UNIVERSIDADE D
COIMBRA

Alexandre Nunes Neto

GENE DELETION AND NANOBODIES STRATEGIES TO TARGET S100B AND IMPROVE RECOVERY IN AN *EX VIVO* DEMYELINATING MODEL

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular com Especialização em Neurobiologia orientada pela Professora Doutora Adelaide Maria Afonso Fernandes Borralho (iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa) e pelo Professor Doutor Carlos Jorge Alves Miranda Bandeira Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra) apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

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The studies presented in this thesis were performed in the Neuron-Glia in Health and Biology group, at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Professor Adelaide Fernandes, PhD, and in collaboration with Professor Claudio Gomes, Faculty of Sciences, Universidade de Lisboa.

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Para ti Pai.

“Choose a Job You Love, and You Will Never Have to Work a Day in Your Life.”

- Confucius

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Resumo

A Esclerose Múltipla (EM) é uma doença crónica, neurodegenerativa e autoimune, caraterizada por processos inflamatórios e desmielinizantes no sistema nervoso central (SNC). Devido a estes processos, começam por aparecer lesões focais em certas regiões do SNC e, dependendo da região, podem surgir diferentes sintomas, resultando no aumento da incapacidade motora e cognitiva à medida que a doença evolui. Apesar dos inúmeros avanços a nível do conhecimento dos mecanismos subjacentes à complexa dinâmica patofisiológica desta doença, a mesma possui uma causalidade multifatorial que torna difícil o desenvolvimento de novas terapêuticas. Neste sentido, a procura de novos biomarcadores e alvos terapêuticos tem sido essencial para o desenvolvimento de novas estratégias para combater a EM. Tomando partido destes, vários avanços têm sido feitos na área da EM, como é o caso da molécula S100B. Em níveis fisiológicos, esta proteína promove a correta diferenciação e maturação de oligodendrócitos, necessários para a correta mielinização do SNC. No entanto, foram encontrados elevados níveis da proteína S100B tanto no soro como no líquido cefalorraquidiano de doentes com EM em fase de diagnóstico, estando também presente nas lesões ativas no SNC. Esta elevação de S100B é também detetada em modelos *ex vivo* de desmielinização, tendo sido demostrado que leva à ativação tanto de astrócitos como de células da microglia, potenciando o processo neurodegenerativo. Recentemente, o nosso grupo demonstrou que a neutralização desta proteína resultava num menor grau de desmielinização, de reatividade glial e consequentemente numa redução da resposta inflamatória.

Desta forma na presente dissertação analisámos em primeiro lugar o efeito neurotóxico desta proteína, através da utilização de culturas organotípicas de cerebelo, provenientes de três linhagens de murganho, nos quais o gene para a S100B está alterado. Assim, através da comparação de culturas provenientes de murganhos expressando a S100B *wild type*, com as de heterozigóticos e ainda de homozigóticos com S100B *knockout*, foi possível notar uma diminuição tanto da desmielinização como da inflamação. Após a comprovação dos efeitos pejorativos desta proteína, procedemos à sua neutralização recorrendo a três Nanocorpos diferentes dirigidos para a S100B. A possibilidade do uso de Nanocorpos específicos para a S100B despertou a nossa curiosidade, devido às várias

características vantajosas destas nanomoléculas, tal como a sua alta capacidade para reconhecer epítopos específicos, a sua maior estabilidade e solubilidade tal como uma imunogenicidade reduzida. A utilização dos Nanocorpos não só levou a uma diminuição da desmielinização, como também a um decréscimo do processo inflamatório. Desta maneira, estes resultados vêm reforçar o papel da S100B na patogénese desta doença e demonstrar que a sua neutralização, pelo uso de Nanocorpos específicos para esta proteína, constitui uma nova possibilidade terapêutica para doenças desmielinizantes inflamatórias como a EM.

Palavras-chave: Esclerose Múltipla; Neuroinflamação; Desmielinização; S100B; Nanocorpos

Abstract

Multiple sclerosis (MS) is a chronic neurodegenerative and autoimmune disease, characterized by inflammation and demyelination of the central nervous system (CNS). Altogether, these processes produce focal lesions in specific CNS regions, and depending on the affected region, different symptoms will appear, leading to an increase in both motor and cognitive disability, dependent on disease progression. Despite the enormous efforts done to unravel the mechanisms underlying MS pathology, the complex onset phenomenon might be due to an interplay of different factors, hampering the development of new therapeutics. The pursuit to find new biomarkers is extremely crucial, in order to find new different ways to tackle this disease. S100B is an inflammatory molecule that has been described as a major player in MS pathology. At physiological levels, S100B promotes differentiation and maturation of oligodendrocytes, required for the correct myelination of the CNS. Nevertheless, S100B has been found at high levels, in both cerebrospinal fluid and serum of MS patients at the diagnosis stage, as well in the active demyelinating lesions. This abnormal S100B overexpression was also detected in *ex vivo* demyelinating models, leading to microglia and astrocyte activation, exacerbating the neurodegenerative process. Recently, our group demonstrated that through S100B neutralization, a lower degree of demyelination, reactive gliosis, and also inflammation were obtained.

First, in the present thesis, we went further to analyse S100B neurotoxic effects using cerebellar organotypic slice cultures from three different mice. S100B heterozygotic and knockout mice demonstrated a decrease in demyelination as well as in inflammation, when compared to wild type mice, evidencing the neurotoxic effect of S100B when overexpressed. Afterwards, we tried to neutralize S100B using three different nanobodies. These small molecules possess several advantages such as extreme specificity to recognize unique epitopes, higher affinity, stability, and solubility than commonly used antibodies, as well as reduced immunogenicity. Interestingly, in the presence of these nanobodies, we observed a reduced degree of demyelination as well as a diminished inflammatory environment. Overall, our results emphasize the already described pejorative roles of S100B on MS pathophysiology, highlighting that S100B

neutralization by the use of nanobodies, might be a promising therapeutic possibility regarding demyelinating and inflammatory diseases as is the case of MS.

Keywords: Multiple Sclerosis; Neuroinflammation; Demyelination; S100B; Nanobodies

Abbreviations

AD	Alzheimer's disease
AGEs	Advanced glycation end-products
APC	Antigen-presenting cell
BBB	Blood-brain barrier
CDR	Complementarity-determining region
CFA	Complete Freund’s adjuvant
CH	Constant domain of the heavy chain
CIS	Clinically isolated syndrome
CL	Constant domain of the light chain
CNS	Central Nervous System
COSC	Cerebellar organotypic slice culture
CSF	Cerebrospinal fluid
DAMPs	Danger-associated molecular patterns
DMTs	Disease-modifying therapies
DN-RAGE	Dominant negative RAGE
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
Fab	Fragment antigen-binding

Fc	Fragment crystallizable region
fl-RAGE	Full-length RAGE
HLA	Human leukocyte antigen
HMBG1	High mobility group box 1
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IgG	Immunoglobulin G
LPC	Lysophosphatidylcholine
MBP	Myelin basic protein
MHV	Mouse hepatitis virus
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NAWM	Normal-appearing white matter
Nb	Nanobody
NF-kB	Nuclear-factor kappa B
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
PAMPs	Pathogen-associated molecular patterns

PLP	Myelin proteolipid protein
PPMS	Primary progressive MS
PRR	Pattern recognition receptors
RAGE	Receptor for advanced glycation end-products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
S100B	Calcium-binding protein S100B
SPMS	Secondary progressive MS
sRAGE	Soluble RAGE
TLR	Toll-like receptor
TMEV	Theiler's murine encephalitis virus
TNF-α	Tumor necrosis factor α
VEPs	Visually evoked potentials
VH	Variable domain of IgG antibody heavy chain
VHH	N-terminal variable domain of the heavy chain-only antibody
VL	Variable domain of the light chain

1. Introduction

1.1. Highlights of Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune disease characterized by inflammation and demyelination of the central nervous system (CNS). This disorder is related to blood-brain barrier (BBB) disruption, letting infiltrating lymphocytes reach the brain, where they attack myelin causing its degradation, oligodendrocyte (OL) malfunction, inflammation, gliosis, as well as neuronal and axonal damage. This relentless attack against myelin sheaths produces focal lesions, found in both grey and white matter of the CNS, including the brain, optic nerve, and spinal cord[1,2]. Depending on the focal lesion region, different symptoms emerge, mainly sensorial and visual disturbances, motor problems, fatigue, and cognitive dysfunctions[3,4]. Magnetic resonance imaging (MRI), one of the most decisive techniques for MS diagnosis, can detect these CNS lesions as well as monitor disease activity alongside MS progression. In most patients, reversible episodes of these neurological dysfunctions, also known as relapses, usually trigger the onset, which can last days or weeks. It is possible to recover entirely but quite often only partially, and overtime, permanent deficits will remain, increasing clinical disability, leading to the progression of the disease[5]. Not all patients follow the same disease profile, different forms of the disease have already been defined, considering its clinical course. Some patients acquire a relapsing-remitting form of the disease, while others, a progressive form. The etiology is not yet established since MS has a multifactorial background of possible causes, although, genetic susceptibility and environmental risk factors play a substantial role, increasing the predisposition to develop MS[6]. The impotence and incapacity of self-care in affected individuals, give rise to a dependence on family and caregivers' help, followed by a significant reduction in life quality with an associated elevated economic burden[7].

1.2. Epidemiology

Nowadays, MS is the most common cause of non-traumatic neurological disability in young adults, affecting almost three times more women than men. Disease onset starts in early adulthood, and the number of affected individuals has been increasing across the years, including now approximately 2.5 million people around the world[8]. MS prevalence differs according to ethnicity and geographic locations. Many studies have already described a positive correlation between MS incidence and higher latitude countries, being North Europe and North America, the ones with the highest values, with 108 and 140 per 100,000 individuals, respectively[7] **(Figure 1.1)**. In Portugal, the prevalence is lower, reaching 56 per 100,000 individuals[9]. Remarkably, environment interactions revealed to be capable of modifying the risk to develop MS when both immigration age and country of residence are combined, since individuals that have migrated to a different country after early adulthood, remain with the disease risk from their first living country[10]. The most reasonable explanation for this correlation between latitude and MS cases is the lack of sun exposure in these regions, which impairs vitamin D production. Even though the specific action mechanism of this vitamin and its role in MS pathology is not well understood, it has been suggested to have a role in the modulation of the immune system[11,12]. There is no apparent cause for this disease, although genetic, lifestyle, and environmental risk factors interaction can alter the probability to develop MS. Factors such as Epstein-Barr virus (EBV) infection, smoking and lack of sun exposure/vitamin D production are the most well-established ones[12]. Regarding genetic influence, there are several genes linked with MS, such as the human leukocyte antigen (HLA) system, in particular, the genetic variant *HLA-DRB1*15:01* allele which has the most substantial impact in MS susceptibility. This gene is involved in antigen recognition by T cells, although the referred variant allows a self-antigen immune attack, targeting myelin and leading to the demyelination of the CNS[4]. Relatively to ethnicity, Caucasian individuals are the ones with the highest MS incidence, while Afro-Caribbean and East-Asia population present the lower incidence[4].

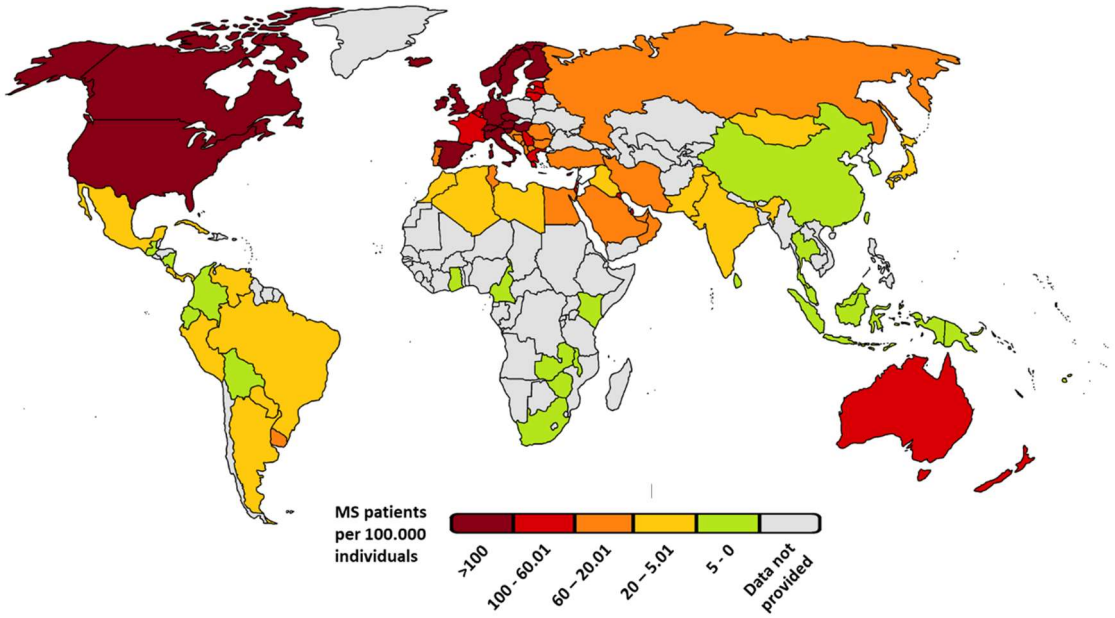

Figure 1.1 - Multiple Sclerosis prevalence around the world. (Adapted from Thompson, A. et al. 2013[7])

1.3. Diagnosis and clinical courses

Diagnosing MS is not simple due to the intrinsic heterogenicity of symptoms and lesions between patients. Several analyses must be done to fulfil MS diagnostic criteria, including the presence of CNS damage, disseminated both in time and space, accessible through MRI. Another analysis includes lumbar puncture, which gives access to oligoclonal immunoglobulin G (IgG) bands determination, found in 90% of patients. Additionally, neurophysiology, a much less invasive method, allows the recording of visually evoked potentials (VEPs) to which MS patients usually have a delayed performance. Finally, blood tests are also used, however, most often with the purpose to rule out other possible diseases[4,5]. Before the beginning of the 21st century, there were four defined forms of MS[13], now, due to imaging technologies progress and the development of new biomarkers for MS, some recent rearrangements were made in the definition of the MS disease phenotypes **(Figure 1.2)**. The recent guidelines indicate that the disease onset is characterized by an initial demyelination attack in a particular region of the CNS, most often in the optic nerve, spinal cord, brainstem, cerebellum or even in the cerebral hemispheres corresponding to a specific clinical debility, directly related with the region affected[6]. The first demyelinating lesion detected in an individual is called clinically

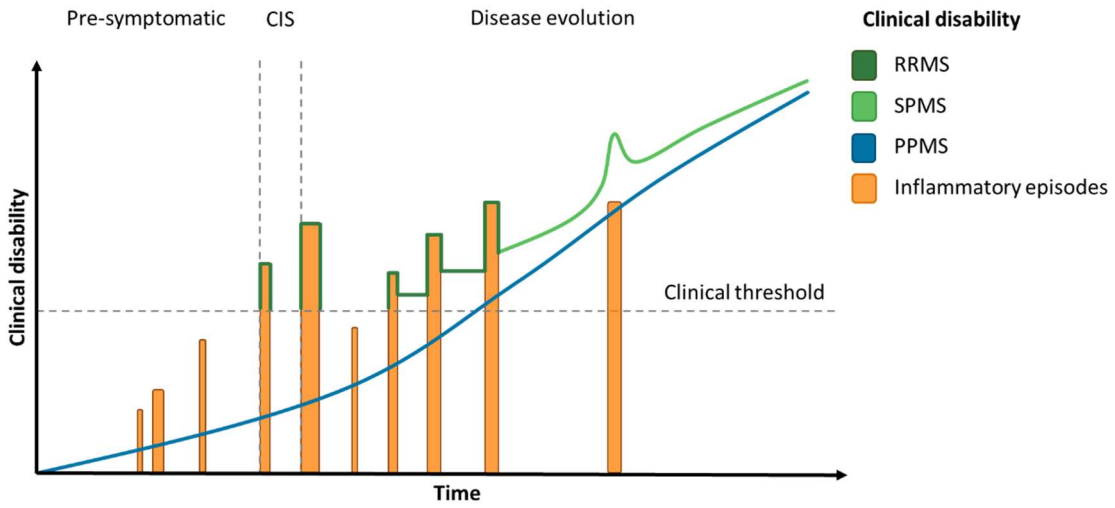

Figure 1.2 - Schematic representation of Multiple Sclerosis clinical courses over time. About 85-90% of patients acquire a Relapsing-Remitting MS (RRMS) form of the disease, characterized by punctual increases in clinical disability due to inflammatory episodes (orange bars), followed by a remission period. In 15-25 years, RRMS patients frequently shift their relapsing-remitting form to a progressive form, termed Secondary Progressive MS (SPMS) (green curve). With much lower incidence, 10-15% of MS patients develop a progressive form of the disease since the onset without evident relapses, also known as Primary-Progressive MS (PPMS) (blue curve). (Adapted from Filippi et al. 2018[6])

isolated syndrome (CIS), which is not considered MS, although the presence of inflammatory and demyelinating processes can eventually lead to the diagnosis of MS if it satisfies the diagnostic criteria. The most prevalent form (85-90%) is relapsing-remitting MS (RRMS), characterized by punctual attacks, also known as relapses, in which neurologic dysfunction symptoms appear. Most often, there is a period of recovering, allowing the symptoms to disappear partially or completely. Therefore, if the symptoms become long-lasting, it may lead to the progression of this neurological disease[14,15]. In 15-25 years, RRMS patients frequently shift their relapsing-remitting form to a progressive form, termed secondary progressive MS (SPMS), wherein a continuous progression of clinical disability without relapses becomes prominent. The other MS type, even though much less prevalent (10-15%), designed as primary progressive MS (PPMS) involves clinical disability increase over time, in other words, a continuous disease progression without relapses[5].

1.4. Multiple Sclerosis pathophysiology

Several cell types are responsible and involved in the insult of the CNS and homeostasis loss, including activated infiltrating macrophages, resident astrocytes, and microglia, releasing molecules such as reactive oxygen species (ROS), reactive nitrogen species

(RNS), cytotoxic and proinflammatory cytokines, in parallel with activation of other invading cells from the peripheral immune system[16]. One of the MS hallmarks is BBB disruption, which permits the access of several activated leukocytes to infiltrate the CNS, triggering myelin damage and inflammatory processes, subsequently establishing focal lesions in both white and grey matter[6], although, the causative origin of it is not yet completely established. The pathology itself is characterized by T-cell-mediated inflammation, along with the release of antibodies by plasmocytes, reactive gliosis, OL dysfunction, known to be the central character for the correct myelination of CNS, neuronal and axonal damage, and the activation of the complement system[17,18]. Together, all these features limit the normal process of remyelination and, subsequently, the neurological repair[19].

1.4.1. Demyelination

OLs are the cells required to produce myelin in the CNS and needed for the correct axonal wrapping, so neurons can promptly propagate action potentials. In demyelinating diseases, the destruction of normal myelin sheets takes place, compromising the normal electrical conduction and comprises two types of demyelination. Primary demyelination is the most common, involving the injury, breakdown, and loss of myelin sheets, usually without axonal damage[20,21]. In secondary demyelination, myelin loss is a result of axonal injury or even its loss[22]. In the initial course of the lesion, perivenous demyelination can be seen and the fusion to confluent demyelinated plaques allows the expansion to the adjacent normal-appearing white matter (NAWM), giving rise to “Dawson fingers” observed by MRI[17].

When a demyelinating episode occurs, several different players can contribute in different ways, therefore augmenting the difficulty to find a specific guilty antigen, responsible for the triggering of the immune reaction in MS[2,23]. Nevertheless, a cascade of an abnormal immune response against self-antigens can be enhanced by the release of cytokines and chemokines, in which the final target corresponds to OLs/myelin[24]. Moreover, remyelination is a natural process that occurs after a demyelinating episode, through the proliferation, migration, and differentiation of oligodendrocyte precursor cells (OPC) into myelinating OLs. This way, new myelin sheaths can be restored to the

denuded axons, protecting them from degeneration and restoring their normal function. Even though, this process can be limited and ineffective if the surrounding environment comprises an inflammatory milieu[25]. Impaired remyelination will therefore cause axonal dysfunction, degeneration, and neuronal cell death, by which accumulating these lesions will result in neurological deficiencies[26]. To create an optimal microenvironment for remyelination, astrocytes are known to recruit microglia, the CNS macrophages, to the lesion site playing a crucial role in the clearance of myelin debris left in the lesion region, through phagocytosis. Such debris were indeed described to impair the correct remyelination process by inhibiting the OPC differentiation[27–30].

1.4.2. Neuroinflammation and glia reactivity

Regarding neuroinflammation, it is known that immune cells, such as autoreactive T cells and B cells, in MS pathology, have the capacity to infiltrate the brain parenchyma, and without strict control of pro-inflammatory cytokines production, glial cell reactivity and subsequently neuronal and axonal damage are prone to occur[16,31]. The inflammatory process itself is usually instigated around capillary venules and veins, and thereafter expands to the surrounding tissues[17]. Although is not clear whether the infiltration of autoreactive T and B lymphocytes, through BBB, is the trigger for MS onset, whether is a secondary event to microglia and macrophage activation as well as the local release of cytokines and self-antigens[32–34]. Most of the pathological examinations can be explained by both theories, due to the heterogeneity of the clinical indicators as well as the medical tools' limitations. Microglia and astrocytes, CNS resident immune cells, play a fundamental role in preserving CNS homeostasis through neuronal support, facilitating synaptic contacts and, thereafter, improving signaling between neurons[35]. In MS, several regulatory systems can be altered, as the case of both microglia and astrocytes, which can undergo both morphological and functional changes. Even if the main goal of both cell types is to recover brain homeostasis, they can switch to a more neurotoxic phenotype, to counteract the insult received, although can even foster demyelination and therefore the formation of gliotic scars, found in inactive lesions[35]. Astrocytes have a star-shape morphology being the most abundant CNS glial cell type and have been described to promote neuronal survival, outgrowth, synaptogenesis, OL metabolic

support, and myelination process[27,36]. Upon brain injury, they can undergo several morphological and functional modifications, becoming activated (the so called reactive astrogliosis). As immunocompetent cells, they can also release neurotoxic mediators, to fight demyelination or other brain injuries, although, in the process, they can become a threat to other cells, since can induce the death of neurons and OLs[37]. Regarding microglia, these cells are the CNS resident macrophages and are known to be the first line of defence against brain injury. Here they can respond to harmful stimuli through not only the recruitment but also the activation of immune cells, acting as antigen-presenting cells (APCs) and thereby initiating the inflammatory response[27]. This microglia reactivity, similarly to astrocytes, can become dangerous to surrounding cells, due to an intensified release of neurotoxic and pro-inflammatory molecules[38]. Further, as mentioned in the previous section, microglia also play a pivotal role regarding myelin debris phagocytosis, needed for remyelination to occur[27–30]. Together, the interplay between astrocytes and microglia, play an important role in the modulation of the immune response, mainly through the production of pro-inflammatory mediators[39,40], although their accumulation can amplify this response, exacerbating demyelination and neurodegeneration[41]. Linked with these lesions and neuroinflammation itself, several molecules have been found deeply altered. The CSF of MS patients has been used as a biomarker duct to measure cytotoxic activity, through the search for specific autoantibodies, such as anti-MOG and anti-MBP antibodies (OL-targeted autoantibodies)[42], neuronal damage-associated light neurofilaments (NFL)[43], cytotoxic molecules as the case of S100B[19,44,45], among other biomarkers[19].

It has already been proposed that the location, shape, and specific patterns of certain active demyelination lesions are congruent with the possibility of being driven by a soluble molecule that can directly or indirectly activate microglia[46]. Astrocytic and microglial activation, along with the intensified release of proinflammatory cytokines have been described to follow lysophosphatidylcholine (LPC)-induced demyelination. Tumour necrosis factor (TNF)-α and interleukin (IL)-1β were reported to be released from astrocytes and microglia when S100B is overexpressed[19,26,45]. Interestingly, this protein has been shown to play a pivotal role in the inflammatory process of MS pathology. Indeed, it was found at high levels in both CSF and serum of MS patients at diagnosis

(Figure 1.3) and seems to promote glial reactivity as well as OL impairments in high concentration[19].

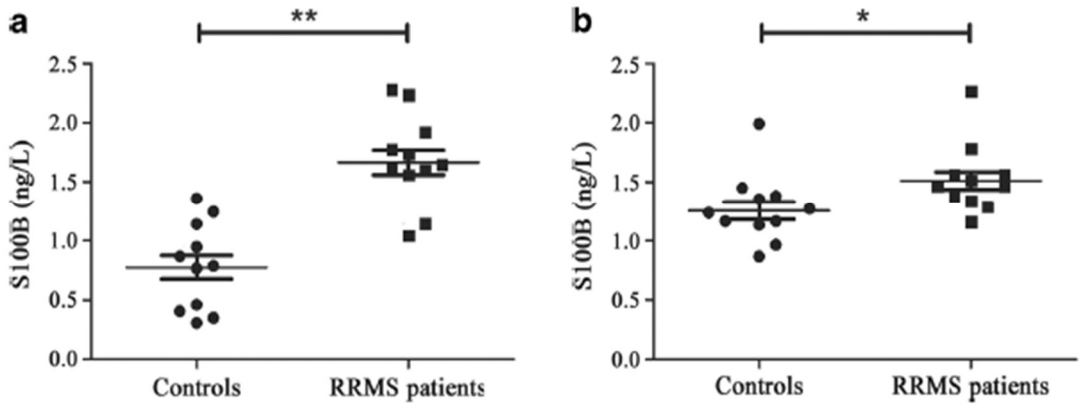

Figure 1.3 - Elevated levels of S100B in both cerebrospinal fluid (a) and serum (b) of patients at the time of diagnosis with RRMS. (Retrieved from Barateiro et al. 2016[19]).

1.4.2.1. S100B

S100B is a small protein involved in Ca^{2+} homeostasis and signaling. The nomenclature of this protein is derived from the technique used to be detected since it has complete solubility in a 100%-saturated solution with ammonium sulphate at neutral pH[47]. From the S100 proteins family, S100B contains two EF-hand helix-loop-helix Ca^{2+} binding domains[48]. Within cells, S100B can be found in the form of homodimer and occasionally as an S100B-S100A1 heterodimer[49]. Being mainly expressed and secreted by astrocytes, it regulates protein phosphorylation, transcription, enzyme activity, metabolism, and, noteworthy, neuron and glia proliferation and differentiation control[44,45]. S100B functions can drastically differ depending on their location. Intracellularly, as a regulator, S100B promotes neuronal proliferation and migration, OL differentiation, modulates cytoskeleton dynamics, required for the correct astrocytic morphology and also inhibits apoptosis[44,50]. Regarding the extracellular compartment, this molecule plays a signaling role, in which, binding to the receptor for advanced glycation end-products (RAGE) or Toll-like receptor 4 (TLR4) and, depending on S100B concentration, ranging from nanomolar (nM) to micromolar (µM), can be neuroprotective and neurotoxic, respectively[51]. In the absence of disease, S100B can promote neurite extension, neuronal survival, astrocytic proliferation, and microglia chemotactic capacity[45]. In neurodegenerative diseases like Alzheimer's Disease (AD) and MS, S100B is

overexpressed and highly released upon injury, by astrocytes. This abnormal secretion leads to an increase in extracellular S100B concentration, reaching μM concentrations[19,52]. In these conditions, S100B binds to its receptor and promotes several neurotoxic effects, as is the case of microglial and astrocyte activation, leading to the release of several pro-inflammatory mediators, the production of ROS, apoptosis induction, and neuronal death[45]. Oligodendrogenesis is impaired too, leading to a delay in remyelination, preventing the normal process of regeneration[26].

1.4.2.2. S100B Receptors

Cell receptors play a pivotal role in the detection of several signals and their consequent transduction to a cellular or tissue response. Some studies have been reporting the influence of specific receptors in certain physiological aspects related to inflammation, activation of the immune system, which when compromised, can both contribute to the development of neurodegenerative diseases and its progression, as is the case of MS. Two pattern recognition receptors (PRR), RAGE and TLR4 which bind S100B, have been repeatedly proving to be contributors to an inflammatory and innate immune response, that may have an effect on the immune status during MS development[19,53–57]. Not only in MS patients but also in MS animal models, both receptors have been found dysregulated[19,53,54,56–58].

1.4.2.2.1. Receptor for advanced glycation end products

From the immunoglobulin super-family, RAGE is a multiligand receptor that can be activated by several ligands such as advanced glycation end-products (AGEs), amyloid-like structures, proinflammatory cytokines, and danger-associated molecular pattern (DAMP) proteins as is the case of S100B and high mobility group box 1 (HMBG1), among others[59–63]. Being a transmembrane receptor, the full-length protein is composed by three major domains: the extracellular fraction, to which ligands bind (one N-terminal V-type and two C-type Ig domains); a single transmembrane helix; and a negatively charged C-terminal tail in the cytosolic region, required for signal transduction[64] **(Figure 1.4)**. Moreover, there are also two additional isoforms of RAGE, the dominant negative RAGE (DN-RAGE), lacking the cytoplasmatic portion, and the soluble RAGE (sRAGE), lacking both transmembrane and cytosolic portions. Both isoforms are unable to transduce the signal

and act like a S100B sequester[65,66]. RAGE is ubiquitously expressed, by diverse cell types, including MS-participating cells: endothelial cells of the BBB; immune cells, comprising T- and B-cells, monocytes/macrophages and dendritic cells; and several CNS cells including microglia and astrocytes[62,67].

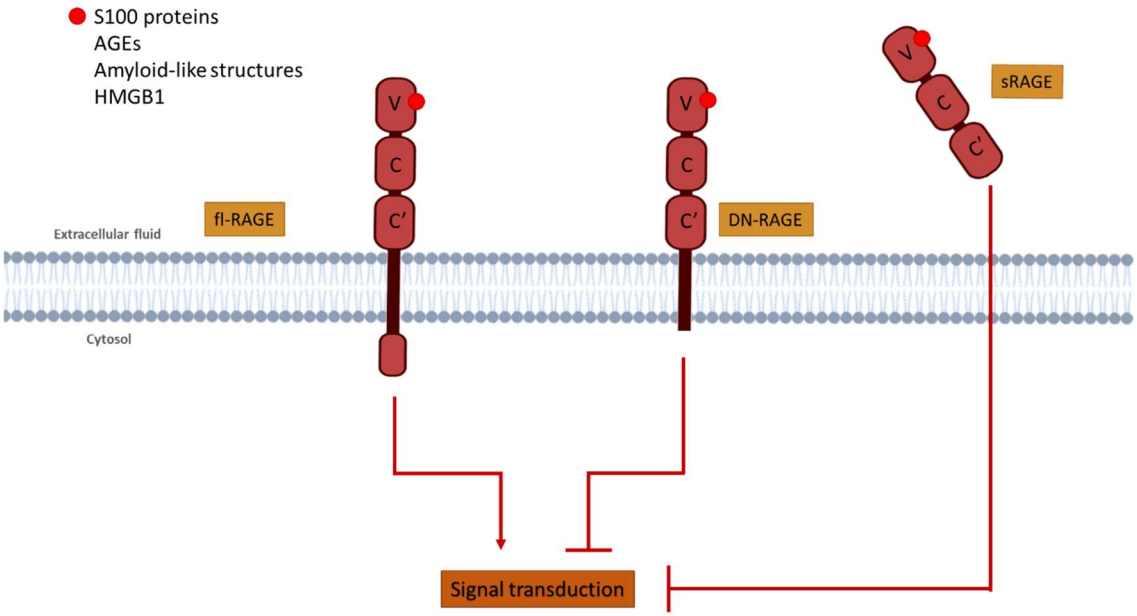

Figure 1.4 - Schematic representation of different forms of RAGE. Full-length RAGE structure (fl-RAGE), the dominant negative isoform of RAGE, lacking cytoplasmatic tail (DN-RAGE), and soluble RAGE, which lacks both cytoplasmatic tail and the transmembrane portion (sRAGE). Both DN-RAGE and sRAGE isoforms are unable to mediate RAGE-mediated cellular activation, derived from RAGE-ligand interaction and act as decoy receptors, capturing RAGE ligands. (Adapted from Ding et al. 2005[65])

As previously depicted, RAGE can exert different roles depending on the ligand and its respective concentration. Nevertheless, RAGE pathways, quite often conduct to the activation of the immune system through different processes, including oxidative stress production, the activation of nuclear factor-kB (NF-kB), leading to the production of pro-inflammatory cytokines such as IL-1β and TNF-α[68,69], which in turn trigger the recruitment of microglia and astrocytes to the lesion, in a RAGE-dependent manner[70,71]. Altogether, it is created a sustained period of cellular activation, spreading cellular dysfunction and consequently, evoking chronic inflammation. In pathological situations, as inflammation, neurodegeneration, nephropathy, and cancer, RAGE has been found upregulated[72]. Interestingly, *post-mortem* brain sections from MS patients showed a consistent increase of RAGE expression by macrophages or microglia, within active demyelinating lesions as well as in the border of chronic active lesions[19]. In addition, blood analysis from MS patients showed a marked overexpression of RAGE in both monocytes and

T-lymphocytes[73]. On the other way, is noteworthy to mention that sRAGE was found downregulated in MS patients[74]. These results highlight the role played by the S100B/RAGE axis in MS, with the duality of RAGE upregulation and sRAGE downregulation, having a harsher impact on MS pathology. Similar results were also found in experimental autoimmune encephalomyelitis (EAE) animal models, where an increase of RAGE expression in the CNS lesions was accompanied by chemotactic immune cell migration to the CNS[75]. Considering the previous results and the fact that the S100B/RAGE axis mediates microglial, astrocytic, and macrophage reactivity as well as migration and proliferation, it is of interest to develop new ways to target these events, in fact, some RAGE-targeted therapies, have been proving to be promising. sRAGE due to the inherent capacity of sequestering S100B can counteract the detrimental effect performed by the full-length isoform of RAGE. sRAGE has been showing to reduce immune cell infiltration and protect, at least partially, the development of EAE in mice. Similar results were reported by the same team, using RAGE antibodies which abrogated EAE induction[75]. Another emergent possibility is DN-RAGE since it is also capable of interfering with RAGE signaling[76]. The use of a RAGE antagonist has also proved to be effective in reducing S100B-induced damaging effects, including lesser astrogliosis, inflammation, and neuronal impairments, while improved OL differentiation and morphological maturation[26].

1.4.2.2.2. Toll-like receptors

Toll-like receptors (TLRs) have been described to be involved in several autoimmune pathologies including MS[77–81]. Present at both cell and endosome membranes, these receptors are expressed by different immune cells such as B- and T-cells, dendritic cells as well as in macrophages[82,83], and do participate in pathogen recognition, coupled with host protection, through the production of pro- and anti-inflammatory cytokines[84] together with APC stimulation[57]. Their primordial purpose seems to be the recognition of different exogenous pathogen-associated molecular patterns (PAMPs), such as lipids/lipopeptides, proteins, and nucleic acids[79], often due to intruding pathogenic microorganisms[54]. Even though, occasionally, TLRs can also recognize endogenous molecules, as DAMPs, enabling autoimmunity triggering, due to improper TLR activation to self-antigens[82]. S100B and HMGB1 are some of the molecules that can be recognized

by TLRs and have been repeatedly depicted to promote proinflammatory signaling through the interaction with TLR4[55,70]. Among TLRs, TLR4 is one, if not the major player in MS pathology. Present at the cell membrane of several immune cells is widely expressed by T helper (Th) 17 cells, being one of the necessary promoters for Th17 differentiation. When activated, TLR4 can promote Th17 infiltration into the CNS[54], through the BBB, fostering CNS injury in MS patients[81]. Moreover, it has also been demonstrated to be involved in myelin damage and to promote inflammation in the spinal cord of EAE mice[54].

1.4.3. Therapeutic strategies against S100B/RAGE axis

As referred before, targeting S100B-related pathogenesis seems to be a promising approach concerning MS treatment. Different molecules have been used to target directly S100B, even with different mechanisms. Although, considering the double way of action of S100B, is important to preserve the neuroprotective role, meaning that we must decrease the extracellular concentrations to physiological levels, without completely abolish S100B expression or erase its entire function.

1.4.3.1. Arundic Acid

Arundic acid is an agent that has been used in the last two decades to inhibit S100B synthesis. Asano and colleagues used arundic acid in a rodent model of focal cerebral ischemia. S100B was overexpressed in astrocytes, leading to reactive astrogliosis along the infarct border. Arundic acid proved to be a powerful inhibitor of S100B synthesis, due to the prevention of infarct expansion and caused an amelioration of neurologic deficits[85]. Another group described similar results regarding the role of arundic acid to downregulate S100B expression in an AD mouse model[86]. More recently this agent was used in an embryonic gut culture system and the results showed a marked prevention of S100B developmental upregulation[87]. Translating to MS research, these studies provide a promising tool that can be used to target S100B, decreasing neuroinflammation.

1.4.3.2. Pentamidine

Pentamidine is an antiprotozoal drug that binds specifically to S100B blocking the interaction with tumor suppressor p53. It has been proved that pentamidine can cause the reduction of S100B, its respective receptor (RAGE) engagement, and proinflammatory cytokine expression[52]. In this way, it was possible to impair neuroinflammation in an AD mouse model[52]. Furthermore, using an *ex vivo* demyelinating mouse model, it was showed that pentamidine is able to prevent both demyelination and inflammation, and even reinforce remyelination[72]. *In vivo* studies were also performed using pentamidine. In EAE animal model of MS, the use of this S100B-binding molecule decreased disease clinical score, enabling a faster recovery. The degree of demyelination and glia reactivity were also reduced, seen in the brain and spinal cord, along with lower values of Inflammation[88,89]. These promising results show the potential effect of pentamidine in neurodegenerative diseases, with higher relevance to MS treatment research.

1.4.3.3. Antibodies

Immunoglobulins, also known as antibodies, are used to target desired molecules, as the case of S100B or RAGE. Regarding structure, antibodies present a Y-shaped form and are produced mainly by B cells. Upon demyelination induction in an *ex vivo* model, using organotypic cerebellar slices, the incubation with anti-S100B antibody, neutralized the S100B effect, reducing demyelination values as well as preventing astrocyte reactivity and pro-inflammatory cytokines and inflammasome molecules expression[19].

1.4.3.4. A promising approach using Nanobodies

Produced by llamas or camels, single domain antibodies or even nanobodies (Nb), can exert inhibitory, agonistic, or antagonistic roles depending on the targeted molecule, through the recognition of a certain epitope or antigen[90]. However, several different characteristics distinguish them from commonly used antibodies. Nanobodies received this nomenclature due to the relatively small molecular size (15kDa)[91]. Corresponding to the N-terminal variable domain of the heavy chain-only antibody (VHH), is homologous to the variable domain of IgG antibody heavy chain (VH) **(Figure 1.5)**. A Nb is composed

by a folded β-sheet with three loops in the regions homologous to the complementarity-determining region (CDR3) of the IgG VH domains[90]. The need to find more molecules, with more advantages and capable to generate better results, was the leading force to the development of these nanoparticles. The extreme specificity to recognize unique epitopes in a subnanomolar scale, the higher affinity, stability and solubility, the resistance upon extreme pH levels, temperature fluctuations, and several different chemical environments, as well as the reduced immunogenicity and toxicity, are some of the benefits that this molecule brings to science, contrasting to the commonly used antibodies[57,92]. These nanoparticles can have very different applications, from studying enzyme mechanism, the inhibition or activation of protein functions via protein-protein interaction modulation, perturbation of protein aggregation, even to the interference with the immune system[90]. Some of these features are directly breaking apart certain difficulties found in MS research. The smaller size, higher solubility, and isoelectric point (pI) allow an easier entrance into CNS, through BBB, as seen in healthy and pathological conditions[93–97], while the reduced immunogenicity due to the absence of Fc region[98], is essential since inflammation and the immune response per se are under analysis. The inhibitory properties of nanobodies are very promising regarding medicine applications as well as for disease diagnosis. Nanobody-mediated S100B targeting will potentially

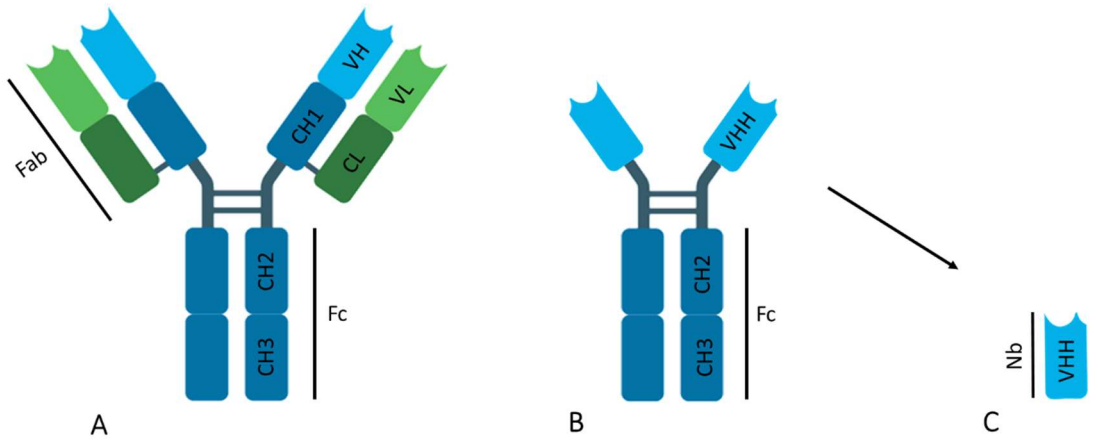

Figure 1.5 - Schematic representation of antibodies and nanobodies. (A) Domain structure of human IgG antibodies, (B) heavy chain only camelid antibodies, from which (C) nanobodies can be obtained. (Adapted from Arezumand et al. 2017[91]). Fab: Fragment antigen-binding; Fc: Fragment crystallizable region; CH: Constant domain of the heavy chain; CL: Constant domain of the light chain; VH: Variable domain of IgG antibody heavy chain; VL: Variable domain of the light chain; VHH: N-terminal variable domain of the heavy chain-only antibody; Nb: Nanobody.

allow the neutralization of S100B and consequent modulation of several pro-inflammatory cytokines involved in MS pathogenesis and possibly the modulation of the immune response, one if not the major challenge in MS research as well as in clinical practice.

1.5. Experimental models to study Multiple Sclerosis and S100B

The design of new therapies would not be possible without the development of experimental models that mimic as much as possible MS pathology. Mice and rats have been the most used animals to create similar MS characteristics, since are easily to manipulate and are phylogenetically closer to humans, increasing translational potential. In MS research, different animal models have been used to study inflammation, autoimmunity, demyelination and remyelination, some better than others, depending on which is the main subject being analyzed, although there is no perfect model that comprises all MS features[99]. The main used models are subdivided into *in vivo* or *ex vivo* groups. *In vivo* models reach closer to human disease features, involving the entire organism and the interplay between organs, tissues and cells makes them more appealing to use, although they are expensive and rises more ethical concerns. In the other hand, *ex vivo* models provide us tools such as easily manipulation of the experimental conditions without many ethical concerns as *in vivo*, although a slightly altered environment prevent the interplay between tissues and organs[99–101]. Additionally, different transgenic mice are also used, not as an MS animal model, but useful regarding the study of different specific players that can play a role in MS pathology.

1.5.1. *In Vivo* models

The use of different *in vivo* models to study MS has been of vital importance to understand its complex pathogenesis since there is high variability among patients regarding the etiology of the disease. To explore different molecular mechanisms and develop new therapies, different animal models have been developed, however, no single experimental model is capable to represent all the complex heterogeneity of clinical, pathological, and immunological characteristics of the disease.

Divided into three main categories, MS animal models can be:

1. Autoimmune and inflammatory models, such as experimental autoimmune encephalomyelitis (EAE).
2. Virus-induced demyelination as the case of both Theiler's murine encephalitis virus (TMEV) and mouse hepatitis virus (MHV).
3. Toxin-induced models, including cuprizone, lysophosphatidylcholine (LPC) and ethidium bromide toxic models.

1.5.1.1. Experimental autoimmune encephalomyelitis (EAE)

The most used and versatile animal model is EAE, an autoimmune model, characterized by T-cell and monocyte infiltration in the CNS, associated with local inflammation, demyelination, axonal loss, and gliosis. EAE is particularly used to study myelin destruction, neuroinflammation, and immune system activation. To induce this model, an active immunization is performed with complete Freund's adjuvant (CFA) assistance, through subcutaneous injections, containing peptides derived from myelin proteins such as myelin proteolipid protein (PLP), myelin OL glycoprotein (MOG) and myelin basic protein (MBP). Therefore, activation of peripheral antigen-specific T cells occurs, allowing these cells to set an immune reactivity against myelin antigens and develop the disease. CFA alone can induce inflammatory processes, immune system activation and a partial BBB permeability, although when pertussis toxin is added, a more severe CNS infiltration occurs and a more consistent form of the disease is obtained[102]. Another way to induce EAE is to inject lymphocytes derived from EAE animals to naive ones, often described as passive immunization[99,103]. Many of the drugs used today by MS patients were developed, tested or validated based on EAE studies, including glatiramer acetate, mitoxantrone, and natalizumab[104,105]. It is of extreme importance to choose the best model at the time of experiment planning. Even so, although several therapeutic approaches showed encouraging results in preclinical studies, they failed and some even caused harm when used in humans[106].

1.5.1.2. Virus-induced model

As previously described in 1.2 - Epidemiology, viruses can take place in MS pathology such as EBV infection. Due to this fact, virus-induced MS animal models were also developed

and are capable to induce MS-like pathogenesis. The most known is TMEV, belonging to the single-stranded RNA picornaviruses, a mouse enteric pathogen capable to cause paralysis and encephalomyelitis. TMEV has two main groups: 1) a highly virulent strain which causes fatal encephalitis and 2) a much less virulent strain. The latter can develop a monophasic and persistent neurologic disease as well as a biphasic form, capable to reproduce chronic demyelinating lesions, being the one used as a chronic-progressive MS animal model[103]. TMEV can infect macrophages as well as CNS glial cells[107–110], during the period of chronic phases. The similarities to human MS as well as the autoimmune response initiated by a viral infection in the CNS are the main features that make this model attractive to study axonal damage, inflammatory-induced demyelination and the determination of immunomodulatory effects on de- and remyelination therapies[111–113]. Even so, it is a difficult model to implement due to the higher variability, intrinsic to each animal used, low standardization, higher mortality rate in infected animals and the safety concerns to manipulate virulent virus strains[114].

1.5.1.3. Toxin-induced model

As listed above, the third MS animal model is toxin-induced, very useful to identify new possible therapies, through the unraveling of the mechanisms underlying de- and remyelination[115], a complement to EAE studies, in which remyelination occurs in a small rate. Metabolic processes implicated in myelin/OL destruction and repair represent essential data, obtainable from this MS model[116,117]. Demyelination can be induced with different compounds, among them, lysolecithin, also known as lysophosphatidylcholine (LPC), and cuprizone are the most used. Demyelination may be elicited by local administration into the CNS white matter via stereotactic surgery or systemic administration and may be used in *ex vivo* models as will be further described.

Lysolecithin is a molecule with detergent-like properties, with particular toxicity to OLs[118], capable to insert itself into the myelin lipidic membrane[119], form micelles[120], convert into lysophosphatidic acid and consequently disturb myelin sheaths structure[99,115]. It is worth mentioning that, in controlled conditions, OL viability, axonal and other cellular components are not altered or compromised during the process of demyelination induction[121]. Moreover, LPC enhances phospholipase A2 activity, increasing the

degradation rate of membrane phosphatidylcholine, a major constituent of myelin[122–124]. After the demyelination process, and as a consequence, T and B cells, microglia and macrophages are recruited to the lesion, initiating the inflammatory process[102,125]. Reactive astrogliosis, axonal instability, and OPCs proliferation and migration are also visible in this model[126]. The good reproducibility and well-defined location of the demyelinated areas allow this toxin-induced model to be an outstanding method to study demyelination and remyelination[127].

Cuprizone model also has mature OLs as the main target, inducing demyelination in a different way than LPC. This toxin is a copper chelator, that when administered promotes copper deficiency. Even though the mechanism is not fully known, it causes specific OL death by disturbing cellular metabolism and subsequent demyelination, without affecting other CNS cells. Animals are fed with 0.2 % cuprizone mixed in chow from 4 to 6 weeks, causing dysfunction on mitochondria, toxicity of OLs inducing substantial demyelination throughout the brain. After this period, the administration of cuprizone is stopped, allowing this way to the differentiation of OPCs into mature OLs, and consequently, the formation of new myelin sheaths to denuded axons[128]. This model is used to discern the underlying mechanisms associated with OL death, OPC migration, differentiation and at last remyelination, although the immune system interaction is absent[129].

1.5.2. *Ex Vivo* models

Research in organs, tissues or cells, obtained from living organisms, have been used for decades as an alternative to *in vivo* assays. *Ex vivo* models allow the control of certain conditions in animal experimentation that would be harder to manage in living animals, with the disadvantage of the slightly altered environment. One of the strengths of these models is the possibility to perform certain tests, for example, therapeutic candidates screening, that could not be possible or even not allowed by ethical issues. With the purpose to analyze different morphological and physiological aspects of brain cells including neurons, OLs, microglia and astrocytes, all of them involved in MS pathology, cerebellar organotypic slice cultures (COSCs) are a resourceful method to perform it.

1.5.2.1. Organotypic slice culture

Considered an important method to reproduce *in vivo*-like characteristics, organotypic slice cultures have been used as an intermediate technique between animal models and cell cultures, in which the three-dimensional architecture of brain environment is maintained and subsequently the contact between the different CNS cells persists[139–141]. Therefore, the preservation of structural features, neuronal connectivity, and electrical and biochemical pathways, makes possible the analysis of processes, such as de- and remyelination as well as the inflammatory response mediated by microglia and astrocytes[142], main players in MS pathology. Altogether, these elements make this method a suitable system to explore cell physiology as well as environmental interactions regarding glial and neuronal cells response, under neurodegenerative conditions as the case of myelin damage[141]. In other aspects, some limitations must be considered when analyzing data. Both astrocytes and microglia have been described to have a strong response when CNS damage takes place, which also happens in the course of slice preparation, including increased proliferation at injury spots and subsequently the formation of glial scar[141]. Another negative component, in contrast to EAE[143], is the absence of immune activity, the main player in MS, since both lymphatic and circulatory systems were lost during slice preparation. Even so, the presence of glial cells and extracellular matrix is an advantage comparing to the co-culture of dissociated cells, which lacks these two components, known to be important in the development, maintenance, and physiology of OLs and myelin[141].

Regarding preparation, slices ranging from 100 µm to 400 µm can be obtained using a microtome[140], thereafter the slices are placed above semi-permeable membranes, with a pore size large enough to allow metabolic substrates and neurotrophic factors to diffuse, as well pharmacological molecules present in the culture medium[144]. Depending on the brain region cultured, including spinal cord[145] and brain regions such as cerebellum[144], forebrain[114], and hippocampus[146] different parameters can be observed, for example, the homogeneity of axon types present in cerebellum, compared with spinal cord axonal variety[114]. In MS research the most common to be used is the cerebellum since it comprises more white matter with specific detectable tracts rather than other brain regions[100,147]. Similar to the method described previously in 1.5.1.3 – Toxin-induced

model, LPC can be added to the medium and induce demyelination without neuronal damage, if in a short period. After a transient demyelinating insult, the cultures recover, however not entirely due to the limited remyelination capacity[148].

1.5.3. Transgenic mice

Different transgenic mice are also used in research, for both *in vivo* and *ex vivo* studies, being very useful to explore different crucial players from specific diseases, as is the case of S100B in MS pathology. Even though S100B does play an important role in CNS development[26], different transgenic mice that overexpressed this specific protein, presented several disruptive features upon challenge. This includes an increased degree of brain damage and periinfarct reactive gliosis (astrocytosis and microgliosis) after permanent focal ischemia[130] as well as an increased susceptibility to perinatal hypoxia-ischemia[131]; higher amyloidosis and gliosis in an AD mouse model[132]; problems concerning learning and memory[133], accompanied by electrophysiological disturbances in the hippocampus[134] among other poorer results when comparing to wildtype mice groups. In the other way, S100B knockout mice have already presented insightful results where it proves that the lack of S100B in the brain can be deleterious promoting epileptogenesis[135] as well as blood-brain barrier permeability and neuron-binding autoantibodies in an age-dependent manner[136]. Nevertheless, S100B depletion did not affect cerebellar development[137], a very important aspect that in this way do not compromise cerebellar studies, as is the case of the present dissertation. Interestingly, an AD mouse model study brought together three different mice regarding S100B expression: S100B-overexpressing mice; wildtype mice; and S100B knockout mice[138]. Contrasting to wildtype and S100B knockout groups, S100B-overexpressing mice group did present very adverse results where glia activation, neuroinflammation, neuronal damage and the loss of several synaptic markers took place, denoting once again the pejorative role of S100B on brain homeostasis or even a worse outcome when mimicking different diseases.

2. Motivation

MS is a neurodegenerative disease for which there is still no cure, only treatments capable to slow down its normal progression. The patient, with the course of the disease, is no more than a spectator with a few possible options to combat this invisible disease that rises deep within. The necessity of different types of assistance, as well as the reduction of quality of life, transform this disease into a nightmare. What people in the health field have done concerning this specific disease, was not only the improvement of the quality of life through different ways, but also the development of new therapeutic strategies, in order to achieve a cure or a level at which the disease progress in much lower fashion. Our group has shown an increased interest in this field over the past years and made several new insights in MS research. S100B protein has captivated our attention in the way as it affects demyelination, oligodendrogenesis, and glial reactivity, most often by the detrimental side.

Beholding the fact that S100B was found upregulated in the CSF of MS patients at the time of diagnosis, our group started to focus on this biomarker with the main goal to develop possible therapeutic strategies. To do this, our group started to uncover some S100B features, using cerebellar organotypic slice cultures (COSC), an *ex vivo* model of demyelination. Analogous to the values found in the CSF from patients, these cultures showed an increase in S100B expression upon a demyelinating insult, elicited by LPC. Additionally, the use of an S100B-directed antibody caused a significant reduction in the degree of demyelination, astroglial reactivity as well as the expression of first-line cytokines and inflammasome-related molecules[19].

Further, making use of primary OL cell cultures, was demonstrated that high concentrations of S100B led to oligodendrogenesis impairment, which was abolished by an antagonist for S100B receptor RAGE the FPS-ZM1[26]. After that, in a more complex

environment using COSCs, RAGE antagonist demonstrated again beneficial effects upon LPC-induced demyelination, such as the correct OPC differentiation and maturation, the prevention of astrogliosis, inflammation, and even neuronal impairment[26].

In the same *ex vivo* model, another S100B-targeted protein, pentamidine, also proved to be valuable since prevented demyelination and the exacerbated expression of pro-inflammatory cytokines[72]. Increasing both complexity and translational power, *in vivo* studies are underway using pentamidine. In EAE animal model of MS, the use of this S100B-binding molecule decreased disease clinical score, enabling a faster recovery. The degree of demyelination and glia reactivity were also reduced in both brain and spinal cord, along with lower values of inflammation[88,89].

All these past results showed that different molecules are capable to diminish S100B-related neurotoxic effects, although, when looking to clinical applicability they may impose some difficulties. Thus, the opportunity to test new small molecules with different remarking advantages as the Nanobodies, urge as a new promising area of study.

3. Aims

The main objective of this study is to better understand S100B-related effects upon LPC-induced demyelination and if by neutralizing this molecule with nanobody-based therapy we could prevent MS-associated pathogenesis. To do so we subdivided the experimental procedure in two specific objectives:

- First, we analysed how S100B depletion would possibly abolish respective neurotoxic effects in the *ex vivo* demyelinating model, commonly used in our lab. For this to happen, we used COSCs from three different mice: wild type; heterozygotic (S100B +/-); and knockout mice (S100B -/-). The LPC-induced demyelination takes place within 18 h and after a period of recovery, we assessed S100B expression, demyelination, oligodendrogenesis, and the inflammatory events.
- Secondly, we evaluated whether the neutralization of S100B, using three different nanobodies, could prevent the pathogenesis observed in the *ex vivo* demyelinating model. To do so, we used COSCs from C57BL/6 wild type mice and induced the LPC insult for demyelination to occur. Along with LPC insult, some cultures were treated with three different nanobodies as well with pentamidine and S100B-directed antibody (already tested by our lab) separately, to compare results. After a 30 h period of recovery, S100B expression, demyelination degree, oligodendrogenesis, glial reactivity, and inflammatory-related molecules were evaluated.

4. Material and Methods

4.1. Animals

To perform the *ex vivo* studies, we used pups with 10 days (P10) from C57BL/6 wild type (WT) mice, as well as from a S100B knockout (S100B -/-) mice and from a S100B heterozygotic (S100B +/-) mice on a B6JTyr;B6N-S100b$^{tm1a(EUCOMM)Wtsi}$/Wtsi genetic background. The colonies were established on Instituto de Medicina Molecular João Lobo Antunes (IMM, Lisbon, Portugal) and the genotyping was done using PCR, in which 3 pairs of primers were used to amplify different DNA regions according to manufacturer's instructions (Wellcome Trust Sanger Institute, Hinxton Cambridge, UK). The animals used in these experiments were kept in a controlled environment (21 ^{o}C and 55-67% humidity in a 12 h light/dark cycles) and were fed with food and water *ad libitum*. Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for the protection of experimental animals). All the procedures were approved by the Institutional Animal Care and Use Committee and the national animal affairs regulatory office (Direção Geral de Alimentação e Veterinária). The best efforts were made to minimize the number of animals used and their suffering.

4.2. *Ex vivo* model of demyelination

In order to assess the role of S100B and S100B-targeting nanobodies in a demyelination situation, we used an established protocol in which cerebellar organotypic slice cultures (COSC) were treated with lysophosphatidylcholine (LPC)[19,148]. Cerebellar parasagittal slices were obtained from postnatal day 10 (P10) mice as described above. Following decapitation, the cerebella were isolated from brains in phosphate buffered saline (PBS),

to which 400 µm sagittal slices were obtained using a McIlwain tissue chopper. To maximize the results, all the optimal slices were transferred into membrane culture inserts with 0.4 µm pores (BD Falcon). Four slices, from different mice, were put together in each insert, in a 6-well cell culture plates, containing 1 mL of medium per well and were kept at 37 °C, in 5% CO_2 controlled atmosphere. The first slice culture medium (Medium 1) used in the experiment, consisted in 50% minimal essential media (MEM; Gibco), 25% heat-inactivated horse serum (Gibco), 25% Earl's balanced salt solution (SIGMA), 6.5 mg/mL glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Biochrom AG), and 1% of both L-glutamine (Sigma-Aldrich) and penicillin/ streptomycin (Sigma-Aldrich), being used in the first 3 days in vitro (DIV), as illustrated in the scheme bellow **(Figure 2.1)**. Afterwards, the medium was completely replaced by a new serum-free media (Medium 2), composed by 98% Neurobasal-A (Gibco), 2% B-27 (Gibco), 36 mM glucose, 25 mM HEPES, 2 mM L-glutamine and 1% U/mL penicillin/streptomycin, to improve neuronal viability and functionality. Half media were replaced every day and slices were maintained for 7 DIV, letting myelination and the clearance of debris to occur, before demyelination induction. To address the first aim, following 7 DIV, slices derived from WT, S100B -/- and S100B +/- mice were exposed to LPC (0.5 mg/mL in serum-free

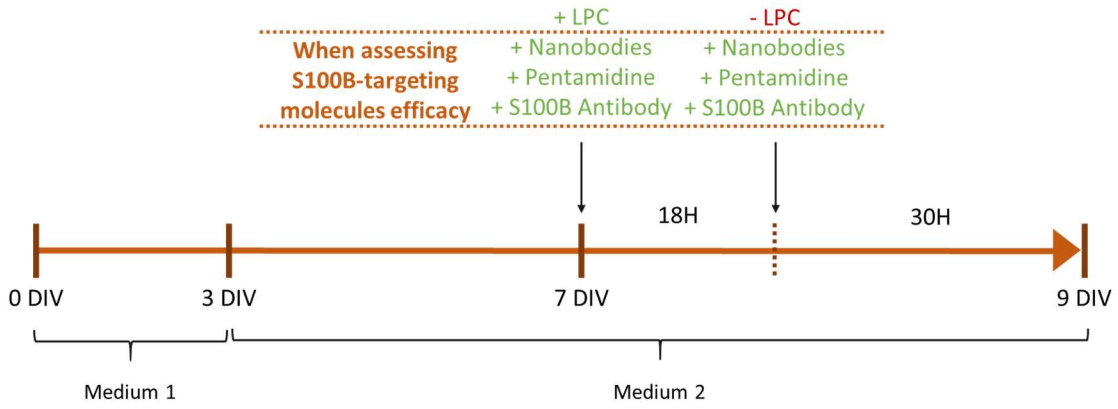

Figure 2.1 - Schematic representation of COSC culture and treatment timepoints . Four cerebellar slices of different animals were placed into a membrane culture in a 6-well cell culture plates, containing 1 mL of the first culture medium (Medium 1) at 37 °C, in a 5% CO_2 controlled atmosphere. At 4 DIV the media was completely removed and replaced by a serum-free media (Medium 2) until 7 DIV. Half of the media was replaced every day. At this timepoint, slices were incubated with LPC (10 µL/mL) to induce demyelination for 18 h, as well as with different treatments when assessing S100B-targeting molecules efficacy. After the period of 18 h, the LPC-containing medium was completely removed and the same medium was replaced, now in the absence of LPC for additional 30 h. When assessing S100B-targeting molecules efficacy, the different treatments were maintained in the 30 h period.

culture media), a demyelinating agent. Following 18 h, the media were collected for ELISA, and new serum-free media replaced and maintained up to 48 h. By 9 DIV, the slices were: (1) prepared for RNA and protein extraction, frozen in RiboZol™ reagent, at -20 °C or (2) fixed in 4% paraformaldehyde (PFA) for immunohistochemistry assays. To address the second aim, assay S100B-targeting molecules efficacy, slices were incubated with LPC in the presence of three different nanobodies (6 nM) and two molecules already tested by our group, Pentamidine (5 mg/mL) and an S100B-directed antibody (1:1000). All assayed molecules were maintained in new serum-free media during recovery period until 48 h post demyelination induction.

The Nanobodies directed to S100B were developed by Professor Claudio Gomes, Faculty of Sciences, University of Lisbon, and the three nanobodies were chosen based on their ability to specifically bind to S100B dimer and tetramer although with different dissociation constants (K_D). Nanobody 2 binds to S100B dimer and tetramer with a $K_D \approx 1$ mM, and Nanobody 16 binds to S100B dimer with a $K_D \approx 0.6$ mM and tetramer with a $K_D \approx 0.2$ mM. Concerning Nanobody 17, the K_D were still not determined.

4.3. Total RNA Extraction and qRealTime-PCR

To evaluate gene expression of several genes of interest, total RNA was extracted from 9 DIV COSC, applying RiboZol™ reagent method, under the recommended conditions (VWR Life Science). In brief, each slice was individually macerated with a power homogenizer to dissociate the tissue, followed by a process of phase separation, RNA precipitation, washing and finally respective dissolving in RNase-free water. NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies) was used to quantify RNA concentration. Samples with 1000 ng of RNA was reversely transcribed into complementary DNA (cDNA), using Xpert cDNA synthesis mastermix kit protocol (GRISP), according to the manufacturer's instructions. Quantitative RealTime-PCR (qRealTime-PCR) was subsequently performed to quantify the expression levels of several genes, in which β-actin was used as an housekeeping gene to normalize the expression of first-line cytokines: tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 10 (IL-10); inflammasome-related molecules: S100B, high-mobility group box protein 1 (HMGB1), toll-like receptor 4 (TLR4), receptor for advanced glycation

end-products (RAGE); and for OL lineage cells: myelin basic protein (MBP), and neuron glia antigen 2 (NG2). Real-time PCR detection system (Applied Biosystems 7300 Fast Real-Time PCR System) was used to perform qRealTime-PCR, using Xpert Fast SYBR mastermix (GRISP). The loading was performed in a 384-well plate in which duplicates were done to each sample, including a no-template control (NTC) as a negative control. The primers' sequences are listed in the **Table 1**. The PCR cycle conditions were previously optimized as follows: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min followed by 40 cycles at 95 ^{o}C for 15 sec and 62 ^{o}C for 1 min.

The analysis of gene expression was performed using Pfaffl modification of the ΔΔCt equation. This equation represents the number of cycles at which the fluorescence reaches the threshold levels of detection (CT). The values of each sample were normalized to the endogenous gene, β-actin, using the $2^{-\Delta\Delta Ct}$ equation. For each sample, the ΔCt was calculated by the difference between the gene of interest Ct value and the mean of β-actin Ct value. Afterwards, ΔΔCt value of each sample was measured through the difference between each sample ΔCt and the ΔCt from the sample defined as reference, in this case the slices that were not incubated with LPC as well as any treatment.

Table 1 - List of primers used in qRealTime-PCR assays.

Gene	Primer	
	Forward	Reverse
β-actin	gctccggcatgtgcaa	aggatcttcatgaggtagt
HMGB1	ctcagagaggtggaagaccatgt	gggatgtaggttttcatttctctttc
IL-1β	caggctccgagatgaacaac	ggtggagagctttcagctcata
IL-10	atgctgcctgctcttactga	gcagctctaggagcatgtgg
RAGE	ttcacgacgaagttccaaacaggt	gttctaggaggactggggtg
S100B	tgtagaccctaacccggagg	tgcatggatgaggaaggcat
TLR4	acctggctggtttacacgtc	gtgccagagacattgcagaa
TNF-α	tactgaacttcggggtgattggtcc	cagccttgtcccttgaagagaacc
MBP	ccatccaagaagaccccaca	cccctgtcaccgctaaagaa
NG2	gggctgtgctgtctgttga	tgattcccttcagctaaggca

HMGB1 - High-mobility group box protein 1; IL- Interleukin; RAGE - Receptor for advanced glycation end-products; TLR4 - Toll-like receptor 4; TNF-α - Tumour necrosis factor α; MBP - Myelin basic protein; NG2 - Neuron glia antigen 2

4.4. Immunohistochemistry

With the purpose to stain and evaluate different cellular and molecular elements such as myelin degradation and glial and astrocytic reactivity through confocal microscopy, 9 DIV COSC were fixated with 4% paraformaldehyde (PFA) for 1 h at room temperature. After the fixation period, the slices were washed 3 times with PBS solution and kept at 4 ^{o}C until the immunostaining procedure. The immunostaining procedure per se consisted in the removal of a slice per insert, through membrane sectioning, and incubation with blocking solution (1 nM HEPES, 2% heat-inactivated horse serum, 10% heat-inactivated goat serum, 1% BSA and 0.25 % Triton X-100 in Hank's balanced salt solution) in a cover glass for 3 h at room temperature. The slices were then incubated with designated primary antibodies, diluted in blocking solution for 48 h, at 4 °C. The slices were then washed 3 times for 20 min each, with washing solution PBS-T (PBS with 0.01 % Triton X-100). This process was followed by an overnight period of incubation with secondary antibodies in blocking solution at 4 °C. The primary and secondary antibodies used are listed in Table 2-3. After secondary antibody incubation the washing process was repeated as described previously, and slices incubated with DAPI (1:1000) to stain the nuclei for 3 min and washed 3 times with washing solution. To prepare the slice to fluorescence/confocal microscopy, Fluoromount-G (Southern Biotech, Birmingham, AL, USA) was used. The images were obtained by Leica DMi8-CS inverted microscope with Leica LAS X software. ImageJ (Fiji Is Just) software was used to process the images.

Table 2 - List of primary antibodies used for immunohistochemistry procedures. Marker, respective target, antibody host, dilution used, brand and reference number are indicated.

Primary antibodies					
Marker	**Target**	**Host**	**Dilution**	**Brand**	**Reference Number**
MBP	Mature oligodendrocytes	Rat	1:200	Biorad	aa82-87
QD9	Decompacted myelin	Mouse	1:100	AbNova	MAB8817
GFAP	Astrocytes	Mouse	1:100	NovoCastra	GFAP-GA5-6035278
Iba-1	Microglia	Rabbit	1:250	Wako	019-19741

MBP – Myelin basic protein; GFAP – Glial fibrillary acidic protein; Iba-1 – Ionized calcium-binding adapter molecule 1

Table 3 - List of secondary antibodies used for immunohistochemistry procedures. Marker, respective target, antibody host, dilution used, brand and reference number are indicated.

Secondary Antibodies					
Antibody	**Target**	**Host**	**Dilution**	**Brand**	**Reference Number**
Alexa 488 anti-mouse	Mouse antibodies	Goat	1:1000	Invitrogen	A10680
Alexa 488 anti-rabbit	Rabbit antibodies	Goat		Invitrogen	A11008
Alexa 594 anti-rat	Rat antibodies	Donkey		Invitrogen	A21209
Alexa 594 anti-mouse	Mouse antibodies	Goat		Invitogen	A11005
Alexa 594 anti-rabbit	Rabbit antibodies	Goat		Invitrogen	A111012

Approximately 20 z-stacks were taken per slice, obtaining different depths of the tissue, from which the ones that had measurable immunoreactivity were merged, reducing possible variations in image acquisition. Focusing on the medullary laminae (branched myelinated fibers) as well as the surrounding granular and grey layers, demyelination, astrogliosis and microglia reactivity were examined.

4.5. Western blot

Protein expression was also evaluated to be combined with gene expression values. Total protein isolation was performed from RiboZol™ treated samples, using the organic phase following chloroform-based phase separation. After protein extraction, total protein quantifications were measured using BCA protein assay kit under manufacture's guidelines (Pierce™ BCA Protein Assay, ThermoFisher Scientific), and stored at -80 °C. Western blot sample were then prepared with a buffer containing 0.25 M Tris-base (pH 6.8), 4% (w/v) sodium dodecyl sulphate, 40% (v/v) glycerol, 0.2 % (w/v) bromophenol blue and 1% (v/v) β-mercaptoethanol following by denaturation for 5 minutes at 100 °C. The samples were then loaded into a Tris-Tricine gel and subsequently transferred to a nitrocellulose membrane (Amersham Biosciences). A blocking solution [1% Tween 20-Tris buffered saline (T-TBS) in 5% (w/v) non-fat milk] were added to membranes for 1 h at room temperature. The next step was to incubate the membranes with the primary antibody overnight at 4 °C. Membranes were then washed and incubated with the same blocking solution used before with the secondary antibody for 1 h at room temperature.

The antibodies used are listed in Table 4 and Table 5. Protein bands were detected using WesternBright Sirius reagent (Advansta) for 1 min and visualized using Using ChemiDoc™ (Bio-Rad Laboratories) equipment. Bands were quantified with ImageLab™ software (Bio-Rad Laboratories) and respective relative intensities normalized to total protein Amidoblack® staining.

Table 4 - List of primary antibodies used in western blot procedures. Marker, respective target, antibody host, dilution used, brand and reference number are indicated.

Primary antibodies					
Marker	**Target**	**Host**	**Dilution**	**Brand**	**Reference Number**
MBP	Mature oligodendrocytes	Mouse	1:800	Millipore	MAB387
NG2	Immature oligodendrocytes	Rabbit	1:500	Millipore	AB5320
S100B	S100B	Rabbit	1:1000	AbCam	ab52642
RAGE	RAGE	Mouse	1:1000	Chemicon	MAB5238
β-actin	β-actin	Mouse	1:5000	Sigma	A5441

MBP – Myelin basic protein; NG2 – Neuron glia antigen 2; RAGE – Receptor for advanced glycation end-products

Table 5 - List of secondary antibodies used in western blot procedures. Marker, respective target, antibody host, dilution used, brand and reference number are indicated.

Secondary Antibodies					
Antibody	**Target**	**Host**	**Dilution**	**Brand**	**Reference Number**
HRP anti-rabbit	Rabbit antibodies	Goat	1:5000	Santa Cruz	sc-2004
HRP anti-mouse	Mouse antibodies	Goat	1:5000	Santa Cruz	sc-2005
HRP anti-rat	Rat antibodies	Goat	1:5000	Santa Cruz	sc-2032

4.6. S100B determination

To evaluate S100B concentration from COSC supernatants, an in-house enzyme-linked immunosorbent assay (ELISA) was performed. The supernatants were incubated overnight at 4 °C on a 96-well plate which was previously coated with a policlonal anti-S100B antibody (2.5 µg/mL, Rabbit MaxPab® affinity purified pAb, Absource) diluted in

blocking buffer (2% BSA in 1x PBS). The coated wells were then blocked for 2 h at room temperature with blocking buffer (2% BSA in 1x PBS). This process was followed by the application of 100 µL of both protein standard and samples and incubated overnight at 4 °C. In the next day, a polyclonal anti-S100B antibody (1 µg/mL, Biotinylated rabbit affinity purified pAb, Absource) diluted in diluent [2% non-fat milk in PBST (0.05 %)] was added and incubated for 2 h at room temperature. The wells were incubated with the secondary reagent (1:5000, Streptavidin-HRP, Sigma-Aldrich) for 1 h at room temperature. To perform the colorimetric reaction, TMB/E Ultra Sensitive Substrate® (Sigma-Aldrich) were used and measured at 450 nm in a microplate absorbance.

4.7. Statistical analysis

All results in this project are presented as mean ± standard error of the mean (SEM). The differences between treatment conditions were evaluated using two-tailed unpaired Student's t-test to compare control *versus* LPC treatment. One-way ANOVA with Tukey post-test was also used when comparing more than 2 treatment conditions, using GraphPad PRISM 6 for Windows. The P values of $P<0.05$ were considered as being statistically significant.

5. Results

5.1. S100B expression is exacerbated after LPC-induced demyelination

The short period in which a relapse takes place combine exacerbated inflammation, demyelination, neuronal and axonal damage, OL malfunction, and reactive gliosis[1]. Known to be part of this homeostatic disruption, S100B can be excessively produced and secreted by astrocytes as a response to cellular damage, reaching extracellular μM concentrations. Contrary to neuroprotective nM concentrations, toxic S100B levels may have harmful consequences in the context of cellular recovery after the insult, probably perpetuating to a certain extent the pathologic condition. After LPC-induced demyelination, this protein is known to be overexpressed and, subsequently, secreted towards extracellular space[19,26], which was confirmed through different techniques. With this in mind, we first evaluated S100B expression from both supernatants and tissue of COSCs, obtained from homozygotic (WT), heterozygotic (HET), and knockout (KO) S100B mice. Regarding WT cultures, the overexpression and secretion of this protein to the extracellular space was evident at 18 h after the disease-like onset when comparing to the respective control (12.27-fold, $P<0.01$), performed through ELISA **(Figure 3.1 A)**. Even though, a marked decrease was noticed 48 h after LPC insult, when evaluating proteins extracted from tissue through Western blot (0.68-fold, $P<0.01$) **(Figure 3.1 B)** or S100B gene expression (0.38-fold, $P<0.001$) **(Figure 3.1 C)**. Regarding HET values, organotypic cultures showed similar results to WT, however subtle variations were noticed. S100B levels in medium, 18 h after LPC insult, were elevated when compared to respective control (6.70-fold, not statistically significant), although not as much as in WT cultures, probably due to the fact that only one allele was being transcribed and subsequently translated **(Figure 3.1 A)**. Concerning tissue-derived proteins (48 h after LPC insult), no

significant differences between control and LPC-induced demyelination were detected in HET cultures, either for protein (0.62-fold) (Figure 3.1 B) or gene expression (1.0-fold) (Figure 3.1 C). At last, KO cultures did not present S100B values in either technique as expected.

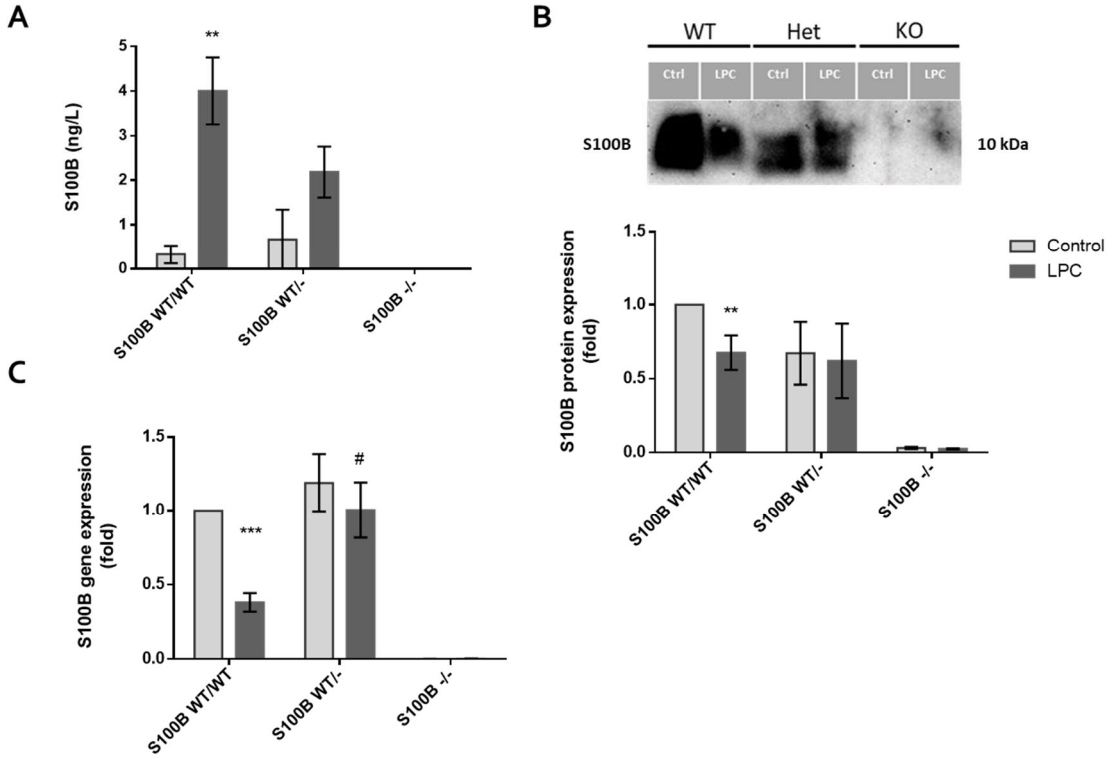

Figure 3.1 - Demyelination induces a substantial release of S100B in cerebellar organotypic slice cultures. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced by fresh medium on cultures for an additional 30 h. (A) Media were collected from cultures, at 18 h post-incubation with LPC, for S100B secretion detection by ELISA. (B) Representative results of S100B expression, from organotypic tissues, at 48 h post-incubation with LPC, analysed by Western blot (top). Graph bars represent respective quantification of S100B (below). Results were normalized to total protein. (C) Relative S100B mRNA levels at 48 h post-incubation with LPC were determined by qRealTime-PCR. The results were normalized to β-actin. Results are mean ± SEM of at least n=3 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (** P<0.01 and *** P<0.001 vs. respective control; # P<0.05 vs. respective condition in WT).

5.2. Demyelination degree is worsened in the presence of S100B

Despite the already described S100B beneficial effects on oligodendrogenesis as well as in the modulation of neurite outgrowth and others[45,149], after brain injury, S100B does exert pejorative roles, affecting the normal function of several brain cells, including OLs[26]. As previously demonstrated, S100B expression is intensified after demyelination,

therefore, we decided to understand how OL lineage cells were reacting upon LPC insult in COSCs, obtained from WT, HET, and KO S100B mice. For these, the transcriptional levels of immature and mature OL markers (NG2 and MBP, respectively) were evaluated by qRealTime-PCR. We observed a significant decline of MBP expression in WT cultures after LPC induction (0.75-fold, $p<0.01$), when comparing to the respective control, suggesting a higher degree of demyelination (Figure 3.2 A). On the other hand, HET and KO cultures maintained similar values to respective controls for both control and LPC-treated slices (~0.71-fold for HET and ~1.2-fold for KO), suggesting a lower level of demyelination in these cultures. In agreement with previous results, we observed an increased expression of NG2 (3.56-fold, not statistically significant) upon demyelination in WT cultures, suggesting that OPC proliferation and recruitment was already occurring 48 h after demyelination induction (Figure 3.2 B). Oppositely, decreased values were observed in HET and KO cultures (1.52-fold and 0.46-fold, respectively), suggestive of a decreased recruitment of OPCs given the lack of demyelinating insult.

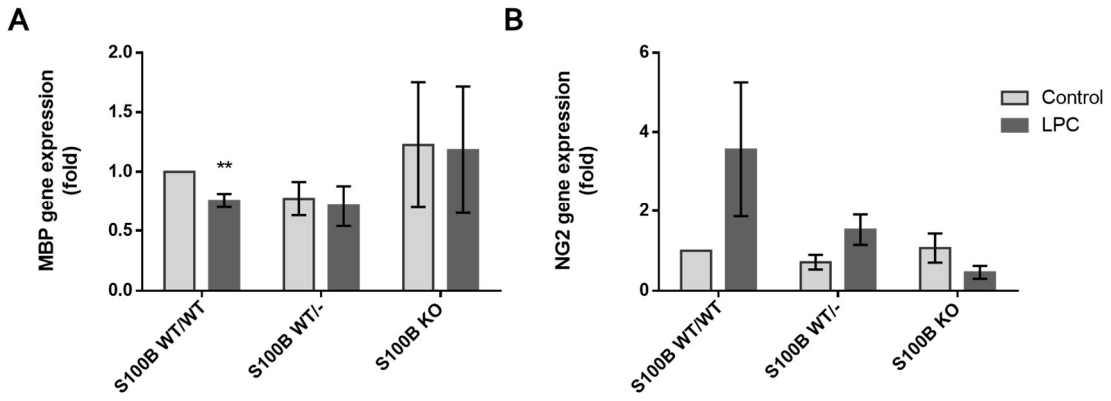

Figure 3.2 - S100B is needed for OL damage and consequent OPC recruitment following demyelination. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced by fresh medium on cultures for additional 30 h. Then, 48 h post-incubation with LPC, samples were processed for analysis of mRNA expression. Relative (A) MBP and (B) NG2 mRNA levels were determined by qRealTime-PCR. Results were normalized to β-actin. Results are mean ± SEM of n=4 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (** $P<0.01$ vs. respective control).

5.3. Pro-inflammatory profile seen in S100B WT cultures following demyelination is prevented in S100B KO cultures

Inflammation is an essential and complex biological process, imperative to overcome different types of cellular damage, as the case of demyelination, being important to improve remyelination ability. Even though, the extent of inflammation can also be detrimental to cell recovery. Astrocytic and microglial activation, along with the intensified release of proinflammatory cytokines have been described to follow LPC-induced demyelination. TNF-α and IL-1β were reported to be released from astrocytes and microglia when S100B is overexpressed[19,26,45]. To analyse the inflammatory profile of our *ex vivo* model, we measured different cytokines, inflammatory mediators, and S100B-related receptors at a transcriptional level, in the three different S100B genotypic mice. The presence of TNF-α and IL-1β, both proinflammatory cytokines, were significantly increased in WT cultures that experienced the demyelinating process when compared to respective controls (2.41-fold, $P<0.05$ and 8.28-fold, $P<0.0001$, respectively) **(Figure 3.3 A-B)**. Alongside, IL-10, an anti-inflammatory cytokine known to suppress TNF-α, IL-1β, and other proinflammatory cytokines production, was also significantly increased compared to respective control (8.16-fold, $P<0.05$), presumably to counterbalance the inflammatory response **(Figure 3.3 C)**. Also HMGB1, which has been associated with active inflammation and found in MS lesions, was found overexpressed in WT after LPC insult (12.65-fold, $P<0.05$) **(Figure 3.3 D)**. RAGE and TLR4, known receptors for S100B and HMGB1, exhibited the same increased pattern in WT condition (14.77-fold and 5.84-fold, respectively), although not significant due to variability **(Figure 3.3 E-F)**. Focusing on both HET and KO conditions, most of the inflammatory mediators were attenuated, with a more pronounced effect in KO cultures. HET cultures showed two different features, both pro- and anti-inflammatory cytokines reached similar values to WT cultures that undergone demyelination (TNF-α 3.25-fold, IL-1β 7.18-fold, and IL-10 15.07-fold) while HMGB1 (7.4-fold), RAGE (6.08-fold, $P<0.05$) and TLR4 (2.54-fold) were reduced comparing with WT cultures. In KO cultures both TNF-α and IL-1β were downregulated when compared to demyelinated WT slices (1.36-fold and

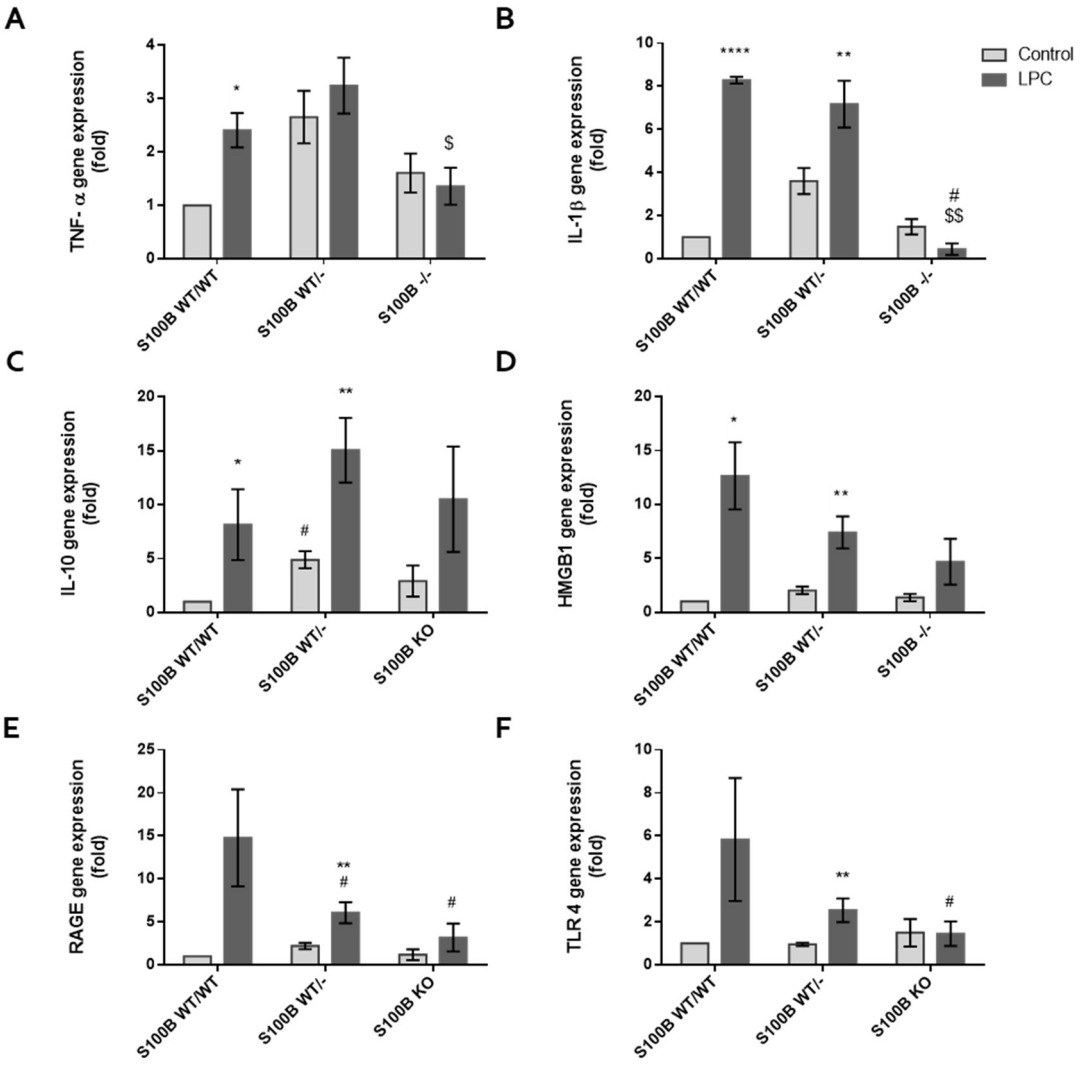

Figure 3.3 – S100B presence worsens the inflammatory profile after LPC insult. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced by fresh medium on cultures for additional 30 h. mRNA samples were collected at 48 h post-incubation with LPC. Relative (A) TNF-α, (B) IL-1β, (C) IL-10, (D) HMGB1, (E) RAGE and (F) TLR4 mRNA levels were determined by qRealTime-PCR. Results were normalized to β-actin. Results are mean ± SEM of at least n=5 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (* P<0.05, ** P<0.01 and **** P<0.0001 vs. control; # P<0.05 vs. respective condition in WT; $ P<0.05 and $$ P<0.01 vs. respective condition in HET).

0.44-fold, P<0.05, respectively), while IL-10 maintained similar levels comparatively to WT cultures (10.5-fold). HMGB1 gene expression also showed a reduced value (4.68-fold), along with RAGE (3.19-fold, P<0.05) and TLR 4 (1.45-fold, P<0.05), when compared to LPC-treated WT cultures. These results denote, again, the strong and pejorative effect of S100B on inflammation, altering cell homeostasis and influencing cell recovery capacity.

Extracellular high concentrations of S100B proteins have been described to result in S100B-RAGE interaction, activating, between others, the pro-inflammatory response by NF-kB[26,150]. After cytokine profile evaluation we went further to analyse this pro-inflammatory pathway activation through Western blot. To assess the relative activation of this pathway, the ratio between phosphorylated NF-kB (pNF-kB) and total NF-kB was performed. Contrarily to expected, we observed that following 48 h of demyelination induction, NF-kB activation was reduced when comparing with each respective control in the three different cultures (Figure 3.4). In WT culture, the decrease was 0.31-fold (P<0.001), in HET culture 0.96-fold and KO culture with 0.74-fold, although not significant for these last cultures. These results may suggest that either we are evaluating NF-kB activation following its window of activation since we already observe

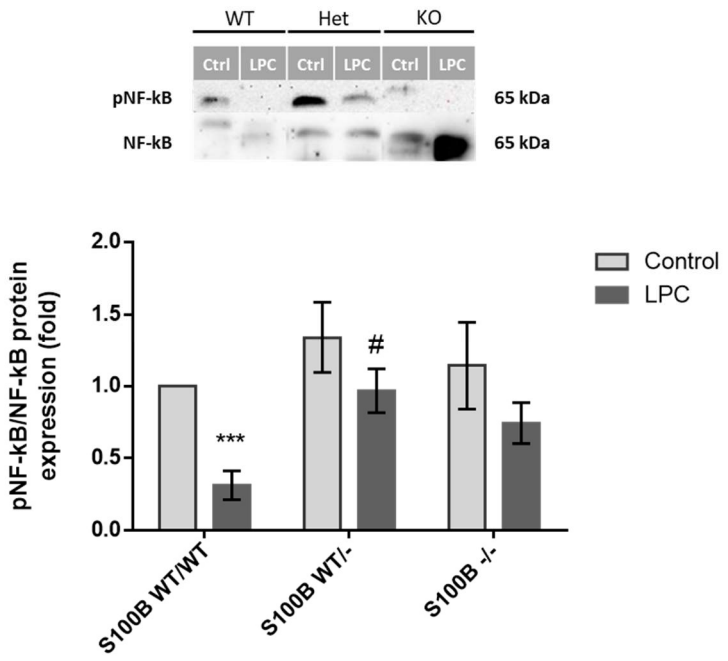

Figure 3.4 – NF-kB proinflammatory pathway seem to be inactive past 48 h of demyelination induction. Cerebellar organotypic slice cultures (COSC) were exposed to lysophosphatidylcholine (LPC) at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced by fresh medium on cultures for additional 30 h. Protein samples were collected at 48 h post-incubation and pNF-kB and total NF-kB protein expression assessed by Western Blot. Representative results of phosphorylated NF-kB (pNF-kB) and NF-kB from organotypic tissues are shown (top). Graph bars represent NF-kB pathway activation through the ratio between pNF-kB and total NF-kB (below). Results were normalized to total protein. Results are mean ± SEM of n=6 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (*** P<0.001 vs. control; # P<0.05 vs. respective condition in WT).

at this time-point high cytokine expression; or that cytokine induction occurs downstream the activation of another intracellular pathway. Overall, these results establish proof of principle that S100B is a crucial player in demyelination pathogenesis.

5.4. Demyelination-induced S100B expression is not affected by the assayed treatments

After confirming S100B neurotoxic effect, we aimed to target this protein using three different nanobodies with micromolar affinity against S100B, alongside with pentamidine and an S100B targeting antibody, two molecules already tested by our group[19,72,89]. For that, COSCs from WT mice were used and different treatments assayed separately. In accordance to previous results, demyelination insult enhanced S100B protein expression and respective release to the extracellular medium 18 h after LPC insult ($P<0.01$) (Figure 3.5 A). Regardless of the treatment used, all cultures suffered S100B increment. Moreover, 48 h after LPC insult, protein values from tissue, as well as S100B gene expression were decreased almost in all treatments (Figure 3.5 B-C), concordant with the results previously described for WT cultures. These results point to a sequestering of S100B at the extracellular level inhibiting its interaction with the respective receptors and consequently its self-induction.

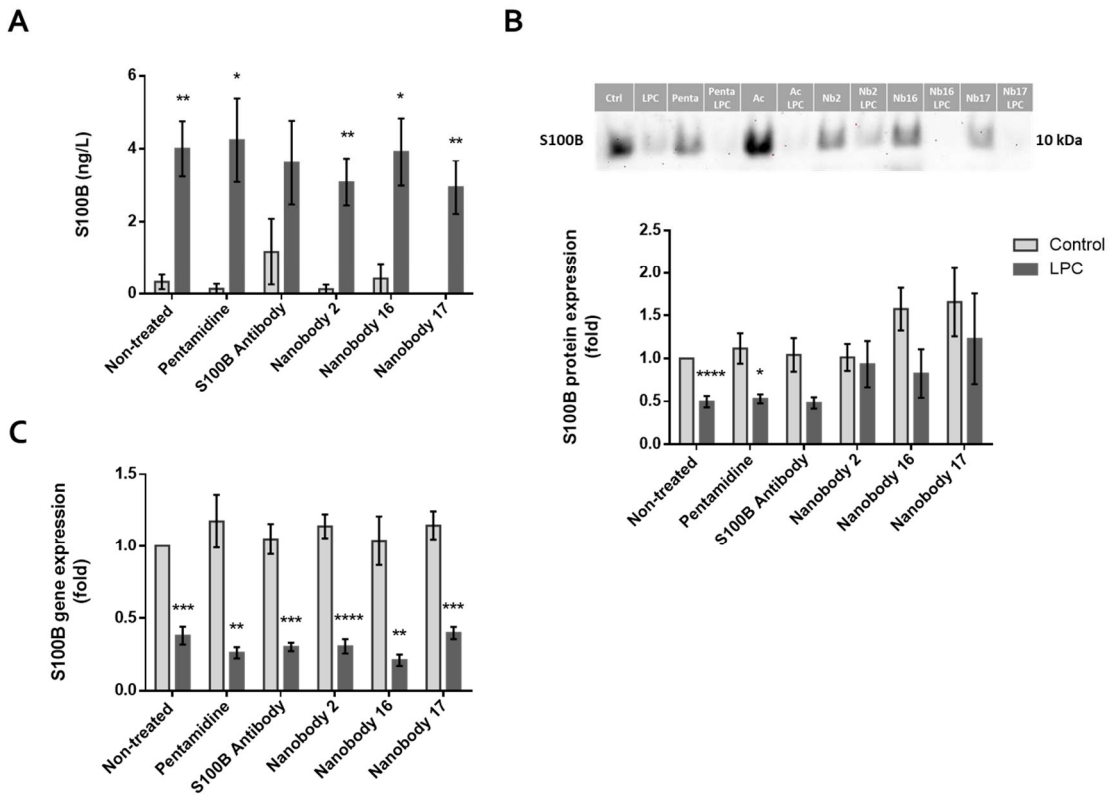

Figure 3.5 - Demyelination induces a substantial release of S100B in cerebellar organotypic slice cultures (COSC) despite of the treatment used. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 2, 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. (**A**) Media were collected from cultures, at 18 h post-incubation with LPC, for S100B secretion detection. (**B**) Representative results of S100B expression, from organotypic tissues, at 48 h post-incubation with LPC, analysed by Western blot (top). Graph bars show the respective quantification of S100B expression (below). Results were normalized to total protein. (**C**) Samples for analysis of mRNA expression were collected at 48 h post-incubation with LPC and S100B gene expression evaluated by qRealTime-PCR. Results were normalized to β-actin. Results are mean ± SEM of at least n=3 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (* P<0.05, ** P<0.01, ***P<0.001 and ****P<0.0001 vs. control).

5.5. Nanobody treatments seem to have a potential beneficial effect on the demyelination degree caused by S100B overexpression

In our *ex vivo* model, demyelination does occur after LPC administration, although, dependent on possible treatment effects, the degree of this insult might be slightly different from non-treated cultures. To evaluate the extent of demyelination, we used two different antibodies through immunohistochemistry, one for mature OLs (MBP) and one for decompacted myelin (QD9), which is observed following demyelination. The representative results shown in **Figure 3.6**, demonstrate that in non-treated slices there

is a marked reduction of MBP staining (red) and increased staining of QD9 (green) upon LPC administration, indicating a high degree of demyelination. Interestingly, although following LPC insult we still see a marked QD9 staining in slices treated with pentamidine and both Nanobody 2 and 17, the MBP positive myelin tracts are maintained, suggesting a preservation of myelin branch structures. Furthermore, Nanobody 16, from all treatments, appears to be the one with less decompacted myelin, also suggesting a lower degree of demyelination. After demyelination induction, we also analysed two different OL lineage markers to evaluate the oligodendrogenesis ability. MBP and NG2 were used for mature OLs and OPC, respectively. After 48 h post-injection with LPC, is possible to observe that MBP values decreased significantly upon demyelination in non-treated

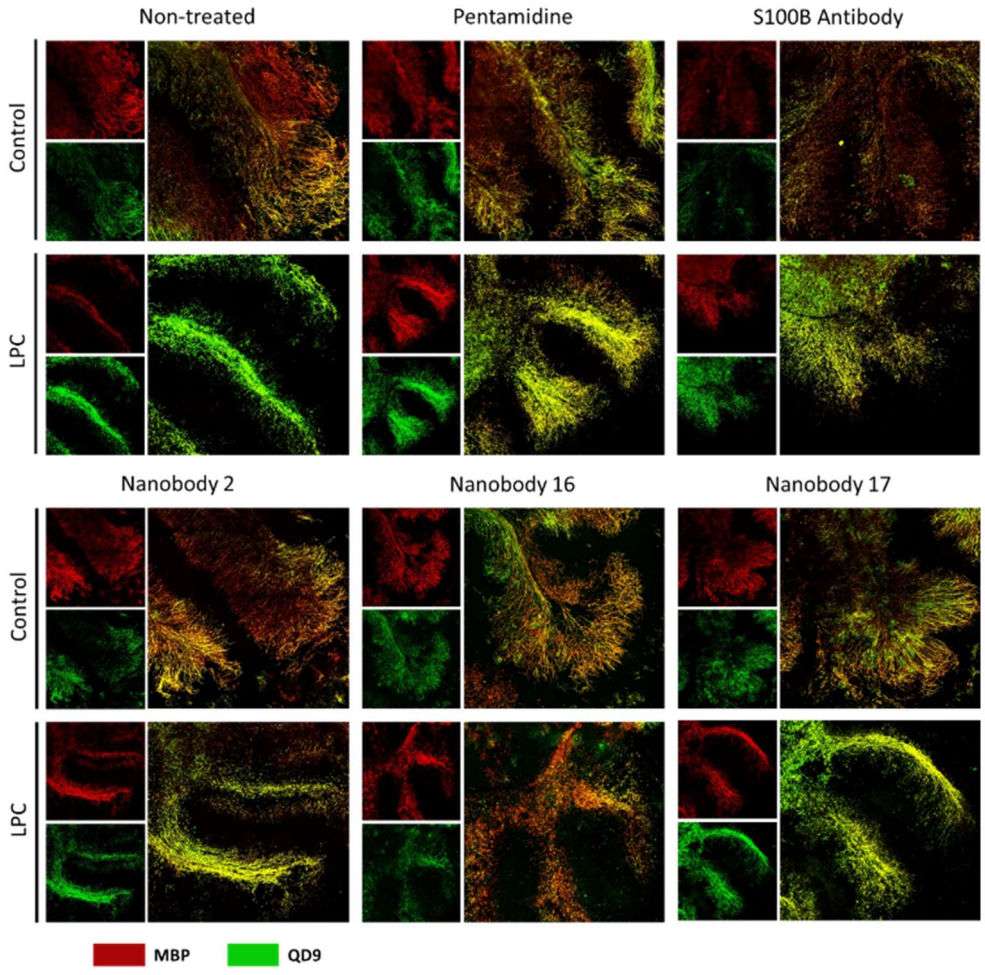

Figure 3.6 – The degree of demyelination in COSCs is apparently lower following nanobody treatment. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 2, 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Double immunostainings were performed in slices fixed at 48 h for mature OLs (anti-MBP antibody, red) and for decompacted myelin (anti-QD9 antibody, green), and representative images are shown.

cultures (0.75-fold, P<0.01), while in each different treatment this accentuated reduction, comparing with respective control, was not verified, except for pentamidine treatment, indicating a higher maintenance of mature OL state even with OL damage (Figure 3.7 A). Considering now NG2 expression, consistent with decreased MBP values in non-treated cultures, these higher values (3.56-fold, not statistically significant) suggest that OPCs were being recruited and proliferating, to help posteriorly on the recovery from the insult (Figure 3.7 B). Curiously these higher NG2 expression was maintained even following pentamidine and S100B antibody, but much reduced in the presence of the nanobodies, namely for Nanobody 2 and 17 (1.71-fold and 0.62-fold, respectively), suggesting a lower necessity of OPC recruitment, and reinforcing the nanobodies efficacy in reducing the S100B neurotoxic effect.

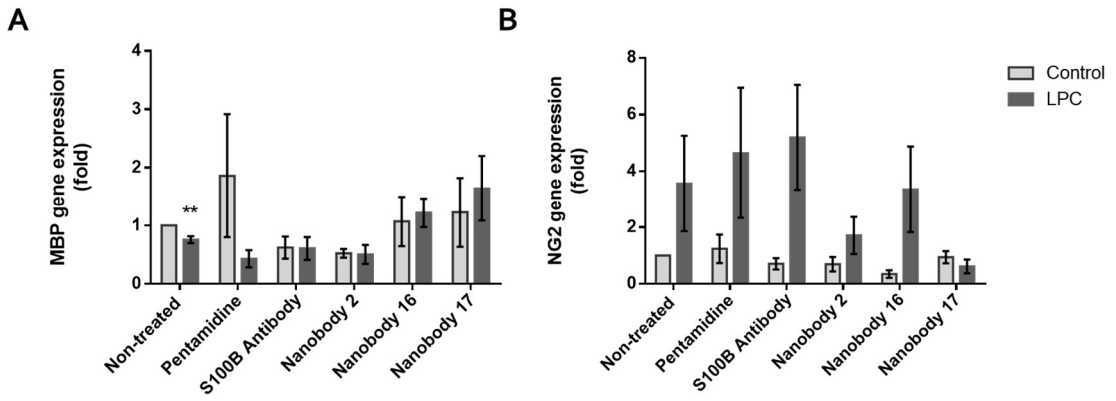

Figure 3.7 – S100B neurotoxic effect change the expression of OL lineage cells-associated genes, which may be partially prevented by Nanobodies treatment. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 2, 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Samples for mRNA expression analysis were collected at 48 h post-incubation with LPC. Relative (A) MBP and (B) NG2 mRNA levels were determined by qRealTime-PCR. Results were normalized to β-actin. Results are mean ± SEM of n=4 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (** P<0.01 vs. control).

5.6. Astrocytic structure loss following demyelination is prevented by nanobodies

CNS injuries are known to be followed by a large set of homeostatic unbalances, affecting different cells. Astrocytes and microglia, CNS resident immune cells, undergo both morphological and functional changes, forming the glial scar. With the purpose to unravel astrocytic changes, an antibody against glial fibrillary acidic protein (GFAP, an astrocytic

specific marker) was used. Typically, higher values of GFAP staining indicate higher astrocytic reactivity, commonly seen in pathological conditions. Nevertheless, LPC-induced demyelination can also cause astrocytic structure loss, as observed in our experiments when compared to the characteristic ramified morphology seen in control, probably due to cellular death **(Figure 3.8)**. Curiously, it seems that, besides S100B antibody and Nanobody 2, all the other treatments were effective in preserving astrocyte morphology, although we may identify an increased GFAP expression in the presence of Pentamidine and Nanobody 17, which may highlight a potential astrogliosis. Further experiments need to be performed to further clarify these astrocytic changes.

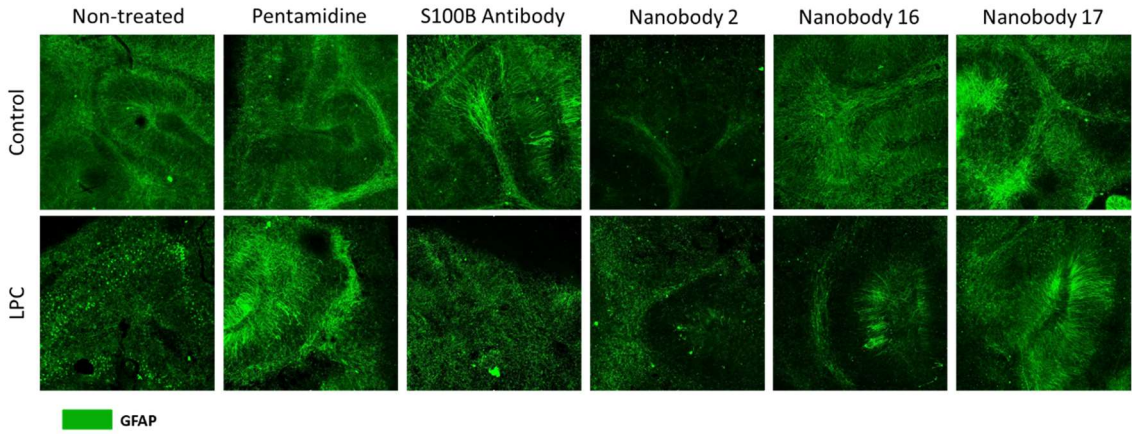

Figure 3.8 - S100B neutralization by Pentamidine and Nanobodies 16 and 17 prevents astrocytic structure loss in the course of demyelination. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 2, 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Immunohistochemistry was performed in slices fixed at 48 h for astrocytes, using an anti-glial fibrillary acidic protein (GFAP, green) antibody and representative images shown from at least n=3 per group.

Moreover, astrocytes are thought to recruit microglia, the CNS macrophages, to the lesion site, playing a pivotal role phagocytosing myelin debris and creating an optimal microenvironment for repair. This process is a fundamental step preceding remyelination, since the presence of myelin debris difficult the normal OPC differentiation[27–30]. In **Figure 3.9**, microglia seems to be homogeneously distributed in control cultures, while in demyelinated cultures it may be observed a subtle migration towards damaged myelin branches or myelin debris. Consistent with this migration, colocalization between microglia and myelin is observable in cultures treated with Pentamidine or S100B antibody after LPC insult, which suggests that microglia may be clearing myelin debris, and potentially increasing remyelination capacity. Regarding

nanobodies treatment we were only able to analyse the effect of Nanobody 16 and 17, due to technical issues, and it seems that microglia staining is reduced following demyelination in the presence of both nanobodies. These results must be further confirmed with new experiments.

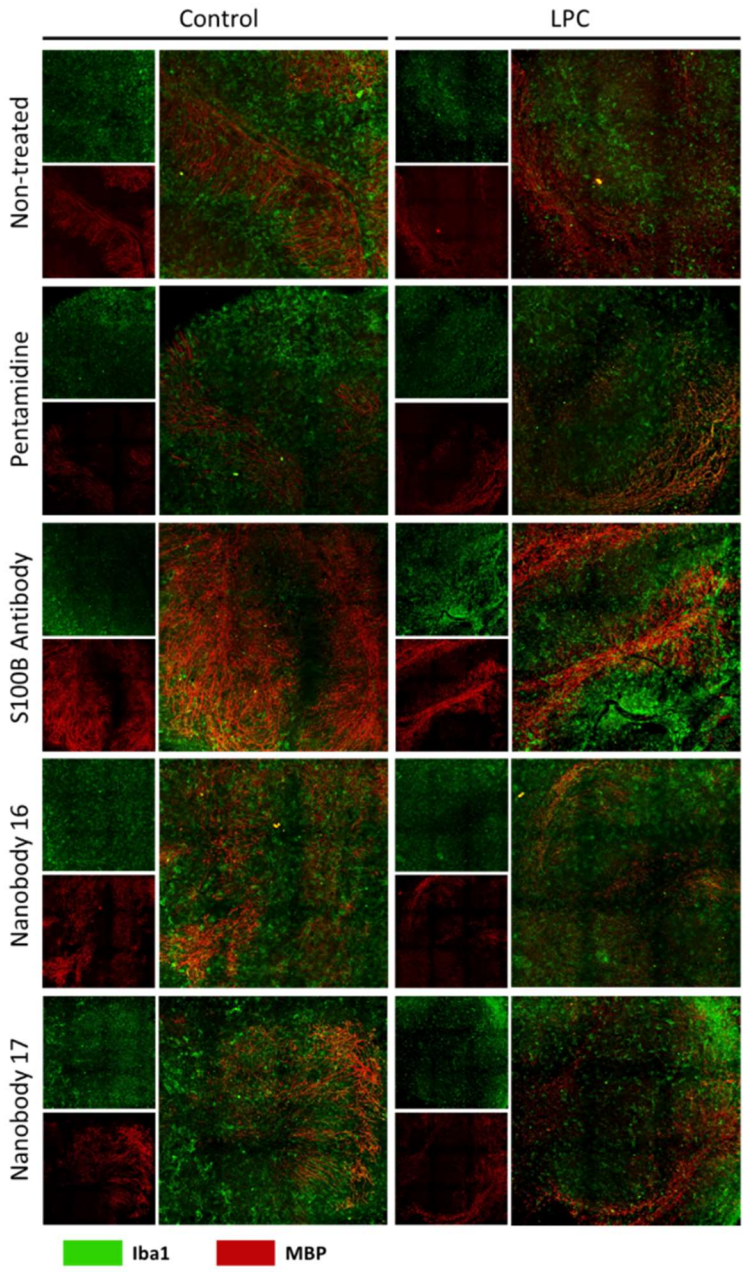

Figure 3.9 – Microglia seems to migrate towards myelin tracts upon demyelination and possibly phagocytose myelin debris. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Double immunostainings were performed in slices fixed at 48 h for microglia using anti-ionized calcium-binding adapter molecule-1 (Iba-1, green) antibody and for mature OLs an anti-myelin basic protein (MBP, red) antibody, and representative images are shown.

5.7. Nanobody 17 seems to revert the pro-inflammatory profile seen following LPC-induced demyelination

In the previous results, we reported that S100B absence in KO cultures did appease the proinflammatory profile found in WT cultures, probably increasing cell recover capacity, maintaining the inflammatory values close to homeostatic conditions to levels similar to those found in cultures that did not suffered demyelination. With this in mind, we further analysed possible fluctuations of gene expression from several inflammatory-related molecules when incubated with the different S100B neutralizing treatments (Figure 3.10). As previously shown, demyelination elicits gene expression of TNF-α (2.41-fold, $P<0.05$), IL-1β (8.28-fold, $P<0.0001$), alongside with IL-10 (8.16-fold, $P<0.05$) and HMGB1 (12.65-fold, $P<0.05$). Also, the S100B receptors are highly expressed as indicated by the increased levels of RAGE (18.53-fold, $P<0.05$) and TLR4 (4.52-fold) upon demyelination. In a global perspective, the different treatments showed interesting results, promoting a downregulation of both proinflammatory cytokines, TNF-α and IL-1β. As Pentamidine (TNF-α 0.6-fold, $P<0.01$ and IL-1β 3.06-fold, $P<0.05$) and S100B Antibody (TNF-α 0.49-fold, $P<0.01$ and IL-1β 4.78-fold, not statistically significant), the three nanobodies provoked a reduction in the expression of TNF-α (Nb2 1.31-fold; Nb16 1.12-fold, $P<0.05$; and Nb17 1.7-fold) and IL-1β (Nb2 5.33-fold; Nb16 3.29-fold, $P<0.05$; and Nb17 1.57-fold, $P<0.01$), reaching similar or even lower values than the former S100B inhibitors. Regarding IL-10, all treatments appeared to maintain elevated values, except for Nanobody 17 (2.81-fold), perhaps indicating a lower level of inflammation to counteract. Concerning HMGB1 expression, Nanobody 17 was the most effective in lowering its expression when compared to LPC-induction in the absence of any treatment (1.7-fold, $P<0.05$). RAGE receptor expression markedly decreased in all treatments, with a higher efficacy for Pentamidine (4.76-fold, $P<0.05$), Nanobody 2 (3.2-fold, $P<0.05$), Nanobody 16 (2.6-fold, $P<0.05$) and Nanobody 17 (0.87-fold, $P<0.01$) suggesting that S100B signal transduction may be abrogated. Concerning TLR4, the high variability suggests a reduced involvement of this receptor in the demyelination pathogenesis.

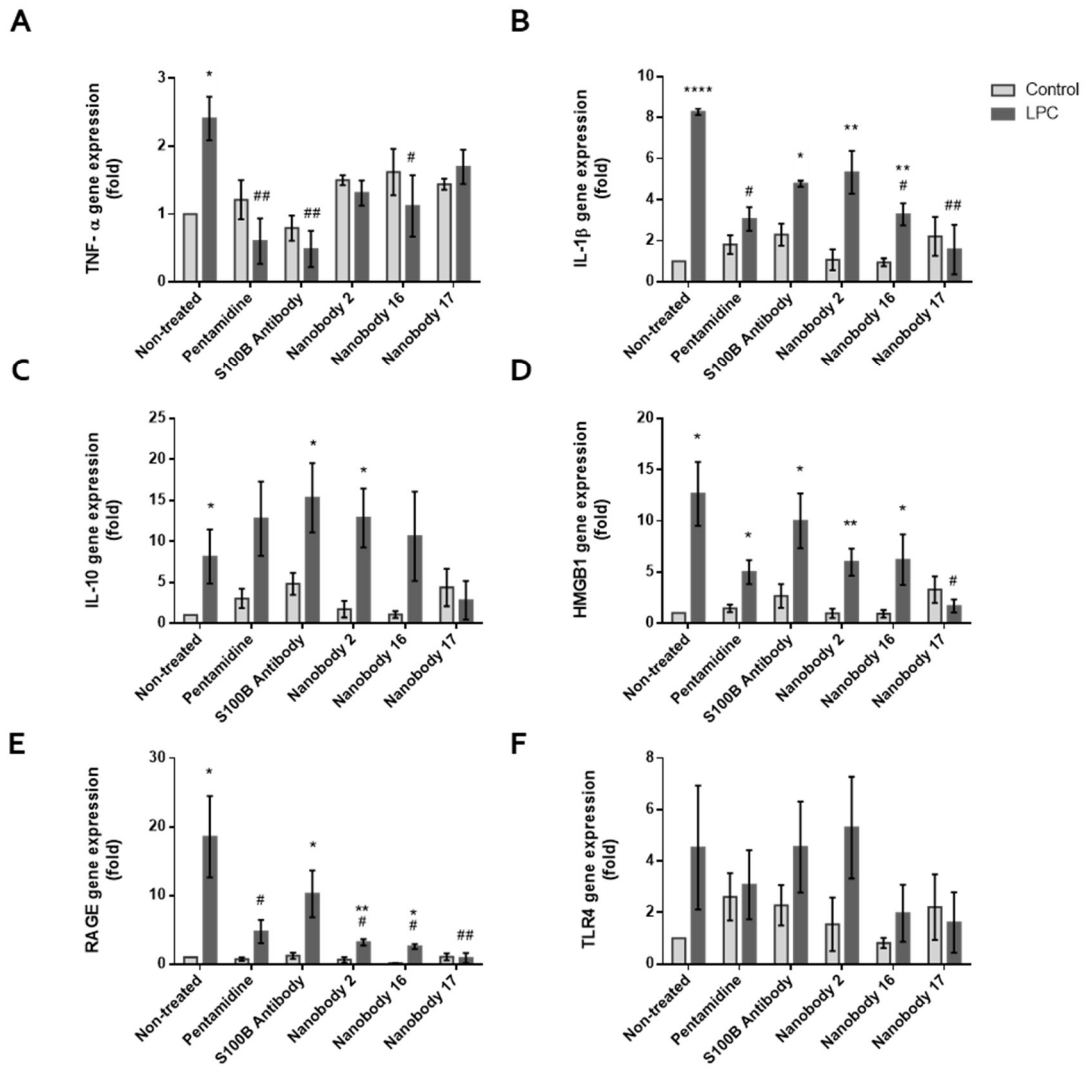

Figure 3.10 - More pro-inflammatory profile is reverted upon treatments in LPC-induced demyelination cultures. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Samples for mRNA expression analysis were collected at 48 h post-incubation with LPC. Relative (A) TNF-α, (B) IL-1β, (C) IL-10, (D) HMGB1, (E) RAGE and (F) TLR4 mRNA levels were determined by qRealTime-PCR. Results were normalized to β-actin. Results are mean ± SEM of n=5 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (* P<0.05, ** P<0.01 and **** P<0.0001 vs. control; # P<0.05, ## P<0.01 vs. non-treated cultures incubated with LPC).

Once again NF-kB pro-inflammatory pathway was evaluated through Western blot, to ascertain its involvement in the induction of cytokine expression under this demyelinating insult. As previously examined in **Figure 3.4**, we observed a reduced NF-kB activation upon LPC-demyelination (0.31-fold, P<0.001), and no changes from control values were observed for each treatment assayed **(Figure 3.11)**. These results suggest once more that further studies are needed to clarify whether NF-kB activation is occurring at other time-point or not involved in cytokine induction following LPC injury.

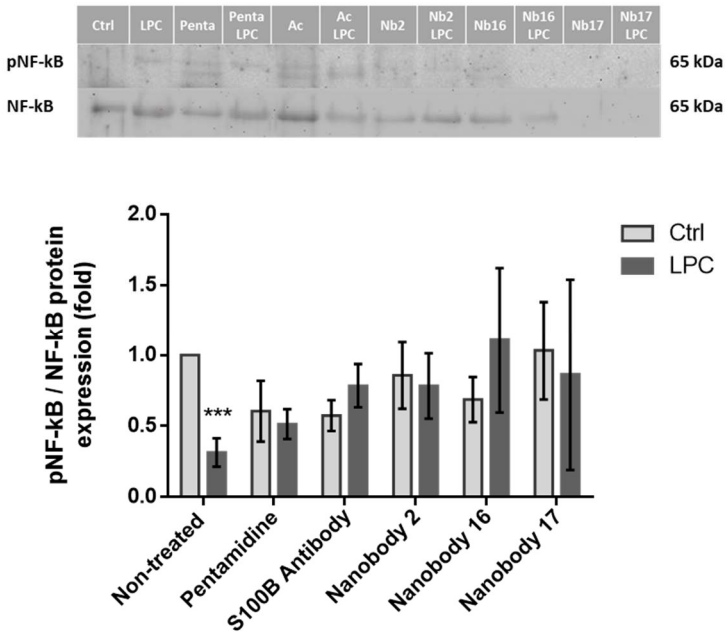

Figure 3.11 – Different treatments seem to abrogate NF-kB proinflammatory pathway inhibition past 48 h of demyelination induction. COSC were exposed to LPC, in the presence of each treatment separately (Pentamidine, S100B Antibody, Nanobody 16 and 17), at 7 days in vitro (DIV) for 18 h. After this period, fresh medium (LPC-depleted) was replaced on cultures and treatments maintained for additional 30 h of recovery. Protein samples were collected at 48 h post-incubation and pNF-kB and total NF-kB protein expression assessed by Western Blot. Representative results of phosphorylated NF-kB (pNF-kB) and NF-kB from organotypic tissues are shown (top). Graph bars represent NF-kB pathway activation through the ratio between pNF-kB and total NF-kB (below). Results were normalized to total protein. Results are mean ± SEM of n=3 per group. One-way ANOVA with Tukey post-test or t test was used to determine the statistical significance as appropriate (*** P<0.001 vs. control; # P<0.05 vs. respective condition in WT).

Overall, these results suggest a beneficial role of S100B-targeting Nanobodies in the prevention of demyelination, glial reactivity, and inflammatory response upon LPC injury, highlighting the need of further studies to better clarify which Nanobody has a higher efficacy.

6. Discussion

MS is a chronic autoimmune disease of the CNS, characterized by neuroinflammation, reactive gliosis, demyelination, axonal loss, and neuronal degeneration[1,2]. As consequence, MS patients face an increase in both motor and cognitive decline over time, depending on how active this disease can be[151,152]. Always with this in mind, several approaches have been made to reduce disease severity, delaying as much as possible the disease progression. S100B has been proving to be one promising therapeutic target since it has been repeatedly described to be deeply involved in MS pathogenesis[19,45,153]. As a starting point, our group showed that S100B was found upregulated in both CSF and serum from MS patients at the time of diagnosis of RRMS, as well as in the active MS lesions from *post-mortem* brain tissues[19]. Besides, this past study also revealed that, in the same *ex vivo* model of demyelination used in the present dissertation, S100B higher expression did worsen the degree of demyelination, provoked higher astrocytic reactivity along with a more pro-inflammatory environment. Furthermore, our group used three different molecules to neutralize S100B neurotoxic effects: (1) an S100B-specific antibody (*ex vivo*)[19]; (2) FPS-ZM1, an antagonist for S100B receptor RAGE (*ex vivo*)[26]; and at last (3) S100B-binding drug Pentamidine (both *in vivo* and *ex vivo*)[72,88,89]. Although all molecules demonstrated promising results diminishing S100B pejorative effects, the possibility to use Nanobody-mediated therapy against S100B for the first time piqued our curiosity.

Initially, we began to evaluate high S100B effects upon demyelination using an *ex vivo* model, commonly used in our laboratory. To accomplish this objective, we used COSCs which provide a complex multicellular environment from different mice groups: homozygotic (WT), heterozygotic (HET), and knockout (KO) S100B to further understand S100B effects through its partial or complete absence (HET and KO cultures, respectively). Within this goal, we first analysed S100B expression and secretion, upon demyelination,

as a step to validate our disease model. Our results in WT mice proved that S100B expression and secretion were significantly amplified 18 h after demyelination induction, although, 30 h after the previously measured time point, S100B transcriptional and translational levels markedly decreased to values below control. These last two results might appear contradictory to those obtained formerly, even though, two different circumstances must be taken into consideration. The time point used was 30 h after LPC incubation stopped, in which inhibitory processes might already be taking place, possibly as a counteracting response to the already increased values of S100B seen before, and over time, a reduction in the protein level. Another important aspect is the fact that S100B protein analysis was done from the slice itself, where an exacerbated secretion is known to be taking place, perhaps leaving the intracellular compartment with lower levels of S100B. Regarding HET cultures, as expected, the levels of S100B expression/secretion were also higher than respective control 18 h after LPC induction, although, reached only half of the concentration seen in WT cultures, presumably since only on allele was being transcribed and subsequently translated. Interestingly, 30 h after LPC incubation stopped, both gene and protein expression values were similar to respective controls, leading us to presume that HET cultures were under a lower level of inhibition than WT cultures, probably because these values were not so increased as WT ones. At last, KO cultures did not present values in either technique as expected.

Concerning demyelination, it is known that higher values of S100B promote microglial and astrocyte reactivity, leading to the release of nitric oxide and TNF-α, being even more detrimental for OLs[45,154]. We further evaluated how OL lineage cells were reacting upon LPC insult in COSCs. Predictably, WT cultures revealed lower values of MBP, accompanied by an increase in NG2 expression. This result suggests that demyelination indeed occurred and is now followed by OPC recruitment, counteracting mature OL damage, or even respective cell death. The partial or complete absence of S100B in cultures (HET and KO cultures, respectively) resulted in stable values of MBP as well as NG2, suggesting that these cultures were not so affected as in the presence of increased values of S100B in WT cultures. Nevertheless, is important to mention that S100B's complete absence was described to impair the correct differentiation of OPCs into myelinating OLs, since S100B

expression at physiological levels is required for a proper differentiation into mature OLs[50].

The third component analysed was the inflammatory panel, since MS pathophysiology is known to detain a complex engagement of inflammatory mediators. As previously mentioned, astrocytes and microglia activation, during demyelination periods, lead to the production of pro-inflammatory cytokines, as also does higher concentrations of S100B[2,41,45,71]. Curiously, S100B expression is regulated by TNF-α, IL-1β among other mediators[155], and is known to transduce its pro-inflammatory signaling, as well as HMGB1, via both RAGE and TLR4 receptors (when overexpressed), leading to the activation of NF-kB pro-inflammatory pathway[150]. In turn, this pathway promotes the release of more pro-inflammatory cytokines such as TNF-α and IL-1β[150], generating an endlessly inflammatory loop. Consonant with previous results, a clear pro-inflammatory picture is present in WT cultures that undergone demyelination. The expression of first-line cytokines was markedly increased upon demyelination in WT cultures as is the case of TNF-α, IL-1β, and HMGB1, already described to follow this pattern in similar circumstances[19,45,53,154,156]. Interestingly, both RAGE and TLR4 receptors were also found overexpressed in demyelinated cultures. The former receptor has been described to be upregulated by extracellular S100B at higher concentrations[157], highlighting again the inflammatory loop. Moreover, IL-10, an anti-inflammatory cytokine, was also found overexpressed, probably as a counteracting response to the ongoing inflammatory process. As mentioned previously, the NF-kB pathway is downstream to S100B-RAGE signaling. Although a higher degree of activation was expected, we observed the opposite. One possible explanation might be the non-involvement of this pathway in this specific inflammatory panel, whereas others may be taking place[71]. Another possible explanation might be directly related to the time point used for this analysis. Since we measured 30 h after the LPC insult ended, this pro-inflammatory pathway might already be subjected to an inhibitory process, even with a possible activation in a previous period to the reported. To strengthen this hypothesis, and as mentioned previously in this section, IL-10 can inhibit several inflammatory mediators, including NF-kB pathway[158]. Regarding HET cultures, both pro- and anti-inflammatory cytokines maintained the increased values as seen in WT cultures, while HMGB1 and both receptors had their

values decreased, yet with a significant increment to respective controls. Interestingly, in HET cultures seems to be present a compensatory effect, relative to the cytokines analysed, since respective upregulation was probably due to other inflammatory mediators, as HMGB1. Given the fact that S100B and respective receptors were slightly downregulated, comparatively to WT cultures that undergone the demyelinating insult, HMGB1 could be continuously contributing to both RAGE and TLR4 signaling. At last, the complete absence of S100B in KO cultures resulted in the reduction of almost all inflammatory mediators, except IL-10. In line with this, the NF-kB activation showed once more to be decreased, probably due to both hypotheses mentioned above. These results denote, again, the strong and pejorative effect of S100B on inflammation, influencing cell recovery capacity.

Beyond the validation of our *ex vivo* model, we went further to tackle this neurotoxic protein using three different nanobodies, all of them with micromolar affinity against S100B. Given the fact that our group already used different molecules to neutralize S100B, and all demonstrated to reduce S100B toxic effects efficiently, we used both pentamidine and S100B-directed antibody, along with the three nanobodies, giving the chance to compare results between different treatments. Following a similar path as the one used to validate our model, we began through the analysis of S100B expression upon treatments. After 18 h of LPC incubation, the secretion of S100B to the extracellular medium remained at higher values in all treatments, as observed in non-treated cultures. Considering that all treatments exert neutralizing roles directly to extracellular S100B protein, it was expected to not affect its levels, at least initially. Once more, S100B transcriptional and translational values decreased 48 h after LPC induction, as seen in non-treated cultures, recalling the already suggested possibilities. The time point used was 30 h after LPC incubation stopped, in which, inhibitory processes might already be taking place, possibly as a counteract response to the already increased values of S100B seen before. Another important aspect is the fact that S100B protein analysis was done from the slice itself, where an exacerbated secretion is known to be taking place, perhaps leaving the intracellular compartment with lower levels of S100B. Altogether, these three different results suggest that none of the treatments had a significant effect preventing S100B production, nevertheless, the possibility of a posterior inhibition of S100B

production, through the sequestering of extracellular S100B by each treatment must not be discarded, since was already proved to occur by Santos et al., 2018[26].

Switching to demyelination analysis, the representative images seem to reveal interesting results. Decompacted myelin, stained with QD9 antibody, is increased in non-treated slices that were subjected to LPC insult, although appears to be reduced upon different treatments. Besides pentamidine and both Nanobody 2 and 17 presented a marked QD9 staining after demyelination, the MBP positive tracts were maintained, suggesting a higher preservation of myelin branch structures. Furthermore, Nanobody 16, from all treatments, appears to be the one with less decompacted myelin, suggesting an even lower degree of demyelination. To strengthen our previous results, we evaluated how OL lineage cells were reacting upon LPC insult. In non-treated slices, 48 h after LPC induction, MBP values decreased while NG2 increased, which corroborate the fact that not only demyelination occurred, but also OPC recruitment was ongoing. This accentuated MBP reduction was not verified upon treatments incubation, indicating higher maintenance of mature OL state even with OL damage. Curiously, higher NG2 values were maintained even following pentamidine and S100B antibody, although much reduced in the presence of nanobodies, namely for Nanobody 2 and 17, suggesting a lower necessity of OPC recruitment, and reinforcing the nanobodies efficacy to reduce S100B neurotoxic effect. Altogether, the three nanobodies seemed to reduce S100B neurotoxic effect on demyelination, through its blockage at this time point, suggesting that S100B must be involved in the exacerbation of demyelination upon LPC insult. This way, using nanobodies to neutralize extracellularly S100B toxic effects, seems to be the best therapeutic approach since a complete deletion of S100B may be detrimental to the correct OPC differentiation into myelinating OLs[26], as already mentioned in this discussion. Finally, is imperative to point out that extending the period of culture would allow us to discern which treatment would have better results, regarding remyelination potential as well as the sequelae extent.

Moving forward, we aim to explore both astrocytic and microglial activation upon demyelination, in the presence of different treatments, since reactive gliosis is a common feature of MS pathology[151]. S100B is known to exert extracellular roles through RAGE engagement in both astrocytes as well in microglia[70,71,153]. Regarding astrocytic reactivity,

this protein can cause reactive gliosis through an autocrine loop in a RAGE-dependent manner[70], despite the fact that S100B is mainly produced and secreted by astrocytes. Due to higher expression of S100B in cultures, has already been described to provoke an increase in astrocytic activation, seen by a reduction of cell extension and inflated cell body[19]. Not only astrocytic activation was reported, astrocyte apoptotic cell death has also already been described[159]. With this in mind, our results from non-treated cultures upon LPC induction revealed what appears to be a generalized cell death across the entire culture, in which the clear astrocytic morphology seen in respective control is completely lost. Our treatments showed different outcomes, depending on the treatment used. Curiously, it seems that, besides S100B antibody and Nanobody 2, all the other treatments were effective in preserving astrocyte morphology. We may identify an increased GFAP expression in the presence of Pentamidine and Nanobody 17, suggesting a potential astrogliosis, although, the reduction of cell extension and inflated cell body, characteristics of reactive astrocytes, were not observed. Further experiments need to be performed to further clarify these astrocytic changes.

Turning now our focus to microglia, this CNS resident macrophage is known to be recruited to the lesion by astrocytes. Besides the intrinsic role of cytokine production, microglia play a pivotal role in the clearance of myelin debris left in the lesion region, through phagocytosis, creating an optimal microenvironment for remyelination to take place[27–30,160]. Our results appear to suggest not only that microglia migrated towards OL branches/myelin debris as well as these cells colocalize with MBP, in Pentamidine and S100B antibody treatment upon LPC insult. This might be suggestive that phagocytosis was occurring, potentially increasing remyelination capacity. Curiously, both Nanobody 16 and 17 seemed to reduce microglia staining following LPC damage, highlighting the need for additional studies to specifically address microglia reactivity under these circumstances.

It is widely known that microglia activation promotes the establishment of a pro-inflammatory environment, via cytokine secretion[27,45,71]. To evaluate the possible changes in the inflammatory panel by each treatment, we analysed different cytokines, known to be involved in MS pathology. Remembering KO culture values for the different inflammatory mediators we saw an appease of the pro-inflammatory profile. With the

same purpose, we assessed the same inflammatory mediators hoping to achieve similar results. Overall, the different treatments showed interesting results, preventing the higher values observed for non-treated cultures. S100B neutralization by nanobodies seems to effectively abrogate S100B-related inflammation, seen through lower levels of TNF-α and IL-1β. Nanobody 17 stands out on their IL-10 value since was the only one in which this anti-inflammatory cytokine reached control values, suggesting a decreased state of inflammation. Despite the relative decrease of HMGB1 in all treatments, Nanobody 17 showed again its high effectiveness, since HMGB1 was also closer to control values, suggesting that respective expression may be dependent on RAGE engagement by S100B[26]. RAGE receptor expression was markedly decreased in all treatments, suggesting that S100B signal transduction was being blocked and therefore, downregulating RAGE expression. Nevertheless, the high variability for TLR4 expression seems not to be a crucial receptor in these injury process. Finally, when we evaluated NF-kB pathway activation, we noticed that all treatments did not show significant differences to respective controls, while in non-treated cultures an accentuated decrease was noticed. These results highlight the need for further analysis of NF-kB activation at different time-points, to further clarify its involvement in S100B-associated exacerbation of inflammation upon demyelination. Altogether these results corroborate the pejorative role of S100B-RAGE axis, creating an inflammatory environment in our experimental model, which can be definitely attenuated by nanobodies.

7. Conclusion

Past results showed that the abnormal S100B expression contributes to MS pathology, since, through its blockage using different S100B targeted treatments, it was possible not only to decrease the disease clinical score in EAE, as well as to promote better outcomes regarding demyelination degree, reactive gliosis and inflammation using *ex vivo* models. Taken together, these results show that S100B can be considered not only a potential new biomarker for MS as well as a promising therapeutic target. The current work aimed to unravel more evidence of S100B-associated pathogenesis in MS, using an *ex vivo* model of demyelination, as well as the possibility to tackle this protein using nanobodies, as a potential therapeutic strategy to reduce CNS damage while improving recovery.

First, we evaluated S100B neurotoxic effects using COSCs from three different mice. The partial or complete absence of S100B within cultures, using heterozygotic and knockout mice as source, demonstrated a decrease in demyelination as well as in inflammation, when compared to wild type counterparts, evidencing the neurotoxic effect of S100B when overexpressed. We then tried to neutralize S100B, using three different nanobodies, leading to interesting results. In the presence of these nanobodies, we observed a reduced degree of demyelination as well as a diminished inflammatory environment, as seen in the first set of experiments through genetic depletion of S100B, indicating an effective abrogation of S100B detrimental effects by nanobodies. Overall, our results emphasize the already described pejorative roles of S100B on MS pathophysiology, highlighting that S100B neutralization by the use of nanobodies, might be a new promising therapeutic possibility regarding demyelinating and inflammatory diseases as is the case of MS.

8. Future perspectives

As in any study, there is always space to discover more about a certain subject. To follow up this study, several other analyses would be crucial to step closer to the understanding of the complex role of S100B in MS. Only this way would allow us to design new and more specific therapeutics to tackle S100B pejorative effects. To continue, would be interesting to evaluate the different S100B species, as a way to better understand the relationship between their oligomerization and subsequently RAGE engagement affinity, and with this in mind, select the best nanobody to be used. Moreover, extended periods of culture with more time points to be evaluated would be necessary to disclosure certain unanswered questions, such as the remyelination potential of each treatment as well as respective modulation of reactive gliosis. Then, based on the *ex vivo* results, we could move into *in vivo* studies that will be decisive to evaluate the effect of the most promising Nanobody on EAE-associated disease clinical scores, in addition to a detailed neuroinflammatory analysis and may even allow a neurological assessment such as electrophysiology, cognitive and behavioural tests.

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