the disturbance of axonal Ca^{2+} homeostasis [108]. The detailed mechanism of the EAE-induced neuropathology and the subsequent paralysis is still in the focus of ongoing research. According to the prevailing knowledge, auto-reactive CD4 T cell subpopulations of Th1 and Th17 cells recognize myelin antigens and are therefore the major drivers in lesion formation in the CNS, leading to further immune cell infiltration, demyelination, neuronal degeneration,

and loss [62, 109-111]. The MS phases of peak, remission, and relapse do not correlate with widespread neuronal loss but rather with reversible axonal damage and de-/re-myelination events. Accordingly, immune cell-mediated neuronal damage in the EAE model is reversed during the remission phase [105]. This immune cell-mediated axonal damage has also been shown in the absence of demyelinated lesions, which supports the suggestion that inflammation-induced axon degeneration is of crucial importance for the neuropathological outcome of MS and EAE disease [1].

1.7 Interaction between neurons and T cells

T cells respond in an antigen-specific manner via their TCRs, which recognize specific peptide fragments presented by APCs via MHC molecules. This signal transduction facilitates T cell activation, proliferation, and the differentiation of effector function. Neurons express MHC class I complexes, which are recognized by the CD8 T cell subset. Antigen presentation via MHC I and CD8 T cell recognition primarily take place during viral infection. CD8 T cell activation and the accompanied inflammatory environment have been shown to cause distortion of neuronal function and increased susceptibility to apoptosis and CD8 T cell-mediated neuronal death [112, 113]. CD4 T cells, which are the predominant T cell subset initiating the onset and progression of EAE, recognize antigens that are presented via MHC II. MHC II expression is absent in neurons but is present in APCs such as DCs and macrophages and under inflammatory conditions even in CNS-resident microglia and astrocytes [114]. Therefore, CD4 T cells do not recognize antigens presented by neurons but nevertheless are in a constant crosstalk with neuronal cells via a variety of surface molecules and secreted factors. This crosstalk can be deleterious as well as beneficial for neurons [115, 116]. Activated CD4 T cells in particular are capable to promote neurite outgrowth [117] and produce neurotrophins, e.g. BDNF, which is assumed to contribute to axonal protection [115]. The induction of regulatory T cell differentiation, which is supported by neuron derived TGFβ, is an impressive example of neuroprotective neuron-CD4 T cell interaction. TGFβ, which is produced by neurons, contributes to the differentiation and maintenance of regulatory T cells and thereby of a local anti-inflammatory microenvironment, which in turn provides neuroprotection through attenuation of microglial and macrophage activation [118, 119]. However, in general T cells are considered to be the major effector cell population during autoimmune attacks in the CNS, leading to exacerbation of the

inflammatory reaction and subsequent tissue damage. CD4 T cells can cause neuronal apoptosis through the TNF-related apoptosis-inducing ligand (TRAIL) pathway [120, 121]. They can also induce oscillatory calcium alterations, which lead to calcium overload and eventual neuron death [122]. First evidence demonstrates that encephalitogenic T cells directly cause axonal dysfunction in the EAE model [105]. Th1 and Th17 cells induce neuronal damage via induction of microtubule destabilization of axons in vitro [123]. Th17 cells inhibit the axonal pro-survival Akt-signaling by their repulsive guidance molecule A (RGMa), which causes axon loss [124]. However, it has been shown that even axons themselves release pro-inflammatory factors such as the ECM molecule matrilin-2, which exacerbates neuroinflammation [125]. These studies suggest that the interaction between the CNS-infiltrating CD4 T cells and axons can be deleterious and may represent a target for therapeutic attempts in MS.

1.8 The neuronal cell adhesion molecule L1

Cell adhesion molecules (CAMs) are cell surface proteins participating in cell-cell recognition, cell adhesion and cell communication and are thereby crucial during CNS development. They recognize and represent attractive or repulsive cues in growth cones and guide axon extension, and for this reason play an important role in neuronal migration and connectivity [126]. CAMs are also intimately involved in the myelination of axons in the central and peripheral nervous system [127, 128]. Several protein families of CAMs with distinct characteristics and functional properties have been identified. Among these families are cadherins, integrins, selectins, and the immunoglobulin superfamily (IgSF) [129-132]. The IgSF-CAMs all include at least one immunoglobulin-like (Ig-like) domain [133], which consists of two β-sheets arranged in a β-strand and connected by disulfide bridges that stabilize the entire structure [134]. In addition to the Ig-like domain, IgSF-CAMs consists of two to five fibronectin (FN) type III-like domains, some of which are connected to the cell surface by transmembrane domains or glycosylphosphatidylinositol anchors. IgSF-CAMs with a cytoplasmic tail transduce extracellular signals to the interior of the cell [129, 135]. The neuronal cell adhesion molecule L1, also termed L1CAM, belongs to the L1 family as part of the IgSF. The L1 family in mammals includes four different members: L1, close homologue of L1 (CHL1), Neurofascin, and the NgCAM-related cell adhesion molecule (NrCAM). Proteins of the L1 family are assembled of six immunoglobulin-like domains, five fibronectin-like

repeats, a transmembrane domain, and a highly conserved intracellular domain [136-139]. All members of the L1 family are associated with the development of the CNS. They are involved in the processes of cell migration, myelination, axon guidance, and axon growth [137, 140-142] as well as in neuronal repair processes [143].

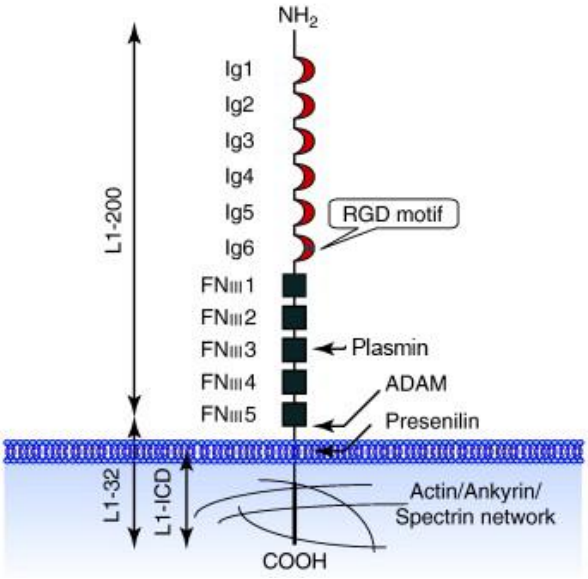

Fig. 1.2 Schematic illustration of the L1 protein structure

The extracellular domain of L1 consists of Ig-like domains 1-6 and FNIII-like repeats 1-5. Positions of RGD motif and proteolytic cleavage sites are indicated. The cytoplasmic tail links L1 to the actin/ankyrin/spectrin network. Adapted from Kiefel *et al* [144].

The extracellular domain of L1 includes 21 N-glycosylation sites [145] distributed over the Ig-like and the FNIII-like domains. The sixth Ig-like domain displays one RGD motif in humans and two in mice [146] and it is composed of Arg-Gly-Asp amino acids [147]. The binding of this motif with integrins or its cleavage by metalloproteases activates intracellular signalling pathways different from those resulting from homophilic interactions [148]. The extracellular part features two metalloprotease cleavage sites, one plasmin cleavage site at the third FNIII-like domain and one disintegrin and metalloproteinase domain-containing protein (ADAM) cleavage site between the fifth FNIII-like domain and the transmembrane part [149]. L1 cleavage by ADAM and in particular by ADAM10 leads to the shedding of the L1 ectodomain, which plays an important role in the cell migration during the CNS development and in tumor cell motility [149, 150]. The cytoplasmic tail of L1 can bind AP2, which plays a role in L1 endocytosis [151]. The tail also features phosphorylation sites that activate the

MAKPK/ERK pathway, which is one of the important pathways involved in neurite outgrowth [152].

High levels of L1 are expressed in the developing CNS, but its levels decrease in the adult brain. L1 is up-regulated in subpopulations of regenerating and sprouting axons after CNS injury [153]. Beneficial effects for the long-term functional recovery after spinal cord injury have been demonstrated by transgenic L1 overexpression [154], recombinant L1 proteins [155] and small molecule agonists of L1 [156]. Conversely, loss of L1 function is detrimental for the CNS development; over 200 reported pathological gene mutations lead to a developmental disorder referred to as L1 syndrome, which involves a broad range of phenotypic features (for example, hydrocephalus, spastic paraplegia, and mental retardation) [157]. The molecular arrangement of the L1 extracellular domain enables complex homo- and heterophilic interactions in *cis* and *trans* with numerous binding partners [158]. These interactions regulate L1-dependent cell adhesion and recognition and potentiate intracellular signaling cascades. In neurons, L1 is capable to form a holoreceptor as a subunit together with Neuropilin-1 (Nrp-1) and PlexinA. This holoreceptor regulates axon repulsion and branching in response to the extracellular soluble factor Semaphorin3A (Sema3A) [158-160]. Several studies report that the interaction between L1 and Nrp-1 is not restricted to neurons, but also exists in *trans* as for example between ovarian cancer and mesothelial cells [161]. Sema3A is known to act as a repulsive axon guidance cue but is also capable to induce neuronal apoptosis [162]. L1 is crucial for Sema3A-induced downstream signaling and growth cone collapse [163, 164]. Since pharmacological inhibition of Sema3A preserves injured axons and promotes remyelination after spinal cord injury [165], the induction of Sema3A in neuronal cell bodies of corresponding demyelinated axons implicates a pathogenic role in MS [166].

1.9 Regulation of L1 expression

The expression of L1 in neurons has been shown to depend on the repressor element-1 silencing transcription factor (REST) [167]. REST is a zinc finger transcription factor that binds to RE-1/neuron-restrictive silencer element sequences on the DNA and thereby coordinates neuronal induction and differentiation by regulating numerous neuronal genes. It functions as a scaffolding element for other transcription regulating complexes and DNA-binding proteins and hence activates, represses, and silences gene

expression under specific conditions [168, 169]. REST coordinates neuronal induction and differentiation and regulates the balance between neuronal differentiation and maintenance of the functional adult neuronal stem cell pool [168]. However, even in mature neurons the levels of REST can increase in pathophysiological conditions, e.g. during epilepsy and ischemic stroke [170, 171]. Low REST levels in neurons are associated with extensive L1 expression. Strikingly, very low REST levels enable expression of the Nova2 brain-specific spicing factor, which produces the full length splicing variant of L1, regarded as neuronal L1 [172]. Therefore, L1 expression regulation by REST occurs by two mechanisms, transcriptional and by alternative splicing processes.

L1 protein is predominantly expressed in axons. Its synthesized, folded, and matured in the endoplasmatic reticulum (ER) and via further processes in the Golgi apparatus [173-175]. The mature L1 protein is first transiently inserted into the somato-dendritic plasma membrane; then becomes internalized by endocytosis, and sorted via endosomes to the axonal compartment. The insertion into the axonal membrane is followed by its stabilization via adapter molecules which link L1 to the cytoskeleton and thereby facilitates the enrichment of L1 in axons [152, 176, 177].

1.10 Non-neuronal L1 expression

It has become evident that the L1 family proteins are also expressed by non-neuronal cells including various types of cancer cells [157, 178-180]. Additionally, it has been shown that several types of immune and ECs also express L1 [181, 182]. Some studies have indicated that L1 is expressed by particular populations of leukocytes in the periphery, among them granulocytes, B cells, T cells, monocytes, and dendritic cells [183, 184]. There is a variety of functional implications of L1 expressed in leukocytes. L1 expressed by B cells promotes homotypic cell aggregation and mediates cell adhesion in lymphoid organs [183]. Pancook et al. demonstrated that L1 is expressed in human monocytes and different myeloid cell lines and that its expression is enhanced by interferon-γ (IFNγ) treatment but not by lipopolysaccharides (LPS) [184]. In contrast, peripheral blood mononuclear cell (PBMC) derived human DCs were found to be negative for L1 expression in the immature state and do not induce L1 in response to IFNγ stimulation. However, LPS- and TNFα-induced functional maturation of DCs was accompanied by a significant *de novo* expression of L1 [184]. The expression of L1 has

also been confirmed in murine DCs, where L1 was shown to regulate transendothelial migration and trafficking. It has been proposed that these processes are facilitated by L1 recognition of the integrin$\alpha_v\beta_3$ receptor on ECs [182, 184, 185]. Furthermore, it has been suggested that recombinant L1 and L1 expression by DCs have co-stimulatory properties to potentiate the T cell response to unspecific T cell receptor stimulation and antigen presentation [186]. Regarding the general concept of thymocyte maturation, immature thymocytes are characterized by their simultaneous expression of $CD4^+$, $CD8^+$ and low levels of the T cell receptor CD3. During the maturation process, T cells differentiate into single positive $CD4^+CD8^-$ (T helper cells) and $CD8^+CD4^-$ (cytotoxic T cells), which express high levels of CD3 [187]. Mature $CD3^{high}$ T cells have been shown to express L1, whereas L1 is absent in immature thymocytes. These findings suggested that L1 is a T cell maturation marker [187]. The major difference between L1 expression in neurons and in most other cell types are the alternative splicing variants of L1 pre-mRNA [188]. The L1 gene has 29 exons, in which the first contains a 5'-untranslated sequence (exon 1a). The neuronal splicing variant of L1 utilizes the entire other 28 coding exons, whereas splicing variants without exon 2 and 27 are found exclusively in non-neuronal cells [189]. Exon 27 encodes for the RSLE motif that is known to have implications for the clathrin-mediated endocytosis of L1. Exon 2-coded motive (YEGHHV) is required for L1 binding to different neuronal ligands, and therefore it is likely to be important during the CNS development [190].

L1 expression in immune cells is in the majority of cases associated with cell type-specific stimuli for activation, differentiation, and maturation. It has been shown that L1 expression is up-regulated in epithelial cells from patients suffering from the inflammatory bowel disease in response to macrophage produced TGFβ [191]. L1 expression is linked with poor survival prognosis in different human tumor samples and cognate cell lines [192]. This and several other cancer-related studies have revealed that L1 expression in tumor cells promote increased Il-1β expression and the activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via integrin-FAK-Src-Akt signaling [193]. Both Il-1β and NF-κB are critical factors during the inflammation process [144, 194]. Interestingly, L1-mediated NF-κB activation requires the integrin-FAK-Src-Akt signaling pathway, which in turn is dependent on L1-integrin binding [193]. Altogether these studies point to the involvement of L1 in inflammatory processes. However, to date non-neuronal L1-associated mechanisms have only been

shown in oncological studies, whereas their role in the context of neurons and immune cells has only been speculated but not confirmed yet [148, 195].

Taken together, neuroinflammation in the CNS is determined by CNS-infiltrating T cells and APCs that initiate an inflammatory cascade, which eventually results in the demyelination and degradation of axons. The underlying crosstalk between the immune and nervous system is among other mechanisms mediated by contact-dependent processes, which involves by a variety of receptors and cell adhesion molecules including L1.

1.11 Objectives

The crucial role of L1 in axon biology as well as the apparent L1 expression in immune cells raised the question whether neuronal L1 plays a role in immune-mediated axonal injury. We hypothesize that L1 is a potential link between neurons and CD4 T cells that facilitates the interaction between the CNS-infiltrating immune cells and axons. This thesis focuses on the role of L1 in human MS and the murine EAE model and mimicked the situation of neuroinflammation using *in vitro* co-cultures of primary cortical neurons and CD4 T cells.

Hence, the following issues were studied in this thesis:

- L1 expression in human brain tissue samples of healthy donors and MS patients
- Expression regulation and role of L1 in EAE-induced neuroinflammation
- Role of L1 in CD4 T cell-mediated axonal degeneration in vitro characterization of microglia/macrophage and DC expression and regulation of L1 *in vitro*
- Role of L1 expression by microglia/macrophages and DCs during EAE-induced neuroinflammation

2 Materials and Methods

1.1. Materials

2.1.1 Chemicals and Substances

3,3'diaminobenzidine	Dako, Germany
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, USA
acetone	Sigma-Aldrich, USA
acrylamide / bis-acrylamide	Carl Roth, Germany
Amersham Full-Range Rainbow Marker	Thermo Scientific, USA
ammonium chloride	Sigma-Aldrich, USA
ammonium persulfate	Sigma-Aldrich, USA
bovine serum albumin	Carl Roth, Germany
brefeldin A	eBioscience,
collagenase/dispase	Roche, Switzerland
cytosine-arabinoside	Sigma-Aldrich, USA
DNase	Roche, Switzerland
Dorbene	Zoetis, USA
Dulbecco's Modified Eagles Medium	Gibco, USA
ethanol	Sigma-Aldrich, USA
ethylenediaminetetraacetic acid	Carl Roth, Germany
Eukitt mounting medium	O. Kindler GmbH, Germany
fetal calve serum	Gibco Life Technologies, USA
gelatin	Sigma-Aldrich, USA
glycine	AppliChem, Germany
H_2O absolute	B. Braun, Germany
HBSS Ca^{2+} Ma^{2+}	Gibco Life Technologies, USA
Histopaque 1083	Sigma-Aldrich, USA
hydrochloric acid fuming	Carl Roth, Germany
hydrogen chloride (HCl)	Carl Roth, Germany
Immu-Mount	Thermo Scientific, USA
isopropyl alcohol	Hedinger, Germany

ketamine	Bayer, Germany
L-glutamin	Gibco Life Technologies, USA
lipopolysaccharide	Sigma-Aldrich, USA
luxol fast blue	Sigma-Aldrich, USA
MACS Neuro Brew-21	Miltenyi biotec, Germany
MACS Neuro Medium	Miltenyi biotec, Germany
Magic Marker XP Western Standard	Invitrogen, USA
methyl alcohol	AppliChem, Germany
milk powder, no-fat	Carl Roth, Germany
monopotassium phosphate	Carl Roth, Germany
new donkey serum	BioRad, USA
new goat serum	BioRad, USA
NP-40	ThermoFisher Scientific, USA
paraformaldehyde (PFA)	Merck, Germany
penicillin/streptomycin	Gibco Life Technologies, USA
penicillin/streptomycin	Gibco Life Technologies, USA
Percoll	Sigma Aldrich, USA
poly-D-lysine	Sigma-Aldrich, USA
potassium chloride	Carl Roth, Germany
propidium iodid	eBioscience, USA
protease inhibitor	Roche, Switzerland
Rosswell Park Memorial Institute-1640 medium	Life Technologies, USA
sodium chloride	Carl Roth, Germany
sodium citrate dihydrate	Merk, Germany
sodium dihydrogenphosphate (NaH_2PO_4)	Carl Roth, Germany
sodium dodecylsulfate	Carl Roth, Germany
sodium phosphate	Sigma-Aldrich, USA
sonylphenoxypolyethoxylethanol	AppliChem, Germany
sucrose	Sigma-Aldrich, USA
tetramethylethylenediamine (TEMED)	Carl Roth, Germany
tris-hydroxymethyl-aminomethane (tris)	Carl Roth, Germany
Triton X-100	GE Healthcare, USA

trypsin/EDTA	Sigma-Aldrich, USA
Tween 20	Carl Roth, Germany
xylazine	Bayer, Germany
Xylene Roti-Histol	Carl Roth, Germany
β-2-mercaptoethanol	Sigma-Aldrich, USA

Tab. 2.1 Chemicals and substances

2.1.2 Buffers and culture media

Solution	**Composition**
blocking/permeabilization buffer (IHC)	5 % (v/v) NGS, 0.5 % BSA, 0.1 % (v/v) Triton-X 100, ad. PBS
blotting transfer buffer	25 mM tris, 192 mM glycine, 20 % (v/v) methyl alcohol, ad. H_2O,
electrophoresis running buffer	247 mM tris-HCl, 840 mM glycine, 35.6 mM SDS, ad. H_2O
lymphocyte culture medium	RPMI-1640, 10 % (v/v) FCS, 1 % (v/v) P/S
neurobasal culture medium (NB)	MACS Neuro Medium, 1 % (v/v) MACS Neuro Brew-21; 1 % (v/v) P/S; 2.5 µM L-glutamin
phosphate buffered saline	137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2PO_4, 1.5 mM KH_2PO_4, ad. H_2O, pH 7.4
phosphate buffered saline with Tween 20	137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2PO_4, 1.5 mM KH_2PO_4, 0.1 % (v/v) Tween 20, ad. H_2O, pH 7.4
radioimmunopreceipitation assay buffer	50 mM tris-HCl pH 7.5, 150 mM NaCl, 0.5 % (v/v) NP-40, 1 mM EDTA, ad. H_2O,
laemmli buffer	0.5 M tris-HCl pH 6.8, 10 % (w/v) SDS, 20 % (v/v) glycerine, 5 % (v/v) β-mercap-toethanol, ad. H_2O
separation buffer	150 mM tris, 4 % (v/v) SDS, ad. H_2O, pH 8.8
stacking buffer	50 mM tris, 4 % (v/v) SDS, ad. H_2O, pH 6.8

tris-buffered saline	50 mM tris, 150 mM NaCl, 2 mM HCl, ad. H_2O, pH 7.4
tris-buffered saline incl. Tween 20	50 mM tris, 150 mM NaCl, 2 mM HCl, 0.1 % (v/v) Tween 20, ad. H_2O, pH 7.4
fix/perm buffer for FACS staining	Foxp3/Transcription Factor Staining buffer SET (eBiosciences, USA)
permeabilization buffer for FACS staining	Foxp3/Transcription Factor Staining buffer SET (eBiosciences, USA)
erythrocytes lysis buffer	155 mM NH_4Cl, 150 mM tris, 0.1 mM EDTA
MACS buffer	2 % (v/v) FCS, 1 mM EDTA, PBS
FACS buffer	0.5 % (w/v) BSA, 1 mM EDTA
citrate buffer	0.3 % (w/v) sodium citrate dihydrate ad. H_2O, pH 6/9

Tab. 2.2 Buffer und culture media

2.1.3 Instrumentation and Devices

AutostainerLink 48	Dako, Germany
Stemi 305 binocular microscope	Zeiss, Germany
Centrifuge 5804R	Eppendorf, Germany
EVOS FL Cell Imaging System	Thermo Scientific, USA
FACS Canto II	Beck & Dickenson, USA
GE NanoVue spectrometer	GE Healthcare Systems, USA
bright field microscope	Hund-Wilovert, Germany
Heracell CO_2 incubator	Heraeus, Germany
Galaxy 1705 Incubator	Eppendorf, Germany
K5001 Immunostainer	Dako, Germany
Light Cycler 480	Roche, Switzerland
LSM 510 confocal microscope	Zeiss, Germany
MACS separator	Miltenyi, Germany
magnetic mixer	Heidolph, Germany
Master Thermo-Cycler	Eppendorf, Germany
Mini 1-D Electrophoresis chamber	BioRad, USA
Mini Trans-Blot System	BioRad, USA

ErgoOne electronic pipette	StarLab, Germany
Neubauer hemocytometer	Paul Marienfeld, Germany
Odyssey Sa Imaging System	LI-COR Biosciences, USA
Pipet Boy	Hirschmann, Germany
Hera safe	Thermo Scientific, USA
PowerPac HC P25	BioRad, USA
ThermomixerR	Eppendorf, Germany
vibratome	Thermo Scientific, USA
Vortex Reax control	Heidolph, Germany
QIAxcel Advanced	Qiagen, Germany
water bath E100	Lauda-Brinkmann, USA
SP8 confocal microscope	Leica, Germany

Tab. 2.3 Instrumentation and devices

2.1.4 Consumables

cell culture flasks	GreinerBio-One, Germany
Eppendorf tubes	GreinerBio-One, Germany
Falcon tubes	GreinerBio-One, Germany
filter paper	Whatmann, UK
nitrocellulose membrane	Merck Millipore, USA
object slides	Thermo Scientific, USA
PCR plate seals	ThermoFisher Scientific, USA
PCR plates	ThermoFisher Scientific, USA
petri dish	GreinerBio-One, Germany
pipette tips	GreinerBio-One, Germany
serological pipettes	GreinerBio-One, Germany
cell strainer	Miltenyi, Germany
MACS LS column	Miltenyi, Germany
cell culture plates	GreinerBio-One, Germany
needles	BD Biosciences, USA
syringes	Braun, Germany

Tab. 2.4 Consumables

2.1.5 Antibodies

Immunogen	Conjugate	Host	Clone	Producer
anti-goat IgG	AlexaFluor488	donkey	-	Invitrogen, USA
anti-goat IgG	IR Dye 680RD	donkey	-	LI-CORE, USA
anti-mouse IgG	IR Dye 680RD	goat	-	LI-CORE, USA
anti-mouse IgG	AlexaFluor488	goat	-	Invitrogen, USA
anti-mouse IgG	IR Dye 800RD	donkey	-	LI-CORE, USA
anti-rabbit IgG	IR Dye 680RD	goat	-	LI-CORE, USA
anti-rabbit IgG	AlexaFluor568	goat	-	Invitrogen, USA
anti-rabbit IgG	AlexaFluor568	donkey	-	Invitrogen, USA
anti-rabbit IgG	AlexaFluor488	goat	-	Invitrogen, USA
anti-rat IgG	AlexaFluor488	goat	-	Invitrogen, USA
anti-rat IgG	AlexaFluor633	goat	-	Invitrogen, USA
anti-rat IgG	AlexaFluor568	goat	-	Invitrogen, USA
APP	-	mouse	22C11	Millipore, USA
CD11b	PE Cy7	rat	M1/70	eBioscience, USA
CD11c	APC	rat	N418	eBioscience, USA
CD28	-	hamster	37.51	BD Biosciences, USA
CD28	-	mouse	CD28.2	BD Biosciences, USA
CD3	-	hamster	145-2C11	BD Biosciences, USA
CD3	-	mouse	OKT3	eBioscience, USA
CD4	Vio450	rat	RM4-5	BD Biosciences, USA
CD4	AlexaFluor647	rat	RM4-5	BD Biosciences, USA
CD4	FITC	rat	RM4-5	eBioscience, USA
CD4 human	FITC	mouse	SK3	BD Biosciences, USA
CD45	eflour605	rat	30-F11	eBioscience, USA
CD45R (B220)	AlexaFluor700	rat	RA3-6B2	eBioscience, USA
CD8	FITC	rat	HIT8a	BD Biosciences, USA
CD8 human	FITC	mouse	SK1	BD Biosciences, USA
FoxP3	PE Cy7	rat	FJK-16S	eBioscience, USA
GAPDH	-	mouse	6C5	Acris, USA
GFAP	-	rabbit	Z0334	Dako, Germany

Iba1	-	rabbit	-	Wako, Germany
IFNγ	Vio450	rat	XMG1.2	eBioscience, USA
IFNγ	-	rat	XMG1.2	eBioscience, USA
IgG isotype	-	rat	-	Sigma-Aldrich, USA
IL-10	-	rat	-	eBioscience, USA
IL-12	-	rat	C18.2	eBioscience, USA
IL-17	PE	rat	TC11	BD Biosciences, USA
IL-4	-	rat	11B11	eBioscience, USA
L1cam	-	rabbit	-	Laboratory of Fritz Rathjen, MDC, Berlin
L1cam	-	rat	555	Laboratory of Peter Altevogt, DKFZ, Heidelberg
L1cam	-	goat	C-20	Santa Cruz Biotech, USA
L1cam	PE	rat	555	Miltenyi, Germany
MAC3	-	rat	M3/84	BD Pharming, USA
MAP2	-	mouse	HM-2	Abcam, USA
MHC II	biotin	rat	M5/114	eBioscience, USA
NeuN	-	mouse	A60	Millipore, USA
pan-NF	-	mouse	SMI-312	Covance, USA
TNFα	AlexaFluor700	rat	MP6XT22	eBioscience, USA
βIII-tubulin	-	mouse	TUJ1	Covance, USA

Tab. 2.5 Antibodies

2.1.6 Kits

DNA Extraction Kit	Thermo Scientific, USA
DreamTaq Green	Thermo Scientific, USA
EAE MOG_{35-55}/CFA Emulsion PTX Kit	Hooke, USA
EAE $PLP_{139-151}$/CFA Emulsion PTX Kit	Hooke, USA
EnVision Detection Systems Peroxidase/DAB	Dako, Germany
Fast Start Taq DNA Polymerase Kit	Roche, Switzerland
Hot Start Core Kit	Jena Bioscience, Germany
Lowry Protein Assay Kit	BioRad, USA

MACS CD4 MicroBeads, human	Miltenyi, Germany
MACS CD4 MicroBeads, mouse	Miltenyi, Germany
MACS CD8 MicroBeads, human	Miltenyi, Germany
Maxima Hot Start Kit	Thermo Scientific, USA
Periodic Acid-Schiff Stain Kit	DAKO, Germany
QuantiNova SYBR green PCR Kit	Thermo Scientific, USA
QuantiScript Reverse transcription Kit	Qiagen, Germany
RNeasy Micro Kit	Qiagen, Germany
RNeasy Mini Kit	Qiagen, Germany
SYBRgreen Kit	Thermo Scientific, USA
Tissue Dissociation Kit-Postnatal Neurons	Miltenyi, Germany

Tab. 2.6 Kits

2.1.7 PCR primer

Target	**Sequence (5'-3')**
HPRT	Fw: TCC AGC GTC CGA TGA
	Rev: AAT GTG ATG GCC TCC CAT CTC CTT CAT GAC
βIII-tubulin	Fw: GCG CAT CAG CGT ATA CTA
	Rev: TTC CAA GTC CAC CAG AAT
L1CAM	Fw: TGG ATG AAC AGC AAG ATT
	Rev: TGA ATG ATT GTC TGA GGT
REST	Fw: AGC CTT GCC AGT ACG AAG C
	Rev: CTT CAG CTG TGC CAT GTA GTG
TNFα	Fw: TCT CAG TTC TAT GGC CC
	Rev: GGG AGT AGA CAA GGT ACA AC
TGFβ	Fw: CTT CAA TAC GTC AGA CAT TCG GG
	Rev: GTA ACG CCA GGA ATT GTT GCT A
IL-1β	Fw: GTG CTG TCG GAC CCA TAT GAG
	Rev: CAG GAA GAC AGG CTT GTG CTC
IL-6	Fw: TCG TGG AAA TGA GAA AAG AGT TG
	Rev: TAT GCT TAG GCA TAA CGC ACT AG
β-actin	Fw: ACC CAC ACT GTG CCC ATC TA
	Rev: GCC ACA GGA TTC CAT ACC CA

Syn-Cre	Fw: GCG GTC TGG CAG TAA AAA
	Rev: GTG AAA CAG CAT TGC TGT CAC
LysM-Cre	Fw: CCC AGA AAT GCC AGA TTA CG
	Rev: GCG GTC TGG CAG TAA AAA
CD11c-Cre	Fw: CCG GTC GAT GCA ACG AGT GA
	Rev: GGC CCA AAT GTT GCT GGA
LysM-WT	Fw: CTT GGG CTG CCA GAA TTT CTC
	Rev:TTA CAG TCG GCC AGG CTG AC
L1-floxP	Fw: GAG CCA CCT GTC ATC ACG GAA
	Rev: CAT GGA TAA GAG GTT CTA GCA CTC
actin	Fw: CTA GGC CAC AGA ATT GAA
	Rev: GTA GGT GGA AAT TCT AGC
L1-Δexon2	Fw: CAC AGA GAC TGA GCT GGC AA
	Rev: AGG TGG CTC TAG CAA TTC GT
CD3 ζ-chain	Fw: CTG CTA CTT GCT AGA TGG AAT
	Rev: TCT CTT CGC CCT AGA TTG AGC

Tab. 2.7 Primer oligonucleotides

2.1.8 Recombinant proteins

GM-CSF	PeproTech, USA
IFNγ	PeproTech, USA
IL-10	PeproTech, USA
IL-12	PeproTech, USA
IL-13	PeproTech, USA
IL-18	PeproTech, USA
IL-1β	PeproTech, USA
IL-23	PeproTech, USA
IL-4	PeproTech, USA
IL-6	PeproTech, USA
IL-6	PeproTech, USA
M-CSF	PeproTech, USA
TGFβ	PeproTech, USA

TNFα	PeproTech, USA

Tab. 2.8 Recombinant proteins

2.1.9 Software

ImageJ	NIH Image, USA
FlowJo	FlowJo, USA
GraphPad Prism	GraphPad Software, USA
ImageStudio	LI-CORE, USA
Illustrator CC	Adobe, USA
Photoshop CC	Adobe, USA

Tab. 2.9 Software

2.2 Methods

2.2.1 Human brain tissue

Frozen tissue blocks of three MS patients and two non-neurological controls were obtained from the Netherlands Brain Bank, Amsterdam, the Netherlands (coordinator Dr. I Huitinga). The study of human samples was approved by the institutional ethics review board (VU University Medical Center, Amsterdam, Netherlands) and all donors or their next of kin provided written informed consent for brain autopsy, use of material and clinical information for research purposes. The MS patients (one female, two males) were 63.3 ± 8.3 (SD) years old and displayed secondary progressive MS. Non-neurological controls (two females) were 83 ± 1.4 (SD) years old. The post-mortem delay of tissue dissection was 7.25 h ± 1.08 h (SD) for MS patients and 6.05 h ± 1.23 h (SD) for non-neurological controls. Tissue block from parieto-temporal cortex were processed for cryostat sectioning

Immunohistochemistry of human brain samples was essentially performed as described before [196]. Briefly, cryosections were air-dried and fixed in cold acetone for 10 min followed by 45 min blocking in tris-buffered saline (TBS), pH 7.4, containing 5 % (v/v) goat serum, 1 % (v/v) bovine serum albumin (BSA), 0.2 % (v/v) Triton-X100 at room temperature (RT). Polyclonal antibodies recognizing human L1 (1:500) [197] and the pan-neurofilament (NF, SMI-312, 1:1000) antibody were applied in blocking solution overnight at 4°C. Sections were washed and incubated with secondary AlexaFluor-

conjugated antibodies for 60 min at RT, washed with TBST and counterstained for cell nuclei with 4',6-diamidino-2-phenylindole dihydrochlorid (DAPI, 1:10000 in TBS for 10 min at RT and sealed with fluorescence protecting mounting medium. Images were sequentially acquired for DAPI, NF- and L1-immunolabeling in deep cortical layers V and VI and from large diameter axons in the subcortical white matter tissue using a SP8 (Leica) confocal laser-scanning microscope.

2.2.2 Isolation and immunostaining of human peripheral blood T cells

PBMCs were isolated from blood samples of healthy donors using Percoll density gradient centrifugation and magnetic-activated cell sorting (MACS). Therefore, blood cells were pelleted by centrifugation with 550 *g* for 5 min. Erythrocytes were lysed with a Tris-NH4Cl solution for 3 min at RT and pelleted again. The cell pellet was resuspended in Percoll-solution (40 % [v/v], diluted in Iscove's Modified Dulbecco's Medium [IMDM]) and carefully layered on top of Percoll-solution (70 % [v/v], diluted in phosphate buffered saline [PBS]). The Percoll-gradient was centrifuged for 30 min with 750 *g* at RT. Lymphocytes were obtained from the interphase, washed in MACS buffer and separated in CD4 and CD8 T cell populations using the CD4 and CD8 human MicroBeads sorting kit (Miltenyi). Separated cells were cultured in full Roswell Park Memorial Institute-1460 culture medium (RPMI) and stimulated using plates coated with anti-CD3 (OKT3, 2.5 µg/mL in PBS) and anti-CD28 (CD28.2, 2 µg/mL in PBS) antibodies overnight. T cells were collected and fixed with paraformaldehyde (4 % [w/v] in PBS), washed two times with deionized water (centrifugation: 550 *g*, 5 min, 4°C). 15 µl of the cell suspension (1x10^6 cells/ml) were smeared on gelatin-coated slides and dried on a low temperature heating plate. Immunocytochemistry was performed as described above (2.2.1), using primary antibody to human CD4 (SKT3, 1:100), CD8 (SK1, 1:100) and antibodies recognizing human L1 (1:500). Images were sequentially acquired for DAPI, CD4/CD8 and L1-immunolabeling using a SP8 confocal laser scanning microscope (Leica).

2.2.3 Mouse lines

C57BL/6J and SJL mice were purchased from Charles River Laboratories. Mice with conditional neuron-specific L1 depletion were generated by crossing genetically modified C57B6/J mice bearing a Cre-recombinase gene driven by the synapsine

promoter (Syn^{Cre}) with mice mutant for a loxP-flanked L1CAM gene locus ($L1^{fl/fl}$). The Generation of Syn^{Cre} [198] and L1-floxed mice [199] was previously described. In all experiments littermates carrying a SynCre allele but lacking loxP-flanked alleles served as controls. Double-fluorescent Cre-reporter mice [B6.Cg-Tg (CAG-DsRed,-eGFP)5Gae/J] were purchased from The Jackson Laboratory and crossed with Syn^{Cre} or $Syn^{Cre}L1^{fl/fl}$ mice. The generation of the transgenic mouse line M3 (L1 tg M3) overexpressing human L1CAM has been described before [200]. In all experiments, mice heterozygous for the transgenic allele were compared to WT control littermates. All animals were housed and maintained in a controlled environment (12 h dark/light cycle, 23°C, 55 % humidity) with food and water ad libitum. Mice were randomized and experimental performance and data acquisition were done in a blinded and unbiased fashion. All animal experiments were approved by the Animal Care and Ethics Committee of the Landesuntersuchungsamt Rheinland-Pfalz (G10-1-008/G15-1-020).

2.2.4 Genotyping of transgenic mice

All offspring mice were ear marked and ear tissue was used to verify specific transgenes. The DNA was extracted from ear tissue using the Tissue Fast to PCR Kit, which extracts the DNA proteolytically for 10 min at RT and enzyme subsequently denatured for 3 min at 95°C using a thermoblock heater. DNA segments were amplified using ready-to-use PCR Kits: Dream Taq Green (CAG, CD11c-Cre), FastStart Taq DNA Polymerase (L1-floxP), Hot Start Core Kit (Syn-Cre, LysM-Cre, LysM-WT) with gene specific oligonucleotide primers in an automatic Mastercycler gradient device (Eppendorf). Amplification of an actin gene segment was used as a positive control. PCR synthesized amplicons were analyzed for length and amount of transcripts by capillary electrophoresis (QIAxcel, Qiagen)

2.2.5 Neurological assessment

Neurological impairment of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice was assessed by a composite neuroscore adapted from Tsenter *et al*. [201] and included the counting of foot misplacements on 1, 1.5, and 3 cm wide beams, and the ability of mice to hold on a round and a square stick, resulting in a maximum score of 8 points.

2.2.6 EAE induction and clinical scoring

EAE immunization and scoring of clinical symptoms was performed as described previously [202]. 8-10 weeks old mice were immunized subcutaneously with 200 µg of the MOG_{35-55} peptide or SJL mice were immunized with 200 µg of $PLP_{139-151}$ emulsified in CFA and supplemented with 0.4-1 mg of heat-inactivated *mycobacterium tuberculosis* H37Ra. Each mouse received 200 ng of pertussis toxin, intraperitoneally injected at the time of immunization and 24 hours later. Sham mice received CFA only. Mice were monitored for clinical symptoms daily and signs of EAE were translated into clinical scores as indicated Tab.1. According to animal welfare regulations mice with a score above 3.5 were sacrificed (Tab. 2.10).

Symptoms	**Score**
no detectable signs of EAE disease	0
tail weakness	0.5
complete tail paralysis	1
weak rightning reflex	2
unilateral complete hind limb paralysis	2.5
complete bilateral hind limb paralysis	3
complete hind limb paralysis and partial forelimb paralysis	3.5
total paralysis of forelimbs and hind limbs	4
death	5

Tab. 2.10 Clinical symptoms and translation to scores in the EAE model

2.2.7 Antibody treatment of mice in the EAE model

Mice were treated with rat anti-mouse L1 antibodies (clone: 324) and control rat isotype IgG antibodies. The antibodies were administered by tail vein injection of 200 µl (1 mg/ml, diluted in PBS) at day 4 and day 8 after immunization with MOG_{35-55} peptide. The function blocking of the anti-L1 antibody (clone: 324) was confirmed by aggregation assays using HEK293T cells transfected with a pcDNA3 vector coding for murine L1 [203]. L1-mediated cell-cell aggregation was assayed as described [204, 205] in the presence of anti-L1 or rat isotype IgG (50 µg/ml). The average size of aggregates treated with anti-L1 was reduced to 26.2 % ± 4.97 % (SEM) normalized to cells treated with the rat IgG isotype control (data not shown, aggregation assays were performed by Wiesia Bobkiewicz, AG Schäfer, Universitätsmedizin Mainz, Germany).

2.2.8 Generation of primary cortical neuron cultures

Neuron cultures were prepared from the cerebral cortex of C57BL/6J mice embryos (E17-E18) taken out from sacrificed mother mice followed by immediate decapitation. Brain tissue was dissected, cortices were separated and blood vessels and meninges were removed. A single cell suspension was prepared by enzymatic papain dissociation using the Neuronal Tissue Dissociation Kit–Postnatal Neurons (Miltenyi) according to the manufacturer's guidelines. The enzymatic reaction was inhibited with BSA containing PBS (0.5 % [w/v]) and centrifuged with 300 *g* for 5 min. The cell pellet was resuspended in neurobasal medium (NB), applied on a cell strainer (40 µm) and the number of cells was determined in a Neubauer hemocytometer. $5x10^5$ cells/mL for gene expression and protein experiments and $2x10^5$ cells/mL for immunohistochemical analysis were seeded on poly-D-lysine (PDL) coated cell culture plates (50 µg/mL in H_2O) and grown for 11 days at 37°C and 5 % CO_2. Glial cell proliferation was inhibited by the addition of cytosine-arabinoside (ara-c, 2 µM) at day 3 until day 6 of neuron cultures. Appropriate concentration of ara-c was determined by pilot experiments (Fig. 2.1). The purity of neuron cultures was monitored by cell culture immunostaining using antibodies for the neuronal nuclei (NeuN) marker (A60, 1:500) and the glial fibrillary acidic protein (GFAP, Z0334, 1:500). Over 99 % of detected cells were immunoreactive for NeuN and <1 % of cells were GFAP-positive. The neurobasal culture medium was exchanged one day prior to experiments to fresh NB medium.

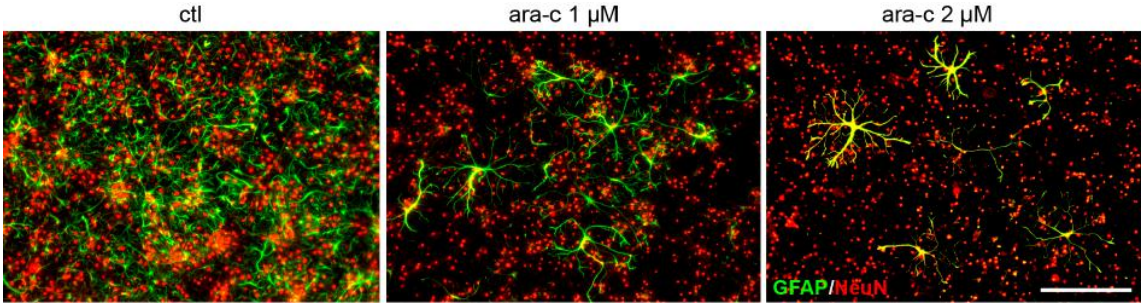

Fig. 2.1 Ara-c mediated inhibition of astrocyte proliferation in primary neuron cultures
Representative images of cell cultures immunostained with antibodies specific for the astrocyte marker GFAP and the neuronal marker NeuN. Verification of remaining astrocytes in control cultures and treated with ara-c (1 µM and 2 µM). Scale: 50 µm.

2.2.9 Isolation and stimulation of CD4 T cells

CD4 T cells were obtained from spleen and lymph nodes of 8-12 weeks old C57BL/6J mice. Cervical, sagittal and axillary lymph nodes and spleen were dissected and

processed for MACS negative sorting of CD4 T cells using the CD4 T cell isolation Kit according to the manufacturer's guidelines (Miltenyi). In short, spleen and lymph node cells were incubated with non-CD4 T cell biotin-antibody cocktail and anti-biotin magnetic micro-beads. The cell suspension was applied to a MACS LS column placed in the magnetic field of a MACS separator (all Miltenyi). All non-CD4 T cells remain on the separation column, whereas unlabeled CD4 T cells were found within the eluate. The purity of CD4 T cells (>95 %) was routinely tested by flow cytometry. The stimulation of the isolated CD4 T cells was performed for 24 hours in complete RPMI-1640 culture medium using plates coated with anti-CD3 (37.51, 3 µg/mL in PBS) and anti-CD28 (145-2C11, 2.5 µg/mL in PBS) antibodies [206].

2.2.10 Co-culture experiments

Unstimulated or stimulated CD4 T cells were added to the enriched and differentiated neuron cultures (11 days *in vitro* [DIV]) in a 1:1 cell ratio. After 2 h of co-culturing, CD4 T cells were removed by culture rinsing with PBS and used for cell culture immunostaining or processed for neuron-specific expression analysis. Therefore, cultured cells were detached using trypsin/EDTA and CD4 T cell depletion was performed by MACS sorting using the CD4 T Cell Isolation Kit according to the manufacturer's guidelines (Miltenyi). The neuronal cell suspension was routinely tested for remaining CD4 T cells using flow cytometry (<5 % CD4 T cells relative to the live gate population, Fig. 2.2). The eluate, containing the neurons, was pelleted and lysed for protein or mRNA expression assays.

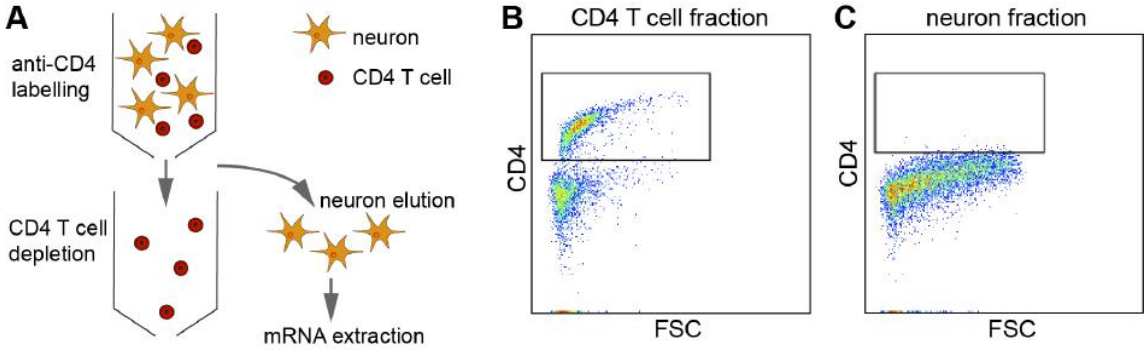

Fig. 2.2 CD4 T cell depletion in neuron-CD4 T cell co-cultures

(A) CD4 T cell depletion in neuron-CD4 T cell co-cultures using MACS method. The depletion was verified by flow cytometry analysis of the CD4 T cell fraction (B, positive control) and of the eluted neuron fraction (C).

2.2.11 Analysis of axonal degeneration in primary neuron cultures

Cells were seeded and cultured on PDL coated glass cover slips with $2x10^5$ cells/mL, grown for 11 days and co-cultured with CD4 T cells as described above (paragraphs 2.2.9, 2.2.10). L1 blocking experiments were carried out in the presence of a rat monoclonal antibody specific to L1 (555) [207] or an isotype-specific rat IgG control antibody (Sigma-Aldrich) using 50 µg/ml in NB medium. The antibodies and CD4 T cells were simultaneously administered to the neuron cultures. The function blocking activity of the antibody was confirmed by aggregation assays using HEK293T cells transfected with a pcDNA3 vector coding for murine L1 [203]. L1-mediated cell-cell aggregation was assayed as described [204] in the presence anti-L1 or rat IgG isotype control antibodies (50 µg/ml). The average size of aggregates treated with anti-L1 was reduced to 45.7 % ± 9.65 % (SEM) relative to cells treated with the rat IgG isotype control (data not shown, aggregation assays were performed by Wiesia Bobkiewicz, AG Schäfer, Universitätsmedizin Mainz, Germany). CD4 T cells were removed after 2 hours of co-culture by repeated rinsing with PBS and cover slips with neurons were processed for immunocytochemistry as described in paragraph 2.2.13. Anti-βIII-tubulin (TUJ1) immunostained neuron cultures were randomly imaged using confocal microscopy and processed for line measurement (ImageJ) of β-III-tubulin immunofluorescence along axons. The mean line scan intensity of all axons of three random images was calculated for each independent experiment and condition. The mean of all replicates per condition represents the axon integrity and was normalized to the respective mock control condition (set as 100 %).

2.2.12 Cell adhesion assay

Anti-CD3/CD28 stimulated CD4 T cells were labeled with caboxyfluorescein succinimidyl ester (CFSE, 1 µM in RPMI-1460) for 10 min at 37°C and washed two times with complete RPMI-1640 culture medium. L1 blocking experiments were carried out in the presence of a rat monoclonal antibody specific to L1 (555) [207] or an isotype rat IgG control antibody using 50 µg/ml in NB medium. The antibodies were simultaneously administered to the neuron cultures as the CD4 T cells. After 30 min of incubation at 37°C and 5 % CO_2, cultures were thoroughly rinsed with NB medium for three times to remove non-adherent cells. Cultures were randomly imaged using fluorescence microscopy and the number of adherent CD4 T cells was quantified using the ImageJ

software particle count. Three independent experiments were performed in triplicates of all conditions.

2.2.13 Immunocytochemistry

Cells were seeded and cultured on PDL coated glass cover slips. For immunostaining, cover slips were washed with PBS, fixed with PFA (4 % [w/v] in PBS) for 10 min at RT in blocking/permabilization buffer (5 % (v/v) NGS, 0.5 % BSA, 0.1 % (v/v) Triton-X100 in PBS) for 1 h at RT. Cultures were incubated with the primary antibodies anti-MAP2 (HM-2, 1:500), anti-NeuN (A60, 1:500), anti-βIII-tubulin (TUJ1, 1:2000), anti-L1 (1:500), anti-CD4 (RM4.5, 1:100) followed by species specific AlexaFluor-conjugated secondary antibodies. All samples were stained for cell nuclei using DAPI diluted in PBS (1:10,000) incubated for 10 min at RT and sealed with mounting medium. Stained cell culture samples were imaged using confocal microscopy (LSM 510, Zeiss).

2.2.14 Generation of primary microglia cultures

Primary microglia cultures were prepared from the cerebral cortex of newborn C57BL/6J mice pups (P1-P2). Brain tissue was dissected, meninges were removed and cells were dissociated using the Neuronal Tissue Dissociation Kit according to the manufacturer's guidelines, similar as for the primary neuron cultures described before (2.2.8). Isolated cortical cells were seeded on PDL coated culture flasks and cultivated in complete Dulbecco's Modified Eagle Medium (DMEM) for 12 days to allow microglial differentiation. Microglia cells were detached from the mixed cell layer by shaking of culture flasks for 4 h with 300 rpm on an orbital shaker, adapted from previously described procedure [208]. $2.5x10^5$ cells/ml were seeded on culture plates, maintained for 48 hours, and treated with either LPS (100 ng/mL), IFNγ, TNFα, IL-1β, IL-4, IL-13, IL-10, TGFβ, M-CSF or, GM-CSF (all 20 ng/mL) for 24 h. Cells were detached from culture plates using trypsin/EDTA and prepared for flow cytometry analysis.

2.2.15 Generation of bone marrow derived macrophages and dendritic cells

Bone marrow was isolated from the femur and tibia of adult C57Bl/6J mice. Therefore, mice were sacrificed by cervical dislocation, femur and tibia were dissected and muscles and tendons were removed. Bone ends were cut off and bone marrow were flushed out with PBS using a C26 cannula syringe. The cell suspension was applied to a cell strainer

(70 µm) to remove any remaining bone and tissue fragments and centrifuged for 5 min at 550 *g* at 4°C. Erythrocytes were lysed with a Tris-NH4Cl solution for 3 min at RT. Bone marrow cells were seeded with a density of 1x 10^6 cells/ml in complete RPMI-1640 culture medium containing M-CSF (20 ng/ml) for macrophage differentiation or GM-CSF (10 ng/ml) and IL-4 (5 ng/mL) to differentiate DCs. Macrophages were differentiated for 7 days and DCs for 10 days with ½ volume medium change every 3 days. Differentiated macrophages were detached from flasks using trypsin/EDTA and seeded with 2.5x10^5 cells/ml, maintained for 24 h, and treated with either LPS (100 ng/mL), IFNγ, TNFα, IL-1β, IL-4, IL-13, IL-10, TGFβ, M-CSF or GM-CSF (all 20 ng/mL) for 24 h. Differentiated weak-/non-adherent DCs were obtained and seeded with 5x10^5 cells/mL in complete RPMI-1640 culture medium supplemented with GM-CSF (10 ng/mL) and stimulated with LPS (100 ng/mL) or TNFα (20 ng/mL) for 48 h to induce maturation.

2.2.16 Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed according to standard procedures. In brief, tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer and equal amounts of proteins (50 µg/lane), determined by using Lowry protein assay Kit, were diluted in laemmli buffer and denaturized at 95°C for 5 min. Proteins were resolved by SDS-PAGE (current: 40 mA) using poly-acrylamide gels (10 % [v/v], Tab. 2.11). Resolved proteins were transferred to nitrocellulose membranes using a tank blotting system in transfer buffer with conditions of 380 mA for 80 min. All membrane washing steps were performed using TBST buffer. Unspecific antibody binding was prevented by blocking with a non-fat milk solution (5 % [w/v] in TBST) or BSA solution (5 % [w/v] in TBST). Primary antibodies specific to L1 (C20, 1:200), REST (07-579, 1:500), total and phosphorylated ERK1/2 (#91015, #91075, 1:1000) and GAPDH (6C5, 1:2000) were diluted in blocking buffer and incubated over night at 4°C. After washing with TBST, membranes were incubated with secondary antibodies conjugated to infrared fluorescence dyes (IRDye, 1:15,000) for 1 h at RT. Protein bands were densitometrically quantified using an Odyssey imaging device and ImageStudio software (LICOR).

gel	composition
stacking gel	50 % (v/v) H_20, 25 % (v/v) poly-acrylamide solution, 25 % (v/v) stack buffer, 0.15 % (w/v) APS*, 0.1 % (v/v) TEMED**
separation gel	40 % (v/v) H_20, 33 % (v/v) poly-acrylamide solution, 33 % (v/v) seperation buffer, 0.15 % (w/v) APS*, 0.1 % (v/v) TEMED**

Tab. 2.11 Composition of SDS-PAGE gels

*APS (ammonium persulfate), **TEMED (tetramethylethylenediamine)

2.2.17 Gene expression analysis

Preparation of mRNA from tissue or cell lysates was performed using the RNeasy Kit according the manufacturer's guidelines. The mRNA concentration was determined using a nanodrop device at a wavelength of 260 nm. 0.5 µg of mRNA was transcribed into cDNA by the QuantiScript Reverse Transcription Kit following the manufacturer's guidelines. The cDNA was simultaneously amplified and quantified by real time detection of the nucleic acid intercalating SYBR Green Kit using the Light Cycler 480. Relative levels of mRNA expression were calculated by the efficiency corrected $2^{-\Delta\Delta Ct}$ method [209]. Calculated by the formula displayed below, using Ct values of the gene of interest (target) and the appropriate housekeeping gene of the treated sample (treated) and of the untreated control (control), calculated with the gene specific amplification efficiency. The values correspond to the x-fold regulation of the mRNA expression of a target gene, normalized to a housekeeping gene and related to an untreated control.

$$\text{x-fold regulation} = (\text{efficiency}_{\text{target}})^{\Delta Cp \text{ target (control-treated)}} / (\text{efficiency}_{\text{reference}})^{\Delta Cp \text{ reference (control-treated)}}$$

Data from CNS tissue was analyzed by the number of total mRNA copies related to a target specific standard curve [210] and normalized to an appropriate housekeeping gene [210].

2.2.18 Isolation of mononuclear cells from CNS

Anaesthetized animals (ketamine 415 mg/kg, xylazine, 9.7 mg/kg) were disinfected with ethanol (70 % [v/v]), fur was cut open along the midline and the sternum was removed for heart exposure. The right heart atrium was opened and mice were transcardially perfused with PBS. Brain and spinal cord were dissected and stored in IMDM. The CNS tissue was chopped into pieces using a scalpel and transferred to IMDM

medium supplemented with collagenase (360 U/ml), DNAse (200 U/ml) and collagenase/dispase (5 µg/ml). The suspension was incubation for 30 min at 37 °C for the proteolytic-dissection, applied to a cell strainer (70 µm) and centrifuged with 550 *g* for 5 min at 4°C. The cell pellet was resuspended in Percoll-solution (40 % [v/v], diluted in IMDM) and carefully layered on top of percoll-solution (70 % [v/v], diluted in PBS). The percoll-gradient was centrifuged for 30 min at 750 *g* at RT. Mononuclear cells were collected from the interphase of the gradient, washed in RPMI-1640 medium and counted using a hemocytometer for further staining and flow cytometry analysis.

2.2.19 Cell surface staining for flow cytometry analysis

Isolated cells from the murine CNS were used for flow cytometry analysis of cell type specific surface molecules using flow cytometry. Staining and washing steps were performed in FACS buffer and centrifugation with 550 *g* for 5 min at 4°C. Unspecific Fc-receptor binding was blocked using Fc-block antibodies following addition of cell surface marker specific antibodies diluted in FACS buffer and incubation for 10 min at 4°C. Labeled cells were washed, resuspended in FACS buffer analyzed using a FACSCanto II device. Dead cells were identified using propidium iodide (PI, 0.1 µg/µl), added to the cell suspension immediately before measurement.

2.2.20 Intracellular staining for flow cytometry analysis

Lymphocyte subsets were identified by their specific cytokine and/or transcription factor expression. Therefore, isolated cells from the CNS were stimulated with cell culture plate bound anti-CD3 (37.51, 3 µg/ml) and anti-CD28 (145-2C11, 2.5 µg/ml) antibodies for 4 h in the presence of brefeldin A (5 µg/ml) to inhibit secretion of produced cytokines which leads to their intracellular enrichment. Cells were then stained for specific cell surface marker molecules as described in paragraph 2.2.19, fixed and permeabilized using fix/perm-buffer (eBiosciences) overnight. All following staining and washing steps were carried out in perm-buffer (eBiosciences). Unspecific Fc-receptor binding was inhibited by incubation with Fc-block antibodies. After 10 min of blocking, specific antibodies for the intracellular staining were applied and incubated for 30 min at 4°C. Unbound antibodies were removed by washing with perm-buffer, following cell-resuspension in FACS buffer and flow cytometry analysis using a FACSCanto II device. Flow cytometry data sets were analyzed using FlowJo software.

2.2.21 Immunohistochemistry

For immunohistochemistry of murine CNS sections, mice were transcardially perfused with PBS, followed by paraformaldehyde (4 % [w/v] in PBS) fixation, brains and spinal cord dissection and overnight post-fixation with paraformaldehyde (4 % [w/v] in PBS). The fixed tissue were then incubated in sucrose solution (30 % [w/v] in PBS) at 4°C for 16 h, frozen and stored at -21°C and cut to 25 µm sections using a cryotome. The sections were blocked with blocking buffer for 1 h at RT and incubated with primary antibodies specific to CD4 (RM4-5, 1:100) and L1 [197] (1:500) overnight at 4°C. Unbound primary antibodies were removed by washing with PBST, followed by incubation with AlexaFluor fluorescence-conjugated secondary antibodies (1:500) for 1 h at RT and cell nuclei staining with DAPI (1:10,000). The immunostained sections were imaged using laser scanning confocal microscope (LSM510, Zeiss).

2.2.22 Histopathology

For histopathological analysis of EAE experiments, mice were anaesthetized (ketamine 415 mg/kg, xylazine, 9.7 mg/kg) and transcardially perfused with PBS followed by perfusion with paraformaldehyde (4 % [w/v] in PBS). Spinal cords were removed, post-fixed in paraformaldehyde (4 % [w/v] in PBS) at 4°C and cut in 7 mm to 10 mm thick transverse segments prior to embedding in paraffin (in collaboration with Tanja Kuhlmann, Institut für Neuropathologie, Universität Münster). Microtome cut sections (4 µm thick) were stained by luxol fast blue combined with the periodic acid Schiff method (LFB-PAS). The LFB-PAS and macrophage-3 antigen (MAC3) staining were analyzed by determination of the effected (stained) area normalized to the overall white matter of the spinal cord section, respectively.

APP immunoreactive axons in EAE lesions were counted and normalized to the lesion area. Immunohistochemistry was performed using a biotin-streptavidin peroxidase technique and an automated immunostainer (AutostainerLink 48, Dako). First, sections were rehydrated: 2x Xylene, 2x ethonal (~100 % [v/v]), 1x ethanol (96 % [v/v]) and 2x ethonal (70 % [v/v]), 5 minutes each and subsequent washing in PBS. The rehydrated sections were incubated in citrate buffer (pH 6.0/9.0) for antigen retrieval at 95°C for 20 min. Primary antibodies were specific to Mac3 to identify macrophage infiltration or to the amyloid precursor protein (APP) to identify degenerating axons. 3,3´diaminobenzidine was used as color substrate and sections were mounted after

dehydration. For dehydration, sections were treated with following steps: 2x ethanol (70 % [v/v]), 1x ethanol (96 % [v/v]), 2x ethanol (~100 % [v/v]) and 2x Xylene, 5 min incubation for each step. For axon loss examination, sections were stained with anti-NF antibody following secondary AlexaFluor fluorescence-conjugated antibodies and counterstained with DAPI (1:10,000) to visualize EAE plaques by accumulations of infiltrated cells. NF-labelled axons were counted within cell plaques and in corresponding homotypic tissue regions lacking plaques of infiltrated cells using the particle count plugin of ImageJ. The axon loss was calculated by the number of axons in lesions relative to the number of axons in homotypic tissue and displayed in percent of lost axon.

2.2.23 Statistics

Normal distribution of analyzed value was tested with the Shapiro-Wilk test. Unless indicated otherwise, *p* values of non-parametric data sets were calculated by the Mann-Whitney U-test and *p* values of parametric data sets were calculated using the Student's t-test. $p<0.05$ was considered to indicate a significant difference. All results are shown as mean value ± SEM.

3 Results

3.1 L1 is expressed on axons in MS brain tissue and human T cells

L1 has been characterized as a neural cell adhesion and recognition molecule that is important to regulate axonal growth and guidance. Its expression predominates in neurons but has been further reported in human immune cells including T cells [181, 211]. We therefore investigate the pathological relevance of the L1 expression in neurons and immune cells during neuroinflammatory processes, as L1 has not been investigated in this context so far. The injury of axons is a major factor of neurological disability in MS, and it primarily takes place in the white matter (WM) of the CNS. Axonal injury occurs already at the onset of the disease, triggered by the inflammatory processes in the CNS. Although MS is considered to be a WM disease, MS-related grey matter (GM) lesions have also been reported. GM lesions differ from lesions within WM regions: they are less inflammatory and are less infiltrated by macrophages and lymphocytes. However, significant demyelination and neuronal loss have been demonstrated within GM lesions [212]. L1 is a cell adhesion molecule known to promote cell-cell contacts by *trans*-homophilic interaction [213]. To investigate L1 expression during MS-related inflammatory processes in the CNS, we used brain cryosections from MS patients and non-pathological control individuals and examined the L1 expression in the cortical tissue and in human T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors.

The examination of the cellular and tissular distribution of L1 by immunohistochemistry using anti-L1 and pan-NF antibodies revealed L1 expression in cortical GM tissue and in subcortical WM axons in tissue samples of the parieto-temporal cortex from MS patients and non-neurological controls (Fig. 3.1A, B). Moreover, L1 was expressed by CD4 and CD8 T cells isolated from human blood of healthy donors, as demonstrated by immunocytochemistry and immunoblotting (Fig. 3.1C, D). The expression of L1 in CD4 and CD8 T cells was further confirmed by detection of *L1* mRNA in the respective cells (Fig. 3.1E). Thus, L1 is expressed in MS brain tissue and human T cells which implies a role in the crosstalk between the nervous and immune system.

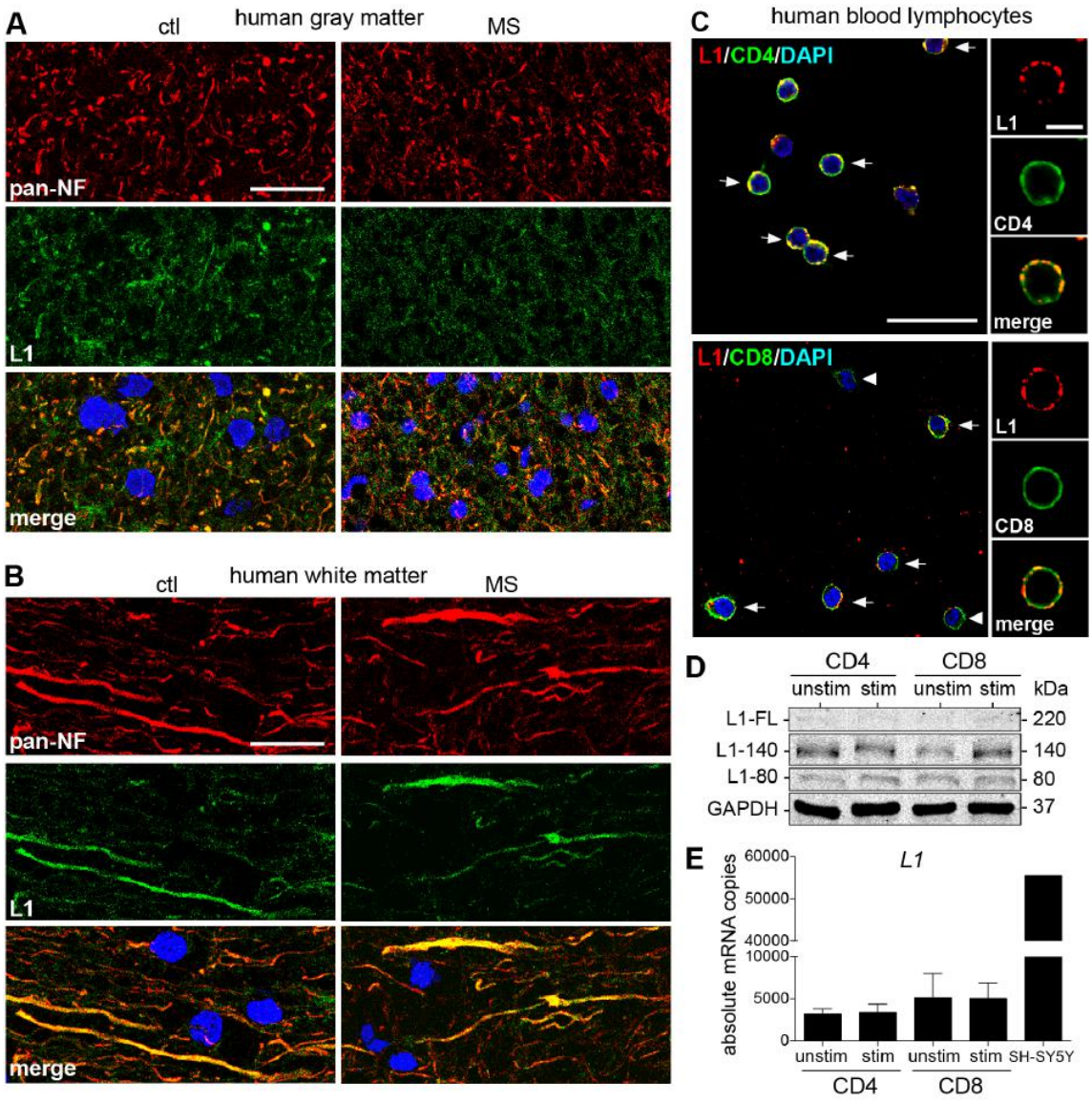

Fig. 3.1 L1 is expressed on axons in MS brain tissue and human blood T cells

(A, B) Representative double-immunostaining for L1 and pan-NF in gray matter and white matter areas of cortical tissue from MS patients and non-neurological controls and counterstained with DAPI. (C) Representative double-immunostaining for L1 and T cell subset marker CD8 and CD4 of enriched T cells from human PBMCs. (D) Immunoblotting of full length (~220 kDa) and cleaved L1 (~140 kDa, ~80 kDa) of lysates from human CD4 and CD8 T cells that were isolated from PBMCs. GAPDH (~37 kDA) served as loading control. (E) Gene expression analysis of absolute *L1* mRNA copies (in 0.5 µg total mRNA) of human CD4 and CD8 T cells separated from PBMCs (n=4). *L1* expression in human neuron-like SHSY5Y cells served as positive control. Values are displayed as mean ± SEM. Scale: 10 µm (A, B), 20 µm, 5 µm (C).

3.2 Neuroinflammation causes down-regulation of neuronal L1 expression

To elucidate the role of L1 in neuroinflammation, we used the MOG_{35-55} immunized active EAE model in C57Bl/6J mice, which is commonly used as the EAE model with a chronic disease progression [47]. Spinal cord tissue of mice displaying pronounced paralytic symptoms was processed for further analysis. Protein lysates of cervical spinal cord tissue were analyzed for L1 protein expression in EAE and sham mice. $PLP_{139-151}$ immunized EAE in mice with SJL background were used as the model with characteristic RRMS disease phases of peak, remission, and relapse phase in order to investigate *L1* mRNA expression levels associated with the respective disease phases. The amount of L1 protein was significantly reduced in the spinal cords of MOG_{35-55} immunized mice at the peak of EAE disease (Fig. 3.2A). The analysis of *L1* gene expression during the peak, remission, and relapse stages of the $PLP_{139-151}$ EAE model revealed the most pronounced L1 reduction during the peak stage, unchanged expression during the remission phase, and again significant reduction during relapse (Fig. 3.2B). The RE1-Silencing Transcription Factor (REST) is known to be an inducible repressor of L1 transcription [167]. Therefore, the expression of *L1* mRNA and its repressor *REST* were analyzed to verify possible implications of *REST*-mediated *L1* regulation during neuroninflammation in the murine CNS. The down-regulation of *L1* during the peak stage coincides with the up-regulation of *REST*. Accordingly, the *L1/REST* ratio was significantly reduced in animals with EAE in comparison to sham controls (Fig. 3.2C, D, E).

Gene expression of *L1* and the T cell receptor (TCR) zeta chain was analyzed in the cortex, cerebellum, and spinal cord to ascertain whether the *L1* mRNA regulation correlates with the presence of CNS-infiltrating T cells or occurs as an overall expression pattern in the CNS of animals during EAE. Since infiltrating leucocytes also express L1 and therefore contribute to the detected *L1* levels, mRNA expression of the L1 splicing variant that lacks exon 2 (Δ2-*L1*), which is considered to be specific for non-neuronal L1 expression was examined [188]. *L1* was similarly expressed in the cortex and cerebellum of sham and EAE mice. *TCR-zeta* mRNA was almost absent in the cerebral cortex and modestly increased in the cerebellum (Fig. 3.2F, G). The EAE-induced *L1* reduction occurred exclusively in the spinal cord, accompanied by a strong presence of *TCR-zeta* transcripts, which suggest strong infiltration of T cells in this tissue (Fig. 3.2H).

T cells are known to express L1 under specific conditions in the periphery [183] but have never been studied in association with acute EAE plaques. L1 expression in CD4 T cells was further verified by flow cytometry, and these results were combined with the analysis of CD8 T cells, which play a minor role during EAE disease but are the predominant T cell subset in MS lesions [41]. Flow cytometry analysis confirmed that L1 is expressed by CNS-infiltrating CD4 T cells, peripheral CD4 T cells and to a lower percentage by CD8 T cells isolated from spleen (Fig. 3.2I). The expression level of non-neuronal *L1* mRNA was just a fraction of the total *L1* expression level in the spinal cord and was therefore negligible for the total *L1* regulation analysis. Thus, the overall reduced *L1* expression levels in EAE mice were predominantly due to down-regulation of neuronal L1, which goes along with increased L1 expression in non-neuronal cells, including T cells (Fig. 3.2J).

Moreover, we used immunohistochemical experiments to investigate the spatial relationship between T cells and axons expressing L1. Spinal cord sections stained for L1, βIII-tubulin, and CD4 demonstrated the expression of L1 on neurons in grey and white matter areas and the expression of L1 in CD4 T cells in the spinal cord of mice during EAE (Fig. 3.2K, L). Moreover, we found a substantial infiltration of CD4 T cells into the spinal cord, which was accompanied by an apparent reduction of L1 immunoreactivity in the gray and white matter of mice with EAE compared to control mice (Fig. 3.2M). Plaques of CNS-infiltrating CD4 T cells were co-located with patches of increased L1 expression (Fig. 3.2M). A more detailed examination of the inflammatory plaques revealed L1-expressing CD4 T cells located adjacent to degenerating axons that expressed L1 too. This suggests that L1 is involved in the interaction between CD4 T cells and neurons due to its homophilic and heterophilic binding capacity (Fig. 3.2N).

To sum up, these findings show that T cell infiltration correlates with down-regulation of neuronal L1 in the murine spinal cord during experimental neuroinflammation and confirm L1 expression in murine CD4 T cells and spinal cord axons at the same time.

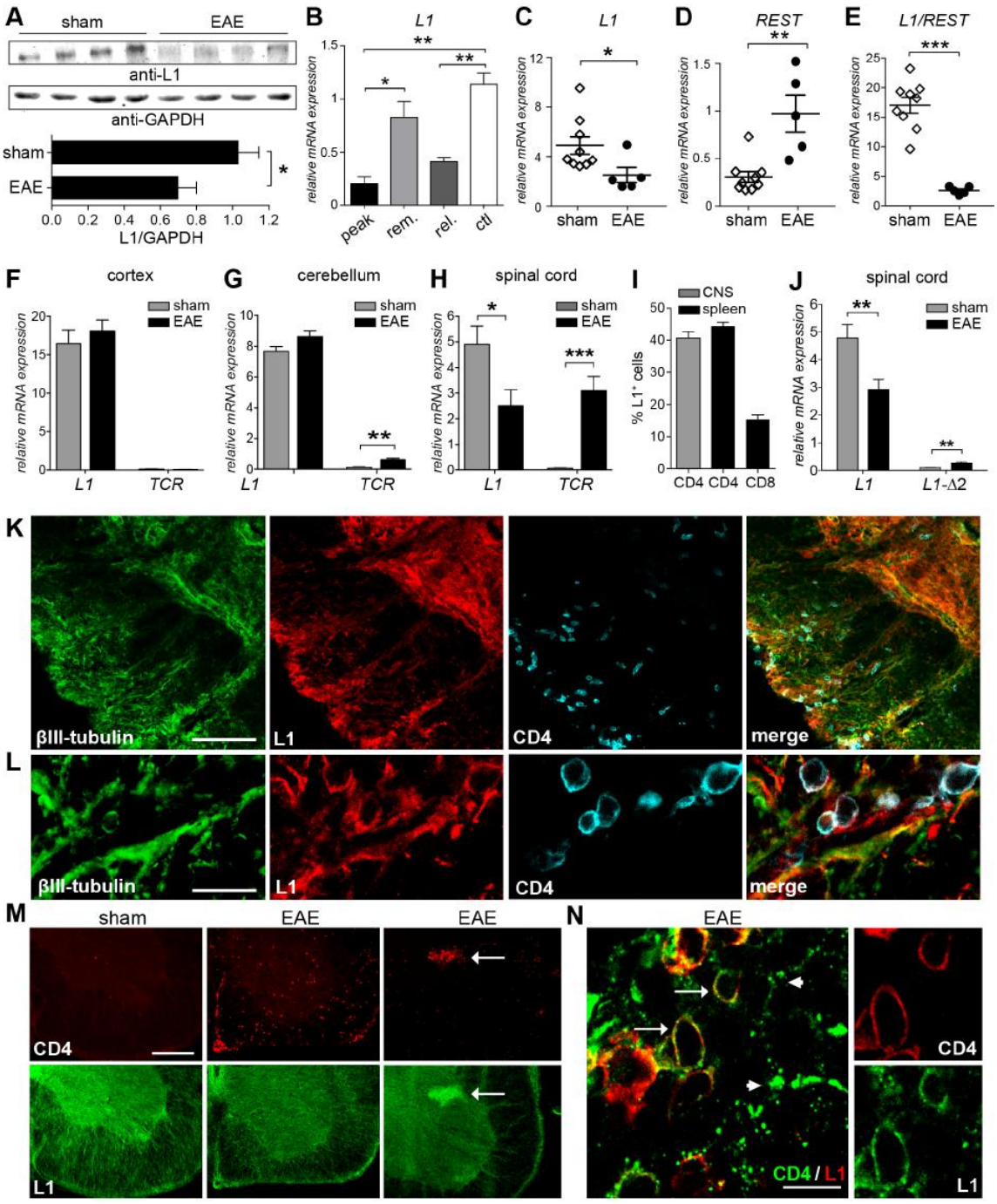

Fig. 3.2 Neuroinflammation causes down-regulation of neuronal L1 expression

(A) Representative immunoblot displays full-length L1 protein (~220 kDa) in EAE compared to sham mice spinal cord lysates (normalized to GAPDH [~37 kDa], n=8/condition). (B) *L1* mRNA expression was analyzed at the peak, remission (rem) and relapse (rel) disease stage in the spinal cord of $PLP_{139-151}$ immunized mice during EAE and in control (ctl) animals (normalized to *β-actin*, n=3/group). (C-E) *L1* and *REST* mRNA expression and the *L1/REST* ratio in the spinal cord at disease peak of MOG_{35-55} immunized EAE compared to sham mice (normalized to *βIII-tubulin*, sham: n=9; EAE: n=5). (F-H) Gene expression analysis of *L1* and *TCR zeta chain* in cortex, cerebellum, and spinal cord of EAE mice at peak of the disease and in sham animals (sham: n=9, EAE: n=5). (I) Flow

cytometry analysis of L1 expression in CD4 T cells isolated from the CNS of EAE mice and in CD4 and CD8 T cells isolated from spleen (n=7/group). Gating strategy: live gate (FSC-SSC)/living cells (IP^-)/$CD45^+$/$CD4^+$ or $CD8^+$, $L1^+$ in % of the T cell subset, respectively. (J) Gene expression analysis of *L1* and a non-neuronal *L1* splicing variant (*Δ2-L1*) in the spinal cord of sham and EAE mice (sham: n=9, EAE: n=5). (K, L) Triple-immunostaining for L1, pan-NF, and CD4 in spinal cord sections of EAE mice. (M, N) Double-immunostaining for L1 and CD4 T cells in control and EAE mice. CD4 T cells (arrows) and neighboring degenerating axons (arrowheads) express L1. Values were displayed as mean ± SEM, *p* values (**p*<0.05; ***p*<0.01; ****p*<0.001) were calculated by unpaired student`s t-test (A, B) or Mann-Whitney *U* test (C, D, E, G, H, J). Scale: 50 µm (K), 200 µm (M), 10 µm (L, N).

3.3 Activated CD4 T cells cause down-regulation of neuronal *L1* expression

The reduced levels of L1 protein expression as well as *L1* mRNA in our *in vivo* neuroinflammation model raised the question whether the L1 regulation is associated with the presence of infiltrating CD4 T cells. The complex environment at sites of EAE plaques, which includes CNS-resident cells and a variety of CNS-infiltrating immune cell subsets, does not allow to investigate detailed cell type-specific interactions. We therefore established a neuron-CD4 T cell co-culture model that enables monitoring of neuronal mRNA expression changes. We used enriched and differentiated primary cortical neurons in co-culture with CD4 T cells isolated from lymph nodes and spleen. The CD4 T cells were used unstimulated or unspecifically stimulated overnight with plate-coated antibodies against CD3 (T cell receptor stimulation) and CD28 (co-stimulation). Co-cultures were incubated for 2 hours and either investigated using immunocytochemistry or cultures were lysed for immunoblotting and mRNA analysis. CD4 T cell depletion was performed using magnetic cell sorting to enable neuron-specific mRNA analysis. We further used flow cytometry to determine stimulation-associated L1 expression in CD4 T cells. The immunoblotting experiments were performed to investigate whether *REST* up-regulation and *L1* down-regulation also occur on protein level and to examine phosphorylation regulation of the extracellular signal-related kinase (ERK), which has been associated with REST regulation and/or stabilization in recent studies [214, 215].

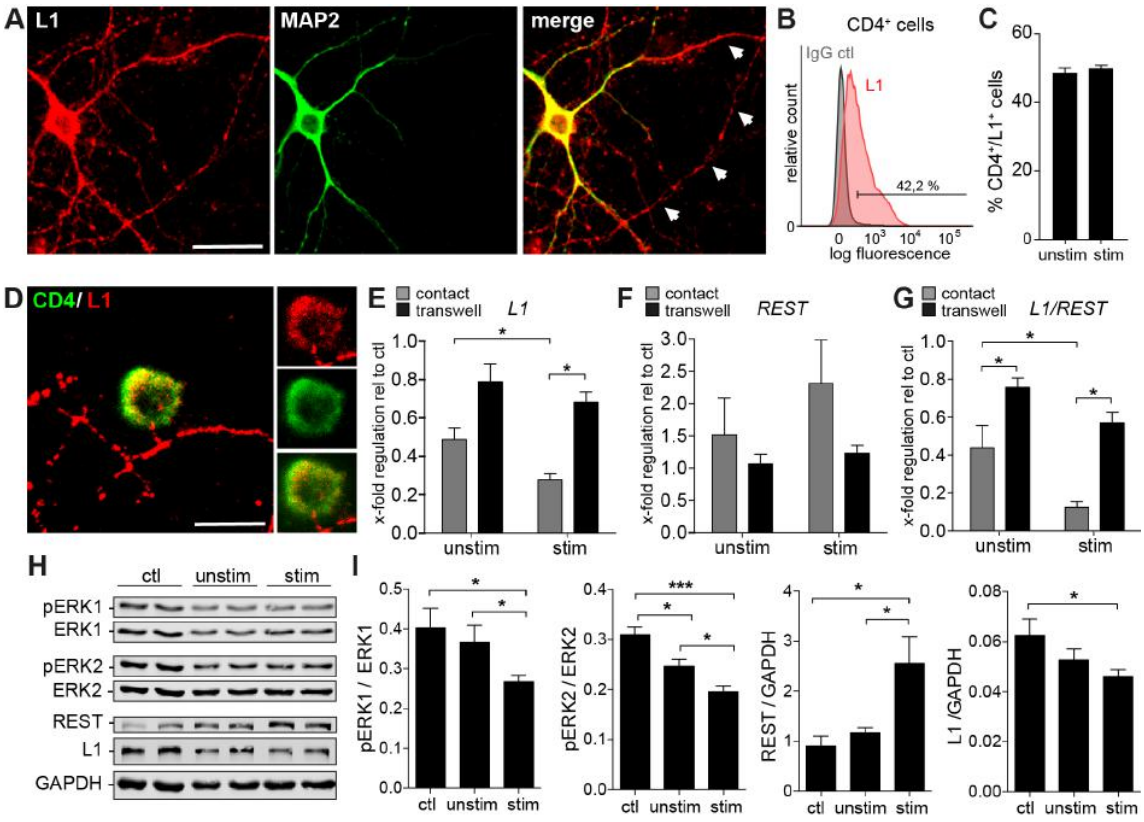

Fig. 3.3 Activated CD4 T cells cause down-regulation of neuronal L1 expression

(A) Double-immunostaining of primary cortical neuron cultures for L1 and the dendrite marker microtubule-associated protein 2 (MAP2). Arrowheads indicate axonal expression of L1. (B) Flow cytometry analysis showing representative analysis of L1 expressing CD4 T cells related to isotype control antibody stained cells. Gating strategy: live gate (FSC-SSC)/living cells (PI^-)/$CD45^+$/$CD4^+$/$L1^+$. (C) Quantification of the flow cytometry analysis of L1 expression in unstimulated or anti-CD3/CD28 stimulated CD4 T cells. $L1^+$ cells in % of the $CD4^+$ T cell population (n=6/group). (D) Double-immunostaining showing L1 expression in CD4 T cells and axons in neuron-CD4 T cell co-cultures. (E-G) Expression of *L1* and *REST* mRNA in neurons co-cultured with unstimulated or anti-CD3/CD28 stimulated CD4 T cells, either in direct contact or separated by transwell inserts (n=4/group). (H) Representative immunoblot of neuron lysates after co-culture with mock control, unstimulated, or anti-CD3/CD28-stimulated CD4 T cells, using antibodies specific for phosphorylated ERK (pERK), total ERK (tERK), (~44 kDa ERK1, ~42 kDa ERK2), REST (~180 kDa), L1 (~220 kDa), and GAPDH loading control (~37 kDa). (I) Quantification of protein band intensities (n=7/condition). Values are displayed as mean ± SEM, p values were calculated by Mann-Whitney U test (*p<0.05, ***p<0.001). Scale: 20 µm (A), 5 µm (D).

Immunocytochemistry experiments demonstrated L1 expression in neurons, including axonal L1 expression (Fig. 3.3A) and L1 expression on CD4 T cells in co-culture (Fig. 3.3D). Moreover, the CD4 T cells were in physical contact with L1-expressing axons (Fig. 3.3D). However, the stimulation of the CD4 T cells did not influence the percentage

of L1-expressing CD4 T cells (Fig. 3.3B,C). Gene expression assays revealed a reduction of neuronal *L1* expression after co-culture with CD4 T cells that was significantly more pronounced in neurons co-cultured with stimulated CD4 T cells than with unstimulated T cells (Fig. 3.3E). Co-cultures with transwell inserts, which prevent direct cell-cell contact but allow exchange of soluble factors, indicated unchanged neuronal *L1* level, which suggests a contact-depended induction of neuronal *L1* down-regulation (Fig. 3.3E). *REST* mRNA levels were up-regulated under conditions that simultaneously induced decreased *L1* expression (Fig. 3.3E, F), thus confirming the hypothesis of *REST* to be a repressing transcription factor of neuronal L1 transcription and resulting in a significantly reduced *L1*/*REST* expression ratio in neurons contacted by stimulated CD4 T cells (Fig. 3.3G). To further investigate how stimulated T cells affect neuronal expression of *L1* and *REST* in co-cultures, we performed immunoblotting for the phosphorylated ERK (pERK) in neuron lysates after co-culture with CD4 T cells. The immunoblotting revealed a significantly reduced phosphorylation of ERK1 and ERK2 in neurons following co-cultivation with stimulated CD4 T cells. Correspondingly, REST was up-regulated and the L1 expression was reduced in comparison to mock-treated neurons (Fig. 3.3H, I).

These results confirmed an inverse relationship between L1 and REST expression at protein level and suggest that stimulated CD4 T cells inhibit ERK1/2 phosphorylation and thereby induce REST-mediated reduction of L1 expression in neurons.

3.4 CD4 cell subsets does not differ in L1 expression and initiation of neuronal L1 reduction

Our previous results demonstrated L1 expression on CD4 T cells in the spinal cord of mice during EAE. Among other molecules, all CD4 T cells express the characteristic TCR and its associated co-receptor CD4. However, they can be divided into different subpopulations on the basis of different cytokine expression patterns [216]. To characterize L1 expression in distinct T cell subpopulations and to test their capability to down-regulate neuronal L1 murine CD4 T cells were isolated from spleen and differentiated into Th1, Th2, Th17, and Treg cell populations *in vitro*. The differentiated T cell subsets were co-cultured with neurons for two hours, and the neuronal mRNA expression changes were analyzed.

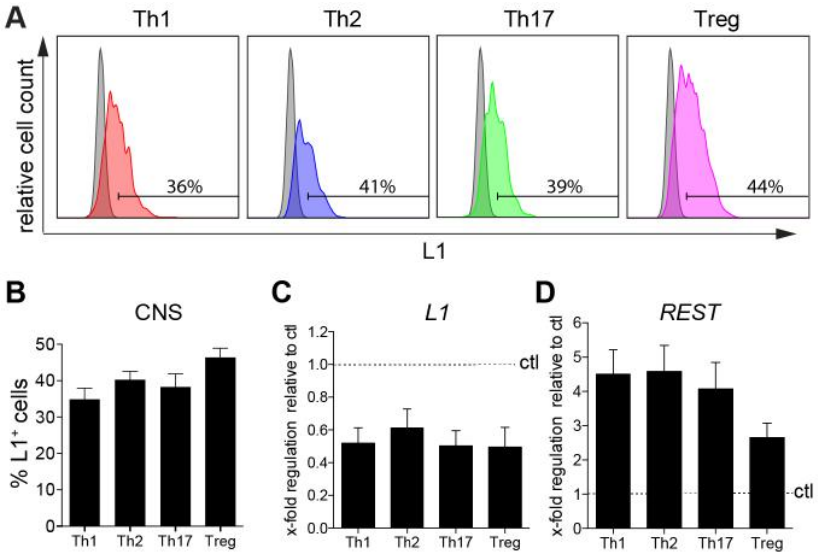

Fig. 3.4 L1 expression and induction neuronal L1 regulation is not different between CD4 T cell subsets
(A) Representative histograms of L1 expression in CD4 T cell subsets analyzed by flow cytometry. Gating strategy: live gate (FSC-SSC)/CD45$^+$/CD4$^+$ and IFNγ$^+$ (Th1), IL-10$^+$FoxP3$^-$ (Th2), IL-17$^+$ (Th17), FoxP3$^+$ (Treg). L1$^+$ cells are displayed in % of the respective subset. Cells stained with an equally fluorocrome-conjugated isotype control antibody served to determine the negative population (gray). (B) Quantification of the L1 expression in CD4 T cell subsets isolated form the CNS of EAE mice (n=7/subset). (C, D) x-fold regulation of *L1* and *REST* mRNA expression in neurons co-cultured with Th1, Th2, Th17 or Treg CD4 T cell subsets relative to mock control (n=6/group). All values are displayed as mean ± SEM.

The flow cytometry analysis revealed a similar percentage of L1-expressing cells in all CD4 T cell subsets with a tendency of more L1-expressing Tregs (Fig. 3.4A, B). The co-culture experiments confirmed the reduction of neuronal *L1* mRNA levels and the increase of *REST* in co-culture experiments with subset-unspecific CD4 T cells. However, there were no differences in neuronal *L1* reduction between the different CD4 T cell subset-neuron co-cultures and simultaneous induction of *REST* in all groups (Fig. 3.4C,D). Thus, the L1 expression in CD4 T cells and the CD4 T cell induced regulation of neuronal L1 expression is not different between CD4 T cell subsets.

3.5 Blocking L1 inhibits CD4 T cell adhesion to neurons and axonal degeneration

T cells have been reported to cause axonal dysfunction at the onset of neurological deficits in the murine EAE model as well as axonal fragmentation and microtubule destabilization *in vitro* [105, 123]. The neuron-CD4 T cell co-culture model revealed neuronal *L1* reduction in a contact-dependent fashion. The *trans*-homophilic binding

capability of L1 [205] together with its expression on neurons and T cells suggested L1-mediated cell-cell adhesion between neurons and T cells. To test this hypothesis, we labeled stimulated T cells with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured them with neurons for 30 minutes in the presence of L1-blocking or isotype control antibodies. We further used the co-culture model to test whether stimulated CD4 T cells induce axonal degeneration of neurons *in vitro* and whether L1 is involved in this process. Therefore, differentiated neurons were co-cultured with unstimulated or stimulated CD4 T cells in the presence of L1-blocking antibodies or isotype IgGs for 2 hours, following immunhistochemical analysis of βIII-tubulin, which indicates changes in the microtubule network that are associated to axon degeneration.

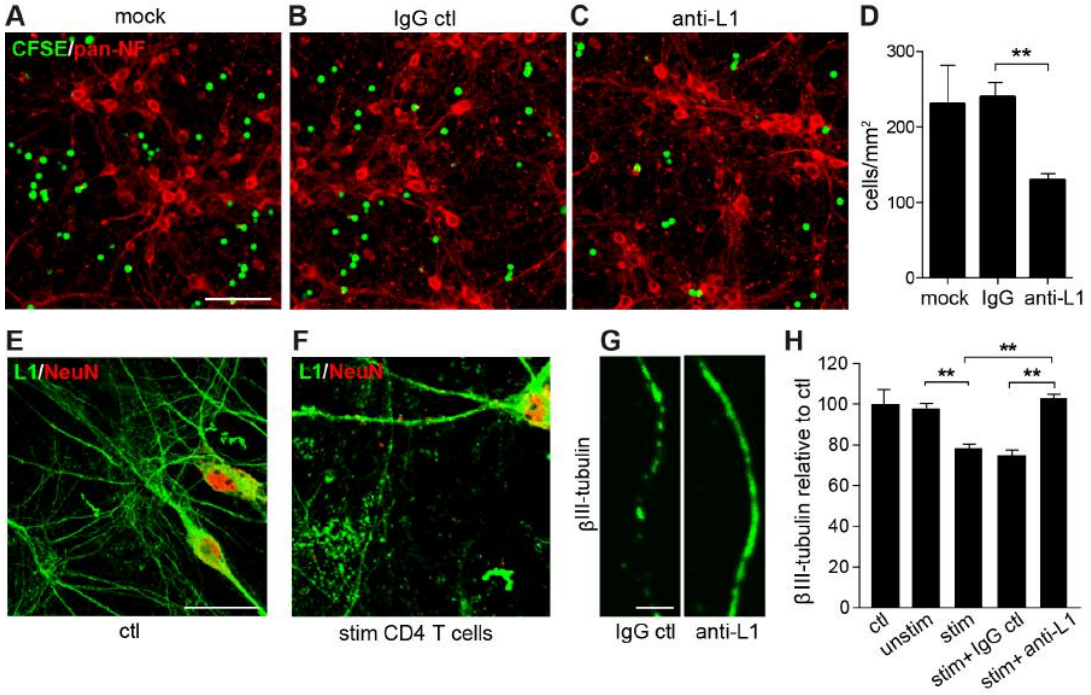

Fig. 3.5 Antibody mediated blocking of L1 reduces adhesion of CD4 T cells to neurons and axonal injury

(A-C) Representative images of CFSE-labeled CD4 T cells and differentiated neurons immunostained with pan-NF antibody after 30 min of co-culture together with mock-, rat IgG ctl- or anti-L1 (clone: 555) antibody treatment. (D) Count of adherent CD4 T cells per mm² in mock, rat IgG and anti-L1 antibody treated conditions (n=6/condition). (E, F) Double-immunostaining for L1 and NeuN in control neuronal cultures and 2 hours after addition of anti-CD3/CD28 stimulated CD4 T cells. (G) Representative βIII-tubulin-immunostained axons of T cell-neuron co-cultures treated with rat IgG ctl or anti-L1 antibodies. (D) Axonal βIII-tubulin immunofluorescence of neurons co-cultured with unstimulated or stimulated CD4 T cells and treated with rat IgG control or anti-L1 antibodies (n=6/condition),βIII-tubulin immunofluorescence is normalized to untreated neuron cultures (set as 100 %). Values display mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (**$p<0.01$). Scale bar: 150 µm (A), 20µm (E), 5 µm (G).

CFSE-labeled CD4 T cells were co-cultured with differentiated neurons for 30 minutes, and non-adherent cells were removed by repeated culture rinsing. Strikingly, the blocking of L1 via antibody-treatment significantly reduced CD4 T cell adhesion to neurons. L1 blocking decreased the number of detected cells by almost 50 % in comparison to isotype IgG- and mock-treated conditions (Fig. 3.5A-D). Cell culture staining for L1 and the neuronal marker NeuN in co-cultures of primary neurons and anti-CD3/CD28-stimulated CD4 T cells revealed axonal degeneration of L1-expressing neurons after 2 hours (Fig. 3.5E-H). Axon degeneration appears as fragmentation into axon segments, whereas axon integrity remained stable under control conditions (Fig. 3.5G). Neurons co-cultured with unstimulated CD4 cells did not exhibit any differences in axonal βIII-tubulin immunoreactivity, which was analyzed by line scan measurements. Co-cultures with stimulated CD4 T cells revealed a striking reduction of axonal βIII-tubulin immunoreactivity, which remained unchanged in presence of IgG isotype control antibodies. Interestingly, the presence of L1-blocking antibodies prevented the loss of axonal βIII-tubulin immunoreactivity, keeping axon integrity at the levels of the mock condition (Fig. 3.5H). Thus, the experiments revealed detrimental effects of stimulated CD4 T cells on the axon integrity in co-cultures and suggest that L1 is involved in this process by facilitating T cell adhesion to neurons.

3.6 Blocking L1 alleviates disease severity and axonal pathology in EAE

Our results revealed that the application of an L1-blocking antibody *in vitro* reduced adhesion of CD4 T cells to cultured neurons and prevented CD4 T cell-induced axonal injury. In order to investigate the beneficial effect of L1 inhibition *in vivo*, we intravenously administered L1-blocking antibodies to C57BL/6J mice during the early phase of EAE on day 4 and day 8 after immunization. This therapeutic approach was performed in two independent cohorts using L1 antibodies (clone: 324), which has already been reported to efficiently block L1 binding capacities and associated functional implications *in vitro* [149, 217]. The clinical score of the individual EAE mice were monitored daily and summarized to a mean clinical score. The mice were sacrificed at the peak stage of the disease and analyzed for immune cell infiltration using flow cytometry and for axonal degeneration and loss using immunohistochemical methods.

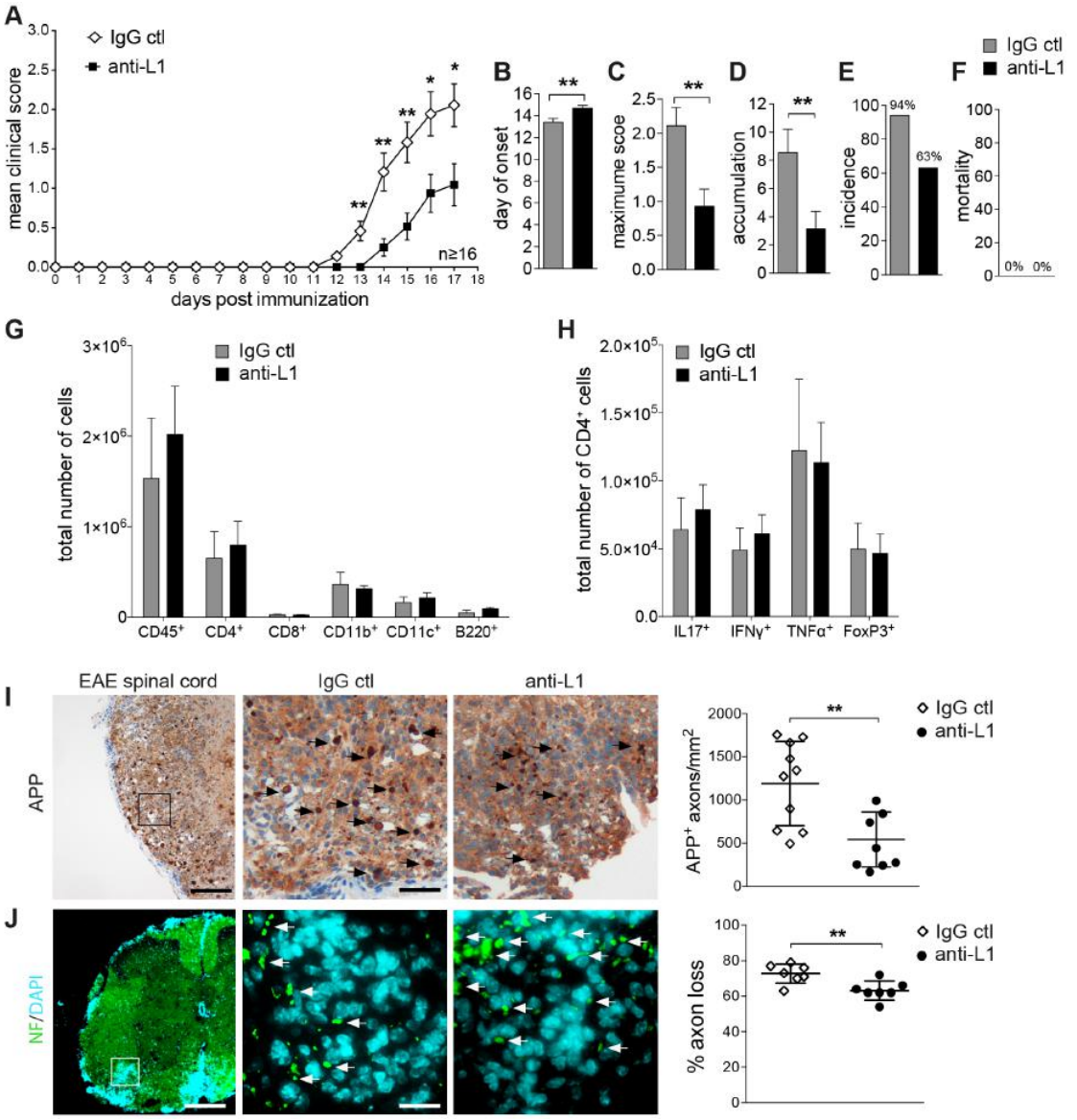

Fig. 3.6 Antibody mediated blocking of L1 alleviates disease severity and axonal pathology in EAE

(A) EAE progression curve following MOG_{35-55} induced EAE in C57Bl6/J mice, which were intravenously injected with rat IgG isotype control or anti-L1 antibodies (clone: 324) at day 4 and day 8 after immunization. Data points indicate mean clinical scores of all mice per group including mice of two independent experimental cohorts. (B-E) Diagrams exhibit specific EAE progression characteristics in detail, including the day of onset (B), maximum score (C), score accumulation (D), disease incidence (E) and mortality (F). (G, H) Flow cytometry analysis of CNS infiltrated immune cells at peak stage of EAE. (G) Total number of immune cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/living cells (IP^-)/$CD45^+$ and $CD4^+$, $CD8^+$, $CD11b^+$, $CD11c^+$or $B220^+$ (n=5/subset). (H) Total number of CD4 T cell subset cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/$CD45^+$/$CD4^+$ and IL-17^+, $IFN\gamma^+$, $TNF\alpha^+$or $FoxP3^+$ (n=5/subset). (I) anti-β-amyloid protein (APP) immunohistochemistry indicates degenerating axons by means of accumulating APP spheroids (arrows). Quantification of APP-immunoreactive axons per mm^2 at lesion sites (n=10/condition). (D) Pan-NF staining indicates axons at sites of EAE lesions and in homotypic areas without plaques. Plaques were identified by atypical cell accumulation

indicated by DAPI stained nuclei. Axon loss represents pan-NF immunoreactive axons in plaques normalized to axons in homotypic areas without infiltration plaques (set as 100 %, IgG ctl n=9, anti-L1 n=10). Values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*$p<0.05$, **$p<0.01$). Scale bar: 500 µm, 50 µm (I); 1 mm, 20 µm (J)

The anti-L1 antibody treatment significantly attenuated clinical EAE scores compared to rat IgG control treated mice (Fig. 3.6A). The disease onset was delayed, and the maximum score, the score accumulation and the disease incidence were reduced; however, there were no changes in the mortality rate (Fig. 3.6B-F). There are a variety of different lymphocyte subpopulations in the periphery that can be characterized by specific cell surface markers. Our experiment focused on lymphocyte and monocyte cell populations, which were already reported to be relevant during EAE [47]. The results indicated no significant changes in the number of distinct T lymphocyte subpopulations, including $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, $CD11b^+$ microglia and macrophages, $CD11c^+$ DCs, and the $B220^+$ B cell subpopulation (Fig. 3.6G). Also, no change in the CD4 T cell subsets was observed between anti-L1 antibody and IgG ctl treated mice. We determined the number of Th17 ($IL\text{-}17^+$) and Th1 (IFNγ) effector T cells, which considered to be the major drivers of EAE progression [61]. The cytokine TNFα primarily has pro-inflammatory properties and is secreted by different subsets of CD4 T cells. The expression of TNFα, which acts as one of the major mediators during inflammatory processes, by $CD4^+$ T cells is also of vital importance in the neuroinflammatory process during EAE [218]. Transcription factor FoxP3 is a specific marker of regulatory T cells, which counteracts effector T cell activity and pro-inflammatory action of surrounding antigen-presenting cells and phagocytes [219] (Fig. 3.6H). Axonal degeneration and the relative axon loss were assessed by immunohistochemistry of spinal cord sections using the axonal injury marker APP and neurofilament staining, respectively. The quantification of the stained spinal cord sections indicated a reduced number of APP immunoreactive axons in the lesions of anti-L1 antibody treated mice (Fig. 3.6I). In addition, the axon loss was significantly more pronounced in anti-L1-treated mice compared to control IgG-treated animals (Fig. 3.6J, axon loss in IgG ctl: 71 ± 3 %, anti-L1: 58 ± 4 %, mean ± SEM), indicating that blocking of L1 prevents axonal degeneration and loss. Hence, the antibody-mediated blocking of L1 alleviates the EAE severity and prevents axonal pathology during EAE disease.

3.7 Generation of transgenic mice with neuron specific L1 depletion

So far, the results indicated L1 to be expressed in neurons and CD4 T cells, and co-culture experiments as well as administration of an L1-blocking antibody in EAE suggested L1 to be implicated in CD4 T cell-mediated axonal injury. To investigate the role of neuronal L1 in neuroinflammation, mice mutants for neuronal L1 depletion were generated.

To circumvent the severe neurological phenotype of constitutively L1 deficient mice that have been reported in other studies [220, 221] and to relate results to neuron-specific effects, a mouse strain that exhibits an incomplete neuron-specific L1 knockout was generated. Therefore, a mutant for a Cre-recombinase, which is driven by the neuron-specific synapsin (Syn^{Cre}) promoter, was crossed with a mutant for a loxP-flanked L1 gene locus ($L1^{fl/fl}$), resulting in the $Syn^{Cre}L1^{fl/fl}$ mouse strain (Fig. 3.7A). To characterize the newly generated $Syn^{Cre}L1^{fl/fl}$ mouse strain, we examined its L1 expression in comparison to respective Syn^{Cre} control littermates using immunoblotting of protein lysates from distinct CNS parts. Since functional L1 mutants in humans and mice have often been reported to cause neurological impairments [220, 221], $Syn^{Cre}L1^{fl/fl}$ and Syn^{Cre} control littermates were examined for neurological disabilities using a behavioral test that displays a composite neuroscore. Moreover, the immunoblotting data was verified by immunohistochemistry experiments in the spinal cord of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ animals.

The immunoblots revealed a substantial reduction of L1 protein expression in the cortex, hippocampus, brain stem, and spinal cord of $Syn^{Cre}L1^{fl/fl}$ mice, while expression was almost unchanged in the cerebellum (Fig. 3.7B,C). Strikingly, in the EAE mouse model, the brain stem and spinal cord are the major sites of immune cell infiltration and axonal injury [222, 223]. L1 expression was reduced in these brain regions down to 25 % of L1 protein levels Syn^{Cre} control mice (Fig. 3.7C). Neither Syn^{Cre} control littermates nor L1 depleted $Syn^{Cre}L1^{fl/fl}$ mice exhibited any apparent neurological abnormalities (Fig. 3.7D). Immunohistochemistry of the L1 expression in the spinal cord displayed a decreased L1 immunoreactivity within the grey and white matter (Fig. 3.7E-J) that is similar to the L1 reduction pattern after experimental neuroinflammation in C57BL/6J wild type mice (Fig. 3.2E). Altogether, $Syn^{Cre}L1^{fl/fl}$ mice did not display obvious neurological abnormalities but strongly reduced L1 protein expression in CNS regions considered to be major sites of axonal injury in the EAE mouse model.

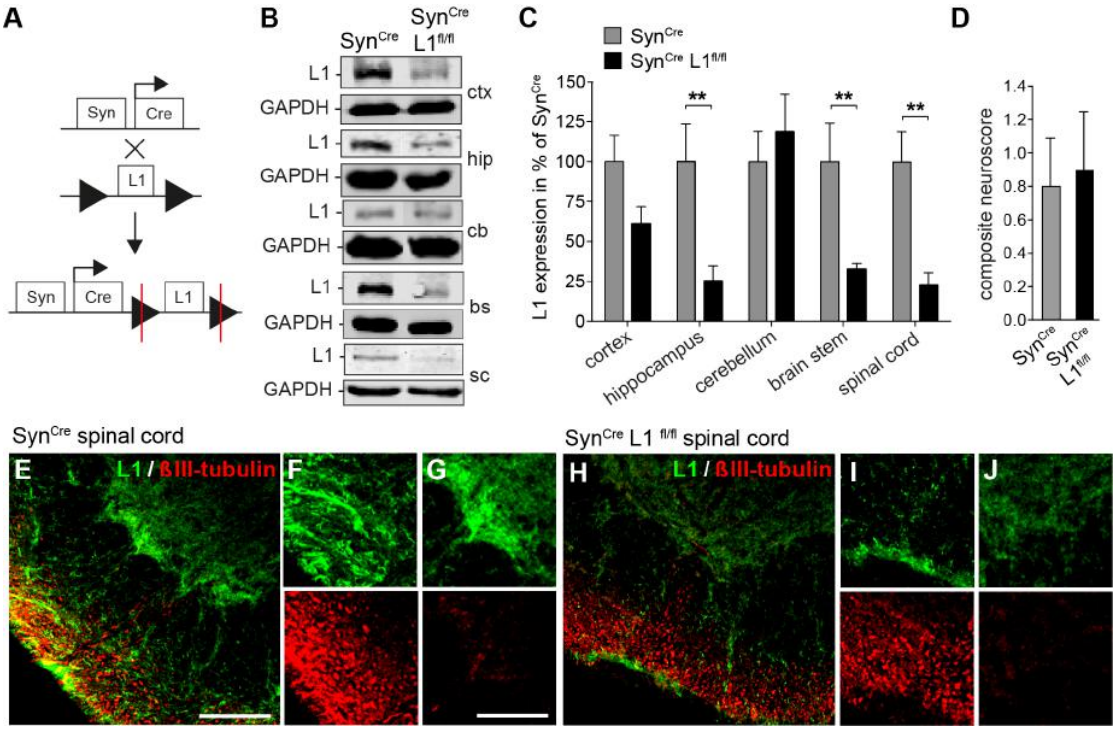

Fig. 3.7 Generation of mice with a neuron specific L1 depletion

(A) Crossing scheme of the $Syn^{Cre}L1^{fl/fl}$ mice strain generation describes cross breeding of mice with a synapsin promoter driven Cre-recombinase with mice bearing a loxP-flanked L1 gene locus. Red lines symbolize depletion of the L1 gene though Cre-recombination activity in $Syn^{Cre}L1^{fl/fl}$ mice. (B) Representative immunoblots showing L1 expression in different brain regions of Syn^{Cre} controls and $Syn^{Cre}L1^{fl/fl}$ littermates. L1 (~220 kDa), and GAPDH loading control (~37 kDa). Analyzed CNS parts, ctx: cortex, hip: hippocampus, cb: cerebellum, bs: brain stem, sc: spinal cord. (C) Densitometric immunoblot quantification of L1 expression normalized to GAPDH in $Syn^{Cre}L1^{fl/fl}$ relative to Syn^{Cre} control mice (set as 100 %, n≥4/genotype). (D) Composite neuroscore showing the pre-experimental absence of neurological signs in Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice (maximum score: 8, n=10/group). (E-J) anti-L1/anti-βIII-tubulin double-immunostaining of the ventral spinal cord from $Syn^{Cre}L1^{fl/fl}$ and Syn^{Cre} mice. White matter in detail (F, I) and grey matter regions (G, J). Values are displayed as mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (**$p<0.01$). Scale: 100 µm (E, G).

3.8 Fluorescent reporter mice indicate Cre-recombination events

Crossing mice from the $Syn^{Cre}L1^{fl/fl}$ line with transgenic double-fluorescent reporter mice enabled an unambiguous characterization of synapsin-Cre-mediated recombination events in different regions of the CNS. Driven by an actin promoter, the dsRed fluorescent protein is ubiquitously expressed in all cells where Syn^{Cre}-mediated recombination is absent. The dsRED gene insert is, similarly to the L1 gene, flanked by floxP sides, which allows to indicate Syn^{Cre}-recombination events in neurons by the exchange of dsRED to eGFP expression [224].

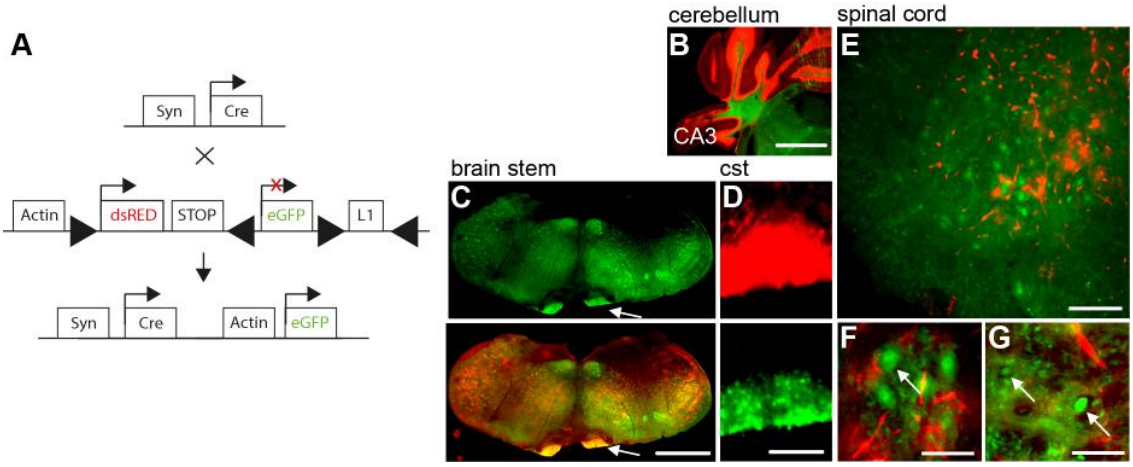

Fig. 3.8 Double fluorescent reporter mice display Syn^{Cre}-mediated recombination events

(A) Crossing scheme of Syn^{Cre}L1$^{fl/fl}$ animals with double fluorescent report mice (β-Actin-dsRED$^{fl/fl}$-eGFP). (B-G) Images showing Syn^{Cre}-mediated recombination events in different brain regions as indicated by the exchange of red (dsRED) to green (eGFP) fluorescence. (B-D) Cre-mediated recombination events in the cerebellum, brain stem and the corticospinal tract. (E-G) Cre-mediated recombination events in the ventral spinal cord (E), magnifications of motor neurons (F) and white matter axons (G). Scale: 1 mm (B, C), 200 µm (D), 50 µm (E, F), 5 µm (G).

The fluorescent reporter system indicated that there was no Cre-recombination in the cerebellum (Fig. 3.8B), which has been associated with the neurological phenotype of constitutive L1-deficient mice [225, 226]. Slices of the brain stem display a widespread distribution of recombination (Fig. 3.8C), including the corticospinal tract. The detailed view however revealed a clear majority of dsRED-fluorescent axons and a minor number of axons affected by recombination (Fig. 3.8D). In the spinal cord, the eGFP expression indicates an extended number of cells affected by recombination (Fig. 3.8E), including motor neurons (Fig. 3.8F) and axons (Fig. 3.8G).

Together with the findings shown in Fig. 3.7, Syn^{Cre}L1$^{fl/fl}$ mice did not display obvious neurological abnormalities but exhibited strongly reduced L1 protein expression in those regions that are major sites of axonal injury in the EAE mouse model.

3.9 Neuronal L1 depletion attenuates EAE severity and axonal injury

To test the hypothesis that L1 plays a role in CD4 T cell-mediated neuroinflammation, four independent cohorts of Syn^{Cre}L1$^{fl/fl}$ mice and their respective Syn^{Cre} control littermates were subjected to a MOG$_{35-55}$ peptide-immunized EAE. The clinical score of EAE mice were monitored in a daily fashion and summarized to a mean clinical score per genotype group. The strong association between immune cell infiltration, disease

induction and progression raised the question whether there are differences between the CNS-invading immune cell subsets of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice. To address this question, we analyzed the number of infiltrated cells in the CNS during EAE by processing CNS tissue from Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice for flow cytometry analysis by means of subset-specific cell surface markers. CD4 T cell subsets were distinguished by their subset-specific cytokine expression and specific transcription factors.

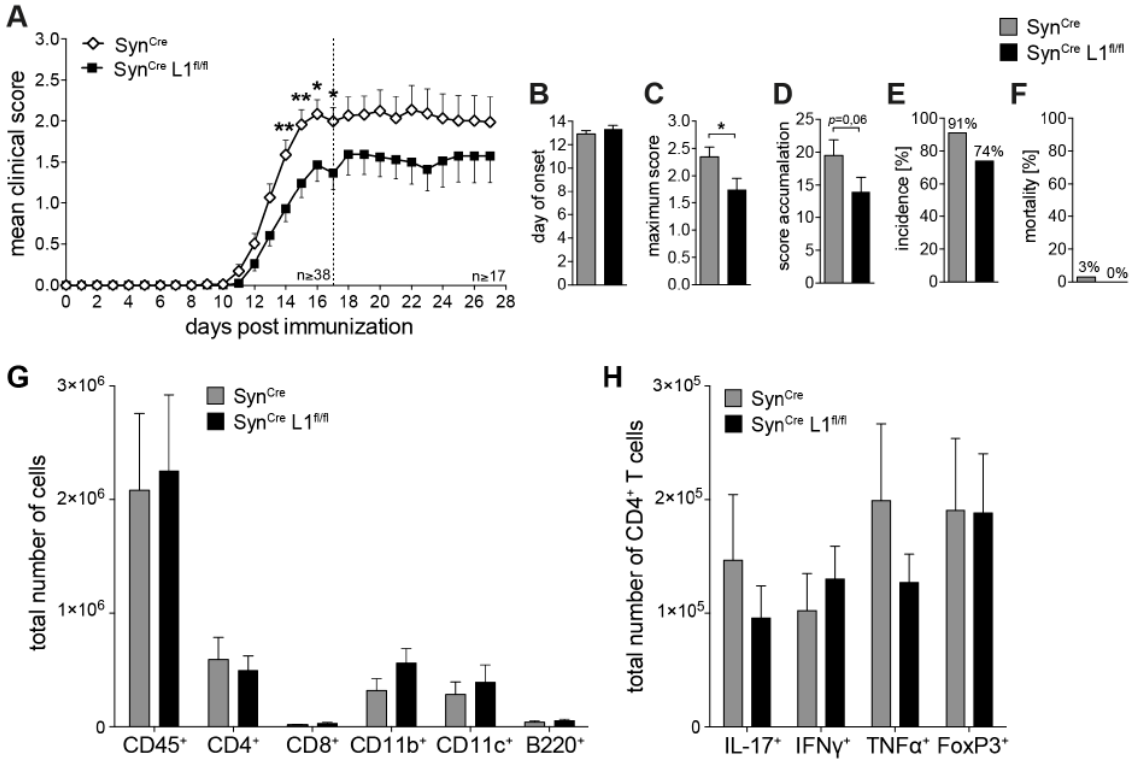

Fig. 3.9 Neuronal L1-depletion attenuates EAE severity despite unchanged immune cell infiltration

(A) EAE progression curve following MOG_{35-55} induced EAE induction in $Syn^{Cre}L1^{fl/fl}$ mice and Syn^{Cre} control animals. Data points indicate mean clinical scores of all mice per group including mice of four independent experimental cohorts. (B-E) Diagrams display specific EAE progression characteristics in detail including the day of onset (B), maximum score (C), score accumulation (D), disease incidence (F) and mortality (E). (G, H) Total number of $CD45^+$ cells and respective subpopulations isolated from CNS. (G) Flow cytometry analysis of CNS infiltrated immune cells during EAE. (G) Total number of immune cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/living cells (IP^-)/$CD45^+$ and $CD4^+$, $CD8^+$, $CD11b^+$, $CD11c^+$ or $B220^+$ (n=5/subset). (H) Total cell number of CD4 T cell subset cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/$CD45^+$/$CD4^+$ and $IL\text{-}17^+$, $IFN\gamma^+$, $TNF\alpha^+$or $FoxP3^+$ (n=5/subset). Values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*$p<0.05$; **$p<0.01$).

$Syn^{Cre}L1^{fl/fl}$ mice exhibited an attenuated mean clinical score in comparison to Syn^{Cre} controls. The significant differences occurred from day 14 to day 17 after active immunization. The beneficial effect of the neuronal L1 depletion was observed in four individual cohorts and the grouped data. The progression curve of both groups displays a chronic course after reaching the peak stage of the disease and does not show considerable signs of remission (Fig. 3.9A). The day of onset represents the first clinical symptoms, was the same for Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice (Fig. 3.9B). In contrary, there were significant differences of the maximum score, which represent the mean of the highest score reached over the entire EAE progression (Fig. 3.9C). A similar trend was demonstrated by the mean score accumulation (Fig. 3.9D). Slight differences of the disease incidence and mortality were observed between Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice (Fig. 3.9E, F). The flow cytometry analysis of CNS-infiltrating immune cells revealed no alterations in the number of immune cells, including T cells, macrophages, DCs, B cells, and different CD4 T cell subsets in Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice (Fig. 3.9G, H).

3.10 Gene expression profiles of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice during EAE

The gene expression analysis of C57Bl/6J mice in EAE and in *in vitro* experiments with primary neurons in co-culture with stimulated CD4 T cells revealed a reduction of L1 expression that coincides with an increased expression of REST. Gene expression analysis clarified, if there are differences *L1* and *REST* expression and between important inflammatory marker between Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice during EAE.

Spinal cords were obtained at the peak stage of EAE disease and processed for mRNA expression analysis using quantitative real-time PCR (qRT-PCR) to determine *L1* and *REST* expression. *L1* and *REST* were either analyzed in samples that represent the mean clinical score of the respective group or in a score-matched fashion to enable a score-dependent and independent analysis of *L1* and *REST* regulation during inflammatory conditions in the CNS. The *L1* expression level in naïve Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice confirmed the *L1* depletion in $Syn^{Cre}L1^{fl/fl}$ animals on mRNA level (Fig. 3.10A), which was shown on protein level using immunoblotting (Fig. 3.7B). *L1* mRNA expression in $Syn^{Cre}L1^{fl/fl}$ mice was reduced to one third of the expression level in naïve Syn^{Cre} controls. The experiments with $Syn^{Cre}L1^{fl/fl}$ mice in EAE and respective control animals also confirmed the results obtained with WT C57B/6J mice, which indicated a significant reduction of *L1* expression in the spinal cord of EAE animals at the peak stage of the

disease (Fig. 3.2B, C). Moreover, the gene expression analysis of the *L1* repressor molecule *REST* indicated a significant up-regulation of *REST* in Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice at the peak of EAE (Fig. 3.10A), thus confirming the results shown previously (Fig. 3.2D). Interestingly, the diagrams that display animals with a matched EAE score (mean score: 2.1 in both groups) revealed an EAE-induced reduction of *L1* in Syn^{Cre} mice that, however, remains significantly higher than in $Syn^{Cre}L1^{fl/fl}$ mice during EAE (Fig. 3.10B). These findings emphasize that even though L1 is significantly reduced in all mice during EAE, there is still a significant difference of *L1* expression between Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ at the peak of EAE. The genotype-dependent reduction of *L1* expression does not impact the *REST* expression in mice in the control and/or EAE group (Fig. 3.10B).

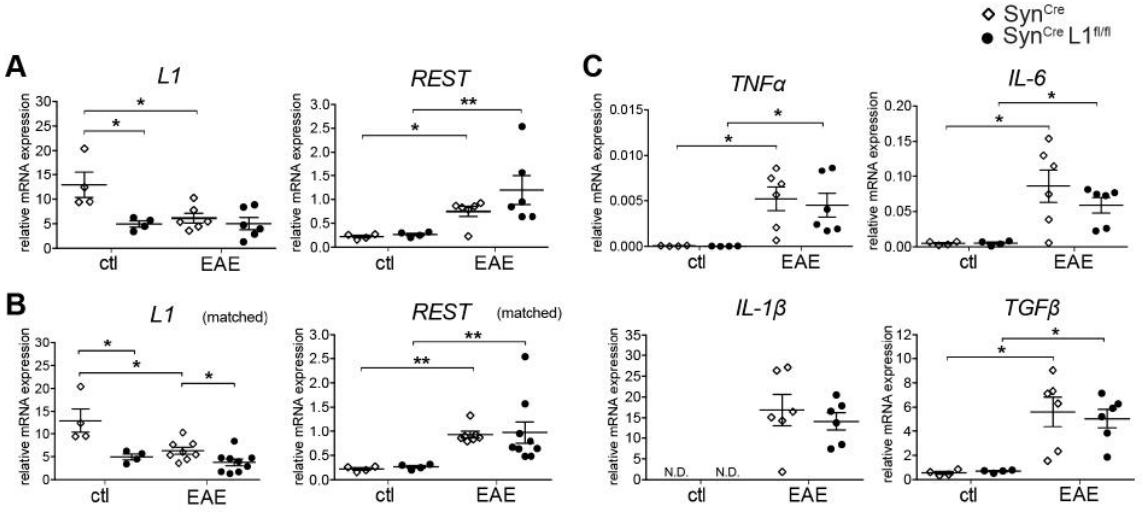

Fig. 3.10 Gene expression analysis of sham and EAE treated Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice

(A,B) mRNA expression assay of *L1* and *REST* at EAE disease peak, (A) between sham (ctl, n=4/genotype) and EAE animals (n=6/genotype) with genotype-related mean clinical scores (Fig. 3.9A, Syn^{Cre} score: 2.2; $Syn^{Cre}L1^{fl/fl}$ score: 1.6) or (B) of controls (n=4/genotype) and EAE animals with matched mean clinical scores (mean score of both groups: 2.1, n=9/genotype). (C) mRNA expression assay of inflammatory cytokines *TNFα, IL-6, IL-1β* and *TGFβ* at EAE disease peak. All diagrams display control (n=4/genotype) and EAE groups (n=6/genotype) of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice with genotype-related mean clinical scores (Fig. 3.9A, Syn^{Cre} score: 2.2; $Syn^{Cre}L1^{fl/fl}$ score: 1.6). Data points represent individual animals and bars represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (**p*<0.05; ***p*<0.01).

The mRNA expression of the pro-inflammatory cytokines *TNFα*, *IL-6, IL-1β,* and the anti-inflammatory cytokine *TGFβ* was significantly increased during EAE, indicating the ongoing inflammation in the spinal cord. However, it remained unchanged in comparison of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice (Fig. 3.10C) and thus implicated no genotype related impact on the ongoing inflammation during EAE.

3.11 Neuronal L1 depletion alleviates axonal injury

Extensive infiltration of macrophages into the CNS is a critical characteristic of MS in humans and in the EAE mouse model [227]. During EAE, CNS-infiltrating macrophages accumulate in high numbers and form plaques, which result in extended lesion sites in the gray matter of the spinal cord. The de-myelination of axons due to detrimental processes in EAE lesions is considered to be the major reason for the neuropathological outcome in the EAE model. Another factor contributing to the outcome of the EAE are inflammatory processes, induced by infiltrating immune cells that lead to axonal degeneration and loss [47]. To investigate these pathological factors, $Syn^{Cre}L1^{fl/fl}$ and Syn^{Cre} mice were sacrificed at the peak of EAE disease, and their spinal cords were processed to perform histological analysis using anti-macrophage antigen-3 (MAC3) immunostaining, demyelination-indicating luxol fast blue (LFB) staining, and immunostaining of the β-amyloid precursor protein (APP), which indicates degenerating axons. Neurofilament (NF) staining was further used to determine axon loss in EAE plaques that were identified by abnormal DAPI-stained cell nuclei accumulations and displayed as axon loss in EAE plaques relative to the number of axons in homotypical spinal cord regions without immune cell infiltration.

Anti-MAC3 immunohistochemistry indicates areas of infiltrating macrophages and CNS-resident microglia cells. Acute EAE plaques are characterized by an extended MAC3 immunoreactivity [227]. Accordingly, spinal cord slices of Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice exhibited large areas of MAC3 immunoreactivity with a trend towards a reduced MAC3-cell infiltration in $Syn^{Cre}L1^{fl/fl}$ mice in comparison to Syn^{Cre} controls, which was, however, not significant (Fig. 3.11A). The LFP staining revealed a similar result with demyelinated plaques in both groups and a trend towards a reduced demyelination in $Syn^{Cre}L1^{fl/fl}$ mice (Fig. 3.11B). Degenerated axons were identified by the accumulation of APP, displayed by anti-APP antibody-immunoreactive spheroids in EAE plaques in the spinal cord. The analysis revealed significantly reduced numbers of APP-immunoreactive axons within plaques of $Syn^{Cre}L1^{fl/fl}$ mice in comparison to Syn^{Cre} animals (Fig. 3.11C). The number of axons was dramatically decreased in EAE plaques of both groups; however, the axon loss was less pronounced in $Syn^{Cre}L1^{fl/fl}$ (63 ± 2 %, mean ± SEM) when compared to Syn^{Cre} mice [73 ± 2 %, mean ± SEM (Fig. 3.11D)|. Thus, the results suggest that the neuronal depletion of L1 did not affect immune cell infiltration or level of demyelination but attenuated axonal degeneration and loss in neuroinflammation.

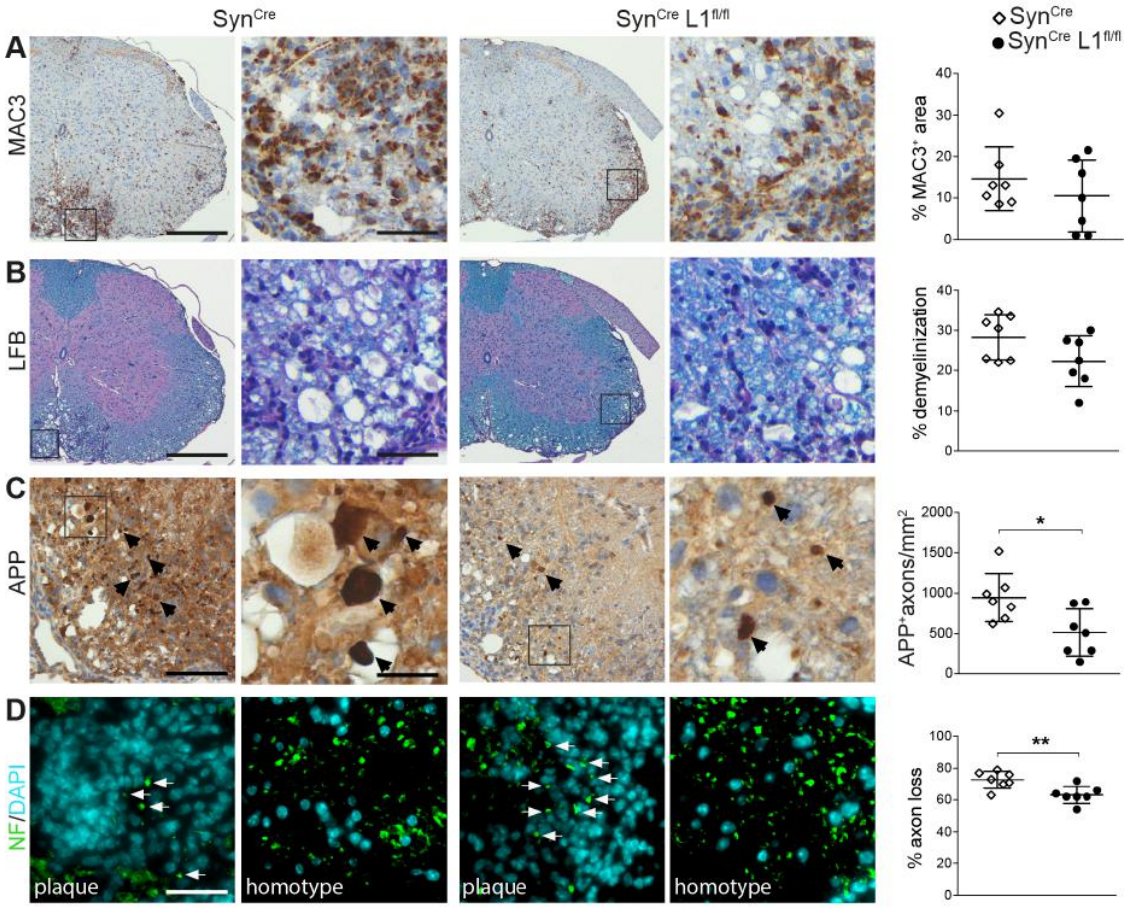

Fig. 3.11 Neuronal L1 depletion alleviates EAE induced axon degeneration and loss

Histologic assessment of spinal cord sections from Syn^{Cre} and $Syn^{Cre}L1^{fl/fl}$ mice at peak of MOG_{35-55} induced EAE. (A) Anti-macrophage antigen-3 (MAC3) immunohistochemistry indicates infiltrated/migrated macrophages and microglia. Quantitative assessment of the MAC3 immunoreactive area relative to the total grey matter area of spinal cord sections (set as 100 %, n=7/condition). (B) Areas missing the Luxol Fast Blue (LFB) staining indicate demyelination within the gray matter of the spinal cord. The quantification displays the demyelinated area relative to the total gray matter area (set as 100 %, n=7/group). (C) Anti-APP immunohistochemistry indicates degenerating axons by means of accumulating APP spheroids (arrow) at lesion sites (n=7/group). (D) Pan-NF staining indicates axons at sides of EAE lesions and homotypic areas without infiltrated cells. Lesions were identified by atypical cell accumulation indicated by DAPI-stained nuclei. Axon loss represents pan-NF immunoreactive axons in lesions normalized to pan-NF immunoreactive axons in homotypic areas (set as 100 %, n=7/group). All data points represent individual animals and bars represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*p<0.05, **p<0.01). Scale bar: 500 µm (A, B), 50 µm (detail A, B), 50 µm (C, D) 10 µm (detail C).

3.12 Transgenic overexpression of L1 did not influence EAE severity

Experiments with an L1-blocking antibody and with mice depleted in neuronal L1 using EAE revealed a significantly attenuated disease severity. These results suggested that increased levels of L1 may lead to a more severe disease progression in EAE. To test this hypothesis, transgenic mice which carry the entire human *L1* gene sequence knockin driven by the normal murine *L1* promoter (L1 tg M3) were induced with MOG_{35-55} and the EAE progression compared to WT control animals.

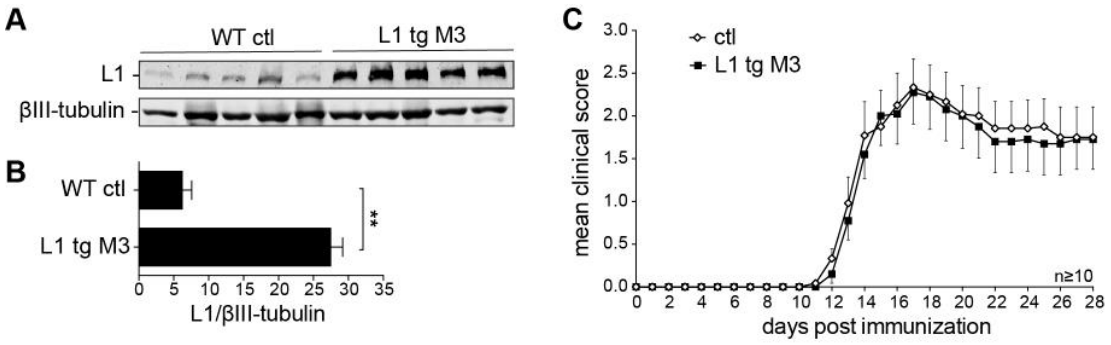

Fig. 3.12 Overexpression of L1 did not influence EAE severity

(A, B) Immunoblot of WT control and L1 overexpressing mice (L1 tg M3) demonstrates marked overexpression of full-length L1 protein (~220 kDa) in the spinal cord of L1 tg M3 mice. βIII-tubulin (~55 kDa) served as loading control. (B) Densitometric quantification of the immunoblot for L1 expression normalized to βIII-tubulin (n=5/group). (C) EAE progression curve following MOG_{35-55} induced EAE in L1 tg M3 and WT control mice (n≥10/group). Data points indicate the mean clinical scores of all mice per group. All values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (**$p<0.01$).

The immunoblotting revealed a substantial increase of L1 expression in the spinal cord of L1 tg M3 mice compared (27.5 ± 1.7; mean AU ± SEM) to WT control mice (6.2 ± 1.3; mean AU ± SEM) (Fig. 3.12A, B). The overexpression of L1 did not cause any differences in the onset or progression of the EAE disease. Both groups reached the peak of disease at the same point of time after immunization and did not exhibit differences during the chronic disease progression or remission phase (Fig. 3.12C). These results indicate that increased L1 expression levels do not aggravate disease severity in EAE.

3.13 Non-neuronal cells display L1 expression in MS and EAE lesions

Even though L1 is predominantly considered to be a neuronal protein, there are evidences of human and rodent peripheral lymphocytes expressing L1 under specific conditions [182, 185]. However, to our knowledge no studies have addressed L1 expression in non-neuronal cells during multiple sclerosis or in the EAE mouse model. Therefore, immunohistochemical experiments with cortex slices of human MS patients were performed to ascertain whether L1 expression also occurs in chronic active MS lesions by CNS-infiltrating immune cells or CNS-resident non-neuronal cells. Moreover, EAE plaques in the spinal cord, which are identified by substantial CD4 T cell infiltrates, were examined for L1 expression by immunohistochemistry.

Immunohistochemistry in slices of chronic active MS lesions revealed L1-expressing cells in the so-called “active rim” area (Fig. 3.13A). The active rim area is a radially expanding area that is characterized by a strong presence of macrophages and large areas of demyelination. It is located at the advancing edge of the plaque around the inactive lesion center. The surrounding tissue is referred to as normal-appearing white matter (NAWM) [40]. L1-expressing cells in the active rim area displayed an amoeboid morphology with few extensions, suggesting the cells to be microglia, macrophages and/or dendritic cells (Fig. 3.13B, C). Double-immunostaining indicated that L1 is expressed in MHC II-immunolabeled cells (Fig. 3.13D). Together with their morphological appearance this observation, suggested that L1-expressing cells are activated macrophages.

Similarly to the situation in chronic MS lesions, immunohistochemistry of spinal cord sections of mice during EAE revealed strong L1 expression in acute plaques. In line with the morphologic observations in chronic active MS lesions, the morphology of L1-expressing cells in acute EAE plaques suggests them not to be of neuronal origin (Fig. 3.13E).

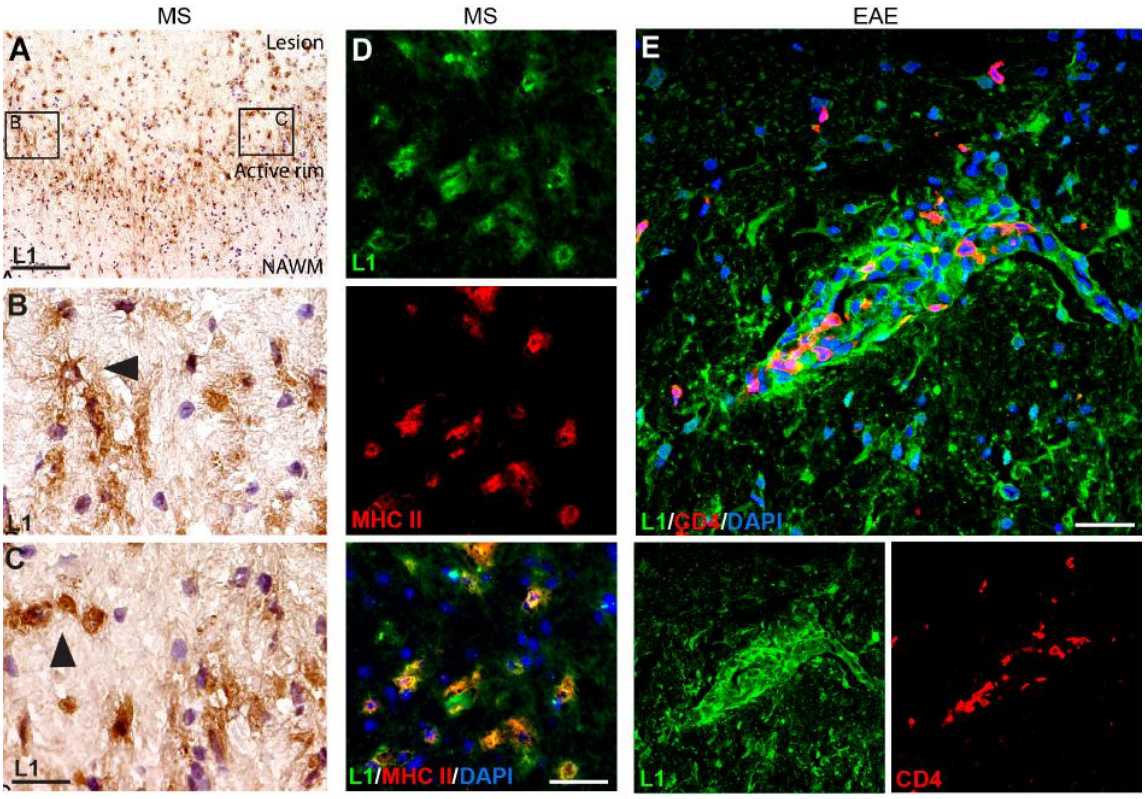

Fig. 3.13 Non-neuronal cells display L1 expression in MS and EAE lesions

(A) Immunohistochemical analysis of brain slices from MS patients showing differential L1 expression within the normal appearing white matter (NAWM) and sites of chronic lesions, including the active rim area. (B, C) High-power magnification of L1 expressing cells within the active rim area in chronic MS lesions. (D) Double-immunostaining of brain slices from human MS patients using antibodies against L1 and MHC II. (E) Representative images of double-immunostained sections from C57BL/6J mice at peak stage of MOG_{35-55} induced EAE using antibodies against L1 and CD4. Scale bar: 200 µm (A), 10 µm (C, D), 20 µm (E).

3.14 L1 expression in EAE lesions is not co-localized with GFAP

Astrocytes are the most abundant cell type in the CNS. They do not express MHC II under healthy conditions, but have been reported to exhibit expression of MHC II during specific inflammatory conditions [228]. Our results indicated a pronounced L1 expression in chronic MS lesions and acute EAE plaques (Fig. 3.13). In acute EAE plaques, MHC II expressing cells include CNS-resident cells like astrocytes and microglia, and infiltrated lymphocytes such as T cells, DCs and macrophages. To identify or exclude potential cell populations that express L1, spinal cord slices taken from mice at the peak stage of EAE were analyzed using immunohistochemistry in order to detect L1 in combination with the astrocyte marker - glial fibrillary acidic protein (GFAP).

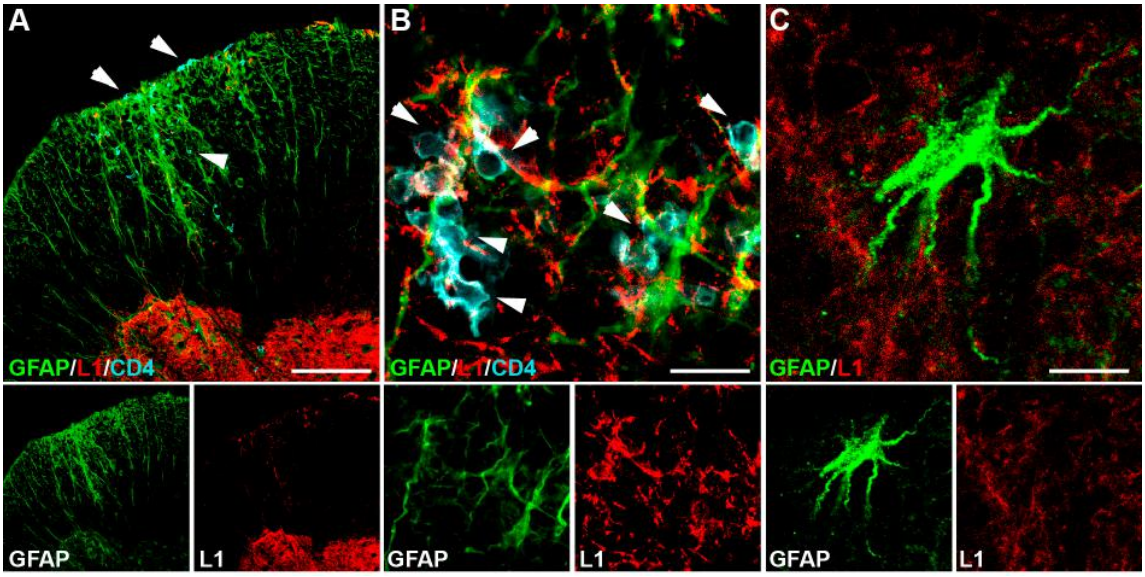

Fig. 3.14 L1 expression in EAE lesions is not co-localized with GFAP

(A-C) Triple-immunostaining of spinal cord sections from C57BL/6J mice at peak stage of the MOG_{35-55} induced EAE using antibodies against L1, CD4 and GFAP. EAE plaques were identified by extensive CD4 T cell infiltration (arrows). Scale bar: 100 µm (A), 20 µm (B), 5 µm (C).

The most pronounced L1 expression in spinal cord sections were observed in gray matter regions with no GFAP-immunoreactive astrocytes (Fig. 3.14 A). A more detailed view of EAE lesion sites revealed L1 immunoreactive cells, which were, however, not co-localized with GFAP-immunoreactive astrocytes (Fig. 3.14B, C). Therefore, astrocytes could be ruled out as L1-expressing cells in acute EAE plaques.

3.15 L1 expression in EAE plaques is co-localized with Iba1

Macrophages and microglia (which are also called "brain macrophages") are very important players in EAE induction and progression as they have the ability to produce and secrete inflammatory mediators, and activate and differentiate T cells via antigen presentation. Previous studies reported that depletion of macrophages and microglia results in a marked attenuation of EAE severity [227]. With regards to the morphologic features of L1-expressing cells seen in EAE plaques and chronic MS lesions (Fig. 3.13A, E) and the co-expression of L1 and MHC II in respective MS plaques (Fig. 3.13D), microglia/macrophages appeared likely to be the cell population expressing L1. Immunohistochemical experiments were carried out using double-immunolabelling for L1 and the ionized calcium-binding adaptor molecule 1 (Iba1) as the most common marker to identify microglia/macrophages to determine possible L1 expression in these cells in spinal cord plaques of mice at the peak of EAE.

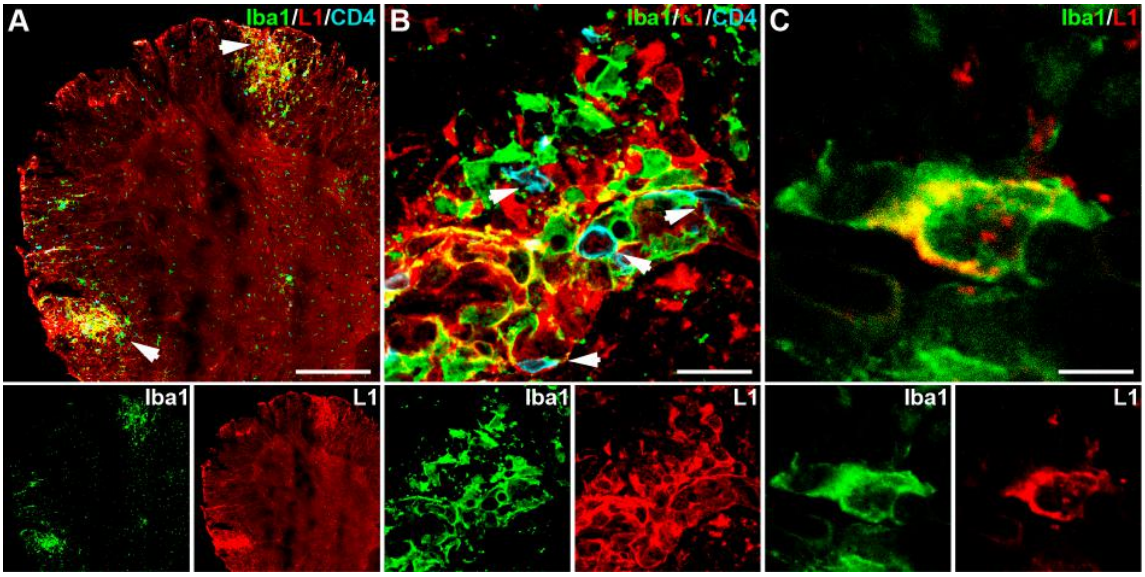

Fig. 3.15 L1 expression in EAE plaques is co-localized with Iba1

(A-C) Triple-immunostaining of spinal cord sections from C57BL/6J mice at peak stage of the MOG_{35-55} induced EAE using antibodies against L1, CD4 and the microglia/macrophage marker Iba1. EAE plaques were identified by extensive CD4 T cell infiltration (arrows). Scale bar: 200 µm (A), 20 µm (B), 5 µm (C).

The immunohistochemical experiments revealed that some, even though not all, of the Iba1-immunoreactive cells express L1 (Fig. 3.15A-C). These findings suggest that microglia/macrophages do not express L1 in a constitutive fashion, but rather induce L1 expression under specific conditions. To address this issue, primary microglia and bone marrow-derived macrophages were treated with different inflammatory stimuli for 24 hours and tested for L1 expression using flow cytometry. Lipopolysaccharide (LPS) is considered to be one of the most competent pro-inflammatory stimuli of cells that express the toll-like receptor 4 (TLR4), including microglia and macrophages. Moreover, the cells were stimulated with the pro-inflammatory cytokines IFNγ, TNFα, and IL-1β. The macrophage colony-stimulating factor (M-CSF) and the granulocyte-macrophage colony-stimulating factor (GM-CSF) are growth factors, which cause stem cell differentiation into macrophages, DCs, and granulocytes, induce cell proliferation and survival, and are also potent stimulatory factors during inflammatory processes. IL-4, IL-13, IL-10, and TGFβ have predominantly anti-inflammatory and regulatory features.

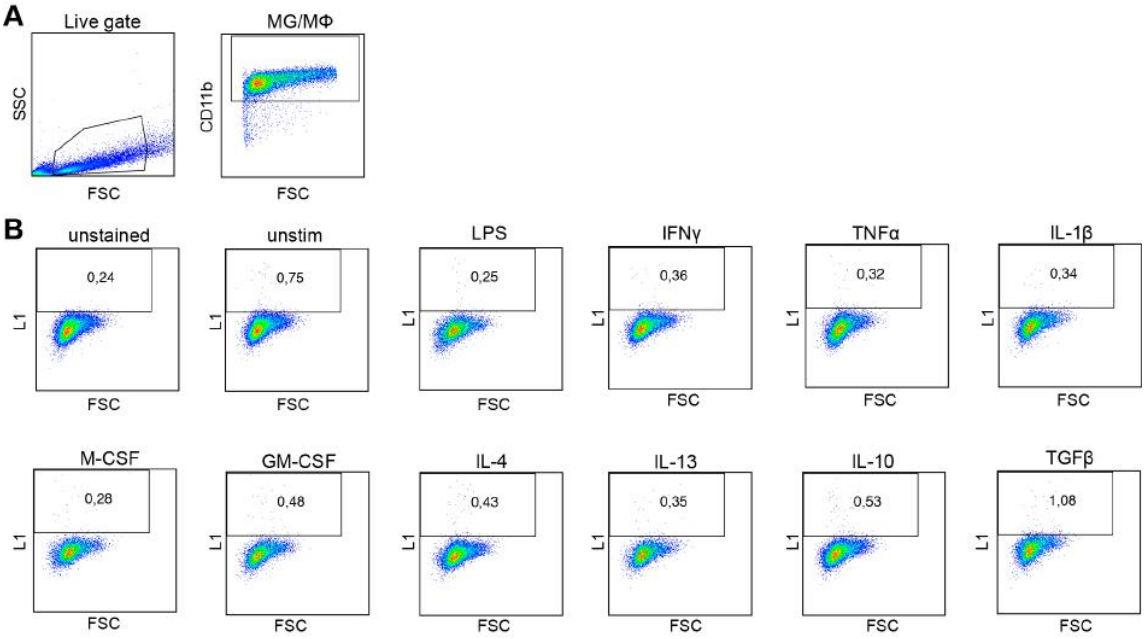

Fig. 3.16 Microglia and macrophages do not express L1 after *in vitro* stimulation with inflammatory factors
Representative flow cytometry analysis of primary microglia cultures (MG) and bone marrow derived macrophages (Mϕ). (A, B) Gating strategy: live gate (FSC-SSC)/CD11b^{+}/L1^{+}. (B) L1 expression analysis of microglia and macrophages unstimulated and after stimulation with inflammatory factors for 24 h.

Primary microglia and macrophages were cultured, differentiated, and stimulated with inflammatory mediators; however, the induction of L1 expression failed. Stimulating the cultured cells with LPS or pro-inflammatory cytokines IFNγ, TNFα, IL-1β, and growth factors M-CSF and GM-CSF ascertained that none of the mentioned factors induced L1 expression. Stimulation with the anti-inflammatory cytokines IL-4, IL-13, IL-10, and TGFβ also did not induce any L1 expression in the respective cells (Fig. 3.16B).

3.16 L1 depletion in microglia/macrophages attenuates EAE severity

Our immunohistochemical experiments revealed clusters of L1 expressing cells in EAE plaques and suggested that L1 expression is expressed by microglia and/or macrophages (Fig. 3.13E). To date, very little is known about the conditions and functional implications of L1 expression in microglia/macrophages. Mice that are transgene for a Cre-recombinase driven by the LysM promoter (LyM^{Cre}) are an appreciated model to specifically deplete genes in microglia and/or macrophages [229, 230]. To test the hypothesis that L1 expressed in microglia/macrophages plays a role during EAE-induced neuroinflammation, LysM^{Cre}L1$^{fl/fl}$ and LysM^{Cre} littermate control

mice were subjected to MOG_{35-55} immunized EAE. The clinical score of EAE progression was monitored daily and summarized to a mean clinical score per genotype group. The microglia/macrophage-specific depletion of L1 raised the question whether there are differences in the number of infiltrated macrophages or the associated T cell populations. To address this question, we analyzed the number of cells from specific immune cell subsets in the CNS during EAE by processing CNS tissue of both genotype groups by flow cytometry. CD4 T cell subsets were distinguished by their subset-specific cytokine expression and transcription factors.

The $LysM^{Cre}L1^{fl/fl}$ mice exhibited attenuated EAE severity in comparison to $LysM^{Cre}$ control animals. Significant differences of the EAE severity between genotype groups were clearly observable at the peak stage from day 17 after MOG_{35-55} immunization and over the entire chronic disease progression. The progression curves of both groups display a chronicle course after reaching the peak stage and exhibit only slight signs of remission with no differences between $LysM^{Cre}$ and $LysM^{Cre}L1^{fl/fl}$ mice. The beneficial effect of L1 depletion in microglia/macrophages was observed in two individual cohorts and the grouped data (Fig. 3.17A). The day of onset was the same between $LysM^{Cre}$ and $LysM^{Cre}L1^{fl/fl}$ mice (Fig. 3.17B). In contrary, we found significant differences in the maximum EAE score (Fig. 3.17C). Similar results were observed in the score accumulation and $LysM^{Cre}$ mice exhibited a slightly higher EAE incidence rate and mortality than $LysM^{Cre}L1^{fl/fl}$ animals (Fig. 3.17E, F). The flow cytometry analysis of CNS-infiltrating immune cells indicated a significant reduction in the amount of CD4 T cells in $LysM^{Cre}L1^{fl/fl}$ mice. None of the other analyzed cell populations showed any significant differences between both groups, including $CD8^+$ cytotoxic T cells, $CD11b^+$ microglia and macrophages, $CD11c^+$ dendritic cells, $B220^+$ B cell subpopulation, and MHC II^+ positive antigen-presenting cells (Fig. 3.17G). Even though the number of infiltrating CD4 T cells had diminished, CD4 T cell subpopulations were not significantly different in $LysM^{Cre}L1^{fl/fl}$ and $LysM^{Cre}$ mice, but exhibited the same trend towards reduced cell numbers as the overall CD4 T cell population (Fig. 3.17H). These results suggested that EAE-induced L1 expression in microglia/macrophages contributes to EAE disease severity.

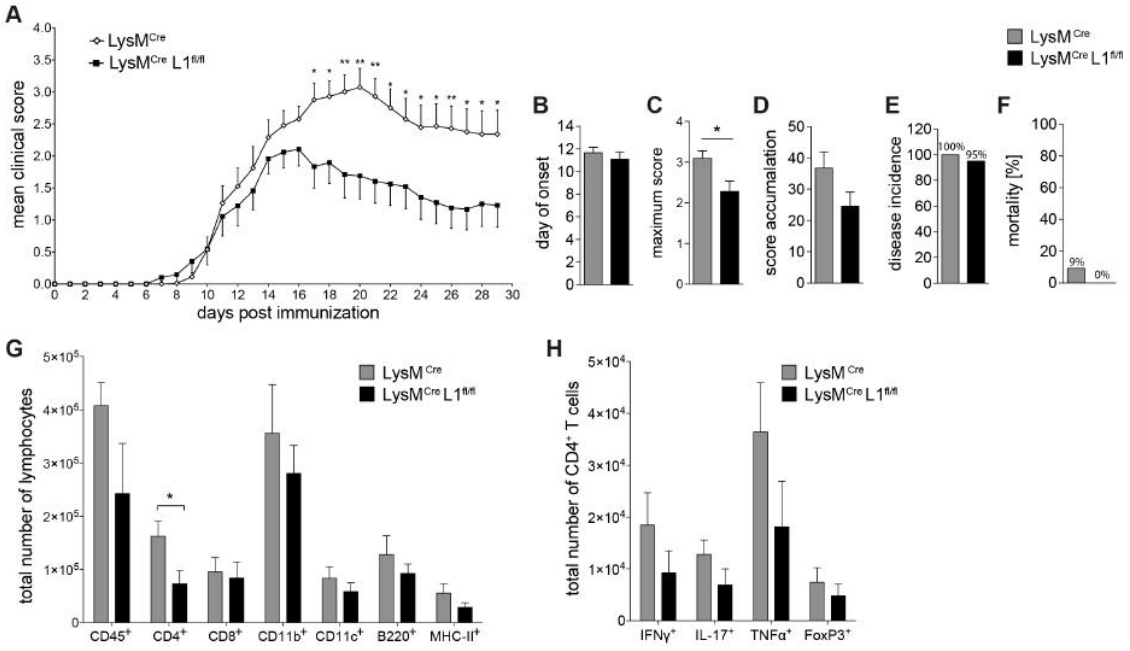

Fig. 3.17 L1 depletion in microglia/macrophages attenuates EAE severity

(A) Diagram displays EAE progression curve following MOG_{35-55} immunized active EAE in $LysM^{Cre}L1^{fl/fl}$ and $LysM^{Cre}$ control mice. Data points indicate the mean clinical scores of all mice per group including two independent experimental cohorts ($LysM^{Cre}$ n≥17, $LysM^{Cre}L1^{fl/fl}$ n≥14). (B-E) Diagrams exhibit specific EAE progression characteristics in detail, including day of disease onset (B), maximum score (C), score accumulation (D), disease incidence (F) and mortality (E). (G, H) Flow cytometry analysis of CNS infiltrated immune cells during EAE. (G) Total number of immune cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/living cells (IP^-)/$CD45^+$ and $CD4^+$, $CD8^+$, $CD11b^+$, $CD11c^+$, $B220^+$ and MHC II^+ cells (n=5/subset). (H) Total cell number of CD4 T cell subset cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/$CD45^+$/$CD4^+$ and $IFN\gamma^+$, IL-17^+, $TNF\alpha^+$ or $FoxP3^+$ (n=5/subset). Values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*$p<0.05$; **$p<0.01$).

3.17 L1 expression is co-localized with CD11c in the spinal cord during EAE

DCs are considered to be the most efficient professional antigen-presenting cell population with a constitutive expression of MHC II [59]. DCs have been shown to influence EAE induction and progression by presenting myelin antigens to CD4 T cells in peripheral lymph nodes and by re-activating infiltrated CD4 T cells in the CNS [79, 231]. Furthermore, previous work demonstrated L1 expression in peripheral DCs of the skin (Langerhans cells) [182]. Thus, DCs potentially constitute a part of the L1-expressing cell population in acute EAE plaques. Immunohistochemical experiments using CD11c as the most common marker to identify DCs were used to determine the L1 expression of DCS within the murine spinal cord at the peak of EAE.

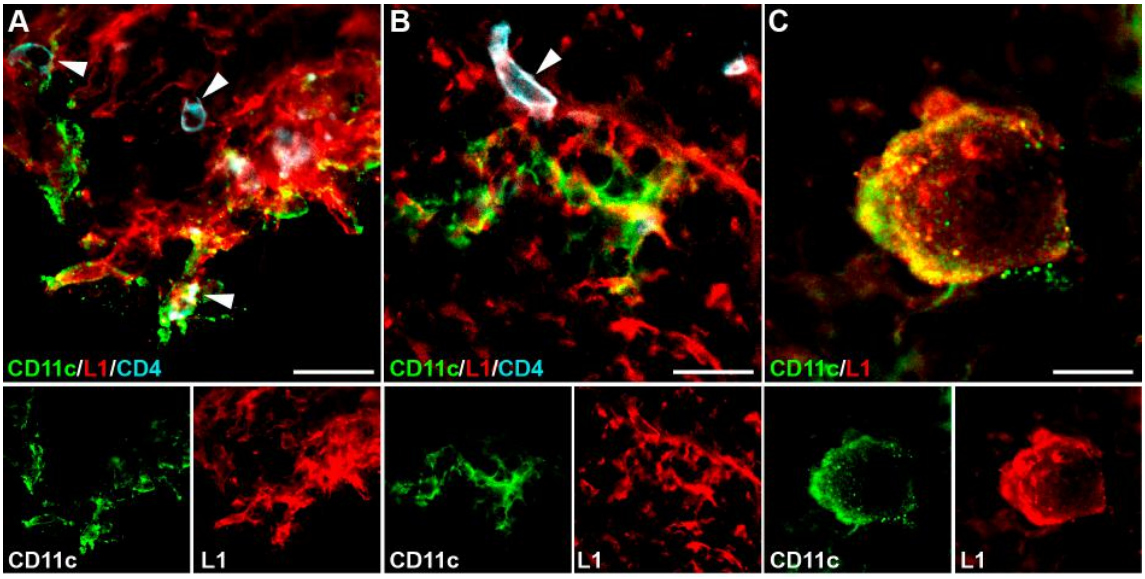

Fig. 3.18 L1 expression is co-localized with CD11c in the spinal cord during EAE

(A-C) Triple-immunostaining of spinal cord sections from C57BL/6J mice at peak stage of MOG_{35-55} induced EAE using antibodies against L1, CD4 and the dendritic cell marker CD11c. EAE plaques were identified by extensive CD4 T cell infiltration (arrows). Scale bar: 200 µm (A), 10 µm (B), 5 µm (C).

The immunohistochemical experiments revealed that most, even though not all, of the CD11c positive cells expressed L1 (Fig. 3.18A-C). These findings suggest that DCs are part of the observed L1-expressing cells.

3.18 Maturation of DCs coincides with L1 expression *in vitro*

Our immunohistochemical experiments revealed L1 expression in DCs under yet undetermined conditions or DC maturation state. To address this issue, the maturation of bone marrow-derived DCs was stimulated with LPS or TNFα treatment and tested for a maturation-related induction of L1 expression *in vitro*. The process of DCs maturation and activation is dynamic and does not allow to distinguish distinct cell populations. We used a basic classification model that divides immature DCs (iDC) and mature DCs (mDC) by means of MHC II and CD86 expression levels as both are considered to be reliable markers of the DC maturation status [232].

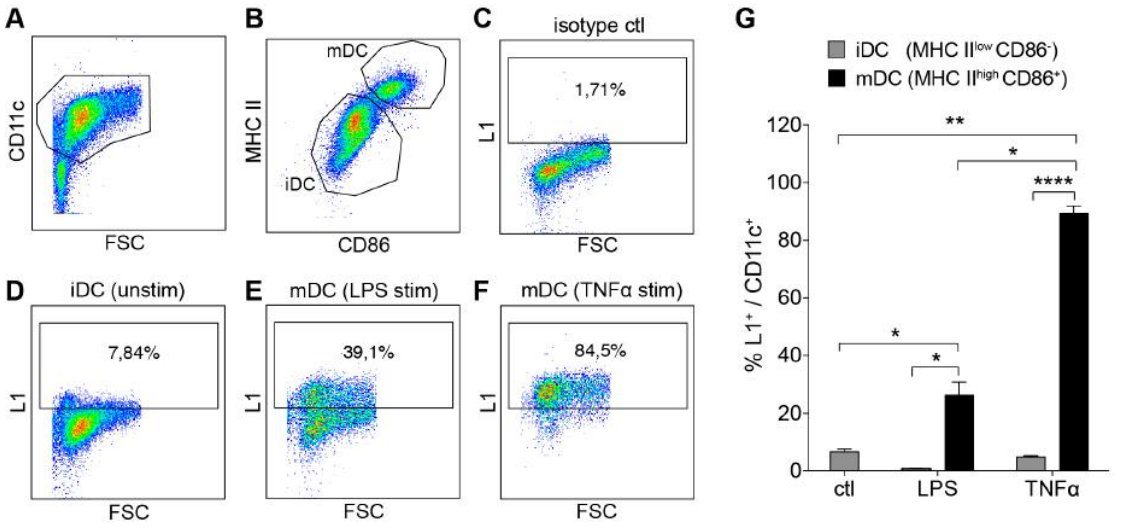

Fig. 3.19 TNFα and LPS induced maturation of DCs coincides with induction of L1 expression

(A-F) Representative flow cytometry analysis of the L1 expression in bone marrow derived DCs after LPS or TNFα induced maturation. Gating strategy: live gate (not shown), (A) $CD11c^+$, (B) MHC II^{low} $CD86^-$ were referred as immature DCs (iDC) and MHC II^{high} $CD86^+$ were referred as mature DCs (mDC). (C) L1 positive gating was defined using DCs stained with an isotype control antibody. (D) Representative L1 expression of unstimulated iDCs, (E) LPS stimulated mDCs, (F) TNFα stimulated mDCs. (G) Quantification of the flow cytometry data (ctl n=4, LPS: n=4/group, TNFα n=8/group). Values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*p<0.05; **p<0.01, ****p<0.0001).

The flow cytometry analysis of DCs revealed that L1 is induced after stimulation with LPS and TNFα. Immature DCs (MHC III^{low} $CD86^-$) do not express considerable level of L1 (Fig. 3.19D, G). The maturation of DCs (MHC II^{high} $CD86^+$) coincides with the L1 expression, both induced by LPS (Fig. 3.19E, G) and TNFα stimulation. Almost all TNFα-stimulated mature DCs occurred to be L1-positive (Fig. 3.19F, G).

3.19 L1 depletion in dendritic cells attenuates EAE severity

Our immunohistochemical experiments revealed clusters of L1-expressing cells in EAE plaques in the spinal cord and thus suggested that L1 is at least in parts expressed by DCs. Very little is known about the expression conditions and functional implications of L1 expression in DCs. However, some studies suggest that L1 is crucial for the endothelial transmigration of DCs in the periphery and hypothesize that there might bean impaired CNS-infiltration of L1-depleted DCs in EAE [182]. In contrast, plate-coated recombinant L1 was reported to possess co-stimulatory features during CD4 T cell activation and L1 co-stimulation was observed during the T cell response to

allogeneic L1 expressing DCs *in vitro* [186]. We generated a mouse line that lacks L1 expression in CD11c positive DCs by crossing mice that are transgenic for a CD11c promoter-driven Cre-recombinase ($CD11c^{Cre)}$ with mice that bear a floxP site flanked L1 gene ($CD11c^{Cre}L1^{fl/fl}$). The DC-specific L1 depletion was confirmed in bone marrow-derived DC cultures from $CD11c^{Cre}L1^{fl/fl}$ compared to $CD11c^{Cre}$ controls, which were stimulated for maturation by TNFα treatment and analyzed by flow cytometry. The flow cytometry analysis of L1 expression in TNFα-stimulated bone marrow-derived DCs revealed an apparent induction of L1 expression in cells obtained from $CD11c^{Cre}$ but not from $CD11c^{Cre}L1^{fl/fl}$ mice, which confirms the CD11c specific L1 depletion in the newly generated mouse line (Fig. 3.20A).

To test the hypothesis that L1 expression in DCs is involved in EAE pathology, $CD11c^{Cre}L1^{fl/fl}$ mice and $CD11c^{Cre}$ littermate controls were subjected to a MOG_{35-55} immunized EAE. Strikingly, $CD11c^{Cre}L1^{fl/fl}$ mice exhibited an attenuated EAE severity in comparison to $CD11c^{Cre}$ controls beginning at day 16 after MOG_{35-55} immunization and remaining over the entire chronic disease progression. The progression curve of both groups displayed a chronicle course with only slight signs of remission. The beneficial effect of DC L1 depletion was observed within two individual cohorts and all grouped data (Fig. 3.20B). The day of onset was the same for $CD11c^{Cre}$ and $CD11c^{Cre}L1^{fl/fl}$ mice (Fig. 3.20C). We observed significant differences in the maximum score (Fig. 3.20D) and score accumulation (Fig. 3.20E). $CD11c^{Cre}$ mice exhibited a slightly higher EAE incidence rate and mortality than $CD11c^{Cre}L1^{fl/fl}$ animals (Fig. 3.20F, G).

The flow cytometry analysis of immune cells that have infiltrated the CNS indicated a significant reduction of the number of $CD4^+$ T cells, $CD8^+$ T cells, and $CD11c^+$ DCs in $CD11c^{Cre}L1^{fl/fl}$ mice in comparison to $CD11c^{Cre}$ controls. Other cell populations did not show any significant differences between both groups, including $CD11b^+$ microglia/macrophages, $B220^+$ B cell populations, and MHC II^+ antigen-presenting cells (Fig. 3.20H). Even though the number of CNS-infiltrating CD4 cells was reduced, numbers of CD4 T cell subpopulations were not significantly different between $CD11c^{Cre}$ and $CD11c^{Cre}L1^{fl/fl}$ mice. However, they exhibited the same tendency of reduced cell numbers as the overall CD4 T cell population (Fig. 3.20I). Thus, L1 depletion in DCs attenuated the EAE severity and caused a reduced infiltration of T cells and DCs.

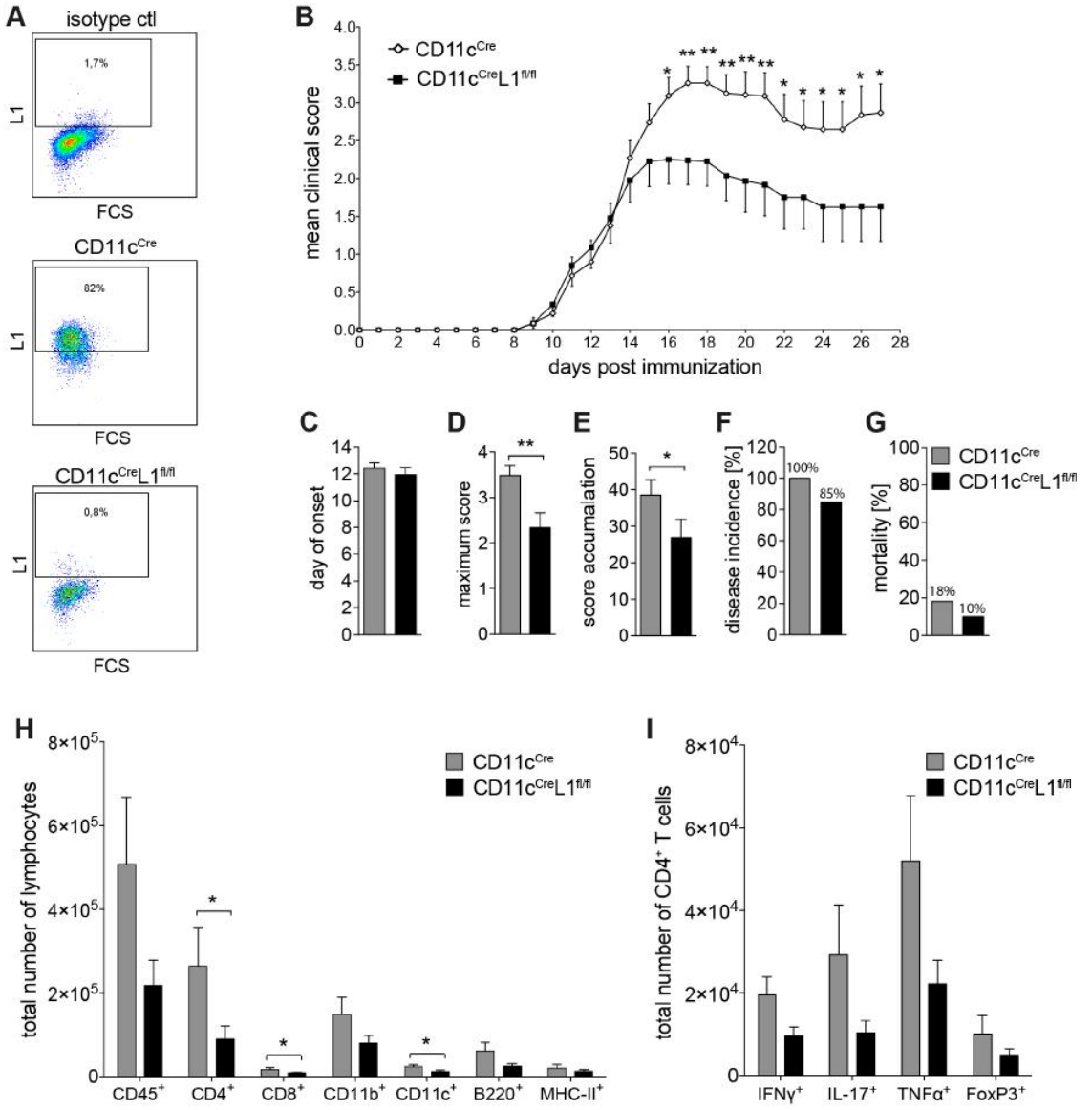

Fig. 3.20 L1 depletion in dendritic cells attenuates EAE severity

(A) Representative flow cytometry analysis of bone marrow derived DCs from CD11c^{Cre}L1$^{fl/fl}$ mice and CD11c^{Cre} controls, DCs were stimulated with TNFα to induce maturation and L1 expression. Gating strategy: live gate (FSC/SSC)/CD11c^{+} and L1^{+} expression related to cells stained with isotype control antibody. (B) Diagram showing EAE progression curve following MOG$_{35-55}$ induced EAE in CD11c^{Cre}L1$^{fl/fl}$ and CD11c^{Cre} control mice. Data points indicate the mean clinical scores of all mice per group including two independent experimental cohorts. (C) Diagrams display specific EAE progression characteristics in detail, including day of disease onset (C), maximum score (D), score accumulation (E), disease incidence (F) and mortality (G). (H, I) Flow cytometry analysis of CNS infiltrated immune cells during EAE. (H) Total number of immune cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/living cells (IP^{-})/CD45^{+} and CD4^{+}, CD8^{+}, CD11b^{+}, CD11c^{+}, B220^{+} and MHC II^{+} cells (n=5/subset). (I) Total cell number of CD4 T cell subset cells isolated from the CNS. Gating strategy: live gate (FSC-SSC)/CD45^{+}/CD4^{+} and IFNγ^{+}, IL-17^{+}, TNFα^{+} or FoxP3^{+} (n=5/subset). Values represent mean ± SEM, *p* values were calculated by Mann-Whitney *U* test (*p<0.05; **p<0.01).

4 Discussion

This thesis revealed a novel role of L1 in the crosstalk between the immune system and the CNS. Experiments with various conditional L1-deficient mouse lines subjected to EAE and neuron/T cell co-cultures implicate L1 in inflammatory processes and immune-mediated neuronal injury. The results further suggest a role of L1 in MS, the most common chronic inflammatory disease of the CNS.

MS causes devastating disabilities with only limited treatment options and no available cure. The initiatory event of the autoimmune pathology is the invasion of CNS by encephalitogenic T cells through the BBB, which causes focal areas of inflammation, demyelination, axonal damage, and neuronal loss during MS and also in the EAE mouse model [47, 48, 102, 233]. Cell adhesion molecule L1 is a neural IgSF member that is crucial in the CNS development. Although IgSF molecules contactin-2 [234] and neurofascin [235] which are closely related to L1 have been identified as autoimmune targets in the CNS, L1 itself has not been implicated in MS or related animal models of neuroinflammation. A great variety of deleterious mutations in the L1 gene locus has been described. They cause several neurological disorders such as X-linked hydrocephalus, spastic paraplegia and mental retardation, MASA syndrome and X-linked agenesis of the corpus callosum (jointly referred to as the L1-syndrome). The beneficial role of neuronal L1 in axon myelination [236], re-myelination processes [237], neuronal survival [238], locomotor recovery after neuron injury [239], and on long-term functional recovery after spinal cord injury has been shown in many studies by using transgenic L1 expression [154], recombinant L1 proteins [155], or small molecule L1 agonists [240]. Therefore, the function of L1 has been considered to ameliorate neural repair and regeneration in experimental lesion models. However, the role of L1 in these processes seems to depend on genetic background and age of the used model animals and on the cellular and molecular context of regenerating and sprouting axons [143].

To the best of our knowledge, L1 had not been investigated in neuroinflammation models such as EAE which mimics some aspects of MS.

In this study, L1 expression in neurons within gray matter and white matter lesions of MS patients was demonstrated for the first time. In line, we found that L1 is expressed in axons and infiltrating T cells in the murine spinal cord. Simultaneous expression of L1 both in neurons and T cells, which physically interact during neuroinflammation [102, 241], suggests a role of L1 in MS pathology. The results of this thesis show that

EAE-induced neuroinflammation in mice causes a massive CNS-infiltration of CD4 T cells, which coincides with an overall reduction of L1 expression in the spinal cord on both mRNA and protein level at peak of the disease. The remitting-relapse EAE model, induced by $PLP_{139-151}$ immunization, indicated an adaptive regulation of L1 expression, which correlated with different disease stages. Similarly to the situation during EAE, where the transcriptional repressor REST was conversely regulated to L1 expression, activated CD4 T cells co-cultured with primary neurons caused a down-regulation of neuronal L1 and concurrent up-regulation of REST and *in vitro*. In addition, antibody-mediated blocking of L1 reduced the number of T cells adhering to neurons, attenuated the T cell-induced axon degeneration *in vitro,* and mitigated EAE severity *in vivo*.

The fact that L1 is expressed in neurons and CD4 T cells along with its homophilic binding capacity [207] suggests L1-mediated cell-cell interaction during neuroinflammatory processes. Both MS in humans and EAE in mice are characterized by detrimental processes induced by infiltrating immune cells that cause axon degeneration and neuronal loss [37, 40, 47]. The attenuated EAE progression in the newly generated mice strain, exhibiting neuron-specific L1-depletion indicated that L1 down-regulation in response to neuroinflammation has a beneficial effect for the disease outcome. Moreover, L1 expression in mononuclear cells in chronic active MS lesions and in active EAE plaques further enforces the hypothesis that L1 is involved in neuroinflammation in the CNS. The cell type-specific L1 depletion in $LysM^+$ (microglia/macrophages) or $CD11c^+$ (DCs) cells in transgenic mice lines significantly alleviated the severity of EAE. These findings suggested an L1-mediated interaction between neurons and T cells and the involvement of L1 in the immunology of APCs; thereby L1 participates in both neuronal and immune processes during initiation and response to neuroinflammation.

4.1 L1 is expressed on axons in MS tissue and human T cells

The crucial role of neuronal L1 expression during the development of the CNS is unequivocal. Mutations in the L1 gene and its subsequent loss of function result in neurodevelopmental disorders jointly referred to as L1 syndrome [150, 242]. Although the neuronal L1 expression decreases during postnatal development towards maturity L1-expressing axons remain in the adulthood [243]. L1 expression in the cerebral cortex tissue and specifically in neuronal cells of healthy human adults has been demonstrated

by the Human Protein Atlas Project using proteomics and immunohistochemical data [244]. L1 has been studied with in models of spinal cord [154, 155] and peripheral nerve injury [245] but its expression pattern and the regulation of neuronal L1 in other brain injury models and autoimmune disease is still elusive.

We observed L1-expressing axons in non-pathological control individuals and in lesions of MS patients. Neuronal L1 has not been implicated in MS or related animal models of neuroinflammation yet. However, IgSF member contactin-2, which is closely related to L1, is recognized as an autoantigen by autoantibodies and Th1/Th17 cells during gray matter MS pathology and has been confirmed by studies using an adoptive transfer of contactin-2 specific T cells in the EAE rat model [246, 247]. Moreover, neurofascin (another L1 family member) has been identified as a target for autoantibody-mediated axonal injury [248, 249]. CD4 and CD8 T cells play the key role in MS pathogenesis. However, CD8 T cells by far outnumber CD4 T cells in white matter lesions and were frequently found in the brain parenchyma, whereas CD4 T cells displayed a more perivascular distribution [250]. Even though CD4 T cells represent a minority of the CNS-infiltrating T cells, they are still considered to be crucial pathogenic effectors in MS and primarily have been described as critical cell population in active MS lesions [251] and chronic active gray matter lesions in the spinal cord [121].

Our experiments with immunoblotting and mRNA analysis of isolated human peripheral blood lymphocytes verified reports that human CD3 T cells express L1 [183] by using immunoblotting and mRNA analysis of isolated human peripheral blood lymphocytes. In line, Ebeling *et al*. reported L1 expression in human T cells and claimed that CD4 T cells form the L1-expressing T cell population and found only a few L1-positive CD8 T cells [181]. The results of this thesis go along with these reports, although we not only detected L1-expressing CD4 but also L1-positive CD8 T cells isolated from PBMCs (with no indication of the frequency of L1-expressing cells).

The avβ3 integrin is expressed by many different cell types, including ECs [252], natural killer cells [253], and in B cell tumors [254], and it is a late activation antigen for mouse T cells [255]. L1 has been demonstrated to be a cellular binding partner for integrins, e.g., for mouse fibronectin receptor α5β1 [217] and the αvβ3 integrin in humans [256]. Interestingly, the avβ3 integrin is expressed by many different cell types, including ECs [252], natural killer cells [253], and in B cell tumors [254], and it is a late activation antigen for mouse T cells [255]. Hubbe *et al.* found that L1 plays a role in the binding of

T cells to ECs. Binding of human L1 to αvβ3 integrin and the L1 presence on the T cell surface have been suggested to be important for T cell adhesion and migration [185]. The αvβ3 integrin have been linked to Th17 cell induced EAE induction in mice and αvβ3 blocking experiments revealed the critical impact of αvβ3 on the disease severity [257, 258]. The underlying mechanism of the αvβ3 integrin during autoimmune processes of EAE or MS is still an issue of ongoing investigation. However, the cell-cell interaction via L1- αvβ3 integrin binding seems to be a promising mechanism for further examinations.

4.2 Neuronal L1 is adaptively regulated during experimental neuroinflammation

To date, very little is known about situation-adapted L1 regulation. Studies on the fetal alcohol syndrome revealed an influence of ethanol on developmental neurotoxicity via the induction of reduced L1 expression, altered cell surface distribution, impaired signal transduction, and impaired interaction with the cytoskeleton [259]. Our experiments revealed down-regulation of L1 on mRNA and protein level at the peak of EAE disease and the correlation between EAE disease stages with L1 gene expression regulation in the relapsing-remitting EAE model. Our results indicate that reduction of L1 expression exclusively occurs in CNS regions massively infiltrated by T cells and with severe neuroinflammation, particularly the spinal cord. Apart from full length neuronal L1, a non-neuronal splicing variant of L1 that lacks exon 2 and 27 was found. Cancer cells and B cells have been shown to express the L1 Δ2/Δ 27 splicing variant [188, 260, 261]. Even though the spinal cord is massively infiltrated by L1-expressing T cells, the mRNA analysis of full-length *L1* and a non-neuronal splicing variant lacking exon 2 [262] revealed that the neuronal *L1* expression was down-regulated in EAE but was still ten-fold higher than the expression of non-neuronal *L1* variant. In turn, a three-fold up-regulation of non-neuronal *L1* was observed in EAE compared to sham mice. Thus, the overall reduced L1 expression levels in EAE result from a predominant down-regulation of neuronal L1, which goes along with increased L1 expression from non-neuronal cells, presumably including T cells, microglia/macrophages, and DCs.

In vitro, *L1* mRNA levels rapidly dropped in neurons upon contact with activated CD4 T cells. Simultaneously, gene expression of *REST*, which is known to repress neuronal L1 transcription [167], was up-regulated in the EAE mouse model and in neuron-CD4 T cell co-cultures. The repressive transcription factor REST coordinates neuronal induction

and differentiation and regulates the balance between neuronal differentiation and maintenance of the functional adult neuronal stem cell pool during the CNS development [168]. REST is scarcely expressed by mature neurons under physiological conditions. However, its induction has been shown under pathological conditions such as ischemia and decreases transcription of many neuronal genes [263]. Transgenic REST knockdown mice demonstrated to be more vulnerable to neurotoxic factors [264], but REST has also been reported to prevent neuronal network hyperexcitability [265], indicating that REST is not an obligate pathogenic trigger but rather supports neuronal homeostasis [266].

Recent studies revealed that reduced ERK1/2 activity causes *REST* mRNA up-regulation in a toxin treated human neuron-like cell line [170]. Moreover, ERK activity promotes the degradation of REST protein complexes in neuronal progenitor cells, whereas de-phosphorylation of ERK leads to sustained levels of REST [267]. In agreement with previous studies that revealed that pro-inflammatory Th17 cells inhibit ERK phosphorylation in cultured neurons [124], we found that stimulated T cells reduced the phosphorylation of ERK1/2 of neurons in co-cultures. Therefore, CD4 T cell-mediated inhibition of neuronal ERK1/2 phosphorylation may enhance the REST complex, which represses L1 transcription. Even though our results indicate that L1 expression is regulated via a signaling pathway that involves ERK1/2 and REST, L1 has previously also been shown to mediate intracellular signaling via ERK1/2 activation itself. In fact, the depletion of L1 expression by using siRNA decreased the proliferation and migration of gastric cancer and melanoma cells, while ERK phosphorylation decreased. In turn, L1 overexpression correlated with ERK activation-associated tumor growth and motility [195, 268]. Moreover, lung cancer cells, experimentally inhibited in L1 expression, were declined in constitutive ERK activation [269]. These findings demonstrate the role of L1-mediated ERK phosphorylation and the associated bad prognosis for patients with high-level L1-expressing tumors [194]. In line, L1 mediated ERK activation has also been shown to be in involved neuronal migration and process outgrowth [270, 271].

By combining our findings and the reported association of ERK phosphorylation and REST expression/stability, we assume a self-enhancing signaling cascade, which starts with CD4 T cell-initiated neuronal inhibition of ERK signaling, followed by REST up-regulation and L1 down-regulation that potentially enhances itself and leads to significantly reduced L1 expression and ERK-phosphorylation levels. Such condition-

related positive-/negative-feedback loops of signaling pathways that include ERK have been described earlier and are crucial for the intracellular signal transduction and are involved in the down-stream expression regulation of various genes [272].

Therefore, impaired ERK activation seems to be linked to reduced neuronal L1 expression level during neuroinflammation which might be beneficial for neuronal survival. Conversely, ERK activation has been reported to promote cell death during neurodegenerative disease, e.g., Alzheimer's disease, Parkinson disease, and after ischemia [273-275] but a the detrimental role of ERK signaling for neuron survival is not unambiguous. It has also been associated with neuronal survival in different studies [276, 277], which is why it is assumed that the difference between ERK-mediated life-and-death signaling depends on the duration, magnitude, and/or compartmentalization of ERK activity [278]. Thus, the reduction of neuronal L1 expression and the inverse relationship to REST regulation during experimental neuroinflammation in context with the published data indicates that inhibition of ERK is involved in REST-mediated L1 down-regulation. This pathway may represent an adaptive mechanism of neurons in response to T cell attacks.

4.3 L1 is involved in T cell adhesion and axon degeneration

Even though L1 is considered to be a neuronal molecule, there is evidence of lymphocytes expressing L1, among them CD4 T cells in humans and mice [181, 185]. Our flow cytometry data on cells isolated from spleen and CNS demonstrated that CD4 T cells and to a lower extent CD8 T cells express L1. L1 expression in CD4 T cells has been demonstrated, different CD4 T cell subsets have not been investigated for subset specific L1 expression. In our experiments, Th1, Th2, Th17, and regulatory CD4 T cell subsets exhibited similar L1 expression rates, indicating no specific L1 expression due to CD4 T cell subset-differentiation, but rather by T cell maturation processes like previously published by Kowitz *et al*., who reported that mature $CD3^{high}$ T cells exhibit a high level of L1 expression [183]. We identified L1-expressing CD4 T cells in EAE plaques in spinal cords and primary CD4 T cells that were isolated from lymph nodes and spleen by using immunohistochemistry and flow cytometry. L1 expression in CD4 T cells has already been reported by several studies, however very little is known about the conditions of L1 regulation and the functional implications of L1 expressed by T cells. Some studies associate L1 with co-stimulatory capacities in CD4 T cells activation [186] and leukocyte

cell adhesion [185]. Studies of Pancook *et al*. and Ebeling *et al.* demonstrated that lymphocyte L1 recognizes integrin $\alpha v\beta_3$ on ECs and suggested that L1 supports lymphocyte migration and extravasation through binding to this integrin [181, 184]. The strong dependency of CD4 T cells to bind to specific integrins during CNS-infiltration was shown with Th1 cells that rely on α4β1 integrin-binding and Th17 cells that bind the αLβ2 integrin in order to enter the CNS-parenchyma [241, 279]. Moreover, a recent study demonstrated that neuronal cell adhesion molecules other than L1, in this case ninjurin-1, plays important role in the activated T cell crawling along the endothelium and in the transmigration into the CNS, and that ninjurin-1 depletion attenuates the susceptibility for EAE in mice [280, 281].

Even though L1 is capable of heterophilic binding, the fact of L1-L1 homophilic interaction is well established, and it has been demonstrated that L1 molecules bind to each other with high affinity while being insensitive to environmental conditions, such as low temperatures or changes in the extracellular Ca^{2+}-concentration [282, 283]. Interestingly, the Δ2 L1 non-neuronal slicing variant has been shown to not interact with other Δ2 L1 but with full length neuronal L1 [284], which might be the reason why T cells do not adhere together but to neurons via Δ2 L1 – full length L1 interaction. In the EAE model, L1 protein expression was reduced but not abolished in axons, including injured ones that were in close vicinity to CD4 T cells, which also expressed L1. Given the *trans*-homophilic binding capacity of L1 and the co-stimulatory effect on T cell activation *in vitro* [186], these findings suggest an L1-mediated interaction between neurons and CD4 T cells, which possibly modulates the immune and neuronal response during interaction.

Similarly to the situation *in vivo*, neurons co-cultured with activated CD4 T cells display neurodegenerative changes of the microtubule network [285] In addition, Th17 cells, which are the primary effector cells of the EAE disease, were shown to cause rapid axonal microtubule destabilization that results in axon fragmentation [123]. Our *in vitro* experiments showed that neuronal *L1* mRNA is down-regulated by CD4 T cells in a contact-dependent manner and that antibody mediated blocking of L1 was sufficient to prevent T cell-induced axonal degeneration and CD4 T cell adhesion to neuron cultures. This implies the existence of ligand-receptor complexes between T cells and neurons that is associated with L1 expression. In agreement with previous work [286], we found that unstimulated T cells express L1, but in contrast to L1-expressing activated T cells,

they did not cause axonal degeneration. This suggests that *trans*-homophilic interactions of L1 between T cells and neurons do not directly trigger axonal degeneration but facilitate other deleterious processes, presumably by supporting T cell adhesion to neurons. Miller *et al*. confirmed that destabilization of neurites by T cells is contact-dependent and suggested that the neurite-degenerating mechanism is mediated by lymphocyte-produced lytic granules and just a bystander effect of the direct cell-cell interaction [123].

To our knowledge, no studies have focused on L1 expression and function in disease with immune-mediated neurodegeneration or *in vitro* experiments so far and therefore no treatment approaches have been investigated yet. However, L1 has come into focus in oncology research, and studies using L1-blocking antibodies as a treatment approach have demonstrated promising results of L1 inhibition [287]. Woltering *et al*. demonstrated a prolonged survival and reduced tumor burden after L1 antibody therapy by attracting tumor-infiltrating macrophages to L1-expressing and antibody-marked tumors. These macrophages were activated via Fc-receptor binding to L1 antibodies, which leads to enhanced phagocytosis and cytotoxicity and therefore activates immunological effector mechanism to block tumor growth [288]. Another study reported an anti-tumor growth effect by L1-blocking antibodies *in vivo* and suggested the suitability of L1 as a target for chemosensitization and of L1-interfering antibodies as an appropriate tool to increase the therapeutic response in pancreatic and ovarian carcinoma [289]. It has been proven that a prolonged high-dose application of L1 antibodies do not lead to any apparent side effects in terms of behavior, weight, blood enzyme markers, and histochemical appearance of organs [289]. Strikingly, the treatment of EAE mice with L1 function-blocking antibodies in our experiments revealed a significant attenuation of disease severity and reduced axonal degeneration and loss. We could therefore extend the clinical spectrum for the possible application of L1-blocking antibodies to reduce the immune cell mediated axonal degeneration during disease like MS. However, also our results demonstrate the striking efficacy of L1-blocking antibodies in the EAE model, the applicability in humans is still elusive. There are several MS treatment attempts, which used intravenous infusion of antibodies that indicated to interpret results from animal experiments with caution. Problems of immunogenicity against the foreign antibodies, an infusion reaction and the insufficient CNS penetration of the injected antibodies restrict the antibody treatment opportunities

of MS [290]. Negative side effects could also by the systematic blocking of L1 which block crucial functions of L1 as well. Even though L1 is not as important during adulthood as during development, it has been shown to be crucial for regenerative processes in the CNS [291].

To unravel the role of neuronal L1 in neuroinflammation, mice mutants for neuronal L1 depletion were generated. A constitutive L1-knockout mice strain would not have been an appropriate model to be tested in EAE experiments, since they display many phenotypic features of the L1 syndrome, including hydrocephalus, corpus callosum agenesis, malformation of the corticospinal tract, and deficits in locomotor function [292-294]. Law *et al*. circumvented these restrictions with conditional knockout mice, in which L1 gene was inactivated in the adult brain by crossing transgene L1-floxP mice to a calcium/calmodulin-dependent kinase II promoter-driven Cre-recombinase mouse line [295]. In the latter, overt morphological and behavioral abnormalities were absent, though they exhibited an increased basal excitatory synaptic transmission, decreased anxiety, and altered place learning. Thus, mice conditionally deficient in L1 expression in the adult brain display mild abnormalities, which are completely different to mice lacking L1 in a constitutive fashion [295]. These findings demonstrate that L1 is not only important during development but also during adulthood.

We generated neuron-specific L1 knockdown mice which exhibited reduction of neuronal L1 (but not complete ablation) in distinct CNS regions of adult mice by crossing mice with a floxP side-flanked L1 gene locus ($L1^{fl/fl}$) [296] and mice transgenic for a Cre-recombinase driven by synapsin promoter (Syn^{Cre}) [295]. Syn^{Cre} mice were shown to express a detectable level of Cre-recombinase, starting at E12.5, once neuronal cells have left the mitotic cycle, migrate towards their position along the dorsal-ventral axis of the neural tube, and extend their axons. The Cre-recombinase reaches its highest expression level and distribution at E18.5 [296]. The assessment of a composite neuroscore of the newly generated mouse line indicated no constitutive neurological impairments, which enabled undistorted experiments absence of L1 syndrome features. There were no apparent signs of developmental impairments in $Syn^{Cre}L1^{fl/fl}$ mice and the L1 expression level in the brain stem and the spinal cord, which represent the major sites of axonal injury in the EAE model [206], exhibited the most pronounced reduction of L1 expression. Strikingly, these mice displayed attenuated EAE severity, axon injury,

and axon loss. Flow cytometry of immune cell subsets from the CNS of both genotype groups revealed no differences of CNS-infiltrating mononuclear cells and different CD4 T cell subsets in the CNS. L1 is crucial for axonal myelination during developmental processes [297] and has been shown to promote re-myelination after nerve experimental injury [156, 245, 298]. We also did not observe any genotype-related differences of EAE-induced demyelination. These results rule out the possibility that a reduced immune response or de-myelination/re-myelination processes are responsible for the attenuated EAE severity and suggest a neuron-specific mechanism that associates L1 reduction with axon preservation.

In contrast to the beneficial role of L1 reduction in our experiments, other studies demonstrated positive effects of neuronal L1 expression and function triggering in adult animals. Studies with heterozygous L1-deficient mice indicated an autism-like phenotype and assumed that molecules known to trigger the functions of L1 may become valuable in the treatment of autism [299]. Other studies indicated beneficial effects of L1 function-triggering antibodies after nerve injury [298] and an improved recovery and ameliorated re-myelination after spinal cord injury in adult mice [154, 245]. We used L1-overexpressing mice generated by Doberstein *et al*. (L1 tg M3) [289] in the EAE model to investigate whether the overexpression of L1 turns the beneficial effect that we found after neuronal L1 depletion into a more severe disease progression. However, we demonstrated a high overexpression of L1 in the spinal cord in comparison to WT controls, the model failed to show any differences between L1-overexpressing and control littermates. Nevertheless, the EAE model clearly differs from most other CNS injury models due to the massive influx of immune cells [241]; in addition, L1 expression level and associated interaction properties with neurons in this model underline possible detrimental implications of neuronal L1 expression and clarifies the critical difference from other models where L1 expression might be beneficial. We assume that these results are the consequence to the already quite exhausted EAE severity of the control animals that is unlikely to be increased even when there are circumstances that would enforce the disease progression. Another reason could be that the endogenous levels of L1 might be sufficient to reach a saturation point in terms of axonal degeneration. Moreover, it is the human L1 gene that has been inserted in this mouse line, and even though L1 is highly conserved between the different species, it is possible that human L1 does not interact with mouse L1 as efficiently as the mouse L1 to itself.

Finally, it is often quite challenging to enhance disease severity in an experimental setup, and since the overall goal is to cure or reduce pathological conditions, we focused on the antibody treatment approach and the conditional L1 knockout experiments.

Neurons are commonly considered to play the "victim role" during inflammatory neurodegeneration, there are however numerous studies outlining the influence of neurons in the adaption and modulation processes during immune cell attacks. The structure and functional properties of neural cell adhesion molecule (NCAM) is similar to L1, it is expressed on the surface of neurons, astrocytes, and microglia [300, 301], and has a crucial role in cell-cell adhesion, synaptic plasticity, and neurite outgrowth [302]. Neurons are able to modulate glial immune responses via homophilic NCAM binding [303], thereby influencing glia cell activation and promoting reduced production of glial TNFα and nitric oxides [300, 304]. CD200, CD22 and CD47 are further neuronal surface molecules with the potential to inhibit glia cell activation by supporting direct cell-cell contact and associated signaling cascades [305]. These findings highlight the vital role of neurons in the adaptation and modulation of the immune response of adjacent cells via cell-cell contact-dependent interactions. Infiltrating CD4 T cells and neurons cannot interact via antigen presentation due to the lack of neuronal MHC II expression. Although CD4 T cells are absent from MHC II/TCR signaling, they have a critical impact on neuronal survival and inflammation-induced apoptosis through the TNF-related apoptosis-inducing ligand (TRAIL) pathway [120]. T cells were also shown to induce oscillatory calcium alterations in neurons, leading to a critical calcium overload that is mediated by NMDA-/AMPA-receptors [122]. In line with our results, studies on T cell-mediated neurodegeneration revealed that in absence of specific antigens polyclonal activated T cells are sufficient to induce axon degeneration and subsequent neuronal death in a process that requires T cell activation and direct cell-cell contact with no similar detrimental effects caused by unstimulated T cells [123, 306]. This process is contact-dependent and does not involve neuronal MHC I mediated antigen presentation but rather other receptor molecules, such as FasL, LAF-1, and CD40 [306], which might be supported by L1-mediated neuron-T cell adhesion.

Recently, reduced L1 expression has been linked to neuroprotection during the CNS development in female mice heterozygous for L1 [307]. It has been suggested that a shift from the preferential homophilic binding mode of L1 to heterophilic interactions modulates cell death signaling pathways [308]. A growing number of proteins that

undergo heterophilic interactions associated with protein complexes, including L1, have been identified. Sema3A holoreceptor is one of these receptor complexes, consisting of L1, neuropilin-1 (NRP1), and plexin [309, 310]. Sema3A is known to act as a repulsive axon guidance cue but is also capable to induce neuronal apoptosis [311]. Interestingly, L1 is crucial for Sema3A-induced downstream signaling and growth cone collapse [310, 312]; it also plays a role in the induction of Sema3A in neuronal cell bodies of corresponding demyelinated axons, and this process has been implicated in the pathogenesis of MS [166]. Neuronal sema3A (either secreted or membrane bound) interferes with T cell activation and proliferation. By forming a complex with NRP1 and Plexins, Sema3A prevents T cell response over-activation by inhibiting T cell receptor polarization and signal transduction by down-regulation of mitogen-activated protein kinase (MAPK) signal cascades [313]. Notably, in contrast to the common assumption that T cell-neuron interactions have a detrimental impact, there is evidence that these interactions can even be beneficial for neurons. Activated CD4 T cells can promote neurite outgrowth and potentially produce neurotrophic factors such as BDNF and the IL-4 cytokine [115, 117, 314].

Another type of contact-dependent neuronal modulation of inflammatory processes is the induction of Tregs, which are important regulators of CNS homeostasis during healthy and pathological conditions and counterbalance inflammation [219]. Elevated level of IFNγ and TNFα during neuroinflammation leads to neuronal expression of TGFβ, CD80, and CD86, which induce encephalitogenic CD4 T cells to differentiate into Tregs [118, 315]. Even though neuron-induced contact-dependent Treg induction was shown to attenuate EAE progression [118], in our experiments the flow cytometry analysis of CD4 T cell subsets from the spinal cord of $Syn^{Cre}L1^{fl/fl}$ mice did not reveal any changes in the number of Tregs, indicating that L1 is not involved in this process. Within this context, it appears more likely that L1 facilitates interaction between neurons and T cells and thereby permits a local transmission of damaging neuromediators and T cell-induced pro-apoptotic signals.

4.4 L1 is expressed by APCs in MS and EAE lesions

To date, it is generally unknown whether and how the L1 molecule contributes to immunological processes and inflammation. However, some studies revealed that L1 is expressed by particular populations of leukocytes in the periphery, among them

granulocytes, B cells, T cells, monocytes, and DCs [183, 184]. In contrast to the widely dispersed down-regulation of L1 in neurons in the presence of CD4 T cells during neuroinflammation in mice, non-neuronal L1- expressing cells accumulate at sites of acute EAE plaques in the spinal cord of mice and in the active rim area in cortex lesions of MS patients. Co-immunostaining for L1 and MHC II (combined with CD11c as the DC marker and Iba1 as the marker for microglia/macrophages) in cortical sections obtained from MS patients and L1 staining in spinal cord sections obtained from EAE mice suggested that the expression of L1 in human and mice CNS lesions is expressed by APCs, namely DCs and/or macrophages. Microglia, macrophages, and DCs are of crucial importance in MS pathogenesis. Microglia and macrophages are able to produce and respond to a variety of different inflammatory cytokines, to impair the functioning of the BBB, to present antigens to T cells, to act as phagocytes, and to damage the myelin sheath [227]. DCs, which are the most efficient antigen-presenting cells, have been identified in healthy nervous system, and they have the potential of CNS antigen-sampling. DCs have also been found in MS lesions and have been shown to be functionally abnormal in patients with MS [316].

L1 expression in activated human monocytes and monocyte-derived macrophages has been shown before. Furthermore, it has been claimed that microglia as CNS tissue-specific macrophages do not express L1 *in situ* or in cell culture [184, 317]. We discovered L1-expressing cells in brain sections from MS patients that displayed a macrophage-like appearance and were positive for MHC II expression, suggesting that these cells are activated macrophages. Our findings go along with the common assumption that CNS-infiltrating activated macrophages form an active rim at the expanding edge of chronic active lesions during MS pathogenesis [40]. We are the first to show that these cells, at least in part, express L1 during MS, and we corrobarated these findings with the detection of L1-expressing $Iba1^+$ macrophages in the spinal cord of EAE mice. Although Pancook *et al.* reported that IFNγ stimulation results in a significant induction of L1 expression in human monocytes and in myelomonocytic cell lines, our *in vitro* studies failed to induce L1 expression in cultured murine microglia and bone marrow-derived macrophages. This suggests that L1 expression in macrophages is induced under specific environmental conditions and is not a constitutive lineage-associated marker. Functional implications of L1 expression in monocytes and macrophages are vague. It has been speculated that L1 expression in these cells may

contribute to cell-cell adhesion in association with their effector function and that L1 may contribute to the effector cell extravasation since L1 in these cells is recognized by ECs via integrin-$\alpha_v\beta3$ [181, 184]. The severity of EAE correlates with the number of infiltrating monocytes, which evolve into activated macrophages within the parenchyma of the CNS [318]. The antigen-presentation properties of resident and CNS-infiltrating APCs are crucial for CD4 T cell activity since they need to be reactivated to access the neuropil [319].

T cell migration strictly depends on their activation status. Naïve CD4 T cells do not readily migrate into the CNS because of their lack of appropriate adhesion molecule and chemokine receptor expression [320]. Besides their role of antigen presentation to T cells, microglia and macrophages also have effector cell properties and participate in the pathogenesis of MS and EAE. They actively cause myelin breakdown and phagocytize myelin proteins within CNS lesions [321]. Activated microglia secrete MMP-2 and MMP-9, which are capable of degrading MBP epitopes within the CNS [322, 323] and are also active in the breakdown of the BBB [324]. We used transgenic $LysM^{Cre}L1^{fl/fl}$ mice lacking L1 expression in monocytes, macrophages, microglia, and granulocytes (myeloid cell lineage) to examine whether L1 expression in these cells influences the disease progression in the EAE mouse model. LysM-Cre driven recombination was shown to have a deletion efficiency of 83-98 % in mature macrophages and close to 100 % in granulocytes. A partial depletion (<16 %) was detected in $CD11c^+$ splenic DCs, which are closely related to the monocyte/macrophage lineage. T cells and B cells were spared of the recombination [325]. Under naive conditions, $LysM^{Cre}L1^{fl/fl}$ mice did not display phenotypical abnormalities, however, the EAE model revealed a significant attenuation of the disease severity.

Of note, the invasion of monocytes has been shown to be strongly associated with EAE progression, which could be prevented by blocking the CNS entry to circulating inflammatory monocytes in the periphery [318]. The alleviated EAE severity suggests that L1 expression in microglia/macrophages have a relevant implication in their migration behavior and/or effector functions. However, we could not detect differences in the number of microglia/macrophages within the CNS, which contradicts the hypothesis that L1 is relevant in the CNS infiltration by monocytes.

Instead of this, L1 expression may influence effector functions of microglia/macrophages at lesion sites in the CNS. A study of Balaian *et al.* [186] reported

that L1 expressed by APCs or plate-coated recombinant L1 together with TCR-stimulating anti-CD3 antibodies functions as a co-stimulatory molecule for T cell activation and proliferation. Therefore, it is possible that the attenuated severity of EAE might be caused by the abated capability of L1-depleted microglia/macrophages to re-activate CNS-infiltrating T cells. Moreover, there is evidence that activated macrophages cause neuronal damage in co-cultures in a contact-dependent manner [326], suggesting that L1 might promote macrophage-neuron contact and adhesion therefore supports the effector cell-induced neuronal degeneration and loss. We were not able to detect L1 expression in cultivated murine microglia and bone marrow-derived macrophages, though we accomplished to induce L1 in bone marrow-derived DCs while stimulating them with LPS and TNFα, which are both commonly known to induce DC maturation [327-329]. Immature DCs in culture, identified by MHC II^{low} $CD86^{-}$, did not express L1 but did so after stimulation with LPS and to an even higher extent after TNFα stimulation. Mature dendritic cells characterized by MHC II^{high} $CD86^{+}$ were nearly all L1 positive, suggesting that L1 is induced during DC maturation and therefore relevant for DC migration towards lymph nodes and/or antigen presentation in them rather than for the environmental surveillance or the antigen uptake. Further *in vitro* studies could clarify whether the expression of L1 in microglia/macrophages and in DCs is involved in antigen presentation and impacts the activation/proliferation of CD4 T cells in the periphery and/or in the CNS during neuroinflammation.

In addition to experiments with L1 depletion in microglia/macrophages, we are also the first to show an attenuated severity of EAE in mice transgenic for L1 depletion in dendritic cells. DCs are present in healthy CNS and increase in number and activation level in patients with MS [330]. Increased numbers of DCs in cerebrospinal fluid, accompanied by higher levels of co-stimulatory molecules and MHC II expression, were primarily seen during the early phases of MS, suggesting an involvement in the disease onset. The presence of DCs in brain tissue and demyelinated MS lesions is apparent but unobtrusive and without a substantial difference in anatomic distribution between MS and normal tissue, which is in line with the suggestion that DCs primarily influence MS during the disease onset by perpetuating the pro-inflammatory T cell response upon CNS entry [316]. In the EAE mouse model, DCs can derive from CNS resident resting microglia cells [79], and peripheral DCs infiltrate into the inflamed CNS [331]. Although DCs do not seem to be as relevant in the progression of EAE as macrophages, they were

shown to be solely sufficient to initiate EAE, and their CNS recruitment correlates with the disease severity [81, 231]. We found L1-expressing DCs in the CNS during EAE by using immunohistochemistry and flow cytometry. L1 expression in DCs has already been reported in the periphery of humans and mice [182, 186]. We used transgenic mice with L1 depletion in $CD11c^{+}$ cells and revealed a significant attenuation of the severity of EAE in these mice in comparison to controls. Moreover, $CD11c^{Cre}L1^{fl/fl}$ mice had reduced numbers of CD4 T cells, CD8 T cells, and DCs in the CNS during EAE, suggesting L1 involvement in DC-mediated CD4/CD8 T cell activation and DC infiltration into the CNS.

Similarly to the situation with microglia/macrophages, very little is known about the functional role of L1 expression in DCs. Since DCs are the most efficient antigen-presenting cell population, which modulates activation, differentiation, and proliferation of T cells, possible implications of L1 during antigen presentation are of interest. To our knowledge, there is just one study that has examined the functional implication of L1 during antigen presentation, which puts forward the hypothesis that recombinant L1 have co-stimulatory properties on T cells. They also showed a reduced PBMC response to stimulation with L1-expressing DCs after inhibition of L1 binding by L1-blocking antibody treatment [186]. Another study demonstrated that L1 is involved in transendothelial migration and the trafficking of dendritic cells through the monolayers of either lymphatic or blood vessel ECs [182]. L1 has been shown to be one among numerous adhesion molecules induced by inflammatory stimuli in ECs [332]. Therefore the DC-EC interaction is likely to be promoted by homophilic L1 binding [333]. Moreover, there are potentially L1-interacting binding partners in the group of integrins, which have been also shown to have properties to bind to each other [334-336]. The L1-integrin interaction is further supported by the homophilic L1-L1 binding, which was shown to promote integrin induction and recruitment [337].

To sum up, our data together with the few studies focused on L1 expression on DCs suggest that L1 is a relevant factor for DC migration and presumably also for antigen presentation and therefore relevant during EAE induction, which is in line with our observations.

4.5 Conclusion and outline

To conclude, it is now evident that L1 expression is not only relevant during the development of the CNS but is also involved in various processes during adulthood, including the crosstalk between the central nervous system and the immune system. We revealed an unexpected role of L1 in the pathological interaction between neurons and activated T cells. Neuron-specific depletion of L1 reduced disease severity and axonal pathology in EAE, which supports the hypothesis that the down-regulation of neuronal L1, mediated by the transcription repressor REST, is an adaptive mechanism to promote neuronal self-defense in response to neuroinflammation. We also demonstrated the involvement of non-neuronal L1 expression by certain mononuclear cells, namely T cells, microglia/macrophages and dendritic cells, to be relevant for the immune cell–neuron crosstalk and the efficient effector functions during neuroinflammation. We provided first evidence and further discuss that L1 facilitates the adhesion of effector immune cells to axons and therefore enables detrimental processes originated by the effector cells and promoted by the close engagement to neurons. We further assume that L1 may facilitate antigen presentation signaling of macrophages and DCs. Therefore, L1 homophilic or heterophilic interaction in *cis* and *trans* might play a role in the initiation of it or during the antigen presentation process itself. To address this issue, transgenic mice with L1 depletion in CD4 T cells would be useful and would provide interesting opportunities in combination with the already established $LysM^{Cre}L1^{fl/fl}$ and $CD11c^{Cre}L1^{fl/fl}$ mice lines. *In vitro* antigen presentation and migration assays of T cells and APCs from transgenic mouse lines could clarify the relevance of L1 expression in different combinations and therefore provide an overview of cell type-specific L1 implications during immunological processes. Moreover, imaging methods with transgenic reporter mice or fluorescence staining methods could reveal alterations in the distribution in the periphery and the CNS of L1 depleted immune cells during healthy and pathological conditions. However, it would also be of further importance to clarify the relevance of data obtained in the murine system in translation to human diseases. We have provided first evidence of L1 to be present on axons during multiple sclerosis, but could not prove if the L1 associated processes in mouse also hold up to humans. Investigation of the axonal L1 expression level of healthy individuals and MS patients in a statistically relevant number would be required to reveal if the neuronal L1 expression during MS is similarly reduced in humans as we have observed in mice.

5 Summary

Even though the central nervous system (CNS) is generally regarded as an immune privileged organ, attacks of infiltrated immune cells during autoimmune diseases as Multiple Sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) lead to CNS injury. The invasion of encephalitogenic T cells into the CNS is considered to be the initiatory event of the autoimmune pathology that causes a direct interaction of cells from the immune system and the CNS. This study demonstrates a novel role of the cell adhesion molecule L1 (L1) in the crosstalk between immune cells and neurons. For the first time, the adaptive regulation of neuronal L1 expression and the role of L1 expressed by mononuclear cells in a model of neuroinflammation have been described. L1 expression was found in axons in MS lesions and in human T cells, as well as on axons and adjacent CD4 T cells in acute spinal cord EAE plaques. The mRNA expression levels of L1 correlated with the disease stages during the relapsing-remitting EAE model and were confirmed by a significant L1 reduction at the peak disease stage in chronic EAE. The reduction of L1 expression coincided with the up-regulation of its transcriptional repressor *REST* (RE1-Silencing Transcription Factor). Stimulated CD4 T cells induced L1 down- and REST up-regulation in neurons and caused a severe axonal injury in a contact-dependent manner in co-cultures. CD4 T cell adhesion to neurons and the subsequent axonal injury were prevented by L1-blocking antibodies. In line, antibody-mediated blocking of L1 in wild type C57BL/6J mice as well as a genetic depletion of L1 in neurons (synapsin^{Cre} x L1$^{fl/fl}$) attenuated the severity of EAE and reduced the axonal pathology, while the immune cell infiltration remained unchanged. Moreover, antigen-presenting cells (APC) in chronic active lesions of MS patients and in EAE plaques expressed L1. The cell type-specific L1 depletion in microglia/macrophages (lysozyme-M^{Cre} x L1$^{fl/fl}$) and dendritic cells (CD11c^{Cre} x L1$^{fl/fl}$) attenuated the severity of EAE. The findings of this thesis suggest that neuronal reduction of L1 is beneficial during neuroinflammation and thereby represents an adaptive process of neuronal self-defense that limits immune cell-mediated axonal injury. L1 expression in APCs appeared to be crucial for an efficient disease induction and progression. Altogether our data identified L1 as a relevant factor in neuroinflammation and implicate cell-type specific functions of L1 in neurons, T cells, and APCs during inflammatory disease progression in the CNS.

6 Zusammenfassung

Obwohl das zentrale Nervensystem (ZNS) im Allgemeinen als immunpriviligiertes Organ gilt, verursachen attackierende Immunzellen schwerwiegende Schäden des ZNS bei Autoimmunkrankheiten wie der Multiplen Sklerose (MS) beim Mensch und der experimentellen autoimmune Encephalomyelitis (EAE) in der Maus. Die Invasion von encephalitogenen T Zellen in das ZNS ist das initiale Ereignis der autoimmunen Pathologie, was eine direkte Interaktion von Zellen aus dem Immunsystem und dem ZNS zur Folge hat. Wir zeigen hier eine bisher unbekannte Rolle des neuronalen Zelladhäsionsmoleküls L1 für die Interaktion zwischen Immunzellen und Neuronen. In dieser Arbeit konnte zum ersten Mal eine adaptive Regulation der L1 Expression in Neuronen und auf mononuklearen Zellen in der EAE gezeigt werden. L1 ist auf humanen T Zellen und auf Axonen von MS Patienten exprimiert und konnte auf CD4 T Zellen und angrenzenden Axonen im Rückenmark von EAE Mäusen nachgewiesen werden. Die Expressionspegel von L1 korrelierten mit den schubförmig-remittierenden Krankheitsstadien im $PLP_{139-151}$ immunisierten EAE Model. Die L1 Regulation wurde zusätzlich durch die signifikante Reduktion von *L1* im Rückenmark auf dem Krankheitshöhepunkt einer MOG_{35-55} induzierten EAE bestätigt und war einhergehend mit einer verstärkten Expression des transkriptionellen L1 Repressors *REST* (RE1-Silencing Transcription Factor). Stimulierte CD4 T Zellen induzierten eine neuronale Herunterregulation von L1 und Hochregulation von REST und eine Schädigung von neuronalen Axonen in *in vitro* Co-Kulturen. Die Adhäsion von CD4 T Zellen an Neurone und der damit assoziierte axonale Schaden konnte durch L1-blockierende Antikörper signifikant vermindert werden. Übereinstimmend mit diesen Ergebnissen bewirkte die antikörpervermittelte Blockierung von L1 in wildtyp C57BL/6J Mäusen, sowie Experimente mit Mäusen in denen neuronenspezifisch L1 depletiert wurde ($Synapsin^{Cre}$ x $L1^{fl/fl}$) eine Verminderung des Krankheitsverlaufs und der axonalen Pathologie im EAE Model, wobei die Infiltration von Immunzellen unverändert blieb. Unabhängig von neuronalem L1 konnte in mononuklearen Zellen in chronisch aktiven Läsionen von MS Patienten und in aktiven EAE Plaques eine L1 Expression nachgewiesen werden. Die zelltypspezifische Depletion von L1 auf Mikroglia/Makrophagen (Lysozym-M^{Cre} x $L1^{fl/fl}$) und auf dendritischen Zellen ($CD11c^{Cre}$ x $L1^{fl/fl}$) bewirkte einen signifikant verminderten Krankheitsverlauf der EAE, und eine reduzierten Infiltration von CD4 T Zellen in des

Rückenmark. Die Ergebnisse dieser Arbeit lassen darauf schließen, dass die reduzierte Expression von L1 während einer Neuroinflammation vorteilhaft ist und damit einen adaptiven Prozess der neuronalen Selbstverteidigung als Reaktion auf T Zell-Infiltration darstellt und immunzell-vermittelte axonale Schädigung verhindert. Die L1 Expression von mononuklearen Zellen scheint hingegen entscheidend für eine effiziente Krankheitsinduktion dieser Effektorzellen zu sein. Zusammengefasst beschreiben diese Daten die L1 Expression von Neuronen, T Zellen und mononuklearen Zellen als kritischen Faktor mit zelltypspezifischer Bedeutung für Neuroinflammation im ZNS.

7 References

1. Funfschilling U., Supplie L.M., Mahad D., Boretius S., Saab A.S., Edgar J., Brinkmann B.G., Kassmann C.M., Tzvetanova I.D., Mobius W., Diaz F., Meijer D., Suter U., Hamprecht B., Sereda M.W., Moraes C.T., Frahm J., Goebbels S., and Nave K.A., *Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity.* Nature, 2012. **485**(7399): p. 517-21.
2. Karram K., Chatterjee N., and Trotter J., *NG2-expressing cells in the nervous system: role of the proteoglycan in migration and glial-neuron interaction.* J Anat, 2005. **207**(6): p. 735-44.
3. Ackerman S., in *Discovering the Brain*. 1992: Washington (DC).
4. Zhu D., Zhang D., Faraco C., Li K., Deng F., Chen H., Jiang X., Guo L., Miller L.S., and Liu T., *Discovering dense and consistent landmarks in the brain.* Inf Process Med Imaging, 2011. **22**: p. 97-110.
5. Lewis T.L., Jr., Courchet J., and Polleux F., *Cell biology in neuroscience: Cellular and molecular mechanisms underlying axon formation, growth, and branching.* J Cell Biol, 2013. **202**(6): p. 837-48.
6. Friede R.L., *The relationship of body size, nerve cell size, axon length, and glial density in the cerebellum.* Proc Natl Acad Sci U S A, 1963. **49**: p. 187-93.
7. Kirkcaldie M.T. and Collins J.M., *The axon as a physical structure in health and acute trauma.* J Chem Neuroanat, 2016. **76**(Pt A): p. 9-18.
8. Riera Romo M., Perez-Martinez D., and Castillo Ferrer C., *Innate immunity in vertebrates: an overview.* Immunology, 2016.
9. Janeway C., *Immunobiology : the immune system in health and disease*. 6th ed. 2005, New York: Garland Science. xxiii, 823 p.
10. Koretzky G.A., *Multiple roles of CD4 and CD8 in T cell activation.* J Immunol, 2010. **185**(5): p. 2643-4.
11. Stout R.D. and Bottomly K., *Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages.* J Immunol, 1989. **142**(3): p. 760-5.
12. Romagnani S., *Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease.* Int J Clin Lab Res, 1991. **21**(2): p. 152-8.
13. Korn T., Bettelli E., Oukka M., and Kuchroo V.K., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
14. Zhu J. and Paul W.E., *Heterogeneity and plasticity of T helper cells.* Cell Res, 2010. **20**(1): p. 4-12.
15. Tai X., Van Laethem F., Pobezinsky L., Guinter T., Sharrow S.O., Adams A., Granger L., Kruhlak M., Lindsten T., Thompson C.B., Feigenbaum L., and Singer A., *Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells.* Blood, 2012. **119**(22): p. 5155-63.

16. Shrikant P. and Benveniste E.N., *The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation.* J Immunol, 1996. **157**(5): p. 1819-22.

17. Mendez-Fernandez Y.V., Hansen M.J., Rodriguez M., and Pease L.R., *Anatomical and cellular requirements for the activation and migration of virus-specific CD8+ T cells to the brain during Theiler's virus infection.* J Virol, 2005. **79**(5): p. 3063-70.

18. Carson M.J., Doose J.M., Melchior B., Schmid C.D., and Ploix C.C., *CNS immune privilege: hiding in plain sight.* Immunol Rev, 2006. **213**: p. 48-65.

19. Galea I., Bechmann I., and Perry V.H., *What is immune privilege (not)?* Trends Immunol, 2007. **28**(1): p. 12-8.

20. Wakim L.M., Woodward-Davis A., and Bevan M.J., *Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence.* Proc Natl Acad Sci U S A, 2010. **107**(42): p. 17872-9.

21. Ballabh P., Braun A., and Nedergaard M., *The blood-brain barrier: an overview: structure, regulation, and clinical implications.* Neurobiol Dis, 2004. **16**(1): p. 1-13.

22. Chen Y. and Liu L., *Modern methods for delivery of drugs across the blood-brain barrier.* Adv Drug Deliv Rev, 2012. **64**(7): p. 640-65.

23. Hein T. and Hopfenmuller W., *[Projection of the number of multiple sclerosis patients in Germany].* Nervenarzt, 2000. **71**(4): p. 288-94.

24. Sellner J., Kraus J., Awad A., Milo R., Hemmer B., and Stuve O., *The increasing incidence and prevalence of female multiple sclerosis--a critical analysis of potential environmental factors.* Autoimmun Rev, 2011. **10**(8): p. 495-502.

25. Talley C.L., *The emergence of multiple sclerosis, 1870-1950: a puzzle of historical epidemiology.* Perspect Biol Med, 2005. **48**(3): p. 383-95.

26. Hafler D.A., Slavik J.M., Anderson D.E., O'connor K.C., De Jager P., and Baecher-Allan C., *Multiple sclerosis.* Immunol Rev, 2005. **204**: p. 208-31.

27. Keegan B.M. and Noseworthy J.H., *Multiple sclerosis.* Annu Rev Med, 2002. **53**: p. 285-302.

28. Correale J., Fiol M., and Gilmore W., *The risk of relapses in multiple sclerosis during systemic infections.* Neurology, 2006. **67**(4): p. 652-9.

29. Sospedra M. and Martin R., *Immunology of multiple sclerosis.* Annu Rev Immunol, 2005. **23**: p. 683-747.

30. Ramsaransing G.S. and De Keyser J., *Benign course in multiple sclerosis: a review.* Acta Neurol Scand, 2006. **113**(6): p. 359-69.

31. Compston A. and Sawcer S., *Genetic analysis of multiple sclerosis.* Curr Neurol Neurosci Rep, 2002. **2**(3): p. 259-66.

32. Hemmer B., Nessler S., Zhou D., Kieseier B., and Hartung H.P., *Immunopathogenesis and immunotherapy of multiple sclerosis.* Nat Clin Pract Neurol, 2006. **2**(4): p. 201-11.

33. Oksenberg J.R., Baranzini S.E., Barcellos L.F., and Hauser S.L., *Multiple sclerosis: genomic rewards.* J Neuroimmunol, 2001. **113**(2): p. 171-84.

34. Panitch H.S., *Influence of infection on exacerbations of multiple sclerosis.* Ann Neurol, 1994. **36 Suppl**: p. S25-8.

35. Giovannoni G., Cutter G.R., Lunemann J., Martin R., Munz C., Sriram S., Steiner I., Hammerschlag M.R., and Gaydos C.A., *Infectious causes of multiple sclerosis.* Lancet Neurol, 2006. **5**(10): p. 887-94.

36. Kornek B. and Lassmann H., *Neuropathology of multiple sclerosis-new concepts.* Brain Res Bull, 2003. **61**(3): p. 321-6.

37. Popescu B.F. and Lucchinetti C.F., *Pathology of demyelinating diseases.* Annu Rev Pathol, 2012. **7**: p. 185-217.

38. Calabrese M., Filippi M., and Gallo P., *Cortical lesions in multiple sclerosis.* Nat Rev Neurol, 2010. **6**(8): p. 438-44.

39. Geurts J.J. and Barkhof F., *Grey matter pathology in multiple sclerosis.* Lancet Neurol, 2008. **7**(9): p. 841-51.

40. Popescu B.F., Pirko I., and Lucchinetti C.F., *Pathology of multiple sclerosis: where do we stand?* Continuum (Minneap Minn), 2013. **19**(4 Multiple Sclerosis): p. 901-21.

41. Babbe H., Roers A., Waisman A., Lassmann H., Goebels N., Hohlfeld R., Friese M., Schroder R., Deckert M., Schmidt S., Ravid R., and Rajewsky K., *Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction.* J Exp Med, 2000. **192**(3): p. 393-404.

42. Booss J., Esiri M.M., Tourtellotte W.W., and Mason D.Y., *Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis.* J Neurol Sci, 1983. **62**(1-3): p. 219-32.

43. Wucherpfennig K.W., Newcombe J., Li H., Keddy C., Cuzner M.L., and Hafler D.A., *Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions.* Proc Natl Acad Sci U S A, 1992. **89**(10): p. 4588-92.

44. Weber M.S., Hemmer B., and Cepok S., *The role of antibodies in multiple sclerosis.* Biochim Biophys Acta, 2011. **1812**(2): p. 239-45.

45. Prineas J.W. and Graham J.S., *Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown.* Ann Neurol, 1981. **10**(2): p. 149-58.

46. Dendrou C.A., Fugger L., and Friese M.A., *Immunopathology of multiple sclerosis.* Nat Rev Immunol, 2015. **15**(9): p. 545-58.

47. Constantinescu C.S., Farooqi N., O'brien K., and Gran B., *Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS).* Br J Pharmacol, 2011. **164**(4): p. 1079-106.

48. Kurschus F.C., *T cell mediated pathogenesis in EAE: Molecular mechanisms.* Biomed J, 2015. **38**(3): p. 183-93.

49. Frischer J.M., Bramow S., Dal-Bianco A., Lucchinetti C.F., Rauschka H., Schmidbauer M., Laursen H., Sorensen P.S., and Lassmann H., *The relation between inflammation and neurodegeneration in multiple sclerosis brains.* Brain, 2009. **132**(Pt 5): p. 1175-89.

50. Kutzelnigg A., Lucchinetti C.F., Stadelmann C., Bruck W., Rauschka H., Bergmann M., Schmidbauer M., Parisi J.E., and Lassmann H., *Cortical demyelination and diffuse white matter injury in multiple sclerosis.* Brain, 2005. **128**(Pt 11): p. 2705-12.

51. Steinman L. and Zamvil S.S., *Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis.* Trends Immunol, 2005. **26**(11): p. 565-71.

52. Farooqi N., Gran B., and Constantinescu C.S., *Are current disease-modifying therapeutics in multiple sclerosis justified on the basis of studies in experimental autoimmune encephalomyelitis?* J Neurochem, 2010. **115**(4): p. 829-44.

53. Gold R., Linington C., and Lassmann H., *Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research.* Brain, 2006. **129**(Pt 8): p. 1953-71.

54. Flugel A., Berkowicz T., Ritter T., Labeur M., Jenne D.E., Li Z., Ellwart J.W., Willem M., Lassmann H., and Wekerle H., *Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis.* Immunity, 2001. **14**(5): p. 547-60.

55. Furtado G.C., Marcondes M.C., Latkowski J.A., Tsai J., Wensky A., and Lafaille J.J., *Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis.* J Immunol, 2008. **181**(7): p. 4648-55.

56. T Hart B.A., Hintzen R.Q., and Laman J.D., *Preclinical assessment of therapeutic antibodies against human CD40 and human interleukin-12/23p40 in a nonhuman primate model of multiple sclerosis.* Neurodegener Dis, 2008. **5**(1): p. 38-52.

57. T Hart B.A., Hintzen R.Q., and Laman J.D., *Multiple sclerosis - a response-to-damage model.* Trends Mol Med, 2009. **15**(6): p. 235-44.

58. Bailey S.L., Carpentier P.A., Mcmahon E.J., Begolka W.S., and Miller S.D., *Innate and adaptive immune responses of the central nervous system.* Crit Rev Immunol, 2006. **26**(2): p. 149-88.

59. Guermonprez P., Valladeau J., Zitvogel L., Thery C., and Amigorena S., *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. **20**: p. 621-67.

60. Bailey S.L., Schreiner B., Mcmahon E.J., and Miller S.D., *CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE.* Nat Immunol, 2007. **8**(2): p. 172-80.

61. Domingues H.S., Mues M., Lassmann H., Wekerle H., and Krishnamoorthy G., *Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis.* PLoS One, 2010. **5**(11): p. e15531.

62. Edwards L.J., Robins R.A., and Constantinescu C.S., *Th17/Th1 phenotype in demyelinating disease.* Cytokine, 2010. **50**(1): p. 19-23.

63. Weiner H.L. and Selkoe D.J., *Inflammation and therapeutic vaccination in CNS diseases.* Nature, 2002. **420**(6917): p. 879-84.

64. Sprent J., *Antigen-presenting cells. Professionals and amateurs.* Curr Biol, 1995. **5**(10): p. 1095-7.

65. Fabriek B.O., Van Haastert E.S., Galea I., Polfliet M.M., Dopp E.D., Van Den Heuvel M.M., Van Den Berg T.K., De Groot C.J., Van Der Valk P., and Dijkstra C.D., *CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation.* Glia, 2005. **51**(4): p. 297-305.

66. Bauer J., Huitinga I., Zhao W., Lassmann H., Hickey W.F., and Dijkstra C.D., *The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis.* Glia, 1995. **15**(4): p. 437-46.

67. Tran E.H., Hoekstra K., Van Rooijen N., Dijkstra C.D., and Owens T., *Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice.* J Immunol, 1998. **161**(7): p. 3767-75.

68. Aloisi F., *Immune function of microglia.* Glia, 2001. **36**(2): p. 165-79.

69. Ransohoff R.M., *Microglia and monocytes: 'tis plain the twain meet in the brain.* Nat Neurosci, 2011. **14**(9): p. 1098-100.

70. Aloisi F., Ria F., Penna G., and Adorini L., *Microglia are more efficient than astrocytes in antigen processing and in Th1 but not Th2 cell activation.* J Immunol, 1998. **160**(10): p. 4671-80.

71. Bessis A., Bechade C., Bernard D., and Roumier A., *Microglial control of neuronal death and synaptic properties.* Glia, 2007. **55**(3): p. 233-8.

72. Ransohoff R.M. and Cardona A.E., *The myeloid cells of the central nervous system parenchyma.* Nature, 2010. **468**(7321): p. 253-62.

73. Cardona A.E., Pioro E.P., Sasse M.E., Kostenko V., Cardona S.M., Dijkstra I.M., Huang D., Kidd G., Dombrowski S., Dutta R., Lee J.C., Cook D.N., Jung S., Lira S.A., Littman D.R., and Ransohoff R.M., *Control of microglial neurotoxicity by the fractalkine receptor.* Nat Neurosci, 2006. **9**(7): p. 917-24.

74. Constam D.B., Philipp J., Malipiero U.V., Ten Dijke P., Schachner M., and Fontana A., *Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia.* J Immunol, 1992. **148**(5): p. 1404-10.

75. Nimmerjahn A., Kirchhoff F., and Helmchen F., *Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo.* Science, 2005. **308**(5726): p. 1314-8.

76. Carson M.J., Reilly C.R., Sutcliffe J.G., and Lo D., *Mature microglia resemble immature antigen-presenting cells.* Glia, 1998. **22**(1): p. 72-85.

77. Heppner F.L., Greter M., Marino D., Falsig J., Raivich G., Hovelmeyer N., Waisman A., Rulicke T., Prinz M., Priller J., Becher B., and Aguzzi A., *Experimental autoimmune encephalomyelitis repressed by microglial paralysis.* Nat Med, 2005. **11**(2): p. 146-52.

78. Wu L. and Liu Y.J., *Development of dendritic-cell lineages.* Immunity, 2007. **26**(6): p. 741-50.

79. Fischer H.G. and Reichmann G., *Brain dendritic cells and macrophages/microglia in central nervous system inflammation.* J Immunol, 2001. **166**(4): p. 2717-26.

80. Serafini B., Columba-Cabezas S., Di Rosa F., and Aloisi F., *Intracerebral recruitment and maturation of dendritic cells in the onset and progression of experimental autoimmune encephalomyelitis.* Am J Pathol, 2000. **157**(6): p. 1991-2002.

81. Greter M., Heppner F.L., Lemos M.P., Odermatt B.M., Goebels N., Laufer T., Noelle R.J., and Becher B., *Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis.* Nat Med, 2005. **11**(3): p. 328-34.

82. Whartenby K.A., Calabresi P.A., Mccadden E., Nguyen B., Kardian D., Wang T., Mosse C., Pardoll D.M., and Small D., *Inhibition of FLT3 signaling targets DCs to ameliorate autoimmune disease.* Proc Natl Acad Sci U S A, 2005. **102**(46): p. 16741-6.

83. Weller R.O., Galea I., Carare R.O., and Minagar A., *Pathophysiology of the lymphatic drainage of the central nervous system: Implications for pathogenesis and therapy of multiple sclerosis.* Pathophysiology, 2010. **17**(4): p. 295-306.

84. De Vos A.F., Van Meurs M., Brok H.P., Boven L.A., Hintzen R.Q., Van Der Valk P., Ravid R., Rensing S., Boon L., T Hart B.A., and Laman J.D., *Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs.* J Immunol, 2002. **169**(10): p. 5415-23.

85. Fabriek B.O., Zwemmer J.N., Teunissen C.E., Dijkstra C.D., Polman C.H., Laman J.D., and Castelijns J.A., *In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology.* J Neuroimmunol, 2005. **161**(1-2): p. 190-4.

86. Kooi E.J., Van Horssen J., Witte M.E., Amor S., Bo L., Dijkstra C.D., Van Der Valk P., and Geurts J.J., *Abundant extracellular myelin in the meninges of patients with multiple sclerosis.* Neuropathol Appl Neurobiol, 2009. **35**(3): p. 283-95.

87. Van Zwam M., Huizinga R., Melief M.J., Wierenga-Wolf A.F., Van Meurs M., Voerman J.S., Biber K.P., Boddeke H.W., Hopken U.E., Meisel C., Meisel A., Bechmann I., Hintzen R.Q., T Hart B.A., Amor S., Laman J.D., and Boven L.A., *Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE.* J Mol Med (Berl), 2009. **87**(3): p. 273-86.

88. Deshpande P., King I.L., and Segal B.M., *Cutting edge: CNS CD11c+ cells from mice with encephalomyelitis polarize Th17 cells and support CD25+CD4+ T cell-mediated immunosuppression, suggesting dual roles in the disease process.* J Immunol, 2007. **178**(11): p. 6695-9.

89. Kimelberg H.K., *Functions of mature mammalian astrocytes: a current view.* Neuroscientist, 2010. **16**(1): p. 79-106.

90. Nair A., Frederick T.J., and Miller S.D., *Astrocytes in multiple sclerosis: a product of their environment.* Cell Mol Life Sci, 2008. **65**(17): p. 2702-20.

91. Zeinstra E., Wilczak N., Streefland C., and De Keyser J., *Astrocytes in chronic active multiple sclerosis plaques express MHC class II molecules.* Neuroreport, 2000. **11**(1): p. 89-91.

92. Zeinstra E., Wilczak N., Chesik D., Glazenburg L., Kroese F.G., and De Keyser J., *Simvastatin inhibits interferon-gamma-induced MHC class II up-regulation in cultured astrocytes.* J Neuroinflammation, 2006. **3**: p. 16.

93. Constantinescu C.S., Tani M., Ransohoff R.M., Wysocka M., Hilliard B., Fujioka T., Murphy S., Tighe P.J., Das Sarma J., Trinchieri G., and Rostami A., *Astrocytes as antigen-presenting cells: expression of IL-12/IL-23.* J Neurochem, 2005. **95**(2): p. 331-40.

94. Tan L., Gordon K.B., Mueller J.P., Matis L.A., and Miller S.D., *Presentation of proteolipid protein epitopes and B7-1-dependent activation of encephalitogenic T cells by IFN-gamma-activated SJL/J astrocytes.* J Immunol, 1998. **160**(9): p. 4271-9.

95. Aloisi F., Ria F., and Adorini L., *Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes.* Immunol Today, 2000. **21**(3): p. 141-7.

96. Fischer H.G. and Bielinsky A.K., *Antigen presentation function of brain-derived dendriform cells depends on astrocyte help.* Int Immunol, 1999. **11**(8): p. 1265-74.

97. Hailer N.P., Heppner F.L., Haas D., and Nitsch R., *Astrocytic factors deactivate antigen presenting cells that invade the central nervous system.* Brain Pathol, 1998. **8**(3): p. 459-74.

98. Song J.H., Bellail A., Tse M.C., Yong V.W., and Hao C., *Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis.* J Neurosci, 2006. **26**(12): p. 3299-308.

99. Lee S.J., Zhou T., Choi C., Wang Z., and Benveniste E.N., *Differential regulation and function of Fas expression on glial cells.* J Immunol, 2000. **164**(3): p. 1277-85.

100. Suvannavejh G.C., Dal Canto M.C., Matis L.A., and Miller S.D., *Fas-mediated apoptosis in clinical remissions of relapsing experimental autoimmune encephalomyelitis.* J Clin Invest, 2000. **105**(2): p. 223-31.

101. Zepp J., Wu L., and Li X., *IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease.* Trends Immunol, 2011. **32**(5): p. 232-9.

102. Siffrin V., Vogt J., Radbruch H., Nitsch R., and Zipp F., *Multiple sclerosis - candidate mechanisms underlying CNS atrophy.* Trends Neurosci, 2010. **33**(4): p. 202-10.

103. Trapp B.D. and Nave K.A., *Multiple sclerosis: an immune or neurodegenerative disorder?* Annu Rev Neurosci, 2008. **31**: p. 247-69.

104. Wang J.T., Medress Z.A., and Barres B.A., *Axon degeneration: molecular mechanisms of a self-destruction pathway.* J Cell Biol, 2012. **196**(1): p. 7-18.

105. Shriver L.P. and Dittel B.N., *T-cell-mediated disruption of the neuronal microtubule network: correlation with early reversible axonal dysfunction in acute experimental autoimmune encephalomyelitis.* Am J Pathol, 2006. **169**(3): p. 999-1011.

106. Sorbara C.D., Wagner N.E., Ladwig A., Nikic I., Merkler D., Kleele T., Marinkovic P., Naumann R., Godinho L., Bareyre F.M., Bishop D., Misgeld T., and Kerschensteiner M., *Pervasive axonal transport deficits in multiple sclerosis models.* Neuron, 2014. **84**(6): p. 1183-90.

107. Nikic I., Merkler D., Sorbara C., Brinkoetter M., Kreutzfeldt M., Bareyre F.M., Bruck W., Bishop D., Misgeld T., and Kerschensteiner M., *A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis.* Nat Med, 2011. **17**(4): p. 495-9.

108. Siffrin V., Radbruch H., Glumm R., Niesner R., Paterka M., Herz J., Leuenberger T., Lehmann S.M., Luenstedt S., Rinnenthal J.L., Laube G., Luche H., Lehnardt S., Fehling H.J., Griesbeck O., and Zipp F., *In vivo imaging of partially reversible th17 cell-induced neuronal dysfunction in the course of encephalomyelitis.* Immunity, 2010. **33**(3): p. 424-36.

109. Hedegaard C.J., Krakauer M., Bendtzen K., Lund H., Sellebjerg F., and Nielsen C.H., *T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis.* Immunology, 2008. **125**(2): p. 161-9.

110. Herz J., Zipp F., and Siffrin V., *Neurodegeneration in autoimmune CNS inflammation.* Exp Neurol, 2010. **225**(1): p. 9-17.

111. Kebir H., Kreymborg K., Ifergan I., Dodelet-Devillers A., Cayrol R., Bernard M., Giuliani F., Arbour N., Becher B., and Prat A., *Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation.* Nat Med, 2007. **13**(10): p. 1173-5.

112. Medana I.M., Gallimore A., Oxenius A., Martinic M.M., Wekerle H., and Neumann H., *MHC class I-restricted killing of neurons by virus-specific CD8+ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway.* Eur J Immunol, 2000. **30**(12): p. 3623-33.

113. Neumann H., Schmidt H., Cavalie A., Jenne D., and Wekerle H., *Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha.* J Exp Med, 1997. **185**(2): p. 305-16.

114. Shaked I., Porat Z., Gersner R., Kipnis J., and Schwartz M., *Early activation of microglia as antigen-presenting cells correlates with T cell-mediated protection and repair of the injured central nervous system.* J Neuroimmunol, 2004. **146**(1-2): p. 84-93.

115. Kerschensteiner M., Gallmeier E., Behrens L., Leal V.V., Misgeld T., Klinkert W.E., Kolbeck R., Hoppe E., Oropeza-Wekerle R.L., Bartke I., Stadelmann C., Lassmann H., Wekerle H., and Hohlfeld R., *Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation?* J Exp Med, 1999. **189**(5): p. 865-70.

116. Moalem G., Leibowitz-Amit R., Yoles E., Mor F., Cohen I.R., and Schwartz M., *Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy.* Nat Med, 1999. **5**(1): p. 49-55.

117. Pool M., Rambaldi I., Darlington P.J., Wright M.C., Fournier A.E., and Bar-Or A., *Neurite outgrowth is differentially impacted by distinct immune cell subsets.* Mol Cell Neurosci, 2012. **49**(1): p. 68-76.

118. Liu Y., Teige I., Birnir B., and Issazadeh-Navikas S., *Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE.* Nat Med, 2006. **12**(5): p. 518-25.

119. Reynolds A.D., Stone D.K., Hutter J.A., Benner E.J., Mosley R.L., and Gendelman H.E., *Regulatory T cells attenuate Th17 cell-mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease.* J Immunol, 2010. **184**(5): p. 2261-71.

120. Aktas O., Smorodchenko A., Brocke S., Infante-Duarte C., Schulze Topphoff U., Vogt J., Prozorovski T., Meier S., Osmanova V., Pohl E., Bechmann I., Nitsch R., and Zipp F., *Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL.* Neuron, 2005. **46**(3): p. 421-32.

121. Vogt J., Paul F., Aktas O., Muller-Wielsch K., Dorr J., Dorr S., Bharathi B.S., Glumm R., Schmitz C., Steinbusch H., Raine C.S., Tsokos M., Nitsch R., and Zipp F., *Lower motor neuron loss in multiple sclerosis and experimental autoimmune encephalomyelitis.* Ann Neurol, 2009. **66**(3): p. 310-22.

122. Nitsch R., Pohl E.E., Smorodchenko A., Infante-Duarte C., Aktas O., and Zipp F., *Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue.* J Neurosci, 2004. **24**(10): p. 2458-64.

123. Miller N.M., Shriver L.P., Bodiga V.L., Ray A., Basu S., Ahuja R., Jana A., Pahan K., and Dittel B.N., *Lymphocytes with cytotoxic activity induce rapid microtubule axonal destabilization independently and before signs of neuronal death.* ASN Neuro, 2013. **5**(1): p. e00105.

124. Tanabe S. and Yamashita T., *Repulsive guidance molecule-a is involved in Th17-cell-induced neurodegeneration in autoimmune encephalomyelitis.* Cell Rep, 2014. **9**(4): p. 1459-70.

125. Jonas A., Thiem S., Kuhlmann T., Wagener R., Aszodi A., Nowell C., Hagemeier K., Laverick L., Perreau V., Jokubaitis V., Emery B., Kilpatrick T., Butzkueven H., and Gresle M., *Axonally derived matrilin-2 induces proinflammatory responses that exacerbate autoimmune neuroinflammation.* J Clin Invest, 2014. **124**(11): p. 5042-56.

126. Togashi H., Sakisaka T., and Takai Y., *Cell adhesion molecules in the central nervous system.* Cell Adh Migr, 2009. **3**(1): p. 29-35.

127. Takeda Y., Murakami Y., Asou H., and Uyemura K., *The roles of cell adhesion molecules on the formation of peripheral myelin.* Keio J Med, 2001. **50**(4): p. 240-8.

128. Martini R. and Carenini S., *Formation and maintenance of the myelin sheath in the peripheral nerve: roles of cell adhesion molecules and the gap junction protein connexin 32.* Microsc Res Tech, 1998. **41**(5): p. 403-15.

129. Brummendorf T. and Rathjen F.G., *Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily.* Curr Opin Neurobiol, 1996. **6**(5): p. 584-93.

130. Sonderegger P. and Rathjen F.G., *Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily.* J Cell Biol, 1992. **119**(6): p. 1387-94.

131. Williams E.J., Furness J., Walsh F.S., and Doherty P., *Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin.* Neuron, 1994. **13**(3): p. 583-94.

132. Tessier-Lavigne M. and Goodman C.S., *The molecular biology of axon guidance.* Science, 1996. **274**(5290): p. 1123-33.

133. Williams A.F. and Barclay A.N., *The immunoglobulin superfamily--domains for cell surface recognition.* Annu Rev Immunol, 1988. **6**: p. 381-405.

134. Wang J.H., *The sequence signature of an Ig-fold.* Protein Cell, 2013. **4**(8): p. 569-72.

135. Waxman S.G., Kocsis J.D., and Stys P.K., *The axon : structure, function, and pathophysiology*. 1995, New York: Oxford University Press. xv, 692 p., 2 p. of plates.

136. Rathjen F.G. and Schachner M., *Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion.* EMBO J, 1984. **3**(1): p. 1-10.

137. Rathjen F.G., Wolff J.M., Frank R., Bonhoeffer F., and Rutishauser U., *Membrane glycoproteins involved in neurite fasciculation.* J Cell Biol, 1987. **104**(2): p. 343-53.

138. Volkmer H., Leuschner R., Zacharias U., and Rathjen F.G., *Neurofascin induces neurites by heterophilic interactions with axonal NrCAM while NrCAM requires F11 on the axonal surface to extend neurites.* J Cell Biol, 1996. **135**(4): p. 1059-69.

139. Holm J., Hillenbrand R., Steuber V., Bartsch U., Moos M., Lubbert H., Montag D., and Schachner M., *Structural features of a close homologue of L1 (CHL1) in the mouse: a new member of the L1 family of neural recognition molecules.* Eur J Neurosci, 1996. **8**(8): p. 1613-29.

140. Brummendorf T., Kenwrick S., and Rathjen F.G., *Neural cell recognition molecule L1: from cell biology to human hereditary brain malformations.* Curr Opin Neurobiol, 1998. **8**(1): p. 87-97.

141. Lemmon V., Farr K.L., and Lagenaur C., *L1-mediated axon outgrowth occurs via a homophilic binding mechanism.* Neuron, 1989. **2**(6): p. 1597-603.

142. Hortsch M., *Structural and functional evolution of the L1 family: are four adhesion molecules better than one?* Mol Cell Neurosci, 2000. **15**(1): p. 1-10.

143. Schafer M.K. and Frotscher M., *Role of L1CAM for axon sprouting and branching.* Cell Tissue Res, 2012. **349**(1): p. 39-48.

144. Kiefel H., Pfeifer M., Bondong S., Hazin J., and Altevogt P., *Linking L1CAM-mediated signaling to NF-kappaB activation.* Trends Mol Med, 2011. **17**(4): p. 178-87.

145. Hoja-Lukowicz D., Link-Lenczowski P., Carpentieri A., Amoresano A., Pochec E., Artemenko K.A., Bergquist J., and Litynska A., *L1CAM from human melanoma carries a novel type of N-glycan with Galbeta1-4Galbeta1- motif. Involvement of N-linked glycans in migratory and invasive behaviour of melanoma cells.* Glycoconj J, 2013. **30**(3): p. 205-25.

146. Gast D., Riedle S., Kiefel H., Muerkoster S.S., Schafer H., Schafer M.K.E., and Altevogt P., *The RGD integrin binding site in human L1-CAM is important for nuclear signaling.* Experimental Cell Research, 2008. **314**(13): p. 2411-2418.

147. D'souzaa S.E., Ginsbergb M.H., and Plow E.F., *Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif.* Trends Biochem Sci., 1991. **16**: p. 246–250.

148. Kiefel H., Bondong S., Hazin J., Ridinger J., Schirmer U., Riedle S., and Altevogt P., *L1CAM: a major driver for tumor cell invasion and motility.* Cell Adh Migr, 2012. **6**(4): p. 374-84.

149. Mechtersheimer S., Gutwein P., Agmon-Levin N., Stoeck A., Oleszewski M., Riedle S., Postina R., Fahrenholz F., Fogel M., Lemmon V., and Altevogt P., *Ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins.* J Cell Biol, 2001. **155**(4): p. 661-73.

150. Schafer M.K. and Altevogt P., *L1CAM malfunction in the nervous system and human carcinomas.* Cell Mol Life Sci, 2010. **67**(14): p. 2425-37.

151. Herron L.R., Hill M., Davey F., and Gunn-Moore F.J., *The intracellular interactions of the L1 family of cell adhesion molecules.* Biochem J, 2009. **419**(3): p. 519-31.

152. Haspel J. and Grumet M., *The L1CAM extracellular region: a multi-domain protein with modular and cooperative binding modes.* Front Biosci, 2003. **8**: p. s1210-25.

153. Schäfer M.K.E. and Frotscher M., *Role of L1CAM for axon sprouting and branching.* Cell Tissue Res, 2012. **349**(1): p. 39-48.

154. Jakovcevski I., Djogo N., Hölters L.S., Szpotowicz E., and Schachner M., *Transgenic overexpression of the cell adhesion molecule L1 in neurons facilitates recovery after mouse spinal cord injury.* Neuroscience, 2013. **252**: p. 1-12.

155. Roonprapunt C., Huang W., Grill R., Friedlander D., Grumet M., Chen S., Schachner M., and Young W., *Soluble Cell Adhesion Molecule L1-Fc Promotes Locomotor Recovery in Rats after Spinal Cord Injury.* J Neurotrauma, 2003. **20**(9): p. 871-882.

156. Kataria H., Lutz D., Chaudhary H., Schachner M., and Loers G., *Small Molecule Agonists of Cell Adhesion Molecule L1 Mimic L1 Functions In Vivo.* Molecular Neurobiology, 2015: p. 1-23.

157. Schäfer M. and Altevogt P., *L1CAM malfunction in the nervous system and human carcinomas.* Cell Mol Life Sci, 2010. **67**(14): p. 2425-2437.

158. Castellani V., De Angelis E., Kenwrick S., and Rougon G., *Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A.* EMBO J, 2002. **21**(23): p. 6348-57.

159. Bechara A., Nawabi H., Moret F., Yaron A., Weaver E., Bozon M., Abouzid K., Guan J.L., Tessier-Lavigne M., Lemmon V., and Castellani V., *FAK-MAPK-dependent adhesion disassembly downstream of L1 contributes to semaphorin3A-induced collapse.* EMBO J, 2008. **27**(11): p. 1549-62.

160. Castellani V., Falk J., and Rougon G., *Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM.* Mol Cell Neurosci, 2004. **26**(1): p. 89-100.

161. Stoeck A., Schlich S., Issa Y., Gschwend V., Wenger T., Herr I., Marme A., Bourbie S., Altevogt P., and Gutwein P., *L1 on ovarian carcinoma cells is a binding partner for Neuropilin-1 on mesothelial cells.* Cancer Lett, 2006. **239**(2): p. 212-26.

162. Ben-Zvi A., Manor O., Schachner M., Yaron A., Tessier-Lavigne M., and Behar O., *The Semaphorin Receptor PlexinA3 Mediates Neuronal Apoptosis during Dorsal Root Ganglia Development.* The Journal of Neuroscience, 2008. **28**(47): p. 12427-12432.

163. Castellani V., De Angelis E., Kenwrick S., and Rougon G., *Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A.* EMBO Journal, 2002. **21**(23): p. 6348–6357.

164. Bechara A., Nawabi H., Moret F., Yaron A., Weaver E., Bozon M., Abouzid K., Guan J.-L., Tessier-Lavigne M., Lemmon V., and Castellani V., *FAK-MAPK-dependent adhesion disassembly downstream of L1 contributes to semaphorin3A-induced collapse.* EMBO Journal, 2008. **27**(11): p. 1549-1562.

165. Kaneko S., Iwanami A., Nakamura M., Kishino A., Kikuchi K., Shibata S., Okano H.J., Ikegami T., Moriya A., Konishi O., Nakayama C., Kumagai K., Kimura T., Sato Y., Goshima Y., Taniguchi M., Ito M., He Z., Toyama Y., and Okano H., *A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord.* Nat Med, 2006. **12**(12): p. 1380-1389.

166. Williams A., Piaton G., Aigrot M.-S., Belhadi A., Théaudin M., Petermann F., Thomas J.-L., Zalc B., and Lubetzki C., *Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis?* Brain, 2007. **130**(10): p. 2554-2565.

167. Kallunki P., Edelman G.M., and Jones F.S., *Tissue-specific expression of the L1 cell adhesion molecule is modulated by the neural restrictive silencer element.* J Cell Biol, 1997. **138**(6): p. 1343-54.

168. Gao Z., Ure K., Ding P., Nashaat M., Yuan L., Ma J., Hammer R.E., and Hsieh J., *The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells.* J Neurosci, 2011. **31**(26): p. 9772-86.

169. Qureshi I.A., Gokhan S., and Mehler M.F., *REST and CoREST are transcriptional and epigenetic regulators of seminal neural fate decisions.* Cell Cycle, 2010. **9**(22): p. 4477-86.

170. Formisano L., Guida N., Valsecchi V., Pignataro G., Vinciguerra A., Pannaccione A., Secondo A., Boscia F., Molinaro P., Sisalli M.J., Sirabella R., Casamassa A., Canzoniero L.M., Di Renzo G., and Annunziato L., *NCX1 is a new rest target gene: role in cerebral ischemia.* Neurobiol Dis, 2013. **50**: p. 76-85.

171. Spencer E.M., Chandler K.E., Haddley K., Howard M.R., Hughes D., Belyaev N.D., Coulson J.M., Stewart J.P., Buckley N.J., Kipar A., Walker M.C., and Quinn J.P., *Regulation and role of REST and REST4 variants in modulation of gene expression in in vivo and in vitro in epilepsy models.* Neurobiol Dis, 2006. **24**(1): p. 41-52.

172. Mikulak J., Negrini S., Klajn A., D'alessandro R., Mavilio D., and Meldolesi J., *Dual REST-dependence of L1CAM: from gene expression to alternative splicing governed by Nova2 in neural cells.* J Neurochem, 2012. **120**(5): p. 699-709.

173. Araki K. and Nagata K., *Protein folding and quality control in the ER.* Cold Spring Harb Perspect Biol, 2011. **3**(11): p. a007526.

174. Cooper G., *The Cell: A Molecular Approach. 2nd edition.* 2000.

175. Vitale A., Ceriotti A., and Denecke J., *The Role of the Endoplasmic Reticulum in Protein Synthesis, Modification and Intracellular Transport.* Journal of Experimental Botany, 1993. **44**(266): p. 1417-1444.

176. Wisco D., Anderson E.D., Chang M.C., Norden C., Boiko T., Folsch H., and Winckler B., *Uncovering multiple axonal targeting pathways in hippocampal neurons.* J Cell Biol, 2003. **162**(7): p. 1317-28.

177. Schafer M.K., Schmitz B., and Diestel S., *L1CAM ubiquitination facilitates its lysosomal degradation.* FEBS Lett, 2010. **584**(21): p. 4475-80.

178. Primiano T., Baig M., Maliyekkel A., Chang B.D., Fellars S., Sadhu J., Axenovich S.A., Holzmayer T.A., and Roninson I.B., *Identification of potential anticancer drug targets through the selection of growth-inhibitory genetic suppressor elements.* Cancer Cell, 2003. **4**(1): p. 41-53.

179. Ben Q.W., Wang J.C., Liu J., Zhu Y., Yuan F., Yao W.Y., and Yuan Y.Z., *Positive expression of L1-CAM is associated with perineural invasion and poor outcome in pancreatic ductal adenocarcinoma.* Ann Surg Oncol, 2010. **17**(8): p. 2213-21.

180. Boo Y.J., Park J.M., Kim J., Chae Y.S., Min B.W., Um J.W., and Moon H.Y., *L1 expression as a marker for poor prognosis, tumor progression, and short survival in patients with colorectal cancer.* Ann Surg Oncol, 2007. **14**(5): p. 1703-11.

181. Ebeling O., Duczmal A., Aigner S., Geiger C., Schollhammer S., Kemshead J.T., Moller P., Schwartz-Albiez R., and Altevogt P., *L1 adhesion molecule on human lymphocytes and monocytes: expression and involvement in binding to alpha v beta 3 integrin.* Eur J Immunol, 1996. **26**(10): p. 2508-16.

182. Maddaluno L., Verbrugge S.E., Martinoli C., Matteoli G., Chiavelli A., Zeng Y., Williams E.D., Rescigno M., and Cavallaro U., *The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells.* J Exp Med, 2009. **206**(3): p. 623-35.

183. Kowitz A., Kadmon G., Eckert M., Schirrmacher V., Schachner M., and Altevogt P., *Expression and function of the neural cell adhesion molecule L1 in mouse leukocytes.* Eur J Immunol, 1992. **22**(5): p. 1199-205.

184. Pancook J.D., Reisfeld R.A., Varki N., Vitiello A., Fox R.I., and Montgomery A.M., *Expression and regulation of the neural cell adhesion molecule L1 on human cells of myelomonocytic and lymphoid origin.* J Immunol, 1997. **158**(9): p. 4413-21.

185. Hubbe M., Kowitz A., Schirrmacher V., Schachner M., and Altevogt P., *L1 adhesion molecule on mouse leukocytes: regulation and involvement in endothelial cell binding.* Eur J Immunol, 1993. **23**(11): p. 2927-31.

186. Balaian L.B., Moehler T., and Montgomery A.M., *The human neural cell adhesion molecule L1 functions as a costimulatory molecule in T cell activation.* Eur J Immunol, 2000. **30**(3): p. 938-43.

187. Havran W.L., Poenie M., Kimura J., Tsien R., Weiss A., and Allison J.P., *Expression and function of the CD3-antigen receptor on murine CD4+8+ thymocytes.* Nature, 1987. **330**(6144): p. 170-3.

188. Jouet M., Rosenthal A., and Kenwrick S., *Exon 2 of the gene for neural cell adhesion molecule L1 is alternatively spliced in B cells.* Brain Res Mol Brain Res, 1995. **30**(2): p. 378-80.

189. Takeda Y., Asou H., Murakami Y., Miura M., Kobayashi M., and Uyemura K., *A nonneuronal isoform of cell adhesion molecule L1: tissue-specific expression and functional analysis.* J Neurochem, 1996. **66**(6): p. 2338-49.

190. De Angelis E., Brummendorf T., Cheng L., Lemmon V., and Kenwrick S., *Alternative use of a mini exon of the L1 gene affects L1 binding to neural ligands.* J Biol Chem, 2001. **276**(35): p. 32738-42.

191. Schafer H., Struck B., Feldmann E.M., Bergmann F., Grage-Griebenow E., Geismann C., Ehlers S., Altevogt P., and Sebens S., *TGF-beta1-dependent L1CAM expression has an essential role in macrophage-induced apoptosis resistance and cell migration of human intestinal epithelial cells.* Oncogene, 2013. **32**(2): p. 180-9.

192. Bondong S., Kiefel H., Hielscher T., Zeimet A.G., Zeillinger R., Pils D., Schuster E., Castillo-Tong D.C., Cadron I., Vergote I., Braicu I., Sehouli J., Mahner S., Fogel M.,

and Altevogt P., *Prognostic significance of L1CAM in ovarian cancer and its role in constitutive NF-kappaB activation.* Ann Oncol, 2012. **23**(7): p. 1795-802.

193. Kiefel H., Bondong S., Pfeifer M., Schirmer U., Erbe-Hoffmann N., Schafer H., Sebens S., and Altevogt P., *EMT-associated up-regulation of L1CAM provides insights into L1CAM-mediated integrin signalling and NF-kappaB activation.* Carcinogenesis, 2012. **33**(10): p. 1919-29.

194. Kiefel H., Bondong S., Erbe-Hoffmann N., Hazin J., Riedle S., Wolf J., Pfeifer M., Arlt A., Schafer H., Muerkoster S.S., and Altevogt P., *L1CAM-integrin interaction induces constitutive NF-kappaB activation in pancreatic adenocarcinoma cells by enhancing IL-1beta expression.* Oncogene, 2010. **29**(34): p. 4766-78.

195. Itoh K. and Fushiki S., *The role of L1cam in murine corticogenesis, and the pathogenesis of hydrocephalus.* Pathol Int, 2015. **65**(2): p. 58-66.

196. Nijland P.G., Michailidou I., Witte M.E., Mizee M.R., Van Der Pol S.M., Van Het Hof B., Reijerkerk A., Pellerin L., Van Der Valk P., De Vries H.E., and Van Horssen J., *Cellular distribution of glucose and monocarboxylate transporters in human brain white matter and multiple sclerosis lesions.* Glia, 2014. **62**(7): p. 1125-41.

197. Rathjen F.G. and Schachner M., *Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion.* EMBO J, 1984. **3**(1): p. 1-10.

198. Zhu Y., Romero M.I., Ghosh P., Ye Z., Charnay P., Rushing E.J., Marth J.D., and Parada L.F., *Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain.* Gene Dev, 2001. **15**(7): p. 859-876.

199. Law J.W.S., Lee A.Y.W., Sun M., Nikonenko A.G., Chung S.K., Dityatev A., Schachner M., and Morellini F., *Decreased Anxiety, Altered Place Learning, and Increased CA1 Basal Excitatory Synaptic Transmission in Mice with Conditional Ablation of the Neural Cell Adhesion Molecule L1.* J Neurosci, 2003. **23**(32): p. 10419-10432.

200. Doberstein K., Harter P.N., Haberkorn U., Bretz N.P., Arnold B., Carretero R., Moldenhauer G., Mittelbronn M., and Altevogt P., *Antibody therapy to human L1CAM in a transgenic mouse model blocks local tumor growth but induces EMT.* Int J Cancer, 2015. **136**(5): p. 25.

201. Tsenter J., Beni-Adani L., Assaf Y., Alexandrovich A.G., Trembovler V., and Shohami E., *Dynamic changes in the recovery after traumatic brain injury in mice: effect of injury severity on T2-weighted MRI abnormalities, and motor and cognitive functions.* J Neurotrauma, 2008. **25**(4): p. 324-33.

202. Jolivel V., Luessi F., Masri J., Kraus S.H.P., Hubo M., Poisa-Beiro L., Klebow S., Paterka M., Yogev N., Tumani H., Furlan R., Siffrin V., Jonuleit H., Zipp F., and Waisman A., *Modulation of dendritic cell properties by laquinimod as a mechanism for modulating multiple sclerosis.* Brain, 2013. **136**(4): p. 1048-1066.

203. Runker A.E., Bartsch U., Nave K.-A., and Schachner M., *The C264Y Missense Mutation in the Extracellular Domain of L1 Impairs Protein Trafficking In Vitro and In Vivo.* J. Neurosci., 2003. **23**(1): p. 277-286.

204. Marx M., Diestel S., Bozon M., Keglowich L., Drouot N., Bouché E., Frebourg T., Minz M., Saugier-Veber P., Castellani V., and Schäfer M., *Pathomechanistic*

characterization of two exonic L1CAM variants located in trans in an obligate carrier of X-linked hydrocephalus. Neurogenetics, 2012. **13**(1): p. 49-59.

205. Christaller W.A., Vos Y., Gebre-Medhin S., Hofstra R.M., and Schafer M.K., *L1 syndrome diagnosis complemented with functional analysis of L1CAM variants located to the two N-terminal Ig-like domains.* Clin Genet, 2016.

206. Siffrin V., Brandt A.U., Radbruch H., Herz J., Boldakowa N., Leuenberger T., Werr J., Hahner A., Schulze-Topphoff U., Nitsch R., and Zipp F., *Differential immune cell dynamics in the CNS cause CD4+ T cell compartmentalization.* Brain, 2009. **132**(Pt 5): p. 1247-58.

207. Appel F., Holm J., Conscience J.F., Von Bohlen Und Halbach F., Faissner A., James P., and Schachner M., *Identification of the border between fibronectin type III homologous repeats 2 and 3 of the neural cell adhesion molecule L1 as a neurite outgrowth promoting and signal transducing domain.* J Neurobiol, 1995. **28**(3): p. 297-312.

208. Schildge S., Bohrer C., Beck K., and Schachtrup C., *Isolation and culture of mouse cortical astrocytes.* J Vis Exp, 2013(71).

209. Pfaffl M.W., *A new mathematical model for relative quantification in real-time RT–PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.

210. Larionov A., Krause A., and Miller W., *A standard curve based method for relative real time PCR data processing.* BMC Bioinformatics, 2005. **6**: p. 62.

211. Kadmon G., Montgomery A.M., and Altevogt P., *L1 makes immunological progress by expanding its relations.* Dev Immunol, 1998. **6**(3-4): p. 205-13.

212. Bo L., Geurts J.J., Mork S.J., and Van Der Valk P., *Grey matter pathology in multiple sclerosis.* Acta Neurol Scand Suppl, 2006. **183**: p. 48-50.

213. Wei C.H. and Ryu S.E., *Homophilic interaction of the L1 family of cell adhesion molecules.* Exp Mol Med, 2012. **44**(7): p. 413-23.

214. Formisano L., Guida N., Laudati G., Boscia F., Esposito A., Secondo A., Di Renzo G., and Canzoniero L.M., *Extracellular signal-related kinase 2/specificity protein 1/specificity protein 3/repressor element-1 silencing transcription factor pathway is involved in Aroclor 1254-induced toxicity in SH-SY5Y neuronal cells.* J Neurosci Res, 2015. **93**(1): p. 167-77.

215. Nesti E., Corson G.M., Mccleskey M., Oyer J.A., and Mandel G., *C-terminal domain small phosphatase 1 and MAP kinase reciprocally control REST stability and neuronal differentiation.* Proc Natl Acad Sci U S A, 2014. **111**(37): p. E3929-36.

216. Luckheeram R.V., Zhou R., Verma A.D., and Xia B., *CD4(+)T cells: differentiation and functions.* Clin Dev Immunol, 2012. **2012**: p. 925135.

217. Ruppert M., Aigner S., Hubbe M., Yagita H., and Altevogt P., *The L1 adhesion molecule is a cellular ligand for VLA-5.* J Cell Biol, 1995. **131**(6 Pt 2): p. 1881-91.

218. Batoulis H., Recks M.S., Holland F.O., Thomalla F., Williams R.O., and Kuerten S., *Blockade of tumour necrosis factor-alpha in experimental autoimmune encephalomyelitis reveals differential effects on the antigen-specific immune response and central nervous system histopathology.* Clin Exp Immunol, 2014. **175**(1): p. 41-8.

219. Anderton S.M. and Liblau R.S., *Regulatory T cells in the control of inflammatory demyelinating diseases of the central nervous system.* Curr Opin Neurol, 2008. **21**(3): p. 248-54.

220. Dahme M., Bartsch U., Martini R., Anliker B., Schachner M., and Mantei N., *Disruption of the mouse L1 gene leads to malformations of the nervous system.* Nature Genetics, 1997. **17**(3): p. 346-349.

221. Cohen N.R., Taylor J.S.H., Scott L.B., Guillery R.W., Soriano P., and Furley A.J.W., *Errors in corticospinal axon guidance in mice lacking the neural cell adhesion molecule L1.* Current Biology, 1998. **8**(1): p. 26-33.

222. Ben-Nun A., Kaushansky N., Kawakami N., Krishnamoorthy G., Berer K., Liblau R., Hohlfeld R., and Wekerle H., *From classic to spontaneous and humanized models of multiple sclerosis: Impact on understanding pathogenesis and drug development.* Journal of Autoimmunity, 2014. **54**: p. 33-50.

223. Siffrin V., Radbruch H., Glumm R., Niesner R., Paterka M., Herz J., Leuenberger T., Lehmann S.M., Luenstedt S., Rinnenthal J.L., Laube G., Luche H., Lehnardt S., Fehling H.-J., Griesbeck O., and Zipp F., *In Vivo Imaging of Partially Reversible Th17 Cell-Induced Neuronal Dysfunction in the Course of Encephalomyelitis.* Immunity, 2010. **33**(3): p. 424-436.

224. De Gasperi R., Rocher A.B., Sosa M.A., Wearne S.L., Perez G.M., Friedrich V.L., Jr., Hof P.R., and Elder G.A., *The IRG mouse: a two-color fluorescent reporter for assessing Cre-mediated recombination and imaging complex cellular relationships in situ.* Genesis, 2008. **46**(6): p. 308-17.

225. Fransen E., D'hooge R., Van Camp G., Verhoye M., Sijbers J., Reyniers E., Soriano P., Kamiguchi H., Willemsen R., Koekkoek S.K., De Zeeuw C.I., De Deyn P.P., Van Der Linden A., Lemmon V., Kooy R.F., and Willems P.J., *L1 knockout mice show dilated ventricles, vermis hypoplasia and impaired exploration patterns.* Hum Mol Genet, 1998. **7**(6): p. 999-1009.

226. Saghatelyan A.K., Nikonenko A.G., Sun M., Rolf B., Putthoff P., Kutsche M., Bartsch U., Dityatev A., and Schachner M., *Reduced GABAergic transmission and number of hippocampal perisomatic inhibitory synapses in juvenile mice deficient in the neural cell adhesion molecule L1.* Mol Cell Neurosci, 2004. **26**(1): p. 191-203.

227. Benveniste E.N., *Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis.* J Mol Med (Berl), 1997. **75**(3): p. 165-73.

228. Stuve O., Youssef S., Slavin A.J., King C.L., Patarroyo J.C., Hirschberg D.L., Brickey W.J., Soos J.M., Piskurich J.F., Chapman H.A., and Zamvil S.S., *The role of the MHC class II transactivator in class II expression and antigen presentation by astrocytes and in susceptibility to central nervous system autoimmune disease.* J Immunol, 2002. **169**(12): p. 6720-32.

229. Cho S.H., Chen J.A., Sayed F., Ward M.E., Gao F., Nguyen T.A., Krabbe G., Sohn P.D., Lo I., Minami S., Devidze N., Zhou Y., Coppola G., and Gan L., *SIRT1 deficiency in microglia contributes to cognitive decline in aging and neurodegeneration via epigenetic regulation of IL-1beta.* J Neurosci, 2015. **35**(2): p. 807-18.

230. Goren I., Allmann N., Yogev N., Schurmann C., Linke A., Holdener M., Waisman A., Pfeilschifter J., and Frank S., *A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage*

ablation on wound inflammatory, angiogenic, and contractive processes. Am J Pathol, 2009. **175**(1): p. 132-47.

231. Sagar D., Lamontagne A., Foss C.A., Khan Z.K., Pomper M.G., and Jain P., *Dendritic cell CNS recruitment correlates with disease severity in EAE via CCL2 chemotaxis at the blood-brain barrier through paracellular transmigration and ERK activation.* J Neuroinflammation, 2012. **9**: p. 245.

232. Villadangos J.A., Cardoso M., Steptoe R.J., Van Berkel D., Pooley J., Carbone F.R., and Shortman K., *MHC class II expression is regulated in dendritic cells independently of invariant chain degradation.* Immunity, 2001. **14**(6): p. 739-49.

233. Smith M.A., Perry G., Zhu X., and Haoudi A., *Neurodegenerative diseases: mechanisms and therapies.* J Biomed Biotechnol, 2006. **2006**(3): p. 47539.

234. Derfuss T., Parikh K., Velhin S., Braun M., Mathey E., Krumbholz M., Kümpfel T., Moldenhauer A., Rader C., Sonderegger P., Pöllmann W., Tiefenthaller C., Bauer J., Lassmann H., Wekerle H., Karagogeos D., Hohlfeld R., Linington C., and Meinl E., *Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals.* Proceedings of the National Academy of Sciences, 2009. **106**(20): p. 8302-8307.

235. Mathey E.K., Derfuss T., Storch M.K., Williams K.R., Hales K., Woolley D.R., Al-Hayani A., Davies S.N., Rasband M.N., Olsson T., Moldenhauer A., Velhin S., Hohlfeld R., Meinl E., and Linington C., *Neurofascin as a novel target for autoantibody-mediated axonal injury.* The Journal of Experimental Medicine, 2007. **204**(10): p. 2363-2372.

236. Wood P.M., Schachner M., and Bunge R.P., *Inhibition of Schwann cell myelination in vitro by antibody to the L1 adhesion molecule.* J Neurosci, 1990. **10**(11): p. 3635-45.

237. Haney C.A., Sahenk Z., Li C., Lemmon V.P., Roder J., and Trapp B.D., *Heterophilic binding of L1 on unmyelinated sensory axons mediates Schwann cell adhesion and is required for axonal survival.* J Cell Biol, 1999. **146**(5): p. 1173-84.

238. Chen S., Mantei N., Dong L., and Schachner M., *Prevention of neuronal cell death by neural adhesion molecules L1 and CHL1.* J Neurobiol, 1999. **38**(3): p. 428-39.

239. Roonprapunt C., Huang W., Grill R., Friedlander D., Grumet M., Chen S., Schachner M., and Young W., *Soluble cell adhesion molecule L1-Fc promotes locomotor recovery in rats after spinal cord injury.* J Neurotrauma, 2003. **20**(9): p. 871-82.

240. Kataria H., Lutz D., Chaudhary H., Schachner M., and Loers G., *Small Molecule Agonists of Cell Adhesion Molecule L1 Mimic L1 Functions In Vivo.* Mol Neurobiol, 2015: p. 1-23.

241. Liblau R.S., Gonzalez-Dunia D., Wiendl H., and Zipp F., *Neurons as targets for T cells in the nervous system.* Trends Neurosci, 2013. **36**(6): p. 315-24.

242. Weller S. and Gartner J., *Genetic and clinical aspects of X-linked hydrocephalus (L1 disease): Mutations in the L1CAM gene.* Hum Mutat, 2001. **18**(1): p. 1-12.

243. Akopians A., Runyan S.A., and Phelps P.E., *Expression of L1 decreases during postnatal development of rat spinal cord.* J Comp Neurol, 2003. **467**(3): p. 375-88.

244. Uhlen M., Fagerberg L., Hallstrom B.M., Lindskog C., Oksvold P., Mardinoglu A., Sivertsson A., Kampf C., Sjostedt E., Asplund A., Olsson I., Edlund K., Lundberg E.,

Navani S., Szigyarto C.A., Odeberg J., Djureinovic D., Takanen J.O., Hober S., Alm T., Edqvist P.H., Berling H., Tegel H., Mulder J., Rockberg J., Nilsson P., Schwenk J.M., Hamsten M., Von Feilitzen K., Forsberg M., Persson L., Johansson F., Zwahlen M., Von Heijne G., Nielsen J., and Ponten F., *Proteomics. Tissue-based map of the human proteome.* Science, 2015. **347**(6220): p. 1260419.

245. Guseva D., Zerwas M., Xiao M.F., Jakovcevski I., Irintchev A., and Schachner M., *Adhesion molecule L1 overexpressed under the control of the neuronal Thy-1 promoter improves myelination after peripheral nerve injury in adult mice.* Exp Neurol, 2011. **229**(2): p. 339-52.

246. Derfuss T., Linington C., Hohlfeld R., and Meinl E., *Axo-glial antigens as targets in multiple sclerosis: implications for axonal and grey matter injury.* J Mol Med (Berl), 2010. **88**(8): p. 753-61.

247. Derfuss T., Parikh K., Velhin S., Braun M., Mathey E., Krumbholz M., Kumpfel T., Moldenhauer A., Rader C., Sonderegger P., Pollmann W., Tiefenthaller C., Bauer J., Lassmann H., Wekerle H., Karagogeos D., Hohlfeld R., Linington C., and Meinl E., *Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals.* Proc Natl Acad Sci U S A, 2009. **106**(20): p. 8302-7.

248. Mathey E.K., Derfuss T., Storch M.K., Williams K.R., Hales K., Woolley D.R., Al-Hayani A., Davies S.N., Rasband M.N., Olsson T., Moldenhauer A., Velhin S., Hohlfeld R., Meinl E., and Linington C., *Neurofascin as a novel target for autoantibody-mediated axonal injury.* J Exp Med, 2007. **204**(10): p. 2363-72.

249. Ng J.K., Malotka J., Kawakami N., Derfuss T., Khademi M., Olsson T., Linington C., Odaka M., Tackenberg B., Pruss H., Schwab J.M., Harms L., Harms H., Sommer C., Rasband M.N., Eshed-Eisenbach Y., Peles E., Hohlfeld R., Yuki N., Dornmair K., and Meinl E., *Neurofascin as a target for autoantibodies in peripheral neuropathies.* Neurology, 2012. **79**(23): p. 2241-8.

250. Hauser S.L., Bhan A.K., Gilles F., Kemp M., Kerr C., and Weiner H.L., *Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions.* Ann Neurol, 1986. **19**(6): p. 578-87.

251. Traugott U., Reinherz E.L., and Raine C.S., *Multiple sclerosis: distribution of T cell subsets within active chronic lesions.* Science, 1983. **219**(4582): p. 308-10.

252. Cheresh D.A., *Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor.* Proc Natl Acad Sci U S A, 1987. **84**(18): p. 6471-5.

253. Rabinowich H., Lin W.C., Manciulea M., Herberman R.B., and Whiteside T.L., *Induction of protein tyrosine phosphorylation in human natural killer cells by triggering via alpha 4 beta 1 or alpha 5 beta 1 integrins.* Blood, 1995. **85**(7): p. 1858-64.

254. Salcedo R. and Patarroyo M., *Constitutive alpha V beta 3 integrin-mediated adhesion of human lymphoid B cells to vitronectin substrate.* Cell Immunol, 1995. **160**(2): p. 165-72.

255. Moulder K., Roberts K., Shevach E.M., and Coligan J.E., *The mouse vitronectin receptor is a T cell activation antigen.* J Exp Med, 1991. **173**(2): p. 343-7.

256. Montgomery A.M., Becker J.C., Siu C.H., Lemmon V.P., Cheresh D.A., Pancook J.D., Zhao X., and Reisfeld R.A., *Human neural cell adhesion molecule L1 and rat homologue NILE are ligands for integrin alpha v beta 3.* J Cell Biol, 1996. **132**(3): p. 475-85.

257. Du F., Garg A.V., Kosar K., Majumder S., Kugler D.G., Mir G.H., Maggio M., Henkel M., Lacy-Hulbert A., and Mcgeachy M.J., *Inflammatory Th17 Cells Express Integrin alphavbeta3 for Pathogenic Function.* Cell Rep, 2016. **16**(5): p. 1339-51.

258. Zhang F., Yang J., Jiang H., and Han S., *An alphanubeta3 integrin-binding peptide ameliorates symptoms of chronic progressive experimental autoimmune encephalomyelitis by alleviating neuroinflammatory responses in mice.* J Neuroimmune Pharmacol, 2014. **9**(3): p. 399-412.

259. Bearer C.F., *L1 cell adhesion molecule signal cascades: targets for ethanol developmental neurotoxicity.* Neurotoxicology, 2001. **22**(5): p. 625-33.

260. Euer N.I., Kaul S., Deissler H., Mobus V.J., Zeillinger R., and Weidle U.H., *Identification of L1CAM, Jagged2 and Neuromedin U as ovarian cancer-associated antigens.* Oncol Rep, 2005. **13**(3): p. 375-87.

261. Reid R.A. and Hemperly J.J., *Variants of human L1 cell adhesion molecule arise through alternate splicing of RNA.* J Mol Neurosci, 1992. **3**(3): p. 127-35.

262. Hauser S., Bickel L., Weinspach D., Gerg M., Schafer M.K., Pfeifer M., Hazin J., Schelter F., Weidle U.H., Ramser J., Volkmann J., Meindl A., Schmitt M., Schrotzlmair F., Altevogt P., and Kruger A., *Full-length L1CAM and not its Delta2Delta27 splice variant promotes metastasis through induction of gelatinase expression.* PLoS One, 2011. **6**(4): p. e18989.

263. Calderone A., Jover T., Noh K.M., Tanaka H., Yokota H., Lin Y., Grooms S.Y., Regis R., Bennett M.V., and Zukin R.S., *Ischemic insults derepress the gene silencer REST in neurons destined to die.* J Neurosci, 2003. **23**(6): p. 2112-21.

264. Yu M., Suo H., Liu M., Cai L., Liu J., Huang Y., Xu J., Wang Y., Zhu C., Fei J., and Huang F., *NRSF/REST neuronal deficient mice are more vulnerable to the neurotoxin MPTP.* Neurobiol Aging, 2013. **34**(3): p. 916-27.

265. Pozzi D., Lignani G., Ferrea E., Contestabile A., Paonessa F., D'alessandro R., Lippiello P., Boido D., Fassio A., Meldolesi J., Valtorta F., Benfenati F., and Baldelli P., *REST/NRSF-mediated intrinsic homeostasis protects neuronal networks from hyperexcitability.* EMBO J, 2013. **32**(22): p. 2994-3007.

266. Zhao Y., Zhu M., Yu Y., Qiu L., Zhang Y., He L., and Zhang J., *Brain REST/NRSF Is Not Only a Silent Repressor but Also an Active Protector.* Mol Neurobiol, 2016.

267. Nesti E., Corson G.M., Mccleskey M., Oyer J.A., and Mandel G., *C-terminal domain small phosphatase 1 and MAP kinase reciprocally control REST stability and neuronal differentiation.* Proc Natl Acad Sci U S A, 2014. **111**(37): p. 2.

268. Yi Y.S., Baek K.S., and Cho J.Y., *L1 cell adhesion molecule induces melanoma cell motility by activation of mitogen-activated protein kinase pathways.* Pharmazie, 2014. **69**(6): p. 461-7.

269. Hai J., Zhu C.Q., Bandarchi B., Wang Y.H., Navab R., Shepherd F.A., Jurisica I., and Tsao M.S., *L1 cell adhesion molecule promotes tumorigenicity and metastatic potential in non-small cell lung cancer.* Clin Cancer Res, 2012. **18**(7): p. 1914-24.

270. Schmid R.S. and Maness P.F., *L1 and NCAM adhesion molecules as signaling coreceptors in neuronal migration and process outgrowth.* Curr Opin Neurobiol, 2008. **18**(3): p. 245-50.

271. Schmid R.S., Midkiff B.R., Kedar V.P., and Maness P.F., *Adhesion molecule L1 stimulates neuronal migration through Vav2-Pak1 signaling.* Neuroreport, 2004. **15**(18): p. 2791-4.

272. Shin S.Y., Rath O., Choo S.M., Fee F., Mcferran B., Kolch W., and Cho K.H., *Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway.* J Cell Sci, 2009. **122**(Pt 3): p. 425-35.

273. Alessandrini A., Namura S., Moskowitz M.A., and Bonventre J.V., *MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12866-9.

274. Perry G., Roder H., Nunomura A., Takeda A., Friedlich A.L., Zhu X., Raina A.K., Holbrook N., Siedlak S.L., Harris P.L., and Smith M.A., *Activation of neuronal extracellular receptor kinase (ERK) in Alzheimer disease links oxidative stress to abnormal phosphorylation.* Neuroreport, 1999. **10**(11): p. 2411-5.

275. Zhu J.H., Kulich S.M., Oury T.D., and Chu C.T., *Cytoplasmic aggregates of phosphorylated extracellular signal-regulated protein kinases in Lewy body diseases.* Am J Pathol, 2002. **161**(6): p. 2087-98.

276. Anderson C.N. and Tolkovsky A.M., *A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside.* J Neurosci, 1999. **19**(2): p. 664-73.

277. Li Z., Theus M.H., and Wei L., *Role of ERK 1/2 signaling in neuronal differentiation of cultured embryonic stem cells.* Dev Growth Differ, 2006. **48**(8): p. 513-23.

278. Subramaniam S. and Unsicker K., *ERK and cell death: ERK1/2 in neuronal death.* FEBS J, 2010. **277**(1): p. 22-9.

279. Rothhammer V., Heink S., Petermann F., Srivastava R., Claussen M.C., Hemmer B., and Korn T., *Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE.* J Exp Med, 2011. **208**(12): p. 2465-76.

280. Ifergan I., Kebir H., Terouz S., Alvarez J.I., Lecuyer M.A., Gendron S., Bourbonniere L., Dunay I.R., Bouthillier A., Moumdjian R., Fontana A., Haqqani A., Klopstein A., Prinz M., Lopez-Vales R., Birchler T., and Prat A., *Role of Ninjurin-1 in the migration of myeloid cells to central nervous system inflammatory lesions.* Ann Neurol, 2011. **70**(5): p. 751-63.

281. Ahn B.J., Le H., Shin M.W., Bae S.J., Lee E.J., Wee H.J., Cha J.H., Lee H.J., Lee H.S., Kim J.H., Kim C.Y., Seo J.H., Lo E.H., Jeon S., Lee M.N., Oh G.T., Yin G.N., Ryu J.K., Suh J.K., and Kim K.W., *Ninjurin1 deficiency attenuates susceptibility of experimental autoimmune encephalomyelitis in mice.* J Biol Chem, 2014. **289**(6): p. 3328-38.

282. Kadmon G., Kowitz A., Altevogt P., and Schachner M., *Functional cooperation between the neural adhesion molecules L1 and N-CAM is carbohydrate dependent.* J Cell Biol, 1990. **110**(1): p. 209-18.

283. Kadmon G., Kowitz A., Altevogt P., and Schachner M., *The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions.* J Cell Biol, 1990. **110**(1): p. 193-208.

284. Jacob J., Haspel J., Kane-Goldsmith N., and Grumet M., *L1 mediated homophilic binding and neurite outgrowth are modulated by alternative splicing of exon 2.* J Neurobiol, 2002. **51**(3): p. 177-89.

285. Shriver L.P. and Dittel B.N., *T-Cell-Mediated Disruption of the Neuronal Microtubule Network : Correlation with Early Reversible Axonal Dysfunction in Acute Experimental Autoimmune Encephalomyelitis.* Am J Pathol, 2006. **169**(3): p. 999-1011.

286. Ebeling O., Duczmal A., Aigner S., Geiger C., Schöllhammer S., Kemshead J.T., Möller P., Schwartz-Albiez R., and Altevogt P., *L1 adhesion molecule on human lymphocytes and monocytes: expression and involvement in binding to αvβ3 integrin.* Eur J Immunol, 1996. **26**(10): p. 2508-2516.

287. Gast D., Riedle S., Issa Y., Pfeifer M., Beckhove P., Sanderson M.P., Arlt M., Moldenhauer G., Fogel M., Kruger A., and Altevogt P., *The cytoplasmic part of L1-CAM controls growth and gene expression in human tumors that is reversed by therapeutic antibodies.* Oncogene, 2008. **27**(9): p. 1281-9.

288. Wolterink S., Moldenhauer G., Fogel M., Kiefel H., Pfeifer M., Luttgau S., Gouveia R., Costa J., Endell J., Moebius U., and Altevogt P., *Therapeutic antibodies to human L1CAM: functional characterization and application in a mouse model for ovarian carcinoma.* Cancer Res, 2010. **70**(6): p. 2504-15.

289. Doberstein K., Harter P.N., Haberkorn U., Bretz N.P., Arnold B., Carretero R., Moldenhauer G., Mittelbronn M., and Altevogt P., *Antibody therapy to human L1CAM in a transgenic mouse model blocks local tumor growth but induces EMT.* Int J Cancer, 2015. **136**(5): p. E326-39.

290. Helliwell C.L. and Coles A.J., *Monoclonal antibodies in multiple sclerosis treatment: current and future steps.* Ther Adv Neurol Disord, 2009. **2**(4): p. 195-203.

291. Aubert I., Ridet J.L., and Gage F.H., *Regeneration in the adult mammalian CNS: guided by development.* Curr Opin Neurobiol, 1995. **5**(5): p. 625-35.

292. Cohen N.R., Taylor J.S., Scott L.B., Guillery R.W., Soriano P., and Furley A.J., *Errors in corticospinal axon guidance in mice lacking the neural cell adhesion molecule L1.* Curr Biol, 1998. **8**(1): p. 26-33.

293. Dahme M., Bartsch U., Martini R., Anliker B., Schachner M., and Mantei N., *Disruption of the mouse L1 gene leads to malformations of the nervous system.* Nat Genet, 1997. **17**(3): p. 346-9.

294. Jakeman L.B., Chen Y., Lucin K.M., and Mctigue D.M., *Mice lacking L1 cell adhesion molecule have deficits in locomotion and exhibit enhanced corticospinal tract sprouting following mild contusion injury to the spinal cord.* Eur J Neurosci, 2006. **23**(8): p. 1997-2011.

295. Law J.W., Lee A.Y., Sun M., Nikonenko A.G., Chung S.K., Dityatev A., Schachner M., and Morellini F., *Decreased anxiety, altered place learning, and increased CA1 basal excitatory synaptic transmission in mice with conditional ablation of the neural cell adhesion molecule L1.* J Neurosci, 2003. **23**(32): p. 10419-32.

296. Zhu Y., Romero M.I., Ghosh P., Ye Z., Charnay P., Rushing E.J., Marth J.D., and Parada L.F., *Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain.* Genes Dev, 2001. **15**(7): p. 859-76.

297. Barbin G., Aigrot M.S., Charles P., Foucher A., Grumet M., Schachner M., Zalc B., and Lubetzki C., *Axonal cell-adhesion molecule L1 in CNS myelination.* Neuron Glia Biol, 2004. **1**(1): p. 65-72.

298. Guseva D., Loers G., and Schachner M., *Function-triggering antibodies to the adhesion molecule L1 enhance recovery after injury of the adult mouse femoral nerve.* PLoS One, 2014. **9**(11): p. e112984.

299. Sauce B., Wass C., Netrakanti M., Saylor J., Schachner M., and Matzel L.D., *Heterozygous L1-deficient mice express an autism-like phenotype.* Behav Brain Res, 2015. **292**: p. 432-42.

300. Chang R.C., Hudson P., Wilson B., Liu B., Abel H., Hemperly J., and Hong J.S., *Immune modulatory effects of neural cell adhesion molecules on lipopolysaccharide-induced nitric oxide production by cultured glia.* Brain Res Mol Brain Res, 2000. **81**(1-2): p. 197-201.

301. Sporns O., Edelman G.M., and Crossin K.L., *The neural cell adhesion molecule (N-CAM) inhibits proliferation in primary cultures of rat astrocytes.* Proc Natl Acad Sci U S A, 1995. **92**(2): p. 542-6.

302. Tian L., Rauvala H., and Gahmberg C.G., *Neuronal regulation of immune responses in the central nervous system.* Trends Immunol, 2009. **30**(2): p. 91-9.

303. Krushel L.A., Tai M.H., Cunningham B.A., Edelman G.M., and Crossin K.L., *Neural cell adhesion molecule (N-CAM) domains and intracellular signaling pathways involved in the inhibition of astrocyte proliferation.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2592-6.

304. Chang R.C., Hudson P., Wilson B., Haddon L., and Hong J.S., *Influence of neurons on lipopolysaccharide-stimulated production of nitric oxide and tumor necrosis factor-alpha by cultured glia.* Brain Res, 2000. **853**(2): p. 236-44.

305. Chavarria A. and Cardenas G., *Neuronal influence behind the central nervous system regulation of the immune cells.* Front Integr Neurosci, 2013. **7**: p. 64.

306. Giuliani F., Goodyer C.G., Antel J.P., and Yong V.W., *Vulnerability of human neurons to T cell-mediated cytotoxicity.* J Immunol, 2003. **171**(1): p. 368-79.

307. Schmid J.S., Bernreuther C., Nikonenko A.G., Ling Z., Mies G., Hossmann K.A., Jakovcevski I., and Schachner M., *Heterozygosity for the mutated X-chromosome-linked L1 cell adhesion molecule gene leads to increased numbers of neurons and enhanced metabolism in the forebrain of female carrier mice.* Brain Struct Funct, 2013. **218**(6): p. 1375-90.

308. Schmid J., Bernreuther C., Nikonenko A., Ling Z., Mies G., Hossmann K.-A., Jakovcevski I., and Schachner M., *Heterozygosity for the mutated X-chromosome-linked L1 cell adhesion molecule gene leads to increased numbers of neurons and enhanced metabolism in the forebrain of female carrier mice.* Brain Structure and Function, 2013. **218**(6): p. 1375-1390.

309. Sawma P., Roth L., Blanchard C., Bagnard D., Cremel G., Bouveret E., Duneau J.P., Sturgis J.N., and Hubert P., *Evidence for new homotypic and heterotypic interactions between transmembrane helices of proteins involved in receptor tyrosine kinase and neuropilin signaling.* J Mol Biol, 2014. **426**(24): p. 4099-111.

310. Bechara A., Nawabi H., Moret F., Yaron A., Weaver E., Bozon M., Abouzid K., Guan J.-L., Tessier-Lavigne M., Lemmon V., and Castellani V., *FAK-MAPK-dependent adhesion disassembly downstream of L1 contributes to semaphorin3A-induced collapse.* EMBO J, 2008. **27**(11): p. 1549-1562.

311. Ben-Zvi A., Manor O., Schachner M., Yaron A., Tessier-Lavigne M., and Behar O., *The Semaphorin Receptor PlexinA3 Mediates Neuronal Apoptosis during Dorsal Root Ganglia Development.* J Neurosci, 2008. **28**(47): p. 12427-12432.

312. Castellani V., De Angelis E., Kenwrick S., and Rougon G., *Cis and trans interactions of L1 with neuropilin-1 control axonal responses to semaphorin 3A.* EMBO J, 2002. **21**(23): p. 6348–6357.

313. Lepelletier Y., Moura I.C., Hadj-Slimane R., Renand A., Fiorentino S., Baude C., Shirvan A., Barzilai A., and Hermine O., *Immunosuppressive role of semaphorin-3A on T cell proliferation is mediated by inhibition of actin cytoskeleton reorganization.* Eur J Immunol, 2006. **36**(7): p. 1782-93.

314. Walsh J.T., Hendrix S., Boato F., Smirnov I., Zheng J., Lukens J.R., Gadani S., Hechler D., Golz G., Rosenberger K., Kammertons T., Vogt J., Vogelaar C., Siffrin V., Radjavi A., Fernandez-Castaneda A., Gaultier A., Gold R., Kanneganti T.D., Nitsch R., Zipp F., and Kipnis J., *MHCII-independent CD4+ T cells protect injured CNS neurons via IL-4.* J Clin Invest, 2015. **125**(2): p. 699-714.

315. Issazadeh S., Navikas V., Schaub M., Sayegh M., and Khoury S., *Kinetics of expression of costimulatory molecules and their ligands in murine relapsing experimental autoimmune encephalomyelitis in vivo.* J Immunol, 1998. **161**(3): p. 1104-12.

316. Wu G.F. and Laufer T.M., *The role of dendritic cells in multiple sclerosis.* Curr Neurol Neurosci Rep, 2007. **7**(3): p. 245-52.

317. Ulvestad E., Williams K., Mork S., Antel J., and Nyland H., *Phenotypic differences between human monocytes/macrophages and microglial cells studied in situ and in vitro.* J Neuropathol Exp Neurol, 1994. **53**(5): p. 492-501.

318. Ajami B., Bennett J.L., Krieger C., Mcnagny K.M., and Rossi F.M., *Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool.* Nat Neurosci, 2011. **14**(9): p. 1142-9.

319. Ransohoff R.M. and Engelhardt B., *The anatomical and cellular basis of immune surveillance in the central nervous system.* Nat Rev Immunol, 2012. **12**(9): p. 623-35.

320. Cose S., Brammer C., Khanna K.M., Masopust D., and Lefrancois L., *Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway.* Eur J Immunol, 2006. **36**(6): p. 1423-33.

321. Bauer J., Sminia T., Wouterlood F.G., and Dijkstra C.D., *Phagocytic activity of macrophages and microglial cells during the course of acute and chronic relapsing experimental autoimmune encephalomyelitis.* J Neurosci Res, 1994. **38**(4): p. 365-75.

322. Cammer W., Bloom B.R., Norton W.T., and Gordon S., *Degradation of basic protein in myelin by neutral proteases secreted by stimulated macrophages: a possible mechanism of inflammatory demyelination.* Proc Natl Acad Sci U S A, 1978. **75**(3): p. 1554-8.

323. Colton C.A., Keri J.E., Chen W.T., and Monsky W.L., *Protease production by cultured microglia: substrate gel analysis and immobilized matrix degradation.* J Neurosci Res, 1993. **35**(3): p. 297-304.

324. Mirshafiey A., Asghari B., Ghalamfarsa G., Jadidi-Niaragh F., and Azizi G., *The significance of matrix metalloproteinases in the immunopathogenesis and treatment of multiple sclerosis.* Sultan Qaboos Univ Med J, 2014. **14**(1): p. e13-25.

325. Clausen B.E., Burkhardt C., Reith W., Renkawitz R., and Forster I., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic Res, 1999. **8**(4): p. 265-77.

326. Arantes R.M., Lourenssen S., Machado C.R., and Blennerhassett M.G., *Early damage of sympathetic neurons after co-culture with macrophages: a model of neuronal injury in vitro.* Neuroreport, 2000. **11**(1): p. 177-81.

327. Abdi K., Singh N.J., and Matzinger P., *Lipopolysaccharide-activated dendritic cells: "exhausted" or alert and waiting?* J Immunol, 2012. **188**(12): p. 5981-9.

328. Brunner C., Seiderer J., Schlamp A., Bidlingmaier M., Eigler A., Haimerl W., Lehr H.A., Krieg A.M., Hartmann G., and Endres S., *Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo.* J Immunol, 2000. **165**(11): p. 6278-86.

329. Granucci F., Ferrero E., Foti M., Aggujaro D., Vettoretto K., and Ricciardi-Castagnoli P., *Early events in dendritic cell maturation induced by LPS.* Microbes Infect, 1999. **1**(13): p. 1079-84.

330. Pashenkov M., Teleshova N., Kouwenhoven M., Kostulas V., Huang Y.M., Soderstrom M., and Link H., *Elevated expression of CCR5 by myeloid (CD11c+) blood dendritic cells in multiple sclerosis and acute optic neuritis.* Clin Exp Immunol, 2002. **127**(3): p. 519-26.

331. Jain P., Coisne C., Enzmann G., Rottapel R., and Engelhardt B., *Alpha4beta1 integrin mediates the recruitment of immature dendritic cells across the blood-brain barrier during experimental autoimmune encephalomyelitis.* J Immunol, 2010. **184**(12): p. 7196-206.

332. Vestweber D., *Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium.* Immunol Rev, 2007. **218**: p. 178-96.

333. Zhao X., Yip P.M., and Siu C.H., *Identification of a homophilic binding site in immunoglobulin-like domain 2 of the cell adhesion molecule L1.* J Neurochem, 1998. **71**(3): p. 960-71.

334. Duczmal A., Schollhammer S., Katich S., Ebeling O., Schwartz-Albiez R., and Altevogt P., *The L1 adhesion molecule supports alpha v beta 3-mediated migration of human tumor cells and activated T lymphocytes.* Biochem Biophys Res Commun, 1997. **232**(1): p. 236-9.

335. Felding-Habermann B., Silletti S., Mei F., Siu C.H., Yip P.M., Brooks P.C., Cheresh D.A., O'toole T.E., Ginsberg M.H., and Montgomery A.M., *A single immunoglobulin-like domain of the human neural cell adhesion molecule L1 supports adhesion by multiple vascular and platelet integrins.* J Cell Biol, 1997. **139**(6): p. 1567-81.

336. Yip P.M., Zhao X., Montgomery A.M., and Siu C.H., *The Arg-Gly-Asp motif in the cell adhesion molecule L1 promotes neurite outgrowth via interaction with the alphavbeta3 integrin.* Mol Biol Cell, 1998. **9**(2): p. 277-90.

337. Silletti S., Mei F., Sheppard D., and Montgomery A.M., *Plasmin-sensitive dibasic sequences in the third fibronectin-like domain of L1-cell adhesion molecule (CAM) facilitate homomultimerization and concomitant integrin recruitment.* J Cell Biol, 2000. **149**(7): p. 1485-502.

8 Appendix

8.1 List of Figures

8.2 List of tables

8.3 Publications

Lutz Menzel, Magdalena Paterka, Robin White, Tanja Kuhlmann, Jack van Horssen, Esther Witsch, Frauke Zipp, Michael K. E. Schäfer, Down-regulation of neuronal L1 cell adhesion molecule expression alleviates inflammatory neuronal injury. *Acta Neuropathologica, Aug 2016 (Epub ahead of print), dio: 10.1007/s00401-016-1607-4*

Lutz Menzel, Lisa Kleber, Carina Friedrich, Larissa Dangel, Irmgard Tegeder, Michael K. E. Schäfer, Progranulin protects against exaggerated axonal injury and astrogliosis following traumatic brain injury. *Glia, Oct 2016 (accepted manuscript)*

Changsheng Huang, Dominik Sakry, Lutz Menzel, Larissa Dangel, Anne Sebastiani, Tobias Krämer, Khalad Karram, Kristin Engelhard, Jacqueline Trotter, Michael K.E. Schäfer, Lack of NG2 exacerbates neurological outcome and modulated glial responses after traumatic brain injury. *Glia, Apr 2016, 64(4) p.507-23. doi: 10.1002/glia.22944*

8.4 Presentations at meetings and conferences

Lutz Menzel, Magdalena Paterka, Frauke Zipp, Michael K. E. Schäfer. Role of the neuronal adhesion molecule L1 during inflammatory processes in the CNS.
Poster presentation at the rmn² meeting in Oberwesel, 2014.

Lutz Menzel, Magdalena Paterka, Robin White, Esther Witsch, Melitta Schachner, Frauke Zipp, Michael K. E. Schäfer. Homeostatic role of neuronal L1 cell adhesion molecule during inflammatory processes in the central nervous system.
Poster presentation at the Society for Neuroscience meeting in Chicago, 2015.

8.5 Acknowledgements

The studies presented in this thesis was funded by the University Medical Center of the Johannes Gutenberg-University Mainz and by the Deutsche Forschungsgesellschaft within the Sonderforschungsbereich CRC 1080 “Molecular and cellular mechanisms of neuronal homeostasis”.

First, I would like to thank the department for anesthesiology, namely [redacted] and [redacted].
In particular, special thanks to [redacted] for the opportunity to work in his team, for setting up the whole project and for the continuous and patient supervision during all experiments, preparation of publications and the writing of this thesis.
I’m grateful for the help of our technicians [redacted]
Best thanks to [redacted] and [redacted] from [redacted] for helping me with big experiments that I would have been totally overchallenged with. Just as well great thanks to [redacted] for her professional contribution in all EAE experiments and to lend me an ear and a smoke whenever needed.
Also many thanks to our vet [redacted] for taking care of our animals and keeping everything in order while I messed up everything.
Great thanks to [redacted] for being a nice co-worker and an even better friend, cheering me up all the time, introducing me to the city of Mainz and sharing similar drinking habits.
In line, many thanks to all friends for being there for me all the time and for keeping me aware of the world outside of the science cosmos.
Not at least, great thanks to my family for raising me to someone who is in the position to do a doctoral degree in the first place and for supporting me during my whole education and studies period.

8.6 Lebenslauf

Name: Lutz Menzel

Geburtsdatum: 02.10.1985

Staatsangehörigkeit: deutsch

Sprachen:

- Deutsch (Muttersprache)
- Englisch (Verhandlungssicher)

Schulbildung:

- Grundschule am Heidekampgraben Berlin — Sep. 1992 – Juli 1998
- Flatow Oberschule Berlin — Sep. 1998 – Juli 2001
- Friedrich Ludwig Jahn Oberschule Potsdam — Sep. 2001 – Juli 2005
    - Abschluss: Abitur
    - Prädikat „gut“ (2,5)

Wehrdienst:

- Julius Leber Kaserne Berlin — Jan. 2006 – Sep. 2006

Studium:

- Technische Fachhochschule Berlin — Okt. 2006 – Sep. 2007
    - Architektur
- Technische Fachhochschule Berlin — Okt. 2007 – Sep. 2010
    - Biotechnologie
    - Abschluss: Bachelor of Science
      Prädikat „gut“ (2,2)
- Universität Potsdam — Okt. 2010 – Sep. 2012
    - Biochemie und Molekularbiologie
    - Abschluss: Master of Science
      Prädikat „gut“ (1,9)

Praktische Erfahrung und Ausbildung:

- SGS Institut Fresenius — Aug. 2007 – Sep. 2007
- Berliner-Kindl-Schultheiss-Brauerei GmbH — Aug. 2008 – Sep. 2008
- Institut für Zell- und Neurobiologie der Charité Berlin — März 2010 – Sep. 2010
    - Bachelorarbeit: „Zytokine und regulatorische Marker Interferon-γ stimulierter Mikrogliazellen“
- Max-Delbrück-Centrum für Molekulare Medizin, Berlin — Dez. 2011 – Sep. 2012

- Masterarbeit: „Chemosensitivierung Claudin-3 und Claudin-4 exprimierender Tumorzellen mit *Clostridium Perfringens* Enterotoxin"

- Klinik für Anästhesiologie der Universitätsmedizin Mainz — März 2013 – Aug. 2016
  - Dissertation: „Role of the cell adhesion molecule L1 during neuroinflammation in the central nervous system"

8.7 Selbständigkeitserklärung

Ich versichere an Eides statt, dass ich die vorliegende Dissertation selbst und ohne unzulässige Hilfe Dritter verfasst habe und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde und mir der Inhalt der Promotionsordnung des Fachbereichs Biologie der Johannes Gutenberg-Universität Mainz vom 22.12.2003 bekannt ist.

Mainz,

_____

(Lutz Menzel)