Acceleration of microglial clearance function by small TREM2-activating anionic disaccharides

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II. Abbreviations

A	Aβ	β amyloid
	ab	antibody
	AD	Alzheimer's disease
	ALS	amyotrophic lateral sclerosis
	ATP	adenosine triphosphate
B	BBB	blood brain barrier
	BDNF	brain-derived neurotrophic factor
	BSA	bovine serum albumin
	$CaCl_2$	calcium chloride
C	CCR7	chemokine receptor 7
	CCR2	chemokine receptor 2
	CD	cluster of differentiation
	cDNA	complementary DNA
	CNS	central nervous system
	ChABC	chondroitinase ABC
	CHO	chinese Hamster Ovarian cells
	CS	chondroitin sulfate
	CS-A	chondroitin sulfate A (chondroitin-4-sulfate)
	CS-B	chondroitin sulfate B (dermatan sulfate)
	CS- C	chondroitin sulfate C (chondroitin-6-sulfate)
	CS-D	chondroitin sulfate D (chondroitin-2,6-sulfate)
	CS-E	chondroitin sulfate E (chondroitin-4,6-sulfate)
	CSPGs	chondroitin sulfate proteoglycans
	COX-2	cyclooxygenase 2
	CX3CL1	fractalkine
D	DAP12	DNAX activation protein of 12 kDa
	DMEM	Dulbecco's modified Eagle medium
	DMSO	dimethylsulfoxide
	DNA	deoxyribonucleic acid
	dNTP	deoxynucleotide
	DS	disaccharides
	DS-4S	disaccharides sulfate 4
	DS-0S	disacharides non sulfate
	DTT	dithiothreitol
E	EAE	experimental autoimmune encephalomyelitis
	ECM	extracellular matrix
	EDTA	ethylenediaminetetraacetic acid
	ERK	extracellular-signal-regulated kinases
	ESdM	embryonic stem cell derived in precursor microglial
F	FCS	fetal calf serum
	FACS	fluorescence activated cell sorting
	Fig	Figure
	FITC	fluorescein isothiocyanate

GAGs	glycosaminoglycans
GalNAc	*N*-acetylgalactosamine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA	hyaluronic acid
HS	heparin sulfate
HSP60	heat shock protein 60
KS	keratin sulfate
Iba1	ionized calcium binding adaptor molecule 1
IFN-gamma	interferon gamma
IgG	Immunoglobulin
IL-1beta	interleukin-1beta
iNOS	inducible nitric oxide synthase
iPS	induced pluripotent stem cells
ITAM	immunoreceptor tyrosine-based activation motif
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
$MgCl_2$	magnesium chloride
MMPs	metalloproteases
$MnCl_2$	manganese Chloride
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NF-kB	nuclear factor kappa B
NGF	nerve growth factor
NHD	Nasu-Hakola disease
NOS2	nitric oxide synthase 2
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerytin
PLA2	phospholipase A2
PNS	peripheral nervous system
PRRs	recognition receptors
RNS	reactive nitrogen species
RNA	ribonucleic acid
rhIFN-gamma	recombinant human IFN-gamma
ROS	reactive oxygen species
shRNA	small hairpin RNA
STAT3	signal transducer and activator of transcription 3
TLR	toll-like receptor
TGFβ	transforming growth factor β
TNFα	tumour necrosis factor α
TREM2	triggering receptor expressed on myeloid cells 2
U87	glioblastoma cells

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Summary

Triggering receptor expressed on myeloid cells-2 (TREM2) is an innate immune receptor, which is expressed on myeloid cells, like microglia inside the central nervous system. TREM2 signals via the adaptor protein DAP12. Previous studies have suggested that TREM2 plays a protective role in neuroinflammatory diseases. Activation of TREM2 increases phagocytosis and decreases pro-inflammatory microglial responses *in vitro*. However, the ligand which specifically binds to TREM2 is still unknown. Main objectives of this study were to find out structural elements recognized by TREM2 and to elucidate the effects of TREM2 ligand binding in microglia.

To identify possible ligands for TREM2; human glioblastoma cells (U87), mouse glioma cells (GL261), small-cell lung carcinoma (SMA-560) and Chinese hamster ovary cells (CHO) were incubated with a TREM2b-Fc-fusion protein. Binding of TREM2 to target cells was blocked with different polysaccharides. Binding strength under different conditions was analysed by flow cytometry.

First, TREM2b-Fc-fusion protein bound CHO, GL261, SMA-MS and U87 cells. Furthermore, these results show that the binding of the TREM2b-Fc fusion protein to U87 cells can be blocked at different efficiencies by the following glycosaminoglycans: dextran sulfate, chondroitin sulfatecontaining all subtypes of chondrotin suflates, chondroitin sulfate A (CS-A, chondroitin-4-sulfate), and adequan (polysulfated glycosaminoglycan), but not by chondroitin sulfate C (chondroitin-6-sulfate). As a control, U87 cells were pretreated with chondroitinase ABC. In line with the former results the binding of the TREM2b-Fc-fusion protein to the cells was reduced after removal of chondroitin sulfates from the glycocalyx of the target cells. These results confirm chondrotin suflates as ligands for TREM2 receptor. In contrast, pretreatment of U87 cells with sialidase (removal of sialic acids) did not change the binding of TREM2b-Fc-fusion protein. CS-A as an endogenous brain-derived molecule showed efficient binding and was selected as promising stimulating ligand for TREM2 in further experiments.

Next, expression of TREM2 in a mouse microglia cell line was confirmed and the effects of CS-A and the core disaccharide unit of CS-A were analyzed. Treatment of TREM2 receptor expressing microglia with CS-A and the CS disaccharide stimulated phagocytosis of beads. On the other hand, the lentiviral knockdown of TREM2 in mouse microglia decreased the phagocytosis of beads after stimulation with 2 mM CS-A or 0.2 mM chondroitin sulfate

disaccharides DS-4S. Stimulation of microglial cells with CS-A led to an up-regulation of pro-inflammatory cytokines (IL-1ß and TNF-α) and a down-regulation of iNOS. While stimulation of microglial cells with DS-4S led to a down-regulation of pro-inflammatory cytokines (IL-1ß, TNF-α and iNOS).

In summary, these results suggest a new ligand for TREM2. Upon TREM2 stimulation by CS A or DS-4S, microglial cells are phagocytically activated. Taken together, these results suggest a protective role for TREM2 in the central nervous system.

1. Introduction

1.1 Microglia

Microglia, the resident immune cell of the CNS, were first described in 1919 by Pío del Río Hortega (del Rio Hortega, 1919). As suggested by their name, microglia are very small compared to other glia in the CNS and have a heterochromatic nucleus (Fig. 1.1). Microglia have crucial importance for development, defense and regulation of CNS. During the development of the CNS, microglia are important in axonal pruning as well as in the clearance of debris and apoptotic cells (Zacharchuk et al., 1990; Ewers et al., 1996; Mallat et al., 2005). Microglia play a role in protection of the immune-privileged CNS against foreign pathogens and as the resident immune cells, they are the first line of defense. They are found throughout in the adult brain and comprise about 10 % of all brain cells (Ransohoff and Perry, 2009).

Microglial processes are motile which allow them to monitor the environment and respond by changing their morphology and genetic expression profile (Davalos et al., 2005; Dudziak et al., 2005). Microglia can also move within CNS and migrate in response to tissue damage or towards specific chemoattractants released by damaged cells (Blade et al., 2005).

However, microglial cells can also produce neurotrophic and neuroprotective molecules such as nerve growth factor (NGF), indicating a role in neuronal survival, in cases of brain injury. Therefore, they are considered to have a double-edged nature, as they have both beneficial and detrimental effects.

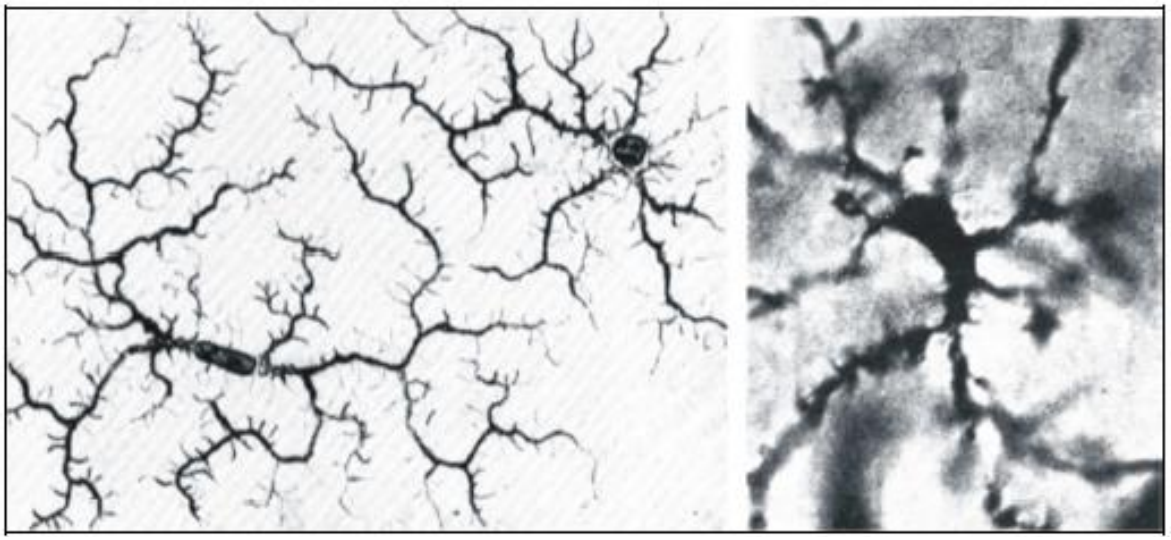

Figure **1.1** - **Left:** Drawing of ramiedied microglia by del Rio Hortega (del Rio Hortega, 1919). **Right:** Photomicrograph of silver stained microglia (del Rio Hortega, 1932).

The origin of resident microglia in the CNS was controversial for many years. In the early 1900s microglia were thought to be of neuroectodermal origin because of its location in the CNS. However, microglia bears certain similarities to cells of the mononuclear phagocyte lineage which exist in other tissues. Indeed, after decades of debate, it is now proven that microglia are of haematopoietic origin and that they are derived from myeloid progenitors from the yolk sac during early development (Fig. 1.2) (Ng et al., 1993; Raivich et al., 1996).

The microglial progenitors cells, which originate from the yolk sac, migrate to the central nervous system (CNS) and develop into early microglia cells (Ginhoux et al., 2010). Another study showed that the microglia can be detected during the development of the CNS in rodents as early as around embryonic days (E8-9), (Hickey and Kimura, 1988). During the last years, evidence arouse that resident microglia emerge through primitive yolk sac macrophages derived from haematopoietic stem cells via a myeloid progenitor state (Fig.1.2) (Ginhoux et al., 2010).

A subset of microglia is derived from bone marrow monocytes, though it has been shown that the transition is a rare event and takes place only under irradiation, lesion or inflammation (Mildner et al., 2007; Kigerl et al., 2009; Shechter et al., 2009). In addition, Ajami *et al* have recently shown that blood-derived monocytes infiltrating the brain during experimental autoimmune encephalomyelitis (EAE) do not contribute to the resident microglia in the CNS (Ajami et al.; Mildner et al., 2007).

These findings go along with older publications which have already shown that yolk sac macrophages can proliferate after migrating into the embryonic tissue (Cossmann et al., 1997).

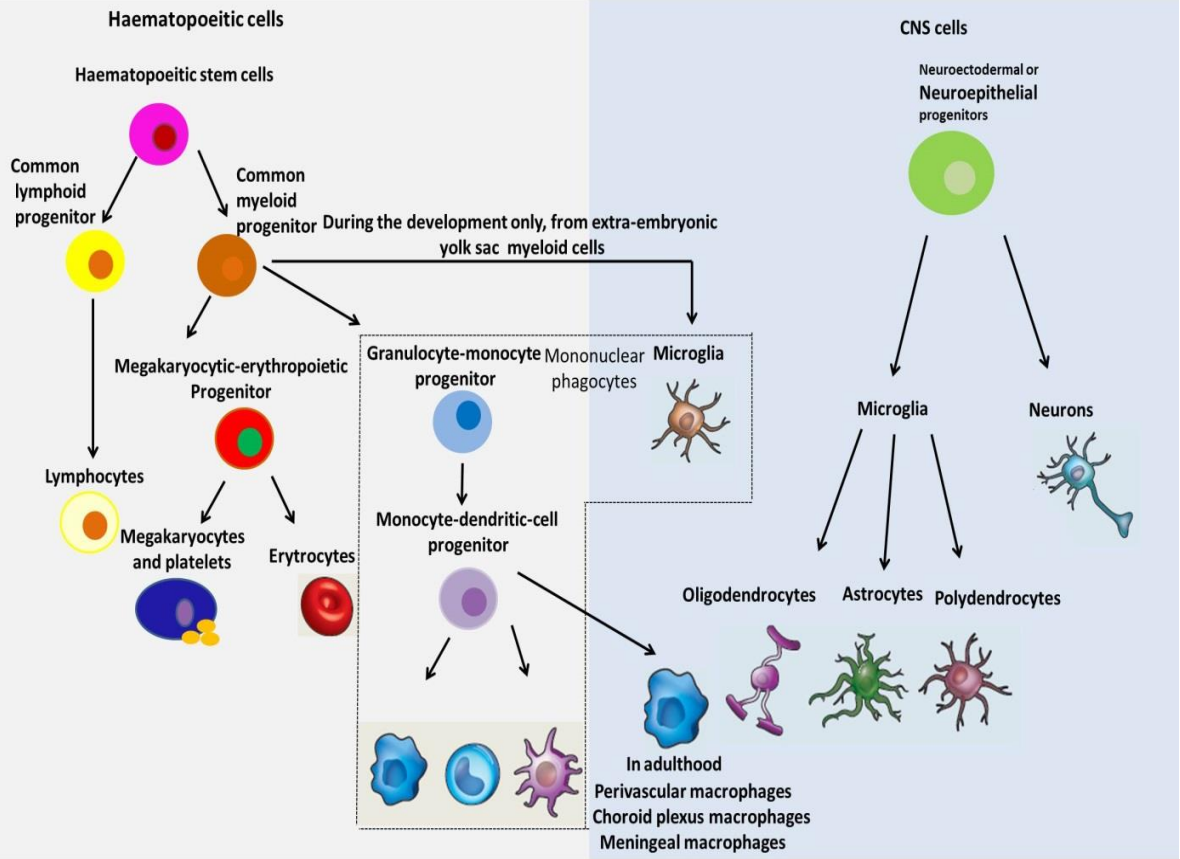

Figure 1.2 - Origin of microglia. Cells from hematopoietic origin and central nervous system are illustrated. The differentiated cells are marked with arrows. Microglia is the only hematopoietic cell found in the parenchyma of the CNS. modified from Ransohoff & Cardona, 2010.

Microglia may be activated by a whole set of structurally unrelated compounds and molecules. Microglia is activated in infectious diseases of the CNS and pathogen components activate microglia *in vitro* (Chi et al., 2001). Microglia detect pathogens by means of pattern recognition receptors (PRRs) on the cell surface, which recognize so-called pathogen-associated molecular patterns (PAMPs), molecular structures associated with pathogens but not with host cells (Xu et al., 2000). Most PRRs are expressed only at low levels on ramified microglia, if at all, and are upregulated upon microglial activation (Chi et al., 2001), and after microglia are activated can have phagocytic capacity ant that is associated with increased release of cytokines and expression of receptors and major histocompatibility complex (MHC) antigens, and in the adult brain microglia can switch inbetween two states (Fig. 1.3).

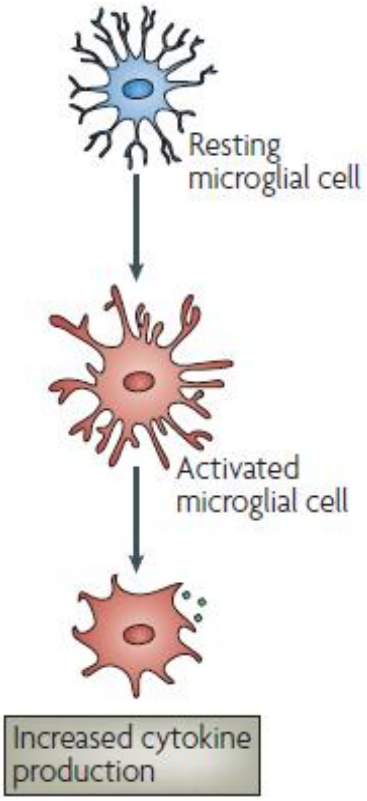

Figure 1.3 – Resting and activated microglia state (Perry et al., 2007).

The most commonly used compound to stimulate microglial activation experimentally *in vitro* and *in vivo* is the bacterial endotoxin lipopolysaccharide (LPS), which is a major component of the outer membrane of gram-negative bacteria (Rietschel and Kimmig, 1994). In addition, microglial receptors initiate phagocytosis of other pathogens which have been opsonised (coated) by soluble components of the immune system (Chi et al., 2001).

Microglia reacts rapidly to local tissue damage, suggesting that a signal from damaged cells can be sufficient to activate microglia (Gehrmann et al., 1991; Morioka et al., 1992). Accordingly, large amounts of ATP that is released mainly by astrocytes under pathological conditions (Ciccarelli et al., 2001) were shown *in vitro* to affect microglial morphology (Honda et al., 2001) as they cause elevation of intracellular Ca2+ (McLarnon et al., 1999), release of cytokines, and activate chemotactic effects (Honda et al., 2001; Davalos et al., 2005). These effects, characteristic of microglial activation, may be mediated via the activation of P2Y purinergic receptor (Honda et al., 2001; Davalos et al., 2005), or opening P2X7 ATP-gated ion channel (Monif et al., 2009). Elevated extracellular K+, also indicative

of tissue damage, depolarizes the membrane potential of microglia and can initiate microglial activation (Colton et al., 1994; Abraham et al., 2001). In addition, inwardly-rectifying K+ channels expressed by microglia (Kettenmann et al., 1990; Norenberg et al., 1994), current through which is enhanced under conditions of elevated K+, may be involved in cytokine-dependent proliferation and differentiation of microglial cells (Schlichter et al., 1996; Shirihai et al., 1996).

Like cells of the mononuclear phagocyte lineage, microglia is also capable of phagocytosing pathogens (Giulian and Baker, 1986; Streit et al., 1988; Rieske et al., 1989). State when the cells are amoeboid and migratory. Phagocytosis of pathogen components and subsequent processing leads to the presentation of antigens by the major histocompatibility complex (MHC) molecules, which are required for T cell activation (Cash et al., 1993). This is accompanied by an upregulation of other molecules associated with activation, including a number of cell adhesion molecules, which allow microglia to further detect and internalize diverse material (Raivich et al., 1999). This removal of apoptotic cells and debris is important in maintaining homeostasis of the CNS. Moreover, the process of phagocytosis induces a general pro-inflammatory phenotype in the microglia.

The main pathway of communication between microglia and other cells of the immune system is through the cytokine system. Microglia expresses cytokines and their receptors and release cytokines (including chemokines) to its environments. This can mediate recruitment of further microglia and peripheral immune cells, and initiate activation of such cells. Cytokines can be grouped as pro–inflammatory and anti-inflammatory cytokines (Kettenmann et al.; Biber et al., 2007).

IFN-γ (Interferron gamma) appears to amplify the pro-inflammatory effects of microglial activators (Colton, 1994; Hausler et al., 2002) and is often used *in vitro* as a co-stimulator for LPS-mediated activation. Microglia expresses receptors for the lymphocyte-derived cytokine interferon-gamma (IFN-gamma) and respond to it both *in vitro* and *in vivo* with an upregulation in microglial expression of MHC molecules, suggesting enhanced antigen presenting ability (Vass and Lassmann, 1990; Loughlin et al., 1992; Panek and Benveniste, 1995; Deckert-Schluter et al., 1999).

The effects of anti-inflammatory cytokines can be largely attributed to the downregulation of pro-inflammatory cytokines or functional antagonism of their effects (Hanisch, 2002). For example, LPS has been shown to induce transforming growth factor β (TGFβ) production by

microglia, through a tumour necrosis factor α (TNF-α) dependent pathway. Since TGFβ was shown to inhibit LPS-stimulated TNFα production (Chao et al., 1995) a self-regulating mechanism can be assumed to exist, with the potential to control inflammation (Choi et al., 2003).

Different microglial receptors and signaling pathways are involved in the detection of and response to different molecules which activate microglia (Pocock and Liddle, 2001). Therefore, it is likely that different microglial activators cause the upregulation of specific genes which are leading to slightly different activated phenotypes. Microglial activation leads to the expression of the so-called death receptor, Fas/CD95 and its ligand FasL/CD95L. This induces the release of reactive oxygen and nitrogen species (ROS), proteolytic enzymes, prostanoids and glutamate. Also, activated microglia expresses inducible nitric oxide synthase (iNOS), which allow the production of the vasodilator and non-specific inflammatory mediator nitric oxide (NO) from L-arginine (Chao et al., 1992; Ding et al., 1997; Possel et al., 2000).

1.2 Triggering receptor expressed on myeloid cells-2 (TREM2)

In the CNS, the innate and adaptive immune responses are strongly reduced, but when have some damage, due to chronic, Triggering receptor expressed on myeloid cells 2 (TREM2) is an innate immune receptor and has an immunoglobulin superfamily domain in their extracellular part with a charged lysine residue in their transmembrane domain. They have also a short cytoplasmatic tail that associates signaling molecule DAP12 containing an ITAM (immunoreceptor tyrosine-based activation motif) (Colonna, 2003), (Fig. 1.4).

TREM2 is expressed on myeloid origin cells such as immature dendritic cells, osteoclasts and microglia (Colonna, 2003). In human, TREM2 is expressed on microglia and a subpopulation of neurons (Sessa et al., 2004). A study using a systemic screen for gene transcripts selectively expressed on resting microglia versus activated microglia/macrophages, was the first to demonstrate TREM2 expression on microglia (Schmid et al., 2002). The same group has shown microglial TREM2 receptor was down-regulated after inflammatory stimulus with LPS/IFNgammaLPSLPS/IFNgammaIFNγ (Schmid et al., 2002). TREM2 signaling after ligation with agonistic monoclonal antibodies on immature dendritic cells has been shown to stimulate maturation and up-regulation of the chemokine receptor CCR7 and some co-stimulatory molecules (Bouchon et al., 2001). Consequently in microglial cells, TREM2

signaling via the adaptor molecule DAP12 stimulates the protein tyrosine kinase ERK (Takahashi et al., 2005). Furthermore, TREM2 can regulate the migratory capacity of microglia (Takahashi et al., 2005). Studies have shown that the tyrosine residues within the immunoreceptor ITAM of the adaptor molecules DAP12 or FcR gamma were required for the inhibitory signaling and the anti-inflammatory signaling of TREM2 in microglia (Neumann and Takahashi, 2007). This would be in line with a novel inhibitory signaling function of the ITAM-containing adaptor molecule DAP12 (Neumann and Takahashi, 2007). TREM2 and DAP12 mutations are leading to Nasu-Hakola disease (see next chapters).

However, apoptotic neural cell membranes are capable to stimulate the TREM2 receptor of microglia, but it is unclear which nervous tissue derived ligand binds to the TREM2 receptor. Extracellular region of mouse TREM2 can bind to bacteria, yeast and bacterial products and binding can blocked by anionic carbohydrate molecules, including dextran sulfate, where TREM2 might function as a sort of scavenger receptor for polyanionic macromolecules (Colonna, 2003; Stefano et al., 2009). Furthermore, the discovery of the ligands for TREM2 receptor will be crucial to verify these beneficial hypotheses and to characterize precisely the contribution of TREM2 receptor to immune and non-immune functions in different neurological diseases.

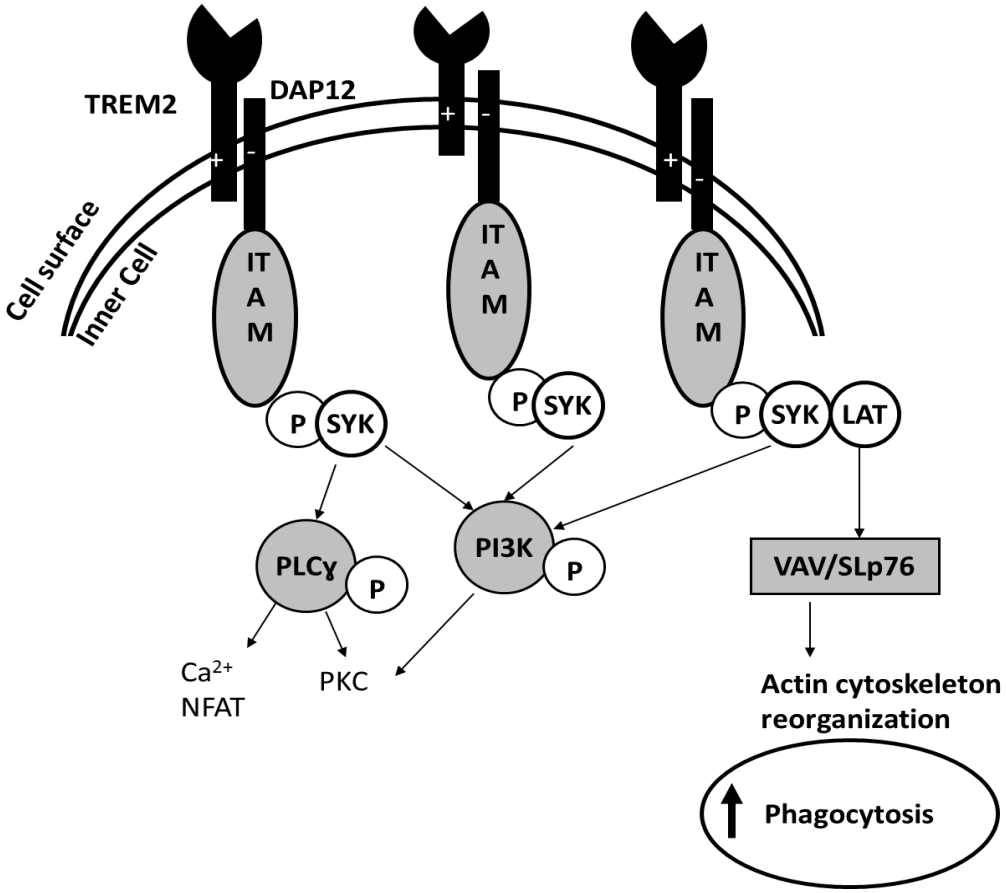

Figure 1.4 - TREM2 signalling cascades after binding of ligand. TREM2 receptor is completely dependent on ITAM domain-containing DAP12. Receptor-domain interaction occurs between charged amino acids (+/-) in their transmembranes regions. Ligand binding to TREM2 induces the formation of multivalent ligand-receptor complexes that trigger full phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) and recruitment of SYK and MAP, mitogen-activated protein kinase. This results in actin cytoeskeleton reorganization and increase of phagocytosis. SYK- spleen tyrosine kinase, PLCγ - phospholipase C-ɣ, PI3K – phosphotidylinositol 3-kinases, LAT – linker for activation of T cells, TREM2 – triggering receptor expression on myeloid cells 2. Modified from (Linnartz and Neumann, 2013).

1.2.1 TREM2 function of primary mouse microglia

It, *in vitro* studies have described TREM2 is a microglial innate immune receptor for phagocytosis of apoptotic neurons (Takahashi et al., 2005). They showed gene transcription and protein expression of TREM2 in cultured murine microglia by RT-PCR and immunohistochemistry, respectively. Stimulation of TREM2 by cross-linking antibodies induced DAP12 phosphorylation, extracellular signal-regulated kinase phosphorylation, and cytoskeleton reorganization (Takahashi et al., 2005). Furthermore, TREM2 stimulation induced phagocytosis of apoptotic material and beads. Knockdown of TREM2 in microglia inhibited phagocytosis of apoptotic neurons and increased gene transcription of tumor necrosis factor-alpha (TNF-alpha) and nitric oxide synthase-2 (NOS2), whereas over-expression of TREM2 increased phagocytosis and decreased microglial pro-inflammatory responses. Thus, TREM2 deficiency of microglia *in vitro* resulted in impaired clearance of apoptotic neurons and inflammation. Cross-linking of TREM2 on primary microglia by a monoclonal antibody did not modulate constitutive gene transcription of TNF-alpha, interleukin-1beta (IL-1beta) and NOS2 (Takahashi et al., 2005). However, stimulation of TREM2 by cross-linking antibodies counter-regulated induction of inflammatory mediators by TLR-stimulation that is also regularly seen in damaged tissue.

1.2.2 Microglial TREM2 is a beneficial phagocytic receptor for silent clearance of apoptotic bodies

TREM2 is a phagocytic receptor that promotes the clearance of apoptotic material by microglia and favors anti-inflammatory cytokine signaling (Takahashi et al., 2005). The TREM2/DAP12 deficiency impairs the clearance of debris arising from sporadic neuronal death. The ensuing accumulation of apoptotic material induces the activation of microglial cells that are no longer alleviated by TREM2/DAP12 and produce neurotoxic pro-inflammatory mediators, inducing further neuronal death and more apoptotic debris (Neumann and Takahashi, 2007). In agreement with a beneficial role of the native microglial TREM2, the laboratory of Professor Neumann established that transfer of myeloid precursor cells transduced with TREM2 ameliorated the disease course of experimental autoimmune encephalomyelitis (EAE) (Takahashi et al., 2007). Likewise, blockade of TREM-2 by an antibody exacerbates experimental autoimmune encephalomyelitis (Piccio et al., 2007).

The ligand of TREM2 is still poorly defined. TREM2 was shown to be a pattern recognition receptor that binds to anionic carbohydrates (Daws et al., 2003) and Hsp60 displayed by damaged cells (Stefano et al., 2009). The detailed characterization of the TREM2 ligands will be an important step to have a candidate molecule for a novel therapy.

1.2.3 Loss of TREM2 function is leading to a monogenic rare disease with presenile dementia and bone alterations

Nasu-Hakola disease (NHD) - also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy is a genetic disorder due to loss-of function mutations in the genes encoding either the TREM2 or its signaling adapter molecule DAP12 (Paloneva et al., 2000). In the brain, expression of DAP12 and TREM2 or their mutated forms are exquisitely restricted to microglia (Paloneva et al., 2001; Roumier et al., 2004; Sessa et al., 2004; Takahashi et al., 2005; Roumier et al., 2008; Wakselman et al., 2008; Thrash et al., 2009). Therefore, the primary cause of NHD can only be an alteration of microglial DAP12/TREM2 function. NHD patients suffer from bone alterations and presenile dementia and death, occuring in the early 40s (Paloneva et al., 2001).

Patients show advanced loss of axons and myelin with axonal spheroids in the frontal and temporal lobes (Paloneva et al., 2000). The most remarkable microscopic alterations are astrogliosis and strong microglial activation in the cerebral white matter lobes (Paloneva et al., 2001). Mouse models of NHD have been obtained by mutating or deleting the TREM2 and DAP12 genes (Tomasello et al., 2000; Turnbull et al., 2006). In contrast to other mouse models of genetic diseases, DAP12-mutated mice recapitulate several of the human symptoms. For instance, DAP12-mutated mice display bone and white matter alterations (Kaifu et al., 2003; Nataf et al., 2005) that are mainly detected at late adulthood (Kaifu et al., 2003). The brain phenotype of TREM2 deficient mice has not been described so far.

1.2.4 TREM2 and Neurodegenerative Disease

Recently an association between the single-nucleotide polymorphism (SNP) rs75932628 in the gene enconding the TREM2 (predicting an R47H substitution) and Alzheimer´s disease in persons of European ancestry was described (Guerreiro et al., 2013; Jonsson et al., 2013). Alzheimer´s disease is a genetically heterogeneous disorder characterized by the coexistence of monogenic and genetically complex forms. The variant R47H of TREM2 roughly triples

the lifetime risk to develop Alzheimer´s disease, but the frequency of this risk variant was reported to be only 0.63% in the Iceland population and somewhat variable across other European populations (Benitez et al., 2011). Another group analyzed how the gene variant (risk allele of rs9394721) affects the risk of Alzheimer´s disease by mapping its effect on the brain by brain magnetic resonance imaging (MRI) in temporal lobes. Then, they demonstrated that the annual rates of brain-volume loss over time in this region were associated with carrying the risk allele of rs9394721, a close proxy for the newly discovered risk variant rs75932628 in TREM2 (Hua et al., 2010).

Several other observations supported that TREM2 variation may confer susceptibility to fromtotemporal dementia and Parkinson´s disease. Microgliosis has been implicated in the pathogenesis of Parkinson´s disease, a neurologic disorder characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta, dopaminergic neurons express ligands that interact directly with TREM2, and TREM2 messenger RNA levels in the substancia nigra are the second highest among all brain regions. In addition, pathological findings in TREM2 variant carriers have shown the presence of abundant Lewy bodies, a hallmark of Parkinson´s disease (Hsieh et al., 2009).

The TREM2 risk variant may affect signaling by TREM2 receptors expressed on microglial cells in the brain, perhaps interfering with anti-inflammatory functions of these cells and their removal of apoptotic tissue.

1.3 Glycosaminoglycans family and classification

The glycosaminoglycans (GAGs) are know to play critical functions in CNS, include inhibition of axonal growth and regeneration, neuritogenic activity, and pathogens infections, the GAGs are the more component of extracellular matrix (Sugahara et al., 2003). GAGs are found many tissues in the cornea, where they are known to regulate neurite growth, regule embryo implantation in the endometrial uterine living during menstrual endothelial cells and also are envolved with tumor progression tumor metastasis, and pathological reparative processes (Laurent et al., 1996; Funderburgh, 2000).

Glycosaminoglycans are linear, sulfated, negatively charged polysaccharides that have molecular weights of rougly 10-100kDa. There are two main types of GAGs. Non-sulfated GAGs include hyaluronic acid (HA), whereas sulfated GAGs include chondroitin sulfate

(CS), dermatan sulfate (DS), keratin sulfate (KS), heparin and heparin sulfate (HS) (Rademacher et al., 1988) and the focus of this work is investigate that CS can binding to TREM2 receptor.

Table 1.1 - Repeating disaccharide units of various Glycosaminoglycans family. Modified from (Sasisekharan et al., 2006)

Glycosaminoglycans	Dissacharides Units	Features
Hyaluronic acid	D-GlcA-β(1 – 4) D-GlcNAc-α(1-4)	• Molecular weight 4- 800 kDa. • Non-sulfated non-covalently attached to proteins in the ECM; also found in bacteria. Chondroitin sulphate • Usually found in synovial fluid, vitreous humour, ECM of loose connective tissue. • Excellent lubricators and shock absorbers.
Chondroitin sulfate	D-GlcA-β(1-3) DGalNAc4S-β(1-4)	• Molecular weight 5-50 kDa. • Most abundant GAG in the body found in cartilage, tendon, ligament, aorta. • Binds to proteins (like collagen) to form proteoglycan aggregates.
Dermatan sulfate	L-IdoA-α(1-3)-D-GalNAc4S-β(1-4)	• Molecular weight 15-40 kDa. • Found in skin, blood vessels, heart valves
Karatan sulfates I and II	D-Gal-β(1-4)-D-GalNAc6S-β(1-3)	• Molecular weight 4-19 kDa. • Most heterogeneous GAG. • KS I is found in the cornea. • KS II is found in cartilage aggregated with CS.

Glycosaminoglycans chains are composed of disaccharide repeating units called disaccharide repeating regions (table 1.1), the repeating units are composed of uronic acid (D-glucoronic acid or L-iduronic acid) and amino sugar (D-galactosamine or D-glucosamine). Hence, GAGs differ according to the type of hexosamine, hexose or hexuronic acid unit that they contain.

Chondroitin sulphate and DS, which contain galactosamine, are called galactosaminoglycans, whereas heparin and HS, which contain glucosamine, are called glucosaminoglycans. The amino sugar may be sulphated on carbon 4 or 6 or on the non-acetylated nitrogen; however, the sugar backbone of GAGs can be sulfated at various positions (Rademacher et al., 1988).

Glycosaminoglycans are large complex carbohydrate molecules that interact with a wide range of proteins involved in physiological and pathological processes in cell adhesion, cell growth and differentiation, cell signaling anticoagulation (Jackson et al., 1991; Casu and Lindahl, 2001).

1.3.1 The role of glycosaminoglycans in the central nervous system

Chondroitin sulfate proteoglycans (CSPGs) consist of a core protein and they are found in extracellular matrix of all cell surface and also in CNS. Most of the CSPGs are secreted from cells and participate in the formation of the extracellular matrix. CSPG are can find attached to matrix protein occurs naturally in the central nervous system, is considered to be a major inhibitor of axonal regeneration and is known to participate in activation of inflammatory response (Rolls et al., 2006).

In the normal central nervous system, CSPGs have a role in development and plasticity during postnatal development and in the adult brain. In the adult, CSPGs play a part in learning and memory. They are also involved in the hypothalamo-neurohypophysial system and also have an important role in CNS injuries and diseases. After CNS injury, CSPGs are the major inhibitory component of the glial scar. Removal of CSPGs improves axonal regeneration and functional recovery. CSPGs may also be involved in the pathological processes in diseases, it is up-regulated after CNS injury and during the course of chronic neurodegenerative disorders such as epilepsy, stroke, Alzheimer´s disease and multiple sclerosis disorders (Viapiano and Matthews, 2006).

The roles of CSPGs in Alzheimer´s disease appear to be multiple. The CSPGs colocalize with both amyloid plaques and neurofibrillary tangles suggesting either that they could be instrumental in the formation of these deposits or that they are a reaction to them (DeWitt et al., 1993; McLaurin and Fraser, 2000). *In vitro* studies demonstrated that CSPGs and GAGs promote Aß fibril formation and binding of GAGs to Aß. It has been shown that CSPGs inhibit the proteolysis of Aß fibrils (Gupta-Bansal et al., 1995). Aß is a very strong stimulant to astrocytic CSPGs production and these CSPGs are particularly inhibitory to neuronal

process outgrowth and may facilitate the decreased axon density and synaptic loss in human Alzheimer´s disease brain (DeWitt et al., 1994; DeWitt and Silver, 1996). How chondroitin sulfate might control neuroinflammation is still unclear.

1.3.2 The structure of chondroitin Sulfate proteoglycans in central nervous system

Chondroitin sulfate glycosaminoglycans (CS-GAGs) are long, linear chains that are formed by repeating disaccharide units (Brooks et al., 2002), can be found also distribuited in the CNS. The disaccharides are polymerized into long chains by the recently identified human chondroitin synthase (Kitagawa et al., 2001) and the protein chondroitin polymerizing factors (Kitagawa et al., 2003). The chondroitin sulfates are then modified by sulfation and the positions of sulfation define the type of CS (Properzi et al., 2003). The CS disaccharides can be monosulfated in the 4 or 6 position of the GalNAc residue (CS-A and CS-C, respectively) or disulfated in the 2 and 6 position of the GalNAc, respectively (CS-D), and in the 4 and 6 position of the GalNAc (CS-E) (Fig. 1.5) and the terminology of CS group in table 1.2 (Sugahara et al., 2003) (Table 1.2).

It has been shown that the disaccharidic (DS) products of CSPG degradation; 6-sulfated disaccharide(CSPG-DS), induces axonal growth and activates microglia to adopt a neuroprotective phenotype (Rolls et al., 2004). Other work in literature demonstrate that disacharidic degradation product of this glycoprotein (CSPGDS) participate in the modulation of the inflammatory responses and can, therefore, promote recovery in immune-induced neuropathologies of the CNS, such as experimental autoimmune encephalomyelitis (EAE) and experiemntal autoimmune uveitis (EAU). It was shown that CSPGDS markedly alleviated the clinical symptoms of EAE and attenuated T cell motility. Furthermore, they decrease secretion of the cytokines interferon-gamma and tumor necrosis factor-alpha (Rolls and Schwartz, 2006). Literature shows that treatment of the visual cortex of adolescent rats at postnatal day P70 with ChABC (chondroitnase sulfate ABC, an enzyme from the bacteria *Proteus vulgaris*, that catalyzes the removal of CS-GAG chains of CSPGs) (Prabhakar et al., 2005) showed reactivation of ocular dominance plasticity after the critical period (Pizzorusso et al., 2002). However, other studies suggest that ChABC drive normalization of ocular dominance, visual acuity and dendritic spine density in the adult rats as shown by anatomical, electrophysiological and behavioral methods (Pizzorusso et al., 2006).

Other works show that administration of CSPG-DS, but not CS-A, in mice, after onset of clinical symptoms of EAE, was able to suppress the disease. Further studies demonstrated that CS-A up-regulated STAT3 and IL-23 expression and thus increased IL-17 producing T cells induceing a Th1 response. CSPG-DS treatment both *in vivo* and *in vitro* decreased TNF-alpha production from splenocytes. In vitro and in vivo studies indicated that CSPG-DS treatment in EAE mice significantly blocked migration of lymphocytes. Whereas CS-A increased lymphocyte infiltration in the mice brain and induced Th1 response (Zhou et al.).

Table 1.2 - Terminology of chondroitin sulfate group.

Letter identification	Site of sulfation	Systematic name
Chondroitin Sulfate	• carbon 4 of the N-acetylgalactosamine (GalNAc) sugar • carbon 6 of the GalNAc sugar • carbon 2 of the glucuronic acid and 6 of the GalNAc sugar • carbons 4 and 6 of the GalNAc sugar	Chondroitin sulfate 4 – 6 -2,6 -4 ,6 sulfate
Chondroitin sulfate A	• carbon 4 of the N-acetylgalactosamine (GalNAc) sugar	Chondroitin-4-sulfate
Chondroitin sulfate C	• carbon 6 of the GalNAc sugar	Chondroitin-6-sulfate
Chondroitin sulfate D	• carbon 2 of the glucuronic acid and 6 of the GalNAc sugar	Chondroitin-2,6-sulfate
Chondroitin sulfate E	• carbons 4 and 6 of the GalNAc sugar	Chondroitin-4,6-sulfate

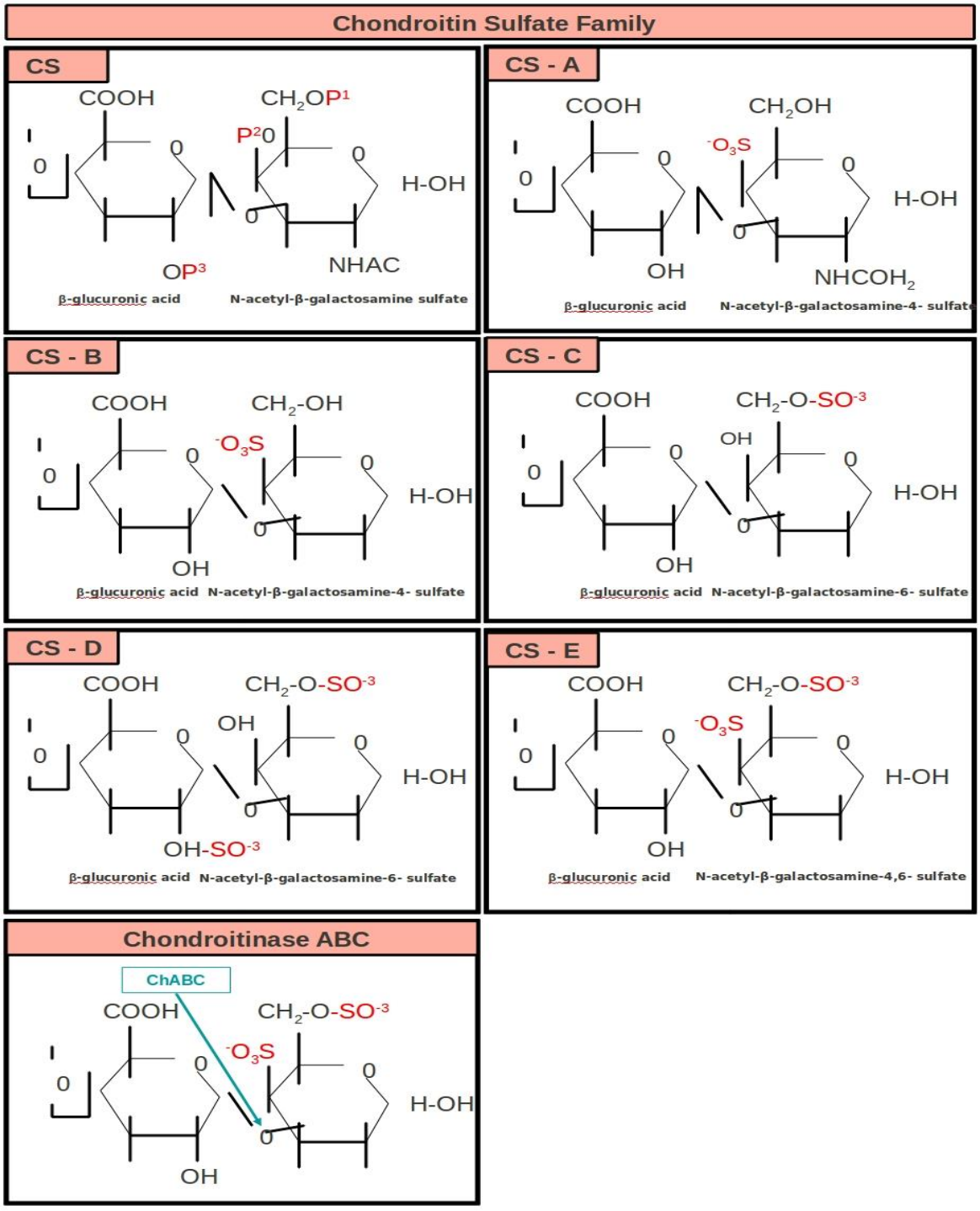

Figure 1.5 - Structures of chondroitin sulfate family and chondroitinase ABC digestion.

2. Aim

Triggering receptor expressed in myeloid (TREM) cells 2, a receptor expressed by myeloid cells, osteoclasts and microglia, is known to play a protective role in bones and brain. Mutations of the receptor or of its coupling protein, DAP12 sustain in fact a genetic disease affecting the two organs, the polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy (PLOSL or Nasu-Hakola disease). So far, specific agonist(s) of TREM2 have not been identified, and that ligands can binds to TREM2 are largely unknown. Heat shock protein 60 (Hsp60) is a mitochondrial chaperone is already been shown can binds to TREM2 (Stefano et al., 2009), and another work showed that TREM-2 binds to lipooligosaccharides of Neisseria gonorrhoeae and is expressed on reproductive tract epithelial cells (Quan et al., 2008).

However, the present thesis proposes to find specifics structures can be recognize by TREM2 receptor.

The first aim is try to identify of activating anionic polysaccharides binding to TREM2. Specific agonists of TREM2 have not been identified so far. Anionic polysaccharides with binding specificity to TREM2 and containing a high number of polymers (> 30 saccharides) might have the capacity to cross-link two or several TREM2 receptors, thus stimulating the signaling cascade. Anionic polysaccharides from different sources and different degrees of polymerization were tested by blocking the binding of a TREM2-Fc-fusion protein to the glycocalyx of altered cells (e.g. glioma cells).

The second aim is to investigate TREM2 function in mouse microglial line. Furthermore, the anionic polysaccharides should be tested in their capacity to stimulate TREM2 function such as phagocytosis in cultured mouse microglia with a normal or knockeddown expression of TREM2. Recently, the laboratory of Dr. Neumann established a protocol for the differentiation of mouse embryonic stem cells to microglia cells (ESdM). The differented mouse microglial cells were transduced with lentivirus to knockdown TREM2 using shRNA. Cells were challenged with chondroitin sulfate or disaccharide sulfate or not sulfated disaccharides. Phagocytic activity was monitored by flow cytometry analysis and inflammatory gene transcripts of microglia were determined by RT-PCR.

3. Materials and Methods:

3.1 Materials

3.1.1 Buffers and Solutions

Table 3.1 – 1X Phosphate buffered saline (PBS)

1X DPBS (Phosphate buffered saline (PBS)	GIBCO-Germany
Calcium chloride ($CaCl_2$)	GIBCO-Germany
Magnesium chloride ($MgCl_2$)	GIBCO-Germany

Table 3.2 – Reverse Transcription mix

Reverse transcription (RT) mix	
1 µl Hexanucleotide Mix (10X)	Roche, Germany
1 µl Deoxynucleotide triphosphate (dNTP) mix (10mM)	Sigma, Germany
2 µl Dithiothreitol (DTT) mix (10 mM)	Invitrogen, Germany
4 µl 5X RT 1st Strand Buffer	Invitrogen, Germany
1 µl RT enzyme (200 U/ml)	Invitrogen, Germany
ad 20 µl ddH2O	Roth, Germany

Table 3.3 - Quantitative real time-polymerase chain reaction (qRT-PCR)-mix

Quantitative real time-polymerase chain reaction (qRT-PCR)-mix	
3 µl Complementary Deoxyribonucleic Acid (cDNA) (200ng/µl)	
1 µl SYBR Green Master Mix (2x)	Applied Biosystems, USA
2 µl Primer mix (10 pmol/µl)	Life Technologies
10.5 µl ddH2O	Roth, Germany

Table 3.4 - 10x (0.125 M) Phosphate buffered saline (PBS), pH 7.3

10x (0.125 M) Phosphate buffered saline (PBS), pH 7.3	

0.007 M $NaH_2PO_4*H_2O$	Roth, Germany
0.034 M $NaH_2PO_4*7H_2O$	Roth, Germany
0.6 M NaCl	Roth, Germany
ad 1 liter ddH_2O	Roth, Germany

Tale 3.5 – 2x HBS (Hepes buffered salina)

2x HBS	
8g NaCl	Roth, Germany
0.38g KCl	Roth, Germany
0.1g Na2HPO4	Roth, Germany
5g Hepes	Roth, Germany
1g Glucose	Roth, Germany
up to 500 ml ddH2O	Roth, Germany
adjust pH to 7.05	

3.1.2 Medium used for culture of cells

Table 3.6 – Medium type for different cell lines

Medium type	**Content**	
Freezing Medium	50 % Fetal bovine serum (FCS)	Gibco, Germany
	40 % cell medium	
	10 % DMSO	Sigma, Germany
MEF Medium **HEK cells**	DMEM with L-glutamine and 4.5g/l D-glucose, without sodium pyruvate	Gibco, Germany
	0.1 mM NEAA	Gibco, Germany
	1 mM sodium pyruvate	Gibco, Germany
	2 mM L-glutamine	Gibco, Germany
	10 % FCS	Gibco, Germany
N2 Medium **ESdM cells**	DMEM/F12 [1:1] with L-glutamine and HEPES [15mM]	Gibco, Germany
	1 % N2 supplement	Gibco, Germany
	100 µg/ml penicillin/streptomycin	Gibco, Germany

U87 Medium	**DMEM with high glucose**	**Gibco, Germany**
	10% FCS	Gibco, Germany
	1% penicillin/streptomycin	Gibco, Germany

GL261 Medium	DMEM/F12 (1:1) with L-glutamine	Gibco, Germany
	1% penicillin/streptomycin	Gibco, Germany
CHO Medium	MEMalpha	Gibco, Germany
	10% FCS	Gibco, Germany
LB medium	10g Tryptone 5g yeast extract 10g NaCl 950 mL deionized water Adjust the pH of the medium to 7.0 using 1N NaOH	Roth, Germany Sigma, Germany Roth, Germany Roth, Germany

3.1.3 Other cell culture reagents

Table 3.7 – Cell culture reagents

Reagents	
Opti-MEM	Gibco, Germany
Trypsin-EDTA (0.25%)	Gibco, Germany
Poly-L-lysine	Sigma, Germany
PBS (1X)	Gibco, Germany
Tryptan blue	Gibco, Germany
Isopropanol	Sigma, Germany
Fluoresbrite polychromatic red 1.0 micron microspheres (the beads for phagocytosis assay)	Polyscience, USA
Neuraminidase (sialidase)	Roche, Germany
Magnesium II Chloride (MgCl2)	Sigma, Germany
Manganese II Chloride (MnCl2)	Sigma, Germany
Bovine serum albumin (BSA)	Sigma, Germany
Ethanol	Roth, Germany
Hexanucleotide Mix (10x)	Roche, Germany
Puromycin	PAA, Germany
RNeasy Mini Kit	Qiagen, Germany
RNAse free DNAse Kit	Qiagen, Germany
SuperScript® III Platinum® Two-Step qRT-PCR Kit	Invitrogen, Germany
HiPure Plasmid Filter Purification Kits	Invitrogen, Germany
Lipofectamine	Invitrogen, Germany
Propidium iodide	Sigma, Germany
Trypsin	Gibco, Germany

3.1.4 Glycosaminoglycan Reagents

Table 3.8 – Glycosaminoglycans reagents

Components	Company
Disaccharides (from CS-A derived – DS-4S)	Sigma, Germany
Chondroitin sulfate sodium salt from shark cartilage	Sigma, Germany
Chondroitin sulfate sodium salt A from bovine cartilage	Sigma,Germany
Chondroitin sulfate sodium salt C from shark cartilage	Nacalai tesque, Germany
Adequan IM – polysulfated Glycosaminoglycan 500mg/5mL	Novartis
Dextran sulfate from Leuconostoc.	Sigma, Germany
Chondroitinase ABC	Seikagaku, Germany
Chondroitin disaccharide not sulfated (DS-0S)	Sigma, Germany
Chondroitin disaccharide sulfated position 4S (DS-4S)	Sigma, Germany

3.1.5 Vectors

Table 3.9 – Packing plasmids

Packaging Plasmids	
Packaging plasmid PLP1 ViraPower ™	Invitrogen, UK
Packaging plasmid PLP2 ViraPower	Invitrogen, UK
Packaging plasmid PLP3 ViraPower ™	Invitrogen, UK

Table 3. 1.1 - shRNA Plasmids used for knockdown of TREM2 in ESdM cell line

Clone1:TRCN0000179471	Mouse NM_026192.1-1754s1c1 (Plasmids1)
Vector	pLKO_TRC005
Species	Mus musculus
Sequencing Primers Forward	5'CCGGGCCTTCTTCTCTTGTTGTCATCTCGAG ATGACAACAAGAGAAGAAGGCTTTTTG-3'
Sequencing Primers Reverse	5'AATTCAAAAAGCCTTCTTCTCTTGTTGTCAT CTCGAGATGACAACAAGAGAAGAAGGC-3'
Hairpin sequence	5'CCCGGGCCTTCTTCTCTTGTTGTCATCTCGA GATGACAACAAGAGAAGAAGGC-TTTTTTG-3'
Antibiotic	Ampicilin

Clone2:TRCN0000184478	Mouse NM_026192.1-897s1c1 (Plasmids2)
Vector	pLKO_TRC005
Species	Mus musculus
Sequencing Primers Forward	5'CCGGGCTGAATTACAAACGGTCCGACTCGAG TCGGACCGTTTGTAATTCAGCTTTTTG-3'
Sequencing Primers Reverse	5'AATTCAAAAAGCTGAATTACAAACGGTCCGA CTCGAGTCGGACCGTTTGTAATTCAGC-3'
Hairpin sequence	5'CCCGGGCTGAATTACAAACGGTCCGACTCGA GTCGGACCGTTTGTAATTCAGC-TTTTTTG-3'
Antibiotic	Ampicillin

Clone3: TRCN0000244544	Mouse NM_026192.2-2405s21c1 (Plasmids3)
Vector	pLKO_TRC005
Species	Mus musculus
Sequencing Primers Forward	5'CCGGCCTTGCGGGAATAGAATTATTCTCGA GAATAATTCTATTCCCGCAAGGTTTTTG-3'
Sequencing Primers Reverse	5'AATTCAAAAACCTTGCGGGAATAGAATTAT TCTCGAGAATAATTCTATTCCCGCAAGG-3'
Hairpin sequence	5'CCGGCCTTGCGGGAATAGAATTATTCTCGA GAATAATTCTATTCCCGCAAGG-TTTTTG-3'
Antibiotic	Ampicillin

3.1.6 Proteins and Antibodies

Table 3.1.2 – Fusion Proteins for flow cytometry

Proteins	Species Reactivity	Dilution	Company
TREM2 - Fc fusion protein	Mouse	25ug	R&D, Germany
Siglec F – Fc fusion protein	Mouse	25ug	R&D, Germany

Table 3.1.3 – Primary and Isotype antibody for flow cytometry

Antibody	Species Reactivity	Dilution	Company
TREM2	Mouse	1:100	R&D, Germany
Isotype IgG1	Rat	1:100	BD Biosciences, Germany

Table 3.1.4 – Secondary antibody for fusion protein

Protein	Host	Directed Against	Dilution	Company
Fluorescein FITC	Goat	Mouse Fc gamma	10µg	Jackson ImmunoResearch, USA

Table 3.1.5 – Secondary antibody for flow cytometry

Antibody	Host	Directed Against	Dilution	Company
PE	Goat	Mouse	1:100	Jackson ImmunoResearch, USA

3.1.7 Primers used for qRT-PCR

Oligonucleotides were obtained from Life Technologies.

Table 3.1.6 – Primers used for qRT-PCR

Name	Forward Sequence	Reverse Sequence
TREM2	5'TGCAAGGTCCCCTCCGGC3'	5'GGGCTTGGGACAGGGCTGTGA3'
IL-1ß	5'CTTCCTTGTGCAAGTGTCTG3'	5'CAGGTCATTCTCATCACTGTC3'
iNOS	5'AAGCCCCGCTACTACTCCAT3'	5'GCTTCAGGTTCCTGATCCAA3'
TNF-α	5'TGATCCGCGACGTGGAA3'	5'ACCGCCTGGAGTTCTGGAA3'
GAPDH	5'ACAACTTTGGCATTGTGGAA3'	5'GATGCAGGGATGATGTTCTG3'

3.1.8 Cells lines

Table 3.1.7 – Different cells lines

Cells Type	Received From
ESdM cell line	ESdM cells were already established using a protocol for microglia precursor cell line differentiation from murine embryonic stem cells was established by group of Prof. Harald Neumann (Napoli et al., 2009).
U87 cells MG	Human glioblastoma-astrocytoma cells (abcam).
CHO cells	Chinese hamster ovarien cells (Life technologies).
HEK cells 293FT	Human Embryonic Kidney 293 cells (Life technologies).
GL261	Mouse glioma cell line (Hertie-Institute for clinical neurology, Germany)
SMA560-MS	Small-cell lung carcinoma (Hertie-Institute for clinical neurology, Germany)
Competent cells	DHαE^{TM} cells (Invitrogen)
ESdM - DAP12 cells	ESdM cells were already established using a protocol for microglia precursor cell line differentiation from murine embryonic stem cells was established by group of Prof. Harald Neumann, but that cells was also performed by Dr. Liviu Bodea (Napoli et al., 2009).
ESdM DAP12 Full Length	DAP12 Full Length ITAM signalling overexpressed.
ESdM DAP12 Truncated	ITAM signaling motifs deleted.

ESdM GFP	Control vectors were inserted in lentiviral expression plasmids containing an internal ribosome entry site (IRES) enhanced green fluorescence protein (eGFP).

3.1.8 Consumables Material

Material	Company
Aluminium folie	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Cell strainer	BD Biosciences, NJ,USA
Corning cell scraper	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Cryovials [2 ml]	Nunc GmbH & Co KG, Wiesbaden, Germany
Falcon tubes [15 ml]	Cellstar, Greiner Bio One, Frickenhausen, Germany
Falcon tubes [50 ml]	Sarstedt Ag & CoKG, N.rnbrecht, Germany
Filtropur [0.25 µm, 0.4 µm]	Sarstedt Ag & CoKG, N.rnbrecht, Germany
Graduate pipette Tips [10 µl,100 µl, 1000 µl]	Starlab GmbH, Ahrensburg, Germany
Lab-Tek Chamber Slide w/Cover	Thermo Scientific, USA
Latex gloves	Ansell Healthcare Europe NV, Brussels, Belgium
Microscope cover glasses	P. Marienfeld GmbH, Lauda-K.nigshofen, Germany
Nitrile gloves	Ansell Healthcare Europe NV, Brussels
Parafilm	Bemis Flexible Packaging, WI, USA
Pasteur pipettes	Brand GmbH & Co KG, Wertheim, Germany
Petri dishes	Sarstedt AG & Co KG, Nurnbrecht, Germany
Pipettes [5 ml, 10 ml, 25 ml]	Sarstedt AG & Co KG, Nurnbrecht, Germany
Safe-seal micro tubes [0.5 ml,1.5 ml, 2 ml]	Sarstedt AG & Co KG, Nurnbrecht, Germany
Syringes	BD Biosciences, NJ,USA
Tissue culture dishes	TPP, Switzerland
Vacuum driven disposable bottle top filter	Millipore Corporation, Bitterica, MA, USA
0.45 µm pore size filter stericup	(Millipore, USA)
10 µl, 100 µl, 1000 µl pipette tips	(Eppendorf, Germany)
15 and 10 cm2 tissue cell culture flask	(Sarstedt, Germany)
6-well-plate	(Cellstar, Germany)
5, 10 and 25 ml disposable plastic pipette	(Costar, Germany)
FACs tube 5mL 12mm	(Sarstedt, Germany)

PCR tubes	(Biozym Diagnostics, Germany)
Cell scraper	(Sarstedt, Germany)

3.1.10 Equipment

EQUIPMENT	
- 20 °C freezer	Liebherr, Bulle, Switzerland
+ 4 °C fridge	Liebherr, Bulle, Switzerland
Apotome	Carl Zeiss AG, Jena, Germany
BD FacsCalibur	BD Biosciences, NJ,USA
Cell Mate II [pipette boy]	Thermo Fisher Scientific Inc, NH, USA
Eppendorf Mastercycler epgradient	Eppendorf AG, Hamburg, Germany
Hera cell 150 [incubator]	Heraeus Holding GmbH, Hanau, Germany
Hera freeze [- 80.C freezer]	Heraeus Holding GmbH, Hanau, Germany
Hera safe [laminar-air flow workbench]	Kendro Laboratory Products GmbH, Langenselbold, Germany
Megafuge1.0R [centrifuge]	Heraeus Holding GmbH, Hanau, Germany
Nanodrop 1000 Spectrophotometer	PeqLab Biotechnologie GMBH Erlangen, Germany
Pipettes [10µl, 100µl, 1000µl]	Eppendorf AG, Hamburg, Germany
Systec D-150 [autoclave]	Systec GmbH, Wettenberg, Germany
Thermomixer compact	Eppendorf AG, Hamburg, Germany
Water bath WB/OB7-45	Memmert G
Real time thermocycler ABI Prism 5700 Seqeunce Detection System,	Applied Biosystems, UK
Vortex 2X2	(VelpScientifica, Germany)
Centrifuges Sorvall Discovery 90SE, Megafuge, Biofuge Fresco	(Hitachi, Germany), (1.OR. Heraeus, Germany), (Heraeus, Germany)

3.1.11 Kits

KIT	**Company**
HiPure plasmid filter DNA purification kit	Life Technologies™
MiniElute PCR Purification Kit	Qiagen, Germany
QIAquick Gel Extraction Kit	Qiagen, Germany
QIAprep Spin Miniprep Kit	Qiagen, Germany

Rneasy® Mini Kit	Qiagen, Germany

3.1.12 Software/Databases

Software	Producer
AxioVision SE64, Rel 4.9.1	Carl Zeiss AG, Jena, Germany
Cellquest Pro	BD Biosciences, NJ,USA
CorelDRAW Graphics Suite x5	Corel, Germany
FlowJo 6.4.7	Tree Star, USA
Gene Designer 2.0	DNA2.0, Inc. USA
Geneious 6.1.6	Biomatters Ltd, New Zealand
Mendeley	Mendeley Ltd, USA
Microsoft Office 2010	Microsoft, USA

3.2 Methods:

3.2.1 Flow cytometry analysis of microglia line

Mouse microglial line (ESdM – embryonic stem cell derived microglial precursor cells) cells were collected using a cell scraper or trypsin. Cells were stained with TREM2 antibody (table 3.1.3 for 60 minutes (min) on ice. After washing with PBS, samples were incubated with a corresponding secondary antibody for 30 min on ice in darkness (table 3.1.5). Corresponding isotype control antibody (table 3.1.3) and non-stained cells were used as negative controls. Analysis was performed with a FACS calibur flow cytometer and FlowJo Software.

3.2.2 Maxiprep

The E. coli competent cells (Invitrogen) were thawn on ice for 15 min and 1µg if each DNA plasmids for TREM2 knockdown (see above table 3.1.1) were added to competent cells and mixed. After incubation for 30 min on ice, cells were heat shocked for 45 seconds in a water bath at 42°C, and then transferred back on ice. 900 µL of LB medium were added, mixed and transferred to culture tubes and were shaken for 1 hour in 37 °C at 250 rpm. After spining down, the bacteria were resuspended in 100 µl of LB medium and distributed on a plate with specific antibiotics. Then, the bacteria were incubated overnight at 37ºC. One single colony

was used for high-copy number of plasmids and incubated with 250 ml LB medium with respective antibiotics of plasmids and incubated overnight at 37ºC. Then, the purification of DNA plasmid was performed by HiPure Plasmid Filter Purification Kits (Invitrogen, K2100-27).

3.2.3 RT-PCR and RNA Isolation

Cells were homogenized in 350 µl of ß-mercaptoethanol and 70 % ethanol. Subsequently, RNA was isolated either using the RNeasy® mini Kit, following manufacturer's specifications. The RNA content was measured using a Nanodrop spectrophotometer. For cDNA production 50 µg total RNA was reverse transcribed using SuperScript III reverse transcriptase and hexameric random primers (table 3.1.8).

For the evaluation of transcriptional levels of cytokines and expression of TREM2 receptor in microglial cell line, Real Time - PCR was performed using 600 ng of cDNA, SYBR GreenER qPCRSuperMix Universal and 400 nM mouse primers (table 3.1.6) into a final reaction volume of 25 µL.

For amplifications (table 3.1.9), a Mastercycler epgradient S® was used and the results were evaluated with the manufacturer's software. Amplification specificity was confirmed by melting curve analysis of GAPDH and the quantification was carried out using the ΔΔCt method.

Table 3.1.8 – Reverse transcription

Prepare RT Mix (I)	**Component**	**Amount**	**Manufacturer**	**Registered office**
	RNA	11 µL (50 µg)		
	Hexanucleotids (mM)	1 µL	Roche	Grenzach-Wyhlen, DE
	dNTPs (mM each)	1 µL	Paqlab	Erlangen, DE
➢ **Start RT Program**	**Temperature**	**Time**		
	65°C	5 min		
	4°C	1 min		

	4°C	pause		
Add RT Mix (II)	**Component**	**Amount**	**Manufacturer**	**Registered office**
	Forwards Strand Buffer 5x	4 µL	Invitrogen, LifeTechnolgies GmbH	Darmstadt, DE
	DTT (0.1 M)	2 µL	Invitrogen, LifeTechnolgies GmbH	Darmstadt, DE
	SuperScript III	1 µL	Invitrogen, LifeTechnolgies GmbH	Darmstadt, DE
➢ **Continue RT Program**	**Temperature**	**Time**		
	25°C	5 min		
	55°C	1h		
	70°C	15 min		
	4°C	∞		

Table 3.1.9 – RT-PCR Program

Step	**Temperature**	**Time**	**Cycles**
1. Initial denaturation	95°C	10 min	
2. Denaturation	95°C	15 s	40x
3. Annealing	60°C	30s	40x
4. Elongation	72°C	30s	40x
5. Inactivation	95°C	10 min	
6. Melting curve	60°C-95°C	20 min	
7. Final	95°C	15 s	
8. Store	4°C	∞	

3.2.1 Viral particle production and lentiviral transfection of TREM2 knockdown

The 293FT-HEK cells were kept in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 1% penicillin/streptomycin and 4 mM glucose at 37°C. Viral particles were produced in 15 cm dishes precoated with poly-L-Lysine. 25 µg of targeting plasmid together with each of the three packaging helper plasmids were co-transfected using Lipofectamine to 6.5 million 293FT-HEK cells and incubated for 20-25 minutes at room temperature (table 3.2.1). Cells were transfected in Trans-MEF-medium (MEF medium without antibiotics containing 5% FCS). Medium was replaced at 6 - 16 hours post-transfection. Viral supernatant (10 ml) was collected at 48 - 72 hours post-transfection. Viral particles were then centrifuged using a Megafuge (Heraeus, Germany, 1300 rpm) for 3 min at 4°C. The viral particle pellet was resuspended in 5 ml MEF medium. Viral particles were either immediately applied to transduce cells or stored at -80°C. Cells having been incubated with lentiviral plasmids to knock-down TREM2 were analyzed by RT-PCR to confirm the knock-down of TREM2 gene transcripts.

Table 3.2.1 – Lentivirus generation (quantities per 15 cm2 dish)

Step	Component	Amount
Plasmid Mix	Plasmid of interest	25 µg
	pLP1 (pMDL gag/pol RRE)	25 µg
	pLP2 (pRSV-Rev)	12.5 µg
	pLP3	15 µg
	Lipofectamine	90 µl
	Medium	7 ml

3.2.2 Phagocytosis assay

For analysis of phagocytosis; 100,000 cells were seeded to each well of a 6-well-plate in N2 medium. At the second day, cells were incubated with different concentrations of CS-A or with dissacharides (DS-4S). After 2 hours of incubation with CS-A or 1 hour with sulfated DS(4S) and non-sulfated DS(0S). Then, those cells were labeled with microsphere beads (fluoresbrite Polycromatic Red microspheres 1.0um, Polyscience, 18660). Afterwards, 2.5 µl/2 mL microsphere beads were added to

each well for 30 minutes, washed with PBS and analyzed by FACS calibur TM flow cytometer (BD Biosciences).

3.2.1 Chondroitinase ABC activity assay

The chondroitinase ABC (ChABC) was used (Seikagu Biobusiness – 100330); where U87 cells (1 million) were pretreated with 50, 100 and 250 mU/ml of ChABC and then incubated for 1 or 24 hours at 37°C. ChABC removes the family of chondroitin sulfates from the glycocalyx of glioma cells. Afterwards U87 cells were washed twice with PBS and incubated with a TREM2b-Fc fusion protein for 60 minutes and after with a FITC-conjugated secondary antibody directed against the Fc part of TREM2b-Fc fusion protein for 30 minutes at 4 °C. Cells were washed twice in PBS and resuspended in 500 µl PBS. Live gating was performed by adding propridium iodide. Analysis was done with a FACS calibur flow cytometer (BD Biosciences).

3.2.2 Removal of sialic acid by Neuraminidase treatment

The U87 cells were treated with neuraminidase (Roche, Germany), an enzyme that can cleave and remove the sialic acids from the glycocalyx of glioma cells. The enzyme was added to the medium at a concentration of 2.5 mU/ml. Cells were incubated at 37 ºC for 2 hours. The cells were collected and incubated with Siglec F-Fc fusion protein or with TREM2b-Fc fusion protein protein for 1 hour at 4 ºC. Siglec F can recognize the sialic-containing Lewis-X glycosides residues. Results were analyzed with a FACS calibur flow cytometer (BD Biosciences) and FlowJo software.

3.2.3 Binding of TREM2b-Fc fusion protein or Siglec F-Fc fusion protein with polysaccharides U87 cells and FACS analysis

Cells were collected by a cell scraper, centrifuged and the pellet was resuspended in 500 µl of U87 medium without FCS and incubated with the 8 µg of TREM2b Fc chimera or Siglec F Fc chimera for 1 hour at 4 °C. For blocking of the TREM2 binding, polysaccharides were added to the incubation step with the Fc chimera at different concentrations of chondroitin sulfate sodium salt from shark cartilage (Sigma – C4384); chondroitin sulfate A sodium salt from bovine trachea (Sigma – C9819); dextran sulfate sodium salt from Leuconostoc ssp.; Adequan canine (Polysulfated Glycosaminoglycan – Novartis Animal Health) (table 3.8). Afterwards, cells were washed twice with PBS and incubated in a FITC-conjugated secondary antibody directed against the Fc part for 30 minutes at 4 °C. Cells were washed twice in PBS and

resuspended in 500 µl PBS. Live gating was performed by adding propidium iodide. Analysis was done with a FACS calibur TM flow cytometer (BD Biosciences).

3.2.4 Binding of TREM2b-Fc fusion protein or Siglec F-Fc fusion protein to mouse or human glioblastoma cells

Cells were collected and the cell pellet was resuspended in 500 µl of U87 cells medium and incubated with the 8 µg of TREM2b Fc chimera or Siglec F - Fc chimera for 1 hour at 4 °C. Afterwards, cells were washed twice with PBS and incubated in a FITC-conjugated secondary antibody directed against the Fc part for 30 minutes at 4 °C. Cells were washed twice in PBS and resuspended in 500 µl PBS. Live gating was performed by adding propidium iodide Analysis was done with a FACS calibur TM flow cytometer (BD Biosciences).

3.2.5 Analysis of pro- and anti- inflammatory cytokine gene transcripts by real-time PCR

RNA was isolated with the RNeasy Mini Kit from ESdM cells after stimulation with 2 mM of CS-A or 0.2 mM of DS-4S, 0.2 mM of DS-0S for either 30 minutes, 1 or 2 hours. And the preparation of cDNA, trancriptase reverse and qRT-PCR were already described in tables 3.1.8 and 3.1.9.

3.2.6 Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5 Software. For two groups, students t-test was used, for data with more than two groups, analysis of variance (ANOVA) followed by Bonferroni test was used. Data are presented as mean +/- SEM of at least three independent experiments.

4. Results:

4.1 Binding of TREM2-Fc fusion protein to the cell surface

Recently, it was shown that apoptotic neural cell membrane contain components that can bind to and stimulate TREM2 receptor of microglia (Hsieh et al., 2009). Here, it was also shown that TREM2 can bind to CHO and U87 cells. To confirm the binding of TREM2 to different cell lines, purified mouse TREM2b-Fc-fusion protein and positive control Siglec F-Fc fusion protein were incubated with GL261, SMA-560, CHO and U87 cells using flow cytometry. The U87 cells are a human primary glioblastoma cell line was obtained from a stage four 44 year-old cancer patient (Clark et al.), the GL261 cells are mouse glioma 261 (Gl261) cells are used frequently in experimental glioblastoma therapy (Szatmari et al., 2006), SMA-560 cells are Small-cell carcinoma also known as small-cell lung cancer is a type of highly malignant cancer that most commonly arises within the lung (Nasu et al.) and CHO cells are a cell line derived from the ovary of the Chinese hamster.

The Siglec F-Fc fusion protein was used as a positive control because Siglec F is also a microglia cell surface receptor which can recognize sialic acid that are express on altered glycocalyx of glioma or CHO cells and is a monosaccharide with a nine carbon backbone, they occur at the end of sugar chains connected to the surfaces of cells with soluble glycoproteins and glycolipids. The binding of mouse TREM2b-Fc fusion protein or Siglec F-Fc fusion protein to glioma and CHO cells were detected by flow cytometry (Figure 4.1). The results shown the TREM2b – Fc fusion protein and Siglec F – Fc fusion protein can binds to glioma and CHO cells and this binding of TREM2b – Fc fusion protein to glioma and CHO cells are lower fluorescence intensity when compared to positive control Siglec F- Fc fusion protein (Figure 4.1 a, b, c and d).

It was shown that the TREM2b - Fc fusion protein is capable of binding to SMA-560 cells (60.2 ± 4.6 fluorescence units, $p<0.001$), to CHO cells (48.7 ± 5.9 fluorescence units, $p<0.001$) and to GL261 cells (203 ± 2.7 fluorescence units, $p>0.05$), but with lower capacity when compared to U87 cells (310 ± 34.9 fluorescence units, $p<0.001$) (Figure 4.1 e). The positive control Siglec F – Fc fusion protein bound to CHO cells (124 ± 14.4 fluorescence units, $p<0.001$), to SMA-560 cells (250 ± 8.9 fluorescence units, $p>0.05$), to GL261 cells (306

± 6.8 fluorescence units, p>0.05) and to U87 cells (405 ± 3.7 fluorescence units, p<0.001) (Figure 4.1 f).

In summary, these results showed that the mouse TREM2 receptor can bind and recognize structures which are expressed on alter glycocalyx of glioma or CHO cells. In addition, the Siglec F receptor can recognize the sialic acids which are expressed on glycocalyx of glioma or CHO cells.

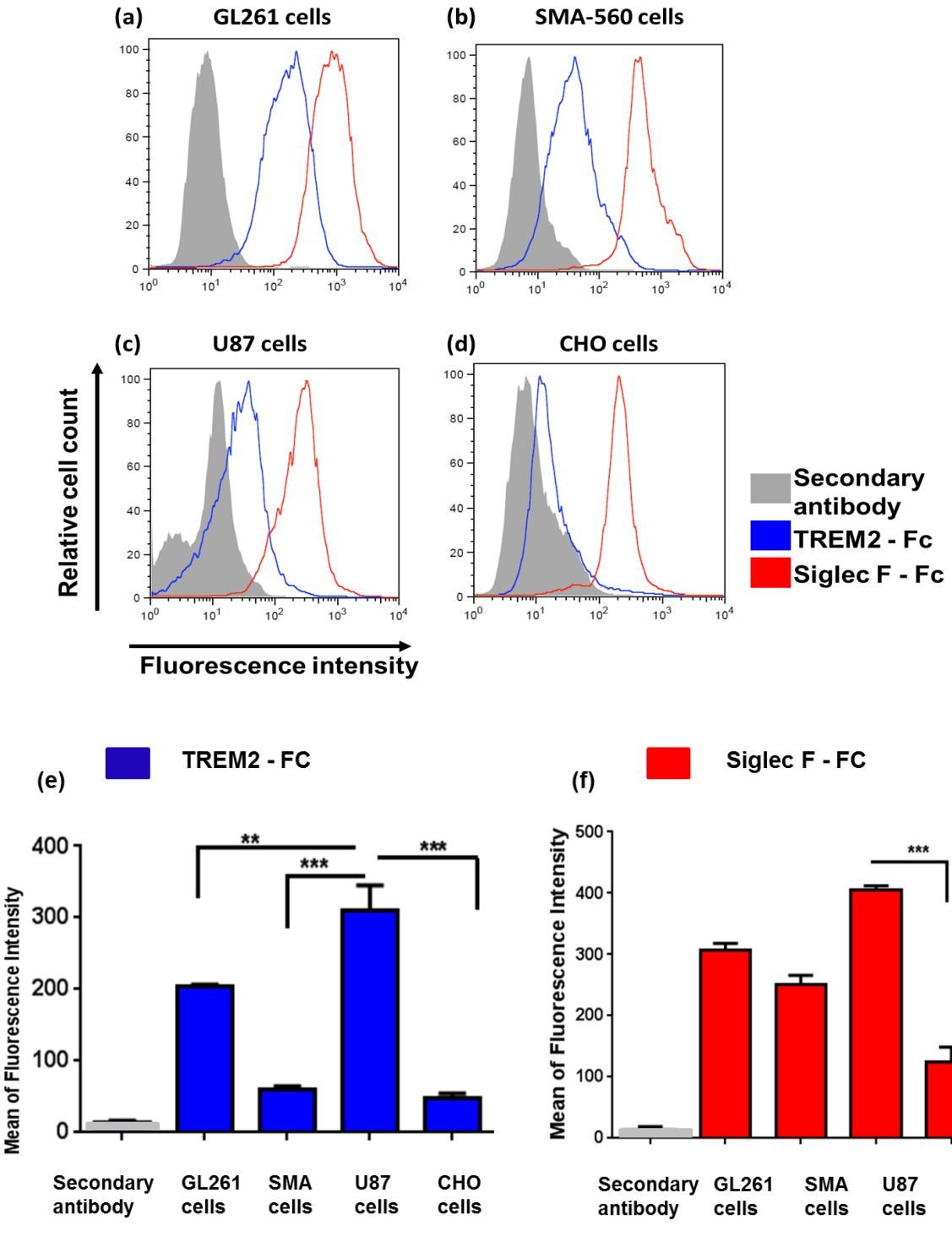

Figure 4.1 - TREM2b-Fc-fusion protein or Siglec F-Fc-fusion protein binding to U87, GL261, SMA-560, and CHO cells. Binding of mouse Siglec F-Fc (red) and TREM2b-Fc-fusion protein (blue) to (a) U87 cells, (b) GL261 cells, (c) SMA MS cells, and (d) CHO cells. Negative control (grey) - secondary antibody. (e and f) Representative images out of three independent experiments. (e) TREM2b-Fc-fusion protein binds to U87, GL261, SMA MS and CHO cells or (f) Siglec F-Fc-fusion protein binds to U87, GL261, SMA MS and CHO cell. Data are presented as mean±SEM, ANOVA – Bonferroni's multiple comparisons using post-hoc test: ***p< 0.001.

4.2 Removing of chondroitin sulfate groups of the glycocalyx of glioblastoma cells by chondroitinase ABC changed the binding of TREM2 to glioma cells

Bacterial ChABC was used to remove the chondroitin sulfate chains from to the glycocalyx of glioblastoma cells.

To test whether the pretreatment of U87 cells with ChABC can change the binding of mouse TREM2-Fc fusion protein to U87 cells, these cells received a pre-treatment with 100 mU/ml of ChABC for 24 hours at 37°C. U87 cells were used because, as shown before, they can increase the binding of TREM2-Fc fusion protein or Siglec F- fusion protein compared to other cell types. The Activity of Chondroitinase ABC of glioma cells can be seen in Figure 4.2 a. U87 cells were treated with 100 mU/ml of ChABC, partial inhibition of the binding of TREM2b-Fc fusion protein was seen when compared to untreated cells (Figure 4.2 b). But, when U87 cells were treated with 100 mU/ml of ChABC and were incubated with the positive control Siglec F-Fc fusion protein, no change in the binding of Siglec F was seen was seen (Figure 4.2 c). This proves that the TREM2 receptor can specifically recognize chondroitin sulfate groups altered.

Here we see that binding of TREM2b-Fc-fusion protein to the glioma cells is significantly reduced after the removal of chondroitin sulfate groups from the glycocalyx of the U87 cells (45.1 ± 2.4 fluorescence units, $p<0.001$) treatment when compared with untreated cells (310 ± 7.1 fluorescence units, $p<0.001$) (Figure 4.2 d). On the other hand, when the glioma cells were treated with ChABC and incubated with the mouse Siglec F-Fc-fusion protein, the results showed no significant change in the binding capacity of Siglec F-Fc-fusion protein (405.3 ± 4.3 fluorescence units, $p>0.05$) when compared with cells receiving no treatment (405.4 ± 4.4 fluorescence units, $p>0.05$) (figure 4.2 e).

The treatment of glioma cells with ChABC reduces the binding of mouse TREM2b-Fc-fusion protein to U87 cells, but does not change the binding of Siglec F-Fc fusion protein to U87 cells.

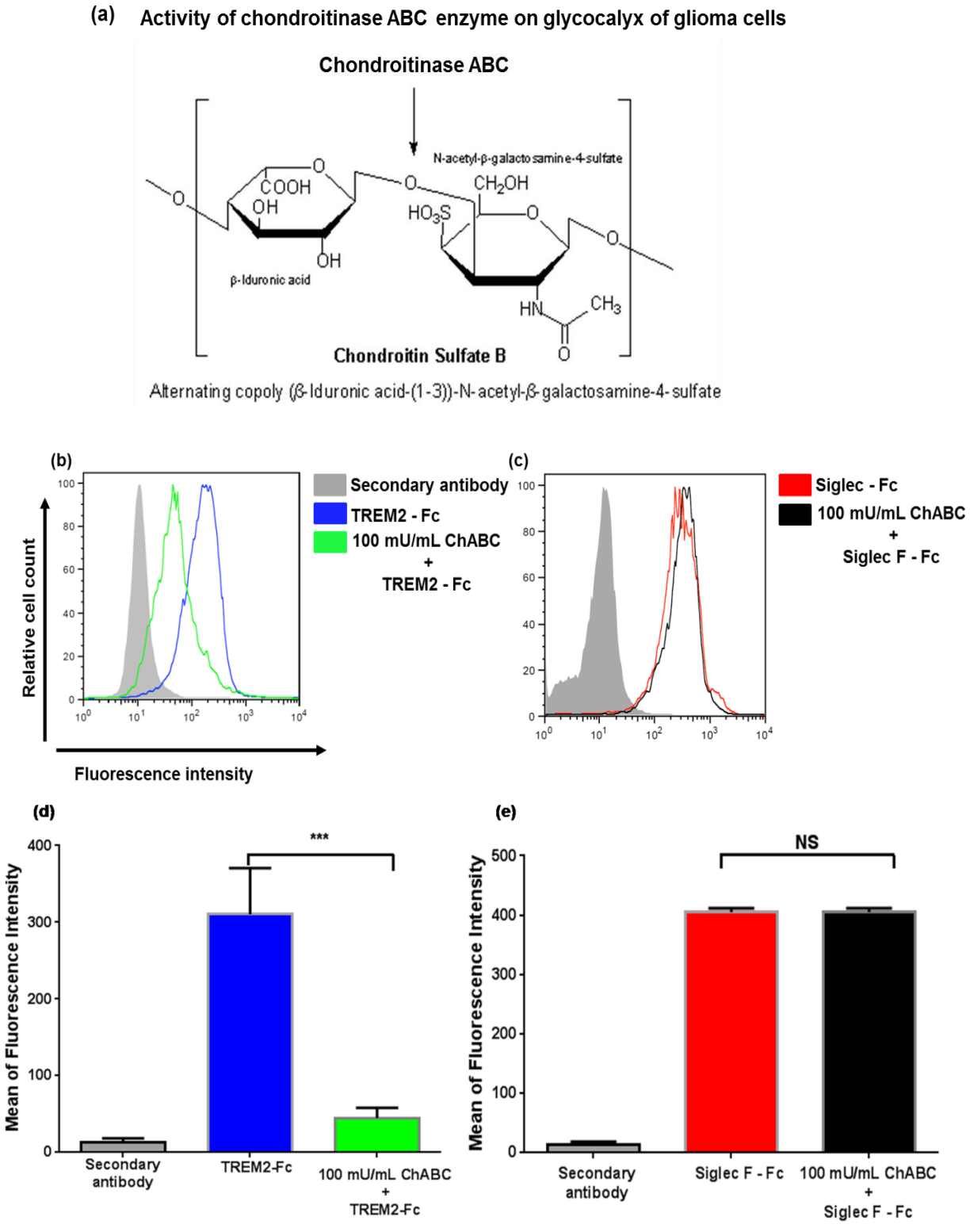

Figure 4.2 – Pretreatment of U87 cells with Chondroitinase ABC after 24 hours (a) Activity of Chondroitinase ABC. Chondroitinase ABC from Proteus vulgaris catalyzes the degradation of CS-A, CS-C, CS and dermatan sulfate mainly into disaccharides. **(b)** Binding of TREM2b-Fc-fusion protein (blue) is partially inhibited after incubation with 100 mU/ml of ChABC for 24 hours (green). **(c)** Binding of Siglec F-Fc-fusion protein (red) is not changed after incubation with 100 mU/mL of ChABC for 24 hours (black). **(d and e)** Graphics represent mean±SEM of fluorescence intensity of three different experiments of ChABC pretreated U87 cells. Secondary antibody control – grey. Data are presented as ANOVA - Bonferroni's multiple comparasions using post-hoc test: ***p<0.001 and NS: Not significant.

4.3 TREM2 is not dependent on removal of sialic acids from the glycocalyx of glioma cells

U87 cells were pretreated with 25 mU/ml sialidase for 2.5 hours at 37°C. Sialidase is a enzyme can hydrolyse alpha-(2->3)-, alpha-(2->6)-, alpha-(2->8)-glycosidic linkages of terminal sialic residues in oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates (Luo et al., 1998). That experiment was performed to prove that the TREM2 can binds specific in chondroitin sulfate group are expressed in glycocalyx of glioma cell and not in other structures. The activity of sialidase enzyme on glycocalyx of glioma cells is shown in Figure 4.3 a. Flow cytometry shows that pretreatment of U87 cells with sialidase reduces the binding of Siglec F-Fc-fusion protein to U87 cells (Figure 4.3 b). On the other hand, sialidase pretreatment of U87 cells does not change the binding of TREM2b-Fc fusion protein to glioma cells (Figure 4.3 c).

The results are given as mean ± SEM of fluorescence intensity of three independent experiments. The pretreatment of glioma cells with sialidase showed significant reduction of the binding of Siglec F-Fusion protein to U87 cells (29.7 ± 9.6 fluorescence units, $p<0.001$) when compared with untreated cells (254 ± 11 fluorescence units, $p<0.001$) (Figure 4.3 d). Interestingly, the glioma cells which were pretreated with sialidase and then incubated with TREM2b-Fc fusion protein showed no significant change the binding of TREM2b-Fc fusion protein to glioma cells (83 ± 9.6 fluorescence units, $p>0.05$) when compared to untreated cells (84 ± 9.7 fluorescence units, $p>0.05$) (Figure 4.3 e).

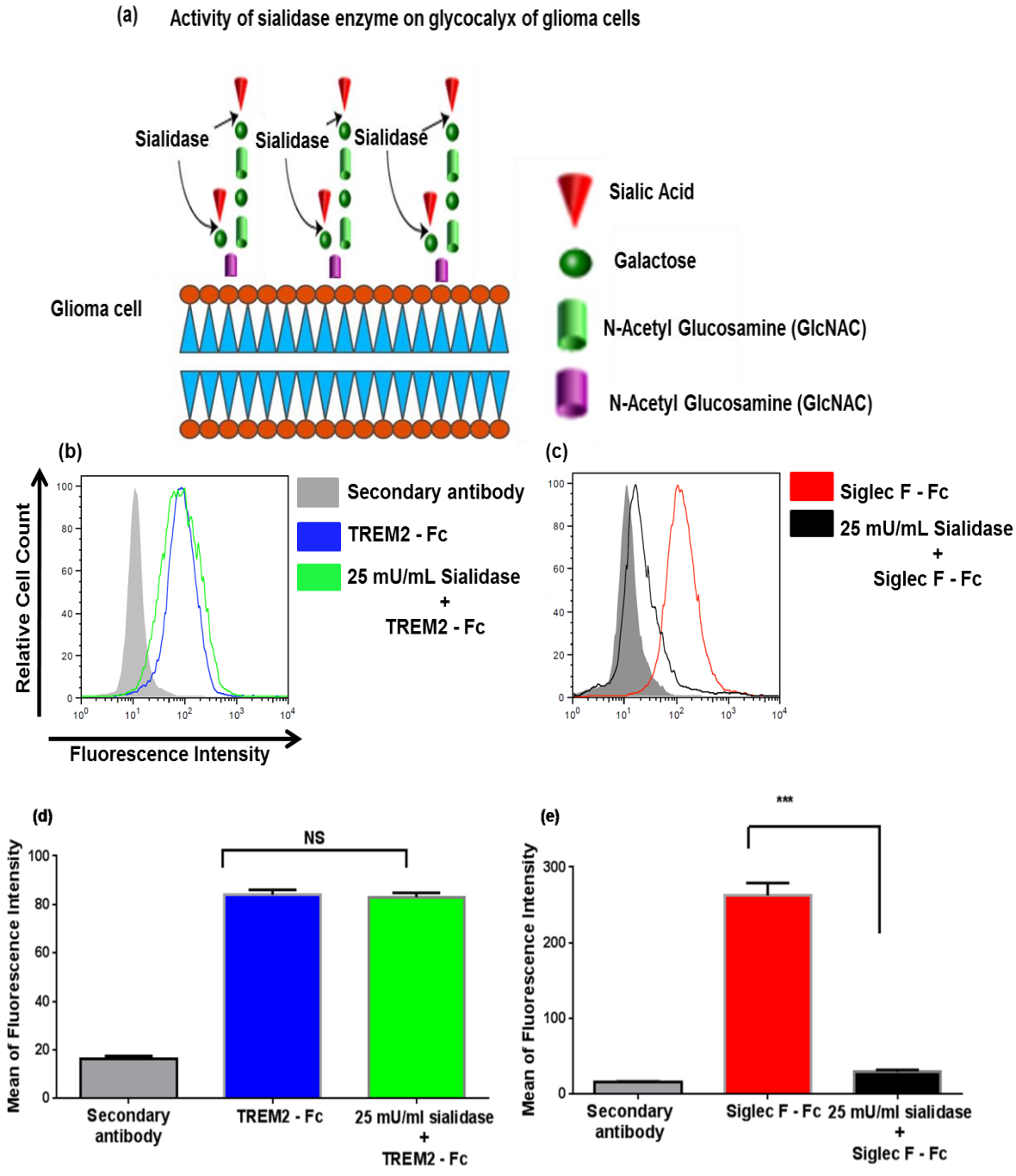

Figure 4.3 – Removal of sialic acids from the glycocalyx of U87 cells does not change the binding of TREM2 to glioma cells. **(a)** Activity of sialidase enzyme. Sialidase hydrolyses alpha-2,3-, alpha-2,6-, and alpha-2,8-glycosidic linkages of terminal sialic acid residues. **(b))** Binding of TREM2b-Fc-fusion protein to U87 cells (blue) did not change after pretreatment with 25 mU/ml of sialidase (green). **(c)** Binding of Siglec F-Fc-fusion protein to U87 cells (red) is nearly completely inhibited after incubation with 25 mU/ml of sialidase (black). **(d)** Graphic represent mean±SEM of fluorescence intensities of three different experiments of Siglec-F-Fc (d) or TREM2b-Fc fusion protein (e) binding after removal of sialic acids. Negative control is secondary antibody (grey). Data are presented as

ANOVA - Bonferroni's multiple multiple comparasions using post-hoc test ***p<0.001 and NS: not significant.

4.4 Chondroitin sulfate and dextran sulfate inhibit the interaction of TREM2b-Fc-fusion proteins with glioma cells

To test possible ligands as binding partners for TREM2, TREM2b-Fc-fusion protein was incubated with different glycosaminoglycans. The following glycosaminoglycans were tested:

1) Dextran sulfate (DXS),
2) Chondroitin polysulfate (Adequan),
3) Chondroitin sulfate A (chondroitin-4-sulfate),
4) Chondroitin sulfate C (chondroitin-6-sulfate),
5) Chondroitin sulfate (CS; have every sulfate group of CS: CS-A, CS-B, CS-C, CS-D and CS-E),

Table 4.1 – Mean±SEM, mean of of fluorescence intensity for inhibition of TREM2bFc-fusion protein binding to glioma cells. In absence of glycosaminoglycans the mean value of the binding of TREM2b-Fc fusion protein for every group is 310 ± 34.9.

Glycosaminoglycan	Concentration (mM)	Fluorescence Intensity (Mean ± SEM)
Dextran sulfate		
	0.2 mM	30.7 ± 6.4
	0.4 mM	31.2 ± 6.0
	1 mM	31.5 ± 5.0
	2 mM	32.3 ± 5.1
	4 mM	31.7 ± 9.0
Chondroitin sulfate		
	4 mM	79 ± 5.1
	8 mM	80.3 ± 2.6
	20 mM	78.8 ± 7.5
Chondroitin sulfate A		
	0.2 mM	49.3 ± 4.7
	0.5 mM	48.5 ± 1.2

	2 mM	52 ± 6.1
	10 mM	45 ± 3.2
Chondroitin sulfate C		
	0.1 mM	303 ± 8.6
	0.2 mM	327 ± 7.5
	0.4 mM	299 ± 5.2
	2 mM	342 ± 8.3
	4 mM	315 ± 7.7
Chondroitin polysulfate Adequan		
	0.3 mM	3.5 ± 8.7
	0.6 mM	4.4 ± 10.9
	1.6 mM	5.8 ± 4.2
	3 mM	13.6 ± 6.2
	6 mM	2.9 ± 7.1
	16 mM	3.8 ± 9.3
	30 mM	8.0 ± 19.7
	60 mM	8.0 ± 19.7
	130 mM	2.7 ± 4.3
	150 mM	2.4 ± 6.0
	200 mM	3.3 ± 8.2
	300 mM	2.9 ± 7.1

Synthetic glycosaminoglycans were tested by flow cytometry (Table 4.1). Results showed that the binding of TREM2b-Fc fusion protein to glioma cells changes after incubation with different glycosaminoglycans when compared to the controls. Binding of TREM2b-Fc fusion protein to glioma cells reduces after incubation with 2 mM of dextran sulfate (Figure 4.4 a), 3 mM of adequan (Figure 4.4 b), 8 mM of CS (Figure 4.4 c), 2 mM of CS-A (Figure 4.4 d). Different concentrations of CS-C cannot change the binding of TREM2b-Fc fusion protein to glioma cells (Figure 4.4 e).

To confirm that the TREM2b-Fc fusion protein cannot bind to other structures which are expressed on glycocalyx of glioma cells, positive control Siglec F-Fc fusion protein was used

and also incubated with 2 mM of CS-A. The results showed that CS-A cannot change the binding of Siglec Fc fusion protein to glioma cells when compared with untreated cells. That proves the TREM2 can bind and recognize specific CS group and not other components (Figure 4.4 f).

Thus, the binding of TREM2b-Fc-fusion protein to the glioma cells is mostly inhibited after incubation of cells with 2 mM of dextran sulfate 32.3 ± 5.1 when compared with untreated cells (control – just incubated with TREM2b-Fc fusion protein), (85.6 ± 2.5 fluorescence units, $p<0.001$). Adequan, a polysulfated glycosaminoglycan which is chemically similar to natural mucopolysaccharides found in cartilaginous tissues, was studied at different concentrations. The results show that the binding of the TREM2-Fc-fusion protein to U87 cells is completely inhibited with 3 mM adequan (13.6 ± 6.3 fluorescence intensity, $p<0.001$), (Figure 4.4 g).

The additionally, the biological substance CS was tested to demonstrate that binding of the TREM2b-Fc fusion protein to U87 cells can be inhibited at different levels of chondroitin sulfate (CS – contains every chondroitin sulfate group). The results show that binding of TREM2-Fc-fusion protein to U87 cells can be significantly partially inhibited after incubation with 8 mM of chondroitin sulfate (80.3 ± 2.6 fluorescence units, $p>0.05$) (Figure 4.4 g).

To confirm that the TREM2 can bind and recognize specific chondroitin sulfate groups and not other structures, Siglec F-fc fusion protein was used as a positive control. The results showed that when the Siglec F-Fc fusion protein was incubated with 2 mM chondroitin sulfate A (150 ± 2.6 fluorescence units, $p>0.05$) cannot inhibited the Siglec F-fc fusion protein binding to glioma cells (Figure 4.4 h) when compared with untreated cells (149 ± 2.5 fluorescence units, $p>0.05$). Together, these results show that the microglial TREM2 can bind to members of glycosaminoglycas such as chondroitin sulfate, which partialy inhibits the binding to glioma cells after incubation of cells with 2 mM chondroitin sulfate A.

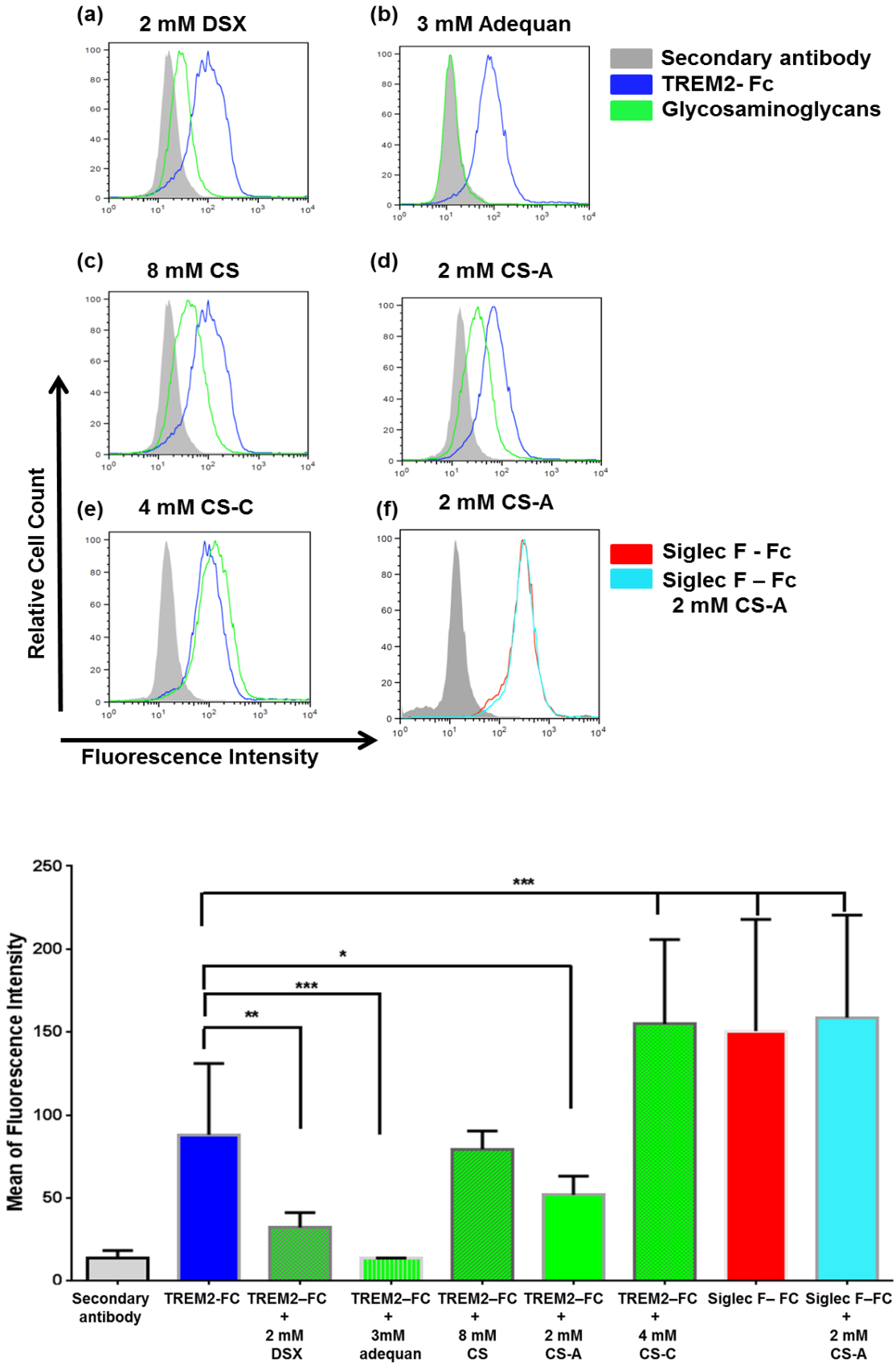

Figure 4.4 - Interaction between TREM2b-Fc-fusion protein and different polysaccharides. (a) Binding of TREM2b-Fc-fusion protein to U87 cells (blue) is partially inhibited after incubation with 2

mM of dextran sulfate (DXS; green). **(b)** Adequan 3mM (green) binds to TREM2b-Fc-fusion protein and can completely inhibit the binding to U87 cells. **(c)** Binding of TREM2b-Fc-fusion protein to U87 cells (blue) is partially inhibited with 8 mM of chondroitin sulfate (CS, green). **(d)** Binding of TREM2b-Fc-fusion protein to U87 cells (blue) is partially inhibited with 2 mM chondroitin sulfate A (CS-A, green). **(e)** Binding of TREM2b-Fc-fusion protein to U87 cells (blue) is not inhibited by 4 mM chondroitin sulfate C (CS-C, green). **(f)** Binding of Siglec F-Fc to U87 cells (red) is not inhibited by 2 mM Chondroitin sulfate A (CS-A, light blue). **(g and h)** Graphic with means±SEM of fluorescent intensities of three different experiments of the binding of the TREM2b-Fc-fusion protein (g) and Siglec F Fc fusion protein (h) to concentrations of different glycosaminoglycans showing the strongest inhibitory effect. Control: secondary antibody (grey). The statistical analyzes compared the values of mean of fluorescence intensity from each single glycosaminlgycans to TREM2b-Fc fusion protein alone. Data are presented as mean±SEM, ANOVA - Bonferroni's multiple comparasions using post-hoc test: ***p<0.001, **p<0.01 and NS: not significant. GAGs: Glycosaminoglycans.

4.4.1 Chondroitin sulfate disaccharides inhibit the binding of TREM2b-Fc fusion protein to glioma cells

The abilities of disaccharides DS-4S and DS-OS to bind to mouse TREM2b-Fc-fusion protein were examined by flow cytometry. The results showed that a concentration of 0.2 mM of DS-4S can partially inhibit binding of mouse TREM2b-Fc-fusion protein to U87 glioma cells. Whereas, 0.2 mM of DS-0S cannot inhibit the binding of mouse TREM2b-Fc-fusion protein to glioma cells (Figure 4.5 a and b).

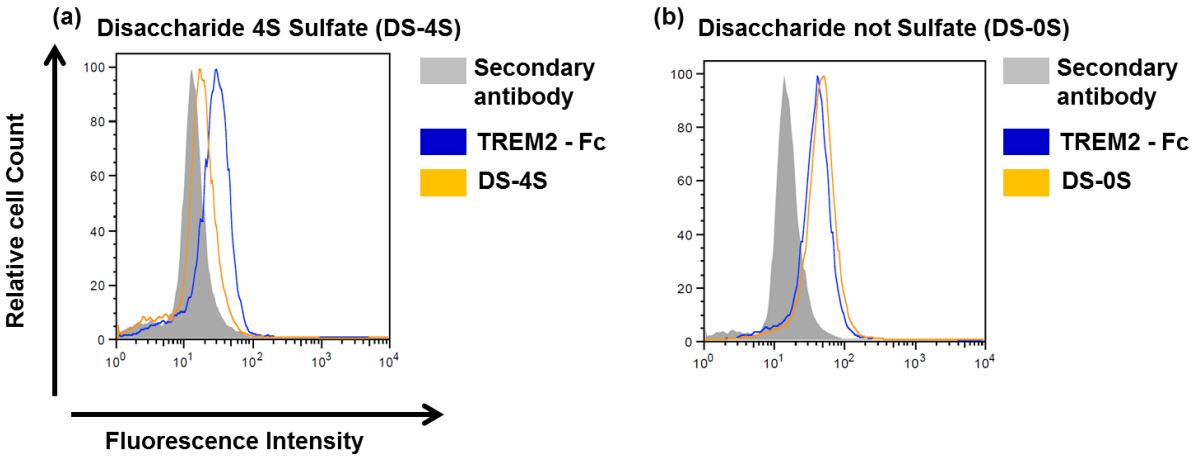

Figure 4.5 – FACS analysis of the binding capacity of disaccharide 4 sulfate or disaccharide non-sulfated to mouse TREM2b-Fc-fusion protein. (a) The TREM2b-Fc-fusion protein binds to U87 cells (blue) and the binding of disaccharides 4 sulfate (DS-4S) to TREM2b-Fc fusion protein is partially inhibited after 0.2 mM DS-4S (orange). **(b)** The binding of disaccharides non-sulfate (DS-

0S) to TREM2b-Fc-fusion protein is not inhibited after incubation with 0.2 mM DS-0S (orange). The TREM2b-Fc fusion protein binds to U87 cells (blue). Control: secondary antibody control (grey).

4.1.1. Analysis of TREM2 function in mouse microglia cell line

Since specific ligands for TREM2 receptor remain unclear, one of the main goals was to find a new ligand for TREM2 and to understand the role TREM2 plays in microglia. For this, microglia cell line (ESdM) was analyzed for expression of TREM2 receptor. Furthermore, TREM 2 expression under stimulation with CS-A or Disaccharides (DS-4S and DS-0s) at varying concentrations and time points was analyzed.

4.1.2. Detection of TREM2 expression in ESdM microglial cell line

To investigate the function of TREM2 in mouse microglia, embryonic stem cells derived microglial precursor cells (ESdM) were used. To detect TREM2 protein levels, flow cytometry and a specific mouse polyclonal antibody directed against TREM2 were used. ESdM cells were showing expression of TREM2 on their surface (Figure 4.6). Thus, data are proving that ESdM microglia cell line has an expression of TREM2 at protein leve.

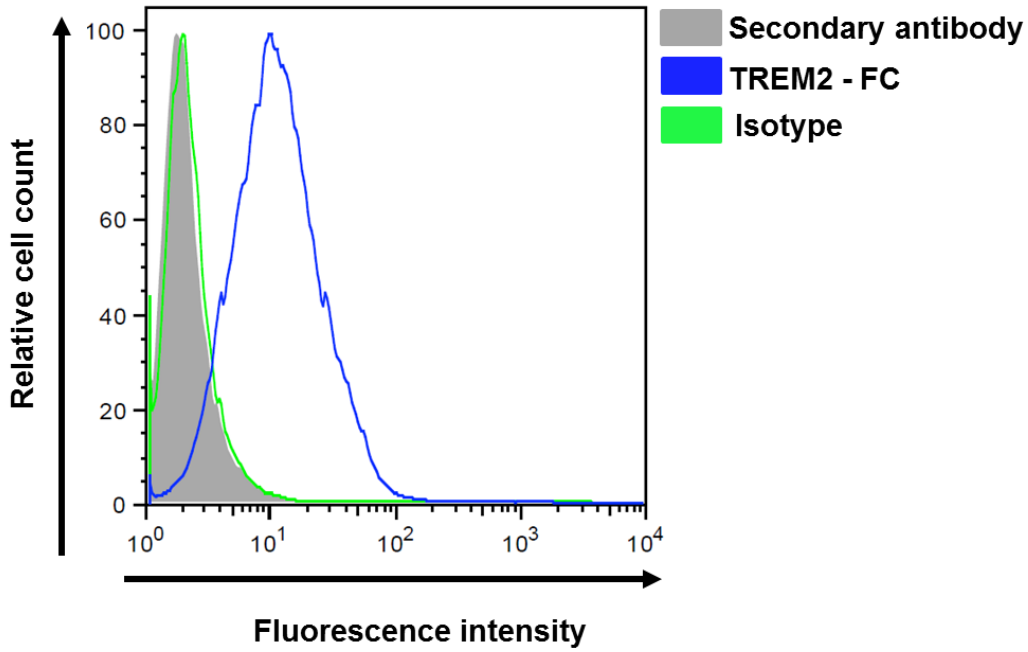

Figure 4.6 – Flow cytometry analysis of TREM2 in ESdM. ESdM cells showed expression of TREM2 when stained with TREM2 antibody (blue). Isotype control (blue). Secondary antibody (grey).

4.1.3. Effects of chondroitin sulfate or chondroitin disaccharide sulfate stimulation on microglia cell line in terms of phagocytosis

The phagocytic capacity of microglia after stimulation with possible ligands of TREM2 receptor was analyzed. Initially, the phagocytosis assay was evaluated after stimulation of ESdM cells with different concentrations of CS-A (blue) (Fig. 4.7a) and during different time points (Fig. 4.7b). The results indicate that 2 mM of CS-A led to a significant increase in ESdM phagocytosis capacity (38.9 ± 22.5 percentage units, $p<0.001$) when compared with untreated cells (black) (21.2 ± 2.3 percentage units, $p<0.001$; Figure 4.7 a). Two hours stimulation of microglia cells with 2 mM of CS-A showed significantly increases of beads phagocytosis (34.9 ± 5.2 percentage units, $p<0.001$) when compared with untreated cells (18.0 ± 3.7 percentage units, $p<0.001$; Figure 4.7 b).

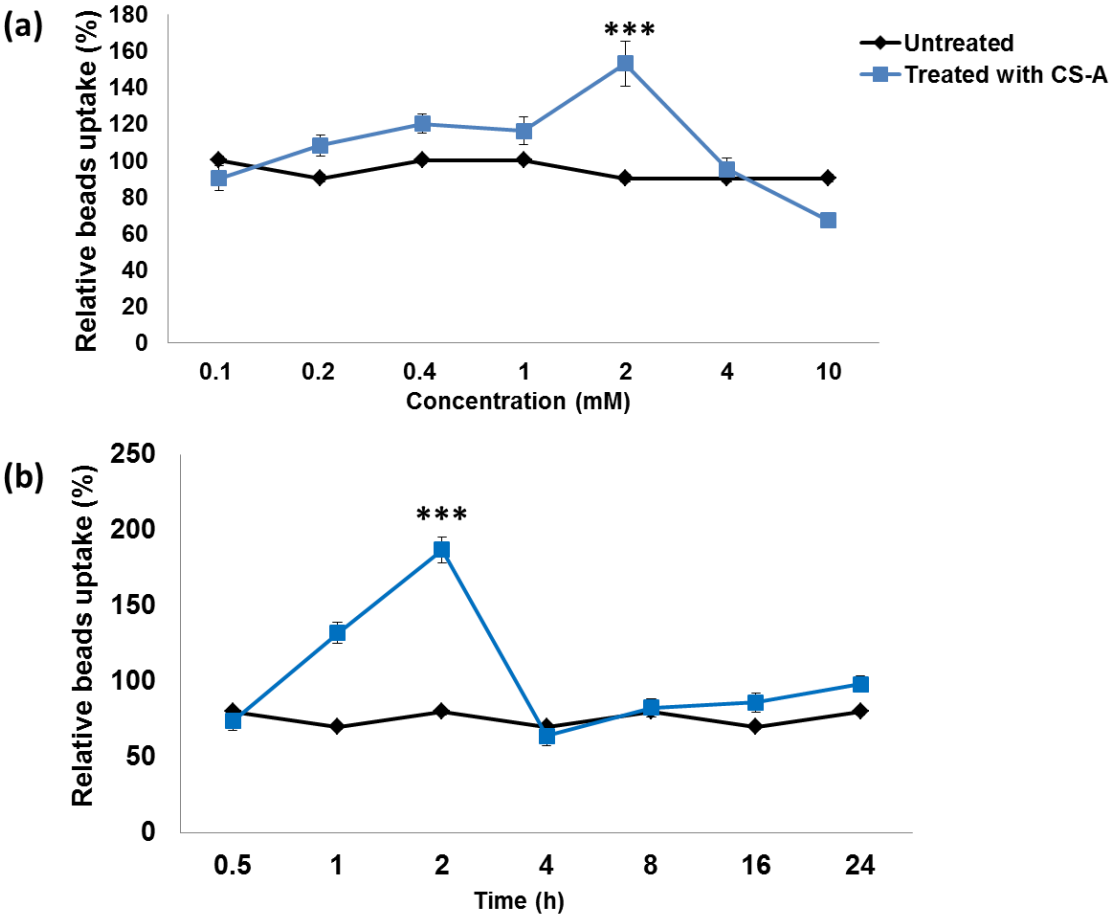

Figure 4.7 – CS-A stimulates phagocytosis in a concentration and time point dependent way. (a) CS-A stimulates phagocytosis in concentration dependent pattern. Relative numbers of phagocytosis in ESdM cells were determined by flow cytometry. ESdM cells were incubated with TREM2 ligand CS-A in different concentration (0.1 mM, 0.2 mM, 0.4 mM, 1 mM, 2 mM, 4 mM and 10 mM) and afterwards with beads. **(b) CS-A stimulates phagocytosis in time dependent way.** ESdM cells were incubated with the TREM2 ligand CS-A (2 mM) for different time intervals (0.5h, 1h, 2h, 4h, 8h, 16h and 24 h and then beads phagocytosis capacity was evaluated by flow cytometry. Data are presented as a mean±SEM. ANOVA - Bonferroni's multiple comparasions using post-hoc test: ***p<0.001.

4.4.2 Chondroitin sulfate disaccharide increases the phagocytosis dependent on time and concentration

DS-4S can bind to TREM2 and increases beads phagocytosis after stimulation with different concentrations (0.001 mM, 0.004 mM, 0.02 mM, 0.01 mM, 0.2 mM, 0.1 mM and 2 mM) and at different incubation times (30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 16 hours and 24 hours). ESdM cells after stimulation with 0.02 mM (42.87 ± 3.8 percentage units, P<0.001) and 0.2 mM (52.3 ± 1.8 percentage units, p<0.001) of DS-4S for 1 hour show a significant increase in beads phagocytosis (orange), when compared with untreated cells (black) 0.02 mM (11.2 ± 2.1 percentage units, p<0.001) and 0.2 mM 10 ± 3.6 percentage units, p<0.001) of DS-4S; Figure 4.8 a).

After stimulation of microglia cells with 0.2 mM of DS-4S for different incubation times (30 minutes 61.4 ± 10.4 percentage units, p<0.001), 1 hour (54.7 ± 7.7 percentage units, p<0.01), 2 hours (0.0 ± 3.9 percentage units, p<0.01) 4 hours (43.5 ± 2.5 percentage units, p<0.01) and 8 hours (53.1 ± 5.2 percentage units, p<0.001) showed significantly increases of beads phagocytosis when compared with untreated cells in respective times (30 minutes 17.0 ± 1.3 , p<0.001, 1 hour 10.4 ± 1.8 p<0.001 , 2 hours 24.3 ± 1.0, 4, p<0.01, 4 hours 22.1 ± 2.7, p<0.01 and 8 hours 23.5 ± 1.7 percentage units, p<0.001; Figure 4.8 b).

The non sulfated disaccharide DS-0S was also tested in ESdM cells with different concentrations and also at different time points of stimulation. The results are not shown here because no difference of beads uptake was observed between the timing and concentrations. In summay, the results here showed that the chondroitin sulfate can stimulate and increase the beads phagocytosis in microglia cell line.

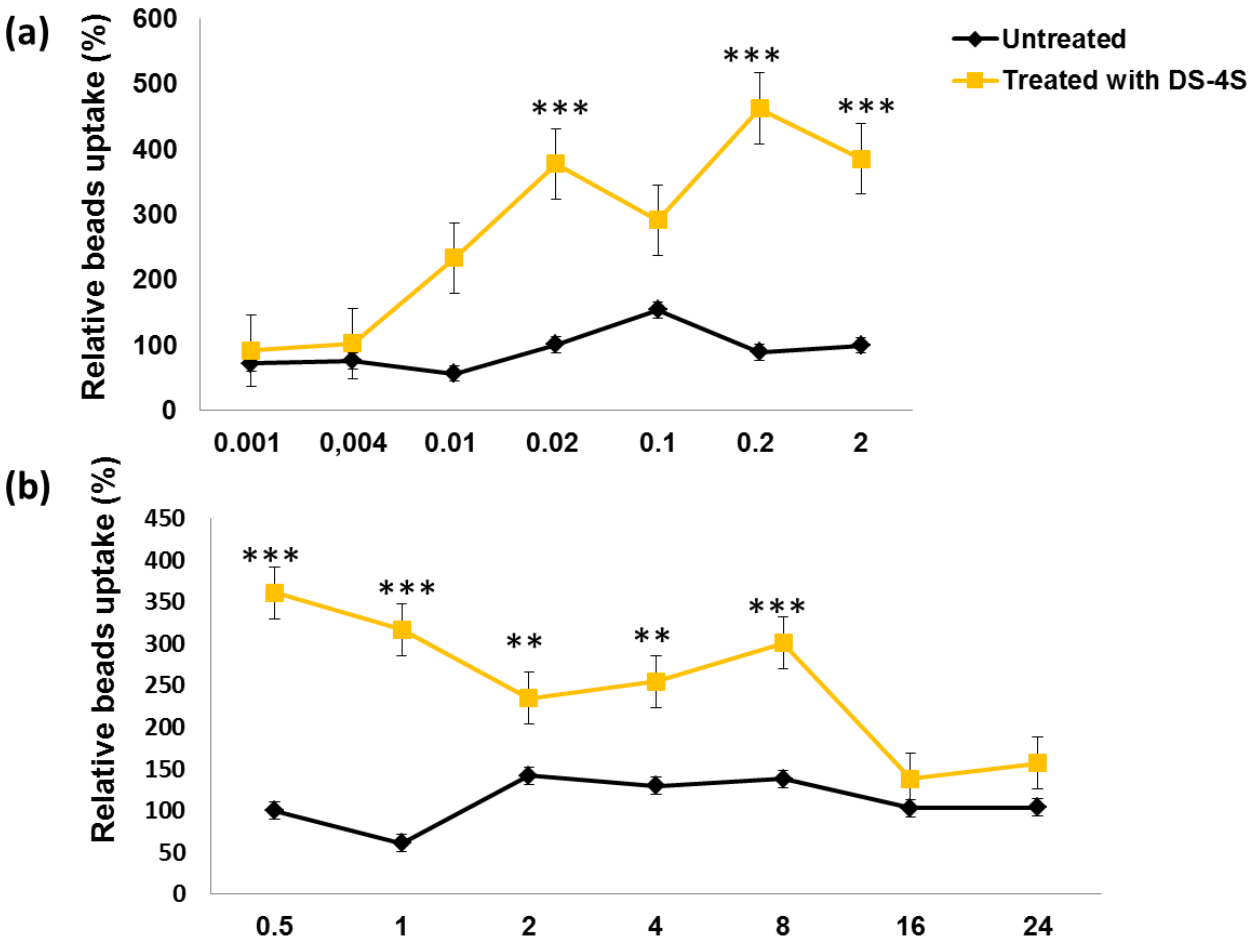

Figure 4.8 – DS-4S stimulates phagocytosis in a concentration and time point dependent way. (a) DS-4S stimulates phagocytosis in concentration dependent pattern. Relative numbers of phagocytosis in ESdM cells were determined by flow cytometry. ESdM cells were incubated with TREM2 ligand CS-A in different concentration (0.001 mM, 0.004 mM, 0.02 mM, 0.01mM, 0.2 mM, 0.1 mM and 2 mM) and afterwards during 30 minutes at 37 ºC with beads. **(b) DS-4S stimulates phagocytosis in time dependent way.** ESdM cells were incubated with the TREM2 ligand DS-4S (0.2 mM) for different time intervals (0.5h, 1h, 2h, 4h, 8h, 16h and 24 h and then beads phagocytosis capacity was evaluated by flow cytometry. Data are presented as a mean±SEM. ANOVA - Bonferroni's multiple comparasions using post-hoc test: ** $p<0.01$, ***$p<0.001$.

4.4.3 Effects of TREM2 knock down on beads phagocytosis

To study the effect of TREM2 on microglial phagocytosis, we used a lentiviral strategy to knockdown TREM2. ESdM cells were transduced with 3 different lentiviral vectors for TREM2 knockdown (shTREM2 – plasmids 1, 2 and 3) or a control vector that expressed a short hairpin scrambled sequence (short hairpin control [shRNA control]).

To confirm the knockdown, TREM2 gene transcripts were analyzed by RT-PCR. Indeed, a lower transcriptional level for TREM2 were detected in ESdM cells transduced with TREM2 knockdown plasmids number 1 (0.17 ± 0.06, $p<0.001$), 2 (0.3 ± 0.06, $p<0.001$) and 3 (0.049 ± 0.06, $p<0.001$) when compared with the shRNA control (1.2 ± 0.008, $p<0.001$; Figure 4.9 a).

Cells were stimulated with different ligands of TREM2 of CS-A (2 mM), DS-4S (0.2 mM) and with DS-0S (0.2 mM) unsulfatedas a control. TREM2 KD P1, P2 and P3 receiving ESdM cells have not shown significant differences of beads phagocytosis after stimulation with 2 mM of CS-A, 0.2 mM of DS-4S or 0.2 mM of DS-0S when compared with untreated TREM2 P1, P2 and P3 knockdown ESdM cells. On the other hand, the shRNA scramble control cells (shRNA vector) shown significant increase of beads phagocytosis after stimulation with 2 mM of CS-A (55.1 ± 1.1 percentage units, $p<0.001$), 0.2 mM of DS-4S (56.2 ± 1.1 percentage units, $p<0.001$) or 0.2 mM DS-0S (57 ± 1.8 percentage units, $p<0.001$) when compared with untreated cells (36.5 ± 0.1 percentage units, $p<0.001$; Figure 4.9 b).

In conclusion, these experiments prove that microglial cells in are dependent on expression of TREM2 receptor on the for beads up-take.

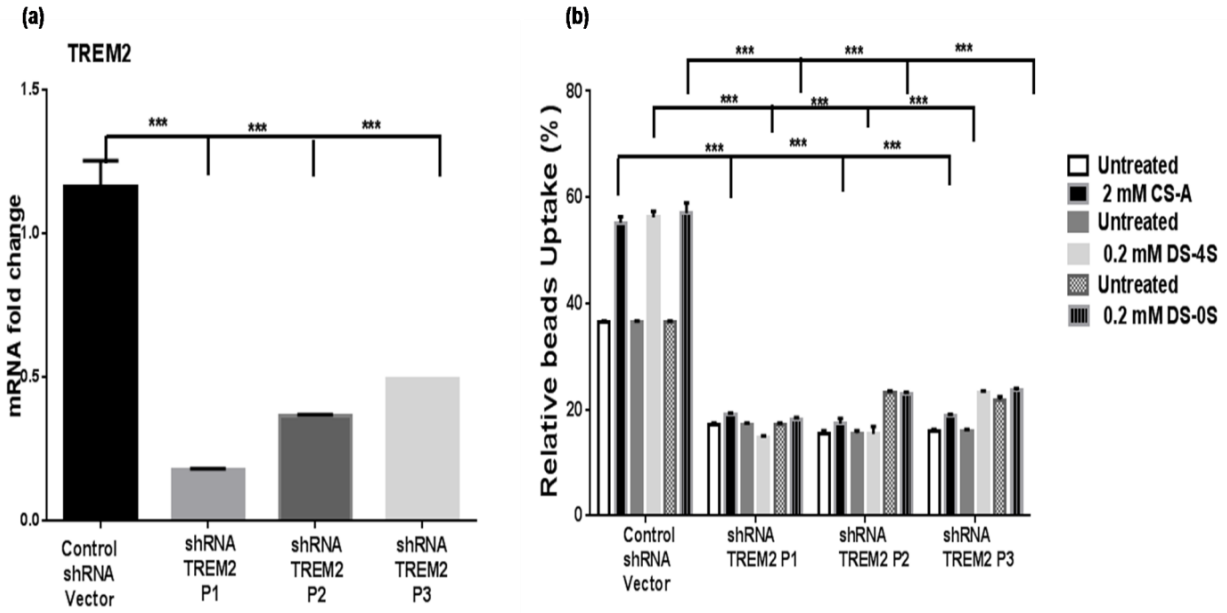

Figure 4.9 – Knockdown of TREM2 in ESdM. (a) RT-PCR analysis of cultured ESdM cells transduced either with a lentiviral vector producing short hairpin RNA specific for TREM2 (shRNA P1, P2 or P3) or irrelevant short hairpin RNAs (shRNA – vector). **(b) Beads uptake after TREM2 knockdown in ESdM cells.** TREM2 knockdown in ESdM cells decreases beads phagocytosis even in the presence of chondroitin sulfate A and chondroitin sulfate disaccharides .The graphic represent mean± SEM of fluorescence intensity from three independent experiments, Data are presented as mean±SEM, ANOVA Bonferroni's multiple comparasions using post-hoc test: ***p<0.001.

4.4.4 Effects of chondroitin sulfate A and chondroitin sulfate disaccharides on DAP12 full length versus truncated DAP12 on microglia

DAP12 is the adaptor molecule of TREM2 and has an immunoreceptor tyrosine based activation motif (ITAM) (Paloneva et al., 2000).

After stimulation of microglial cells with CS-A, beads phagocytosis was increased (Figure 4.10 a). To prove the involvement of TREM2 receptor in phagocytosis, cells were stimulated with CS-A and DS-4S ligands of TREM2), using DS-0S as internal control. ESdM cells over-expressing full length DAP12, ESdM cells having a truncated ITAM domain of DAP12 and ESdM control cells were stimulated with 2 mM of CS-A for 2 hours and with 0.2 mM of DS-4S or DS-0S for 1 hour based on previous results.

After stimulation of DAP12-overexpressing ESdM cells with CS-A (72.3 ± 1.0 percentage units, $p<0.001$) or DS-4S (71.4 ± 2.4 percentage units, $p<0.001$), show significant increase in beads phagocytosis was seen when compared with untreated treated control cells (GFP) (CS-A: 64 ± 1.1, percentage units $p<0.001$, DS-4S: 61.2 ± 0.2 percentage units, $p<0.001$). ESdM cells having a truncated ITAM domain of DAP12 showed significant difference in beads

phagocytosis after stimulation with CS-A (16.9 ± 2.9, percentage units p<0.001), DS-4S (10.3 ± 0.8 percentage units, p<0.001) or DS-0S (13.8 ± 0.8 percentage units, p<0.001) when compared to treated control cells (GFP) (CS-A: 64.1 ± 1.1, p<0.001, DS-4S: 61.2 ± 0.2, p<0.001 and DS-0S: 51.3 ± 3.5 percentage units, p<0.001, respectively). The control cells (GFP) showed significant increase of beads phagocytosis after stimulation with CS-A (64.1 ± 1.4 percentage units, p<0.001 in percentage units), DS-4S (61 ± 0.2 percentage units, p<0.001) or DS-0S (51.3 ± 3.5, percentage units p<0.001) when compared to untreated cells from the same group of cells (GFP) (35.2 ± 1.2, p<0.001, 32 ± 1.2, p<0.001 and 31 ± 1.3, percentage units p<0.001, respectively). In conclusion, these results show that the phagocytosis by microglial cell line is mediated via DAP12 (Figure 4.10).

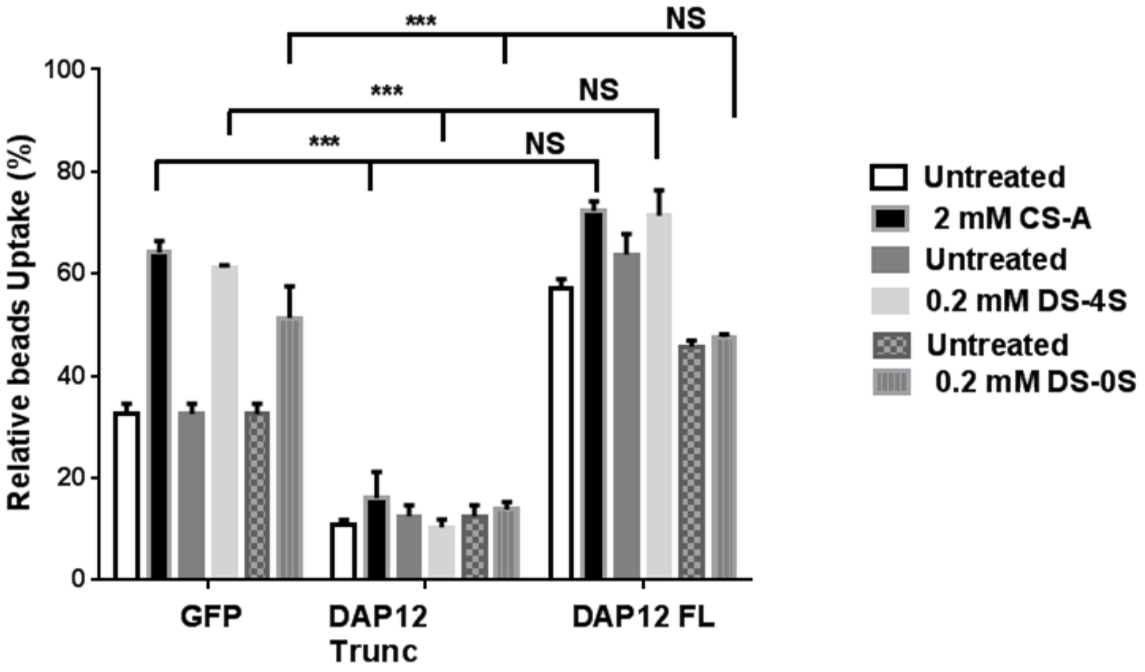

Figure 4.10 – Phagocytosis of beads is dependent on functional DAP12 expression and can be enhanced by CS-A or DS-4S. Over-expression of DAP12 increases beads phagocytosis**.** ESdM GFP cells are controls cells, ESdM DAP12 Trunc cells have truncated ITAM domain and DAP12 Fl. Cells have overexpression of full length. Data are presented as mean±SEM. ANOVA-Bonferroni's multiple comparasions using post-hoc test: ***p<0.001, NS: not significant.

4.4.5 Effects of chondroitin sulfate A and chondroitin sulfate disaccharides on microglia cells by analyses of pro- and anti- inflammatory cytokines

ESdM cells were stimulated with 2 mM CS-A, 0.2 mM chondroitin sulfate disaccharide DS-4S or 0.2 mM unsulfated disaccharide DS-0S for 30 minutes, 1 hour or 2 hours at 37 ºC. Afterwards, transcriptions of pro- and anti- inflammatory cytokines were analyzed by RT-PCR. Values collected from these cells after stimulation with CS-A, DS-4S or DS-0S were normalized to those without stimulation.

The results showed that 2 mM of CS-A significantly increases the transcription of the pro-inflammatory cytokines IL-1ß and TNF-α after 1 and 2 hours of incubation with CS-A (IL-1ß, 1 hour (62 ± 0.33 and 2 hours 352 ± 0.34; Fig. 4.11 a) and TNF-α, 1 hour (62.7 ± 0.47 and 2 hours 353 ± 1.0: fig. 411 b) when compared with unteated cells 1 hour (0.52 ± 0.22 and 2 hours 1.3 ± 0.3). The transcription of iNOS decreased significantly after 30 minutes and stayed decreased after 1 hour and 2 hours of incubation with 2 mM of CS-A when compared with untreated cells (1 hour (0.89 ± 0.03 and 2 hours 1.3 ± 0.3; Fig. 4.11 c), iNOS expression after 30 minutes (0.029 ± 0.06) 1 hour (0.049 ± 0.06) and 2 hours (0.58 ± 0.05) was observed Fig. 4.11 c. Stimulation with CS-A significantly increased the transcription of the anti-inflammatory cytokine IL-10 during the different incubation with different times when compared with untreated cells (1.6 ± 0.6, 30 minutes 7.7 ± 0.5, 1 hour 3.8 ± 0.5 and 2 hours 3.9 ± 0.6; Fig. 4.11 d).

On the another hand, after stimulation of ESdM cells with 0.2 mM of DS-4S the gene expression of pro-inflammatory cytokines of IL-1ß, TNF-α and iNOS were increased. The expression of IL-1β was significantly increased after 30 minutes (9.9 ± 0.3; Fig. 4.12 a), but not significantly increased after 1 hour (1.8 ± 0.3) and 2 hours (1.3 ± 0.3) when compared to untreated cells (1.3 ± 0.6; Fig 4.12 a). The expression of TNF-α was also significantly increased 30 minutes (21.5 ± 0.3) after stimulation with DS-4S when compared to untreated cells (1.3 ± 0.3) and after 1 hour (6.9 ± 0.3) of stimulation, expression of TNF-α was significantly increased when compared to untreated cells (3.1 ± 0.3; Fig. 4.12 b). Interestingly, the expression of pro-inflammatory cytokines of IL-10 was significantly increased after 30 minutes (20.1 ± 0.3) and 1 hour (3.9 ± 0.3) of stimulation with DS-4S in ESdM cells when compared with untreated cells (1.3 ± 0.3, 1.4 ± 0.03, respectively; Fig. 4.12 c).

The results with stimulation of DS-0S on ESdM cells showed no strong effects on the production of proinflammatory cytokines during different times periods after stimulation. The

transcription expression of IL-1β showed significant increase after stimulation for 30 minutes (0.8 ± 0.03) and 2 hours (0.7 ± 0.2) when compared to untreated cells (1.4 ± 0.3 and 0.9 ± 0.03, respectively; Fig. 4.13 a). The transcription expression of TNF-α after stimulation with DS-0S was significantly increased after 30 minutes (0.8 ± 0.03) and 2 hours (1.3 ± 0.1) when compared to untreated cells (1.3 ± 0., 0.9 ± 0.03; Fig 4.13 b). The iNOS transcription expression showed significant increase after 30 minutes (0.8 ± 0.03) of stimulation when compared with untreated cells (1.3 ± 0.3: Fig. 4.13 c). Stimulation with DS-0S does significantly increase the transcription of the anti-inflammatory cytokine IL-10 during the different incubation with 30 minutes (0.4 ± 0.06) and 2 hours (1.3 ± 0.1) when compared with untreated cells (0.6 ± 0.03, 0.5 ± 0.03; Fig. 4.13 d). In the end, these results confirm that the sulfated groups of chondroitin sulfate are maybe involved in microglia phagocytosis mediated via TREM2 receptor.

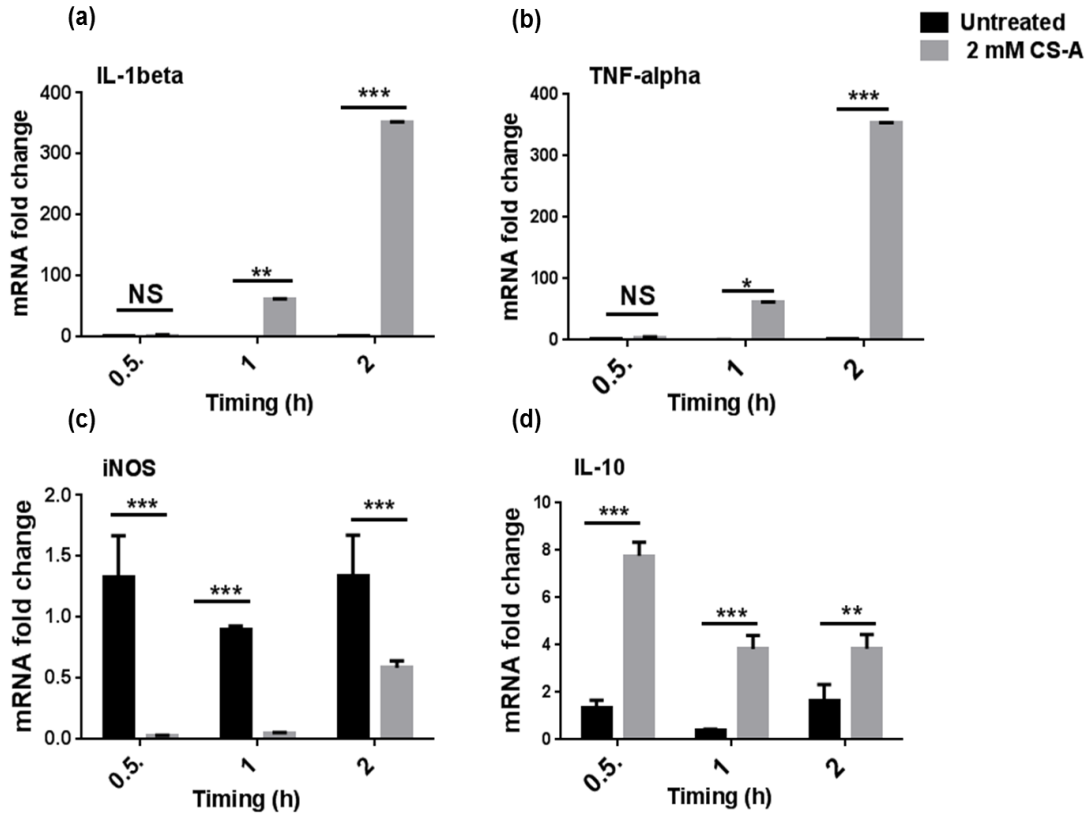

Figure 4.11 – Anti- and pro-inflammatory mRNA profile after stimulation of microglia cell line cells with CS-A. qRT-PCR was carried out to detected IL-1ß, TNF-α, iNOS and IL10 in ESdM cells after stimulation with 2 mM of CS-A during 30 minutes, 1 hour and 2 hours. (a) Microglia cell line show significantly increased of mRNA transcriptional level expression of IL-1ß after stimulation with 2 mM of CS-A during 1 hour and 2 hours (grey) when compared with untreated cells (black). (b) Microglia cell line show significantly increased of mRNA transcriptional level expression of TNF-α after stimulation with 2mM of CS-A during 1 and 2 hours (grey) when compared with untreated

cells(black). (c) iNOS mRNA transcriptional level expression in microglia cell line show decreased when compared with untreated cells after 30 minutes, 1 hour and 2 hours of treatment with 2 mM of CS-A. (d) Treatment of microglia cell line with 2 mM of CS-A after 30 minutes, 1 hour and 2 hours increased transcription of anti-inflammatory cytokine IL-10. Data are presented as mean±SEM. ANOVA-Bonferroni's multiple comparasions using post-hoc test: ***p<0.001, **p<0.01, * p<0.05 and NS: not siginificant.

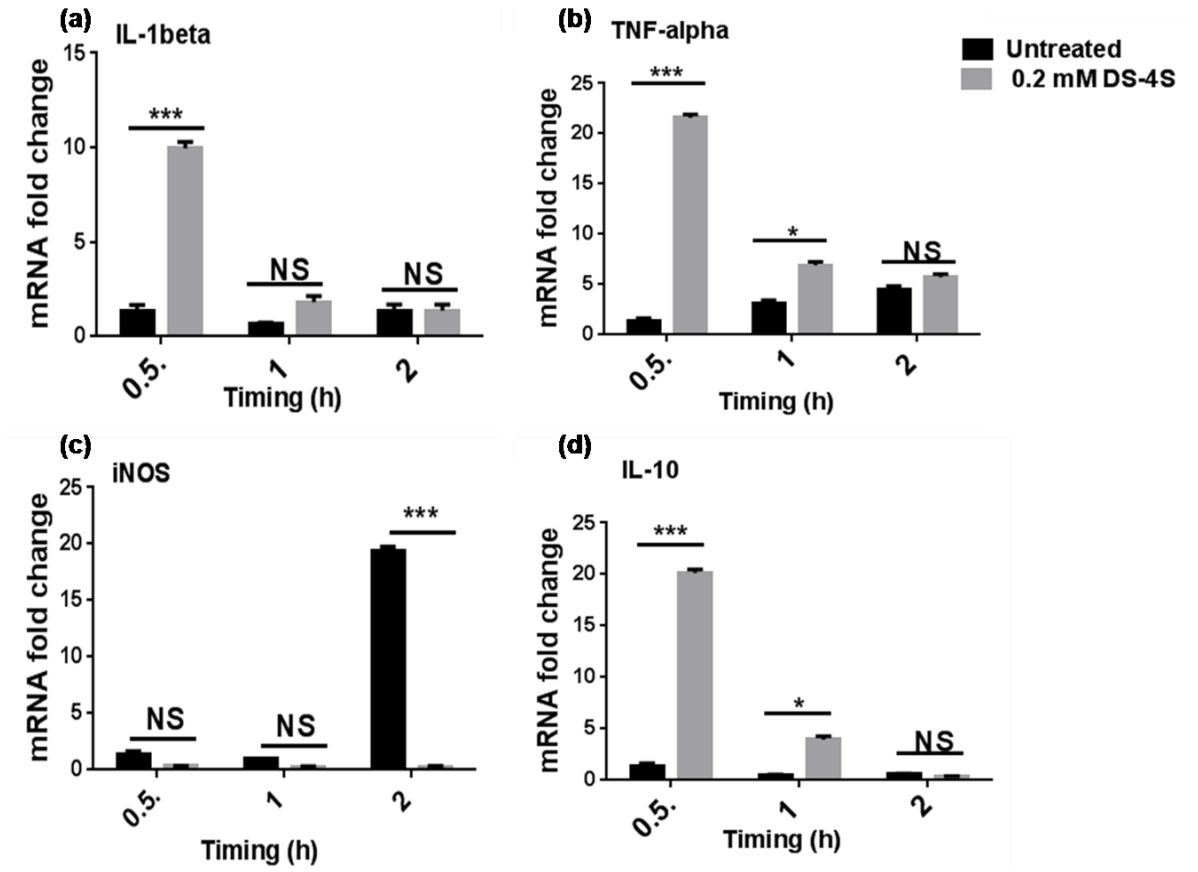

Figure 4.12 – Anti- and pro-inflammatory mRNA expression profile after stimulation of microglia cell line cells with DS-4S. qRT-PCR to detected IL-1ß, TNF-α, iNOs and IL10 in ESdM cells ater stimulated with 0.2 mM of DS-4S during 30 minutes. (a) Microglia cell line show significantly increased of mRNA transcriptional level expression of IL-1ß after stimulation with 0.2 mM of DS-4S during 30 minutes (grey) when compared with untreated cells (black). (b) Microglia cell line show significantly increased of mRNA transcriptional level expression of TNF-α after stimulation with 0.2 mM of DS-4S during 30 minutes and 1 hour (grey) when compared with untreated cells (black). (c) iNOS mRNA transcriptional level expression in microglia cell line show decreased when compared with untreated cells after 2 hours of treatment with 0.2 mM of DS-4S. (d) Treatment of microglia cell line with 0.2 mM of Ds-4S after 30 minutes and 1 hour significantly increased transcription of anti-inflammatory cytokine IL-10. Data are presented as mean±SEM. ANOVA - Bonferroni's multiple comparasions using post-hoc test: ***p<0.001, **p<0.01, * p<0.05 ande NS: not siginificant.

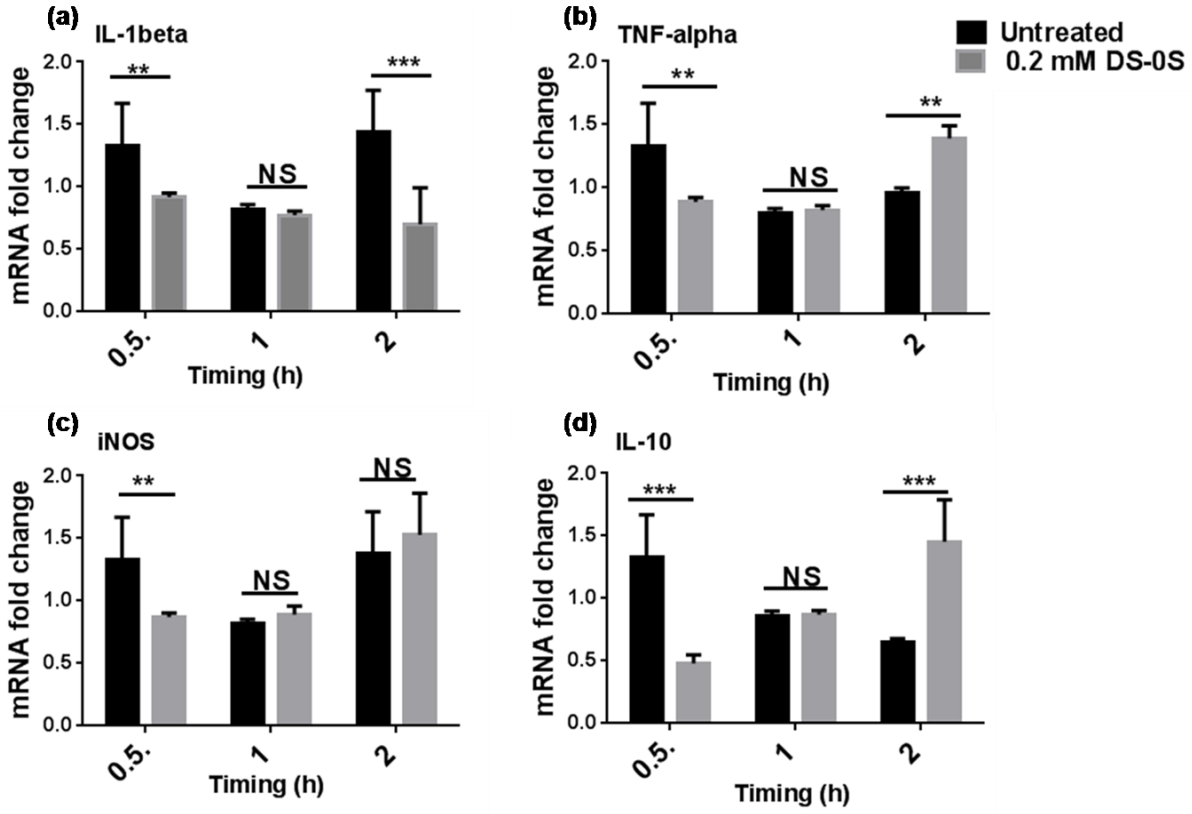

Figure 4.13 – Anti- and pro-inflammatory mRNA expression profile after stimulation of microglia cell line cells with DS-0S. qRT-PCR to detected IL-1ß, TNF-α, iNOs and IL10 in ESdM cells ater stimulated with 0.2 mM of DS-0S during 30 minutes. (a) Microglia cell line show significantly increased of mRNA transcriptional level expression of IL-1ß after stimulation with 0.2 mM of DS-0S during 30 minutes and 2 hours (grey) when compared with untreated cells (black). (b) Microglia cell line show significantly increased of mRNA transcriptional level expression of TNF-α after stimulation with 0.2 mM of DS-4S during 30 minutes and 2 hours (grey) when compared with untreated cells (black). (c) iNOS mRNA transcriptional level expression in microglia cell line show decreased when compared with untreated cells after 30 minutes of treatment with 0.2 mM of DS-0S. (d) Treatment of microglia cell line with 0.2 mM of DS-0S after 30 minutes and 2 hours significantly increased transcription of anti-inflammatory cytokine IL-10. Data are presented as mean±SEM. ANOVA - Bonferroni's-multiple multiple comparasions using post-hoc test: ***$p<0.001$, **$p<0.01$ ande NS: not siginificant.

5. Discussion

Past studies on microglial phagocytic activity were carried out mainly in cell culture systems using various approaches like application of fluorescent proteins and bacteria or yeast particles (Heneka et al., 2006; Mairuae et al. 2007; Koenigsknecht and Landreth, 2004). However, neonatal or immortalized microglia are not reflective of microglial properties in tissue environment. Recently, fluorescent microspheres were not only applied to primary microglia but also injected into the brain of prion diseased models (Hughes et al., 2010).

One of the main characteristics of microglia is phagocytosis of either pathogens or of cells that underwent apoptosis and cellular debris during development and in pathology and regeneration (Fourgeaud and Boulanger, 2007).

In this project I aimed to find new ligands for microglial receptor TREM2, which until now, is yet to be described. Glycosaminoglycans from different sources and having different degrees of polymerization were tested by blocking the binding of mouse TREM2-Fc-fusion proteins to the glycocalyx of altered cells (glioma cells). Furthermore, the glycosaminoglycans were tested in their capacity to stimulate phagocytosis using fluorescent beads in culture of mouse microglia (ESdM – stem cell-derived microglia) cells with a normal or knocked down expression of TREM2.

Therefore I investigated which chondroitin sulfate or chondroitin sulfate disaccharides could bind to TREM2-Fc-fusion protein and inhibit the binding to glioma cells. Interestingly, CS-A showed the better candidate substance can binds to TREM2 receptors. The characterization of the function of CS-A in microglia cells showed that CS-A can increase the beads phagocytosis. Knockdown of TREM2 in microglia decreased the beads phagocytosis in ESdM cells after stimulation with ligands of TREM2.

Couple of years ago it was reported that the microglial TREM2/DAP12 complex is involved in phagocytosis of apoptotic neurons without inflammation (Takahashi et al., 2005). The immune receptor TREM family was discovered by the group of Prof. Colonna and was proven to be expressed together with DAP12 in neutrophils and monocytes (Bouchon et al., 2000). To facilitate a better understanding of the involvement of microglial receptor TREM2 and its co-adaptor molecule DAP12 in phagocytosis after stimulation with CS-A, ESdM cells

overexpressing full length DAP12, truncated DAP12, or control cells were used. Results showed that CS-A increases microglia phagocytosis in a time and concentration dependent manner. Moreover, ESdM cells have less capacity to take up the fluorescent beads compared to the control group even in the presence of CS-A, proving the specificity of CS-A-triggered activation of microglia via the TREM2/DAP12 complex.

Thus, the present study brings new evidence that the ligand for microglial receptor TREM2 could be a member of chondroitin sulfate family (CS-A). Furthermore, data demonstrate an involvement of the chondroitin sulfate ligand in the phagocytosis of microglial cells, proposing a mechanisms that might be used for therapy in the future in neurodegenerative disease.

5.1 Binding of TREM2 to chondroitin sulfate group or chondroitin sulfate disaccharides

Intially, TREM2 was found to bind broadly to bacteria, and that binding was inhibited by anionic glycans (Daws et al., 2003). It was demonstrated that TREM2 interacts with endogenous ligands (Hsieh et al., 2009),. and promotes phagocytosis of apoptotic neurons without activation of TNFα transcription (Takahashi et al., 2005). However an endogenous apoptotic material epitope that can be recognized by TREM2 has not been identified.

Until now, no work in literature has demonstrated binding of CS family to TREM2 microglial receptor. However CS is known to bind other receptors such as CD36 and CD44, that are expressed on macrophages (Ruffell et al.). The report here demonstrats that CS family is a new ligand for TREM2 receptor and the best CS subunit that can bind to TREM2 is CS-A. With the mention that CS-C showed no binding to TREM2 receptor. Interestingly, a report indicated that another member of the TREM receptor family, TREM-like 4 (Treml4), also recognizes apoptotic cells (Hemmi et al., 2009).

In this thesis, it was shown that the mouse TREM2-Fc-fusion protein can recognize specific ligands expressed on the cellular membrane of CHO, GL261, SMA-MS and U87 cells. The glioma cells might have modifications on the glycocalyx, where the TREM2 might bind to (Daws et al., 2003).

The glycosaminoglycans families have been used for therapy in several inflammatory diseases, where CS is a sulfated glycosaminoglycan (GAG) composed of linear chains that are

formed by repeated disaccharide (Rolls et al., 2006). Disaccharides are polymerized into long chains and modified by sulfation. CS disaccharides can be monosulfated at positions 4 or 6 (CS-A and CS-C, respectively) or disulfated at position 2 and 6 (CS-D) and positions 4 and 6 (CS-E). CS is usually found attached to proteins, as part of a glycosaminoglycan and participates in the formation of the extracellular matrix. CS is considered to be a major inhibitor of axonal regeneration and is known to participate in activation of inflammatory responses (Rolls et al., 2006). In experimental autoimmune encephalomyelitisCS-C promotes recovery and neuronal survival, effects that were associated with inhibition of NF-kB nuclear translocation (Vallieres and du Souich). Our results show that CS-C can not bind to TREM2 receptor, but CS-A can bind and activate it. In studies using a combined *in vivo* model of chronic arthritis and atherosclerosis, intraperitoneal bovine CS reduced NF-kB nuclear translocation in peripheral blood mononuclear cells (Vallieres and du Souich; Herrero-Beaumont et al., 2008) and patienst who received treatment with 1000mg/Kg/day of CS orally for 63 days were partially prevented from developing osteoarthritis (Seed et al., 2009). The 6-sulfated disaccharide (chondroitin sulfate C) induces axonal growth and activated microglia to adopt a neuroprotective phenotype (Rolls et al., 2004). Thus, disaccharidic degradation of CS participates in the modulation of the inflammatory responses and could, therefore, promote recovery in immune-induced neuropathologies of the CNS, such as experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU) (Rolls et al., 2006). It has been shown that disaccharides obtained from CS markedly alleviate the clinical symptoms of EAE and attenuate T cell motility. Furthermore, there is decreased secretion of cytokines such as interferon-gamma and TNFα (Rolls et al., 2006).

Another study has shown that DS-stimulated microglia possessed increased beads phagocytic capacity but lacked direct cytotoxic effects such as secretion of NO (Ebert et al., 2008). Analysis by microarray showed up-regulation of 29 genes that had anti-inflammatory and neuroprotective functions, when microglial cells were stimulated with DS, but when microglia cells were stimulated with IFN-γ the 25 genes were down-regulated (Ebert et al., 2008). This study is in agreement with my results, where I have demonstrated that chondroitin sulfate disaccharides can bind to TREM2 receptors. Two different disaccharides were used in my study, disaccharides sulfated in position 4 (DS-4S) and as control - disaccharides which are not sulfated (DS-0S). The non-sulfated disaccharides showed no binding to TREM2 receptor. This means that the sulfate group can interfere with the binding to TREM2 receptor. But it is still unclear how the disaccharides can bind to TREM2.

In future it will be interesting to perform new experiments with different groups of disaccharides provided from CS-C (DS-6), from CS-D (DS-2,4) and from CS-E (DS-4,6) to confirm the binding of disaccharides to TREM2 dependent on the sulfate-group position. Another interesting question to be analyzed in the future is to elucidate whether chondroitin sulfate D (CS-D) or chondroitin sulfate E (CS-E) can bind to TREM2 receptor.

5.2 TREM2 receptor binding of chondroitin sulfate group from the glycocalyx of glioblastoma cells

The enzyme ChABC has been demonstrated to be an effective treatment to promote axonal regeneration after spinal cord injury. In the cerebellum, degradation of CS GAG chains by treatment with ChABC promoted structural plasticity of Purkinje axons (Garcia-Alias et al., 2006). Recently, it has been shown after C4 dorsal spinal cord injury that rats that received both ChABC and task-specific rehabilitation showed the best recovery in skilled paw (Wang et al., 2007). The ChABC has been used for treatment of recovery in different neurodegenerative diseases. In spinal cord injury, treatment with ChABC degraded CS-GAG at the injury site, up-regulated a regeneration-associated protein in the injured neurons, and promoted regeneration.

Here I investigated whether TREM2 receptor could still bind to glioma cells after removal of the CS group of the glycocalyx on cells treated by chondroitinase (ChABC). The results showed that after pre-treatment of glioma cells with ChABC, the binding of TREM2 to U87 cells was partially inhibited. In contrast, when the glioma cells were treated with sialidase, an enzyme that could cleave the sialic acid groups of the glycocalyx of the target cells, no change of TREM2 binding to glioma cells was observed. As known the U87 cells is a human primary glioblastoma cells and have alter glycocalix where expressed alterated amounds of glycosaminoglycans. These results confirmd that CS groups are present on the glycocalyx of U87 glioma cells and can be digested and removed by chondroitinase ABC and consequently influence the binding of TREM2 receptor to U87 cells. This also suggests that the new ligand of TREM2 receptor is chondroitin sulfate and not another component of the extracellular matrix of glioma cells.

5.3 TREM2 is not dependent on removal of sialic groups of the glycocalyx of glioma cells

Sialic acid is neuraminic acid, a monosaccharide with a nine-carbon backbone (Varki and Schauer, 2009). Sialic acids are found widely distributed in animal tissues and to a lesser extent in other organisms, ranging from plants and fungi to yeasts and bacteria, mostly in glycoproteins and gangliosides (Schauer, 2000). They are found at the end of sugar chains connected to the surfaces of cells and soluble proteins (Schauer, 2000). Here I found that sialic acid can not be recognized by TREM2. This was confirmed after treatment of glioma cells with sialidase did not change the binding capacity of the TREM2 to glioma cells.

5.4 Analysis of TREM2 function in mouse microglia cell line

Microglia have been implicated as active contributors to neuronal damage in neurodegenerative diseases, in which the over-activation and dysregulation of microglia might result in serious damage to the CNS and production of neurotoxic components. The microglia cells can produce pro-inflammatory cytokines and reactive oxygen species. Microglia are assumed to remove synapses in the developing brain and take part in neuronal pruning in the postnatal brain.

To mimic the *in vivo* neuro-inflammatory situation, the ESdM cell culture was stimulated with CS-A and the capacity to take up beads by phagocytosis was analyzed. Data obtained showed an increase of beads phagocytosis after stimulation with CS-A compared to unstimulated ESdM cells. The ESdM microglial cells are well known to phagocytose cellular debris during inflammation in CNS (Napoli et al., 2009). The analysis *in vitro* showed that ESdM cells stimulated with CS-A demonstrated activation of TREM2 signaling leading to increased phagocytosis.

Next, knockdown of TREM2 receptor in ESdM was achieved by lentiviral transduction. TREM2 knockdown ESdM decreased the beads phagocytosis when compared to the control.

The hypothesis investigated within this thesis is that TREM2 expressed on microglial cells bind to CS-A and act further via ITAM motifs of DAP12 increasing microglial phagocytosis capacity. The results confirm this hypothesis. Indeed, ESdM cell line expressed TREM2 and bound CS-A leading to increased phagocytosis via DAP12. All these data indicate that

TREM2 might be important for clearance of cellular debris and prevention of neurodegenerative processes.

Recently, it was demonstrated that TREM2 deficiency results in impaired and delayed osteoclasts differentiation with reduced bone resorption capability in vitro (Otero et al., 2010). Furthermore, lentiviral vectors were successfully used for specific knockdown of TREM2 gene transcripts by small hairpin RNAs in ESdM cells. These results suggest that TREM2 deficiency or knockdown can dysregulate and reduce microglial phagocytosis, which can affect the inflammatory progress.

Another study demonstrated that disacchrides have a protective effect during the development of neurodegenerative diseases. They showed that disaccharides stimulate the production of anti-inflammatory cytokines, but did not demonstrate how this small molecule could be involved in this mechanism. I now demonstrate, in this study, that the disaccharides can bind TREM2 receptor. I stimulated the ESdM cells with disaccharides sulfated in position four (DS-4S) and with disaccharides which were not sulfated (DS-0S).T he sulfated disaccharides can increase beads phagocytosis when compared to the control cells. That suggests a protective effect of disaccharides, since TREM2 activated by disaccharides increases phagocytosis and might induce the production of anti-inflammatory cytokines.

However, I also tested the capacity of ESdM microglial cells to produce anti and pro inflammaotry cytokines after stimulation with the ligands of TREM2 (DS-4S, DS-0S and CS-A). Interestingly, I confirmed what has been shown before in literature, namely that glycosaminoglycans have both protective and deleterious effects. MRNA level of pro-inflammatory cytokines (IL-1ß, TNF-α and iNOS) and an anti-inflammatory cytokine (IL-10) after stimulation with CS-A were increased. It is known that cytokines play a potential role in neurodegenerative diseases, when microglia remain in a chronically activated state. Direct injection of the cytokines IL-1α, IL-1β and TNF-α into the CNS resulted in local inflammatory responses and neuronal degradation. This is in contrast with the potential neurotrophic (inducing growth of neurons) actions of these cytokines during acute neuroinflammation (Willenborg and Staykova, 2003). It is known that the microglia population can be neuroprotective by degrading amyloid-beta plaques in AD by complement receptor (Crehan et al, 2012.). Stress models in murine found that microglia mainly recruit macrophages from the periphery that then transform into phagocytes in the brain (Wohleb et al., 2013). Therefore most of the phagocytes that were associated with plaques in the mouse

brain came from the bone marrow (A. R. Simard and S. Rivest, 2004). These findings show that microglia are committed to an inflammatory response with a lower phagocytotic capacity than newly recruited phagocytes. In mouse models of AD it was demonstrated that anti-inflammatory drugs like minocycline improve cognitive functions and reduce the activation of microglial cells, but do not alter the amyloid plaques deposition and distribution (R. Fan, F. Xu, M. L. Previti, J. Davis, 2007). Therefore it is believed that an acute neuroinflammatory response is generally beneficial to the CNS, since it tends to minimize further injury and contributes to repair of damaged tissue. The opposite is the case for a chronic stimulation: chronic neuroinflammation is most often detrimental and damaging to nervous tissue. Thus, whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on the duration of the inflammatory response. The progressive deposition of amyloid in AD disease might provide a chronic stimulus to microglial cells. Also the chemotactic functions of amyloid-β to attract microglia contribute further to the ongoing inflammatory process (Lue et al., 2001).

Nasu-Hakola disease, also referred to as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) is a rare recessive human genetic disorder (Verloes et al., 1997; Bianchin et al., 2004). Nasu-Hakola disease is characterized by early onset cognitive dementia and bone cysts (both evident by the third decade). Individuals with this disease die by thefourth or fifth decade of life. Seminal studies by Peltonen and colleagues identified loss of function mutations in two separate genes as the causes of this rare disorder: namely, TREM2 and DAP12, also referred to as killer-cell activating receptor-associated protein (KARAP) and TYROBP (Paloneva et al., 2000; Paloneva et al., 2002; Klunemann et al., 2005).

TREM2 is an orphan receptor that has recently been implicated in limiting the pro-inflammatory activation state of macrophages and promoting expression of molecules associated with presenting antigen to T-lymphocytes (Bouchon et al., 2000; Bouchon et al., 2001; Daws et al., 2001; Daws et al., 2003). TREM2 belongs to the class of receptors lacking an intracellular signaling tail. To date, only a single molecule has been identified that serves to mediate TREM2 triggered intracellular signaling: DAP12 (Lanier et al., 1998; Lanier and Bakker, 2000; Daws et al., 2001; Prada et al., 2006).

Two types of data suggest that TREM2 and DAP12 may play either a direct or indirect role in oligodendrocyte development and function. First, histological analysis of adult CNS tissue

from DAP12 knock-out mice in studies by two separate groups revealed modest, diffused hypomyelination in anterior brain regions that was associated with substantial reductions in CNS microglia (Kaifu et al., 2003; Roumier et al., 2004; Nataf et al., 2005). Subsequent gene profiling studies of CNS mRNA isolated from DAP12KO mice by a third group revealed decreased expression of selected myelin transcripts (Kiialainen et al., 2007). These data were initially interpreted to suggest a fundamental role for microglia in supporting oligodendrocyte development and/or myelination. In support of this hypothesis, TREM2 and DAP12 expression near white matter tracts has been detected by in situ hybridization analysis in post-natal developing brain (Kiialainen et al., 2005).

In this study I showed that the activation of phagocytosis in ESdM cells after stimulation with ligands of TREM2 is dependent on the co-receptor DAP12. For this analysis, different ESdM cell lines were developed by lentiviral transduction to overexpress either full length functional DAP12, truncated non-functional DAP12 (in which the ITAM signaling motif was deleted) or a control vector. The backbone vector was designed with a GFP expressing cassette.
After stimulation of ESdM with CS-A, DS-4S or DS-0S and incubation with beads, ESdM in which the ITAM signaling motif was deleted showed decreased capacity to take up beads, while the ESdM with DAP12 overexpression showed increased phagocytosis of beads compared with control cells. This finding confirms that the DAP12 co-receptor is involved in phagocytosis of microglia cells via TREM2 receptor ligands.

In conclusion, in this work a new ligand for TREM2 stimulation *in vitro* was identified. CS-A and chondroitin sulfate disaccharides (DS-4S) activated phagocytosis of microglial cells via TREM2 and were dependent of DAP12-ITAM signaling. Clearance of cellular debris via CS acting on TREM2 might fulfill a protective role in the central nervous system (CNS).

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8. Curriculum Vitae

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Education and Professional Experience

2010-present: PhD in Molecular Biology

Coordinator: Prof. Dr. Harald Neumann at the Institute Reconstructive Neurobiology, University of Bonn, LIFE & BRAIN Center, Germany. Member of the Department Medical Neuroscience, the international Graduate School of Theorical and Experimental Medicine (THEME), University of Bonn, Germany.

Thesis title: "Acceleration of microglial clearance function by small TREM2-activating anionic disaccharides".

2013 – LabStatistics Course

Institute of Medical biometry, Informatics, and Epidemiology (IMBIE), Bonn Germany.

2012 – Flow cytometry workshop

Institute of Molecular Medicine and Experimental Immunology (IMMEI), Bonn Germany.

2006-2008: MSc in Science in Immunology.

Coordinator: Dr. Niels Olsen Saraiva Câmara at the Department of Immunology, Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil.

Thesis title: "Cellular answer to th1/th2 and its modulation in the secondary kidney inflammatory wound to ischemia and reperfusion".

2000-2004 –Bachelor of Science in Biomedicine

Thesis title: "Production and characterization of monoclonal antibodies against the serine 1650 phosphorylation site in the tail domain of myosin-Va"

Coordenator: Prof. Dr. Roy Larson

University of Barão de Mauá in Ribeirão Preto, São Paulo – Brazil.

Academic Honors and Fellowships

2005-2006- Technical Training

At University Federal of Sao Paulo – UNIFESP, Department of Immunology, Parasitology and Microbiology – Sao Paulo, Brazil.

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2010- till present time: PhD in Molecular Biology

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Teaching Assistance

- **2007 - Teaching Assistance**

  In "Laboratory Methods in Immunology" for pharmacy students, under the supervision of Dr. Lourdes Issac at the Institute of Biomedical Sciences, USP, Sao Paulo, Brazil.

- **2007 – 2008 - Scientific supervision**

  Mayra Carvalho Ribeiro. Involvement of T lymphocytes in the pathogenesis induced by the action of cisplatin nephorotoxic – University of Sao Paulo – USP, Sao Paulo, Brazil, .

- **2008 – Teaching Assistant**

  Immunology within a course in nursing, under the supervision of Dr. Antônio Côndino Neto at the Institute of Biomedical Sciences, USP, São Paulo, Brazil.

Scientific Presentations

1 – "Critical involvement of Th1 cytokines on Renal Tissue Injuries induced by Ischemia and Reperfusion."
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2 – "Renal Ischemia and Reperfusion Injury modulates the Lung Allergic Response Through Th1/th2 balance."
Landgraft, RG; Campanholle, G; Paiva, VN; Silva, RG; Monteiro, RMM; Martins JO; Pacheco-Silva; A, Jancar S; Câmara, NOS.
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3 – "Lung Inflammation is Induced by Renal Ischemic and Reperfusion Injury as Part of the Inflammatory Syndrome."
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4 – "Involvement of Lymphocytes in the pathogeneses of cisplatin-Induced nephrotoxicity in mice."
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5 - "Participation of Th1/Th2 cytokines in ischemia and reperfusion injury."

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6 - "TCR-dependent and –independent pathways in T cell activation during renal ischemia and reperfusion"
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13th International Congress of Immunology
Rio de Janeiro, Brazil – 2007.

7 - "The influence of cytokine milieu and TCR activation on renal tissue injuries induced by ischemia and reperfusion"
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World Congress of Nephrology
Rio de Janeiro, Brazil – 2007.

8 – "Determining the cell-autonomous mechanisms through which Gli3 regulates threedimensional"
V. N. de Paiva, Sandra Blaess
Joint Meeting of the German and Japonese Societies of Developmental Biologists. Dresden, Germany - 2011

9 – "Identification of glycosaminoglicans as binding partner for TREM2"
V. N. de Paiva, Bettina Linnartz-Gerlach, Harald Neumann
EURON and THEME joint PhD meeting
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10 – "Th1 and Th2 Related Cytokines Influence Systemic Inflammatory Responses in Renal Injuries Induced by Ischemia and Reperfusion"
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15th International congress of Immunology
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Publications

1. **Phosphorylation of the alpha subunit of translation initiation factor 2 by PKR mediates translation shut off in the mouse brain during status epilepticus.**

Larissa S. Carnevalli, Catia M. Pereira, Carolina B. Jaqueta, Viviane S. Alves, Vanessa N. Paiva, Krishna M. Vattem, Ronald C. Wek, Luiz Eugênio A. M. Mello and Beatriz A. Castilho.

Biochemical Journal. , p.187 - 194, 2006.
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2. Critical Involvement of Th1 Cytokines on Renal Tissue Injuries Induced by Ischemia and Reperfusion
Vanessa N. Paiva, Monteiro RM, Marques Vde P, Cenedeze MA, Teixeira Vde P, dos Reis MA, Pacheco-Silva A, Câmara NO.

Journal of Immunopharmacology, p.1-5, 2008
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3. Modulation of Lung Allergic Response by Renal Ischemia and Reperfusion
Campanholle G, Silva RC, Martins JO, Landgraf MA, Vanessa N. Paiva, Ferreira RR, Amano MT, Hiyane MI, Cenedeze MA, Pacheco-Silva A, Camara NO, Landgraf RG Cell Physiol Biochem, p.3-4, 2012

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Inflamm Res. 2010 Oct;59(10):861-9, 2010

Doi: 10.1007/s00011-010-0198-0

9. Declaration

I - hereby confirm that this work submitted is my own. This thesis has been written independently and with no other sources and aids than stated. The presented thesis has not been submitted to another university and I have not applied for a doctorate procedure so far.

Hiermit versichere ich, dass die vorgelegte Arbeit – abgesehen von den ausdrücklich bezeichneten Hilfsmitteln – persönlich, selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde. Aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle kenntlich gemacht worden.

Die vorliegende Arbeit wurde an keiner anderen Hochschule als Dissertation eingereicht. Ich habe früher noch keinen Promotionsversuch unternommen.

Vanessa Nunes de Paiva Bonn, 28th of March 2014