Functional analysis of microglial signal regulatory protein β 1 (SIRPβ1)

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Abbreviations

AD - Alzheimer disease	APP - Amyloid Precursor Protein
APL – altered peptide ligand	BBB – blood-brain barrier
BDNF – brain derived neurotrophic factor	BM – bone marrow
BMT – bone marrow transplantation	βTubIII – Beta-tubulin III
CFA – complete Freund's adjuvant	SIV - simian immunodeficiency virus
CMV – cytomegalovirus	CNS – central nervous system
CSF – cerebrospinal fluid	DNA – deoxyribonucleic acid
dsRNA - double-stranded RNA	EAE – experimental autoimmune encephalomyelitis
EDTA – ethylenediaminetetraacetic acid	ELISA – enzyme-linked immunosorbent assay
ES cells – embryonic stem cells	FACS – fluorescence activated cell sorting
FCS – fetal calf serum	FITC – fluoro-isothiocyanate
GDNF – glial cell-derived neurotrophic factor	GFAP – glial fibrillary acidic protein
GFP – green fluorescent protein	eGFP – enhanced green fluorescent protein
HIV-1 – human immunodeficiency virus-1	HSC – hematopoietic stem cell
HSV – herpes simplex virus	IFN-γ – interferon-γ
Ig – immunoglobulin	IL-10 – interleukin-10
ITIM - immunoreceptor tyrosine-based inhibition motifs	ITAM - immunoreceptor tyrosine-based activation motifs
Lin-BM – lineage-negative BM	LTR – long terminal repeat
LT-HSC – long-term hematopoietic stem cells	mAbs - monoclonal antibodies
MBP – myelin basic protein	MHC – multi-histocompatibility complex
MMP – matrix metalloprotease	MOG – myelin oligodendrocyte glycoprotein
MS – multiple sclerosis	pMSCV – murine stem cell virus

NGF – nerve growth factor	nt - nucleotide small
NHD - Nasu Hakola disease	NT-3 – neurotrophin-3
NSCs – neural stem cells	OPC – oligodendrocyte precursor cell
PBS – phosphate buffered saline	PCR – polymerase chain reaction
PE – phycoerythrin	PI - phosphoinositide
PGK – phosphoglycerate kinase	PFA – paraformaldehyde
PLP – proteolipid protein	pll3.7 – plasmid LentiLox 3.7
PTX – pertussis toxin	RNA – ribonucleic acid
RNAi - RNA interference	RISCs - RNA-induced silencing complexes
RBL - Rat basophilic leukemia	shRNA - short hairpin RNA
SIRPs - signal regulatory proteins	SIRPα - signal regulatory protein alpha
SIRPβ - signal regulatory protein beta	SIRPγ - signal regulatory protein gamma
SIN – self-inactivating	siRNAs – small interfering RNAs
ST-HSC – short-term hematopoietic stem cells	TBE – Tris-Borate EDTA
TCR – T cell receptor	TGF-β – transforming growth factor-β
TNF-α – tumour necrosis factor-α	VSV-G – vesicular somatitis virus G-protein
Ψ – retroviral packaging signal	

Summary

Microglial cells are the resident macrophages of the central nervous system (CNS) and thus form the interface between the neural parenchyma and the immune system. Although little is known about microglia in the normal CNS, it is obvious that they are quickly activated in all acute pathological events that might affect the CNS. In this study, the role of signal regulatory protein-β1 (SIRPβ1) in microglia function was investigated by analyzing the effect of SIRP's engagement on phagocytosis, inflammatory responses as well as on intracellular signalling in microglia with specific monoclonal antibodies (mAbs).

The signal regulatory proteins (SIRPs) are a family of transmembrane glycoprotein that are mainly involved in signal transduction cascade and belong to the immunoglobulin (Ig) superfamily. These proteins are expressed in the hematopoietic cells including granulocytes, monocytes, dendritic cells, lymphocytes and the CNS. Although the extracellular domains of SIRPs are highly similar, classical motifs in the cytoplasmic or transmembrane domains distinguish them as either activating (β) or inhibitory (α) isoforms.

The C-terminal intracellular domains of the SIRPα subfamily contain relatively long amino acid sequences (110aa for SIRPα1) that include four tyrosine residues to form two immunoreceptor tyrosine-based inhibition motifs (ITIM). Conversely, SIRPβ1 subfamily contains members that have a short intracellular domain containing only a few amino acids (4aa for SIRPβ1). Despite a short cytoplasmic tail, SIRPβ1 was reported to physically associate with the immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor protein DAP12 in other cell types under the mechanism for signal transduction and regulation of transmigration. SIRPβ1 as well as TREM2 are associated with DAP12 activating molecule and are expressed in microglia. We have shown that microglial SIRPβ1 plays an important role in removal of apoptotic membrane fragments without induction of inflammation. Moreover relation of phagocytosis by SIRPβ1signal has been recently reported in macrophage (JBC).

Functional aspect of SIRPβ1 with respect to ligands is still unknown. We are interested to know how extracellular ligands for SIRPβ1 might regulate cellular function. Protein expression of SIRPβ1 in microglial cells or splenocytes of brain, spinal cord and spleen from 7-8 weeks old mice (n=12) (EAE animal model) and from 12 months old APP transgenic mice (n=6) was analyzed by immunohistochemistry with a purified antibody

directed against SIRPβ1 (SIRPβ-80 and SIRPβ-84 monoclonal antibodies). Gene transcript levels of SIRPβ1 and DAP12 significantly increased in the spinal cord of mice with induced EAE as well as in the brain of APP mice. Totally, SIRPβ1 was detected in microglial cells or splenocytes of all organs investigated with its higher expression level in the spleen. Double labelling with antibodies directed against SIRPβ1 and microglia marker Iba1 (rabbit, 1/200; Wako, Japan) confirmed the microglial structure of the cells, positive stained for SIRPβ1.

To study microglial SIRPβ1 function, we used lentiviral RNA interference technique. Our hypothesis is that like TREM2, SIRPβ1 takes part in maintenance of CNS microenvironment by clearance of apoptotic cells without induction of inflammation.

Microglial activation has been increasingly recognized to contribute to the pathogenesis of several neurodegenerative diseases. Our study suggests that the SIRPβ1 associated with its signalling molecule DAP12 and its stimulation induced phosphorylation of the ITAM motifs that leads to phagocytosis and over-activated microglia in the neurodegenerative disease state. This finding has broad reaching implications, as several pathological hallmark proteins associated with neurodegenerative disease, such as myelin, melanin, prions, and amyloid β are reported to be phagocytized by microglia and ongoing research in our group is showing SIRPβ1 mediates the evidence of microglial phagocytosis. It has also been shown that dying neurons, splenocytes, amyloid β and myelin are phagocytized by microglia, suggesting that SIRPβ1 mediated phagocytosis could be a mechanism of ongoing response to neurodegeneration. SIRPβ1 has not long been defined as a major contributing factor to multiple neurodegenerative diseases.

In summary, our results demonstrate that microglial SIRPβ1 and its associated signalling molecule DAP12 are involved in phagocytosis and that deficiency of SIRPβ1 results in diminished microglial uptake of apoptotic neurons, splenocytes, Aβ-peptide and myline. Thus, understanding the role of SIRPβ1 mediated phagocytosis in the neurodegenerative process may help to elucidate the difference between normal microglial homeostasis and the disease state, offering hope for the generation of novel therapeutic compounds.

1. Introduction

Central nervous system (CNS) epitomizes the largest part of the nervous system. Together with the peripheral nervous system, it has an essential elementary role in the control of behaviour. The CNS is contained within the dorsal cavity, with the brain in the cranial subcavity and the spinal cord in the spinal cavity. There are two main well-defined classes of cells in the nervous system: nerve cell (neuron) and glial cells (glia). Further focus will be on the glial cells, which comprise a major part of my study.

Neurons, the fundamental functional unit of the nervous system, were first recognized in the early 20th century by the Spanish anatomist Santiago Ramony Cajal. Cajal proposed that neurons are discrete cells that communicated with each other via specialized junctions or spaces. He also hypothesized that individual cells recognize each other and transmit signals and that signalling between few interconnected cells never produce a simple behaviour. Nerve cells are main signalling units of the CNS. A typical neuron has four defined regions: the cell body, dendrites, axon and the presynaptic terminals. The cell body (soma) is the metabolic centre of the cell. It contains the nucleus, which stores the genes of the cells as well as endoplasmic reticulum, an extension of the nucleus where the cell proteins are synthesized. The cell body usually gives rise to two kinds of processes: several short dendrites and one long tubular axon. Near its ends tubular axon divides into fine branches that form communication sites with other neurons. The points where two neurons interact or communicate are known as synapses. Those releasing the signal are called presynaptic cells. Neurons constitute about a half the volume of the CNS in vertebrates and glial cells make up the rest (Banati, 2003).

Glial cells form a structural and functional network with complex cell-cell communication pathways that enable fast and slow signalling amongst themselves as well as with neurons. They exert regulatory influence on normal synaptic transmission and alter it in diseased conditions. It is becoming increasingly clear that an understanding of brain function in diseased conditions requires a better account of the highly plastic, disease-associated changes in glial physiology in vivo. Particularly microglia, the brain's ubiquitous but normally inconspicuous immune effector cells are prominently involved in many brain diseases. They respond rapidly and in a highly confined territorial way to subtle, acute and chronic pathological stimuli. Detection of microglial activation provides

diagnostically useful formal parameters of disease, such as the accurate spatial localisation, disease progression and the secondary neurodegenerative or adaptive changes remote from the primary site of disease. The latter has potential relevance for the understanding of disease-induced brain plasticity. Systematic attempts are now undertaken, using positron emission tomography and a ligand with relative selectivity for activated microglia to develop generic imaging tools for a cellular in vivo neuropathology (Banati, 2003).

1.1. Biology of glial cells

Glial cells are derived from a Greek word meaning glue; although in reality glia do not commonly hold nerve cells together. Preferably, they surround the cell bodies, axons and dendrites of neurons. As far as it is known, glias are not directly involved in information processing but they are thought to have several other vital roles:

- Glia cells support neurons; they separate and insulate neuronal groups and synapses.
- Oligodendrocytes and Schwann cells are also type of glial cells, which produce myelin that is used to insulate nerve cell axons, and facilitate conduction of electrical signals.
- There are some glial cells known to be scavengers, removing debris after injury or neuronal death.
- Efficient signalling between neurons promotes the important housekeeping cores by glial cells.
- Some classes of glial cells (radial glia) guide migrating neurons and direct outgrowth of axon during brain development.
- Glial cells actively regulate the assets of the presynaptic terminals at the nerve-muscle synapse of vertebrates.
- Never the less some glial cells like astrocytes play crucial roles in forming an impermeable lining in the brain’s capillaries and venules the blood-brain barrier that prevents toxic substances in the blood.

Some glial cells are known to release growth factors and help to nourish nerve cell. In the vertebrate nervous system, glial cells divide into two major classes: microglia

and macroglia. Macroglia; are broadly categorized into three types, predominantly found in the vertebrate nervous system: oligodendrocytes, Schwann cells and astrocytes (Kandel, 2000).

Oligodendrocytes and Schwann cells are comparatively small cells that have relatively few processes. Functionally, these cells insulate the axon, forming the myelin sheath. Oligodendrocytes envelop on an average about 15 axonal internodes each. Schwann cells occur in the peripheral nervous system. The types of myelin produced by oligodendrocytes and Schwann cells differ to some degree in chemical makeup. Astrocytes, the most abundantly found glial cell, may play an important role in bringing nutrients on the surface of nerve cells in the brain and spinal cord. Thus, some astrocytes place end-feet on the brain's blood vessels and cause the vessel's endothelial cells to from tight junctions creating the protective blood-brain barrier. Astrocytes also help to maintain the proper potassium ion concentration in the extracellular space between neurons. They also take up neurotransmitters from synaptic zone after release and help to regulate synaptic activities by removing transmitters. The role of astrocytes is largely a supporting one (Kandel, 2000).

1.1.1 Origin of microglia

For today's neuropathologist, it must be hard to believe that those microglia were once considered as endangered species. Yet, a few years ago, it was suggested that the existence of the microglia was in doubt and their name should be abandoned. Since the initial comprehensive description of microglia by del Rio-Hortega in 1932, the exact origin of microglia remains the subject of debate (Hong, 2003). What can be regarded as a gross scientific error by today's standards is a long and complicated history of microglia. Discovered independently by Nissl and Robertson, microglia cells were first studied in detail by del Rio-Hortega (Rio-Hortega, 1932). Rio-Hortega also deserves credit for establishing valuable knowledge on the role of microglia in CNS pathology. However, in the following years and mainly due to a lack of cell type-specific markers, controversy arose around microglial embryonic development and their 'nature' as well as their cellular 'identity' was unclear. In the mid-1980s, microglia was "rediscovered" with the advent of immunocytochemistry and lectin markers. Meanwhile, the esoteric debate

that surrounded microglia for decades had given way to research activity involving a broad circle of scientists. As a result, more than thousand papers have been published on microglia over the last few years. The biology and function of microglia are central in many issues in modern neuropathology. In addition, it is becoming increasingly clear that certain molecules and signal regulatory proteins expressed by microglia have the potentiality of serving as diagnostic "sensors" in day-to-day neuropathological practice (Graeber, 1994).

Microglial cells are close cousins of other phagocytic cells including macrophages and dendritic cells. Though the origin of microglia is a matter of debate, some research groups still suggest that microglia derive from mesodermal precursor cells of possibly hematopoietic lineage that enter the brain during the embryonic and early postnatal phases of development (Barron, 1995; Cuadros, 1998). Other research groups shown that mcroglia are derived from myeloid progenitor cells (such as macrophages and dendritic cells) which come from the bone marrow. During embryonic development, however, they migrate in to the CNS to differentiate into microglia. (Streit et al, 2004).

1.1.2 Immune function of microglia

Microglia, the smallest of the glial cells, can act as phagocytes, cleaning up CNS debris. They are representatives of the immune system in the brain and spinal cord. Microglia protects the brain from invading microorganisms and is thought to be similar in nature to macrophages in the blood system (Streit etal, 2004).

The most characteristic feature of microglial cells is their rapid activation in response to even minor pathological changes in the CNS. Microglia activation is a key factor in the defence of the neural parenchyma against infectious diseases, inflammation, trauma, ischemia, brain tumours and neurodegeneration. Microglia activation occurs as a graded response in vivo. The transformation of microglia into potentially cytotoxic cells is under strict control and occurs mainly in response to neuronal or terminal degeneration, or both. Activated microglia cells are mainly scavenger cells but also perform various other functions in tissue repair and neural regeneration. They form a network of immune-alert resident macrophages with a capacity for immune surveillance and control. Activated microglia can destroy invading microorganisms, remove potentially deleterious

debris and promote tissue repair by secreting growth factors. An understanding of intercellular signalling pathways for microglia proliferation and activation could form a rational basis for targeted intervention on glial reactions to injuries in the CNS (Kreutzberg, 1996).

Microglia respond to tissue insult with a complex array of inflammatory cytokines and actions, these actions transcend the historical vision of phagocytosis and structural support that has long been enshrined in the term "reactive gliosis." Microglia are now recognized as the prime components of an intrinsic brain immune system (Streit, 1995), such they have become a focus in cellular neuroimmunology and therefore in neuroinflammation. This is not the inflammation of the adaptive mammalian immune response with its array of specialized T-cells and the made-to-order antibodies produced through complex gene rearrangements. This is, instead, the innate immune system, upon which adaptive immunity is built (Medzhitov, 2000). Chronic microglial activation is an important event in neurodegenerative diseases which can lead to chronic neuronal dysfunction, injury as well as loss and hence to disease progression. The recognition of microglia as the brain's intrinsic immune system and the understanding that chronic activation of this system leads to pathologic sequelae, has led to the modern concept of neuroinflammation. This vision of microglia-driven neuroinflammatory responses with neuropathological consequences has extended the older vision of passive glial responses that are inherent in the concept of "reactive gliosis"(Wolfgang, 2004).

1.1.3 Interaction with other brain cells

Communication among glial populations and between glial and neuronal population as well as immune cells is also based on cytokines (Hanisch, 2002). Hematopoietic cells and the cytokines are produced and are effective in most cell type. Certain members have been postulated to possess neurodevelopment function; other modulates neurotransmitter and neurosecretion or participate in the neuroimmune-endocrine communication (Hanisch, 2001a, Raber, 1998).

Microglia is an integral component of the CNS (Giulian, 1995); (Streit, 2002). Microglia is embedded in a population of most heterogeneous cell type and carries the function of monitoring their well-being (Raivich, 1999, Streit, 2002). There is currently

enormous research progress concerning the ability of the glial cells to communicate with neurons and each other. As much as astrocytes are shown to act as active partners in neuronal information processing beyond their contribution to metabolic and homeostatic support as much microglia appear to collect multiple signal from both glia and neuron for maintaining or switching level of alert and activity (Bezzi, 2001, Polazzi, 2002, Polazzi, 2003). Since the various region and structure of the CNS vary in the type of cells and neurotransmitter phenotype microglia should also be considered having regional variation in sensory and executive features. Age and gender difference can be demonstrated as well (Hanisch, 2002).

Microglia contributes significantly to the mass of anaplastic astrocytomas whereas they are infrequent in low-grade examples and oligodendroglioma. Tumour-microglial interactions in glioma are complex and still poorly understood. The fact that astrocytoma manage to survive and grow in the presence of activated microglia indicates the failure of normal defence mechanisms and suggests that microglia may contribute to tumour progression by favouring the survival of tumour cells and sustaining their proliferation and migration. Recent studies suggest that the capacity of microglia to secrete cytokines, upregulate costimulatory molecules and activate anti-tumour T-cells is insufficient to initiate a normal immune response in the environs of the tumour. It is still unclear whether microglial impaired response in astrocytoma is determined by the molecules secreted by neoplastic cells or if tumour infiltrating microglia are somehow defective. On the other hand, effective microglia-lymphocyte communication for antigen presentation and reciprocal influences are readily inducible upon microglial activation (Aloisi, 2001).

1.1.4 Microglial defence and involvement in cerebral diseases

Signal for microglia activation needs to be translated into pattern of inducible function. Microglia can rapidly response or organize for response by other cells (Beattie, 2002, Draheim, 1999, Häusler, 2002). Cellular changes are believed to be trigged within minutes. Microglia cells can quickly migrate to a site of injury or infection, following chemotactic gradient as built up by endogenous factor or factor released from the irritating material (Hanisch, 2004). Microglia and brain macrophages have been

recognized to play crucial roles in important diseases such as viral infections, autoimmunity and neurodegenerative disorders. HIV encephalitis, multiple sclerosis and Alzheimer's disease are examples where understanding the role of microglia promises to hold essential information concerning disease pathogenesis (Graeber, 1994). Microglias are thought to be highly mobile cells that play numerous important roles in protecting the nervous system. They are also thought to play a role in neurodegenerative disorders such as Alzheimer's disease, dementia, multiple sclerosis and amyotrophic lateral sclerosis. Microglias are responsible for producing an inflammatory reaction (Streit et al., 2004) and as well they are relatively under-recognized, widely distributed cell population within brain parenchyma constituting about 1 to 2% of all cells. They have been well known to neuropathologists since they harbour agents including viruses such as HIV-1, treponema pallidum, fungi and prions during sub-acute or chronic CNS infections. This emphasizes their relationship to macrophages. Parenchymatous microglias are considered to be resident macrophages, and contribute to inflammation within the CNS and development of the glial nodules present in certain viral and fungal CNS infections. Perivascular microglia contributes to the blood-brain barrier. Therefore, pathological abnormalities induced by one agent may compromise the integrity of the blood-brain barrier and permit entry of another pathological agent which, by it self, would not be able to pass the barrier. Microglia are considered to be of bone marrow origin (Streit et al., 2004) but it is still not known when in embryonic life these bone marrow elements enter the CNS and, therefore, the environment to which they are exposed including other embryonic cells and types of trophic factors. Their relationship to blood-borne macrophages remains unclear including presence and timing of activation, ability to phagocytose and phenotypic markers. It is unknown whether they can traverse back across the blood-brain barrier while carrying infected particles, genes or trophic factors.

Microglias are specialized cells in the brain that act as a kind of guard for the immune system. They may serve as “front-line defenders” or as “recruiters” of other immune-system agents. These cells activated early in the course of Alzheimer’s disease, but their function still are not well understood. It is unclear whether;

(1) Activated microglia might be good for “eating up” beta-amyloid, a key suspect in Alzheimer’s;

(2) Activated microglia might be bad because they recruit immune system cells that lead to potentially harmful long-term inflammation; or

(3) Microglia might be best "turned on" at some times and "turned off" at others.

Through observation in regions of the brain severely affected by Alzheimer's disease, certain microglias have numerous cell-surface proteins such as signal regulator protein-beta1 (SIRPβ1), which might be particularly sensitive to beta-amyloid. Based on experiments with cells, we hypothesize that SIRPβ1 is a kind of "switch" that results in microglia being less effective at destroying beta-amyloid and more effective at enlisting immune-system "fighters," called T cells, which cause inflammation.

1.2 Family of signal regulatory protein

Microglial cells are considered the tissue resident macrophages of the CNS (Kreutzberg, 1996). They represent the major resident immunocompetent cell type. Currently, basic and clinical neuroscience develops large interest in microglia biology. This is due to an accumulation of experimental and clinical data on the impact of microglia in neurodegeneration (Streit, 2002a, Hanisch, 2002a, Schwartz, 2003). Various receptors responsible for the specific recognition of targets for phagocytosis by macrophages have been identified (Greenberg, 2002, Platt, 1998). The best characterized is the Fc receptor (FcR), which recognizes the Fc region of IgG bound to antigen, presented on microbial pathogens (Cox, 2001, Ravetch, 2001). The phosphorylated ITAM then serves as a docking site for the tyrosine kinase Syk. Downstream signaling mediated by phosphoinositide (PI) 3-kinase or Rho family small GTP-binding proteins eventually triggers phagocytosis of IgG-coated (opsonized) particles (Cox, 2001, Strzelecka, 1997, Caron 1998; Aderem 1999, Stephens, 2002). One of such receptor family is recognized as signal regulatory protein (SIRPs). The SIRP family has been studied by many groups and has therefore been given various names, as shown in TABLE 1. (Brown, 2006)

Family member	Other names	NCBI Entrez Gene ID	Expression	Extracellular ligands	Function
SIRPα	CD172A, SHPS1, P84, MYD-1, BIT, PTPNS1	140885 (human), 25528 (rat), 19261 (mouse), 327666 (bovine)	Myeloid cells, neurons	CD47, SP-A, SP-D	Inhibitory
SIRPβ	CD172b	10326 (human), 310212 (rat), 381484 (mouse)	Macrophages, neutrophils	ND	Activating
SIRPγ	CD172g, SIRPβ2	55423 (human)	Lymphocytes, natural killer cells	CD47	No signal

The SIRP nomenclature is the most widely used and has recently been summarized (van den Berg, 2005). We used SIRP-gamma rather than SIRPβ2 to distinguish it from SIRPβ1. SIRP-gamma does not associate with DAP12, as observed for SIRPβ1 (van den Berg, 2005).

1.2.1 Structure of SIRP family.

SIRPs family is divided into at least three subfamily isoforms - referred to as SIRP-alpha (α), SIRP-beta (β) and SIRP- gamma (γ) (van den Berg, 2005). Signal regulatory proteins are all contain an N-terminal extracellular domain, a signal transmembrane domain and a C-terminal intracellular domain. Based on the structure of their transmembrane and /or intracellular domain and their potential role in signal transduction, SIRP-α has long intracellular domain that comprises two putative immunoreceptor tyrosine-based inhibition motif (ITIM). Studies have suggested that activation of SIRP-α ITIMs delivered inhibitory signal that negatively regulate cell responses (Kharitonenkov, 1997). In contrast, SIRPβ1 and SIRPγ have only minimal intracellular tails. Studies have shown that SIRPβ1, the predominant form found in human leukocytes, can bind to the immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor protein DAP12 and thus deliver positive regulatory signal (Tomasello, 2000, Jiang, 1999). It is unclear how SIRPγ mediates signal transduction. Although, the intracellular domains of SIRP isoforms are well conserved, the signalling pathways mediated by them are strikingly different. The extracellular domains of all SIRPs share highly homologous amino acid sequence and a similar structure that contain three Ig-like loops (Yuan Liu, 2006) as shown in fig 1.

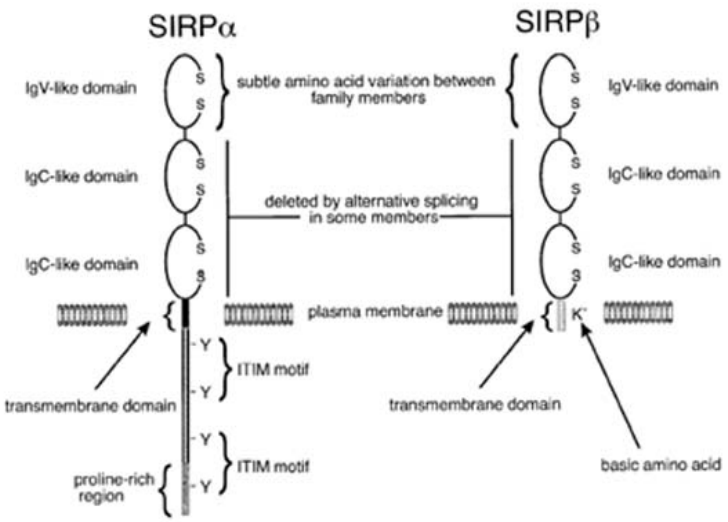


Figure 1 The SIRP family: structural characterisation

The SIRP family consists of two subtypes that differ by the presence or absence of an intracellular domain. The SIRP a subtype possesses a long cytoplamic domain with a proline-rich region near the C terminus and two ITIM motifs that can bind SH2 domain-containing phospatases. SIRPβ1 has no cytoplamsic domain but has a charged lysine residue within its transmembrane domain that is relevant to its association with DAP-12. Both subtypes are similar in their extracellular domains, which contain three Ig-like domains. Individual amino acid differences are evident in the single IgV-like domain when different SIRP family members are compared. (Cant, 2001)

1.2.2 Role of signal regulatory family

Previous studies with cells from rat, mouse, and cattle demonstrated expression of SIRP molecules mainly on neuronal and myeloid cells (Adams, 1998, Timms, 1998). This corresponds well with the strong SIRP expression observed on the surface of human monocytes, granulocytes, and dendritic cells. Further analyses showed that SIRP also expressed on the surface of bone marrow (BM) $CD33^+$ and $CD34^+$ myeloid progenitor cells, a subset of $CD19^+$ B lymphocytes, and on early $CD34^+$, $CD117^+$, and $CD90^+$ hematopoietic stem/progenitor cells. This suggests that SIRP is not only involved in the function of myeloid cells, but may also play an important role in the regulation of stem cell differentiation (Seiffert, 1999). The presence of 3 immunoglobulin-like loops within the extracellular domain of SIRPs (Kharitonenkov, 1997) suggested that this molecule interacts either with a soluble ligand or with a membrane molecule on other cells. Recent studies have shown that SIRP is indeed involved in cell-cell interactions (Kharitonenkov, 1998) and also (Brooke, 1998) demonstrated that the interaction of cattle SIRP on

monocytes with an unknown molecule on $CD4^+$ T cells is important for T-cell activation and proliferation, and Sano et al in 1997 showed that rat SIRP is involved in neuronal contacts, as well as in the outgrowth of rat neurons (Sano, 1997).

1.2.3 SIRPβ1 and involvement of adapter protein DAP12

The signal regulatory proteins (SIRPs) are a family of transmembrane glycoproteins which belong to the immunoglobulin (Ig) superfamily and are expressed in hematopoietic cells including granulocytes, monocytes, dendritic cells and lymphocytes. SIRPβ1 one of the members of SIRP family was reported to be physically associated with the immunoreceptor tyrosine-based activation motif containing adapter protein DAP12. SIRPβ1 has shown to be involved in phagocytosis. Furthermore, gene transcripts of this DAP12 associated protein are down regulated in the central nervous system of the patients with the inflammatory Nasu-Hakola brain disease (unpublished observation, K. Takahashi). Nasu Hakola disease (NHD) also known as membranous lipodystrophy was reported by Nasu (Nasu, 1970) and Hakola (Hakola, 1972). The main symptoms of membranous lipodystrophy are those of a progressive presenil dementia due to sclerosing leukoencephalopathy with skeletal polycystic lesions (Ahn, 1996, Akai, 1977, Mastushita, 1981). NHD is a progressive dementia that presents accompanied by bone cysts and, at random, epilepsy. It is an autosomal recessive hereditary disease and its genetic defect is located on the 19q13.1 chromosome. The genetic mutation was identified at DAP12. It appears that DAP12 is expressed in the microglial activation and the differentiation of macrophages in the central nervous system and at the same time, in the osteoclasts in charge of bone remodelling (Molina-Monasterios, 2003).

SIRPβ1 is a transmembrane protein that possesses three Ig-like domains in its extracellular region and a short cytoplasmic tail (Kharitonenkov, 1997). As mentioned earlier, SIRPβ1 has a basic amino-acid side chain in its transmembrane region that is essential for association of the activating adapter protein DAP12. Cytoplasmic region of SIRPβ1 there are only 6 amino acids and it lacks signalling motifs for association with phosphatases. Instead, this protein associates with DNAX activation protein 12 (DAP12, also known as KARAP) (Deitrich, 2000, Tomasello, 2000).

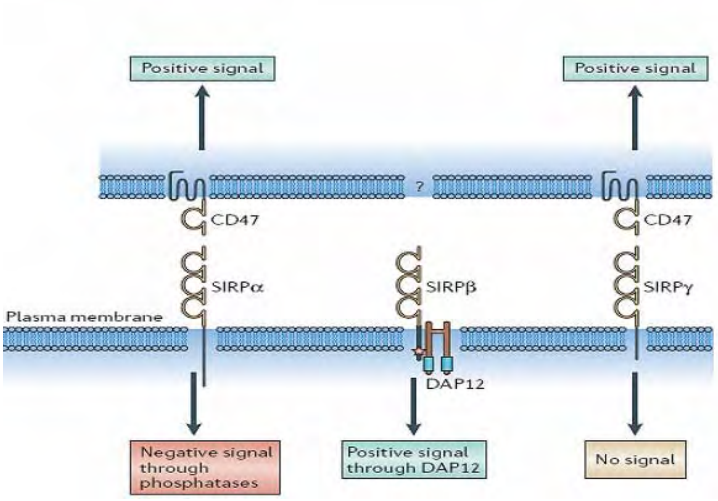


Figure 2. (Brown, 2006)

DAP12 is a dimeric adapter protein that contains an immunoreceptor tyrosine-based activation motif (ITAM) and can associate with SIRPβ1 through an amino acid with a basic side chain in the transmembrane region of SIRPβ1 (Tomasello, 2005, Lanier, 2000) and transmit activating signals. The involvement of other signalling adapter proteins in some cell types has been suggested (Liu, 2005). Recent studies have also shown that SIRPβ1 is expressed as a disulphide-linked dimer (Liu, 2005), whereas SIRPα and SIRPγ are monomeric proteins (Brooke, 2004, Piccio, 2005). It was initially discovered based on its homology to another transmembrane protein, known as SHP substrate-1 (SHPS-1) or SIRPα (Kharitonenkov, 1997). Human SIRPβ expressed in monocytes and granulocytes but not in lymphocytes (Seiffert, 2001). The protein forms a complex with DAP12 in human monocytes or in transfected nonhematopoietic cells (Deitrich, 2000, Tomasello, 2000). DAP12 is a transmembrane protein, which was originally identified based on association with the inhibitory receptors of natural killer cells (Lanier, 1998, Wu, 2000). Its intracellular region contains a single ITAM motif which binds Syk or the tyrosine kinase ZAP-70. The association between SIRPβ1 and DAP12 is thought to be mediated by an ionic interaction between single amino acids of opposite charge (lysine of SIRPβ1 and aspartic acid of DAP12) within the transmembrane regions (Deitrich, 2000, Tomasello, 2000, Cant, 2001). Ligation of SIRPβ1 resulted in the tyrosine phosphorylation of DAP12 and the subsequent recruitment of Syk to the SIRPβ1-DAP12 complex in RBL-2H3 cell transfectants (Tomasello, 2000). SIRPβ1 is therefore implicated as a positive regulator of

hematopoietic cells. To obtain the optimal type and strength of the immune response that is required to eliminating pathogen while minimizing the side effects, the response is regulated through a balance of activating and inhibitory signals that are delivered by receptors on the surface of the cell immune system. One such group of cell-surface receptors is the signal regulatory protein (SIRP) family. In this thesis, we emphasize the molecular aspect of the interaction between the SIRPβ1 and their adapter protein molecule DAP12 and show how they might be involved in immune-cell regulation.

The physical association between SIRPβ1 and KARAP/DAP-12 results in the functional coupling of SIRPβ1 engagement to the recruitment of the protein tyrosine kinase Syk and to serotonin release in rat basophilic leukemia (RBL) cell transfectants (Tomasello, 2000). Signalling pathway activated by SIRPβ1 promotes phagocytosis in macrophages (Fig. 3). Engagement of SIRPβ1 (Tomasello, 2000) by its putative ligand induces the tyrosine phosphorylation of DAP12 and the subsequent recruitment of Syk to DAP12 and its tyrosine phosphorylation. Activated Syk then mediates the tyrosine phosphorylation of SLP-76, which forms a multiprotein complex that triggers activation of the MEK-MAPK-MLCK cascade.

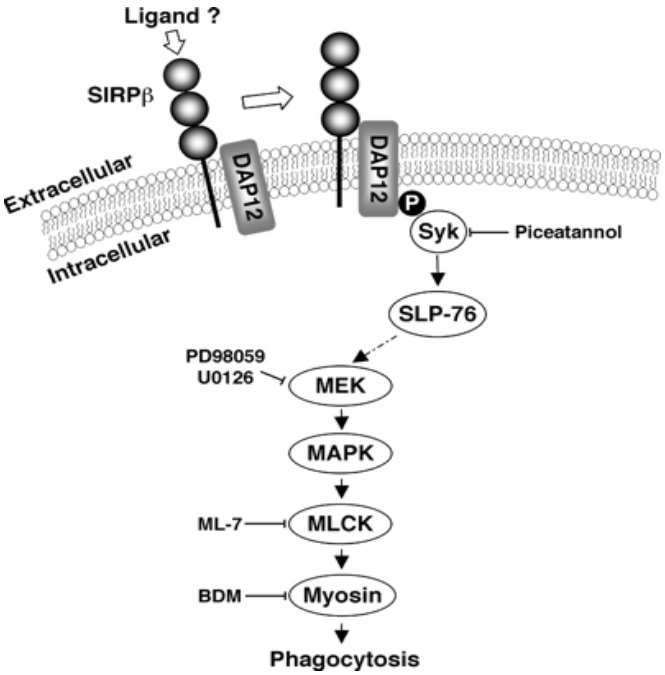


Figure 3. *Proposed model for the signaling pathway underlies the promotion of phagocytosis by SIRPβ1 in macrophages.*

MLC phosphorylation by MLCK increases myosin ATPase activity and elicits the reorganization of the actin cytoskeleton that underlies promotion of the phagocytic response (Hayash, 2004) as shown in figure 3.

Role of SIRPβ1 was partially investigated in macrophage function and the effect of SIRPβ1 engagement was examined by specific monoclonal antibodies (mAbs) on phagocytosis as well as on intracellular signalling (Hayash, 2004). A physiological ligand for SIRPβ1 remains to be identified. The ligand of SIRPβ1 might be a soluble protein, such as IgG or complement, which binds to a phagocytic target. Alternatively, it might be a microbial component such as bacterial lipopolysaccharide or peptidoglycan, both of which recognized by Toll-like receptors on macrophages or dendritic cells (Fujioka, 1996). We investigated the functional role of SIRPβ1 in microglia by analyzing the effect of SIRPβ1 engagement on phagocytosis, inflammatory responses as well as on intracellular signalling in microglia with specific monoclonal antibodies (mAbs).

1.3 Lentiviral vectors

With the human genome sequence in hand and rapid progress in its annotation, gene transfer will become a major tool in functional genomics, animal transgenesis, and gene therapy. The need for effective gene transfer systems is obvious, and number of gene transfer systems are being developed. Among these are lentiviral vectors, which have certain advantages over other vector systems. Lentiviral vectors integrate into the host genome. This is a useful property where a stable and long-term expression of the transgene is required and it obviates the need for repeated vector administration. This approach may be useful for directing gene therapy treatment to specific tissues and organs. In addition, gene delivery to the whole brain to treat global neuronal degenerative disorders could best be accomplished by targeting the viral vector for transport across the blood brain barrier and thus widespread distribution over the whole brain.

1.3.1 Introduction to lentivirus

Lentiviral vectors are a type of retrovirus that can infect both dividing and non-dividing cells because their preintegration complex (virus “shell”) can get through the intact membrane of the nucleus of the target cell. Lentiviruses can be used to provide highly effective gene therapy as they can change the expression of their target cell's gene for up to six months. They can be used for non-dividing or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, and

muscle and liver cells, cell types for which previous gene therapy methods could not be used. HIV is a very effective lentiviral vector because it has evolved to infect and express its genes in human helper T cells and macrophages. The only cells lentiviruses cannot gain access to are quiescent cells (in the G0 state) because this blocks the reverse transcription step (Amado and Chen, 1999). Lentiviral vectors, which are derived from retroviral vector, are considered the ideal gene delivery system. Potential of lentiviral vector was first revealed in 1996 through the demonstration that they could transduce neurons in vivo. Their ability to integrate into the genome of target cells allows for long term gene expression and they do not induce an immunological response and they have a large cloning capacity (up to 10kb). However, retroviral vectors have a major drawback in that they fail to infect nonmitotic cells (Lewis, 1994, Roe, 1993).

1.3.2 Genomic structure of lentiviruses

To obtain a lentiviral gene vector, a reporter gene or therapeutic gene is cloned into a vector sequence that is flanked by LTRs and the Psi-sequence of HIV. The LTRs are necessary to integrate the therapeutic gene into the genome of the target cell, just as the LTRs in HIV integrate the dsDNA copy of the virus into its host chromosome. The Psi-sequence acts as a signal sequence and is necessary for packaging RNA with the reporter or therapeutic gene in virions. Viral proteins, which make virus shells, provided in the packaging cell line, but are not in context of the LTRs and Psi-sequences and so are not packaged into virions. Thus, virus particles are produced that are replication deficient and are designed to be unable to continue to infect their host after they deliver their therapeutic content (Schmidt 2000). The life cycle of the lentivirus is common to all members of the *Retroviridae* family and can be described by the following steps (schematically represented in figure 4):

1- Attachment and entry: the interaction between the virus and the target cell occurs via specific receptors. Once bound to the surface, the viral and cellular membranes undergo fusion. After this fusion step, the virion nucleoprotein is delivered to the cytoplasm where reverse transcription begins.

2- Reverse transcription: synthesis of double stranded DNA from a RNA template carried out by the reverse transcriptase enzyme using cellular tRNA as a primer.

3- Integration: once the DNA synthesized, the viral integrase catalyzes its integration into the host genome and allows the provirus to become a permanent genomic element in the host.

4-Transcription and viral protein synthesis: the first transcription products from the virus code for the REV TAT and NEF. At a later stage, un-spliced and single spliced RNA species are produced.

5-Virion assembly and release: viral genome and structural proteins are packed into the viral particles and released at the plasma membrane.

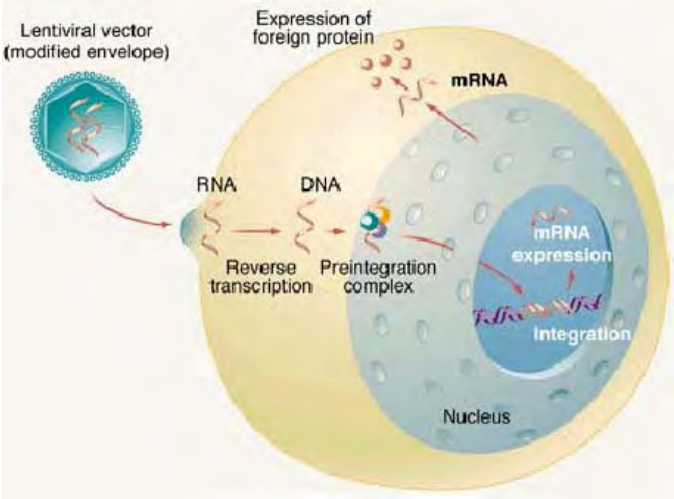


Figure 4. *Schematic representation of a retrovirus life cycle, which can be described in 5 steps from attachment of the virus till the new virion assembly and release.*

Lentiviruses comprise one of three subfamilies of retroviruses, which also include the oncoviruses (RNA tumour viruses) and spuma (or foamy) viruses. Members of all three subfamilies are able to infect humans and are associated with a spectrum of pathogenic potential, from no disease (as in the case of foamy viruses) to cancer (human T leukemia viruses, HTLV), immune deficiency (human immunodeficiency virus, HIV), and neurological disorders (HTLV and HIV). The first lentivirus was isolated in 1904 from a horse with haemolytic anemia and was named equine infectious anemia virus (EIAV) (Vallee, 1904). Since then, related viruses have been isolated from other ungulate species (e.g. - visna-maedi virus from sheep; caprine arthritis/encephalitis virus, CAEV, from goats; bovine immunodeficiency virus from cows, cats feline immunodeficiency virus, FIV nonhuman primates simian immunodeficiency virus, SIV), and humans

(Clements, 1992). Lentiviruses are generally associated with chronic diseases of the immune system and the central nervous system.

All retroviral derived lentiviruses contain minimally three genes, *gag, pol*, and *env*, which encode the structural proteins as well as the enzymes (reverse transcriptase, RNase H, integrase, and protease) required for virus replication. However, lentiviruses contain additional genes that are essential for or contributory to efficient virus replication and persistence. The prototype lentivirus, HIV-1, encodes six additional genes (*tat, rev, vif, vpu, vpr*, and *nef*) (figure 5).

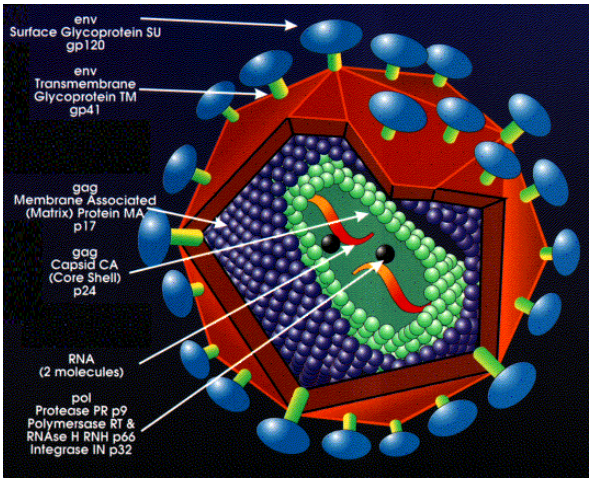


Figure5. *The non-primate lentiviruses contain fewer of these extra genes, but all contain the tat and rev genes, which are responsible for transcriptional and posttranscriptional activation of viral gene expression respectively (http://biology.kenyon.edu)*

1.3.3 Lentiviral vectors for gene therapy

The discovery that human immunodeficiency virus-type 1 (HIV-1) can infect both mitotic and non-mitotic cells (Bukrinsky, 1999, Bukrinsky, 1992) has led to the development of a new class of retroviral vector to be used for gene therapy. These vectors are termed lentiviral vectors, “lenti” being the Latin term for slow, referring to a slow and persistent rate of infection. It is the ability of this newly developed lentiviral system to allow infection of quiescent cells, which has brought a new hope to the field of gene therapy. While a number of lentiviruses have been described, the best understood is HIV-1 and thus most experimental vectors are based on this system. The first lentiviral vectors to use experimentally were not intended for use in gene delivery but for the study of HIV-1 pathogenicity. These vectors normally contained the entire viral genome with the exception of the *env* gene. A reporter gene was expressed in place of *env* and the envelope protein was then expressed by another construct (Page, 1990; Landau, 1991).

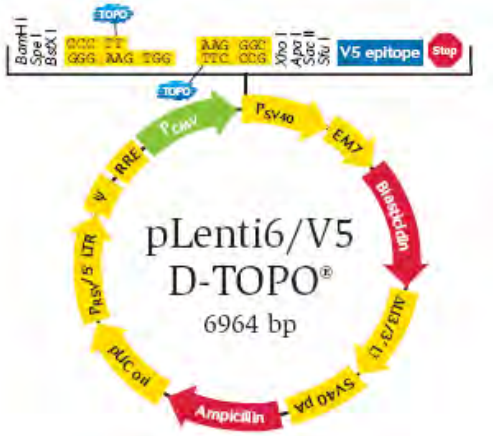


Figure 6 *pLenti6/V5 D-topo plasmid map From Web-site (www.invitrogen.com)*

The development of replication incompetent lentiviral vectors designed for the purpose of gene delivery began with the realization that lentiviral vectors may have some benefit over onco-retroviral vectors. The first generation of lentiviral vectors separated structural genes between two constructs. All cis-acting sequences were contained within a third construct expressing the gene of interest (Parolin, 1994). While this system worked, it gave low viral titers and was limited to the transduction of the natural target cells of HIV-1. Later vectors expressed structural elements on one construct under the control of the cytomegalovirus (CMV) immediate early promoter. Now days it is widely used by many research groups to analyse several mechanisms in central and peripheral nervous system.

The second vector was used to express the envelope protein, normally the vesicular stomatitis virus G-protein (VSV-G). The VSV-G protein confers pantropic activity on viral particles and allows for greater stability (Bartz, 1997; Burns, 1993). The third generation construct contained all required cis-acting sequences, e.g. the 5' and 3' LTRs and ψ, and the gene of interest under control of the appropriate promoter (Naldini, 1996). Such constructs made expression dependent on the presence of trans-acting proteins expressed from the first construct. While being functional, such vectors raised questions about safety, as homologous regions shared between the different constructs anticipated recombination events, which could give rise to replication competent viral particles (Hu, 1990). The second generation of lentiviral vectors tackled this problem by removing large amounts of the viral genome later discovered unnecessary for gene delivery. The use of the third generation of lentiviral gene delivery system is the replacement of the U3 region of the 5' LTR with a constitutively active promoter has allowed the removal of Tat from the system (Kim, 1998). In addition, it was found that Rev could be contributed from a separate vector (Dull, 1998). Thus, the vector expressing

structural and regulatory proteins no longer contains sequences for Tat and Rev. This third generation system is self-inactivating (SIN) to prevent the possible unwanted expression of genes proximal to the site of integration. Such transcriptional read through is due to transcriptional activity of the 5'LTR promoter as well as deficient cleavage and polyadenylation of vector transcripts within the 3'LTR (Swain, 1992). During reverse transcription, the 5'LTR of the resulting viral DNA derived from the 3'LTR of the viral RNA. The SIN system was accomplished by deleting the U3 region of the 3' LTR (Zufferey, 1998). The modified 3' LTR allows viral packaging but self inactivates the 5' LTR for biosafety purposes (Miyoshi, 1998). The element also contains a polyadenylation signal for efficient transcription termination and polyadenylation of mRNA in transduced cells (Iwakuma, 1999; Swain, 1989). An added advantage of this modification is that the elimination of transcription from the viral LTR allows the possibility for tissue specific expression upon the use of the appropriate promoter.

1.3.4 Silencing-lentivirus vector

In the RNAi field, it was discovered that plasmid-based RNAi could substitute for synthetic siRNAs, thus permitting the stable silencing of gene expression (Brummelkamp, 2002). In such systems, an RNA polymerase III promoter used to transcribe a short stretch of inverted DNA sequence, which results in the production of a short hairpin RNA (shRNA) that processed by Dicer to generate siRNAs. These vectors have been widely used to inhibit gene expression in mammalian cell systems (Ventura, 2004). Although the molecular mechanism of siRNA is not completely understood, it is generally believed that siRNA induces the degradation of target mRNA in a sequence-specific manner, leading to posttranscriptional silencing of gene expression. As such, siRNA could potentially be used both as a research tool and as a therapeutic approach. It is clear that siRNA-based gene silencing is still an evolving technology. It remains an open question whether the in vivo application of siRNA in mammals will revolutionize biomedical research and therapeutics or whether it is just one more tool with narrow utility. Indeed, clinical trials using siRNA to treat viral infection and other diseases are appearing on the horizon, the outcome of which will certainly have a tremendous impact on the fate of the entire siRNA field (Check, 2004). RNA interference (RNAi) has

emerged as a powerful tool to silence gene expression, and has rapidly transformed gene function studies across phyla. RNAi operates through an evolutionarily conserved pathway that is initiated by double-stranded RNA (McManus, 2002; Dykxhoorn, 2003). The ability of dsRNA to affect gene expression was already well known in mammals; here, a series of interferon-inducible pathways respond to dsRNA by inhibiting translation through the action of a dsRNA-activated protein kinase (DAI or PKR) (Williams, 1999). The key difference between this response and RNAi was their respective specificity: the PKR response inhibited gene expression globally, whereas RNAi had a specific effect on gene expression. A more specific dsRNA response in mammalian cells had also been proposed to operate through the localized activation of a ribonuclease that responds indirectly to dsRNA (Stark, 1998). The latter pathway might have provided a plausible explanation for RNAi, as it was clear that the targeted mRNA destroyed in response to dsRNA. However, a series of startling observations made it clear that RNAi was mechanistically distinct from any previously known dsRNA response. One of the first indications that RNAi was a novel biological phenomenon was the potency of its effect. The study described in this thesis employs the use of the third generation silencing lentiviral gene delivery system, which well known as LentiLox 3.7. This is a lentiviral vector designed for inducing RNA interference in a wide range of cell types, tissues and organisms. Rubinson et al (2003) have shown this vector to infect and efficiently silence proteins in hematopoietic stem cells and their progeny, and have used infected embryonic stem cells and single cell embryos to create transgenic animals (Douglas, 2003). Current approaches to study gene function such as generating knockout mice are time-consuming and expensive and cannot be applied to human tissues. RNAi has emerged as a rapid and efficient means to manipulate gene function in mammalian cells (McManus, 2002).

Retroviral vectors are efficient, stable gene delivery tools in mammalian cells (Lois, 2001; Scherr, 2002), and recent studies suggest that they can stably express shRNAs in transformed and primary cells (Brummelkamp, 2002). A lentivirus-based vector (pLL3.7) is developed that expresses RNAi-inducing shRNAs under the control of the U6 promoter Fig.7 (Tuschl, 2002).

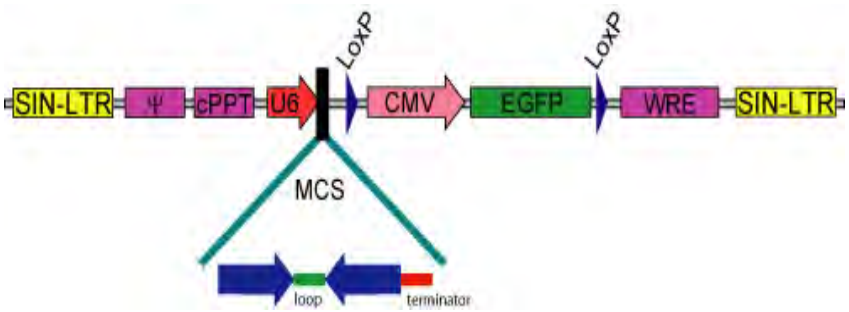
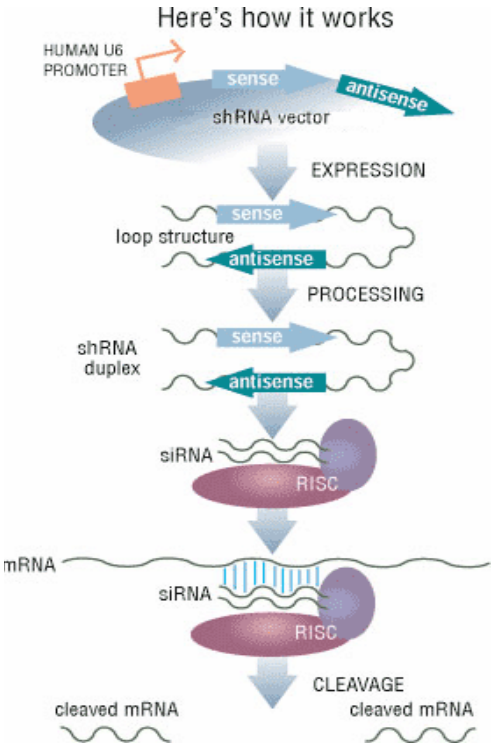


Figure 7. *Schematic diagram of pll3.7 silencing vector.*

This pll3.7 vector was engineered to co-express enhanced green fluorescent protein (EGFP) as a reporter gene, permitting infected cells to be tracked by flow cytometry. Lentiviruses have two key advantages over other gene delivery systems. First, they can infect non-cycling and post-mitotic cells (Naldini, 1998; Naldini, 1996). Second, transgenes expressed from lentiviruses are not silenced during development and can be used to generate transgenic animals through infection of embryonic stem (ES) cells or embryos (Pfeifer, 2002; Lois, 2002). In addition, Takahashi et al. demonstrated in 2005 that TREM2 knockdown microglia with the help of lentivirus-based background vector (pLL3.7) showed reduced phagocytosis. Thus, Deficiency of TREM2 results in impaired uptake of apoptotic neurons and increased production of inflammatory mediators (Takahashi, 2005).

RNA interference (RNAi) is a mechanism in molecular biology where the presence of certain fragments of double-stranded RNA (dsRNA) interferes with the expression of a particular gene that shares a homologous sequence with the dsRNA. RNAi is distinct from other gene silencing phenomena in that silencing can spread from cell to cell and generate heritable phenotypes in first generation progeny when used in *Caenorhabditis elegans* (Guo, 1995). RNAi appears to be a highly potent and specific process which is actively carried out by special mechanisms in the cell, known as the RNA interference machinery. While not all details of this mechanism are known, it appears that the machinery once it finds a double-stranded RNA molecule, gets processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step), Then the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process which separates into two strands. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate

RNA (effector step). dsRNAs direct the creation of small interfering RNAs (siRNAs) which target RNA-degrading enzymes (RNAses) to destroy transcripts complementary to the siRNAs. Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand.



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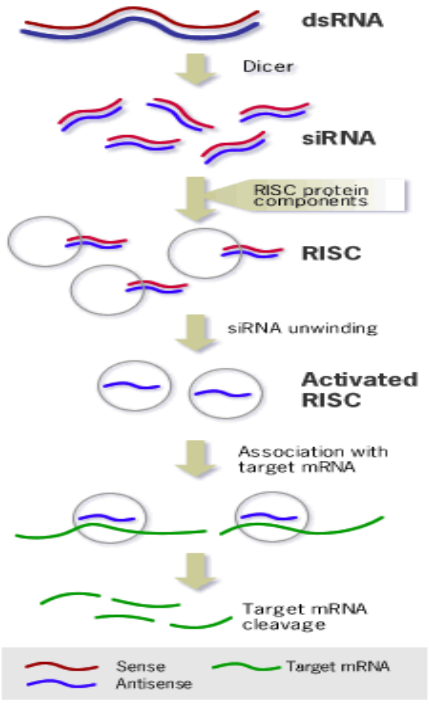


http://www.panomics.com/SR1000.cfm http://www.ambion.com/techlib/append/RNAi_mechanism.htm

***Figure 8**. Scechmetic presentation of silencing mechanism.*

One of the great mysteries surrounding RNAi is how a cell can respond to virtually any incoming dsRNA by efficiently and specifically silencing genes that are homologous to it. Since the seminal discovery of small RNA species, progress in both genetic and biochemical dissection of the silencing process have produced a basic understanding of the interference mechanism (Hammond, 2001).

1.3.5 Production of lentivirus

If a more concentrated lentiviral vector suspension is required, the vector preparation needs to be concentrated. Titers of viruses in general and lentivectors in particular are critically capable of achieving every step from cell binding to expression of the transgene depending on both vector and cell characteristics. Lentiviral vector contains

CMV promoter and a flurorescent protein gene as a reporter, under the control of a promoter that is active in most primary cells (Salmon, 2000). For several years now, 293FT cells have been using as a target. These cells are stable, easy to grow and 100% susceptible to transduction by VSV-G pseudotyped LVs. These are also now commonly used by many laboratories, which thus help comparing titers between them. The production of functional lentiviral particles performed by co-transfection of a packaging cell line with four different vectors as described. As Gag and Pol proteins are not assembled accordingly in murine cells, this procedure is performed in a human cell line (Swain, 1989) , the cell line of choice normally being based on 293FT cells, a human embryonic kidney cell line. For reasons still unknown, many other common laboratory cell lines, e.g.HeLa, successfully transfected and produce large amounts of viral protein but secrete only few viral particles (Haselhorst, 1998). The cell line used in this study is 293FT. The 293F cell line is a fast growing variant of 293 cells. 293FT cells are a variant of 293F stably expressing the SV40 large T antigen allowing for the replication of plasmids containing the SV40 origin of replication, which is present on the transfer vector used. Viral particles are secreted into the culture medium from which they are collected. Transduction of hematopoietic cells cultured in serum free medium is estimated to require anywhere between 10^7 and 10^8 transducing units18, (McTaggart, 2000). The pseudotyping of viral particles with the VSV-G protein allows for great stability compared to those with viral glycoproteins.

VSV-G pseudotyped particles may be stored at 4^oC for 2-3 days, can tolerate a freeze and thaw cycle and may be concentrated 100-fold by ultracentrifugation, all without a significant loss in viral titer (Bartz, 1997). The only disadvantage of using VSV-G pseudotyped virus is its inactivation upon contact with human serum, limiting its experimental use (DePolo, 2000). Secretion of viral particles exists maximal 24hr following transfection and decreases two-fold in the second 24 hours period. Low viral titers may be dealt with by altering culture conditions, e.g. decreasing the temperature to 32^oC as well as using low serum concentration (2%).

1.3.6 Biosafety of lentivirus

The general idea is to avoid contamination with the recombinant virus, which spreads through liquid or aerosol. Therefore, all the work should be contained within the same room. According to the decision of the Institutional Biological Committee, which was approved by the Institutional Safety Committee, lentivirus work is done locally, within the facilities of each department and is under the responsibility of the principle investigator. The development of the third generation lentiviral gene delivery system has addressed many problems of biosafety regarding the use of lentiviral vectors in the laboratory. These lentiviruses are pseudo-coated with VSV-G and are capable of infecting human cells, and thus present important biosafety issues (Douglas, 2003). The separation of cis and trans acting regions of the viral genome onto separate vectors has enabled the use of this system in scientific and clinical research. Progress in the safety level of its use has been achieved by decreasing the level of homogeneity between these vectors. However, the presence of some sequences is required on more than one vector, e.g. approximately 300bp of gag is required on both the packaging and transfer vectors, thus the possibility of recombination, while very small, must still be considered. Although, if such an event was to occur, infected cells could not express viral proteins due the self-inactivating property of the system (SIN), and transport of transcripts to the cytoplasm could not occur in the absence of Rev, which is delivered to packaging cells on its own expression vector. Another concern in clinical research is the possibility of recombination events between engineered virus and natural virus in patients already infected with HIV-1. Studies suggest that if such an event were to occur, the possibility of recombination between the genome of both viruses is quite likely, resulting in the emergence of a new viral species (Jetzt, 2000). The development of SIN lentiviral vectors has decreased the risk of aberrant expression of genes endogenous to the transduced cells, a matter of major concern in clinical gene therapy trials (Marshall, 2003). Debyer described how the field has engineered lentivectors with increasing biosafety both for the lab worker and for the patient. The risk associated with state-of-the-art lentivectors is therefore minimal, although a psychological barrier to use these vectors in the clinic may still have to be overcome. Due to their increased performance, care should be taken to

avoid accidental transduction of the lab worker with potential hazardous genes. The precautions that have to be taken described in detail (Debyser, 2003).

1.4 EAE and APP: Autoimmune and transgenic animal model for function analysis of SIRPβ1

1.4.1 Experimental Autoimmune Encephalomyelitis (EAE)

Animals immunized with components of CNS myelin develop an autoimmune demyelinating disease of the CNS called experimental autoimmune encephalomyelitis (EAE) (Whitehouse, 1969; Alvord, 1984; Zamvil, 1990). This disease is the result of a CD4C T cell–mediated immune response directed at specific proteins within the CNS, and it serves as a model of the human disease multiple sclerosis (MS).

In the case of EAE, progression of disease may be attributed either to sustained autoreactivity directed against an immunodominant priming determinant or to acquired autoreactivity directed against determinants not involved in the initiation of disease. This acquisition of neo-autoreactivity, commonly referred to as epitope spreading, presumably results from endogenous priming with new self-antigens generated from damaged tissue over the course of disease (Lehmann, 1992; McRae, 1995; Yu, 1996; Vanderlugt, 1996).

1.4.2 Molecular pathogenesis of experimental autoimmune encephalomyelitis (EAE)

EAE is an acute or chronic-relapsing, acquired, inflammatory and demyelinating autoimmune disease. The animals are injected with the whole or parts of various proteins that make up myelin, the insulating sheath that surrounds neurons. These proteins induce an autoimmune response in the animals - that is the animal's immune system mounts an attack on its own myelin because of exposure to the injection. The animals develop a disease process that closely resembles MS in humans. Several proteins or parts of proteins (antigens) are used to induce EAE including Myelin Basic Protein (MBP), Proteolipid Protein (PLP), and Myelin Oligodendrocyte Glycoprotein (MOG).

Advantages of EAE:

- EAE is an animal disease; hence it enables researchers (especially immunologists) to study demyelination (the process underlying the symptoms of MS) in ways that would not be morally acceptable in studies of MS in humans.
- It allows researchers to test potential treatments for MS for their efficacy and safety without putting the lives of people at risk.
- It allows researchers to experiment with different ways of inducing EAE to attempt to find potential causes of MS.
- Because the generations times of most of the EAE species are short and because they breed very fast, large populations of such animals can be turned over in short periods.

Disadvantages using EAE:

- EAE is **not** a multiple sclerosis and a number of significant assumptions are made when proposing EAE as an animal model of MS.
- It is undeniable that the animals involved suffer considerably - at the very least they are given the animal equivalent of MS - and questions about the ethics of EAE are inescapable.

Multiple sclerosis (MS) is the most common chronic disease of the CNS. "Self-reactive" T cell, responding to the myelin components of the isolating sheath of the nerve cells, has been implicated in the development of MS. Even though research into MS has been carried out a number of years, the cause of this demyelinating disease is still not known. The EAE model is worldwide used as the only animal model of human MS. There is a strong correlation between "self-reactive" (myelin protein specific) T cells and disease induction in the EAE model of MS. To induce EAE, mice are immunized with myelin protein or peptides and monitored daily for disease development. This protocol induces a strong T cell response and chronic disease in C57Bl/6 mice 12-17 days after immunization.

1.4.3 Alzheimer's disease and its pathological substrate

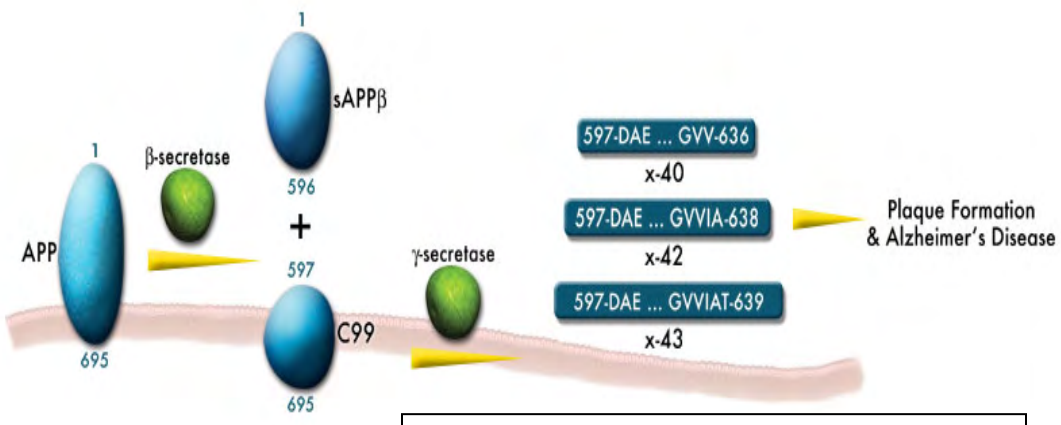
Alzheimer's disease (AD) is the most common cause of dementia. It usually occurs in elderly patients and starts gradually with early signs being forgetfulness,

particularly in remembering recent events and the names of people and things. There may be some other cognitive difficulties early on but nothing overly alarming. Emil Kraepelin first identified the symptoms of the disease as a distinct nosologic entity, and Alois Alzheimer (1906) first observed its characteristic neuropathology. In this sense, the disease was co-discovered by Kraepelin and Alzheimer, who worked in Kraepelin's laboratory. Because of the overwhelming importance Kraepelin attached to finding the neuropathological basis of psychiatric disorders, he made the generous decision that the disease would bear Alzheimer's name (Weber, 1997). Alzheimer himself described the characteristic light microscopic lesions-neurofibrillary tangles and amyloid-bearing senile plaques-in the limbic and cerebral cortices by using the Bielschowsky's method of silver impregnation. The neurofibrillary tangles were eventually shown by electron microscopy to be composed of nonmembrane-bound bundles of paired, helically-wound -10-nm filaments (PHF) in the perinuclear cytoplasm selected neurons (Kidd, 1963; Terry, 1963). The senile or neuritic plaque is a complex, multicellular lesion, the temporal genesis of which is only imperfectly understood. So-called "classical" neuritic plaques consist of a compacted spherical deposit of extracellular, ~8-nm amyloid filaments surrounded by variable numbers of dilated (dystrophic) neuritis, both axonal terminals and dendrites. Many such classical plaques contain apparently activated microglial cells intimately surrounding the amyloid core (Wisniewski, 1989).

1.4.4 Molecular pathology of amyloid precursor protein

AD is a debilitating neurodegenerative condition characterized by the loss of cognitive skills and severe behavioural changes (dementia). The principle protein implicated in the development of Alzheimer's disease is the amyloid precursor protein (APP). Indeed, a small subset of patients exhibiting familial AD exhibit APP mutations; this was the first genetic link to AD identified. Cleavage of APP by proteases known as secretases gives rise to a group of peptide fragments known as Amyloid β (Aβ). Aβ, which forms dense extracellular aggregates called amyloid plaques, has been demonstrated to induce neuronal death, which may ultimately lead to disease. The principle protein implicated in Alzheimer's disease is the transmembrane APP. Multiple APP isoforms, generated by alternative splicing have been described with a 770 amino

acid isoform being the largest and a 695 amino acid isoform being most prevalent in neuronal cells. Aβ is produced by sequential cleavage of APP by proteases called secretases. Proteolysis of APP by β-secretase, which cleaves APP695 after Met-596, produces a large soluble n-terminal fragment (sAPPβ) and a small membrane-bound c-terminal fragment (C99). The C99 fragment is further processed by γ-secretase cleavage within the trans-membrane region at Val-636, Ala-638 or Thr-639 to produce the three Aβ isoforms of 40, 42 or 43 amino acids, designated x-40, x-42 or x-43. The Aβ isoforms can induce neuronal apoptosis and aggregate into amyloid plaques. Progression of Alzheimer's disease is associated with increased levels of the x-42 and x-43 Aβ peptides (Buxbaum, 2004).



http://www.upstate.com/features/app_lp.asp

However, the expression of multiple βAPP isoforms in astrocytes (Siman, 1989; Haass, 1991) and the observation of apparent Aβ fibrils inside microglial cells (Wegial, 1990) have also led to hypotheses that either or both of these cells could contribute to the Aβ deposits. In the model of Alzheimer's disease, (Selkoe 1994) has reviewed some very considerable points to new therapeutic strategies aimed at one or more crucial steps in its molecular progression. First, one could partially inhibit the proteases that liberate Aβ from its precursor. Second, one could decrease the production and release of Aβ by a variety of means other than protease inhibition, for example by pharmacologically diverting some βAPP molecules from an amyloidogenic to a nonamyloidogenic-processing pathway. Third, one could retard the apparent maturation of extracellular Aβ deposits into neuritic plaques, perhaps by interfering with the fibrillogenesis that seems to

accompany this change. Fourth, one could interfere with the activities of microglial cells that contribute to the chronic inflammatory process around the neuritic plaques. This last point is one of our great interests in our hypothetical study for functional understanding of signal regulatory protein-beta1. Fifth, one could attempt to block the molecules on the surface of neurons or their intracellular effectors that mediate the apparent neurotoxic effects of Aβ and the proteins intimately associated with it. None of these pharmacological objectives will be easy to reach. However, the strength of accumulating data emerging from laboratories worldwide suggests that these are rational therapeutic targets which offer the best hope of slowing or arresting the progression of the fundamental disease process early in its tragic course (Selkoe, 1994).

2. Aims of the study

Nasu-Hakola disease known as a membranous lipodystrophy (polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy-PLOSL) is an autosomal recessive hereditary neurodegenerative disease where genetic mutation was identified in DAP12. DAP12 is a 12 kDa transmembrane protein recently recognized as a key signal transduction receptor element in immune cells. We have found that activation signals initiated through DAP12 predicted to play strategic roles in triggering microglial cells. The cytoplasmic domain of SIRPβ1 was reported to physically associate with the ITAM activation motif containing adapter protein DAP12.

Microglia constitute as many as 12% of the cells in the CNS and it seems that DAP12 is expressed in the microglial activation and the differentiation of macrophages in the CNS. The study of particular neurodegenerative disease where DAP12 is dominantly express along with its associated receptors SIRPβ1 could give us a therapeutic outline for understanding of the molecular mechanism for neurodegenerative research. It would be an advantage to understand whether SIRPβ1 could be involved in such diseases and if yes, then what the functional characteristics are. Could SIRPβ1 play any crucial role in Alzheimer's diseases and multiple sclerosis? In order to answer these questions we addressed to determine the functional assignment of SIRPβ1 in microglia.

Therefore, we focused on the following tasks:

1. It was prim importance to confirm expression of SIRPβ1 gene in microglial cells.
2. We tried to understand whether SIRPβ1 is involved in up or down-regulation of important microglial cytokines.
3. Whether stimulation of cytokine or chemokine such as interferon-gamma or beta does up or down-regulate SIRPβ1 and DAP12 levels in microglia.
4. Could silencing of SIRPβ1 in microglia shows any functional imbalances in CNS?
5. Does SIRPβ1 involved in some autoimmune or Alzheimer's like diseases?

3. Materials and Methods

3.1. Materials

3.1.1 Buffers and solutions

- **(10X) 0.125M Phosphate-Buffered Saline (PBS),** pH 7.3

Components	Concentration	Company
$NaH_2PO_4.H_2O$	0.007M	Roth, Germany
$Na_2HPO_4.7H_2O$	0.034M	Roth, Germany
NaCl	0.6M	Roth, Germany
ddH_2O	up to1 litter	Roth, Germany

- **4% paraformaldehyde (PFA),** pH 7.3

Components	Amount	Company
PFA	20g	Sigma, Germany
NaOH	30ml	Roth, Germany
PBS(10X)	50ml	
ddH_2O	up to 1 litter	Roth, Germany

- **Lysis buffer for erythrocytes**

Components	Concentration	Company
NH_4Cl	0.156M	Roth, Germany
$KHCO_3$	0.01M	Roth, Germany
EDTA	$5x10^{-6}$M	Roth, Germany
ddH_2O		Roth, Germany

- **(10X) TBE Buffer**

Components	Concentration	Company
Tris-Base	1.78M	Roth, Germany
Boric Acid	1.78M	Sigma, Germany
EDTA	0.04M	Roth, Germany
ddH_2O	to 2 litters	Roth, Germany

- **(6X) Loading buffer**

Components	Concentration	Company
EDTA	0,5M	Roth, Germany
Sucrose	60%	Fluke Biochemika, Germany
Bromphenol Blue	0,04%	Sigma, Germany
Xylene Cyanole	0,04%	Sigma, Germany
Ficol-400	2%	Bio-Rad, Germany

- **1% Agarose gel**

Components	Amount	Company
Agarose	0.5g	SeaKem,Cambrex, USA
Etidium Bromide or	1.25µl	Roth, Germany
Gel Star (when gel extraction)	4µl	BioWhittaker Molecular Applications, USA

TBE (1X)	50ml	

- **PCR reaction mix** (50 µl sample)

Components	Amount	Company
dNTP mix (10mM)	1µl	Amersham Bioscience, USA
Taq polymerase(100U/20µl)	0.5µl	Roche, Germany
Forward primer (10pmol/µl)	3µl	MWG, Germany
Reverse primer (10pmol/µl)	3µl	MWG, Germany
Buffer (10X)	5µl	Roche, Germany
dd H_2O	37.5µl	Roth, Germany

- **Reverse transcription (RT) mix** (20 µl sample)

Components	Amount	Company
Total RNA	5µg	
Hexanucleotide Mix (10X)	1µL	Roche, Germany
dNTP mix (10 mM)	1µL	Amersham Bioscience, USA
DTT mix (0.1M)	2µL	Invitrogen, Germany
5XRT 1st Strand Buffer	4µL	Invitrogen, Germany
RT enzyme (200U/ml)	1µL	Invitrogen, Germany
dd H_2O	up to 20µl	Roth, Germany

- **Real time RT-PCR** (25 µl sample)

Components	Amount	Company
SYBR Green Master Mix(2x)	12.5µl	Applied Biosystems, UK
cDNA	1µl	
forward and reverse primer pair mix (10 pmol/µl)	1µl	MWG, Germany
ddH_2O	10.5µl	Roth, Germany

- **Digestion reaction mix** (20 µl sample)

Components	Concentration	Company
Enzyme 1 (10U/µl)	0.5µl	Roche, Germany
Enzyme 2 (10U/µl)	0.5µl	Roche, Germany
Buffer (10X)	2µl	Roche, Germany
insert/plasmid	up to1µg	
ddH_2O	up to 20µl	

- **Ligation reaction mix** (10µl sample)

Components	Concentration	Company
T4 Ligase (1U/µl)	1µl	Roche, Germany
Ligation Buffer (10X)	1µl	Roche, Germany
DNA	8µl	
ddH_2O	10µl	Roth, Germany

3.1.2 Cell culture media and reagents

- **293FT cell line medium** (based on human embryonic kidney cell line HEK):

Components	Concentration	Company

DMEM		Gibco, Germany
Fatal Calf Serum	10%	PAN, Germany
1% L-Glutamate	1%	Gibco, Germany
1% Penisilin/Streptomicin	1%	Gibco, Germany
1% Glucose	1%	Sigma, Germany
Geneticin(when expanding)	1%	Gibco, Germany

- **Mixed-glia culture basal medium**

Components	Concentration	Company
BMS		Gibco, Germany
Fatal Calf Serum	10%	PAN, Germany
1% L-Glutamate	1%	Gibco, Germany
1% Penisilin/Streptomicin	1%	Gibco, Germany
1% Glucose	1%	Sigma, Germany

- **BME-based neuronal medium**

Components	Concentration	Company
BMS		Gibco, Germany
B-27	2%	Gibco, Germany
Fatal Calf Serum	1%	PAN, Germany
1% L-Glutamate	1%	Gibco, Germany
1% Penisilin/Streptomicin	1%	Gibco, Germany
1% Glucose	1%	Sigma, Germany

- **Other cell culture reagents**

Opti-MEM (reduced serum medium)	Gibco, Germany
Trypsin-EDTA(1X)	Gibco, Germany
Poly-L-Lysine	Sigma, Germany

3.1.3 Western blotting buffers and reagents

- **Preparation of lysis buffer P**
  10x stock lysis buffer (500mM)

Components	Concentration	Company
Tris-HCl	30.28g	Roth, Germany
all diluted in 1lit ddH_2O	500ml	Roth, Germany

1x stock lysis buffer (50mM)

Components	Concentration	Company
NaCl	120mM	Roth, Germany
EDTA	5 mM	Roth, Germany
NP-40	0.5%	

Stacking gel buffer

Components	Concentration	Company
Tris –HCL, pH-6.8	0.5 M	Roth, Germany
SDS	0.4%	Roth, Germany

Separation gel buffer

Components	Concentration	Company
Tris –HCL, pH-8.8	1.5 M	Roth, Germany
SDS	0.4%	Roth, Germany

Preparation of 100 mM PMSF in Isopropanol **M=174.19 g/mol**

174.19: 1000 ml x 100 = 17,419 g

For 10 ml: 10 ml x 17,419 g: 1000 ml = 0.17419 g.

- **Preparation of proteinase inhibitors**

1. Stock solutions:

Components	Concentration	Company
Aporptinin	10gm/ml	Chemicon, Germany
Hepes	0.01M	Chemicon, Germany
Leupeptin	5mg/ml	Chemicon, Germany
PMSF	100mM	Chemicon, Germany
Isopropanol	10ml	Roth, Germany

2. Working solutions:

- Thaw Aporotinin and Leupeptin on ice.
- Put 2 µl of Aporotinin and 2 µl of Leupeptin together in eppendorf tube (on ice).
- Centrifuge shortly and freeze at -20°C.

I) SDS-Gel Electrophoresis

NuPAGE LDS Sample Buffer (4x) (Invitrogen, cat. number NP0007).

β-mercaptoethanol (Fluka, cat. number 63689).

II) Electrophoresis

- **NuPAGE MOPS SDS Running Buffer:**

Components	Concentration	Company
MOPS	50mM	Roth, Germany
Tris-Base, pH-7.7	50mM	Roth, Germany
SDS	0.1%	Roth, Germany
EDTA	1mM	Roth, Germany

Prestained protein marker (e.g. PageRuler™ Prestained Protein Ladder Plus from Fermentas, cat. number SM1811).

NuPAGE 1ß% Bis-Tris Gel 1.5 mm X 10 well (Invitrogen, cat. number NPO315BOX). **Power Supply.**

XCell *SureLock*™ Mini-Cell unique cam (Invitrogen, cat. number EI0001).

- **Blotting**

1. 10x Transfer Buffer:

Components	Concentration	Company
Tris-HCL	25mM	Roth, Germany
Glycine	192mM	Roth, Germany
ddH_2O	1000ml	Roth, Germany

2. 1x Transfer Buffer

Components	Concentration	Company
10x Transfer buffer	100ml	Roth, Germany
Methanol	200ml	Roth, Germany

ddH_2O	700ml	Roth, Germany

3. Additionally:

Aqua bidest.	Sarstedt, Germany
Plastic tray	Sarstedt, Germany
Plastic tank	Sarstedt, Germany
Forceps	Sarstedt, Germany
Gel knife	Sarstedt, Germany
Magnetic stirrer	Sarstedt, Germany
Blotting membrane, e.g. nitrocellulose membrane	
Filter paper	Bio-Rad
XCell *SureLock*™ Mini-Cell unique cam	
Power Supply	Invitrogen, Germany

- **Blocking the membrane**

Components	Concentration	Company
1x PBS	500ml	Roth, Germany
Tween-20	250µl	Roth, Germany
Milk powder	5g	Roth, Germany

- **Primary antibody incubation**

1. Primary antibody
2. Antibody diluent ECL Advance Blocking Agent (supplied with the ECL Advance Western Blotting Detection Kit from Amersham Biosciences, product code RPN2135).

Washing the membrane

PBS-Tween-20-Solution

- **Secondary antibody incubation**

Components	Concentration	Company
HRP labelled secondary antibody	-	Chemicon, Germany
PBS - Tween-20 in Milk powder	5%	Roth, Germany

- **Washing the membrane**

PBS-Tween-20-Solution …. Roth, Germany

- **Detection**

1. ECL Advance Western Blotting Detection Kit (Amersham Biosciences, product code RPN2135).
2. Photo paper (e.g. Hyperfilm™ECL from Amersham Biosciences, product code RPN3103K).

3.1.4 Antibodies

- **Primary antibodies**

Fluorophore	Specificity	Source	Company
CD11b	Macrophages, Microglia	Rat	BD Pharmingen, DE.
SIRPβ80	-	Rat	Japanese group of Takashi Matozaki.
SIRPβ84	-		

Isotype-IgG	-	Rat	BD Pharmingen, Germany
β-tubulinIII	-	Mouse	Sigma, Germany
IbaI	Microglia	Mouse	
TREM2	-	Mouse	
Flag	-	Mouse	Sigma, Germany
GFP	-	Mouse	BD Pharmingen, Germany
Actin	-	Mouse	
Phospho-tyrosin	-	Mouse	
		Mouse	
Biot-APP	-	Mouse	

- **Secondary antibodies**

Fluorophore	**Specificity**	**Source**	**Company**
FITC	Mouse	Goat	Dianova, DE
FITC	Rat	Goat	Dianova, DE
Cy3	Rat	Goat	Sigma, DE
IgG & IgM, peroxidase	Mouse	Goat	Chemicon, DE

3.1.5 Primer sequences

The following oligonucleotides used for PCR amplification: (All primers purchased from MWG, Germany)

Target	**Accession number**	**Oligo nucleotide**	**Sequence**
SIRP-α	NM_007547	forward	5'-CTGTTTCTGTACAGGAGCCAC-3'
		reverse	5'-CTTCCTCTGGACCTGGACACTAGCATAC-3'
SIRP-β1	NM_001002898	forward	5'-CCCGTTCACAGGAGAACATT-3'
		reverse	5'-CCGGAGACCATAGGTGAAGA-3'
18s	X67238	forward	5'-ATCCATTGGAGGGCAAGTCT-3'
		reverse	5'-CCGCGGTCCTATTCCATTAT-3'
WRE		forward	5'-ACCGGTTAATCAACCTCTGG-3'
		reverse	5'-GGTACCGCGGGGAGGCGGCCCAAA-3'
β-Actin	X55749	forward	5'-TGCGTGACATCAAAGAGAAG-3'
		reverse	5'-TAGAATTACCCGCCAAGCAC-3'
SV-40		forward	5'-CAGCAGGCAGAAGTATGCAA-3'
		reverse	5'-CCTCGGCCTCTGCATAAATA-3'
Egfp		forward	5'-AGCCACAACGTCTATATCATGGCCGAC-3'
		reverse	5'-TCACGAACTCCAGCAGTGACCATGTGAT-3'
DAP12	NM_011662	forward	5'-ATGGGGGCTCTGGACCCCT-3'
		reverse	5'-TCATCTGTAATATTGCCTCTGTGT-3'

Table I. Real time RT-PCR primers

- **Cloning primers**

Target	Accession number	Oligo nucleotide	Sequence
IFN-γ	NM_008337	forward	5'-ACTGGCAAAAGGATGGTGAC-3'
		reverse	5'-TGAGCTCATTGAATGCTTGG-3'
IL-1β	NM_008361	forward	5'-ACAACAAAAAAGCCTCGTGCTG-3'
		reverse	5'-CCATTGAGGTGGAGAGCTTTCA-3'
TNF-α	NM_013693	forward	5'-CCGTCAGCCGATTTGCTATCT-3'
		reverse	5'ACGGCAGAGAGGAGGTTGACTT-3'
TGF-β	NM_011577	forward	5'-AGGACCTGGGTTGGAAGTGG-3'
		reverse	5'-AGTTGGCATGGTAGCCCTTG-3'
NOS2	NM_010927	forward	5'-GGCAAACCCAAGGTCTACGTTC-3'
		reverse	5'-TACCTCATTGGCCAGCTGCTT-3'
IL-10	NM_010548	forward	5'-AGGCGCTGTCATCGATTTCTC-3'
		reverse	5'-TGCTCCACTGCCTTGCTCTTA-3'
Sirpβ1-133	NM_001002898	forward	5'-CCTGCTGATTCGGGTACCTA-3'
		reverse	5'-GACCGGAGACCATAGGTGAA-3'
18s-134	X67238	forward	5'-CATGGCCGTTCTTAGTTGGT-3'
		reverse	5'-GAACGCCACTTGTCCCTCTA-3'
DAP12-120	NM_011662	forward	5'-TGCCTTCTGTTCCTTCCTGT-3'
		reverse	5'-AATCCCAGCCAGTACACCAG-3'
SIRP-α-320	NM_007547	forward	5'-CCGTATCAGTAATGTCACCCCAG-3'
		reverse	5'-CTGTGCTGGAGATGTTGTAGGAG-3'

3.1.6 Consumables

6 and 24-well culture plates	CellStar, VWR International, Germany
15ml tubes	CellStar, VWR International, Germany
50ml tubes	Sarstedt, Germany
5ml, 10ml, 25ml pipettes	Sarstedt, Germany
Chamber slides	Nunc GmbH, Germany
Cryovials	VWR International, Germany
75cm^2 and 175 cm^2 culture flasks	Sarstedt, Germany
5ml polystyrene round-bottom tubes	BD Falcon, USA
3cm,10cm culture dishes	Sarstedt, Germany
Bacteria culture 10ml tube	Sarstedt, Germany
500µl, 1000µL plastic tube	Eppendorf, Germany
PCR tubes	Biozym Diagnostik, Germany
10µl, 100µl and 1000ul tips	Starlab, Germany
96 well- optical reaction plate	Microamp, Applied Biosystems, Germany

5ml, 10ml syringes	Braun, Omnifix, Labomedic, Germany
Needles	100Sterican, Braun, Germany
Stopcock for infusion	Discofix, Braun, Germany
Glass slices for cryosectioning	Menzel-Glacer, Germany
Bottle top filters (0.25µm pore)	Millipore, Germany
Filters (0.45µm and 0.2µm pore)	Filtropur, Sarstedt, Germany

3.1.7 Equipment and software's

Centrifuges	Ultracentrifuge, Sorvall Discovery^{TM} 90SE, Hitachi, Germany Megafuge, 1.OR. Heraeus, Germany Biofuge Fresco, Heraeus, Germany
Cryostat	Micron HM560, Microm Int., Germany
Flow cytometer	FACSCalibur, Becton Dickinson Bioscience, Germany
Electrophoresis gel chambers	Blomed Analitik GmbH, Germany
Power supply	Amersham Bioscience, Germany
Heating block	Stuart Scientific, Germany
Incubators	Heracell240, Heraeus, Germany
Laminar-Air-flow workbench	Herasafe, Heraeus, Germany
Microscopes	Axiovert40CFL, Zeizz, Germany Axiovert200M, Zeizz, Germany Fluoroview1000 Confocal micro., Olympus, Germany
pH-meter	Hanna Instruments, Germany
Photometer	Biophotometer, Eppendorf, Germany
Real time thermocycler	ABI Prism 5700 Sequence Detection System, Applied Biosystems, UK
Thermocycler	T3, Biometra, Germany
Transiluminator	Dark Reader, Clare Chemical Research, Germany
Vortex	$2X^2$, VelpScientifica, Germany
Scale	Sartorious, Germany
-80°C Freezer	Herafreeze, Heraeus, Germany

Magnetic stirrer	Velp Scientific, Germany
Software's	Openlab4.0.1, Improvision, Germany CorelDraw. Graphics Suite 11, Germany Endnote v8,Thomson ISI ResearchSoft, USA Microsoft Office XP, Microsoft USA, USA Olympus FluoView1.4. Olympus, Germany SDS 2.2.2, Applied Biosystems, USA Cellquest Pro, BD Biosciences, USA

3.1.8 Kits and additional reagents

- **DNA and RNA purification kits**

Kit name	Purpose	Company
Endofree Plasmid Maxiprep	Plasmid extraction (up to 500µg)	All from Qiagen, Germany
QIAprep Plasmid Miniprep	Plasmid extraction (up to 20µg)	
Min iElute Gel extraction	DNA (70bp-4kb) extraction from agarose gels	
Mini Elute Clean up	DNA (70bp-4kb) purification from reactions	
RNeasy Mini	Total RNA (up to 100µg) purification	
RNeasy Mini for lipid tissue	Total RNA (up to 100µg) purification from lipid tissue	

- **Additional reagents**

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK)	Charité, Berlin
Pertussis toxin	List Biological Laboratories, USA
Mycobacterium Tuberculosis H37Ra	Difco, USA
Incomplete Freund's adjuvant	Difco, USA
Lipofectamine2000 reagent	Invitrogen, Germany
Ampicillin	Sigma, Germany
LB agar and LB media	Fluke Biochemika, Germany
DMSO	Sigma, Germany
Glycerol	Sigma, Germany
Tissue tek O.C.T. compound	Sakura, NL
β-Mercaptoethanol	Sigma, Germany
Ethanol	Roth, Germany
Propidium Iodide	Sigma, Germany

3.2 Animals

Adult 6-9 week old female C57BL/6 mice were obtained from Charles River, Sulzfeld, Germany. For induction of experimental autoimmune encephalomyelitis (EAE), neuronal culture pre-natal as well as for all mixed glia culture experiments 3 to 4 days old post-natal mice used and further microglias were isolated from these mixed glia cultures.

3.3 Tissue culture

3.3.1 Mixed-glia culture

75cm^2 sterile flasks were used for culture. Flasks were incubated with 7ml of PLL overnight in the incubator at 37^{0}C for 5% of CO_2. Next day, 3 to 4 days old postnatal mice were used for culture. Hippocampus and cortex were isolated into one dish of 35cm^2 containing ice cold 1xPBS and then the meningers were removed and transfered into the sterile 15ml tube containing 5ml of ice cold 1xPBS. Working into the laminar flow the cells were resuspend using 5ml pipette and kept into the ice for 5 min. After 5 minute of incubation on ice, upper part (supernatant) was transfered into a new 15ml tube, carefully and then centrifuged for 7min at 2500 rpm, 4^{0}C. After centrifugation, supernatants were aspirated and pellets were resuspended into the 5 to 6ml of basal medium and then cultured in 10ml mixed-glia culture basal medium containing of 75cm^2 sterile flasks and then cultured flasks were kept into the incubator at 37^0 C for 10% of CO_2.

Mixed-glia culture basal medium were changed twice a week for at least one month for microglia isolation.

3.3.2 Primary cell cultures

Microglia was prepared from the brains of postnatal day 3 to 5 (P3 or P4) C57BL/6 mice as described previously (12). In brief, to collect microglial cells, the cultures were shaken on a rotary shaker (200 rpm) for 2 h. The detached microglial cells were seeded in normal culture dishes for 1 h, and then all nonadherent cells were removed and discarded. Purity of the isolated microglia was > 95% as determined by flow cytometry analysis with antibody directed against CD11b (BD Biosciences). Microglial cells were cultured in basal medium as described above.

3.3.2.1 Staining of cell suspension for flow cytometry analysis

In brief, after 2hrs of mix glia culture flask shaking cell suspension contained supernatant were collected. Centrifuged cell suspension in the culture tube and resuspended the pellet in appropriate volume of primary antibody at recommended dilution into the 1:200. For control, we included an isotype-matched antibody in each

case. After gentle vortex cells were incubated at 4°C for 30 minutes. Then, 1ml of PBS was added to the pellet and centrifuged at 2000 rpm for 5 minutes. Appropriate volume of secondary reagent such as fluorescent molecule and/or conjugated antibody at recommended dilution was mixed carefully and incubated at 4°C for 30 minutes. Finally, stained cells were washed with 1ml of PBS and centrifuge at 2000 rpm for 5 minutes and after centrifuge supernatant was once again discard. Resuspend the pellet in flow Cytometey buffer and adjusting the cell suspension to concentration of $1x10^6$ cells/ml in ice cold 1xPBS in ice bucket and analysis was done with a FACS-Calibur™ flow cytometer (BD Biosciences).

3.3.3 Neuronal culture

Primary neuronal cultures were prepared from hippocampus and cortex of C57BL/6 mice embryos (E15) as described previously (Neumann H, et al, 2002) and (Neumann et al., 1995). Briefly, neurons were isolated from whole brains of embryonic day 16 mice, and the meninges were removed. Cells (5×10^3/ml) were plated into dishes that had been pretreated with poly-L-ornithine (0.5 mg/ml; Sigma, St. Louis, MO) in 0.15 M boric acid. Cells were cultured in chemically defined medium containing basal medium Eagle (BME; Invitrogen, Gaithersburg, MD) with B27 supplement [2% (v/ v), Invitrogen] and glucose [1% (v/v) 45%; Sigma]

3.3.4 Bone-marrow culture

To isolate BM cells, mice were sacrificed, skin and muscle were removed from their hind limbs, ends of the bones were cut off and BM from femora and tibiae was flushed out using a 27 gauge needle and 5 ml syringe, and collected in 5 ml of cold 1X PBS. Erythrocytes were lysed using 1ml/mouse of hypotonic solution for 30 sec. Immediately, PBS was added preventing further lysis of white blood cells and the suspension was centrifuged (5 min. 2000 rpm 4°C). After a second washing step, total BM cells collected. RNA was isolated using RNeasy minikit (Qiagen).

3.4 Lentivirus based vector system

3.4.1 Cloning into lentiviral vectors

Lentiviral vectors of third generation (pLenti6/V5 D-TOPO, Invitrogen) were used for genetic modification of cells. The CMV promoters were used and as well according to requirement were replaced to the phosphoglycerate-kinase (PGK), neuronal specific synapcin1 or microglia specific the ionized calcium-binding adaptor molecule 1 (Iba1) gene. The genomic copy of the Iba-1 gene was located within a segment of the major histocompatibility complex class III region between the Bat2 and TNFα genes. Among the brain cells Iba-1 gene specifically expressed in microglia. Iba-1 protein is therefore suggested to act as an adapter molecule, mediating calcium signals that may function in a monocytic lineage including microglia (Kohsaka, 2005). Downstream the promoter, enhanced GFP (eGFP) reporter gene was inserted into the TOPO cloning sites or for therapeutic approach GFP was cut out using BamHI and XhoI (Roche). The mouse SIRPβ1 gene of 1176 base pairs (NM_001002898) and the mouse DAP12 gene of 345 base pairs (NM_011662) (provided by E. Vivier, Centre d'Immunologie, Marseille, France) were tagged with the GFP gene and cloned into the pLenti6/V5 vector, previously amplified using extended primers was inserted. Accordingly, gene expression was confirmed with transfection of the packaging cell line by fluorescence imaging or RT-PCR (primers detailed in Materials, RT-PCR primers list).

3.4.2 Silencing lentiviral vectors construction

Mostly, in our experimental set-up lentiviral vectors of the third generation, PLL3.7 (provided by L. van Parijs, MIT, Cambridge, MA) and pLenti6/V5 (Invitrogen) were used for the transduction of microglia. The mouse SIRPβ1 gene was derived from reverse-transcribed RNA obtained from primary cultured microglia. To analyze the functional characteristic of SIRPβ1 we tried to knockdown this gene by using short-hairpin RNA interference technique in microglia cells. The correct nature of all cloned sequences was confirmed by automated sequencing (Seqlab) of the vectors. wSIRPβ1 short hairpin RNA sequence 5'-TAA CTG AAG ACG GCA GGT ATT CAA GAG ATA CCT GCC GTC TTC AGT TTT TTT TC-3' and mutant-type mouse SIRPβ1 short

hairpin RNA sequence for control study 5'-TAA GAC TTG AGC GCA GGA ATT CAA GAG ATT CCT GCG CTC AAG TCT TTT TTT C-3' were inserted into the PLL3.7 silencing vector as described by Rubinson et al. (Rubinson, 2003). For overexpression of SIRPβ1, we tag three times FALG tag at the N-terminal of SIRPβ1 gene in the pll3.7 vector.

Lentiviral transduction was performed as described previously (Rubinson, 2003). In brief, PLL3.7 or pLenti6/V5 vectors were purified and then cotransfected together with packaging vectors (Invitrogen) into 293FT cells (Invitrogen). Supernatant was collected after 48hrs, and viral particles in the supernatant were concentrated at 1:100 to 1:300 by ultracentrifugation for 90 min at 25,000 rpm (Sorvall Discovery™ 90SE Centrifuge) and recovered by suspension in PBS. Titers of viral particles ranged between 10^6 and 10^7 U/ml multiplicity of infection. Purified microglia seeded at 2 x 10^5 cells/ml into 24-well plates. Lentiviral particles and 8 μg/ml polybrene (Sigma-Aldrich) were added to the culture and centrifuged for 90 min at 30^0C. Supernatant was removed immediately after infection and replaced with BME medium containing 10% FCS and 50% glial culture supernatant. In all experiments the efficiency of microglial transduction was at least 90% (the number of microglias, expressing the GFP molecule) as determined by immunocytochemistry.

3.4.3 Production of viral particles

The 293FT (Invitrogen) packaging cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 1% penicillin/streptomycin and glucose (Sigma) at 37°C in 10% CO_2.

In order to produce viral particles, 5 million 293FT cells were seeded in 10 cm dishes precoated with poly-L-Lysine (Sigma). After removing the culture medium and washing once with PBS, 5 ml of Opti-MEM medium (Gibco) without antibiotics were added. Cells were transfected using the gene-containing vector together with three packaging helper plasmids and Lipofectamine 2000 reagent (Invitrogen). The principle of this procedure is the entrance of the DNA to the cells after forming lipidic complexes form with the Lipofectamine2000 reagent. Medium was replaced for fresh DMEM 10 hrs post-transfection. Viral supernatant (10ml) was collected at 48-72 hrs post-transfection.

To increase viral titres, particles were concentrated by ultracentrifugation (25000 rpm for 1hr and 30min at 4°C) using a Sorvall Discovery^{TM} 90SE ultracentrifuge. Supernatant was removed, and pellet resuspended by slightly shivering the tubes overnight at 4°C in 250µl of medium. Particles were used immediately for transduction or frizz down in -80^{0}C.

3.5 Microglia transduction

Microglia cells were isolated from mixed-glial culture. The cultures were shaken on a rotary shaker (200-400 rpm) for 2 h. Supernatants contained microglia cells suspension were centrifuged for 1500 rpm for 5 min. at 4°C. After, removal of supernatant 200µl of BME medium were added to the pellet and transduced using fresh or -80°C frozen concentrated viral particle along with concentration of 1µg/µl of Polybrene (final concentration approximately 8 µg/ml polybrene (Sigma-Aldrich)) in to the one well of 24 well- culture-dish. 24 well-culture-dishes were centrifuged at 3000 rpm for 90 min. at 30°C. Media changed to fresh BME after centrifugation.

3.6 Immunocytochemistry

3.6.1 Immunocytochemical labelling of microglia cells

Microglia was fixed in 4% paraformaldehyde for 1 h, blocked by 1% BSA/PBS for 2 h, and then immunostained with a purified monoclonal rat antibody directed against SIRPβ1 (SIRPβ-80 and SIRPβ-84) (provided by Takashi Matozaki, Gunma University, Japan) and a secondary fluorescence goat-anti-rat-Cy3 antibody directed against rat IgG (1:200; Dianova). To identify the cell type, cells were double labelled with monoclonal mouse antibody directed against CD11b followed by a secondary fluorescence FITC-conjugated antibody directed against mouse IgG. Images were collected by confocal laser scanning microscopy with a 40x objective (Olympus).

3.6.2 Immunocytochemical labelling of neuronal cells

Microglia was fixed in 4% paraformaldehyde for 1 h, blocked by 1% BSA/PBS for 2 h, and then immunostained with a purified monoclonal rat antibody directed against

SIRPβ1 (SIRPβ-80 and SIRPβ-84) (provided by Takashi Matozaki, Gunma University, Japan) and a secondary fluorescence goat-anti-rat-Cy3 antibody directed against rat IgG (1:200; Dianova). To identify the cell type, cells were double labelled with monoclonal mouse antibody directed against beta-tubulin1 followed by a secondary fluorescence FITC-conjugated antibody directed against mouse IgG. Images were collected by confocal laser scanning microscopy with a 40x objective (Olympus).

3.6.3 Immunocytochemical labelling of astrocytes

Microglia was fixed in 4% paraformaldehyde for 1 h, blocked by 1% BSA/PBS for 2 h, and then immunostained with a purified monoclonal rat antibody directed against SIRPβ1 (SIRPβ-80 and SIRPβ-84) (provided by Takashi Matozaki, Gunma University, Japan) and a secondary fluorescence goat-anti-rat-Cy3 antibody directed against rat IgG (1:200; Dianova). To identify the cell type, cells were double labelled with monoclonal mouse antibody directed against GFAP followed by a secondary fluorescence FITC-conjugated antibody directed against mouse IgG. Images were collected by confocal laser scanning microscopy with a 40x objective (Olympus).

3.7 Microglia chemokine and cytokine stimulation

To analyze functional properties of SIRPβ1 primary microglia cells were isolated and purified from postnatal mouse brain tissue as reported before. Activation of these cells was done with tumour necrosis factor-alpha (10ng/ml), tumour necrosis factor-beta (10^3U/ml), interferon-gamma (100 U/ml) and LPS (1mg/ml) also incubated for 48 and 72 hours at 37°C in 10% CO_2. After incubation, RNA was isolated and prepared for cDNA. Inflammatory mediators could induce gene transcription of SIRPβ1 and DAP12 in primary microglia were quantitatively analysed by real time PCR.

3.8 Western blotting

For Western blotting, 293 cells (Invitrogen) were cotransfected with the fSIRPβ1 plus DAP12-GFP plasmids. Cells were added to culture dishes coated with the antibody directed against the Flag epitope or control antibody and shortly centrifuged. After 10 min the cells were lysed, and the total protein lysate was analyzed with antibody specific

for tyrosine phosphate (4G10; Upstate Biotechnology) by NuPAGE electrophoresis system (Invitrogen) and ECL Advance Western Blotting Detection Kit (Amersham Biosciences). In a second step, the membrane was reblotted with antibody directed against GFP (BD Biosciences), Flag (Sigma) and beta-actin (AbCam) as a positive control.

3.9 Induction of Experimental Autoimmune Encephalomyelitis

3.9.1 Animals

Adult 6-9 week old female C57BL/6 mice were obtained from Charles River, Sulzfeld, Germany. For induction of experimental autoimmune encephalomyelitis (EAE), mice were injected in the tail base bilaterally with 200 µl of an innoculum containing 100 µg of MOG35-55 (amino acids MEVGWYRSPFSRVVHLYRNGK; Seqlab) and 1 mg *Mycobacterium tuberculosis* H37 Ra (DIFCO, Detroit, MI) in incomplete Freund adjuvant (DIFCO). Pertussis toxin (200 ng; List Biological Laboratories, Campbell, CA) was injected on day 0 and day 2 of immunization. Clinical signs were scored as following: 0, no clinical signs; 1, complete limp tail; 2, complete limp tail and abnormal gait; 3, one hindlimb paralysis; 4, complete hindlimb paralysis; 5, fore- and hindlimb paralysis or moribund. Only mice having disease onset (clinical score of one or more) on day 14 were used for experiments. Mice were sacrificed for histopathology and RNA was isolated at day 14.

3.9.2 Myelin Oligodendrocyte Glycoprotein (MOG) emulsion

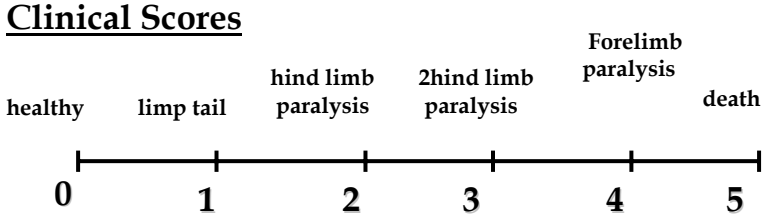
MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; Charité, Berlin) was emulsified with 1 mg of Mycobacterium Tuberculosis H37Ra (Difco, Detroit, MI) in incomplete Freund's adjuvant (Difco). Emulsion was left overnight at 4ºC before mice immunization.

3.9.3 Immunization of animals with MOG_{35-55} peptide

For immunization protocol, adult 6-8 weeks old female C57Bl/6 mice were anesthetized and injected subcutaneously in both inguinal lymph node regions with 200 µg of MOG_{35-55} emulsion.

PTX (200 ng, List Biological Laboratories, Campell, CA) was injected on day 0 (intravenously) and 2 (intraperitoneally). Weight and clinical score of animals was checked daily for at least 30 days thereafter unless moribund

Scoring of clinical symptoms was as follows:



0- Healthy, 1- Complete limp tail, 2- Weakness of hind limbs, 3- Paralysis of hind limbs
4 - Weakness and paralysis of fore limbs and 5 - Death.

Animals with a clinical score less than 1 by day 14 post-immunization were not used for the study.

3.10 Immunohistochemistry

3.10.1 Immunohistochemistry labelling in EAE and APP sections

To demonstrate the presence of SIRPβ1 in microglia cells and splenocytes, we investigated brains, spinal cords and spleens from 7-8 weeks old, female C57BL/6 mice (n=12) in experimental autoimmune encephalomyelitis (EAE) model. The animals were intravenously injected by PBS (n=6, control group) or MOG 35-55 antigen (n=6, experimental group) to induce EAE. The mice were treated in agreement with the German law on the use of laboratory animals. They were observed on daily basis with weight determination and evaluation of the clinical symptoms of EAE. At day 16^{th} (the peak of the disease), the mice were anesthetized with 1.25% solution of avertin. A perfusion fixation of the animals was performed transcardinally with tris buffered saline (TBS) with heparin (pH 7.4) followed by the injection of 0.1 M Phosphate buffered saline

(PBS) (pH 7.4) containing 4% paraformaldehyde. Subsequently, brains, spinal cords and spleens were removed and fixed in a 4% paraformaldehyde solution at 4°C for one day, passed in 15% and 30% sucrose in PBS, frozen in liquid nitrogen and then stored at -20°C. The hemispheres, the cerebellum, the spinal cord and the spleen were cut with a cryostat in 4 µm and 20 µm thick sections on glass slides and stored until use at -20°C.

After blocking for endogenous peroxidase activity with H_2O_2-methanol and for unspecific protein interactions with 10% bovine serum albumin 4µm thick paraffin and 20µm thick fresh-frozen sections were immunostained with a purified antibody directed against SIRPβ1 (SIRPβ-80 and SIRPβ-84; monoclonal rat, 1:200). Primary antibody in paraffin sections was detected with biotinylated secondary antibody, ABC-complex (Biomeda, Foster City, CA, USA), and 3, 3-diaminobenzidine-HCl (Sigma-Aldrich, Taufkirchen, Germany). Counterstaining with hematoxylin was performed afterwards. SIRPβ1 in fresh-frozen sections was detected with Carbocyanin Cy3-labeled anti-rat IgG secondary fluorescence antibody (Dianova, Hamburg, Germany). Sections were mounted in Corbit-Balsam (Hecht, Hamburg, Germany) or in molwiol and viewed with a Leica DMBL light and fluorescence microscope (Olympus, Germany). Images were collected by confocal laser scanning microscopy with 40x or 60x objectives (Olympus). Negative control was carried out.

3.10.2 Immunohistochemistry labelling in brain microglia

To identify the cell type, double-label immunofluorescence and confocal laser scan microscopy were performed with antibodies directed against SIRPβ1 and Iba1 (rabbit, 1/200; Wako, Japan). The primary antibodies were detected with Cy3-labeled anti-rat IgG and FITC--conjugated anti-rabbit IgG (Dianova, Hamburg, Germany) secondary fluorescence antibodies. Sections were mounted in molwiol and viewed with the Olympus laser scanning confocal microscope (Olympus).

3.11 Real-time PCR analysis from EAE and APP mice specimens

For analysis in specific part of mice for SIRPβ1and DAP12 expression following in EAE, APP and control mice were sacrificed. Brain, cerebellum, spinal cord and spleen

were removed carefully. RNA was isolated by the RNeasy Mini Kit lipid tissue (Qiagen) and reverse transcription was performed as described in section (3.11.1). Levels of the same SIRPβ1 and DAP12 were determined separately in EAE, APP and control.

3.11.1 RNA extraction, reverse transcription and real-time PCR quantification

RNA was isolated from cell-culture using RNeasy minikit (Qiagen). The principle of the procedure of this kit is the selective binding of RNA to a silica membrane while the rest of the cell components are washed away.

As starting material from 20,000 to 1 million cells from each cell samples were disrupted by addition of buffer containing β-mercaptoethanol 1% (Sigma) and homogenized to reduce viscosity of lysates. Afterwards, ethanol 70% was added to provide proper binding conditions to the silica-gel membrane of the columns provided by the kit. Finally, RNA molecules longer than 200 nucleotides eluted in 25µl of water to obtain DNA copy (cDNA), reverse transcription (RT) was performed using approximately 3µg of RNA isolated as described above. Superscript III reverse transcriptase enzyme (Invitrogen) and hexamer random primers (Roche) were used for this reaction during 1 hour at 50°C. The product of this synthesis was utilized as template for the relative quantification step. As control of possible genomic DNA contamination, samples without the reverse transcriptase enzyme were prepared.

Real time quantitative Polymerase Chain Reaction (PCR) was performed using an ABI Prism 5700 Sequence Detection System machine (Applied Biosystems), which detects fluorescent signal coming from a reporter during the PCR amplification reaction. SYBR green (emits signal only when intercalates in double stranded DNA) was the fluorescent dye of choice which is included in the PCR master mix (Applied Systems) together with the polymerase enzyme (AmpliTaq Gold). The amplification protocol for the GeneAmp 5700 Sequence Detection System Software (v.2.2) was performed. Cycles were as follows: initial denaturation at 95°C for 10 min, denature at 95°C for 15 sec., annealing and amplification at 60°C for 1 min. repeated 40 times. Non-template control and negative samples from reverse transcription were included. Ribosomal subunit 18s (18s) was used as endogenous gene expression control. After the reaction, the melting curve program was performed to assess the presence of specific products. A peak in the

negative first derivative of the fluorescence versus temperature graph indicated a unique specific product in the amplification reaction.

3.12 Phagocytosis assay

3.12.1 Phagocytosis assay of apoptotic neurons and splenocytes

Primary microglia cells were lentivirally transduced with shSIRPβ1 and shControl. After transduction microglia was cultured for 72h to achieve effective knockdown of SIRPβ1 by RNA interference. Neurons were cultured for 5–10 days and splenocytes for 2 days then okadaic acid at the final concentration of 30 nM was added and incubated for 3 h to induce apoptosis. Neuronal and splenocytes cell membranes were labelled with CellTracker CM-DiI membrane dye at the final concentration of 2µg/µl (Molecular Probes). After incubation, apoptotic neurons and splenocytes were washed two times with 1x PBS and added to the transduced microglial culture at an effector/target ratio of 1:20. At 1 and 24 h after addition of apoptotic neurons and splenocytes, the number of microglia having phagocytosed neuronal and splenocytes cell membranes were counted using a confocal fluorescence microscope (Olympus). Apoptotic cells were counted in three different areas at a 10x magnification. The amount of phagocytosis was confirmed by quantifying phagocytosed cells.

3.12.2 Beads phagocytosis assay

Microglia was transduced with the fSIRPβ1 and the GFP control vector. Cells were cultured on dishes, which are precoated with 10 µg/ml antibody directed against the Flag epitope (Sigma-Aldrich) or 10 µg/ml isotype control antibody (Sigma-Aldrich). After 24 h, 1.00 µm of red fluorescent microsphere beads (Fluoresbrite Polychromatic Red Microspheres; Polysciences Inc.) were added for 1 h. Phagocytosis of microsphere beads by microglia was analyzed by fluorescence microscopy. Furthermore, the percentage of microglia having phagocytosed beads was determined. Because phagocytosis varied from one experiment to the other, the relative change in phagocytosis was determined. Data are shown as the relative change in phagocytosis between microglia cultured on antibody directed against the Flag epitope and control antibody.

3.12.3 Amayloid beta conjugated phagocytosis assay

Microglial cells were prepared from brains of postnatal (P3–P4) C57BL/6J mice. To collect microglial cells, the mixed glia culture were shaken on a rotary shaker (350 r.p.m.) for 2 h. Primary microglia cultured was maintained in a 24-well plate at a density of 3 x 10^5 cells per well. Every individual experiment primary microglia was tranduced with pll3.7 RNA interference mediated Sh-SIRPβ1-GFP plasmid. As a control, Primary microglia were tranduced with ShSIRPβ1-Control-GFP which is scrambled sequence of silencing peptide and an empty plenty-GFP plasmid respectively. These respectively transduced cultures were treated with biotinylated $Aß_{42}$ peptide (10 µg/ml) (human amyloid-β peptide 1–42 conjugated at the N-terminus with biotin) from Bachem (Heidelberg, Germany) for overnight. To demonstrate that uptake of $Aβ_{42}$ involves phagocytosis, cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton-100. Fixed cells were incubated with Cy3-conjugated streptavidin (Amersham). 24-well culture plate were washed with 1x Phosphate buffer saline several times and kept at 4°C in the dark until analysis made under a laser scanning confocal microscopy (Leica LSC, Heidelberg, Germany).

3.12.4 Cy3 conjugated myelin phagocytosis assay.

Primary microglia cultured in a 24-well plate at a density of 3 x 10^5 cells per well were tranduced with pll3.7 RNA interference mediated Sh-SIRPβ1-GFP plasmid. As a control Primary microglia were tranduced with Sh-SIRPβ1-Control-GFP which is scrambled sequence of silencing peptide and an empty plenty-GFP plasmid. Transduced microglia were treated with Cy3-labeled mouse myelin (2 µg/ml) for 6 hrs. After 6hrs incubation in 10% Co_2 at 37^0C cells were washed with 1x Phosphate buffer saline several times to get rid of Cy3-labeled myelin which was not phagocytosed by microglia. Analysis of myelin phagocytosis was done after 24hrs under a laser scanning confocal microscope (LSC; Leica, Heidelberg, Germany).

3.13 Appendix to Materials and Methods

• **RNA Isolation**

Isolation of RNA was performed using the RNeasy Mini Kit (Qiagen, Germany). The protocol used is summarized as follows:

• Take eppendorf with tissue (e.g. 20-30mg in PBS).

• Add 700µl lysis buffer (inc. 1:100 β-mercaptoethanol), use 350µl if less than 20mg of tissue or less than $5x10^6$ cells (lysis buffer lasts approx. 1 month).

• Homogenize with small syringe (use large first if a lot of tissue). Centrifuge (3min, max).

• Mix 600µl of supernatant with 600µl EtOH (70% in dd H_2O).

• Add 700µl into column and centrifuge (15s, 10000rpm), throw away waste, add rest of mixture and repeat.

• Add 350µl wash buffer RW1 and centrifuge (15s, 1000rpm), throw away waste.

• Mix 10µl DNase1 with 70µl RDD buffer, add to filter and leave for 15min.

• Add 350µl wash buffer RW1 and centrifuge (15s, 10000rpm), throw away waste.

• Add 500µl wash buffer RPE (+EtOH) and centrifuge (15s, 10000rpm), remove waste, repeat and centrifuge (2min, max). Remove waste and centrifuge again to dry (1min, max).

• Place column in eppendorf, add 35µl RNase free H_2O (onto filter), leave for 3 min, centrifuge (1min, 10000), throw away column. RNA is collected in eppendorf.

• Measure concentration (µg/ml) and RNA/protein absorption (260/280) using photometer (Eppendorf), and dilute with ddH_2O accordingly to start with about 3µl of sample RNA.

• **Polymerase Chain Reaction (PCR)**

Polymerase chain reaction was performed for the amplification of specific cDNAs in order to obtain gene products for insertion into viral expression constructs, as well as for analysis of sequences inserted into such constructs following mini-prep plasmid preparation.

In cases where correct sequences were required a polymerase with high proofreading ability was used, Vent Polymerase (New England Bio Labs), whereas in cases of analysis a standard Taq Polymerase was used (Roche). A master mix was prepared containing all required reagents (see Materials, buffers and solutions) and aliquot to PCR tubes according to the following protocol:

• Add 47.5µl master mix to each tube.

• Add 2µl of appropriate cDNA (nothing to –ive control).

• Place tubes into PCR machine and choose required program (use heated lid!).

Example of program:

Initial Denaturation Step: 94^{o}C 3min

Cycle Step 1 – Denaturation: 94 ^{o}C 1min

Cycle Step 2 – Annealing 55-60 ^{o}C 1min (dependent upon primers used)

Cycle Step 3 - Elongation 74 ^{o}C 1min (3min for Vent Polymerase)

Repeat cycle steps accordingly between 15 to 35 times.

Final Elongation Step: 74 ^{o}C 3min.

PCR products were either stored at 4^{o}C or ran on a 1% agarose gel for analysis or extraction of the product.

• **<u>Agarose Gel Analysis and Extraction</u>**

In order to observe PCR products they were run on a 1% agarose gel. For analysis, ethidium bromide was added to the gel to allow visualization under a UV-lamp. Where ever extraction of the product was required, "Gelstar" (BioWhittaker Molecular Applications) was used instead of ethidium bromide to allow visualization without the use of an UV- lamp and thus preventing the possibility of mutation. After setting the gel in the chamber (see Materials, buffers and solutions) proceed as follows:

• Add a mixture of 3µl loading dye and 6µl sample to each lane, using a ladder in one lane to evaluate product size.

• Connect to power supply and set to 120V, 110A for 30min.

• Visualize DNA under a UV-lamp.

• For extraction, visualize using a Dark Reader transiluminator, cut out required fragment and extract DNA.

Extraction of DNA from agarose was performed using the QIAquick Gel Extraction Kit (Qiagen) as follows:

• Excise DNA fragment and weigh.

• Add 3xVol buffer QG to 1xVol gel (max 400mg, normally use 450µl).

• Leave at 50oC for 10 min (vortex every 3min).

• Add 1xVol isopropanol (normally use 150µl), invert several times, place in column, centrifuge 1min, discard flow through. (Qiagen column collects fragments within range of 70bp-10Kb).

• Add 500µl QG buffer, centrifuge 1min, and discard flowthrough.

• Add 750µl buffer PE, centrifuge 1min, discard flowthrough, and repeat centrifugation.

• Place column in clean tube, add 10µl EB buffer/H2O, leave 1min, and centrifuge 1min.

• **Real-time RT-PCR**

Pipette reaction mix accordingly prepared (see Materials, buffers and solutions) into wells. Add 1 µl of cDNA. Use a "non-template control" (NTC) well only with master mix. When pipetting, avoid bubbles. Cover the plate, with the plastic lid.

Program on ABI Prism SDS 7000 (Standard Protocol)

Cover T°= 105°C!! Initial denature: 95°C, 10min

Denature: 95°C, 15sec

Annealing: 60°C, 60sec

Amplification for 40 cycles

Final elongation: 72°C, 10min

To asses if a specific product was obtained, perform dissociation curve analysis.

(95°C, 60°C, 95°C ramp rate 2%).

(Alternatively, the PCR outcome can be examined by 3% agarose gel using 5 µl from each reaction)

Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

• **Ligation**

Ligation of insert into plasmid was performed using T4 DNA Ligase (Roche). Ligation reactions were normally carried out at 15oC for at least 3 hours. The reaction mix used was as listed below. Normally a ratio of 1:3 or 1:15 was used for plasmid: insert DNA in a volume of 8µl.

• **Transformation**

Chemically competent bacteria (TOP10 Chemically Competent E.Coli, Invitrogen) were transformed with ligated insert-plasmid DNA and expanded according to the following protocol:

• Defrost chemically competent cells on ice (500µl per tube).
• Aliquot 100µl of cells per transformation and leave on ice for 30min.
• Dilute ligation mix 1:1 and add 0.6µl to 100µl of competent bacteria.
• Heat-shock cells at 42^{o}C in water bath for 1min.
• Return cells to ice for 2min.
• Add 1ml LB medium and incubate in for 45min at 37oC (rotatory shaker, >200rpm).
• Centrifuge for 3min at 7000rpm and remove excess medium.
• Plate onto appropriate selective LB plates and incubate at 37oC overnight.
• Pick colonies and grow in selective LB medium for 10hr at 37oC in shaker.
• Isolate plasmid DNA using mini-prep kit (Qiagen).
• Verify ligation by restriction digest and PCR.
• Prepare high concentrate stock of positive samples using maxi-prep kit (Qiagen).

• **Transfection**

Transfection of cell lines was performed to produce lentiviral particles as well as to confirm the expression of genes cloned into plasmids. Lentiviral particles were produced by co-transfection of the lentiviral plasmid along with plasmids expressing accessory lentiviral genes into a packaging cell line using a lipofectamine-based technique as follows:

• Plate 5x10^{6} 293FT cells in a poly-L-lysine coated 10cm dish one day before transfection to obtain a culture of 80-90% confluence.

• On the day of transfection add antibiotic-free medium to the cells (10ml).

• Prepare DNA-lipofectamine complexes:

9µg packaging mix + 3µg vector in 1.5ml Opti-MEM medium (Gibco).

36µl Lipofectamine 2000 (Invitrogen) in 1.5ml Opti-MEM

leave for 5min at room temperature.

mix gently and leave for 20min at room temperature

• Add transfection mix to cells dropwise, mix gently and leave for 6 hours.

• Add medium containing antibiotics and 2% serum to the transfected cells.

• Remove supernatant 36-72hrs post-transfection and pellet the debris.

As the lentivirus is VSV-G pseudotyped, the supernatant may be stored overnight at 4^{o}C, frozen at -80^{o}C or concentrated by ultracentrifugation (25,000rpm, 4^{o}C, and 90min).

• **Transduction**

A number of different transduction protocols can be used depending upon the target cell. Cells at a confluence of 30-50% were treated with lentiviral supernatant and kept at 37^{o}C overnight. Fresh medium was added to the cells on the following day.

Expression normally reached its peak 48hr post-transduction.

• **Supernatant Spin Infection (cell lines)**

Cells at a confluence of 30-50% in 6-well dishes were treated with lentiviral supernatant, centrifuged (2500rpm, 30^{o}C, 2x - 45min) and kept at 37^{o}C overnight.

Fresh medium was added to the cells on the following day. Expression normally reached its peak 48hr post-transduction and was generally higher than that observed following regular supernatant treatment.

• **Viral Concentrate (primary cell culture and cell lines)**

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4^{o}C in 300µl serum free medium (e.g. Stem Span). The resuspended viral concentrate was added to the target cells and kept at 37^{o}C overnight. Fresh medium was added to the cells on the following day. Expression normally reached its peak 48hr post-transduction and was

generally higher than that observed following regular supernatant treatment or spin infection.

• **Concentrate Spin Infection (primary cell culture and cell lines)**

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4^{o}C in 300µl serum free medium. The resuspended viral concentrate was added to the target cells, centrifuged (2500rpm, 30^{o}C, 90min) and kept at 37^{o}C overnight. Fresh medium was added to the cells immediately after the following centrifugation. Expression normally reached its peak 48hr post-transduction and was generally higher than that observed following other transduction procedures.

Kill Curve

The pLenti6/V5 plasmid expresses the antibiotic blasticidin to allow selection of transduced cells in culture. The following protocol was used to determine the minimum concentration of balsticidin required to kill non-transduced cells:

• Plate cells in a 6-well plate at a confluence of 25%.
• Add blasticidin at various concentrations, e.g. 0, 2, 4, 6, 8, 10 µg/ml.
• Change medium every 3-4 days.
• Observe the percentage of surviving cells.
• Determine the lowest concentration that kills all cells within 10 days of treatment.

Viral Titre Determination

Titration of the viral titre may be performed using FACS analysis to quantify the number of transduced cells in order to estimate the concentration applied to primary hematopoietic cultures. Cells (can be from a cell line like HeLa) are transduced as follows:

• Plate 5x104 cells per well in a 6 well culture dish.
• Prepare 10-fold serial dilutions of viral stock (10-6, 10-5, 10-4, 10-3, 10-2, 0).
• Add to cells in total volume of 2ml medium containing 6µg/ml polybrene.
• Leave overnight at 37^{o}C.
• Add fresh medium and leave overnight at 37^{o}C.

Transduced cells were allowed to culture for 4 days following transduction and then analyzed by FACS (normally best done using a GFP reporter gene construct). Only dilutions yielding to 1-20% GFP-positive cells should be considered for titer calculations. Below 1%, the FACS may not be accurate enough to give a reliable determination of the number of GFP+ cells. Above 20%, the chance for each GFP+ target cell to be transduced twice significantly increases, resulting in underestimation of the number of transducing particles. The following formula is used to calculate the viral titre:

$$\text{Titer (Hela-transducing units / ml)} = \frac{(5\text{x}10^4 \text{ Hela cells}) \text{ x } (\%\text{ GFP-positive cells}/100)}{\text{Volume of supernatant (in ml)}}$$

Protein extraction procedure:

293FT cells were used for protein extraction; all medium were removed from 10cm culture dish and washed once with ice-cold 7ml of 1x PBS with 70 µl PMSF and incubated for 2-3 min. Abrade twice the adherent cells with a cell scraper then slightly resuspend the suspension with 5 ml pipette. Every thing was kept into 15 ml tube and washed the dish with 3 ml PBS and centrifuged 15 ml tube (10 min, 1800 rpm, 4°C). After centrifugation, remove a supernatant and resuspend a pellet in lysis buffer P with proteinase (Shortly before use add 1 ml lysis buffer P into the tube with Aporotinin and Leupeptin.) and cells were lysed for 30 min at 4°C. The lysates were transfer into eppendorf and centrifuged at 15 min, 13000 rpm, 4°C, the resulting supernatant were subjected to measure a protein concentration by Bradford's method or freeze at -20°C. Final concentrations were used for immunoblot analysis.

4. Results

4.1. Flow cytometry analysis of microglial cells isolated from mixed glial culture

To assess the purity of the microglial population from mixed glial cell culture system and to show specificity of the desired cells, we performed flow cytometry analysis. Microglia cells were labelled with biotinylated CD11b as well as with control IgG specific primary antibodies. Staining was followed by a secondary antibody conjugated to a fluorescent molecule.

The microglial cell population was obtained by a specific selection (Fig 4.1). Therefore, the depletion method selected in this work shown to be a trustworthy protocol to obtain a microglial enriched cell population.

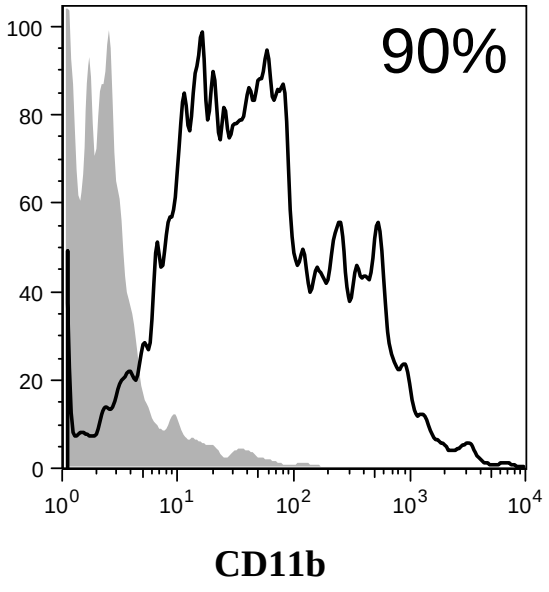


Figure 4.1. Microglial population were found to be positive for CD11b (up to >90%)

4.2. SIRPβ1 and its adaptor protein DAP12 expression analyzed in primary cultured microglia by RT-PCR.

SIRPβ1 in primary microglial cells was completely unknown to scientific community. In the patients with the inflammatory Nasu-Hakola brain disease which identified as a genetic mutation in DAP12. It appears that DAP12 is expressed in case of microglial activation in the CNS. Therefore, we analyzed SIRPβ1 that is DAP12 associated receptor in primary microglial cells. The primary microglia cells were isolated and purified from postnatal mouse brain tissue. Gene transcripts of SIRPβ1, DAP12 and 18s (as housekeeping control gene) were analyzed by RT-PCR in cultured primary microglial cells. Total RNA was prepared from cultures of microglia, neurons, bone marrow derived myeloid cells and splenocytes. Gene transcripts for SIRPβ1 and DAP12 were detected in microglia, bone marrow derived myeloid cells and splenocytes, but not in cultured neurons (Fig. 4.2).

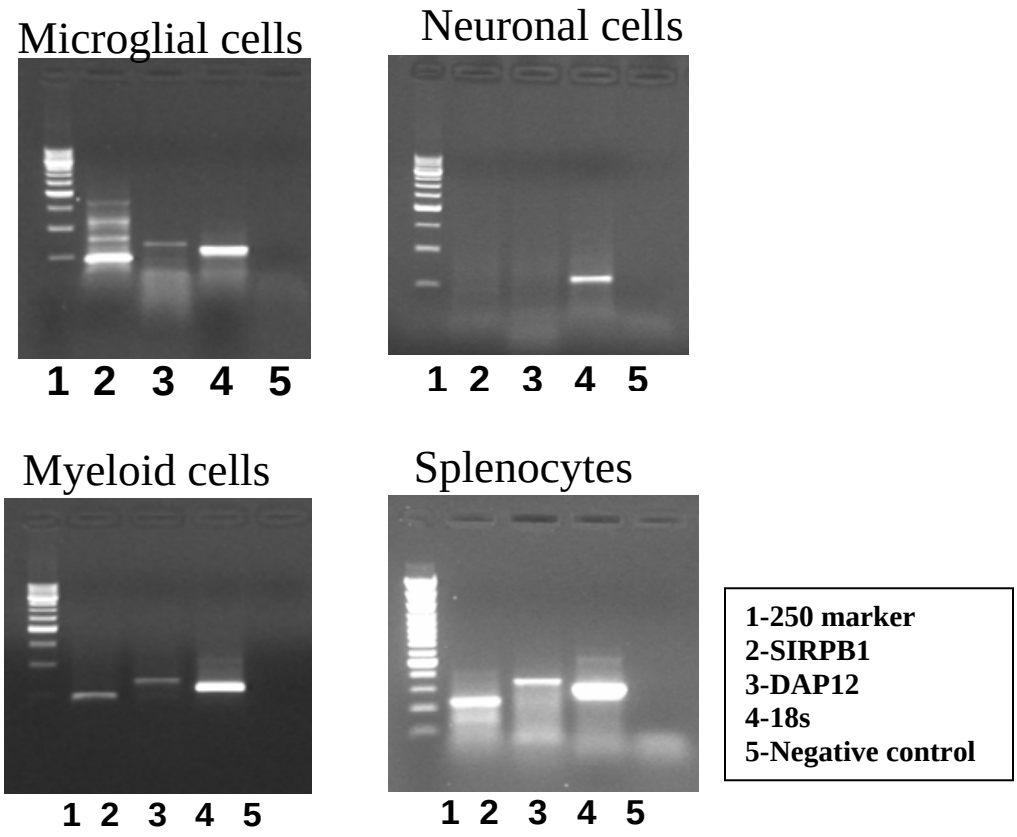


Figure 4.2. *Analysis of SIRPβ1 and DAP12 gene transcripts demonstrated a transcription of SIRPβ1 and DAP12 in microglia, myeloid cells and splenocytes, but not in neurons.*

4.3. SIRPβ1 expression analysis in primary cultured microglia by immunocytochemistry

Subcellular expressions of SIRPβ1 were analyzed in different cell culture system by immunocytochemistry and confirmed by confocal microscopy. CD11b and IgG an isotype control were used as primary antibody for co-immunostaining and confirmed not only staining protocol but also antibodies specificity (Fig. 4.3 A). Immunolabelling of SIRPβ1 was detected as a cytoplasmic staining in cultured mircroglia using monoclonal antibodies directed against SIRPβ1 (SIRPβ80 and SIRPβ84, respectively) (Fig. 4.3 A). Interestingly, no immunostaining of SIRPβ1 was detected in cultured neurons double-labelled with antibodies directed against β-tubulin-III (Fig. 4.3 B and C).

A

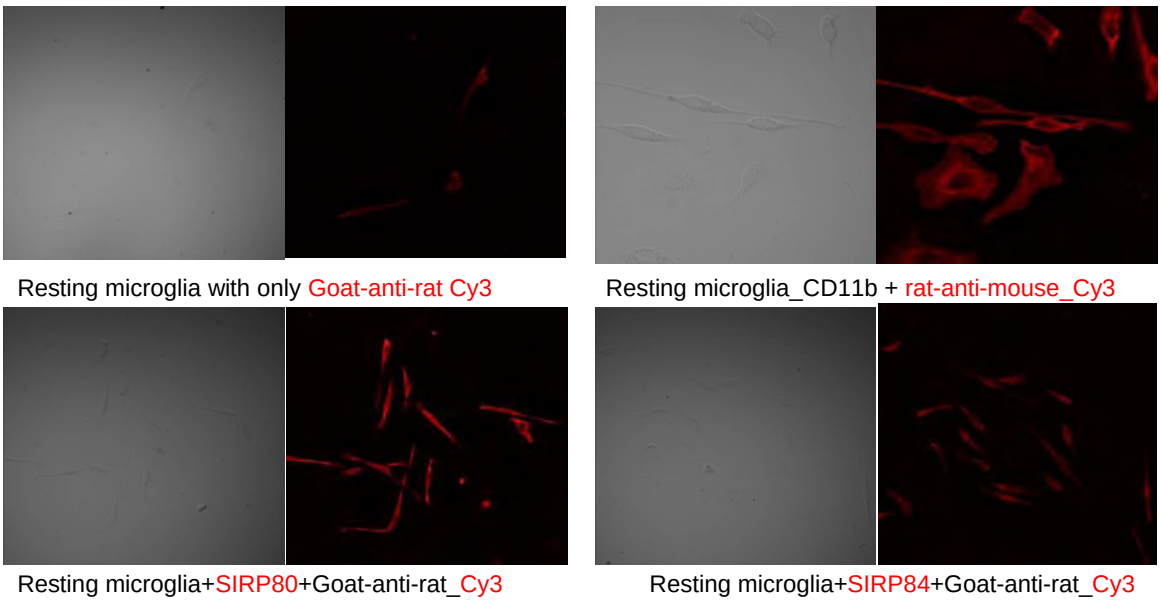


Figure 4.3. (A): *SIRPβ1 protein was detected by immunofluorescence labeling in primary microglia, double-labeled with CD11b. Scale bar: 20μm*

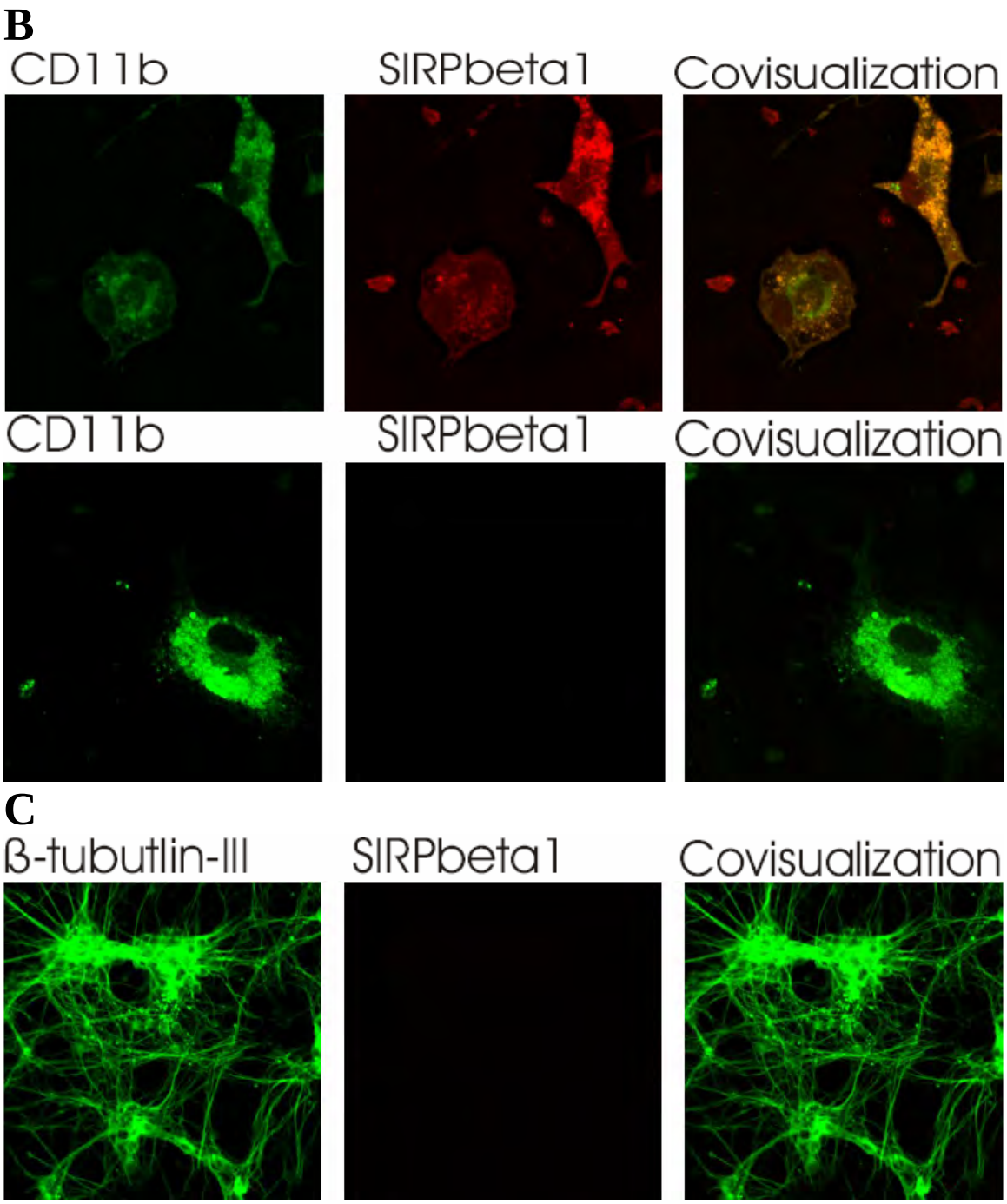
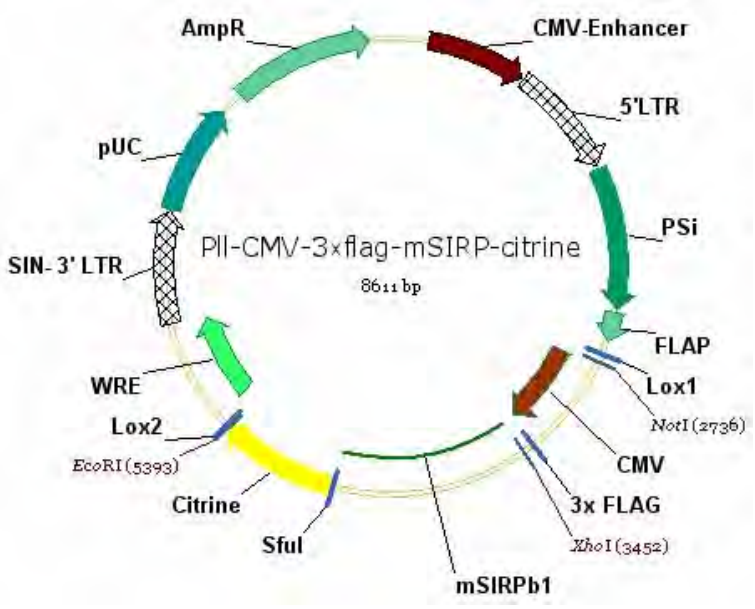


***Figure 4.3. (B and C)**: No SIRPβ1 signal was detected after using a control antibody or in cultured neurons. Scale bars: 20μm.*

4.4. Lentiviral vectors and microglial transduction

Viral vectors were used to genetically manipulate the cell population we were interested, which contain a fluorescent marker (eGFP) (scheme on figure 3.4.1).

A)



B)

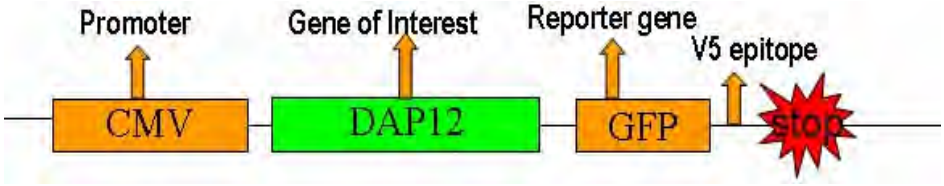


Figure 3.4.1. *Schematic representation of the constructs. Lentiviral vector backbone, the CMV promoter was used for experimental setup. Pll3.7 vector was modified for 3xflag-mSIRPb1 (A) pLenti6/V5 D-topo plasmid was modified for DAP12 gene integration. As well downstream, the eGFP gene was inserted between the TOPO cloning sites (B).*

In these vectors the gene of interest is under the control of the CMV promoter. Although intricacies for modifying cells using lentivirus derived vectors under our experimental conditions were faced before, the system was tested for assessing the percentage of microglial cells expressing the reporter gene after transduction.

First, the vector expressing eGFP was used to transfect the packaging cell line 293FT in order to prepare viral particles. Upon transfection almost 100% of the packaging cell line was positive for eGFP (fig 3.4.2).

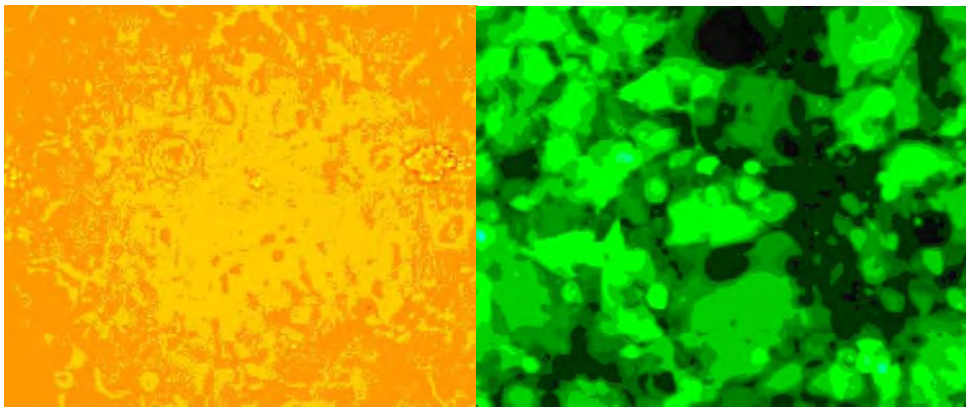


Figure 3.4.2. *Upon transfection using a CMV promoter driven eGFP expression lentiviral construct, the packaging cell line 293FT was 100% positive for eGFP expressing cells. Scale bar: 20 um.*

After selection, isolated microglia was transduced with the eGFP containing viral particles produced as described above in 293FT cells. When using non-ultracentrifuged, freshly collected from the packaging cells line viral supernatant (10^6 TU/ml), less eGFP expression was observed in the microglia (as was checked after 24 hours. Transduced 293FT cell line as procedure control showed 100 % eGFP positive cells (fig 3.4.2). Thereafter, a protocol to augment viral titer (10^8-10^9 TU/ml) was chosen. Viral fresh supernatants were ultracentrifuged and the pellet containing viral particles were resuspended in a low volume of media (500µl). Efficiency of microglial cell transduction using these concentrated viral particles was analyzed by flow cytometry (Fig 4.1). One day after incubation with the concentrated viral particles up to >90 % of cells were positive for eGFP (figure 4.1). Further cell transduction has been in the range of this value.

4.5. Upregulation of SIRPβ1 in primary cultured microglia by interferon's

To begin defining the role of SIRPβ1 expression in functionally modulating microglial activation, we chose to use the common postnatal brain-derived microglia culture system for these studies. We have shown, whether inflammatory mediators could induce gene transcription of SIRPβ1 and DAP12 in primary microglia, we treated the cells with tumour necrosis factor-alpha (10 ng/ml), tumour necrosis factor-beta (10^3U/ml), interferon-gamma (100 U/ml) and LPS (1mg/ml) for 72 hrs at 37°C in 10% CO_2. Indeed, treatment with inflammatory mediator's up-regulated gene transcription of SIRPβ1 and DAP12. At 72 hrs after stimulation with inflammatory cytokines the percentage of microlgia showing SIRPβ1 expression was increased up to 1.7 fold after TNF treatment, up to 3 fold after interferon-gamma and beta treatment and up to 1.5 after LPS treatment.

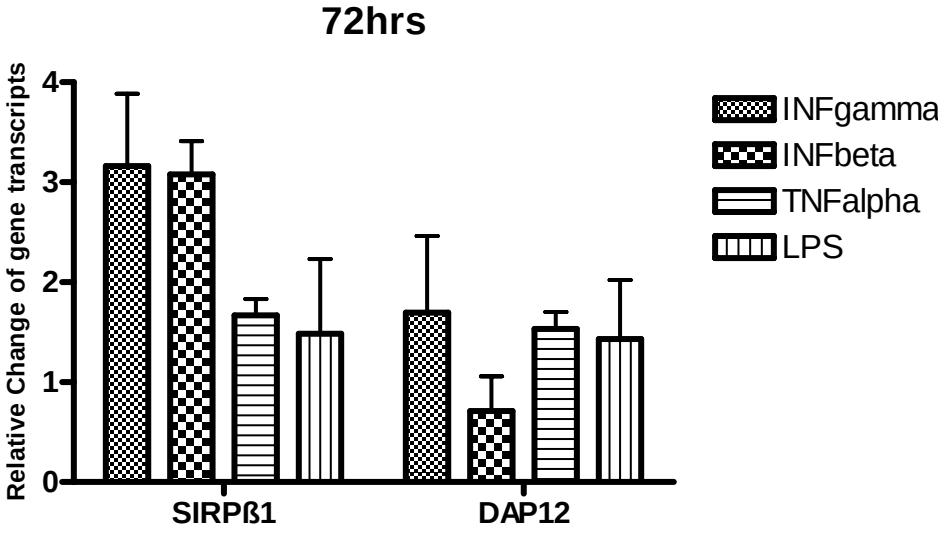
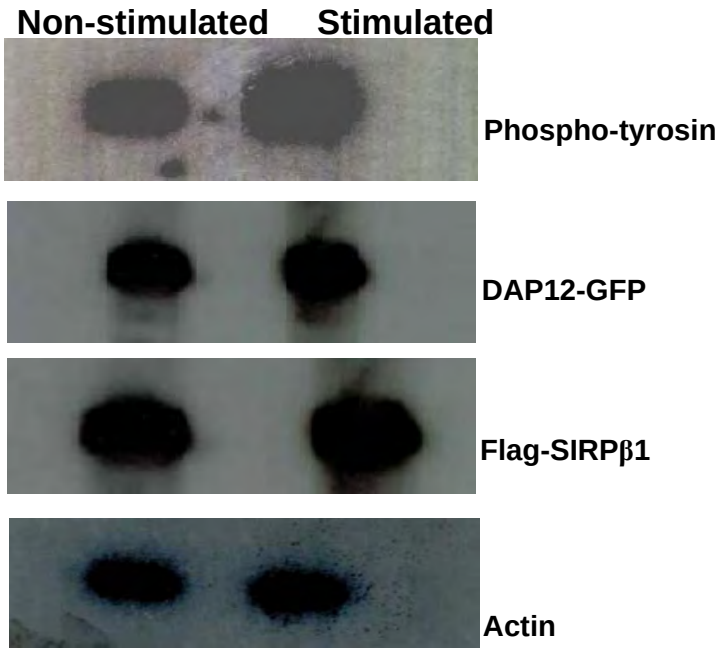


Figure 4.5. *Real-time RT-PCR analysis of primary microglia for SIRPβ1 and DAP12 demonstrated induction of SIRPβ1 by interferon-gamma and beta after 72 hrs treatment.*

4.6. DAP12 phosphorylation analyzed by Western blotting.

C-terminal short cytoplasmic domain of SIRPβ1 interact with its adapter protein DAP12 molecule through phosphotyrosin-binding (PTB) domain through charged amino acid binding. To assess the information at translation levels, we performed some western blotting experiment to analyze signalling of SIRPβ1. Western blot analysis was performed using the NuPAGE electrophoresis system and 10% Bis-Tris gels (Invitrogen). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse polyclonal IgG and IgM (Chemicon, Germany). The Enhanced Chemiluminescence (ECL) Advance Western blotting Detection Kit (Amersham Biosciences) was utilized. We tagged the SIRPβ1 gene at the extracellular region with a flag-tag and stimulated the receptor with flag-tag specific antibodies. Stimulation of flag-tagged SIRPβ1 of a co-transduced cell line 293FT stimulated phosphorylation of DAP12 as determined by Western blotting after immunoprecipitation of DAP12. Thus, SIRPβ1 associated with its signaling molecule DAP12 and stimulation of SIRPβ1 induced phosphorylation of the ITAM motifs.

A)



B)

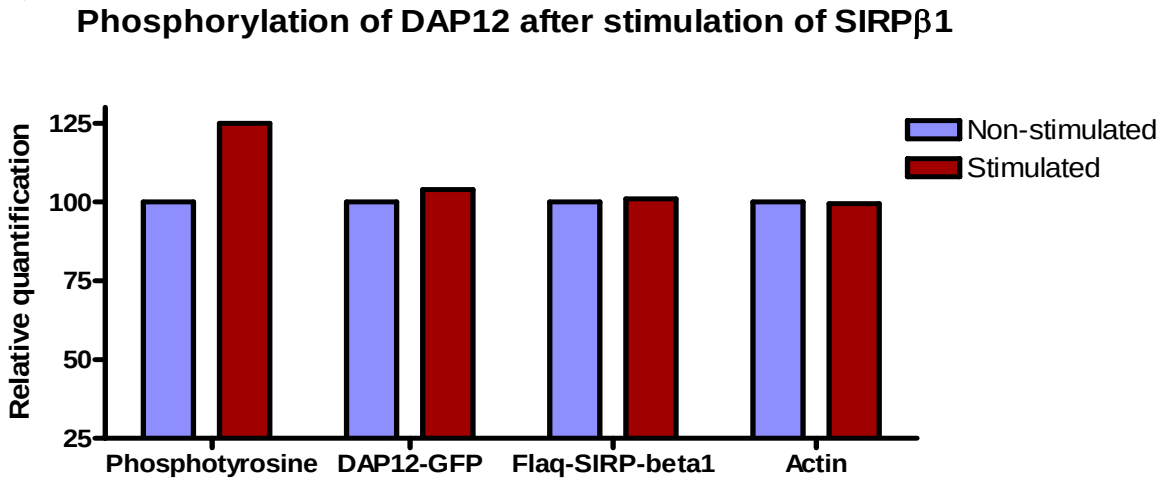


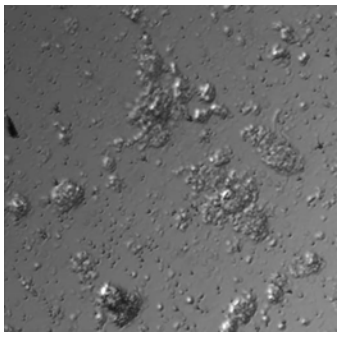
Figure 4.6. *A) Cell line 293 was lentivirally transduced with SIRPβ1 tagged at the extracellular side with a 3x flag epitope and with DAP12 tagged at the intracellular side with GFP. Cells were stimulated by coating the dish with cross-linking antibodies directed against the flag epitope. Protein lysates were analyzed by Western blotting with specific antibodies directed against actin, phosphotyrosin, DAP12-GFP or flag- SIRPβ1. Cross-linking of SIRPβ1 with the flag-specific antibody induced phosphorylation of DAP12.*

B) For quantification, optical density (OD) of the respective bands per experimental group was determined by ImageJ software (NIH) and normalized to the mean values of the untreated bands.

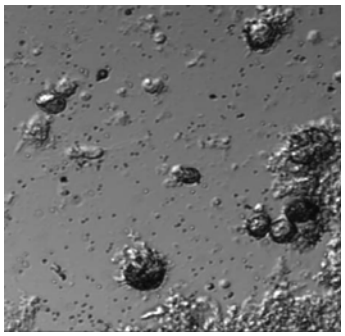
4.7. Increased phagocytic capacity of primary microglia after stimulation of SIRPβ1

Next, we tried to establish a system for better understanding of the parameters that influence the microglial cells for their phagocytic activation. We tried to understand whether SIRPβ1 overexpression into primary microglia cells could show any functional influence or not. First, we lentivirally transduced flag-tagged SIRPβ1 and GFP vector in primary microglia; also beads were added to these cells and stimulated fSIRPβ1 with a flag-tag specific antibody. However, the micoglial cells showed increased phagocytic activity compared to control IgG antibody stimulation. (Figure 4.7)

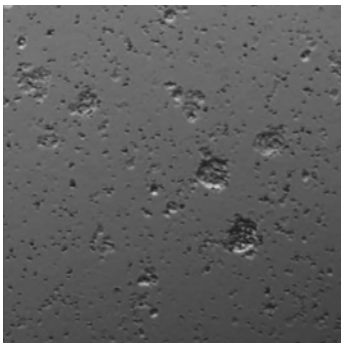
A



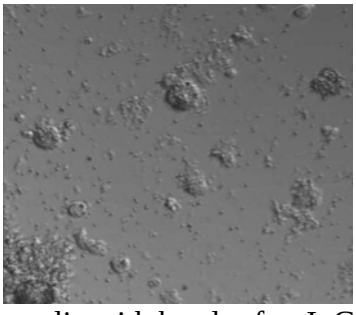
GFP-transduced microglia with beads after flag antibody stimulation



fSIRP-transduced microglia with beads after flag antibody stimulation



GFP-transduced microglia with beads after IgG antibody stimulation



fSIRP-transduced microglia with beads after IgG antibody stimulation

B

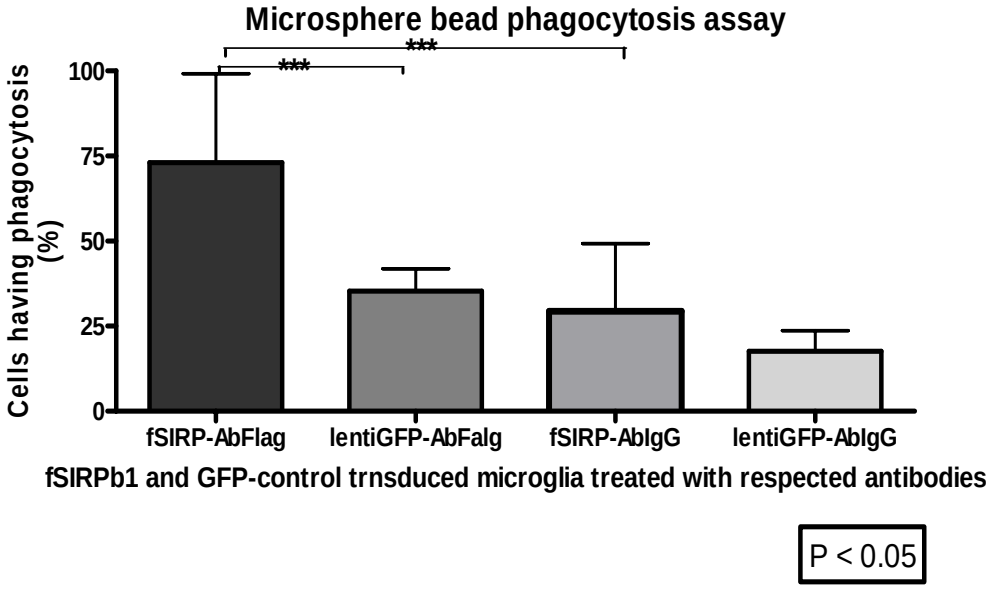


Figure 4.7. *Bead phagocytosis analyses*

A. *Primary microglial cells were transduced with flag-tagged SIRPb1 or a control GFP vector. Cells were cultured on dishes coated with flag-specific antibodies or control antibodies. Phagocytosis of microsphere beads was visualized by confocal microscopy. Scale bar: 20µm.* ***B.*** *Primary microglial cells were transduced with flag-tagged SIRPb1 or a control GFP vector. Cells were cultured on dishes coated with flag or control specific antibodies. Phagocytosis of microsphere beads was analyzed and quantified by flow cytometry. Data are presented as mean ± SEM. Number of independent experiments n=12. ANOVA followed by unpaired t-test: p= <0.05 (fsirpb1 versus gfp control)*

4.8. Induction of experimental autoimmune encephalomyelitis

A mouse model for MS was established. For this purpose, C57Bl/6 female mice were injected with MOG35-55 peptide and developed a progressive relapsing disease with a late mild remission. Disease susceptibility observed in this work is detailed in the table 4.8.

Mouse strain	Incidence	Disease onset	Maximal clinical Score
C57Bl/6 64	65 ± 15%	12 ± 1 days After immunization	3 ± 0.5

Table 4.8. *EAE disease susceptibility of C57Bl/6 mice strain. Incidence (%), mean time of disease onset (days) and mean maximum clinical score ± S.E.M. are shown for 9 independent immunization time points.*

As specified above, the incidence of the disease was between 60% and 70%. Onset of clinical symptoms was observed generally at days 10 to12 after immunization. Maximal clinical score was between 3 and 3.5 and was observed at days 4 to 5 after onset of the symptoms (this was the time point chosen for cell injection in the migration and therapeutic approaches). Only mice that showed onset (score more than 1) of clinical symptoms before day 14 were considered for the following experiments.

A graph showing typical outcome data from four mice in which EAE was induced independently is depicted in figure 4.8. A decrease in the weight can be observed immediately after immunization with a second point of weight loss together with the first clinical manifestations. Minimal weight values coincide with worsening of the clinical symptoms at 4 to 5 days after onset of disease.

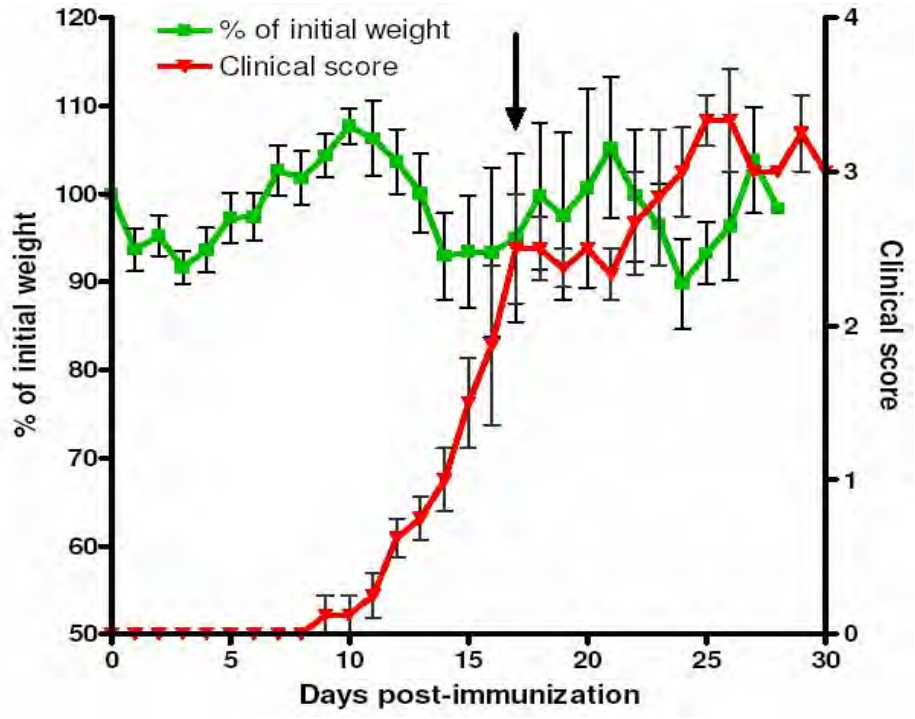


Figure 4.8. *Representative diagram of mean values (± S.E.M.) of relative (to initial) body weight change and clinical score of 4 independent immunized mice during the course of EAE.*
Mice usually lost weight during first 2 days after the first clinical symptoms. This time point coincides with worsening of the clinical score. This arrow represents the EAE time point achieved mice were sacrificed for SIRPβ1 analysis in different region.

4.9. Immunohistochemistry for detection of SIRPβ1 in Iba1 positive microglial cells

Next, we performed immunohistochemical analysis of formalin-fixed, paraffin-embedded spinal cord sections. Double-immunfluorescence labelling with antibodies directed against SIRPβ1 and Iba1 was done. Indeed, SIRPβ1 positive cells (red) were identified as microglial cells showing also staining for Iba1 (green). In detail, 61 % of SIRPβ1 positive cells were double-immunolabelled for Iba1 (Fig. 4.9).

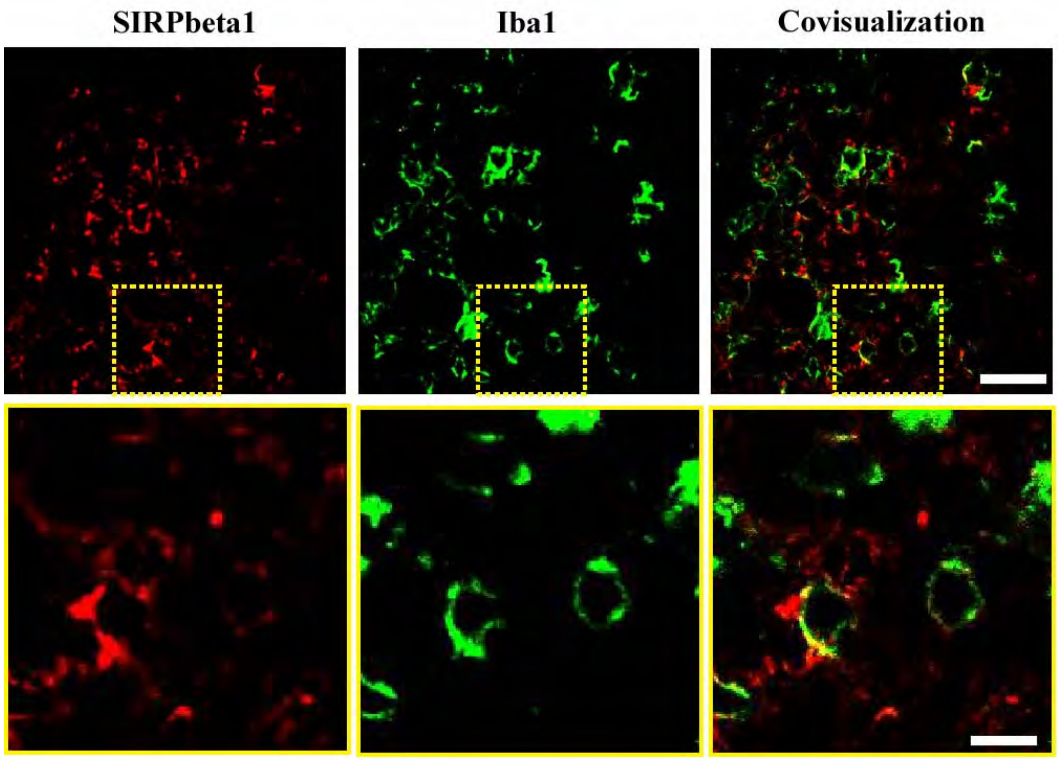
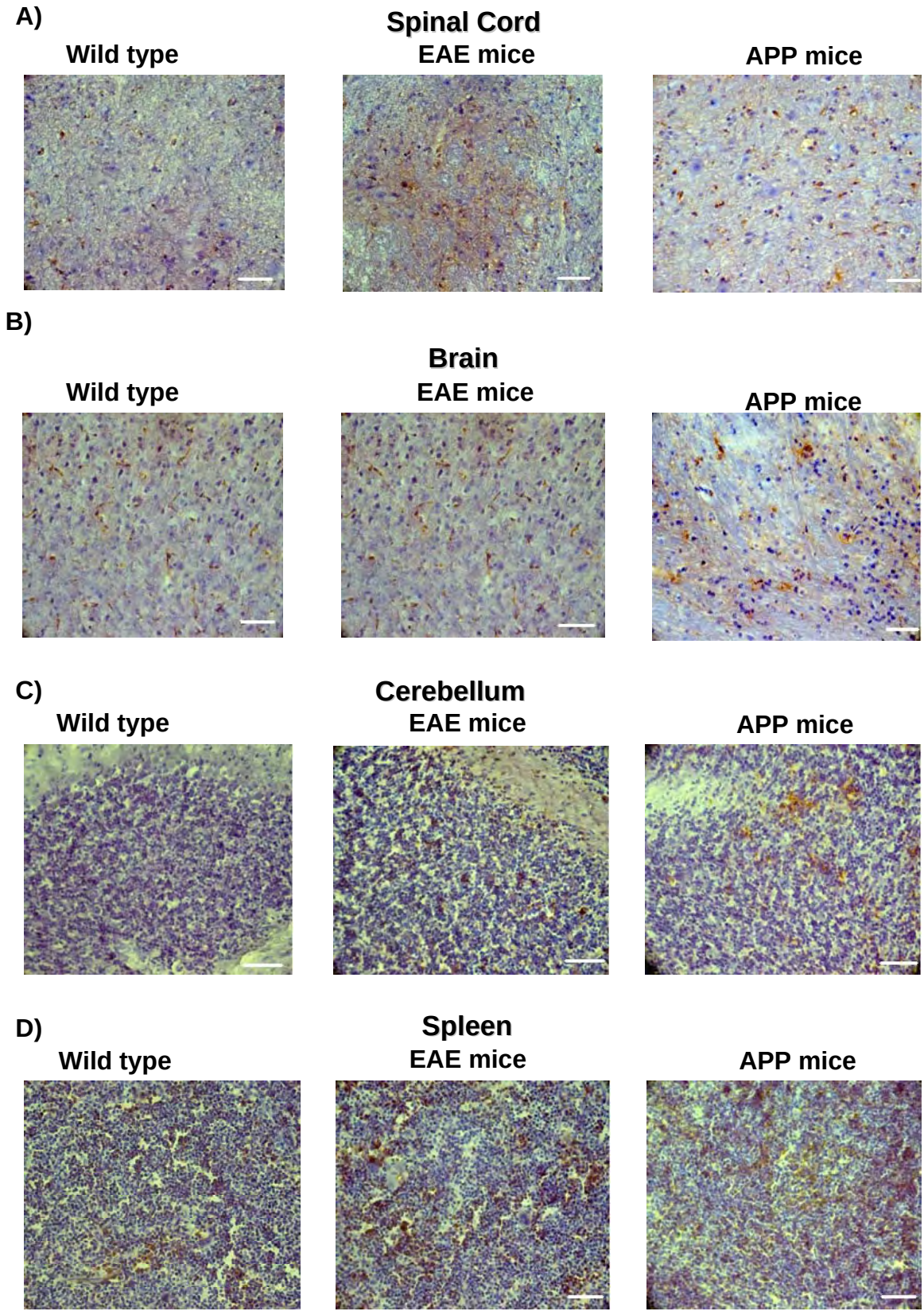


Figure 4.9. *Double-fluorescence labeling with specific antibodies directed against SIRPβ1 (red) and Iba1 (green) demonstrated co-localization of SIRPβ1 and the microglial marker Iba1.*

Image was taken from the spinal cord of EAE mice at day 13 of the disease. Scale bar: 20μm 61% (YSEM) of Iba1 positive cells show SIRPβ1 expression.

4.10. Immunohistochemistry for SIRPβ1 in EAE, APP-transgenic and control mice.

SIRPβ1 expression was found in a subpopulation of microglia in brain, cerebellum, spinal cord as well as in spleen tissues in EAE and APP transgenic mice. Although some apparently resting microglia was weakly positive for SIRPβ1, most of the strongly stained microglia appeared to be in a reactive state as judged by morphology. SIRPβ1immunoreactivity was found in the brain gray matter in EAE, AD and control mice. To analyze the expression of SIRPβ1 in the CNS crayo sections of mice were stained with a rat monoclonal antibody directed against mouse SIRPβ1. All immunoreactive cells had a microglial-like shape. The number of SIRPβ1 positive cells substantially increased during the animal model of MS. Interestingly, the number of SIRPβ1 positive cells in APP transgenic mice was found to be >75 folds increased by quantitative real-time PCR analysis. Mice were immunized with the peptide myelin oligodendrocyte glycoprotein (MOG_{35-55}) to induce EAE and were killed for immunohistochemical analysis at a clinical score of 3 (+/- 0.5). After induction of EAE, SIRPβ1 was detected on 37% cells/ mm2 in the spinal cord, 23% cells/ mm2 in the brain and 10 cells/ mm2 in the cerebellum (Fig. 4.10 E). In normal tissue very few cells were positive for SIRPβ1 (Fig. 4.10). In APP transgenic mice, 34% SIRPβ1 positive cells/ mm2 was detected in the spinal cord, 35% SIRPβ1 positive cells/ mm2 was detected in the brain and 14% SIRPβ1 positive cells/ mm2 was detected in the cerebellum.


A)
Spinal Cord
Wild type
EAE mice
APP mice
B)
Brain
Wild type
EAE mice
APP mice
C)
Cerebellum
Wild type
EAE mice
APP mice
D)
Spleen
Wild type
EAE mice
APP mice

E)

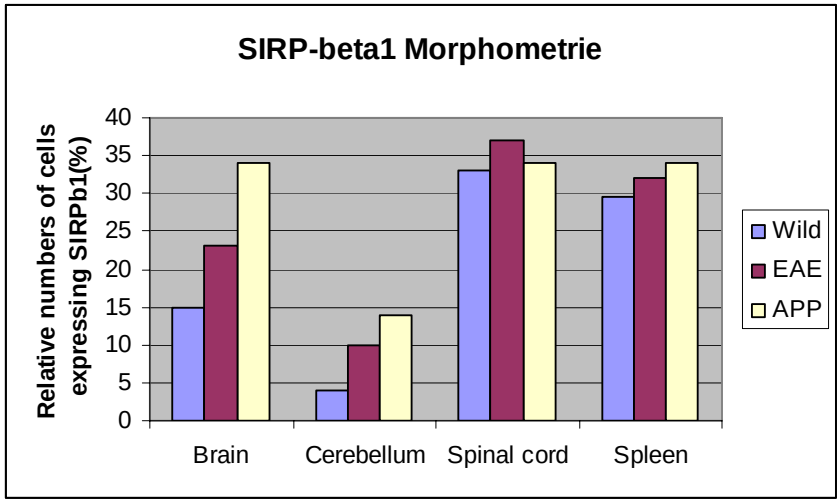
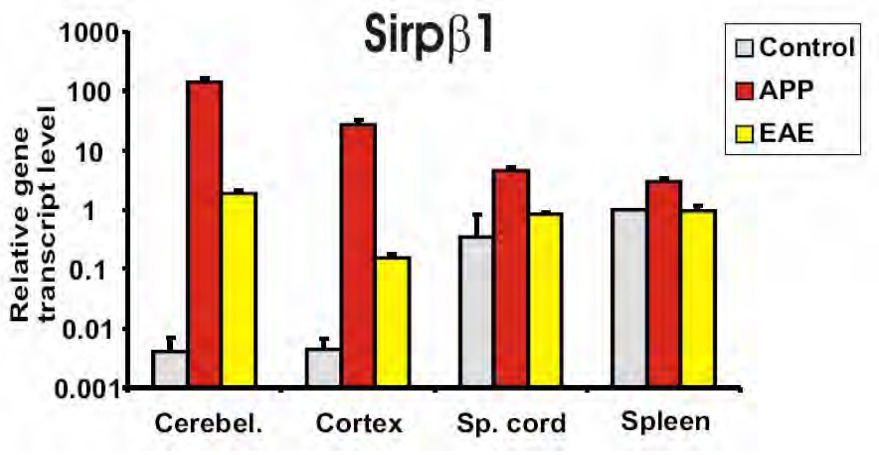


Figure 4.10. A-D *SIRPβ1 is strongly expressed in the spinal cord of EAE mice and in the brain of APP transgenic mice.*
E *Quantification of positive cells in relation to all cells/mm2 in %).*

4.11. Real-time PCR analysis of SIRPβ1 in EAE, APP-transgenic and control mice

Next, we analyzed gene transcription of SIRPβ1 and DAP12 in distinct CNS regions of EAE and APP mice compared with there age-matched control mice by quantitative real-time PCR. Gene transcript levels were normalized to the housekeeping gene 18s rRNA and compared to levels in the spleen of normal mice. Gene transcripts for SIRPβ1 and DAP12 were already detected in the spinal cord of healthy mice (Fig. 4.11). After induction of EAE, gene transcript levels of SIRPβ1 and DAP12 were significantly increased in the spinal cord as well as in the brain and cerebellum. In detail, gene transcripts of SIRPβ1 >5 fold (± 2) higher compared to the spleen of mice showing EAE with a clinical score of 3 (± 0.5) (Fig. 4.11 A and B). Most fascinating finding was observed in APP transgenic mice, where transcript levels of SIRPβ1 were 100 fold (±20) higher compared to the spleen of APP mice (Fig. 4.11 A and B).

A



B

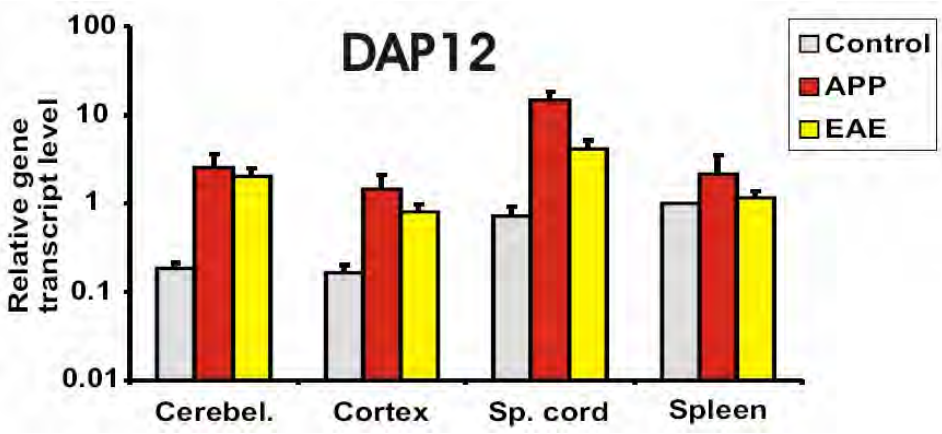


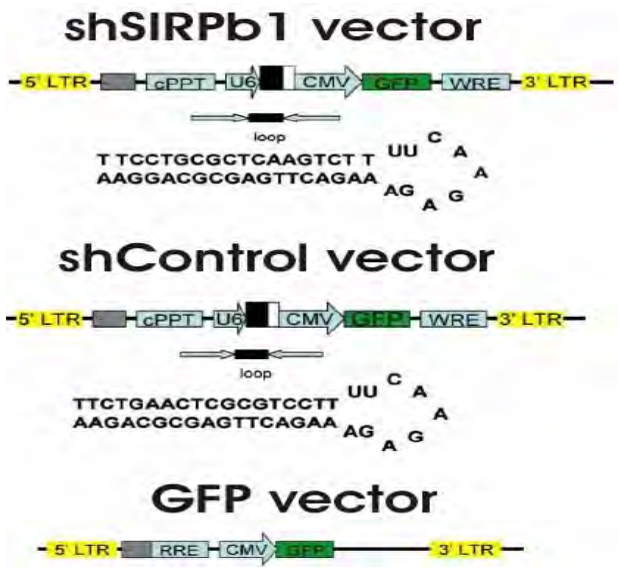
Figure 4.11. *Relative gene transcript levels were determined for SIRPβ1 (A) and DAP12 (B) in cerebellum, cortex, spinal cord, and spleen derived from EAE mice at day 13 after onset of clinical symptoms and APP transgenic mice at month 12 after birth. Gene transcript levels of SIRPβ1 significantly increased in the spinal cord of EAE mice and the cortex of APP transgenic mice, while DAP12 gene transcript levels increased only slightly. Data are shown as mean ± SEM. Number of independent experiments n= 6. ANOVA followed by unpaired t-test: p > 0.05 (SIRPβ1 EAE spinal cord versus cortex, cerebellum and spleen).*

4.12. Silencing of SIRPβ1 in microglia cells.

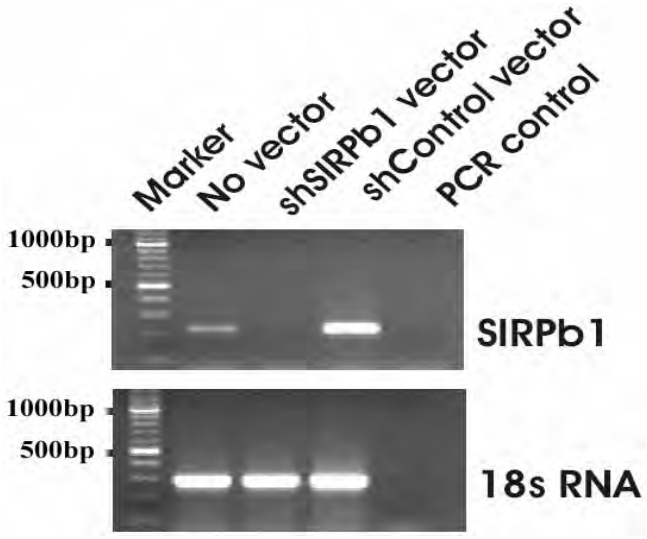
To confirm knockdown of SIRPβ1 gene transcripts, microglial RNA was analyzed at 72 hours after transduction with the lentiviral RNA interference vector.

Indeed, no gene transcripts for SIRPβ1 were detected after 40 PCR cycles in reverse-transcribed RNA of microglia after knockdown of SIRPβ1 (Fig. 4.12B). The control vectors plenty-GFP did not knock down SIRPβ1. Immunohistochemistry was performed to confirm absence of SIRPβ1 expression on microglia after knockdown. Although SIRPβ1 was detected by specific antibodies on control vector–transduced microglia, no expression of SIRPβ1 was observed after transduction of the microglia by the shSIRPβ1 vector (Fig. 4.12 C and D).

A



B



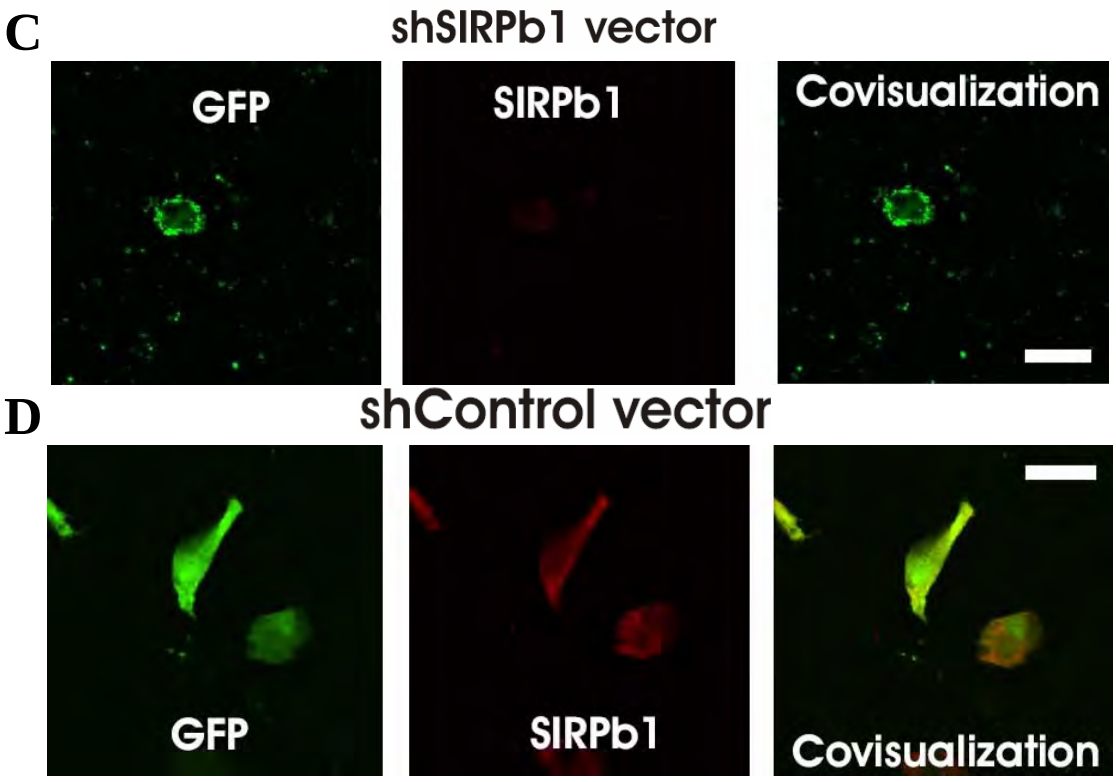


Figure 4.12. *Lentiviral knock-down of SIRPβ1:* ***A.*** *Schematic diagram of short hairpin SIRPβ1, mutated short hairpin SIRPβ1 as a primary control and plenti-eGFP vector as a secondary control. Silencing of SIRPβ1 confirmed by RT-PCR using Sh-SIRPβ1 contained viral supernatant and concentrated viral particles. SIRPβ1 expression was confirmed in resting as well in a short hairpin SIRPβ1 transduced microglias.* ***B.*** *Successful knock-down of SIRPβ1 was confirmed by RT-PCR. No gene transcripts were detected after lentiviral knock down of SIRPβ1, while SIRPβ1 gene transcripts were detected without vector or after shControl vector transduction.* ***C.*** *SIRPβ1 silencing was confirmed by staining in of microglia with specific antibodies (SIRP84) directed against SIRPβ1. Scale bar: 20μm.****D.*** *SIRPβ1 was detected in microglia with specific antibodies (SIRP84) after transduction with the control vector. Scale bar: 20μm.*

4.13. Pathophysiological function of SIRPβ1

4.13.1. Impaired phagocytosis of apoptotic neural cells and splenocytes after lentiviral knock-down of SIRPβ1 in primary microglia.

Primary microglia cells were transduced with silencing and control lentiviral vector. Again, transduction efficiency was always >90%, leading to a loss of SIRPβ1 in

almost all microglial cells after transduction with the shSIRPβ1 vector. Holding confirmation of SIRPβ1 gene presence in sh-control microglia, we further moved to knock-down this gene to put on more functional information in microglia. Up-regulation of phagocytosis without induction of inflammatory cytokines and cytotoxic mediators implies clearance without an inflammatory reaction. Because microglia plays an important role in the removal of apoptotic neurons and splenocytes during development and aging, SIRPβ1 might recognize apoptotic neuronal and splenocyte membranes and participate in their clearance.

To study the pathophysiological function of SIRPβ1, we used a lentiviral strategy to either knock-down SIRPβ1 by RNA interference and found that microglia was transduced with a lentiviral vector expressing either short hairpin SIRPβ1 (shSIRPβ1) RNA, a control vector that expressed a short hairpin scrambled sequence of SIRPβ1 (short hairpin control [shControl]), or GFP as a control (plenti-GFP) shown us that SIRPβ1 is one of the essential genes for phagocytosis in microglia which clarify apoptotic neurons and as well as splenocytes (Fig. 4.13A and B). Neurons and splenocytes were labelled by a red fluorescent membrane dye and pretreated with okaidic acid to induce apoptosis. Microglia transduced with the control vector (shControl) (this work is in progress) or GFP control phagocytosed the red fluorescent–labelled apoptotic neuronal and splenocyte membranes fragments was detected under the fluorescence microscope after 24 hours (Fig. 4.13 A and B). However, an apoptotic membrane fragment was barely detected under the fluorescence microscope after 24 hours, when the SIRPβ1 receptor was knocked down in microglia (Fig. 4.13 A and B). In detail, after 24 hours only 9.9 +1.5% (mean + SEM) of microglia showed phagocytosed neuronal material after knockdown of SIRPβ1, whereas 14.4 + 2.4% of microglia transduced with the plenty-GFPcontrol vector showed phagocytosis (Fig. 4.13 C and D). Similar result was observed after 24 h only 9.3 +1.5% (mean+ SEM) of microglia showed phagocytosed splenocyte material after knock down of SIRPβ1, whereas 21.5 + 1.4% of microglia transduced with the plenty-GFP-control vector showed phagocytosis (Fig. 4.13 C and D)

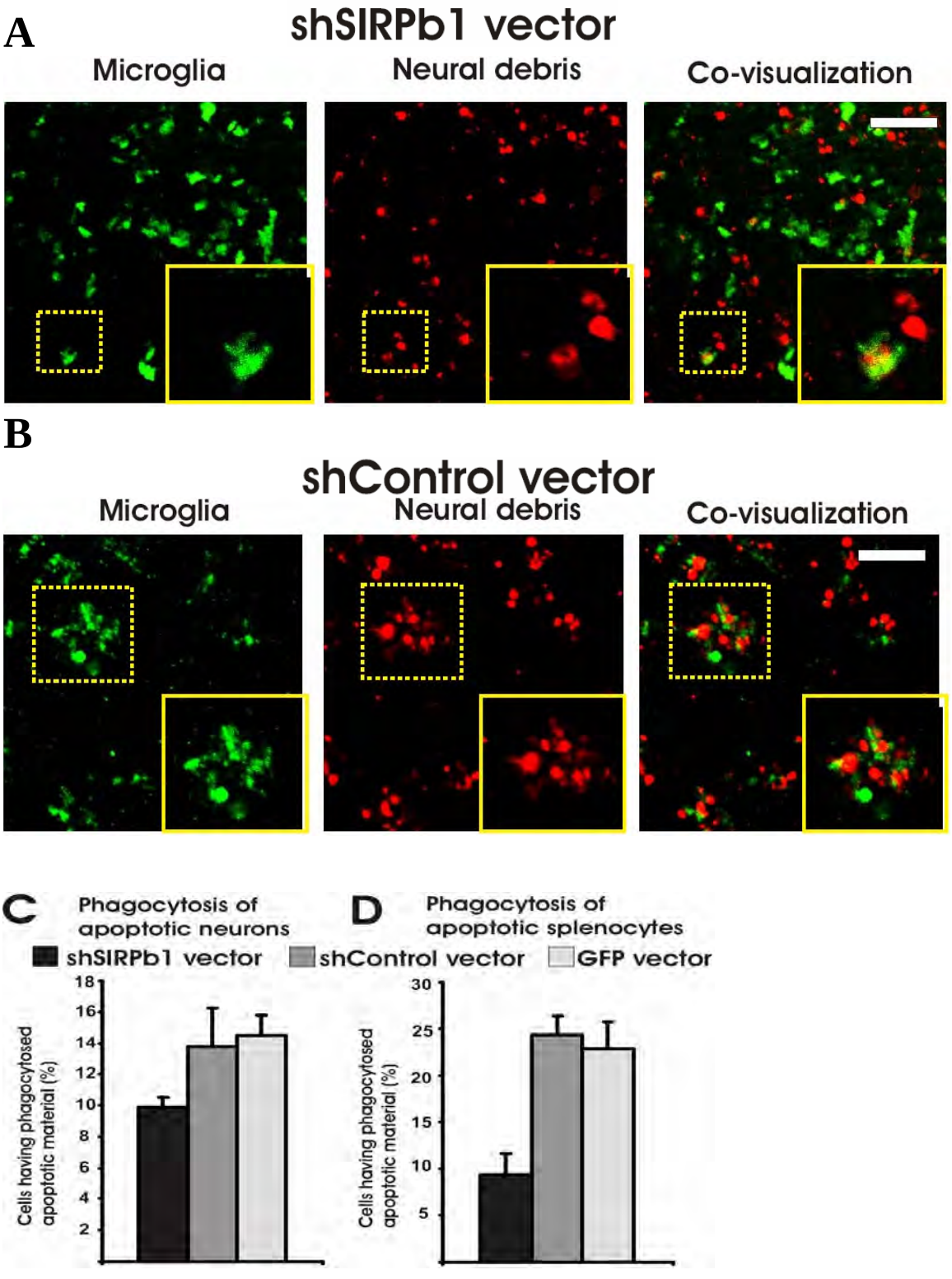


Figure 4.13.1. Phagocytosis of apoptotic neural cells or splenocytes by microglia.

A. *Microglial cells were transduced with short hairpin interference SIRPβ1 vector and challenged with apoptotic neural membranes labelled with a red fluorescent dye. Scale bar: 20μm.*

B. *Microglial cells were transduced with a short hairpin interference control vector and challenged with apoptotic neural membranes labelled with a red fluorescent dye. Scale bar: 20μm.*

C *and* ***D.*** *Statistical analysis at 24 hours after phagocytosis of apoptotic neural cells or splenocytes by microglia lentivirally transduced with a shSIRPβ1, shControl or GFP vector. Data are presented as mean +/- SEM. Number of independent experiments n=18. ANOVA followed by unpaired t-test: p= < 0.05 (shSIRPβ1 versus ShSIRPβ1 control and planti-vector control).*

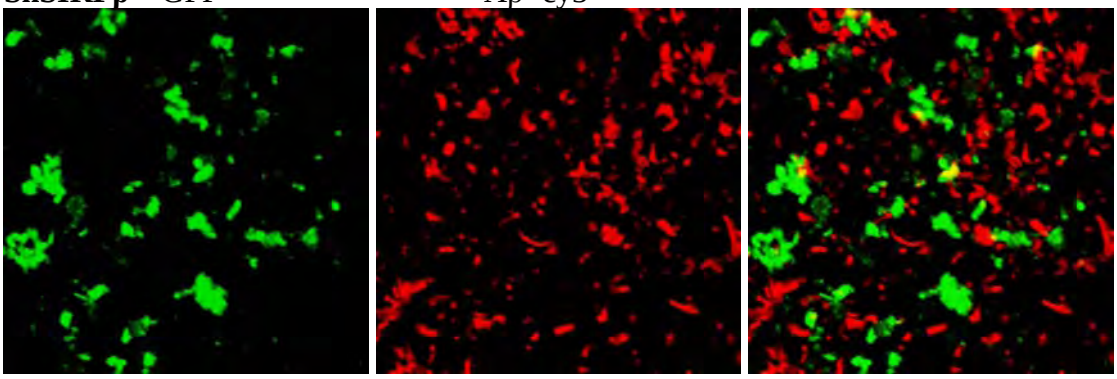
4.13.2. Impaired uptake of β-amyloid after lentiviral knock-down of SIRPβ1 in primary microglia

Primary microglia culture was maintained in a 24-well plate at a density of 3 x 10^5 cells per well. Each individual experiment of primary microglia was transduced with pll3.7 RNA interference mediated Sh-SIRPβ1-GFP plasmid. As a control primary microglia was tranduced with ShSIRPβ1-Control-GFP which contains scrambled sequence of silencing peptide and an empty plenty-GFP plasmid, respectively. These respectively transduced cultures were treated with biotinylated $Aβ_{42}$ peptide (10 μg/ml) (human amyloid β peptide 1–42 conjugated at the N-terminus with biotin) from Bachem (Heidelberg, Germany) overnight. To demonstrate that uptake of $Aβ_{42}$ involves phagocytosis, cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton-100. Fixed cells were incubated with Cy3-conjugated streptavidin (Amersham). These 24-well culture plates were washed with 1x Phosphate buffer saline several times and kept at 4°C in the dark until analysis under a laser scanning confocal microscopy (Leica LSC, Heidelberg, Germany). Under confocal microscopy, we observed that biotinylated $Aß_{42}$ peptide internalization in silenced SIRPβ1 microglia showed respectively 30 % which is 3 fold (+/- 0.5) lower then ShSIRPβ1-control-GFP 81.30 % and plenty-GFP 82 %. (Independent-samples *t* test, *p* values ***<0.05). This gives very

clear declaration that SIRPβ1 is very purposeful receptor, which involved in amyloid plaques clearance by primary microglia cells in Alzheimer's disease.

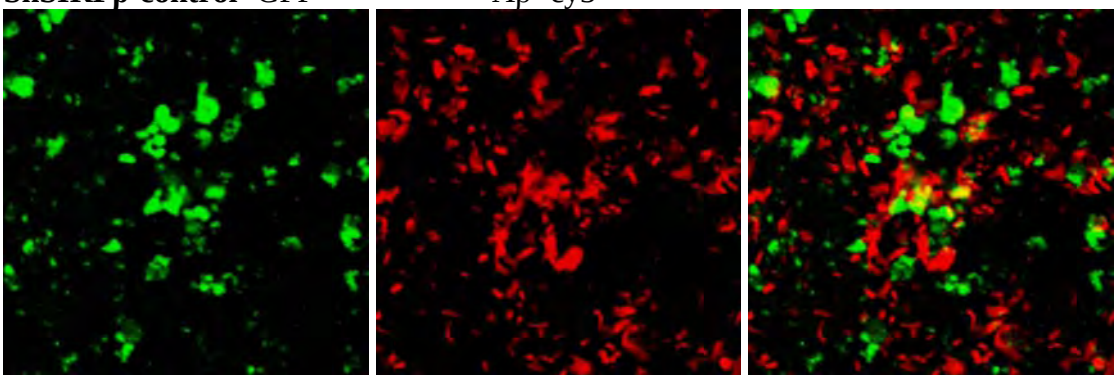
A)

ShSIRPβ – GFP Aβ- cy3

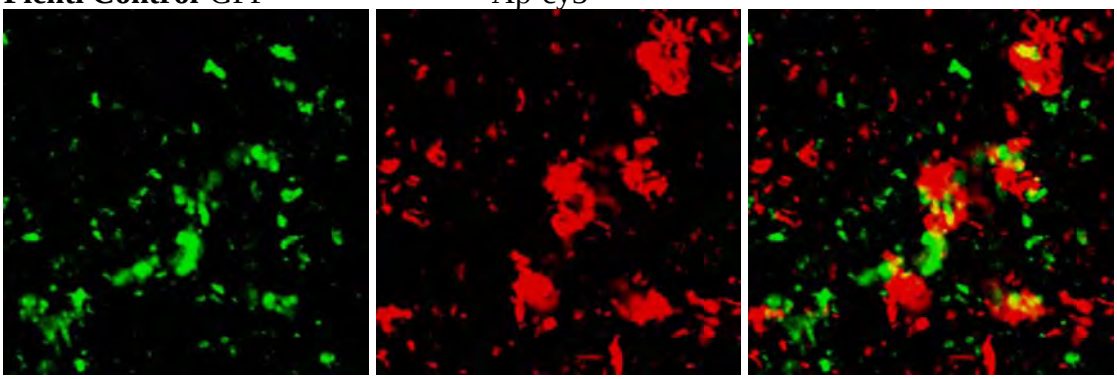


B)

ShSIRPβ-control- GFP Aβ- cy3



Plenti Control-GFP Aβ-cy3



C)

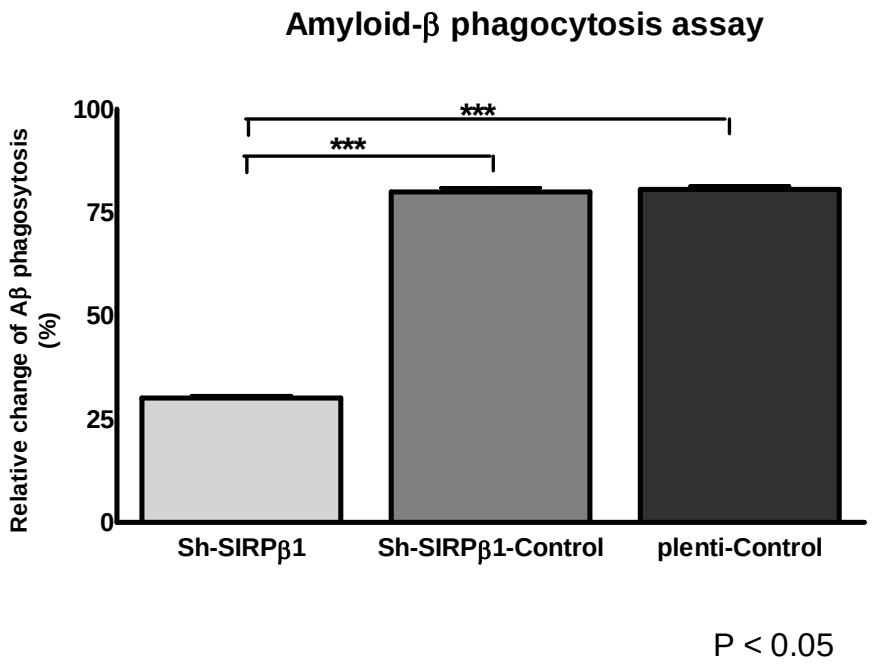


Figure 4.13.2. Phagocytosis of amyloid – beta by microglia.

A. *Microglial cells were transduced with a short hairpin interference SIRPβ1 vector (green) and challenged with biotinilyted amyloid-beta membranes labeled with a red fluorescent (Cy3). Scale bar: 20μm.*

B. *Microglial cells were transduced with a short hairpin interference control vector and normal empty plenty vector (green) challenged with biotinilyted amyloid-beta membranes labeled with a red fluorescent (Cy3). Scale bar: 20μm.*

C. *Statistical analysis 24 hours after phagocytosis of amyloid-beta by microglia lentivirally transduced with a shSIRPβ1, shControl or plenty-GFP vector. Data are presented as mean +/- SEM. Number of independent experiments n=9. ANOVA followed by unpaired t-test: *** p= < 0.05 (shSIRPβ1 versus ShSIRPβ1 control and planti-vector control).*

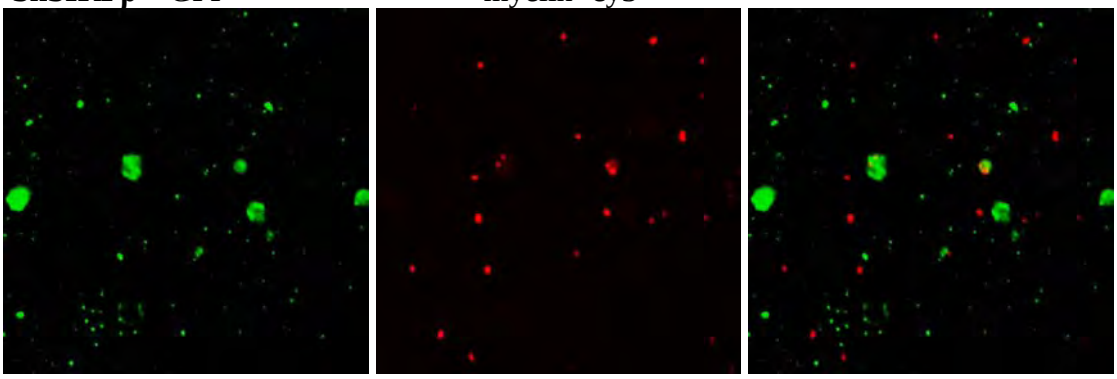
4.13.3. Impaired uptake of basic myelin after lentiviral knock down of SIRPβ1 in primary microglia

Phagocytosis by microglia/macrophages is a hallmark of the MS and Alzheimer's lesions; however, the extent of tissue damage and the type of cell death will

dictate subsequent innate responses. Microglia/macrophages are the key effecter cells that remove damaged myelin sheaths by phagocytosing myelin debris. To confirm the biological functional activity of SIRPβ1 gene, it was analyzed with and without silencing in primary microglial cultures, we transduced them with shSIRPβ1-GFP and Sh SIRPβ1-control-GFP plasmid, respectively and cells were incubated along with 2µg/ml Cy3-labeled myelin. Myelin internalization was measured after for 6 hours of administration by fluorescence confocal microscopy. In general, microglia cells highest internalization was detectable 6 hours after myelin administration and after 24 hours, the fluorescence intensity of Cy3-labeled myelin decreased (Liu, 2006)**.** Under confocal microscope, we observed that myelin internalization in silenced SIRPβ1 microglia shown respectively 20.66 % which is 4 fold (+/- 0.5) lower then ShSIRPβ1-control-GFP 76.45 %. (Independent-samples *t* test, *p* values <0.05).

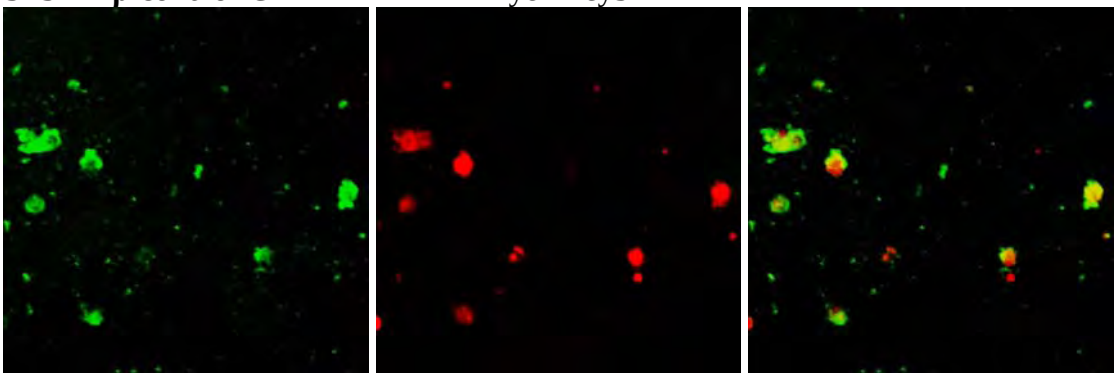
A)

ShSIRPβ – GFP myelin- cy3



B)

ShSIRPβ-control-GFP myelin-cy3



C)

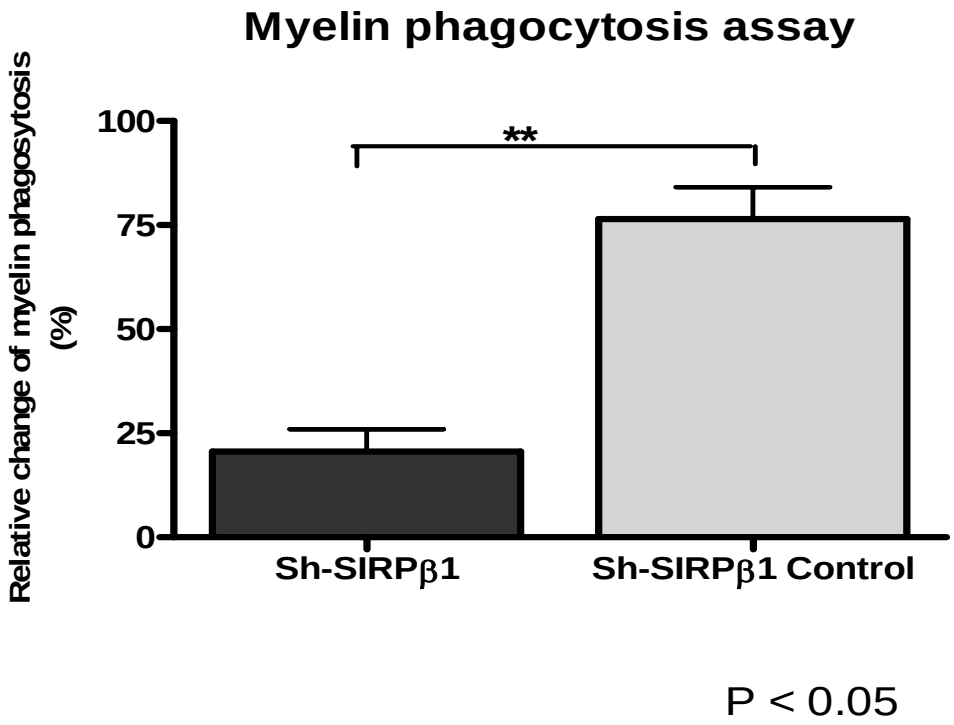


Figure 4.13.3. Phagocytosis of myelin by microglia.

A. *Microglial cells were transduced with a short hairpin interference SIRPβ1 vector (green) and challenged with cy3-conjugated myelin membranes labeled with a red fluorescent. Scale bar: 20μm.*

B. *Microglial cells were transduced with a short hairpin interference control vector (green) challenged with cy3-conjugated myelin membranes labeled with a red fluorescent. Scale bar: 20μm.*

C. *Statistical analysis at 24 hours after phagocytosis of myelin by microglia lentivirally transduced with a shSIRPβ1, shControl or plenty-GFP vector. Data are presented as mean +/- SEM. Number of independent experiments n=9. ANOVA followed by unpaired t-test: ** p= < 0.05 (shSIRPβ1 versus ShSIRPβ1 control and planti-vector control).*

5. Discussion

5.1. Functional characteristics of microglia

Microglia, which may constitute as many as 12% of the cells in the CNS, were described by del Rio-Hortega (1932) as a distinct cell type within the CNS with characteristic morphology and specialized staining characteristics that differentiate them from other glial cells and neurons. Persistent microglial activation is an important constituent of neurodegenerative diseases, and this most likely contributes to neuronal dysfunction, injury, and loss in these diseases. Gene transfer into primary cultured microglia or macrophages by classical transfection procedures is very poor and results in very low transfection efficiency (Mack, 1998). Several factor that potentate the phagocytosis of apoptotic cells have been identified in vitro. These include glucocorticosteroids (Liu et al., 1999), ligation of the CD44 cell surface molecule (Hart et al., 1997), and CD36-gene transfer to semiprofessional phagocytes (Ren et al., 1995). In addition, different cytokines have been shown to regulate the uptake of apoptotic cells by human monocyte derived macrophages (Ren and Savill, 1995) and semiprofessional phagocytes (Walsh et al., 1999). In addition, a phagocytosis-promoting effect of in vivo administered GM-CSF on monocytes and polymorphonuclear leukocytes has been demonstrated in cancer patients (Galati et al., 2000). The understanding of microglia as the brain's intrinsic immune system and the understanding that chronic activation of this system leads to pathologic sequelae has led to the modern concept of neuroinflammation (Streit, 2004). As it was shown earlier by Takahashi (2005) the microglial several factors that potentate the phagocytosis of apoptotic cells.

5.2. Microglial receptor SIRP-β1 involvement in functional understanding

In our studies, we investigated one of such essential protein from microglial cells that were not much under consideration in functional understanding. We aimed to have evidence for the functional properties of microglial SIRPβ1 molecule to clarify its role in the CNS environment. For the first time, we have shown the interaction of signal

regulatory protein beta1 to its adapter protein DAP12, in microglia. In our studies also found that association between DAP12 and SIRPβ1 regulates such phenomena as phagocytosis in microglia.

The immune system must be highly regulated to obtain optimal immune responses for the elimination of pathogens without causing undue side effects. This tight regulation involves complex interactions between membrane proteins and leukocytes. Members of the signal-regulatory protein (SIRP) family, which are expressed mainly by myeloid cells, provide one example of these regulatory membrane proteins. The signal regulatory proteins (SIRPs) are a family of transmembrane glycoproteins, which belong to the immunoglobulin (Ig) superfamily. The (Ig) superfamily structurally contains two G-type Ig subunit and one V-type Ig domain in the extracellular region on N-terminal. DAP12, a dimeric adapter protein that contains an ITAM activation motif that associates with the SIRPβ1 through its basic amino acid side chain in the C-terminal transmembrane region of SIRPβ1, transmits activating signals. However, the natural ligand for SIRPβ1 is unknown; keeping the *in vivo* significance of SIRPβ1 remains unresolved. It is possible that the heterogeneity of amino acid sequence in the extracellular region of SIRPβ1 will result in heterogeneous biological responses to the putative ligand of this protein.

Microglia cells were known to express DAP12 gene and it was reported that TREM2, as its associated protein molecule, regulates microglia function (Takahashi, 2005). For the first time we have shown that, SIRPβ1 is associated with DAP12 in microglial cells as well. We have demonstrated clearly that SIRPβ1 and DAP12 mRNA are present in the microglial cells, myeloid cells and splenocytes but interestingly not in neuron. SIRPβ1 surface expression was not only shown by real-time polymerase chain reaction but also by immunocytochemistry. CD11b and IbaI were used as a positive control, as it is known that they are microglia-macrophage specific surface markers. Immunolabelling of SIRPβ1 was detected as a cytoplasmic staining in cultured mircroglia using monoclonal antibodies directed against SIRPβ1 (SIRPβ80 and SIRPβ84, respectively) which was kindly provided by Japanese research group of Takashi Matozaki. Our microglial transduction was found to be reproducible and the expression was stable. Interestingly, no immunostaining of SIRPβ1 was detected in cultured neurons double-labelled with antibodies directed against β-tubulin-III. Nevertheless, neuronal

cells do express SIRPα that was identified in mouse brain as p84, which is a protein expressed on the surface, and this led to the first identification of a ligand for mouse SIRPα, namely the widely expressed cell-surface protein CD47 (Jiang, 1999).

5.3. Activated microglial cytokines involvement in activation of SIRP-β1 function

In our experiments we have found that activated microglia have shown upregulation of SIRPβ1. We also tried to understand why SIRPβ1 upregulates during microglial activation compared to resting microglia. It has been already shown by several groups that activated microglia have the tendency to upregulate cytokines profile. As we found in primary cultured microglia cytokines such as interferon-gamma (IFN-γ), interferon beta (IFN-β), LPS and TNF-α play major roll in SIRP-β1 upregulation, but the exact mechanisms by which it exerts its beneficial effects are unknown.

Various receptors responsible for the specific recognition of targets for phagocytosis by macrophages have been identified (Greenberg, 2002; Platt, 1998). The best characterized is the Fc receptor (Fc R), which recognizes the Fc region of IgG bound to antigen, presented on microbial pathogens (Ravetch, 2001). The phosphorylated ITAM then serves as a docking site for the tyrosine kinase Syk. Downstream signalling mediated by phosphoinositide (PI) 3-kinase or Rho family small GTP-binding proteins eventually triggers phagocytosis of IgG-coated (opsonized) particles (Stephens, 2002). Role of SIRPβ1 was already investigated in macrophage function and examined the effect of SIRPβ1 engagement by specific monoclonal antibodies (mAbs) on phagocytosis as well as on intracellular signalling (Hayashi, 2004). SIRPβ1 was shown previously to bind DAP12 (Deitrich, 2000; Tomasello, 2000). Furthermore, engagement of SIRPβ1 resulted in the tyrosine phosphorylation of DAP12 and the subsequent recruitment of *Syk* to the SIRPβ1-DAP12 complex in RBL-2H3 cell transfectants (Deitrich, 2000; Tomasello, 2000). We have now shown that engagement of SIRPβ1 induced the tyrosine phosphorylation in 293FT cells. It already elicited that the phosphorylation of Syk, a Syk inhibitor or kinase-negative Syk blocked the promotion of phagocytosis. A Syk inhibitor also blocked the activation of MAPK induced by SIRPβ1 engagement. The tyrosine phosphorylation of DAP12 and subsequent activation of Syk thus appear to contribute to

the promotion of phagocytosis by ligation of SIRPβ1 (Jackman, 1995). To analyze signalling of SIRPβ1, we have shown very transparently that SIRPβ1 associated with its signalling molecule DAP12 and stimulation of SIRPβ1 induced phosphorylation of the ITAM motifs. To achieve this observation, we cloned three time flag nucleotides sequence at the N-terminal of SIRPβ1 and then followed by GFP sequence at C-terminal as a fluorescent reporter gene in to the plenty-lox 3.7 viral vector background. DAP12 was cloned into the pLenti6/V5 D-topo background. For co-transfection of these two vectors, Lepofectamine transfection reagent was applied to achieve efficient transfection of 293FT cells. Flag monoclonal antibodies were used for the phosphorylation to stimulate SIRPβ1 expression and after 48 hours, total protein lysate was utilized for western blotting analysis. It was also demonstrated that engagement of SIRPβ1 induced the tyrosine phosphorylation in our culture system where we shown an appropriate control for this experiment.

Furthermore, the effect of SIRPβ1 stimulation on microglia in our study was relatively selective for phagocytosis, indicating that SIRPβ1 may activate intracellular pathways distinct from other innate immune receptors. We have found that phagocytosis, which is one of the most vital functions of microglia has shown wide interrelation and importance to SIRPβ1 protein. Microsphere beads phagocytosis assay has been done, where microglia were transduced by overexpressing SIRPβ1 by flag-tag SIRPβ1 construct and in parallel stimulated by flag and IgG monoclonal antibodies which demonstrated statistically significant increase in the up-take of microsphere beads upon flag-antibody stimulation in activated microglia. This enables us to explain the necessity of SIRPβ1 protein to microglial functioning.

5.4. Silencing of SIRP-β1 decipher unmarked understanding in microglial functional

In addition, we have used a lentivirus-based vector (pLL3.7) that expresses RNAi-inducing shRNAs under the control of the U6 promoter (Fig.7; (Tuschl, 2002). This vector was engineered to co-express enhanced green fluorescent protein (EGFP) as a reporter gene. We showed that RNA interference for SIRPβ1 by lentiviral small hairpin RNAs reduced SIRPβ1 gene transcripts to levels undetectable by 40 cycles of RT-PCR in

transduced microglia, whereas the gene transcripts of ribosomal 18s as an endogenous control was not affected. Furthermore, knockdown of SIRPβ1 in microglia by RNA interference was confirmed by immunocytochemistry using monoclonal antibodies directed against SIRPβ1 (SIRPβ80 and SIRPβ84, respectively). Knockdown of SIRPβ1 in microglia by RNA interference allowed us to study the role of SIRPβ1 in mature and differentiated microglia. Although we have not observed any effect of the transduction procedure or the expression of nonrelated small hairpin RNAs in our control experiments, we cannot completely exclude that the lentiviral transduction procedure *per se* might influence the phenotype of microglia.

5.5. Microglial receptor SIRP-β1 involvements in neurodegenerative disease

Nasu-Hakola disease (NHD) is a neurodegenerative disease expressed by progressive dementia accompanied by bone cysts and, at random, epilepsy. It is an autosomal recessive hereditary disease where genetic mutation was identified in DAP12 and its genetic defect is located at the 19q13.1 chromosome. It appears that DAP12 is expressed in the microglial activation and the differentiation of macrophages in the CNS as well at the same time, in the osteoclasts in charge of bone remodeling (Molina-Monasterios, 2003). Gene transcripts of this DAP12-associated protein are down-regulated in the CNS of the patients with the inflammatory NHD (Takahashi, 2007). To study intensively these DAP12 expressed activated microglia and their role in neurodegenerative disease we used postnatal three to four days old newborn mice to isolate microglia. We have shown the purity of microglia which isolated from mixed-glial cultures.

We further moved by SIRPβ1 gene silencing to put on more functional information in to microglia. Neuronal loss via apoptosis is a key element in numerous neurodegenerative diseases. It has been shown that microglial cells phagocytose and degrade apoptotic material. In this study we investigated the ability of microglial cells to take up and degrade neuronal apoptotic material, demonstrated a SIRPβ1 receptor mediated pathway for the uptake, and there could be a lysosome and proteasomal mediated degradation of proteins from apoptotic material (figure 10). Up-regulation of

phagocytosis without induction of inflammatory cytokines and cytotoxic mediators implies clearance without an inflammatory reaction. Since we have shown that microglia plays an important role in the removal of apoptotic neurons and splenocytes during development and aging, SIRPβ1 might recognize apoptotic neuronal and splenocyte membranes and participate in their clearance.

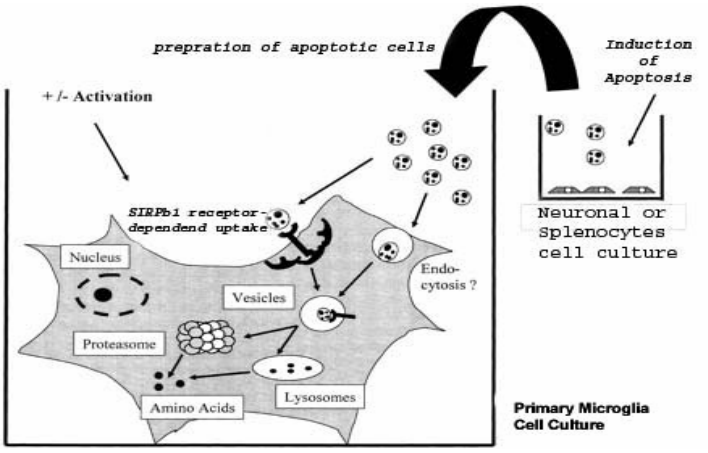


Figure 10. *Scheme diagram of uptake and degradation of apoptotic cells by microglial cells. Induction of apoptotic cells were produced by okaidic acid for apoptosis in neuronal or spleen cells. Apoptotic cells were added to cultures of primary microglial cells. The microglia was able to recognize and to take up apoptotic material. The SIRPβ1 receptor played a key role in this process in our finding. Uptaken apoptotic cells was transported into lysosomes, and proteins were degraded by lysosomal proteases were shown also by (Grune, 2004). Part of the proteins from uptaken apoptotic bodies were degraded by the proteasomal system. Activation status of microglia influenced the uptake and degradation of apoptotic bodies.*

Inflammatory reactions involving microglia are characteristic of several chronic conditions like MS, AIDS, cerebral malaria, tuberculosis and AD. Investigators have devoted considerable efforts to the study of microglial involvement, particularly in MS and AD. In EAE, micoglial paralysis results in the substantial amelioration of the clinical signs and in strong reduction of the CNS inflammation. As well in the case of AD, reactive microglia found to colocalize with neuritic plaques in the cortical region of AD brains (Rogers et al., 1988). In the late neurodegeneration phase of EAE, which goes along with destruction of neurons, activated microglia may have a crucial role. We have shown in this late neurodegeneration phase of EAE and in 6 and 12 months old APP transgenic mice model that microglial activation, which correlate to up-regulation of surface membrane protein SIRPβ1 and its adapter protein DAP12 up to some extent.

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9. Erklärung/Declaration

An Eides statt versichere ich, dass ich die Arbeit mit dem Titel "Therapy apporoach of Neurodegenerative Disorders by Bone Marrow Stem Cells„ selbst und ohne jede Hilfe angefertigt habe, dass diese oder eine ähnliche Arbeit noch keiner anderer Stelle als Dissertation eingereicht wurde. Ich habe früher noch keinen Promotionsversuch unternommen.

This thesis has been written independently and with no other sources and aids than stated.

Bonn, (month) 2007

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- **"Molecular Analysis of CD44 Expression in Experimental Brain Tumour Cell Lines". S. GAIKWAD** and A. Stan; submitted as poster presentation for „48. Jahrestagung der Deutschen Gesellschaft für Neuropathologie und Neuroanatomie e.V."; 08. - 11. Oktober 2003; Berlin, Germany.
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- **"Phagocytosis of apoptotic neural cells by the microglial receptor SIRPb1" S. Gaikwad,** S. Larionov and H. Neumann. Submitted as poster presentation for the "Annual-meeting Bonner forum biomedicine" February 10-12, 2007; Bad Breisig, Germany.
- **"Phagocytosis of neural debris, mylein and beta-amyloid mediated by the microglial receptor SIRPβ1". S. Gaikwad**, S. Larionov, A. Monsonego, T. Matozaki and H. Neumann. Article in preparation.

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God grant me the serenity to accept the things I cannot change, courage to change the things I can, and wisdom to know the difference.

- (Reinhold Niebuhr)

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