“Microglia derived from embryonic stem cells and its application in CNS diseases”

PhD thesis

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Abbreviations

ANOVA - analysis of variance
AP - alkaline phosphatase
APP - amyeloid precursor protein
Arg - Arginase
BBB - blood brain barrier
BDNF - brain derived neurotrophic factor
BME - Basal Medium Eagle
BSA - bovine serum albumine
CCL - chemokine C-C motif ligand
CCR - chemokine (C-C motif) receptor
CD - cluster of differentiation
CFA - complete Freund's adjuvant
CNS - central nervous system
CNTF - ciliary neurotrophic factor
CS - clinical score
CSF – colony stimulating factor
CT - computer tomography
CX3CR1 - Chemokine CXC motive receptor
CX3CL1 - Chemokine CXC motive ligand
DABCO - 1,4-diazabicyclo[2.2.2]octane
DAPI - 4',6-diamidino-2-phenylindole
DAP12 - DNAX activation protein of 12 kDa
DTT - dithiothreitol
DMEM - Dulbecco's Modified Eagle's Medium
DMSO - dimethylsulfoxide
DNA - deoxyribonucleic acid
dNTP – deoxynucleotide triphosphate
EAE - experimental autoimmune encephalomyelitis
EB - embryoid body
EDTA - ethylenediaminetetraacetic acid
EGF - epidermal growth factor
EGFR – epidermal growth factor receptor
ESC - embryonic stem cell
ESdM - embryonic stem cell derived mircoglial precursor
ERKs - extracellular-signal-regulated kinases
FACS - fluorescence activated cell sorting
FCS - fetal calf serum
FGF - fibroblast growth factor
Fig - figure
Fizz - found in inflammatory zone
Fn - fibronectin
G-CSF - granulocyte-colony stimulating factor
GAP43 - growth associated protein 43
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GFAP - glial fibrillar acidic protein
GFP - green fluorescence protein

GM-CSF - granulocyte macrophage colony stimulating factor
hCG - human chorionic gonadotropin
HE – hematoxylin/eosin
HEK – human embryonic kidney
HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSV – herpes simplex virus
Iba1 - ionized calcium binding adaptor molecule 1
ICM - inner cell mass
IFN - Interferon
Ig - immunoglobulin
IGF- insulin-like growth factor
IL - Interleukin
iNOS - inducible nitric oxide synthetase
IP-10 - Interferon gamma-induced protein
iPS - cell induced pluripotent stem cell
ITAM - immunoreceptor tyrosine-based activation motif
ITIM - immunoreceptor tyrosine-based inhibition motif
ITS - Insulin-Transferrin-Selenite
IU – international unit
LB - lysogeny broth
LIF - leukemia inhibitory factor
LFB – luxol fast blue
LOH – loss of heterozygosity
LPS - lipopolysaccharide
MAC – macrophage antigen
MBP -myelin basic protein
MCP-1 - monocyte chemoattactic protein 1
MEF - murine embryonic fibroblast
mg – milligram
ml - millilitre
MHC - major histocompatibility complex
MIG - monokine induced by gamma interferon
MMP – matrix metallo proteases
MOG - myelin oligodendrocyte glycoprotein
MRT - magnetic resonance tomography
MS - multiple sclerosis
NGF – nerve growth factor
nGS - normal goat serum
NK – natural killer
NT-3 - neurotrophin-3
NO - nitric oxide
OA – okadaic acid
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PE - phycoerytin
PDGF - platelet-derived growth factor
Pen/Strep – penicillin/ streptomycin
PET - positron emission tomography
PFA - paraformaldehyde
PMSG - pregnant mare serum gonadotrophin
PLL - Poly-L-lysine

PLP – proteolipid protein
PGK - phosphoglycerate kinase
PTEN - phosphatase and tensin homolog
qRT – quantitative real time
RNA - ribonucleic acid
ROI – reactive oxygen species
RT – real time
SEM - standard error of the mean
SiglecH - sialic acid binding immunoglobulin -like lectin H
SIRP - signal-regulatory-protein
SPECT - Single photon emission computed tomography
SR – scavenger receptor
SSEA - stage-specific embryonic antigen
SVZ – subventricular zone
TAM – tumor associated macrophages
TGF-β - transforming growth factor β
TLR - toll-like receptors
TNFα - tumor necrosis factor alpha
TREM2 - triggering receptor expressed on myeloid cells 2
TRIS –tris(hydroxymethyl)-aminomethane
TRK - tyrosine related kinase
WHO – world health organization
μm - micrometer

1. Introduction

1.1. Microglia

1.1.1. General Introduction

Two main cell types can be found in the central nervous system (CNS): neurons and glial cells. Neurons are exitable cells which can form neuronal networks and transmit information throughout the CNS. Glial cells support and protect neurons, therefore they are known as supporting cells of the nervous tissue. They are roughly divided into two subgroups, macroglia and microglia. Astrocytes and oligodendrocytes belong to the class of macroglia.

Microglia were first described in detail by del Rio Hortega in 1919 (del Rio Hortega, 1919) (Fig. 1.1). They are the resident immune cells which are the first line and main component in the active immune defense of the CNS. They are found throughoutly in the adult brain and compromise about 10 % of all brain cells (Ransohoff and Perry, 2009).

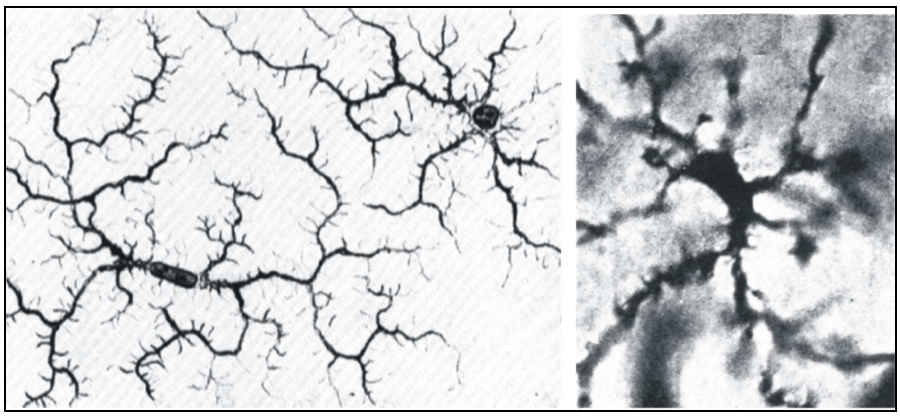

Fig. 1.1: Left: Drawing of ramified microglia by del Rio Hortega (del Rio Hortega, 1919). Right: Photomicrograph of silver stained microglia (del Rio Hortega, 1932).

Microglia respond to injury as well as to brain disease, they are known to scavenge invading microorganisms and dead cells and furthermore act as immune effector cells. Thus, they are key players in innate immunity and brain homeostasis in the immune privileged brain (Rivest *et al.* 2009). Additionally, microglia most likely contribute to the

onset of or increase of neuronal degeneration as well as inflammation in many brain diseases by producing detrimental factors like superoxide anions, nitric oxide (NO) or pro-inflammatory cytokines. However, microglia cells are also able to produce neurotrophic and neuroprotective molecules such as nerve growth factor (NGF), indicating a role in neuronal survival in cases of brain injury. Therefore, they are considered to be of double-edged nature, as they have both beneficial and detrimental effects.

1.1.2. Origin of microglia

During development in mice, microglia populate the brain in two waves. First, ionized calcium binding adaptor molecule 1 (Iba-1) positive cells appear in the neuroepithelium during embryonic development (E9.5-10.5) before blood circulation is established (Alliot *et al.,* 1999). It was shown by Ginhoux et al that these cells are derived from primitive macrophages in the yolk sac (Ginhoux *et al.,* 2010). The second wave takes place from E13.5 until birth, when an increase in Iba-1 positive cells in the brain and spinal cord takes place (Chan *et al.,* 2007). Microglial precursor cells which matured in the CNS during fetal development retain an immature phenotype resembling the M2 subtype of macrophages (see chapter 1.1.5) (Rae *et al.,* 2007).

Microglia were believed to be of mesodermal origin, as originally suggested by del Rio Hortega in 1919. Until today, this hypothesis still lacks concrete evidence and is a matter of debate (Chan *et al.,* 2007). Therefore, it was thought to be possible that microglia can arise from the neuroectodermal lineage like macroglia and neurons as well (Fedoroff *et al.,* 1997). It has been suggested that they also might emerge from circulating blood mononuclear cells (Perry *et al* 1985; Biber *et al* 2007), though microglia are present in the developing CNS before vascularization (Alliot *et al.,* 1999).

A subset of microglia is derived from bone marrow monocytes, though it has been shown that the transition is a rare event and takes place only under irradiation, lesion or inflammation (Shechter *et al.,* 2009, Mildner *et al.,* 2007, Kigerl *et al.,* 2009).

In addition, Ajami *et al* have recently shown that blood-derived monocytes infiltrating the brain during experimental autoimmune encephalomyelitis (EAE) do not contribute to the resident microglia in the CNS (Ajami *et al.,* 2011, Mildner *et al.,* 2007).

During the last years, evidence arouse that resident microglia emerge through primitive yolk sac macrophages derived from haematopoietic stem cells via a myeloid progenitor state (Fig.1.2) (Ransohoff and Cardona 2010; Ginhoux *et al.*, 2010, Alliot *et al.*, 1999).

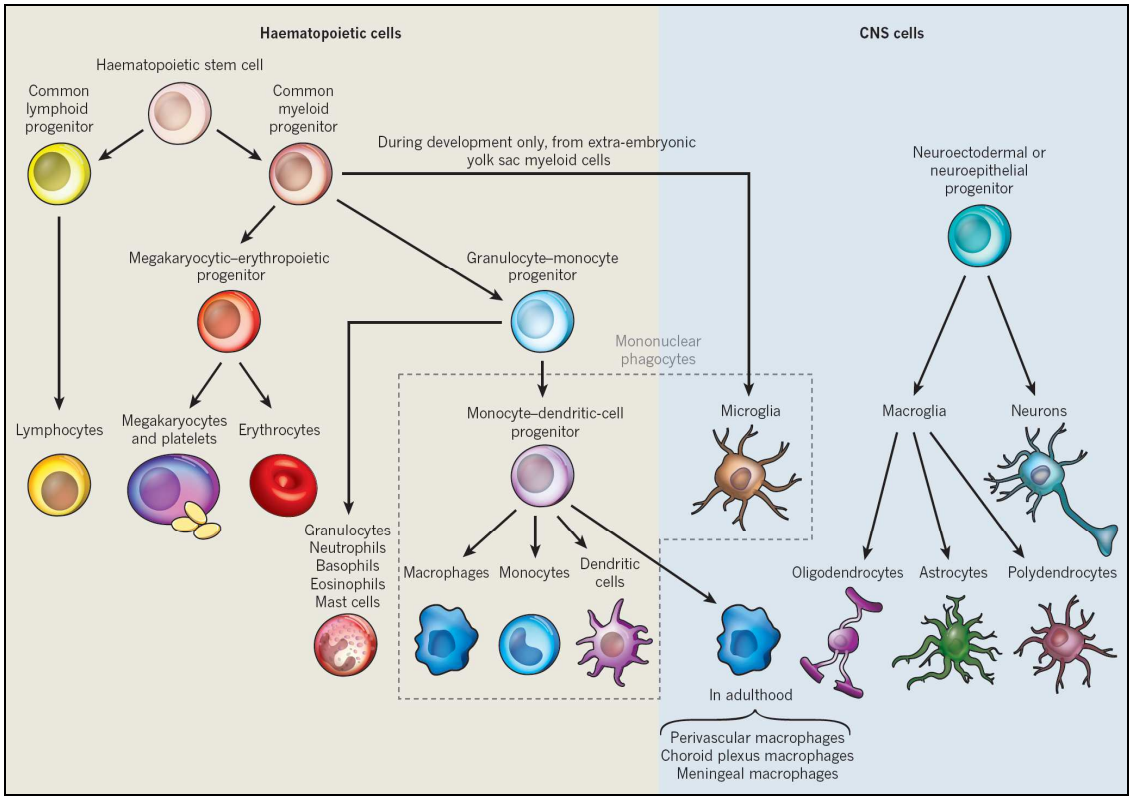

Fig.1.2: Relation of microglia to CNS cells. Microglia are the only cells of haematopoietic origin in the parenchyma of the CNS and migrate there during development (Ransohoff and Cardona, 2010).

These findings go along with older publications which have already shown that yolk sac macrophages are able to proliferate after migrating into the embryonic tissue (Cossmann *et al.*, 1997). Thus, microglia are most likely derived from primitive yolk sac macrophages which differentiate into microglial cells.

1.1.3. Characterization of microglia

Microglia characterization regarding surface expression markers is not a simple task, as they share most markers with macrophages. Nevertheless, microglia have a number of specific properties which distinguish them from other macrophages in the body. Inside

the brain, the density of microglia cells seems to be determined by specific different features of brain regions. For example, density of microglia in mice varies from 5 % in the cerebral cortex to 13 % in the substantia nigra (Lawson *et al.*, 1990). This diversity of microglia continues with their morphology, which ranges from a highly ramified appearance with multiple cell processes to round phagocytic cells. Concerning the proliferation, microglia behave like bone marrow myeloid progenitor cells (Alliot *et al.*, 1991) and share the expression of the stem cell marker cluster of differentiation (CD) 34. Opposed to splenic macrophages or alveolar lung macrophages, adult quiescent microglia express low levels of cell surface marker CD45, major histocompatibility complex (MHC) class II and Fc receptor molecules. However, several studies defined the following surface marker profile for microglia: CD68+, CD45 low, CD11b+, CD11c high, MHC class II+, Iba1+ and F4/80+ (Guillemin and Brew, 2004; Napoli *et al.*, 2009). In addition, microglia express co-stimulatory molecules CD86 and CD40, though in low levels (Aloisi *et al.*, 2000). Furthermore, microglia share some phenotypic and functional features of neural stem cells, including the expression of nestin (Yokoyama *et al.*, 2004). This feature cannot be found in any other population of tissue-resident macrophages.

1.1.4. Microglia states

In the adult brain microglia can switch inbetween two states (Fig. 1.3).

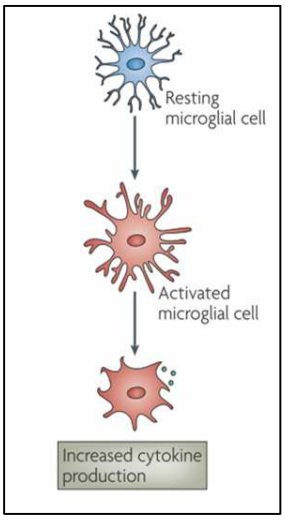

Fig. 1.3: Resting and activated microglia state (Perry *et al.*, 2007)

The first state is the so called “resting” state, in which highly ramified microglia carry out active tissue scanning in the brain. The processes of microglia are continuously moving, scanning the whole space of the CNS parenchyma every few hours (Nimmerjahn *et al.*, 2005). Resting microglia release immunosuppressants as well as neurotrophic factors and establish short time contacts with neurons to check on their health status (Hanisch 2002). They furthermore secrete cytokines and chemokines which are crucial for neuronal survival (Linnartz *et al.*, 2010). This resting state is regulated via immunosuppressive mediators such as Interleukin (IL) 10 or transforming growth factor β (TGF-β) present in the interstial fluid (Ransohoff and Perry, 2009). Furthermore, interaction of microglia with astrocytes and neurons through neurotransmitter receptors plays an important role in microglia state transduction (Graeber *et al.*, 2010, Hanisch and Kettenmann 2007). Especially chemokine CXC motif (CXC) 3 ligand 1 (CX3CL1) secreted by neurons is involved in maintaining the resting state of microglia and is sensed via the CX3C receptor 1 (CX3CR1) expressed by microglia.

The second state is the “activated” state which occurs in response to pathology and a disturbed CNS homeostasis, leading to phagocytosis, migration and release of inflammatory cytokines. A variety of stimuli leading to activation of microglia is known, including viral envelopes, bacterial cell wall components, pathological proteins or serum factors crossing the blood brain barrier (BBB) (Adams *et al.*, 2007, Heppner *et al.*, 2011). Microglia are able to recognize these stimuli through toll like receptor (TLR) 1 to 9 or complement receptor macrophage antigen 1 (MAC-1) (Lehnardt *et al.*, 2010). Upon signals of damaged or non-functional cells, microglia migrate to the affected site and then act as scavenger cells. This migration is mostly mediated by “find-me” signals emitted by dying cells (Blume *et al.*, 2011). Dead cells are recognized through a variety of molecules such as triggering receptor expressed on myeloid cells 2 (Trem2), CD36 or Scavenger Receptor 1B (SR1) (Napoli and Neumann 2009). The process of microglial phagocytosis is mainly regulated by environmental signals. Furthermore, activated microglia are also known to promote neuroprotection via secretion of neurotrophic factors like NGF (Elkabes *et al.*, 1996). The major functions of activated microglia resemble those of tissue macrophages and include phagocytosis, antigen presentation and the production of

chemokines, oxidative radicals or NO (Benveniste *et al.*, 2001).

In the resting state microglia mostly produce neurotrophins, whereas cytokines are produced upon stimuli (Elkabes *et al.*, 1996). For example, cytokine gene transcription is induced by lipopolysaccharides (LPS) or interferon (IFN) γ. An initial release of some cytokines such as tumor necrosis factor (TNF) α has an autocrine impact on further cytokine release. Cytokines play a crucial role in regulation of surface marker expression as a requirement for chemotaxis and cellular interactions like antigen presentation or apoptosis (Hanisch *et al.*, 2002).

In summary, the activated state of microglia is very dynamic and diverse, leading to different functional properties, ranging from neuroprotective to neurotoxic consequences (Biber *et al.*, 2007; Nimmerjahn *et al.*, 2005; Hanisch *et al.*, 2007).

1.1.5. Subdifferentiation of microglia

According to recent studies, immune responses have to be well controlled to have a beneficial effect on neurons. Otherwise, impaired regulation of immune responses can lead to neuronal loss and even result in autoimmune diseases (Butovsky *et al.*, 2005). Thus, pathways involved in pro- and anti-inflammatory processes have to be tightly and precisely regulated.

The double-edged nature of migrolia activation, which can lead to toxic or protective effects in the brain, depends on the secretion of anti- or pro-inflammatory molecules. Beside their activation states, microglia are further divided into a range of different subtypes, of which the M1 and M2 subtype represent the extremes and are distinctive in regards to surface receptors, cytokine production and effector functions (Fig. 1.4) (Mantovani *et al.*, 2004).

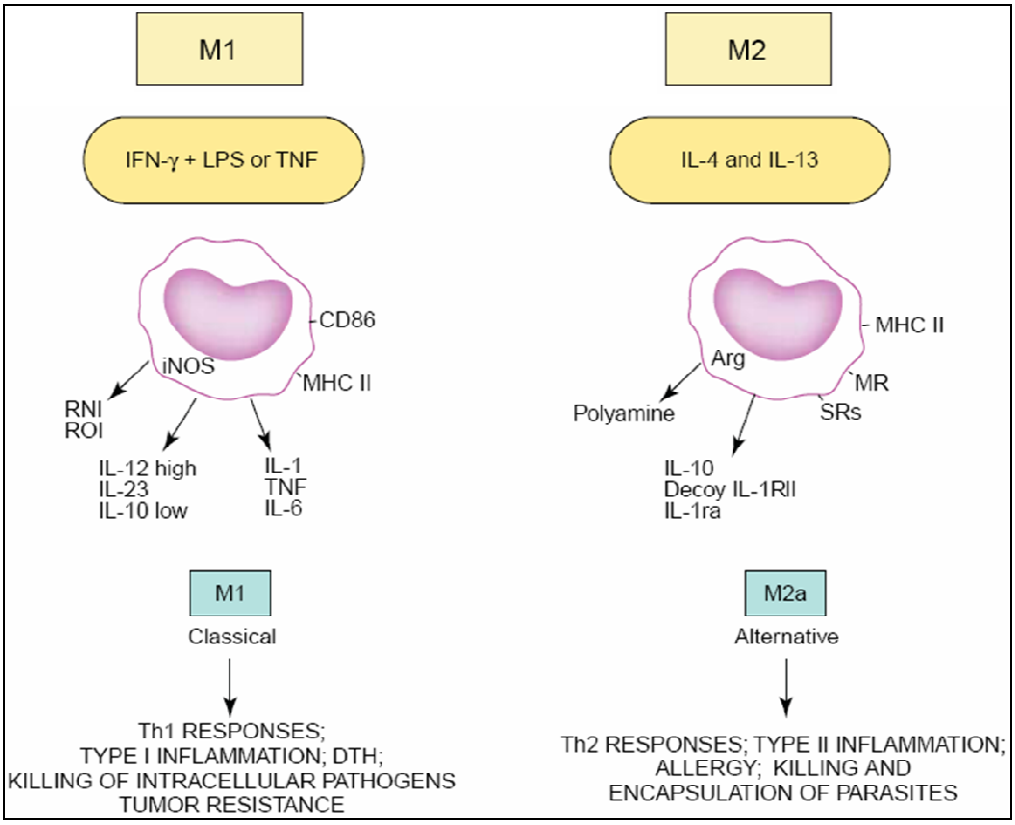

Fig. 1.4: Overview of M1 and M2 microglia subtype properties. Cells develop different functional properties in response to their environment. M1 polarization through IFN-γ and TNF-α leads to expression of iNOS, TNF-α, reactive oxygen intermediates (ROI) and IL-6, which lead to cytotoxic function. M2 polarization through IL-4 and IL-13 leads to increased levels of Arginase (Arg) and Scavenger Receptors (SR) and cells are more prone to immunoregulatory functions (Mantovani *et al*., 2004).

The first one is the type I microglia (M1), which resembles the classically activated pro-inflammatory microglia. This microglia type responds to microbial compounds, pro-inflammatory cytokines and lymphokines secreted by type 1 T helper (Th) cells (Mantovani *et al.*, 2004; Michelucci *et al.*, 2009). Upon stimulation, microglia present higher levels of MHC class I and II, CD80 and CD86 and show enhanced antigen presentation capability (Benveniste *et al.*, 1996). They furthermore respond through a higher motility and increased proliferation rates termed microgliosis (Nimmerjahn *et al.*, 2005, Ransohoff 2007). In addition, they secrete pro-inflammatory cytokines such as IL-1 and TNF-α (Hanisch and Kettenmann, 2007), free radicals like NO and superoxide anions (Goerdt and Orfanos, 1999). Although these molecules are crucial for the defense

of the brain towards pathogens, their expression leads also to a cytotoxic effect and collateral damage to healthy tissue (Ding *et al.*, 1988). M1 stimulation also triggers CXCL10 secretion, which is involved in microglia recruitment via its receptor CXCR3 (Rappert *et al.*, 2004).

The second type is the type II microglia (M2), which resembles the alternatively activated anti-inflammatory subtype (Michelucci *et al.*, 2009; Kigerl *et al.*, 2009). Molecules involved in activation of this subtype are molecules derived from type 2 Th cells like IL-4, IL-10, IL-13 or TGF-β (Goerdt and Orfanos, 1999). In the healthy brain, the alternative activation of microglia is mostly found in presence of apoptotic cells, which require phagocytic activity without inflammatory responses. Type II microglia are known for their anti-inflammatory properties, as they express receptors involved in endocytosis and phagocytosis like SR1 (Stein *et al.*, 1992). Furthermore, M2 polarized microglia have been shown to secrete neuroprotective factors such as insulin-like growth factors (IGF) or NGF (Butovsky *et al.*, 2006). M2 microglia secrete a variety of immune-suppressive cytokines such as TGF-β and IL-10 (Napoli and Neumann, 2009). They play an important role in mediating tolerance and abating inflammatory processes.

This broad spectrum of microglia effects allows a delicate balance of pro- and anti-inflammatory immune responses in the brain. Furthermore, *in vitro* studies showed that the subtypes have different effects on neurogenesis and oligodendrogenesis. Type I microglia activated by IFN-γ decreased neurogenesis and oligodendrogenesis, whereas type II microglia activated by IL-4 had the opposite effect (Butovsky *et al.*, 2006).

1.1.6. Embryonic stem cell derived microglia

During the last years, primary microglia have been commonly used to study microglia function. Several methods for isolation of primary microglia have been established and range from fluorescence activated cell sorting (FACS) to isolation from mixed glial cultures (Ford *et al.*, 1995, Giulian and Baker 1986). All of those methods have the drawback to be time-consuming and to yield only a low number of cells. Thus, they are not suitable for biochemical assays requirering high cell numbers.

Thus, oncogenically transformed microglial cell lines such as BV2 and N9 have been used, which have been immortalized via retroviral transfection with v-raf and v-myc. (Bocchini *et al.,* 1992). The immortalization process and their lack of some microglia properties (Blasi *et al.*, 1990, Horvath *et al.*, 2008) make these cell lines only a poor substitute to study microglia function.

Recently, a differentiation protocol for microglia-like cells from murine embryonic stem cells (ESCs) was described (Tsuchiya *et al.*, 2005). By modification of this protocol, a protocol for ESC differentiation into microglia was established (Napoli *et al.*, 2009; Beutner *et al.*, 2010). The protocol results in stable and pure proliferating cultures of ESC-derived microglial precursor (ESdM). ESdM derived from C57BL/6 ESCs express microglia markers like CD11b, CD11c, CD29, CD45, CD68 and Iba-1, but are negative for stem cell markers like CD117. ESdM are also positive for CD86, indicating that they are capable of antigen presentation. ESdM express CX3CR1 and are migratory active towards the chemokine CX3CL1. Furthermore, ESdM are capable of phagocytosing Aβ and fluorescent microsphere beads. Additionally, ESdM engraft as microglial cells into brain tissue upon transplantation (Napoli *et al.*, 2009). Analysis of the cytokine gene transcription showed a similar inducibility of cytokines of primary microglia and ESdM. Therefore, ESdM can be considered an adequate substitute for primary microglia and can be used in studies aiming at microglia function in neurodegenerative diseases.

1.2. Multiple Sclerosis

1.2.1. General Introduction

Multiple Sclerosis (MS) is a chronic inflammatory neurodegenerative disease which leads to demyelination of neurons in the CNS (Compston and Coles, 2002). With an estimated prevalence ranging between 2 and 150 per 100000, MS is one of the most common neurologic diseases, especially among younger people (Rosati *et al.*, 2001). Until now,

the cause of the disease is not known, although several environmental risk factors like lack of sun exposure have been found (Ascherio *et al.*, 2007).

MS can be classified into four subtypes according to the United States National Multiple Sclerosis Society: the relapsing-remitting, the secondary progressive, the primary progressive, and the progressive-relapsing form. The course of the relapsing-remitting subtype shows relapses with longer time periods of remission inbetween and is the most common form. Secondary progressive MS can be described as a follow-up of the relapsing-remitting form, in which patients begin to show neurologic decline between acute phases without remission phases (Compston and Cole, 2008). The primary progressive form of MS describes a disease course without remission phases after the time point at which the first symptoms appear (Miller *et al.*, 2007). The last common subtype is the progressive relapsing form, which combines a steady neurologic decline with superimposed attacks (Lublin *et al.*, 1996).

The loss of effective signal conduction via the axons can lead to a broad variety of neurological symptoms, including muscle weakness, loss of coordination, visual problems or changes in sensation. Early symptoms include fatigue, double visions and visual disturbance, genereal muscle weakness and tremors. As disease progresses, the symptoms become more severe and can result in blindness, muscle spasm and paralysis (Compston and Cole, 2008).

1.2.2. Pathology and role of microglia in multiple sclerosis

In MS, the immune system carries out a destructive immune response against antigens of the CNS like myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocytic protein (MOG). Subsequently, this reaction leads to demyelination and axonal injury within the white matter of the CNS (Fig 1.5).

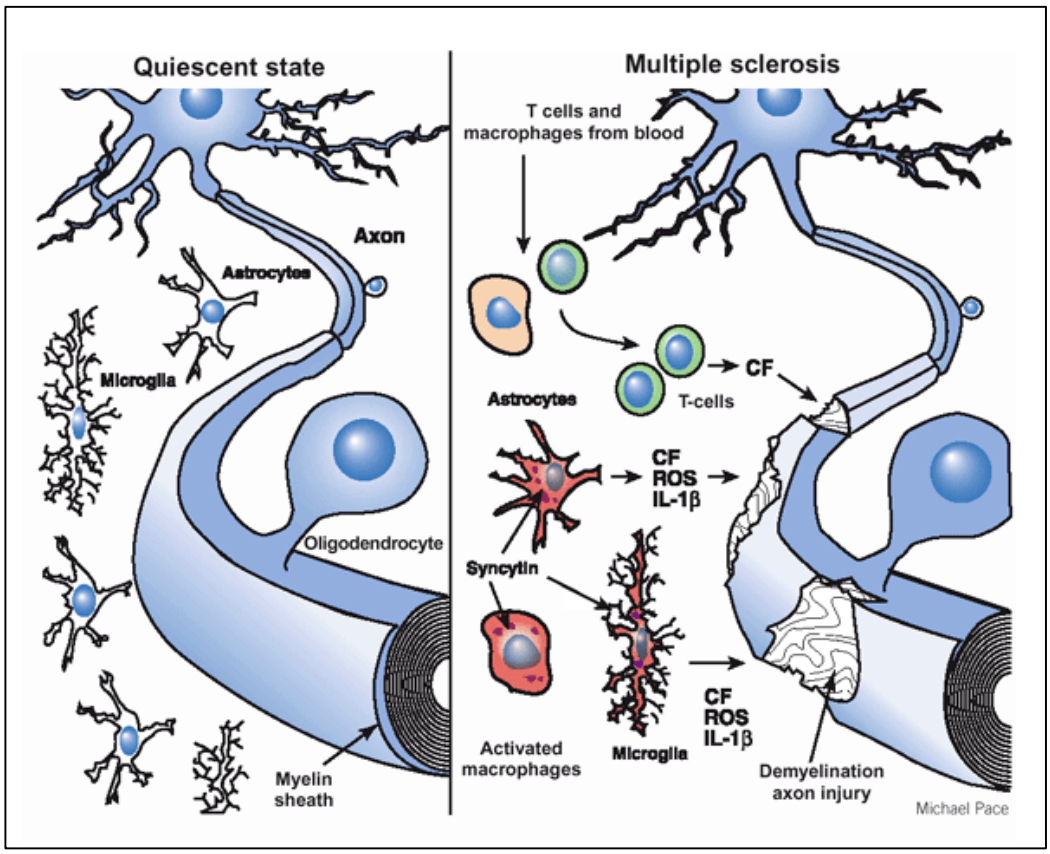

Fig. 1.5: Pathogenesis of MS. Activated T cells and macrophages migrate from the blood stream to the CNS and initiate the inflammatory lesions through cytotoxic factors (CF). Activated microglia contribute to demyelination via secretion of CF, ROS and IL-1β (Mattson and Taub, 2004).

Due to the demyelination, impairment of signal transmission occurs, axons are damaged and neurons are lost (Arnold and Matthews, 2002). Remyelination can occur in early phases of the disease, but the myelin sheath is not rebuilt completely. During the course of MS, scar-like plaques appear around the damaged axons, which are called lesions (Chari *et al.*, 2007).

Apart from the demyelination process, inflammation is another pathological hallmark of MS. The inflammation is mainly mediated by activated autoreactive T cells which invade the brain via the BBB and initiate an inflammatory response against autogens (Compston and Cole, 2002) and attack the myelin sheath (Wucherpfennig and Strominger, 1995). This attack in turn stimulates other immune cells like microglia and macrophages and leads to the secretion of inflammatory cytokines (Cannella and Raine, 1995). Despite of

the numerous beneficial functions of microglia, the activation of microglia has been implicated in the pathogenesis of a variety of CNS diseases. In case of MS, activated microglia cells produce MHC class II and present brain specific antigens to T cells (Dangond *et al.*, 1997). They furthermore produce pro-inflammatroy cytokines, free radicals and matrix metalloproteases (MMP) (Benveniste *et al.*, 1997). Inflammatory cytokines attract immune cells like lymphocytes and trigger the production of adhesion molecules, which in turn facilitate the immigration of cells through the BBB into the CNS. In summary, these processes lead to further recruitment of T cells, B cells and macrophages to the inflammation site. Autoreactive B cells start to produce antibodies against myelin antigens (Steinman *et al.*, 2002). The inflammation process is further increased through histamine release by mast cells (Theoharides *et al.*, 2007). It has been demonstrated that brain regions with a high grade of demyelination also contain high numbers of microglia (Bauer *et al.*, 1994). The crucial role of microglia in MS is further underpinned by the finding that depletion of microglia attenuates EAE in presence of T cells (Huitinga *et al.*, 1990).

1.2.3. Treatment of multiple sclerosis

Until now, there is no known cure for MS, though treatments aim at prevention of attacks and disability. Currently, most treatments involve immunosuppressants. Five medications have been proven as helpful: IFN-1a, IFN-1b, glatiramer acetate, mitoxantrone and natalizumab. All of those decrease the number of attacks in the relapsing-remitting form of MS. However, long term studies of side-effects are still not present (Comi *et al.*, 2009). Glatiramer acetate has been demonstrated to decrease microglial activation and thus have a beneficial impact on disease course (Ratchford *et al.*, 2011).

In attack phases, administration of corticosteroids leads to a decrease of acute symptoms. But this treatment does not have an impact on long term disease course or recovery (Burton *et al.*, 2009).

Current research is focused on better understanding of the course and the pathogenesis of MS. Furthermore, there is still need for therapies for the progressive subtypes of MS. Another major challenge is the improvement of treatments in general, especially concerning neuroprotection and regeneration.

The field of stem cell research has led to new approaches in addressing neuroinflammatory disorders including MS. Several cell types have been used for therapeutic approaches. To give an example, neural stem cells have been reported to migrate to inflammation sites in the CNS and to contribute to functional recovery and tissue repair (Pluchino *et al.*, 2003). A similar beneficial effect was shown using bone marrow cells (Zappia *et al.*, 2005).

1.2.4. Experimental autoimmune encephalomyelitis

For therapeutic studies concerning MS, the most commonly used animal model is the EAE. Like MS, it is an inflammatory demyelinating autoimmune disease which is mediated by T cells. EAE is inducible in common laboratory animals such as mice, rats, rabbits or primates. In mice, myelin proteins or peptides like MBP or MOG emulsified in adjuvant are used for induction of disease. Injection of this mixture leads to an inflammatory response to the protein (Billiau and Matthys, 2001). Co-injection with pertussis toxin decreases the BBB and allows immune cells to infiltrate the CNS tissue (Kerfoot *et al.*, 2004). Depending on the antigen injected, different EAE forms can be induced. With MOG-peptides (aminoacids 30 to 55) the course of EAE will be the relapsing-remitting subtype. Around two weeks after immunization the first clinical symptoms appear, starting with decreased tail tonus and paralysis of the tail. Progression of symptoms is ascending up the body, first affecting the hind limbs and then the forelimbs (Fig. 1.6).

Fig. 1.6: The left mouse displays clinical symptoms of EAE (clinical score of 3 with hind limb paraparesis), whereas the mouse to the right is healthy (Janeway´s Immunology 7th Edition).

Therefore, the symptoms reflect the anatomic location of the inflammatory lesions. In the relapsing-remitting form of EAE, recovery of symptoms can be complete or partial (Elhofy *et al.*, 2002).

Concerning the pathology, EAE mimics important aspects of human MS like progressing demyelination, inflamed lesions and axonal injury (Kornek *et al.*, 2000).

However, EAE cannot be considered to be completely equivalent to human MS, as it differs in time course and nerve inflammation.

1.2.5. Neurotrophin 3

Neurotrophin 3 (NT3) is a member of the neurotrophin family, which is involved in the homeostasis and regulation of the central and peripheral nervous system. Other members of this family are brain derived neurotrophic factor (BDNF) or NGF.

NT3 is closely linked to the survival and maintenance of function of a variety of neural populations (Chen *et al.*, 1997). NT3 interacts with the tyrosine related kinase C (TrK C) with high affinity (Lamballe *et al.*, 1994) and with a lower affinity with TrK B and the low affinity NGF receptor.

Receptors for neurotrophins are expressed on several different cell types, including lymphoid cells, cardiac cells, muscular cells or endothelial cells (Labouyrie *et al.*, 1997; Donovan *et al.*, 1996; Oichi *et al.*, 1997; Ricci *et al.*, 2000). Furthermore, expression of neurotrophin receptors was found in B cells (Kerschensteiner *et al.*, 1999).

The diversity of target cells mirrors the broad range of diverse biological effects. They play a crucial role in axonal outgrowth and neurogenesis as well as in neural survival (Kannan *et al.*, 1992; Memberg *et al.*, 1995). Recent studies indicate that NT3 plays a role in mediating the bidirectional cross-talk between the immune system and the nervous system (Biber *et al.*, 2007). Additionally, neurotrophins were shown to be endogenously expressed in lesion formation in MS (Hohlfeld *et al.*, 2006). The linkage of neurotrophins and severity of MS in the post-relapse phase with complete remission which was shown by Caggiula in 2005 qualifies neurotrophins as promising candidates for studies of therapeutic approaches of MS (Caggiula *et al.*, 2005).

1.3. Glioma

1.3.1. General Introduction

A glioma is defined as a CNS tumor type which arises from glia cells. They are primary tumors, occurring rather seldomly with an estimated prevalence of 15 cases for 100,000 people (American Cancer Society, 2006).

The world health organization (WHO) established a commonly accepted classification for brain tumors. In the recent version from 2007, gliomas represent the major group of brain tumors with 40%. Gliomas can be divided into several groups according to their cell type pendandts glia, astrocytes and oligodendrocytes and their WHO-grade (see table 1.1).

Tab.1.1: Official classification of glioma according to the world health organization (WHO, 2007)

WHO nomenclature	WHO grade	Histopathology
Pilocytic astrocytoma	I	Low cell density, bipolar pilocytic cells Rosenthal fibres
Low-malign astrocytoma	II	Low to minor cell density, fibric or gemistocytic astrocytes, some mitosis, few nuclear atypias
Low-malign oligodendroglioma and oligoastrocytoma	II	Honeycomb-structure, low cell atypia, few mitosis
Anaplastic astrocytoma	III	High cell density, cell and nucleus polymorphisms
Anaplastic oligodendroglioma and oligoastrocytoma	III	Additional honeycomb-structures, mixed forms
Glioblastoma	IV	High cell density, high number of atypias, necrosis, vascular proliferation

Low grade gliomas (grade I and II) are considered as benign and not anaplastic; therefore a good prognosis for the patients can be stated. The average age of the first diagnosis of a low grade glioma is between 30 and 40 years (Ichimura *et al.*, 2004). The medial survival time ranges between 7 to 8 years, depending on the time-point when the tumor changes to a malign tumor through increasing dysplasia. The low graded types of gliomas are growing slowly but infiltrative and can therefore reach an impressive size before first symptoms appear.

Gliomas with a WHO grade with II or higher are anaplastic and malignant. In case of glioblastomas, the median of survival after diagnosis ranges from 2 to 8 months, depending on the age of the patient (Scott *et al.*, 1987; Curran *et al.*, 1993). In this group, glioblastomas are the most prominent tumors and count for 23% of all brain tumors. Median age for first diagnosis is 64 years, the medial survival time with maximal therapy is 14.6 months (Stupp *et al.*, 2005). Glioblastoma can arise *de novo* as primary or from existing lower-grade tumors as secondary glioblastomas (Kleihues *et al.*, 1999), which differ a lot in molecular structures (Tso *et al.*, 2006). Glioblastomas are characterized by rapid and infiltrative growth pattern and are diffcult to target in therapy.

1.3.2. Symptoms of glioma

As virtually every part of the central nervous system can be affected by gliomas, the range of symptoms which they cause is very broad. With an incidence of 50 %, head aches are the most prevalent first symptom (Forsyth *et al.*, 1993). Depending on the histology, the location and growth rate of the tumor, gliomas can cause neurological misfunctions. Approximately 30 % of brain tumor patients show epileptical seizures (Glantz *et al.*, 2003). Most patients suffer additionally from general symptoms like nausea, vomiting, sleepiness, dementia or changes in character properties (Glantz *et al.*, 2000).

In histopathology, the different glioma types show a variety of cellular structures. Lower grade tumors show a wide morphological spectrum with low cell density, typical bipolar pilocytical cells and Rosenthal fibres. Malignant tumors display a high cell density with cell and nuclei atypias, development of vasculature and necrosis.

In regard to pathophysiology, gliomas lead to changes in several brain structures and properties. One major hallmark for the depiction of tumors is the BBB breakdown, which is mediated by tumor capillaries (Dickman *et al.*, 1989).

Tumors with a size of 2 to 3 cm in diameter result in an increase in intracranial pressure, as the brain parenchyma cannot be compressed (Black *et al.*, 1986).

A variety of imaging diagnosis is available, ranging from computer tomography (CT), magnetic resonance tomography (MRT), positron emission tomography (PET) to single photon emission computed tomography (SPECT). In addition with contrast agents, these

methods allow a precise imaging diagnosis. Nevertheless, stereotactic biopsies and liquor diagnostics are performed to fully diagnose a glioma.

1.3.3. Pathology of glioma

As glioma derives from healthy cells in the brain, they hardly express antigens which can be targeted by the immune system of the patient (Li *et al.*, 2008).

Nevertheless, the immune system fights tumor cells after transition, a resistant polulation remains and forms a resistant tumor mass (Dunn *et al.*, 2002).

Gliomas developed several mechanisms to overcome the anti-tumor response of the immune system (Fig. 1.7).

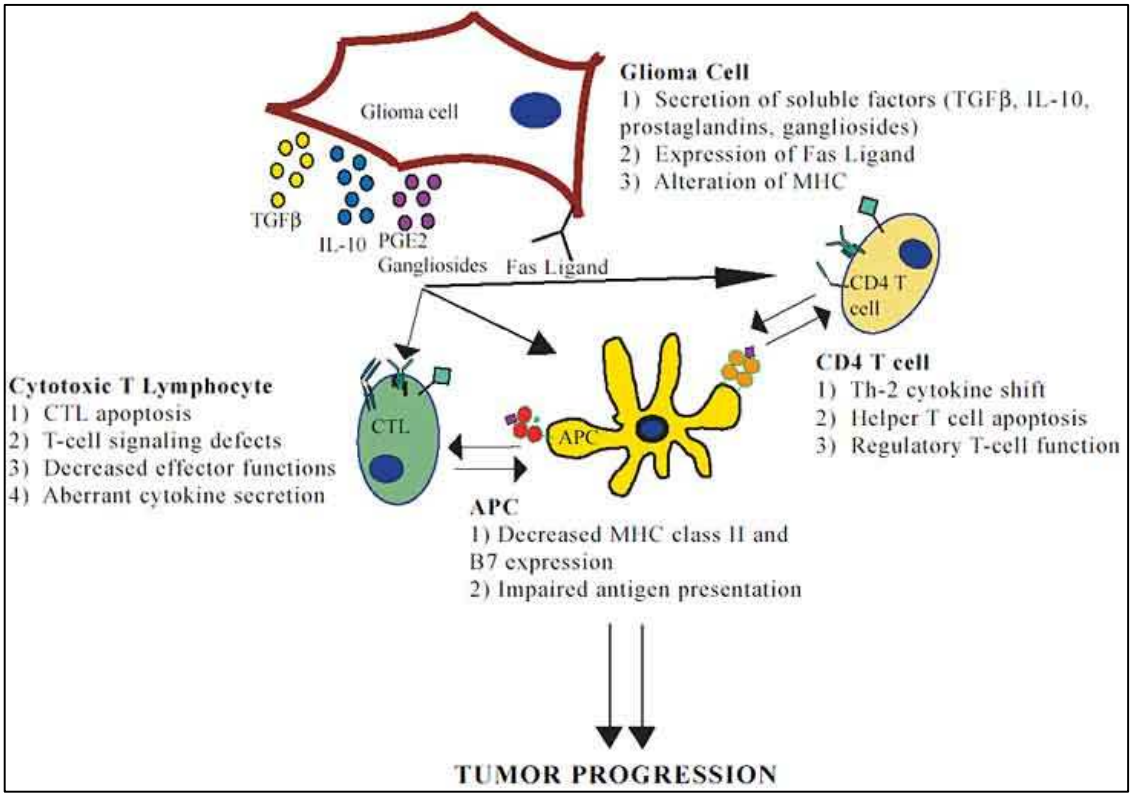

Fig. 1.7: Overview of immune response suppression by glioma cells. Glioma cells secrete factors such as TGF-β and IL-10 which suppress the interaction of and immune answer mediated by T cells and antigen presenting cells (Prins *et al.*, 2004)

Tumor cells in glioma are known to express a variety of immune-suppressive molecules like IL-10, prostaglandin E2 or TGF-β (Wrann *et al.*, 1987; Wiendl *et al.*, 2002; Wischhusen *et al.*, 2002). The secretion of these molecules leads to a local immunosuppression as well as to a systemic immunodeficiency via impeding microglia and T cell activation (Platten *et al.*, 2001). TGF-β inhibits antigen presentation of dendritic cells and other antigen presenting cells, and interferes with natural killer (NK) cells. Furthermore, it inhibits the activation and differentiation of cytotoxic T cells (Weller and Fontana, 1995). Thus, tumor specific antigens are lost and the tumor cells are capable of hiding from the immune system (Vega *et al.*, 2008). It was shown by Hao and colleagues that pro-inflammatory cytokines such as IFN-γ, TNF-α or IL12 and their corresponding receptors are barely expressed in the tumor residuum (Hao *et al.*, 2002).
The sum of these immune-suppressive reactions even results in a variety of healthy cells such as microglia, lymphocytes or endothelial cells which supply the tumor with nutrients and oxygen (Badie and Schartner, 2001; Watters *et al.*, 2005).

1.3.4. Mutations in gliomas

In tumor cells, a wide variety of mutations can be found. The most prominent ones are mentioned below.
The gene p53, also called the tumor suppressor gene, has a prominent function in cell cycle control, DNA repair and apoptosis (Kern *et al.*, 1991). Mutations causing deletion or alterations of this gene are the most common ones in human gliomas, causing increased cell proliferation and angiogenesis (Watanabe *et al.*, 1997).
Another mutation found especially in glioblastomas with a prevalence of 50% are amplifications of the epidermal growth factor receptor (EGFR)-gene (Zhang *et al.*, 2008), which codes for a receptor involved in the stimulation of cell division. Overexpression or alteration causing isoform proteins lead to an increased activity of that protein. Therapies aiming at EGFR were found to sensitize tumor cells to chemotherapy and radiotherapy (Slamon *et al.*, 2001).
Phosphatase and tensin homolog (PTEN) acts as a tumor suppressor gene through its phosphatase properties, which is involved in regulation of the cell cycle (Chu *et al.*, 2004). Mutation in this protein leads to an immense increase in cell division and is found

in most gliomas.

Additionally, loss of heterozygosity (LOH) is a frequent gene alteration in 60-90 % of glioblastoma. This mutation is closely linked to a poor survival rate and is most likely one of the key feature in glioblastoma development (von Deimling *et al.*, 1992).

During the last years, so called tumor stem cells were described for several glioma types (Ignatova *et al.*, 2002; Singh *et al.*, 2003; Yuan *et al.*, 2004; Taylor *et al.*, 2005). These cells share properties with neural stem cells. This fact indicates that gliomas may rise from those cells. Further evidence for this hypothesis was found in mice experiments, as a mutation of p53 in cells of the subventricular zone (SVZ) leads to glioma formation (Uhrbom *et al.*, 2002).

1.3.4. Therapy of glioma

Treatment of glioma is still a major clinical challenge and up to now no curative treatment is at hand. Generally, the therapy of choice depends on several factors like location, cell type and grade of the tumor. Mostly, gliomas are treated with a combination of the following three approaches. Many studies aim at the improvement of the current treatments (Valtonen *et al.*, 1997; Chan *et al.*, 2005; Reardon *et al.*, 2006).

One of the main treatments of glioma is the surgical therapy. A lot of studies showed that the extent of the tumor resection closely correlates with the survival time of the patient (Soffietti *et al.*, 1989; Jeremic *et al.*, 2003; Keles *et al.*, 2004). The patient not only benefits from the removal of tumor tissue, but also from the release of intercranial pressure, which improves further therapeutic approaches. Nevertheless, brain surgery is not capable of removing all infiltrating tumor cells which cause glioma reformation. Radiotherapy is another standard therapy for the treatment of glioma. Nowadays, for smaller gliomas stereotactic radiotherapy is used, in which a precise application of radation to the glioma is possible. But, in respect to long term survival, no effect of radiotherapy alone was found in case of grade II gliomas (Karim *et al.*, 2002).

Chemotherapy aims at the impairment of cell division, which mostly affects fast-dividing cells like tumor cells. Therefore, a variety of cytotoxic chemotherapeutics is used in established combinations and cycles. One of the biggest disadvantages of this therapy is that it not only affects tumor cells but also healthy dividing cells as well. This leads to

hair loss and damage of the intestinal epithelium.

Experimental therapies are mostly located on a molecular level, leading to so called molecular targeted therapy approaches. These approaches aim at the cancer specific signal cascades and molecules to influence proliferation, invasion and angiogenesis (Krause *et al.*, 2005; Adjei *et al.*, 2005). One of the most promising targets is TGF-β. Encouraging data was obtained using a TGF-β antisense oligonucleotide for the treatment of glioblastoma (Schlingensiepen *et al.*, 2006). Animal experiments and *in vitro* data endow this finding (Jachimczak *et al.*, 1996).

Another possible candidate is the platelet derived growth factor (PDGF) receptor. Upon inhibition with imatinib the tumor growth rate decreases and complete remission could be seen in some patients (Dresemann *et al.*, 2005).

A promising approach is the antiangiogenetic therapy, which aims at the inhibition of tumor neovascularization. So far, over 70 compounds have been tested, of which the most promising are Cediranib and Bevacizumab (Batchelor *et al.*, 2007; Vredenburgh *et al.*, 2007).

Studies about immunotherapy involving cytokines like IL-2 or interferons did not result in proof of effectiveness, additionally leading to immense toxic and neurotoxic side-effects (Merchant *et al.*, 1988; Albert *et al.*, 1998). Another approach in this field is the active specific immunotherapy which includes the *in vivo* induction of tumorspecific cytotoxic T lymphocytes. Upon induction, these cells might trigger apoptosis of tumor cells (Kägi *et al.*, 1994).

A variety of genetherapy-strategies has been examined in regard to malign gliomas. Mostly, adenoviral and retroviral vectors are used to deliver therapeutical genes into the glioma site. Examples for that are the oncolytic genetherapy, p53-genetransfer or IFN-β genetransfer (Mineta *et al.*, 1995; Lang *et al.*, 2003; Yoshida *et al.*, 2004).

A therapeutic effect was demonstrated *in vivo* by bone marrow derived cells expressing thymidine kinase of herpes simplex virus (HSV-tk) through bystander-mediated glioma cell killing after gancylovir application (Miletic *et al.*, 2007).

In summary, gene therapy seems to be the most promising approach to conquer malignant glioma in a safe and reliable fashion.

1.3.5. Role of microglia

The properties of microglia can both fight or support tumor cells in the CNS and once more show their double-edged nature.

Microglia and macrophages are known to be attracted by and to infiltrate the glioma via the fractalkine pathway (Umemura *et al.,* 2008; Held-Feindt *et al.,* 2010) and are the most commonly found immune cells in the tumor residuum (Graeber *et al.,* 2002). Recruitment of microglia is mediated through a variety of cytokines such as CXCL1, CCL2 and colony stimulating factor 1 (CSF-1) (Held-Feindt *et al.,* 2010; Lamagna *et al.,* 2006).

These tumor-infiltrating microglial cells obtain an immuno-suppressive phenotype and promote production of TGF-β via an autocrine loop (Umemura *et al.,* 2008). In addition to the immunosuppressive cytokine milieu provided by the glioma cells, this effect leads to T cell tolerance and inactivation of tumor targeted immune responses (Carpentier *et al.,* 2006). It has been demonstrated that tumor-associated macrophages (TAMs) are polarized into the alternatively activated M2 subtype which supports tumor infiltration and growth (Martinez *et al.,* 2008; Mantovani *et al.,* 2001). TAMs secrete anti-inflammatory cytokines like IL-10, IL-23 and therefore establish an immunosuppressive environment (Badie and Schartner, 2001; Wu *et al.,* 2010). They have also been shown to express MMPs which enhance tumor progression through degradation of extra-cellular-matrix (ECM) (Rao, 2003). Via secretion of EGF, microglia are also capable of enhancing tumor angiogenesis and proliferation (Lafuente *et al.,* 1999).

Microglia have also been described to have detrimental effects on glioma cells. After pro-inflammatory stimuli, activated microglia of the M1 subtype can secrete molecules which inhibit glioma growth (Nickles *et al.,* 2008). Mora *et al* observed activated microglia that can induce autophagy-dependent death of glioma cells (Mora *et al.,* 2009). As microglia are phagocytic cells, they are also capable of decreasing glioma cell number through phagocytosis. It has been stated that macrophages are able to phagocyte glioma cells in absence of apoptosis of glioma cells (Fadeel *et al.,* 2004).

In addition to direct functions of microglia in fighting glioma, they are also involved in recruiting NK cells, which play a pivotal role in tumor rejection via induction of tumor cell apoptosis. In the CNS, attraction of NK-cells is for instance mediated by CX3CL1 secreted by microglia (Huang *et al.,* 2006).

2. Materials and Methods

2.1. Materials

2.1.1. Buffers and solutions

10x (0.125 M) Phosphate buffered saline (PBS), pH 7.3
- 0.007 M $NaH_2PO_4*H_2O$ (Roth, Germany)
- 0.034 M $NaH_2PO_4*7H_2O$ (Roth, Germany)
- 0.6 M NaCl (Roth, Germany)
- ad 1 liter ddH_2O (Roth, Germany)

10x Annexin binding buffer
- 100 mM HEPES
- 1,4 M NaCl
- 25 mM $CaCl_2$

10x Tris-Boric Acid-EDTA (TBA) buffer
- 1.78 M Tris-Base (Roth, Germany)
- 1.78 M Boric Acid (Sigma, Germany)
- 0.04 M Ethylenediaminetetraacetic Acid (EDTA) (Roth, Germany)
- ad 2 liter ddH_2O (Roth, Germany)

1 % Agarose gel
- 0.5 g Agarose (Biozym, Germany)
- 1.25 µl Ethidium Bromide (Roth, Germany)
- 50 ml TBE (1x)

4% Paraformaldehyd (PFA), pH 7.3
- 20 g PFA (Sigma, Germany)
- 30 ml NaOH (Roth, Germany)
- 50 ml PBS (10x)
- ad 1 liter ddH_2O (Roth, Germany)

6x Loading buffer
- 0.5 M EDTA (Roth, Germany)
- 60 % Sucrose (Roth, Germany)
- 0.04 % Bromphenol Blue (Sigma, Germany)
- 0.04 % Xylene Cyanole (Sigma, Germany)
- 2 % vol/vol Ficol-400 (Bio-Rad, Germany)

Moviol

4.8 g Moviol (Kremer Pigmente, Germany)
12 g Glycerol (Sigma, Germany)
12 ml ddH_2O (Roth, Germany)
24 ml Tris buffer 0.2 M (Roth, Germany)
1.32 g DABCO (Sigma, Germany)

TRIS

12.1 g Trizma Base (Sigma, Germany)
18.0 g Sodium Chloride (Roth, Germany)
88 ml Hydrochloric Acid 1 M (Roth, Germany)
ad 2 liter ddH_2O (Roth, Germany), adjust to pH 7.4

Reverse transcription (RT) mix

5 µg total ribonucleic acid (RNA)
1 µl Hexanucleotide Mix (10X) (Roche, Germany)
1 µl Deoxynucleotide triphosphate (dNTP) mix (10mM) (Sigma, Germany)
2 µl Dithiothreitol (DTT) mix (10 mM) (Invitrogen, Germany)
4 µl 5X RT 1st Strand Buffer (Invitrogen, Germany)
1 µl RT enzyme (200 U/ml) (Invitrogen, Germany)
ad 20 µl ddH_2O (Roth, Germany)

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

3 µl Complementary Deoxyribonucleic Acid (cDNA) (200 ng/µl)
1 µl SYBR Green Master Mix (2x) (Applied Biosystems, USA)
2 µl Primer mix (10 pmol/µl)
10.5 µl ddH_2O (Roth, Germany)

2.1.2. Cell culture media and reagents

ES medium

Dulbecco's modified Eagle's medium (DMEM) 4.5 g/l D-glucose (Gibco, Germany)
2 mM GlutaMAX (Gibco, Germany)
1 mM Sodium-Pyruvate (Gibco, Germany)
0.1 mM Non-essential-amino acids (Gibco, Germany)
0.05 mM 2-Mercaptoethanol (Gibco, Germany)
15 % vol/vol Fetal Calf Serum (FCS) (Gibco, Germany)
1 µg/l Leukemia Inhibitory Factor (LIF) (Chemicon, USA)

Basal medium

Basal Medium Eagle (BME) (Gibco, Germany)
10 % vol/vol FCS (Gibco, Germany)
1 % vol/vol D-glucose (Sigma, Germany)
2 mM L-glutamine (Gibco, Germany)
100 µg/ml penicillin/streptavidin (pen/strep) solution (Gibco, Germany)

Differentiation medium

DMEM 4.5 g/l D-glucose (Gibco, Germany)
2 mM GlutaMAX (Gibco, Germany)
1 mM Sodium-pyruvate (Gibco, Germany)
0.2 mM Non-essential-amino acids (Gibco, Germany)
0.05 mM 2-Mercaptoethanol (Gibco, Germany)
15 % vol/vol FCS (Gibco, Germany)

Glioma medium

DMEM/F12 (Gibco, Germany)
10 % vol/vol FCS (Gibco, Germany)
100 µg/ml pen/strep solution (Gibco, Germany)

ITSFn medium

DMEM/F12 (Gibco, Germany)
25 µg/ml Insulin (Sigma, Germany)
30 nM Sodiumselenite (Sigma, Germany)
50 µg/ml Transferrin (Sigma, Germany)
5 µg/ml Fibronectin (Sigma, Germany); (add freshly before using medium)

MEF medium

DMEM 4.5 g/l D-glucose (Gibco, Germany)
2 mM L-glutamine (Gibco, Germany)
1 mM Sodium-Pyruvate (Gibco, Germany)
0.1 mM Non-essential-amino acids (Gibco, Germany)
10 % vol/vol FCS (Gibco, Germany)

N2 medium

DMEM/F12 (Gibco, Germany)
1 x N2 supplement (Gibco, Germany)
0.48 mM L-glutamine (Gibco, Germany)
5.3 µg/ml D-glucose (Sigma, Germany)
100 µg/ml pen/strep solution (Gibco, Germany)

Neuronal medium

Basal medium eagle (Gibco, Germany)
1 % vol/vol FCS (Gibco, Germany)
1 % vol/vol D-glucose solution (Sigma, Germany)
2 % vol/vol B27 supplement (Gibco, Germany)

2i medium

50 ml DMEM/F12 (Gibco, Germany)
50 ml Neurobasal Medium (Gibco, Germany)
0.5 ml N2 (100x , Gibco, Germany)
1 ml B27 (50x, Gibco, Germany)
0.5 ml GlutaMax (100x Gibco, Germany)
0.5 ml BSA (5 mg/ml)
50 µl Insulin (20 mg/ml)
200 µl β-Mercapthoethanol (Gibco, Germany)
1 µg/l Leukemia inhibitory factor (LIF) (Chemicon, USA)
10 µl PD0325901 (10mM, Axon Medchem)
30µl CHIR99021 (10mM, Axon Medchem)

2.1.3. Other reagents

- 5x RT 1st Strand Buffer (Invitrogen, Germany)
- Ampicilin (Sigma, Germany)
- Alkaline phosphatase Detection Kit (Millipore, Germany)
- Annexin, biotinylated (Sigma, Germany)
- Bovine serum albumin (BSA) (Sigma, Germany)
- Corbitbalm (Hecht, Germany)
- CX3CL1, recombinant mouse (R&D, Germany)
- Dimethylsulfoxide (DMSO) (Sigma, Germany)
- Endofree Plasmid Maxiprep (Qiagen, Germany)
- Ethanol (Roth, Germany)
- Fibroblast growth factor (FGF) 2, recombinant human (R&D, Germany)
- Fibronectin from bovine plasma (Sigma, Germany)
- Fluoresbrite Polychromatic Red microspheres 1.0 µm (Polyscience, USA)
- G418 antibiotics (Sigma, Germany)
- Gelatin (Fluka, Germany)
- Granulocyte macrophage-colony stimulating factor (GM-CSF), recombinant mouse (Invitrogen, Germany)
- Hexanucleotide Mix (10x) (Roche, Germany)
- IFN-γ, recombinant mouse (R&D, Germany)
- Incomplete Freund's adjuvant (Difco, USA)
- Isopropanol (Sigma, Germany)
- Laminin, natural mouse (Invitrogen, Germany)
- Lysogeny broth (LB) agar and LB media (Fluka, Germany)
- Lipopolysaccharides (LPS ; Sigma, Germany)
- LookOut® Mycoplasma PCR Detection Kit (Sigma, Germany)
- MOG35-55 (MEVGWYRSPFSRVVHLYRNGK) (Charité, Berlin, Germany)
- Mycobacterium Tuberculosis H37Ra (List Biological Laboratories,USA)
- Normal goat serum (nGS) (Sigma, Germany)
- Neurotrophin3,recombinant (R&D, Germany)
- Okadaic acid potassium salt (Sigma, Germany)

- Opti-MEM (Gibco, Germany)
- PBS (1x) (Gibco, Germany)
- Pertussis toxin (List Biological Laboratories,USA)
- Puromycin (PAA, Germany)
- Poly-L-lysine (PLL) (Sigma, Germany)
- Reverse transcriptase hexamer (Roche, Germany)
- RNeasy Mini Kit (Qiagen, Germany)
- RNAse free DNAse Kit (Qiagen, Germany)
- SuperScript® III Platinum® Two-Step qRT-PCR Kit (Invitrogen, Germany)
- SYBR Green Master Mix (2x) (Applied Biosystems, UK)
- TritonX-100 (Sigma, Germany)
- Transferrin bovine (Sigma, Germany)
- Trypsin-EDTA 0.25 % (Gibco, Germany)

2.1.4. Vectors

- PLL- phosphoglycerate kinase (PGK) green fluorescent protein (GFP)-Neomycine-resistance (received from Dr. Isabella Napoli, Bonn, Germany)
- PLL-PGK NT3-Neomycine-resistance
- PLL3.7 vectors (provided by L. van Parijs, MIT, Cambridge, MA)
- Packaging plasmid PLP1 ViraPower ™ (Invitrogen, UK)
- Packaging plasmid PLP2 ViraPower ™ (Invitrogen, UK)
- Packaging plasmid PLP3 ViraPower ™ (Invitrogen, UK)
- TRC hairpin-pLKO.1-puro vector (Broad Institute TRC, Cambridge, USA/ Prof. Dr. Hornung, Institute of Clinical Chemistry and Pharmacology, Bonn, Germany)
- Mission pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA (Sigma, Germany)

2.1.5. Cell lines

ES-C57BL/6-ATCC BL6 (ATCC, Germany)
GL261 (Hertie-Institute for clinical neurology, Germany)
SMA560 (Hertie-Institute for clinical neurology, Germany)
Human Embryonic Kidney (HEK) 293 cells cells (Invitrogen, Germany)

2.1.6. Antibodies

Primary antibodies for immunocytochemistry

Antibody	Host	Dilution	Supplier
Arginase	goat	1:100	Santa Cruz Biotech, USA
β-tubulin-III	mouse	1:500	Sigma, Germany
CD16/32	rat	1:500	BD Biosciences, Germany
CD45	rat	1:500	BD Biosciences, Germany
CD68	rat	1:500	AbD Serotec, USA
CD86	rat	1:500	BD Biosciences, Germany
CD206	goat	1:100	R&D Systems, Germany
GFAP	rabbit	1:1000	DAKO, USA
Iba1	rabbit	1:500	Wako, Germany
iNOS	rabbit	1:500	BD Biosciences, Germany
Nestin	mouse	1:200	Millipore, Germany
NT3	rabbit	1:100	Santa Cruz, USA
Oct 4	mouse	1:250	Santa Cruz, USA
Sox2	rabbit	1:250	Abcam, USA
SSEA1	mouse	1:80	Abcam, USA
GAP43	rat	1:200	DAKO, USA

Secondary antibodies for immunocytochemistry

Antibody	Host	Directed against	Dilution	Supplier
Alexa488	goat	anti mouse	1:500	Invitrogen, Germany
Alexa488	goat	anti rabbit	1:500	Invitrogen, Germany
Alexa488	goat	anti rat	1:500	Invitrogen, Germany
Cy3	goat	anti mouse	1:500	Dianova, Germany
Cy3	goat	anti rabbit	1:500	Dianova, Germany
Cy3	goat	anti rat	1:500	Dianova, Germany

Primary antibodies for flow cytometry

Antibody	Host	Dilution	Supplier
CCR2	mouse	1:80	Abcam, USA
CD11b-biotinylated	rat	1:200	BD Biosciences, Germany
CD11c- biotinylated	hamster	1:200	BD Biosciences, Germany
CD16/32	rat	1:100	BD Biosciences, Germany
CD29	rat	1:200	BD Biosciences, Germany
CD34	rat	1:200	BD Biosciences, Germany
CD36- biotinylated	mouse	1:200	BD Biosciences, Germany
CD45- biotinylated	rat	1:200	BD Biosciences, Germany
CD49- biotinylated	rat	1:200	BD Biosciences, Germany
CD68- biotinylated	rat	1:50	AbD Serotec, Germany
CD80- biotinylated	rat	1:200	BD Biosciences, Germany
CD86- biotinylated	rat	1:200	BD Biosciences, Germany
CD115- biotinylated	sheep	1:40	R&D Systems, Germany
CD117/cKit- biotinylated	rat	1:200	BD Biosciences, Germany
CD133- biotinylated	rat	1:200	eBioscience, USA

CX3CR1	rabbit	1:200	ProSci Inc, USA
F4/80- biotinylated	rat	1:100	AbD Serotec, Germany
Ly6G- biotinylated	rat	1:50	Abcam, USA
MHC II- biotinylated	rat	1:200	BD Biosciences, Germany
SiglecH- biotinylated	rat	1:200	Hycult Biotech. Germany
Trem2	rat	1:200	BD Biosciences, Germany
Isotype IgG1	rat	1:200	BD Biosciences, Germany
Isotype IgG2	rat	1:200	BD Biosciences, Germany
Isotype IgA	mouse	1:200	BD Biosciences, Germany

Secondary antibodies for flow cytometry secondary antibodies

Antibody	**Host**	**Directed against**	**Dilution**	**Supplier**
PE	goat	Streptavidin	1:500	Jackson Immuno, USA
PE	goat	anti rat	1:500	Jackson Immuno, USA
PE	goat	anti rabbit	1:500	Jackson Immuno, USA

2.1.7. Other staining reagents

4',6-diamidino-2-phenylindole (DAPI) (Sigma, Germany)
PKH26, red fluorescent (Sigma, Germany)

2.1.8. Primer for qRT-PCR

Oligonucleotides obtained from Eurofins MWG

Name	Forward sequence	Reverse sequence
Arginase	CAG AAG AAT GGA AGA GTC AG	CAG ATA TGC AGG GAG TCA CC
CCL2	CAG GTC CCT GTC ATG CCT CT	GGG ATC ATC TTG CTG GTG AA
CX3CL1	TGG TGA TCC AGA TGC TTC AT	TGC TCA TCC GCT ATC AGC TA
CXCL9	CTT CCT GGA GCA GTG TGG AG	TGT TGC AAT TGG GGC TTG
CXCL10	CAT CCA CCG CTG AGA GAC AT	CTC AGA CCC AGC AGG ATG AG
Fizz	CTG GTG AGT TCG GGT CAG CTC TGT	CAG TAT TCA CTG GGA CCA TCA GCT G
GAPDH	ACA ACT TTG GCA TTG TGG AA	GAT GCA GGG ATG ATG TTC TG
IL-1β	ACA ACA AAA AAG CCT CGT GCT G	CCA TTG AGG TGG AGA GCT TTC A
IL-10	AAG GAC CAG CTG GAC AAC AT	TCT CAC CCA GGG AAT TCA AA
NOS2	AAG CCC CGC TAC TAC TCC AT	GCT TCA GGT TCC TGA TCC AA
NT3	AAA TAG TCA CAC GGA TGC CA	GGC AAA CTC CTT TGA TCC AT
SiglecH	GTG ACA GAC CTC ACT CAC AGC CC	GGT CGT GGG GCC CAG GGA TA
TGF-β	CAA TTC CTG GCG TTA CCT TG	GCT GAA TCG AAA GCC CTG TA
TNF-α	TCT TCT CAT TCC TGC TTG TGG	AGG GTC TGG GCC ATA GAA CT
Ym1	CAT TCA GTC AGT TAT CAG ATT CC	AGT GAG TAG CAG CCT TGG

2.1.9. Consumables

- 0.22 µm pore size filter stericup (Millipore, USA)
- 10 µl, 100 µl, 1000 µl pipette tips (Eppendorf, Germany)
- 10 ml syringes (Braun, Germany)
- 175 cm^2 tissue cell culture flask (Sarstedt, Germany)
- 5 ml disposable plastic pipette (Costar, Germany)
- 6-well-plate (Cellstar, Germany)
- 75 cm^2 tissue cell culture flask (Sarstedt, Germany)
- 8.0 µm Culture Plate Inserts 12 mm Diameter (Millipore, Germany)
- 10 ml disposable plastic pipette (Costar, Germany)
- 15 ml tubes (Greiner, Germany)
- 24-well-plate (Greiner bio-one, Germany)
- 48-well-plate (Greiner bio-one, Germany)
- 25 ml disposable plastic pipette (Costar, Germany)
- 50 ml plastic tubes (Sarstedt, Germany)
- 5 ml polystyrene round-bottom tubes (BD Falcon, Germany)
- Bottle top filters, 0.25µm pore (Milipore, Germany)
- Cell scraper (Sarstedt, Germany)
- Cell strainer (BD Falcon, Germany)
- Cotton buds (Roth, Germany)
- Chamber slides (Nunc, Germany)
- Cryovials (VWR, Germany)
- Erlenmeyer flask, 250 ml (Schott-Duran, Germany)
- Glass beads 5 mm (Roth, Germany)
- Glass cover slides 24x24 mm (VWR, Germany)
- Glass needles, pulled and polished, 50-100 µm (Hofheim, Germany)
- Glass pasteur pipettes (Brand, Germany)
- Injection needles (Braun, Germany)
- Lab – Tek Chamber Slide w/Cover Permanox Slide Sterile 4 Well (Labomedic, Germany)
- MicroAmp® 96- & 384-Well Optical Adhesive Film (Applied Biosystems, USA)
- MicroAmp optical 96 well plate (Applied Biosystems, USA)
- PCR tubes (Biozym Diagnostics, Germany)
- Petri dishes 100x15 mm (BD Falcon,Germany)
- Tissue culture dish 100x20 mm (Sarstedt, Germany)
- Tissue culture dish 60x15 mm (Sarstedt, Germany)
- Tissue culture dish 150x20 mm (TPP, Germany)
- Tissue Tek (Sakura, USA)
- Tubes 1.5 ml (Eppendorf, Germany)

2.1.10. Equipment

- Axioskop 2 (Zeiss, Germany)
- Axiovert 40 CFL (Zeiss, Germany)
- BD FacsCalibur (BD Bioscience)
- Biofuge Fresco (Heraeus, Germany)
- Canon Powershot G9 (Canon)
- Centrifuges Sorvall Discovery 90SE (Hitachi, Germany)
- Cryostat Microtom HM560 (Microm Int., Germany)
- Electrophoresis gel chambers (Biomed Analytik GmbH, Germany)
- Eppendorf Mastercycler epgradient S (Eppendorf Germany)
- Fluoview1000 Confocal microscope (Olympus,Germany)
- Freezer -80°C Herafreeze (Heraeus, Germany)
- Hamilton syringe (Hamilton, USA)
- Hot bead sterilizer (FST, Germany)
- Incubator HeraCell 240 (Heraeus Germany)
- Laminar air flow workbench Herasafe (Heraeus, Germany)
- Megafuge, 1.OR. (Heraeus, Germany)
- Microm HM560 cryostat (Microm, Germany)
- Neubauer chamber (Brand, Germany)
- Power supply (Amersham Bioscience, Germany)
- RS 2000 X-ray source (Rad Source Technologies, USA)
- Surgical instruments (F.S.T,Germany)
- Thermocycler T3 (Biometra, Germany)
- Vortex 2X (Velp Scientifica, Germany)

2.1.11. Software / Databases

- Openlab4.0.1 (Improvision, Germany)
- CorelDRAW Graphics Suite 11 (Corel, Germany)
- EndNote X1 (Thomson ISI ResearchSoft, USA)
- Microsoft Office (Microsoft, USA)
- Olympus FluoView1.4 (Olympus, Germany)
- SDS 2.2.2 (Applied Biosystems, USA)
- Cellquest Pro (BD Biosciences, USA)
- FlowJo 6.4.7 (Tree Star, USA)
- KaleidaGraph 4.0 (Synergy, USA)
- Graph Pad Prism 5, (Graph Pad Software, USA)
- Axiovision 4.6.3 (Carl Zeiss Imaging Solutions, Germany)
- ImageJ 1.39u (NIH, USA)
- NCBI gene bank

2.1.12. Anesthethics

- Ketamin (Ketanest, Pfizer, Germany)
- Xylazin (Rompun, Bayer, Germany)

Before perfusion and for immunization for EAE experiments, animals were anesthesized with Ketamin/Xylazin i.p. (Ketamin 80 mg/kg and Xylazin 16 mg/kg).

2.1.13. Mice

C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). All mice were kept and bred under pathogen free conditions in a 12 hour light/12 hour dark cycle with access to food and water *ad libitum* in the animal facility.

2.2. Methods

2.2.1. Generation of embryonic stem cell lines

For generation of ESC lines, C57BL/6 mice were used. Females were superovulated with i.p. injection of 5 international units (IU) of pregnant mare serum gonadotrophin (PMSG) at noon and 5 IU of human Chorionic Gonadotropin (hCG) 46 hours later. Breeding pairs were set up and plug-positive females were isolated. Females were sacrificed at day 3.5 post coitus and uteri were flushed with PBS to isolate blastocysts. Blastocysts were seeded to irradiated mouse embryonic fibroblast (MEF) cells in 48-well plates in 2i medium mixed 1:1 with ES medium. Two days later, half of the medium was replaced. 5 days post isolation, cells were trypsinized and transferred to a 24-well plate with MEF layer. Medium was replaced every day. After 10 to 12 days, primary ESC colonies were frozen down and expanded.

2.2.2. Generation of embryonic stem cell derived microglia

The murine ESC line ES-C57BL/6-ATCC (BL6) was used for the differentiation into ESdM. The differentiation protocol was obtained by modifications of a neuronal differentiation method (Lee *et al.*, 2000). The protocol consists of five steps:
(i) cultivation of ESC, (ii) formation and differentiation of embryoid bodies (EBs), (iii) differentiation into neuroectodermal lineage and isolation of myeloid precursor cells, (iv) differentiation into microglial precursor cells and (v) cultivation of ESdM.
Undifferentiated ESC were kept in culture in presence of LIF containing ES medium on a MEF layer. They were trypsinized and cultured on gelantin-coated tissue culture dishes for one day in ES medium with LIF. For EB formation, cells were dissosciated and plated onto bacterial culture dishes in Differentiation medium. After 4 days, the resulting EBs were plated to gelatin coated cell culture dishes. Two days later, selection of nestin-positive cells was initiated using ITSFn medium. After 6 days, cells were expanded in N2 medium containing 1 μg/ml laminin and 5 ng/ml bFGF. Microglial differentiation was triggered by removal of bFGF after 6 days. Laminin was withdrawn after 6 additional days. Three weeks after growth factor withdrawal, ESdM could be isolated by manual picking.

2.2.3. Maintenance and subpolarization of cell lines

ESdM were kept in N2 medium in 5% CO_2 at 37°C. Upon 80 % confluency, cells were splitted with a cell scraper. ESdM could be frozen slowly in N2-medium supplemented with 10 % DMSO and 50 % FCS.

For subpolarization of ESdM, cells were treated with 100 U/ml IFN-γ and 5 ng/ml LPS for 24 hours for M1 or 20 ng/ml IL-4 for 48 hours for M2 subpolarization.

SMA560 and GL261 glioma cells were kept in glioma medium at 37°C and 5% CO2. Upon confluency, cultures were splitted using Trypsin.

2.2.4. Isolation of primary neurons and regeneration assay

Primary neurons were isolated from 15 day old C57BL/6J mice embryos (Gorlovoy *et al.*, 2009). They were cultured in neuronal medium in PLL coated chamber slides at 37°C and 5% CO2. For the regeneration assay, medium was supplemented with different amounts of ESdM culture supernatant, PBS or 10 ng/ml recombinant NT3 and incubated for 48 h. Immunocytochemistry was carried out with antibodies directed against growth associated protein (GAP) 43 and Iba1.

2.2.5. Isolation of primary microglia

Primary microglia were obtained from brains of postnatal (day 3 or 4) C57BL/6 mice (Takahashi *et al.*, 2005). Briefly, meninges were removed and dissociated by trituration. Cells were cultured in basal medium for 2 weeks to form a confluent mixed glial monolayer. To isolate microglial cells, cultures were shaken on a rotary shaker for 3 h at 350 rpm. Detached microglia were seeded on PLL coated culture dishes in N2 medium.

2.2.6. Lentiviral transduction of cells

For lentiviral transduction of ESdM (GFP and NT3) and SMA560 cells (GFP), PLL3.7 vectors were used. Vectors contained a neomycine-resistance gene for selection of successfully transduced cells. GFP and NT3 were cloned under control of a pgk-promoter in the backbone. Calcium-phosphate precipitates were prepared with 25 µg pLP1, 12.5 µg pLP2 and 15 µg pLP3 (Invitrogen) and 25 µg lentiviral vector plasmid of interest in 1.125 ml water and 125 µl $CaCl_2$ (2.5 M). Precipitates were then applied to HEK293FT cells in

MEF medium (5% FCS). After 48 and 72 h, the supernatant was collected, filtered and transferred to target cells. Two days post transduction, cells were selected using 250 µg/ml G418 for GFP and NT3 vectors and with 20 µg/ml Puromycin for shRNA knock-down vectors.

The following plasmids were used for generating knockdown ESdM:

siglech: TRCN0000068083, Target sequence: 5'-GCCCAAATTAACATTAGAGAA-3'

trem2: TRCN0000068094 Target sequence: 5'-GTCATGTACTTATGACGCCTT-3'

Knock-down plasmids for sialic acid binding immunoglobulin -like lectin H (SiglecH) and Trem2 were generous gifts from Prof. Dr. Veit Hornung, University of Bonn. Non-target shRNA Control Plasmid (Sigma, Germany) was used as control for the knock-down experiments.

2.2.7. Immunocytochemistry of cultured cells

For immunocytochemistry, cells were fixed with 4% PFA for 10 min, blocked with 10x BSA containing 5% nGS (for 30 min and stained with various antibodies (see 2.1.6) overnight.

Secondary antibodies were applied for 30 min, cells were washed with PBS and finally stained with DAPI for 30 s and covered with Moviol. Images were taken with a fluorescence microscope (Axioskop2, Zeiss) or a confocal microscope (Fluoview FV1000, Olympus).

2.2.8. Flow cytometry analysis

ESdM were collected using a cell scraper. Fc-receptor blockade was performed with a rat monoclonal antibody against CD16/CD32. Cells were stained with various antibodies (see 2.1.6) for 60 min on ice. After washing with PBS, samples were incubated with secondary antibody for 30 min on ice in darkness. Isotype-matched controls and non-stained cells were used as negative controls. Analysis was performed with a FACSCalibur flow cytometer and FlowJo Software.

2.2.9. Analysis of cytokine gene transcripts by qRT-PCR

RNA was isolated according to the manual using the RNeasy Mini Kit. Afterwards, reverse transcription was performed with SuperScript III reverse transcriptase and hexamer random primers. For quantitative real time (RT)-PCR, 600 ng of cDNA, SYBR Green PCR Master Mix and specific oligonucleotides were used according to the following conditions:

Component	**Amount per reaction**	**Final**
SYBR green mix (2x)	12.5 µl	1 x
cDNA (200 ng/µl)	3 µl	600 ng
Primer (10 pmol/µl)	1 µl each	0.4 pmol/µl
Water	Ad 25 µl	

The quantitative RT-PCR was carried out using the Eppendorf epgradient S Mastercycler with the following programme:

Cycle number	**Denature**	**Anneal**	**Extend**
1	95°C for 10 min	-	-
2-40	95°C for 10 s	60°C for 30 s	72°C for 30 s

Amplification specificity was confirmed via analysis of melting curves. Quantification was performed using the deltadelta-CT method.

2.2.10. RNA Microarray

For RNA microarray, RNA was isolated according to the manual using the RNeasy Mini Kit from Qiagen. 1 mg of RNA of three ESdM or primary microglia cultures was used. Microarray analysis was performed at the LIMES institute, Bonn, Germany by the working group of A. Staratscheck-Jox using the Ambion Illumina system.

2.2.11. Phagocytosis assay

For analysis of phagocytosis capacity of the ESdM, a phagocytosis assay with labeled microsphere beads was performed. $1x10^5$ cells were seeded to each well of a 6-well-plate in N2-medium. For stimulation of the cells, 500 ng/ml LPS were added for 24 h.

Afterwards, 10 µl microsphere beads were added to each well for 1.5 h, washed throughoutly with PBS and analyzed by flow cytometry.

2.2.12. Chemotaxis assay

For the chemotaxis assay, $1x10^5$ cells were transferred into culture plate inserts in an 24-well-plate in N2-medium and incubated for 1 h at 37°C, 5 % CO_2.
Concentrations ranging from 0-20 ng/ml CX3CL1 were added into the well. After 3-4 h, ESdM migrated to the back side of the membrane were counted.

2.2.13. Co-culture of ESdM and glioma cells

For phagocytosis experiments, $1x10^6$ GL261 or SMA560 cells were stained with 2 µl PKH26 in 198 µl Solution C for 5 min at 37°C. $1x10^5$ cells per well of GFP-ESdM or knockdown cells were co-seeded with SMA560 or GL261 in N2 in chamber slides and cultivated for 24 hours. Monocultures of SMA560 and GL261 were used as controls. Slides were fixed with 4 % PFA for 15 min and covered with Moviol. For each well, 5 randomly chosen areas were analyzed using a confocal laser scanning microscope. Number of total cells and of cells with internalized PKH26+ particles was assessed.
For proliferation assay, $1x10^5$ ESdM and GFP-SMA or GFP-GL261 were co-seeded into 6-well dishes. For 5 consecutive days, cell number was counted and GFP+ cells were assessed using a fluorescence microscope.

2.2.14. Annexin V staining

Annexin V staining was performed after co-culture of ESdM with GFP-SMA560 or GFP-GL261 in chamber slides. As a positive control, glioma cells were treated with 100 nmol/ml okadaic acid for 24 hours. Cells were washed once with PBS and incubated with 3 µl biotinylated Annexin V in 200 µl annexin binding buffer for 15 min. After washing with PBS, cells were incubated with Streptavidin-Cy3 and analyzed via fluorescence microscopy.

2.2.15. Induction of experimental autoimmune encephalomyelitis

To prepare the reagent for immunization, 4 mg/ml MOG were diluted to 2 mg/ml in PBS. This mixture was then brought into emulsion with the same volume of complete Freuds Adjuvant (10 mg/ml *M. tuberculosis* in incomplete Freuds Adjuvant) using two syringes and a stopcock. The emulsion was incubated overnight at 4°C.

Female C57BL/6J mice six weeks of age were used for the EAE experiments. 100 µl of activated MOG-emulsion were injected at each inguinal lymph node region at both sides of the anaesthetised animal. 100 µl of Pertussis toxin (5 ng/µl) were injected intraperitoneally at day of induction and 48 h later.

2.2.16. Monitoring of experimental autoimmune encephalomyelitis afflicted animals

After immunization, animal weight and clinical score were controlled on a daily basis. Clinical score was determined according to the following chart:

Clinical score	Symptoms
0	no clinical symptoms
1	limp tail
2	complete limp tail and weakness of hindlimbs
3	paraparesis of at least one hind limb
4	complete hind limbs paraparesis and weakness of forelimbs
5	moribund state or tetraplegia

At the peak of the disease (clinical score of 3, around day 19 post immunization), mice were injected with PBS, GFP-transduced ESdM or NT3-GFP-transduced cells. For cell treatment, 4 million cells in 100 µl PBS were administered intravenously. Mice displaying a clinical score of at least four for a minimum of three days were euthanized and were not taken into account for analysis.

2.2.17. Perfusion of animals

Before perfusion, mice were anesthetized with Ketamin/Xylazin and checked for pain reflexes. Mice were transcardially perfused with 20 ml PBS followed by 20 ml 4% PFA. Spinal cords and brains were dissected and stored in 4% PFA. Neurohistopathology was

carried out by the working group of Prof. Dr. Marco Prinz in Freiburg as described previously (Prinz *et al.*, 2006).

2.2.18. Preparation of cryoslices

For analysis of tissue, cryoslices were prepared. Organs obtained by perfusion of the animals were kept in PFA for 48 h and another 48 h in 30% sucrose solution. Before slicing, tissue was embedded in Tissue Tek and frozen. The frozen tissue was cut into 10 to 20 µm thick slices on super frost slides with a cryostat and stored at -20°C.

2.2.19. Immunohistochemistry of cryoslices

Cryoslices were fixed in 4% PFA immediately after thawing. They were washed with TRIS-buffer and blocked with 10% BSA for 10 min. After washing with TRIS, they were incubated with the first antibody in tris(hydroxymethyl)aminomethane (TRIS) overnight. The slices were stained with fluorescent secondary antibody for 2 h and with DAPI for 5 min. Slides were covered with Moviol.

Assessment of immune cell infiltrates was performed by the working group of Prof. Marco Prinz in Freiburg as described previously (Prinz *et al.* 2006).

2.2.20. Statistics

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

3. Results

3.1. Generation of embryonic stem cell lines

In the first step, ESC lines were generated out of blastocysts obtained from C57BL/6 mice. After extracting the blastocysts out of the uterus horns at day 3.5, they were seeded to a MEF cell layer. After 3 days, the blastocysts hatched and inner cell mass (ICM) expanded during the next days. First trypzination was performed at day 8, the next day ESC colonies formed and primary ESC lines were established (Fig.3.1).

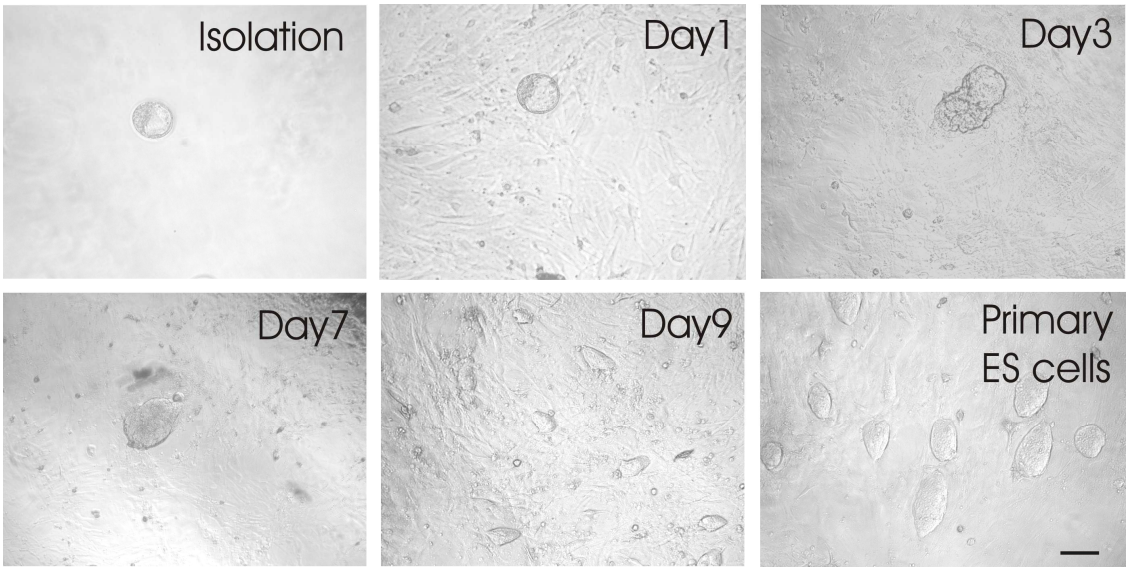

Fig. 3.1: Isolated blastocysts were seeded to a MEF cell layer (Day 1). After a few days, the trophoblast spred out (Day 3), and ICM expanded (Day 7). Primary ESC colonies formed after first trypzination (Day 9). Scale bar 50 μm.

For confirmation of ESC properties, the generated ESC lines were stained with antibodies direct against the stem cell markers Sox2, Oct4 and stage-specific embryonic antigen 1 (SSEA1). Furthermore, ESCs were stained against alkaline phosphatase (AP). The generated ESC lines were positive for all tested markers, therefore were considered to be *bona fide* ESCs (Fig. 3.2).

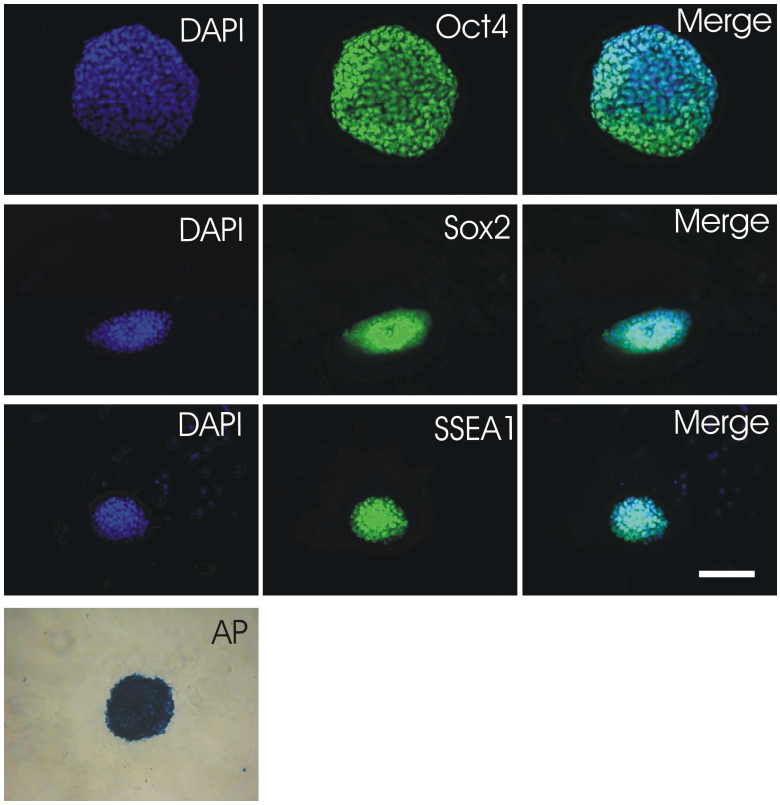

Fig.3.2: Immunocytochemistry of the generated ESC lines using antibodies against stem cell markers Oct4, Sox2 and SSEA1. ESC colonies were costained with DAPI and stained positive for the markers. Colonies were also positive for AP. Scale bar 100 µm.

3.2. Characterization of embryonic stem cell derived microglia

3.2.1. Differentiation of mouse embryonic stem cells into ESdM

By modification of a protocol for neuronal differentiation (Lee *et al.*, 2000) an efficient protocol for microglia precursor cell line differentiation from murine ESCs was established (Napoli *et al.*, 2009; Beutner *et al.*, 2010).

C57BL/6 ESCs were cultured in LIF containing medium on a MEF layer, where they formed typical cellular colonies (Fig. 3.3-A). After dissociation of the ESCs, EB formation was induced with withdrawal of LIF on non-coated petri dishes (Fig. 3.3-B). EBs can be recognized as round cell aggregates which are non-adherent. EBs were plated after 4 days and expanded in ITSFn medium (Fig. 3.3-C). The EBs attached and cells

started to grow out of the plated EBs. In this adherent culture, a high number of nestin positive cells was found (Fig.3.4-A) and expanded in presence of bFGF and laminin. After 7 days, growth factors were withdrawn and cells differentiated into neuronal precursor cells forming a neuronal network.

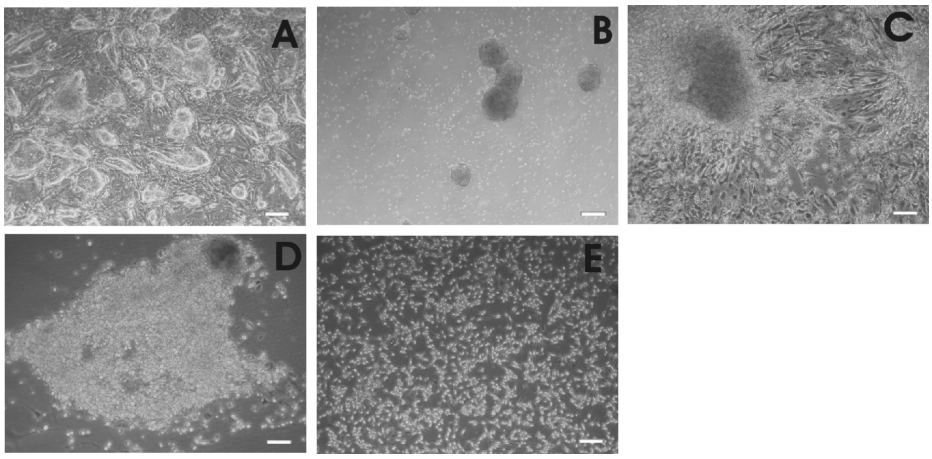

Fig. 3.3: Brightfield pictures throughout the differentiation protocol. A) ESCs on MEF layer. B) EB formation. C) Mixed neuronal culture. D) Microglial precursor culture. E) ESdM in culture. Scale bar 200 µm (A), 100 µm (B-E). Figure derived from Beutner *et al.*, 2010.

In this stage, β-tubulin-III positive cells were found (Fig. 3.4-B) as well as microglia-shaped cells which could be stained for Iba1 (Fig. 3.4-B). Furthermore, cells positive for miroglia marker CD45 and the astrocyte marker glial fibrillar acidic protein (GFAP) were found in this mixed culture, but staining showed no colocalization of these markers (Fig. 3.4-C). Microglia-shaped cells appeared as shiny round cells and started to proliferate in clusters (Fig.3.3-D), outgrowing other cells after three weeks post growth factor-withdrawal. After the last step, the differentiation protocol resulted in a pure ESdM culture (Fig. 3.3-E) which could be stained for Iba1 and CD68 (Fig. 3.4-D).

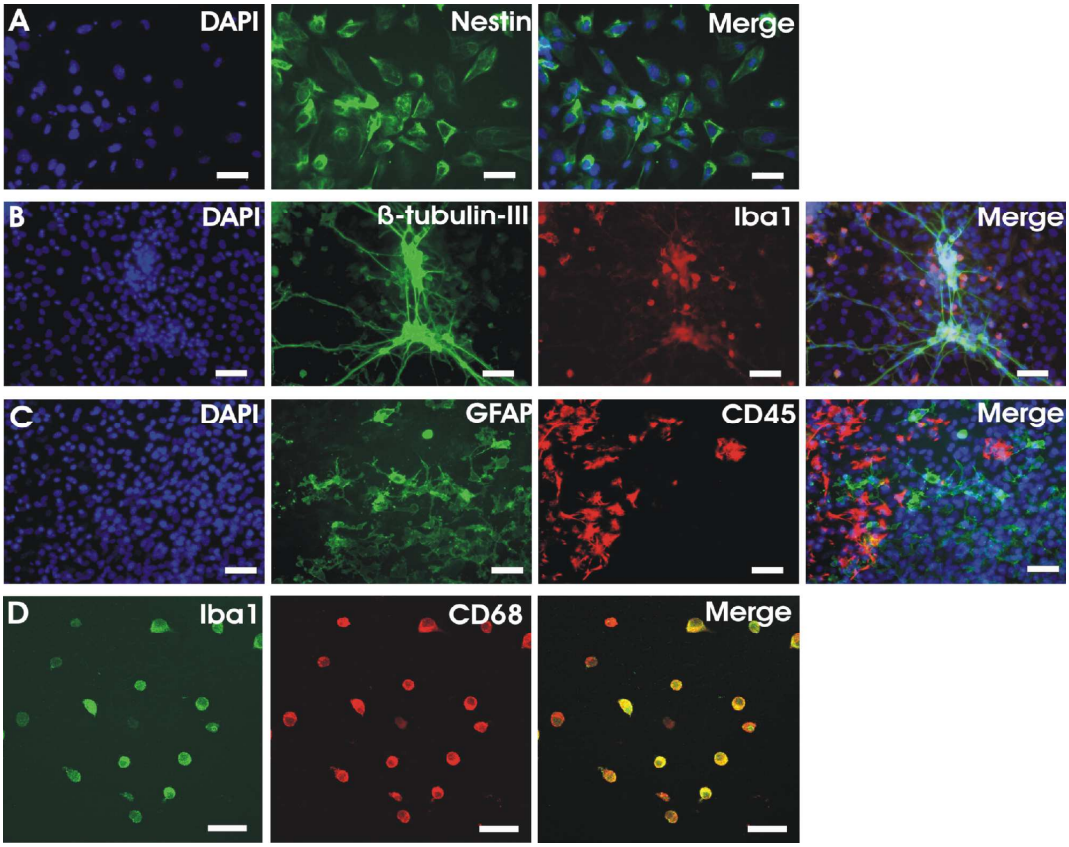

Fig. 3.4: Immunocytochemistry throughout the differentiation protocol. A) Neural precursor cells at the third step (day 13) of the protocol. Cells were positive for nestin and nuclei were labeled with DAPI. B) Cells in a mixed neural culture, stained against Iba1 for microglia-like cells and β-tubulin-III for neuronal cells. C) Double immunostaining of astrocyte marker GFAP and microglia marker CD45 at step 3 of the protocol. No co-localization was visible. D) ESdM immunostained against Iba1 were also positive for CD68. Scale bar 20 µm (A-C), 50 µm (D). Figure derived from Beutner *et al.*, 2010.

3.2.2. Surface molecule expression of ESdM

To define the properties of the obtained ESdM, different experiments were carried out. As a variety of antibodies against microglial surface markers can be purchased, the ESdM were analyzed via flow cytometry to confirm the microglial identity with a variety of different antibodies.

The result of the flow cytometry is summarized in figure 3.5. ESdM showed a high expression level of the microglial surface molecules CD11b, CD11c, CD29, CD45, CD49, CD68, CD80**,** CD86 and CD115. Furthermore, expression of Trem2, F4/80, MHCII and CX3CR1 could be shown. Interestingly, ESdM did not express Ly6G and

chemokine (C-C motif) receptor 2 (CCR2). Stem cell markers like CD34, CD133 and cKit were undetectable or only weakly expressed. Lack of these stem cell population markers indicates that the ESdM are fully committed to differentiation.

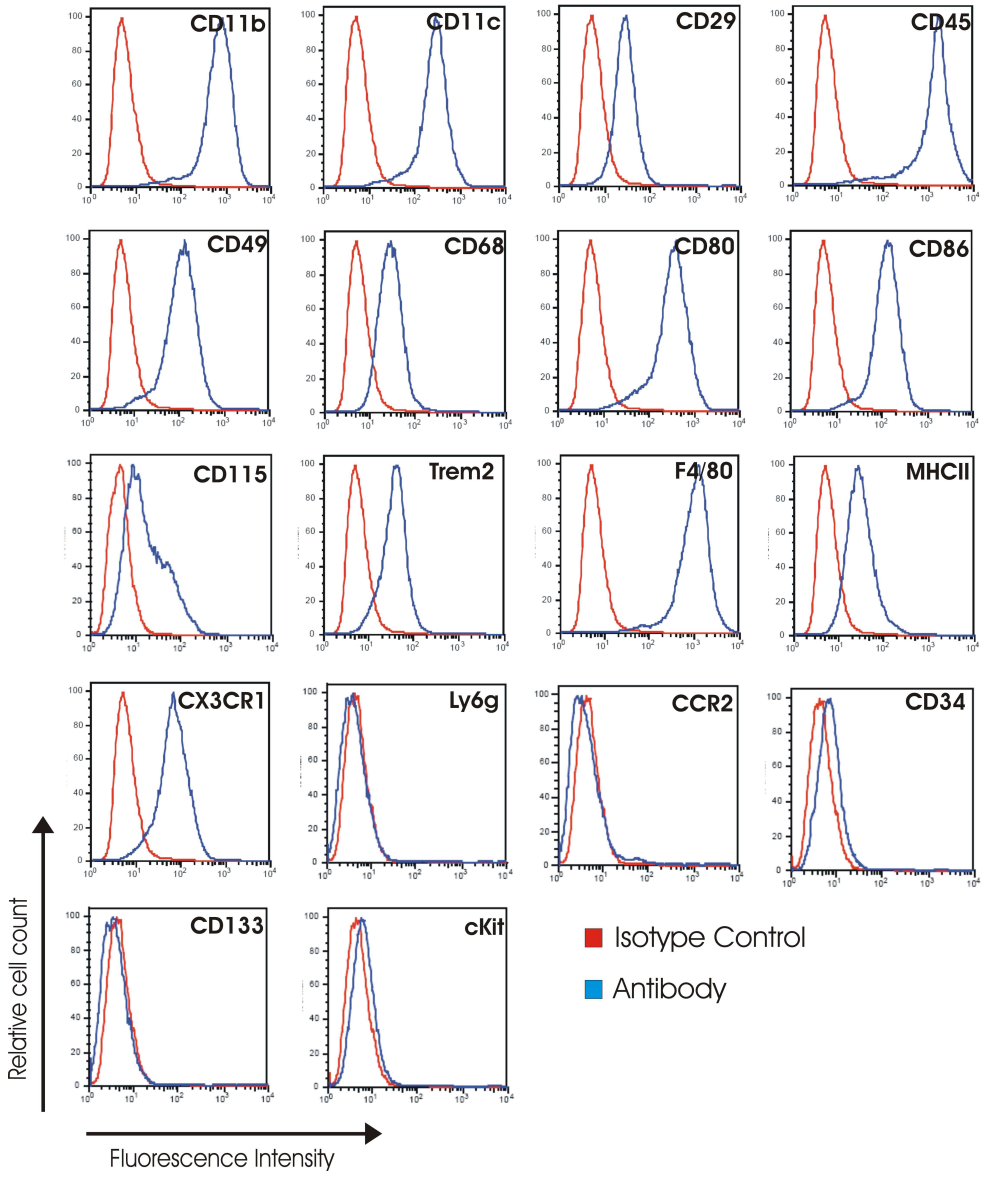

Fig. 3.5: Flow cytometry analysis of ESdM surface markers. Microglial surface markers CD11b, CD11c, CD29, CD45, CD49, CD68, CD80**,** CD86, CD115, Trem2, F4/80, MHCII and CX3CR1 were expressed. ESdM did not express Ly6G and CCR2. Stem cell markers such as CD34, CD133 and cKit were barely or not expressed. Figure modified from Beutner *et al.*, 2010.

3.2.3. Relative cytokine gene transcription of ESdM

One of the most typical features of microglia is the production of pro- or anti-inflammatory cytokines and reactive oxygen species (ROS) upon pathological conditions. Therefore, gene transcript levels of nitric oxide synthase (NOS) 2, TNF-α and TGF-β of ESdM after stimulation for 24 hours with 500 ng/ml LPS or 500 U/ml IFN-γ were analyzed via qRT-PCR (Fig. 3.6-A). Upon treatment with IFN-γ, gene transcripts of NOS2 showed a high increase (1000-fold), $p< 0.005$; whereas TNF-α transcripts were increased 20-fold, $p<0.01$. Treatment with LPS led to a similar, though weaker increase of NOS2 and TNF-α gene transcripts. TGF-β transcripts were almost unaffected by both treatments.

3.2.4. Migration towards fractalkine

Microglia are known to have chemotactical migration properties. Thus, a migration assay using fractalkine CX3CL1 was performed (see Fig. 3.6-B). The relative migration of ESdM in comparison to non-directed migration increased in a dose-dependent manner up to 20 ng/ml, where migration extended to almost 200% of basal migrationl level ($p<0.005$). As a control, 20 ng/ml CX3CL1 were added to both the upper and lower chamber to analyze to which extent the ESdM show undirected migration. This treatment did not lead to an increase in migration.

3.2.5. Phagocytosis assay

Another important functional aspect of primary microglia is the capacity of phagocytosis upon activation. This aspect of microglia function can be mimicked by an *in vitro* phagocytosis assay. The outcome of the phagocytosis assay is shown in figure 3.6-C. Upon stimuli with LPS, the percentage of ESdM showing uptake of two or more microsphere beads was increased from 20.9% for untreated cells to 31.4% for LPS treated cells.

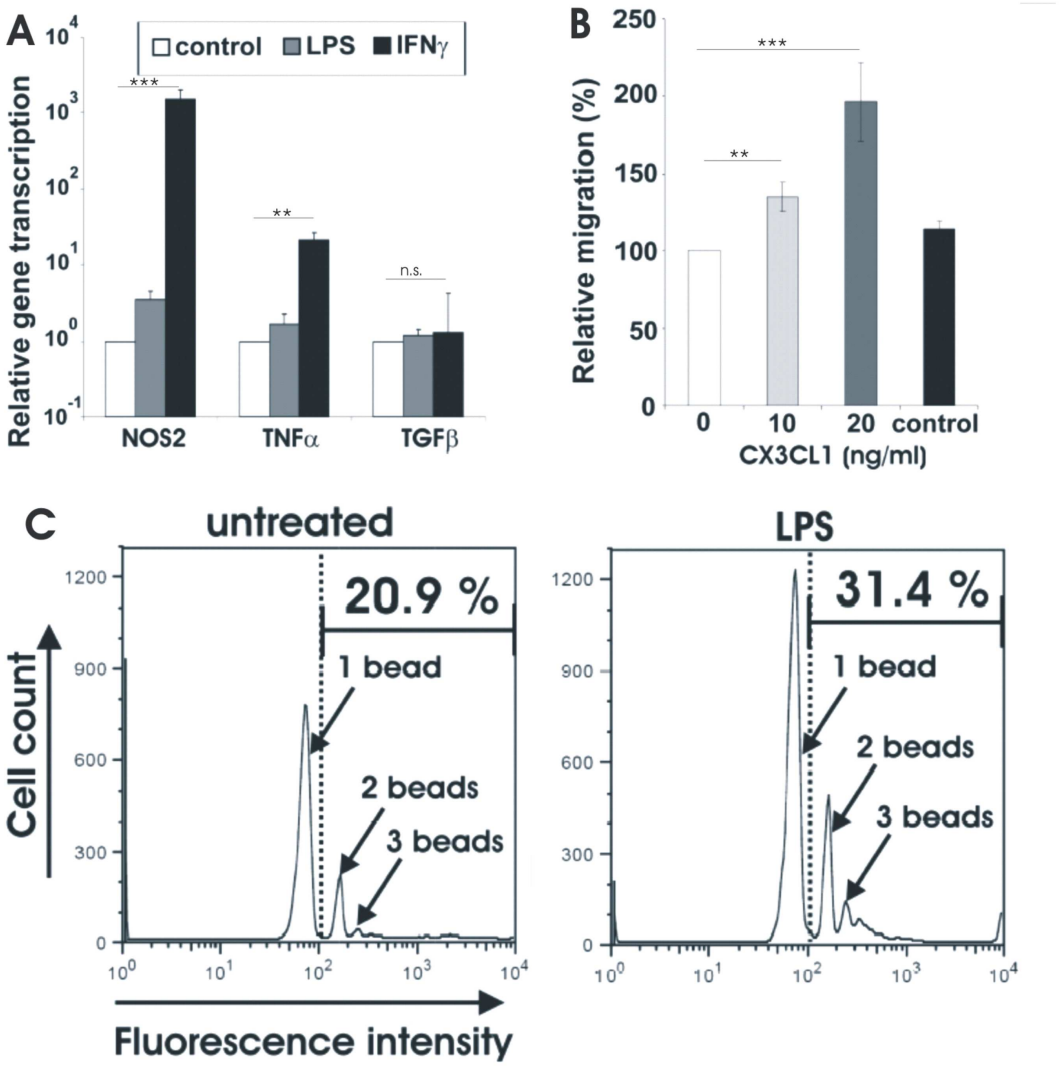

Fig. 3.6: Functional analysis of ESdM. A) Relative cytokine gene transcription of ESdM upon treatment with IFN-γ (500 U/ml) or LPS (500 ng/ml) for 24 hours. Treatment increased transcription of NOS2 and TNF-α, transcription of TGF-β was almost unaffected. Data is presented as mean +SEM, ANOVA ***p<0.005, **p<0.01. B) Migration assay of ESdM towards CX3CL1. ESdM migrated in a dose-dependent manner towards the CX3CL1. Data is presented as mean +SEM, ANOVA: ***p< 0.005; **p<0.01. C) Phagocytosis assay, number of beads taken up was determined by fluorescence intensity. Phagocytic inactive cells are not shown in the histogram. Upon stimulus with 500 ng/ml LPS the uptake of microspheres by ESdM increased. Figure published in Beutner *et al.*, 2010.

3.2.6. RNA microarray of ESdM and primary microglia

To confirm the identity of the ESdM as microglia cells, a RNA microarray was performed to compare the gene expression of primary microglia to ESdM by the lab of Andrea Staratscheck-Jox at the LIMES institute, Bonn, Germany.

Out of 44 000 analyzed genes, about 20 000 genes were found to be expressed in ESdM and primary microglia. Of the genes expressed, over 19 000 were coexpressed by ESdM and primary microglia in a similar dimensions. Only about 1 000 of the expressed genes were not found in both samples and were therefore expressed selectively.

Table 3.1 lists a selection of genes assigned to different cell types. Microglial genes like Trem2, CD206 or MHCI were expressed in a high amount in both ESdM and primary microglia. However, genes assigned to astrocytes like GFAP or neurons like Nestin are only weakly expressed. The same holds true for ESC genes like Sox2 and Myc. These results indicate that ESdM are fully commited to differentiation and share a similar genetic profile with primary microglia.

Tab. 3.1: Selection of genes expressed by ESdM and primary microglia (pMG). Microglial genes like Trem2 or CD206 are expressed by both ESdM and primary microglia in a comparable high amount. Genes assigned to astrocytes, neurons or ESCs were barely expressed.

Gene	Entrez Gene #	ESdM	pMG
Microglia genes			
Mrc1 or CD206 mannose receptor	17533	4107,7	2041,6
H2-Q2 (MHCI) histocompatibility 2	15013	1657,8	1821,8
Cd63 antigen	12512	3462,6	4978,9
Tnf, tumor necrosis factor	21926	298,9	1054,2
Trem2, triggering receptor expressed on myeloid cells 2	83433	5232,8	10981,9
Siglec1, sialic acid binding Ig-like lectin 1	20612	149,3	1134,0
Astrocyte genes			
Gfap, glial fibrillary acidic protein	14580	80,0	78,6
Bmp2, bone morphogenetic protein 2	12156	83,9	86,6
Olig2, oligodendrocyte TF 2	50913	79,3	75,5
Neuron genes			
Nefm, neurofilament	18040	85,0	82,4
Tubb3, tubulin, beta 3	22152	90,1	87,1
Nes, nestin	18008	79,0	191,6
ESC genes			
Nanogpd,similar to Nanog homeobox	634428	78,6	73,5
Sox2,SRY-box containing gene 2	20674	74,8	77,6
Myc, myelocytomatosis oncogene	17869	66,1	72,4

3.2.7. Subdifferentiation of ESdM into neurotoxic and neuroprotective subtypes

For subdifferentiation of ESdM, cells were treated with 100 U/ml IFN-γ and 5 ng/ml LPS or with 20 ng/ml IL-4 for 24 hours, respectively. After stimulation, RNA was isolated and qRT-PCR was performed for several marker genes assigned to M1 or M2 microglia subtypes (see Fig. 3.7).

ESdM treated with IFN-γ and LPS showed a significant upregulation of genes assigned to the M1 microglia subtype like iNOS, CXCL10 and TNF-α.

On the other hand, treatment of ESdM with IL-4 led to an increase in genes assigned to the M2 subtype, among these are arginase I (Arg), IL-10, found in inflammatory zone 1 (Fizz1) and YM1 (Mantovani *et al.*, 2004). Except for Fizz1, the upregulation was specific for IL-4 treatment.

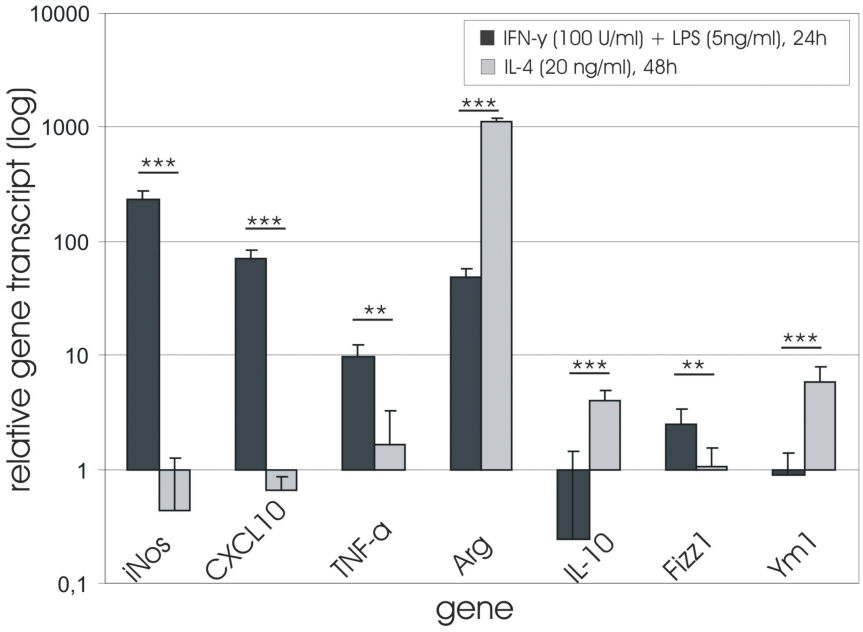

Fig. 3.7: Results of qRT-PCR for genes assigned to M1 or M2 subtype after treatment of ESdM with 100 U/ml IFN-γ and 5 ng/ml LPS or 20 ng/ml IL-4. Treatment with IFN-γ and LPS led to an upregulation of genes from the M1 subtype, namely iNOS, CXCL10 and TNF-α. Upon stimulus with IL-4, ESdM showed higher expression of anti-inflammatory genes assigned to the M2 subtype such as Arg, IL-10, Fizz1, and YM1. Data are presented as mean + SEM and analyzed using one way anova followed by Bonferroni; ***p<0,005, **p<0,01.

For immunocytochemistry, cells were treated for 24 hours with IFN-γ and LPS or for 48 hours with IL-4. The subdifferentiated cells were immunolabelled with antibodies directed against surface markers of M1 and M2 microglia subtypes (Kigerl *et al.*, 2009); (see Fig. 3.8). The treatment with IFN-γ and LPS led to a more intense staining for M1 subtype markers CD86, iNOS and CD16/32. On the other hand, treatment with IL-4 resulted in a slight increase of fluorescence for Arg and CD206, markers assigned to the neuroprotective subtype of microglia.

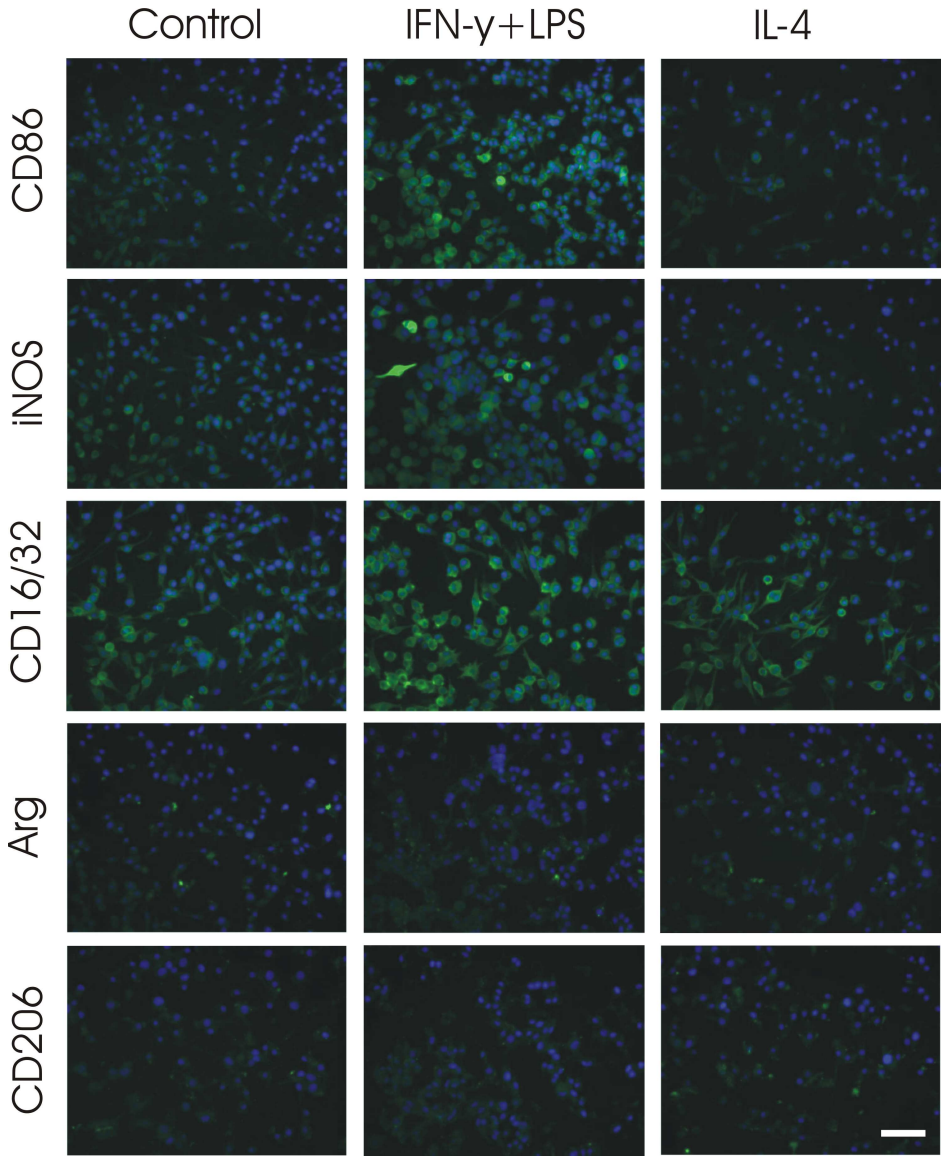

Fig. 3.8: Immunocytochemistry for antibodies directed against surface markers of M1 and M2 subtype. IFN-γ + LPS-treatment resulted in an increase in expression of CD86, iNOS and CD16/32. Treatment with IL-4 led to an increase in expression of Arginase I and CD206. Scale bar 100 µm. Diploma student Celine Ruegsegger contributed to this figure.

3.3. Therapy of experimental autoimmune encephalomyelitis by NT3-GFP-ESdM

3.3.1. Lentiviral transduction of ESdM

For experiments performed to determine the effect of ESdM on EAE afflicted mice, ESdM were transduced with GFP or GFP and NT3 using a lentiviral vector system. NT3-GFP-ESdM cells stained positive for NT3 (Fig. 3.9-A). The constitutive expression of NT3 was also confirmed by qRT-PCR (Fig 3.9-B).

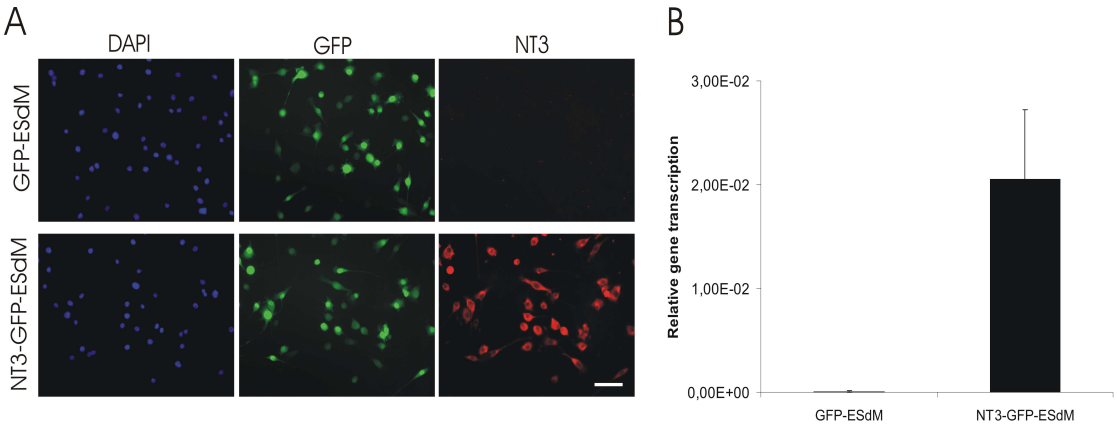

Fig.3.9: A) Immunocytochemistry of NT3-GFP-ESdM in comparison to control GFP-ESdM using an antibody against NT3. Transduced cells showed a strong signal, whereas the control cells did not stain for NT3. Scale bar: 50µm. B) Relative gene transcription of NT3 analyzed via qRT-PCR.

3.3.2. Effects of NT3-GFP-ESdM supernatant on neurons *in vitro*

In order to analyze the beneficial effect of NT3 expressed by ESdM on neurons, primary neuronal cultures were treated with either water, GFP-ESdM supernatant, NT3-GFP-ESdM supernatant or recombinant NT3 (10ng/ml) for 48 hours (see Fig. 3.10). In comparison to supernatant of GFP-ESdM, supernatant of NT3-GFP-ESdMs strongly promoted neurite outgrowth demonstrated by an increased immunoreactivity for GAP43 (Fig.3.10-A). Furthermore, neurons cultured in presence of recombinant NT3 or NT3-GFP-ESdM supernatant appeared to be more viable and showed more axons than those treated with GFP-ESdM or water. Quantification of GAP43+ neurites showed that in water control 3.2% +/- 2.2% of neurites stained positive, treatment with GFP-ESdM resulted in 19.1% +/- 5.0% of GAP43+ neurites. Supernatant of NT3-GFP-ESdM

resulted in 82.2% +/- 3.6%, the positive control of recombinant NT3 led to 90.5% +/- 6.5% of GAP43+ neurites (Fig. 3.10-B).

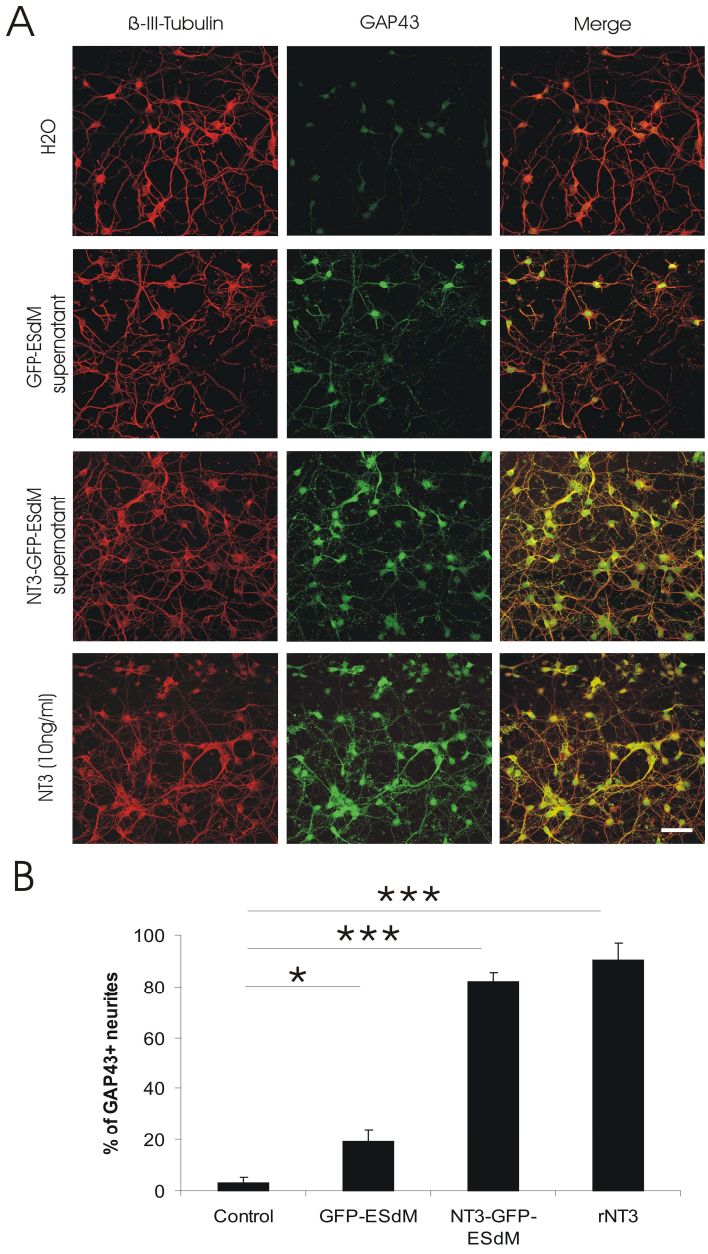

Fig. 3.10: A) Neurons cultured in medium supplemented with water, GFP-ESdM supernatant, NT3-GFP-ESdM supernatant or recombinant NT3 stained for β-III-Tubulin and GAP43. NT3-GFP-ESdM supernatant resulted in an increase in GAP43 staining, indicating neurite growth. Scale bar 100 µm. B) Quantification of GAP43+ neurites. Statistical analysis via ANOVA followed by Bonferroni, *p<0.05; ***p<0.0001.

3.3.3. Migration and engraftment of ESdM into inflammatory spinal cord lesions

EAE was induced by injection of activated MOG-emulsion to both inguinal lymph node regions to female C57BL/6J mice. After about 12 days, the mice displayed the first symptoms of disease, starting with a limp tail. For all experiments, only animals showing symptoms at day 14 and a clinical score of 3 at day 19 were included.

At a clinical score of 3 (20 days post induction), 4 million GFP-ESdM were injected intravenously to investigate the fate of the ESdM. After 24 hours, a small number of GFP positive cells could be found in the lumbal part of the spinal cord. The GFP positive cells were located in close vicinity to the inflammatory lesions of the spinal cord. The number of GFP-ESdM increased over time, indicating a stable engraftment of ESdM in the tissue (Fig. 3.11-B). Furthermore, the GFP-signal of the ESdM colocalized with Iba1-staining, showing that the ESdM engrafted as microglial cells in the nervous tissue. (Fig. 3.11-A).

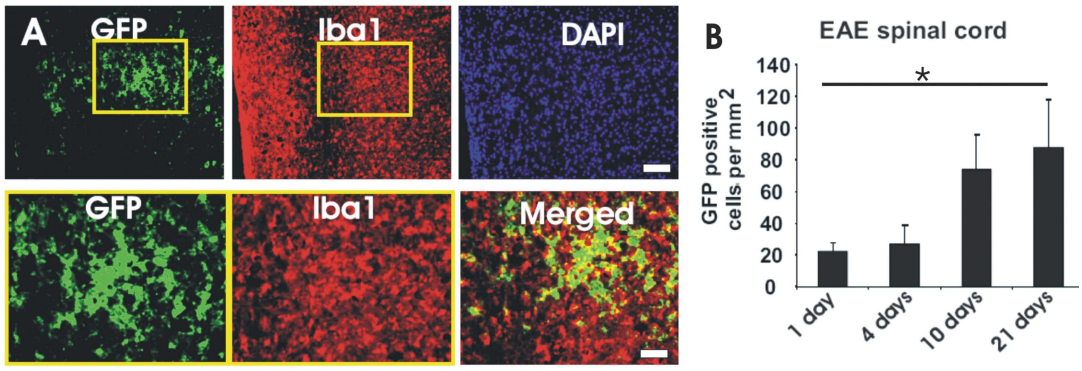

Fig. 3.11: A) Longitudinal cryosections of spinal cord from EAE afflicted mouse, 10 days post injection of GFP-ESdM, stained for Iba1 and DAPI, scale bar 50 µm. B) Quantification of GFP+ cells at several time points after cell injection, data are presented as mean + SEM and analyzed via one way anova followed by Bonferroni; *p<0,05. Vera Lenerz and Ruslan Masgutov contributed to this figure.

3.3.4. Clinical improvement of EAE after transplantation of NT3-GFP-ESdM

At a clinical score of 3 (day 20), EAE-afflicted mice were treated with an intravenous injection of $4x10^6$ GFP-ESdM, NT3-GFP-ESdM or 100 µl PBS. Injection with both ESdM-types had a significant beneficial effect on the clinical symptoms. The mice were found to recover significantly and showed less severe onset of the disease (Fig. 3.12).

Whereas the GFP-transduced ESdM displayed a mild therapeutical effect on EAE-afflicted mice, administration of NT3-GFP-ESdM had an even stronger effect.
Treatment with NT3-GFP-ESdM additionally impeded the tendency to relapse after first recovery. This effect could not be seen upon treatment with GFP-ESdM or PBS.

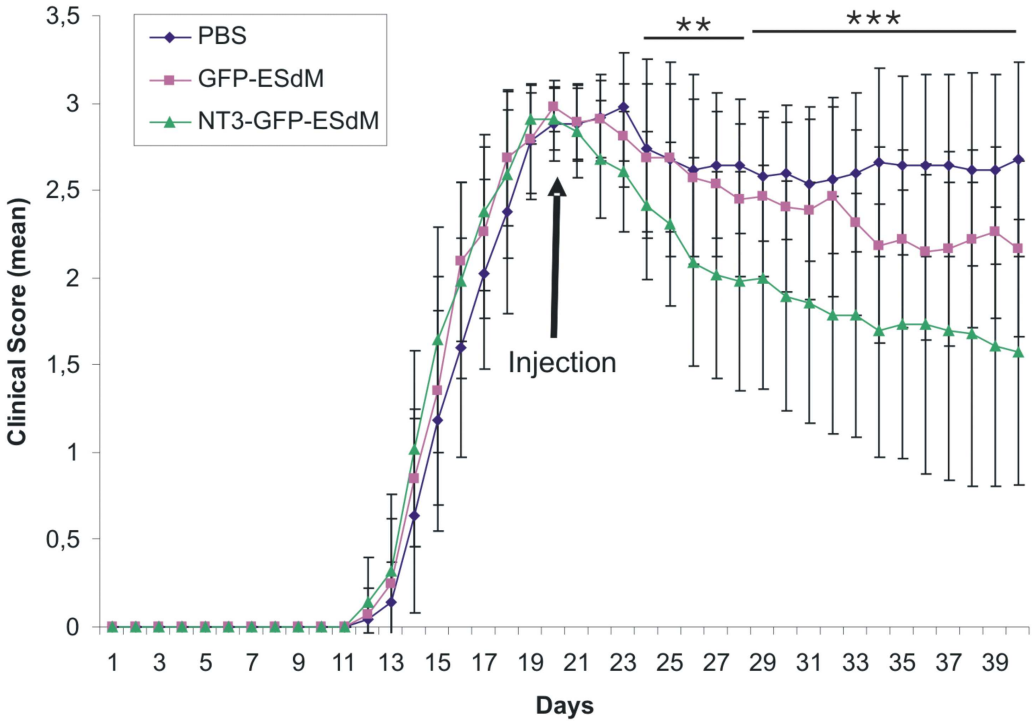

Fig. 3.12: Clinical score of EAE mice until day 40 post induction. Mice were treated with either PBS, GFP-ESdM or NT3-GFP-ESdM, administered at day 20 intravenously. Data are presented as mean +/-SEM, PBS n=25; GFP-ESdM n=27; NT3-GFP n=28, data was analyzed via one-way anova followed by Bonferroni: (**p<0,01 for PBS versus NT3-GFP-ESdM from day 25 to day 30; ***p<0,005 for PBS versus NT3-GFP-ESdM from day 30 to day 40).

3.3.5. Axonal injury and demyelination after transplantation of NT3-GFP-ESdM

40 days post induction of EAE, the mice were perfused and brains and spinal cord were collected for further analysis. The following histopathological stainings and quantifications were performed at the Institute of Neuropathology in Freiburg, Germany by Angela Dann in the working group of Prof. Marco Prinz.
To reveal the impact of the treatment with NT3-GFP-ESdM on the grade of demyelination, cryoslices of the spinal cords were stained with Luxol Fast Blue (LFB);

(Fig. 3.13-A). LFB stains the myelin sheaths in nervous tissue blueish, whereas neurons are stained violet. Treatment with NT3-GFP-ESdM led to a reduction of the demyelinated area from 44.6% +/- 3.5 % (PBS treated control) to 31.8% +/- 2.8 % (Fig.3.13-C).

Axonal damage was obtained via staining against amyeloid precursor protein (APP) (Fig.3.13-B). Axonal damage was decreased in mice treated with NT3-GFP-ESdM (25.1% +/- 2.1 %) in comparison to PBS treated control mice (38.8% +/- 3.2 %) (Fig. 3.13-D).

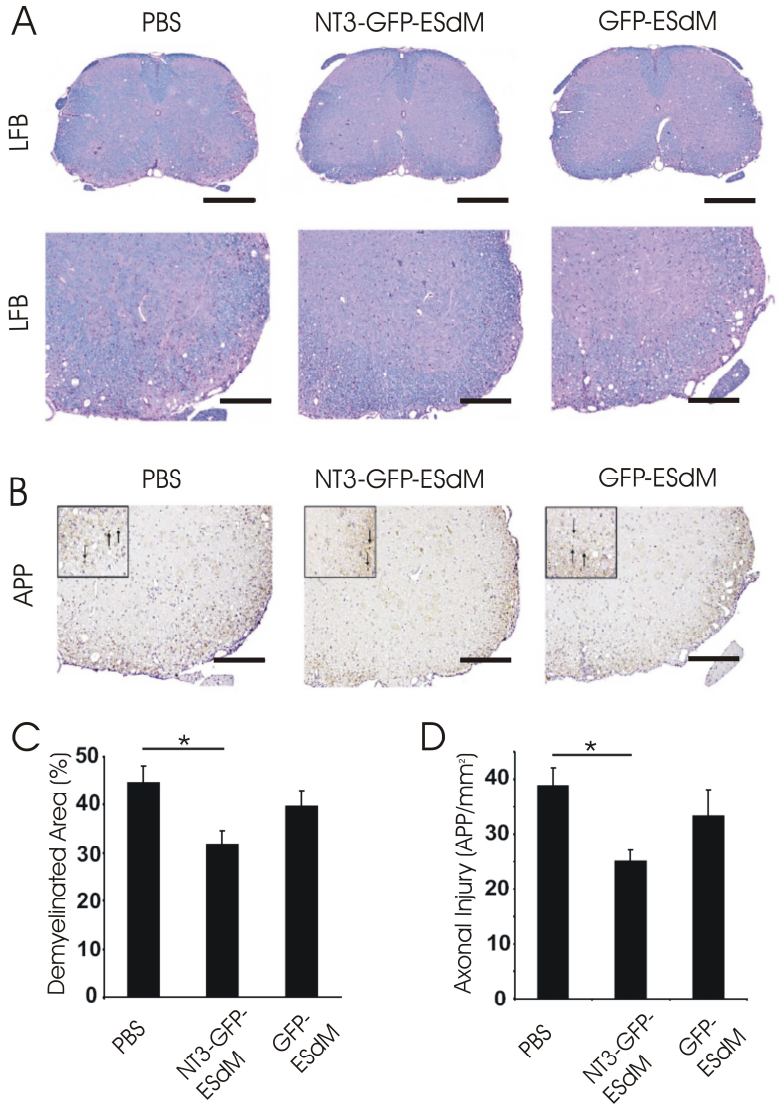

Fig. 3.13: A) Cryoslices of spinal cords derived from EAE afflicted mice 40 days post induction stained with LFB for myelin. Mice were treated with PBS, GFP-ESdM or NT3-GFP-ESdM. LFB staining shows that PBS mice suffered more demyelination in comparison to ESdM-treated mice. Scale bar upper row 500µm; scale bar lower row 200 µm B) Cryoslices of spinal cords derived from EAE afflicted mice 40 days post induction stained for APP, scale bar 200 µm.C) Quantification of the demyelinated area in spinal cord sections, * $p < 0.5$.Data are presented as mean + SEM. D) Quantification of axonal damage, * $p < 0.5$, data are presented as mean + SEM. Data was obtained in collaboration with Prof. Marco Prinz.

3.3.6. Number of invading T cells and macrophages into spinal cord tissue

For further comparison of the disease severity in the three different treatments, the number of inflammatory infiltrates into the nervous tissue was obtained (Fig. 3.14).

Thus, cryoslices of spinal cords were stained with antibodies against macrophage antigen (MAC) 3 for macrophages or CD3, a marker for T cells (Fig.3.14-A, B). For the macrophages, the mice treated with ESdM transduced with GFP or GFP and NT3 show a slightly lower infiltration into the spinal cord. However, differences in macrophage infiltrates are not significant inbetween the three groups. For the T cell infiltrates, the same holds true. No differences could be found regarding the number of T cell infiltrates into the spinal cord within the different treatments (Fig. 3.14-C, D).

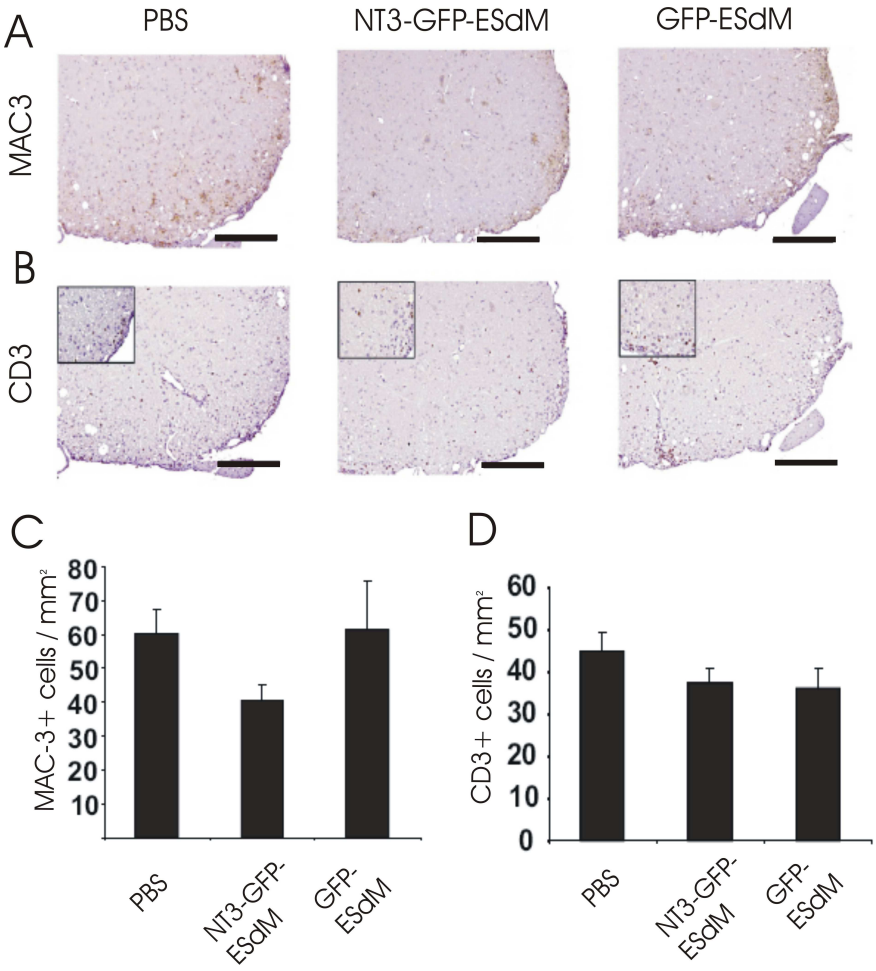

Fig. 3.14: A) Staining of spinal cord cryoslices of EAE afflicted mice 40 days post induction treated with PBS, GFP-ESdM or NT3-GFP-ESdM against macrophages (MAC-3) and B) T cells (CD3). Scale bar 200µm. C) Quantification of macrophages and D) T cells infiltrates in the spinal cord. The differences are not significant. Data are presented as mean + SEM. Data obtained in collaboration with Prof. Marco Prinz.

3.3.7. Anti-inflammatory and growth promoting effect of NT3-GFP-ESdM

To analyze if the NT3-GFP-ESdM had additional beneficial effects by mediating the cytokine milieu in the spinal cord, qRT-PCR was performed with RNA derived from spinal cords four days post injection of PBS, GFP-ESdM or NT3-GFP-ESdM (Fig. 3.15). The cytokine profile obtained by this method revealed that the treatment with NT3-GFP-ESdM resulted in a shift of the cytokine profile towards an anti-inflammatory milieu. A downregulation of the pro-inflammatory cytokines iNOS, TNF-α, IL-1β and TGF-β could be detected upon treatment with NT3-GFP-ESdM. A similar, but weaker effect was found upon treatment with GFP-ESdM in comparison to PBS treated mice. However, TGF-β has not been downregulated after treatment with GFP-ESdM. For the anti-inflammatory cytokine IL-10, an upregulation was detected upon treatment with NT3-GFP-ESdM, as well as for NT3 itself.

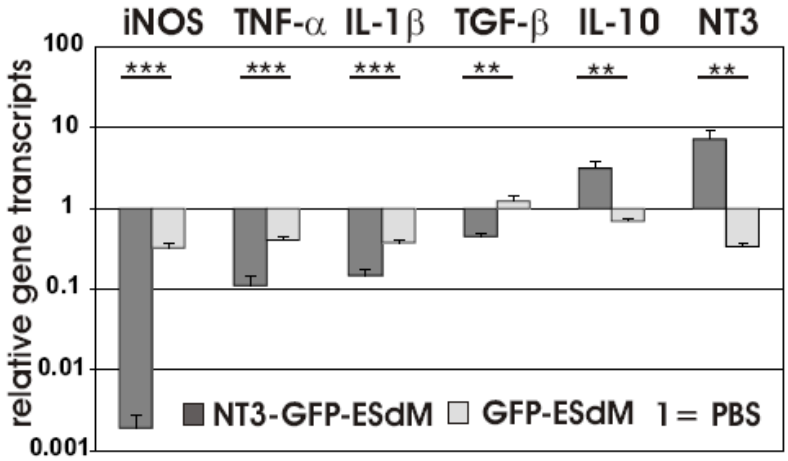

Fig.3.15: Quantitative realtime PCR for cytokine profile 4 days post injection of PBS, GFP-ESdM or NT3-GFP-ESdMs. Injection of NT3-GFP-ESdM resulted in an anti-inflammatory milieu. Data are presented as mean + SEM, ***p<0.005, **p<0.01.

The beneficial impact observed in the *in vitro* experiment for neurite growth effects of NT3 (see chapter 3.3.2) could be seen *in vitro* as well, as NT3-GFP-ESdM had a positive effect on neurons. Cryosections of the spinal cord could be stained for GAP43, a marker for neurite outgrowth, indicating a neuronal regenerative effect (Fig. 3.16-A).

Quantification of GAP43+ cells in the spinal cord showed a significant increase after 4 days post treatment with NT3-GFP-ESdM in comparison to PBS or GFP-ESdM (Fig. 3.16-B).

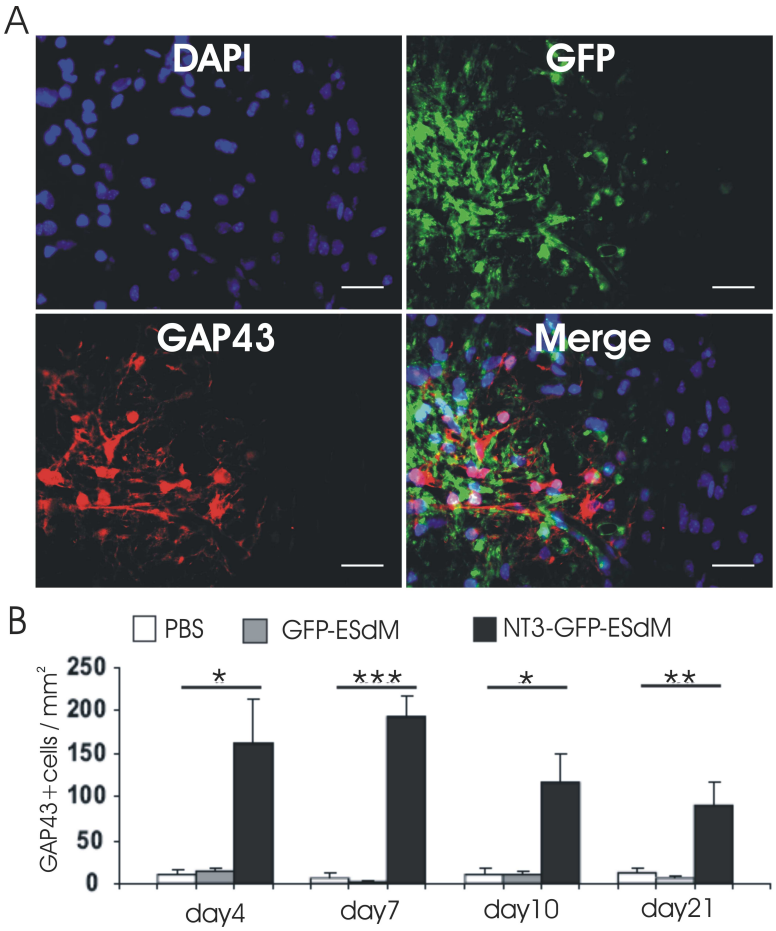

Fig. 3.16: A) Staining of spinal cord cryoslices 4 days post injection of NT3-GFP-ESdM. Slices were immunolabeled with GAP43. Scale bar 50µm. B) Quantification of GAP43+ cells at different time points post injection, treatment with NT3-GFP-ESdM led to an increase of GAP43+ cells. Data are presented as mean + SEM of three independent experiments. Statistical analysis via ANOVA followed by Bonferroni, *p<0.05; **p<0.01; ***p<0.0001. Vera Lenerz contributed to this data.

3.4. Phagocytosis of glioma cells through ESdM

3.4.1. Co-culture of ESdM with SMA560 and GL261 glioma cells

To study the *in vitro* effects of ESdM on glioma cells, co-culture experiments were performed following analysis of glioma cell number and phagocytosis. Lentiviral transduced GFP-SMA560 or GFP-GL261 glioma cells were co-cultured with ESdM and the glioma cell number was determined every day for 5 days (Fig. 3.17). A pure glioma culture was used as a control. In the pure culture, both glioma cell lines displayed an exponential growth, whereas the GFP-GL261 expanded slower than the GFP-SMA560 line. In co-culture with the ESdM, the growth rate of glioma cells was significantly decreased in both experiments. After 5 days, the mono-culture of GFP-SMA560 expanded to $528.8^{x}10^{4}$ +/-$67.8^{x}10^{4}$ cells, in presence of ESdM they reached $247.7^{x}10^{4}$ +/- $87.0^{x}10^{4}$ cells. The GFP-GL261 cell line alone expanded to $149.0^{x}10^{4}$ +/- $23.1^{x}10^{4}$ cells, the ESdM reduced that cell number to $64.0^{x}10^{4}$ +/- $4.6^{x}10^{4}$ cells at day 5.

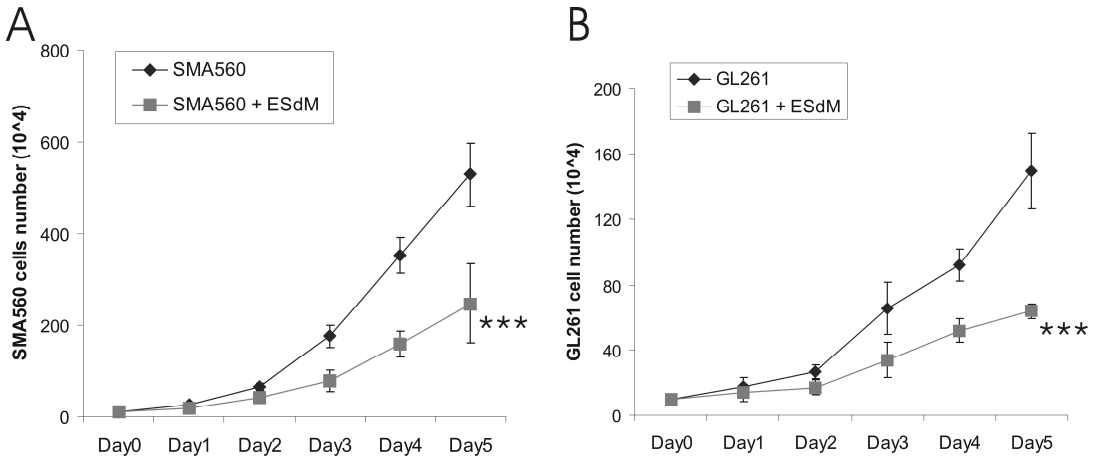

Fig. 3.17: A) Quantification of SMA560 glioma cells in co-culture with ESdM in comparison to a pure SMA560 culture. In co-culture, SMA560 showed a lower expansion of cell number. Data is presented as mean +/- SEM. Statistical analysis via students t-test, ***p<0.0001. B) Quantification of GL261 glioma cells in presence or absence of ESdM. Co-culture with ESdM led to lower GL261 cell numbers. Data is presented as mean +/- SEM. Statistical analysis via students t-test, ***p<0.0001.

To confirm that the reduction of glioma cells in the cell culture was not due to an increase in apoptosis, staining with Annexin V was performed. GFP+ glioma cells were cultured

alone or in presence of ESdM. After 24 hours, the cultures were stained with Annexin V and the number of positive GFP+ cells was determined. In GFP-SMA560 culture, 4.0% +/- 0.7% of cells stained positive for Annexin V, whereas in the co-culture with ESdM 5.8% +/- 3.0 % were apoptotic. For GL261, the mono-culture showed 5.4% +/- 3.9%, the co-culture showed 4.2% +/- 2.0% of apoptotic cells. As a positive control, GFP-SMA560 and GFP-GL261 were treated with 100ng/ml okadaic acid for 24 hours. Treatment resulted in 86.2% +/- 5.8% apoptotic GFP-SMA560 cells and in 86.3% +/- 9.0% apoptotic GFP-GL261 cells (Fig.3.18).

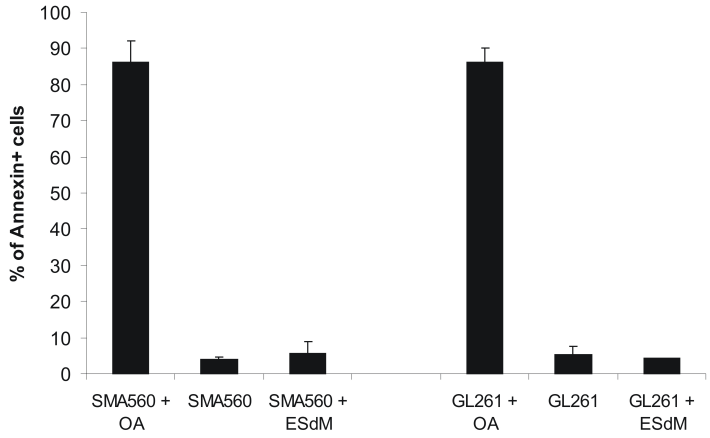

Fig. 3.18: Co-cultures of ESdM and GFP-SMA560 or GFP-GL261 and mono-cultures of the glioma lines were stained with Annexin V after 24 hours. As a positive control, glioma cultures were treated with 100 nmol/ml okadaic acid (OA) for 24 hours. Analysis via ANOVA and Bonferroni test, no significant differences were found inbetween the glioma cultures and the co-cultures with ESdM. Data is presented as mean + SEM.

3.4.2. Phagocytosis of SMA560 and GL261 glioma cells by ESdM

As the reduction of glioma cells was not due to an increase of apoptosis, a phagocytosis experiment was performed. Thus, ESdM were lentivirally transduced with GFP and cultured with PKH26 fluorescent dye labeled SMA560 or GL261 glioma cells. The uptake of glioma cells by ESdM was visualized using confocal microscopy (Fig. 3.19-A). In both cases, GFP-ESdM showed an uptake of fluorescent particles, which indicates phagocytosis of glioma cells through GFP-ESdM. In the co-culture with SMA560 cells, 46.6% +/- 11.1% of the total ESdM cell number showed uptake of PKH26+ particles. For

the co-culture with GL261 glioma cells, an uptake from 60.8% +/- 7.8% was seen (Fig. 3.19-B).

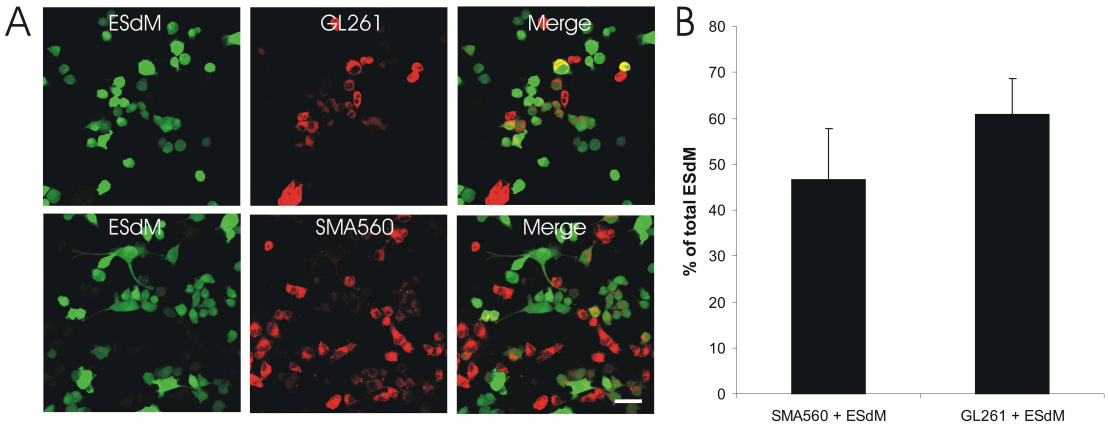

Fig. 3.19: A) Confocal imaging of co-cultures of GFP-ESdM with GL261 or SMA560. In both cases, GFP-ESdM showed uptake of PKH26+ particles. Scale bar: 50 µm.
B) Quantification of GFP-ESdM which showed uptake of PKH26+ particles in co-culture with SMA560 or GL261 glioma cells, data is presented as mean + SEM.

Z-Stack-Imaging revealed that the GFP-ESdM have indeed taken up the glioma cells, as the red signal of PKH26+ particles was located inside the cells (Fig. 3.20)

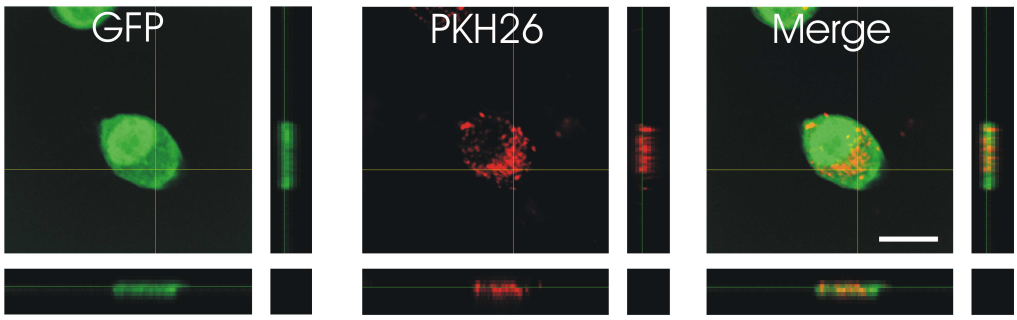

Fig. 3.20: Z-stack confocal imaging of GFP-ESdM with internalized PKH26+ particles. Particles are located inside the GFP-ESdM. Scale bar: 20 µm

3.4.3. Effect of polarized ESdM on glioma proliferation and phagocytosis

To further evaluate the effect of ESdM on glioma cells in co-culture, ESdM were treated for 24 hours with IFN-γ (100 U/ml) + LPS (5 ng/ml) for M1 polarization or with IL-4 (20 ng/ml) for M2 polarization. The subpolarized ESdM were used for the co-culture with GFP-SMA560 or GFP-GL261 to find out if the polarization of ESdM affects glioma growth rate or phagocytosis capability of ESdM. The proliferation assay revealed that the subpolarization into M2 subtype had no significant effects on the growth behaviour of glioma cells in co-culture. GFP-SMA560 grew to 590.9 $^{x}10^{4}$ +/- 84.2 $^{x}10^{4}$ cells in a single culture after 5 days, in co-culture with ESdM that number was reduced to 209.1 $^{x}10^{4}$ +/- 7.9 $^{x}10^{4}$ cells. M2 polarized cells showed a similar result with 217.8 $^{x}10^{4}$ +/- 23.7 $^{x}10^{4}$ GFP-SMA560 cells. Subpolarization of ESdM into M1 subtype decreased the glioma cell number a bit more to 166.1 $^{x}10^{4}$ +/- 18.6 $^{x}10^{4}$ cells (Fig.3.21-A). Although this result did not reach significance, a difference between GFP-SMA560 cell numbers in control ESdM and M1 co-culture could be found at day 3. Similar effects of subpolarization were found in the GFP-GL261 set-up. The single culture reached 253.0 $^{x}10^{4}$ +/- 28.7 $^{x}10^{4}$ cells culture with control ESdM reached 161.7 $^{x}10^{4}$ +/- 24.9$^{x}10^{4}$ cells. Treament of ESdM for M2 subtype was almost equal to control ESdM (162.9 $^{x}10^{4}$ +/- 14.9 $^{x}10^{4}$). Subpolarization into M1 type resulted in an expansion of GFP-GL261 cells to 145.5 $^{x}10^{4}$ +/- 23.2 $^{x}10^{4}$ cells. Again, significance was obtained at day 3 in comparison to non-treated ESdM (Fig.3.21-B).

Quantification of phagocytosis showed that M1 treatement resulted in an increase of phagocytosis in co-culture with SMA560. Control ESdM reached 37.0% +/- 6.1%, M1 ESdM reached 64.5% +/- 13.9%. Treatment for M2 polarization did not have an effect in comparison to the control and resulted in 38.4% +/- 7.5%.

In co-culture with GL261 glioma cells, 62.3% +/- 5.0% of control ESdM showed uptake of PKH26+ particles. M1 treatment increased that value to 79.8% +/- 7.3%. M2 treatment resulted in a slightly lower uptake, 55.2% +/- 1.8% of M2 ESdM showed uptake of red particles (Fig.3.21-C).

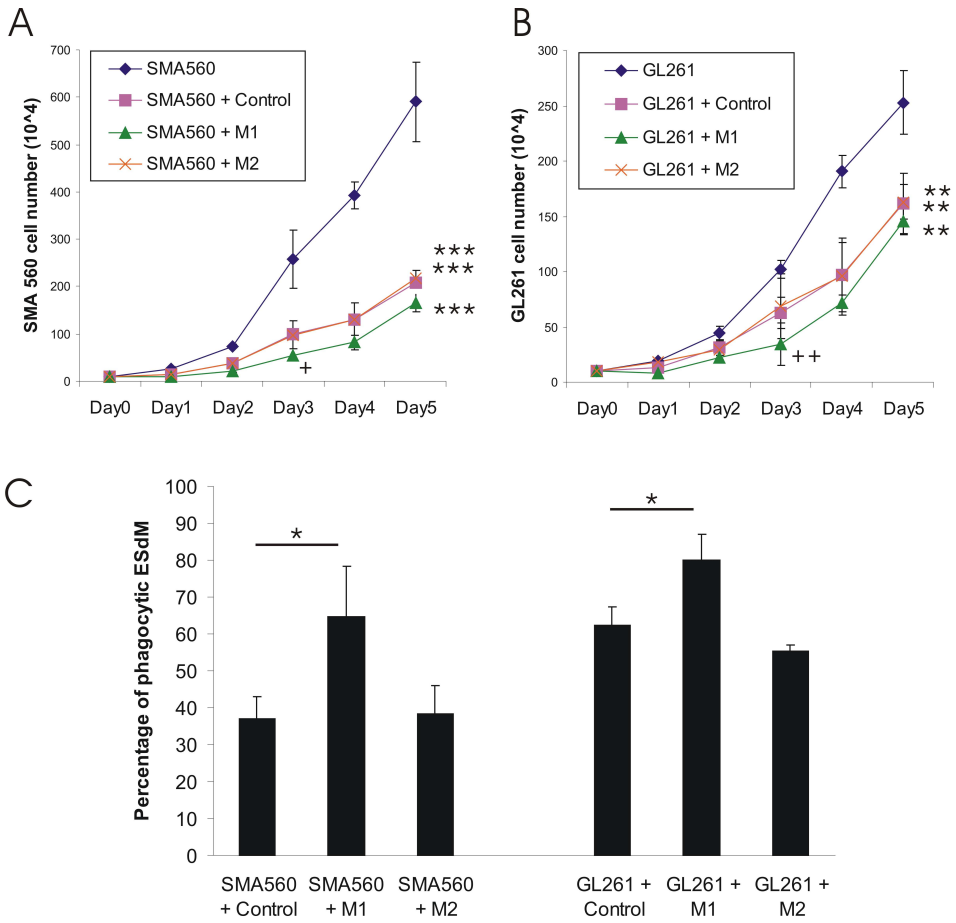

Fig. 3.21: Effects of ESdM on glioma proliferation and phagocytosis after subpolarization into M1 and M2 subtype. A) Quantification of SMA560 cell number in co-culture with ESdM, M1 and M2 polarized ESdM. B) Quantification of the co-culture of ESdM, M1 or M2 ESdM with GL261 cells. Data is presented as mean +/- SEM. Statistical analysis via ANOVA followed by Bonferroni, + $p<0.05$; ++/**$p<0.01$; ***$p<0.0001$. + indicates the significance in comparison to the wildtype ESdM, * in comparison to the glioma mono-culture. C) Quantification of uptake of PKH26+ particles by non-treated ESdM, M1 treated or M2 treated ESdM after 24 hours of co-culture with SMA560 or GL261 glioma cells. Data is presented as mean + SEM. Statistical analysis via ANOVA followed by Bonferroni, *$p<0.05$.

As polarization of ESdM into M1 subtype led to an increase in phagocytosis of glioma cells, analysis was performed in regard to phagocytosis-associated receptors. FACS analysis confirmed that M1 polarized ESdM showed a higher expression of SiglecH and Trem2 (Fig. 3.22) in comparison to non-stimulated cells.

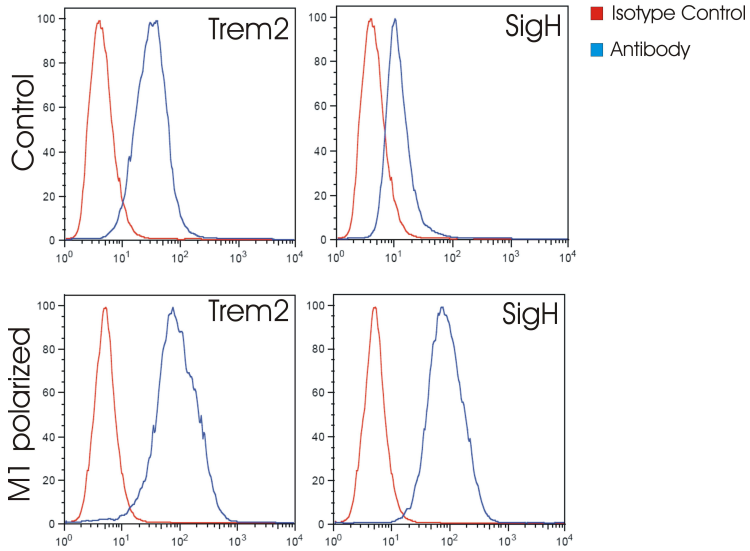

Fig. 3.22: FACS analysis for Trem2 and SiglecH expression of untreated ESdM control and ESdM treated with IFN-γ (100 U/ml) + LPS (5 ng/ml) for M1 subpolarization. Stimulation resulted in a higher expression of Trem2 and SiglecH.

3.4.4. Effects of DAP12 in glioma proliferation and phagocytosis through ESdM

The protein DNAX activation protein of 12 kDa (DAP12) is known to play an important role in phagocytosis, as it is associated with phagocytic receptors such as SiglecH and Trem2. Therefore, the effects of DAP12 overexpression and DAP12 dominant negative mutation with lacking immunoreceptor tyrosine-based activation motif (ITAM) domain were analyzed in regard to glioma cell proliferation and phagocytosis of glioma cells through GFP-ESdM.

The proliferation assay revealed that the cell number in both cell-cultures is decreased through ESdM. The mono-culture of SMA560 expanded to $553.1^{x}10^{4}$ +/-$60.0\ ^{x}10^{4}$ cells, in presence of ESdM to $322.9\ ^{x}10^{4}$ +/- $26.9\ ^{x}10^{4}$ cells. The overexpression of DAP12 further decreased the SMA560 cell number to $246.9\ ^{x}10^{4}$ +/- $18.2\ ^{x}10^{4}$ cells, whereas the DAP12 lacking ITAM ESdM only had little but significant effect on the glioma cell number of $409.4\ ^{x}10^{4}$ +/- $15.5\ ^{x}10^{4}$ cells. The GL261 cell line alone expanded to $99.3\ ^{x}10^{4}$ +/- $6.4\ ^{x}10^{4}$ cells, the ESdM reduced cell number to $61.1\ ^{x}10^{4}$ +/- $2.5\ ^{x}10^{4}$ cells. In co-culture with DAP12 lacking ITAM ESdM GL261 culture almost reached cell numbers like in the mono-culture with $91.7\ ^{x}10^{4}$ +/- $4.5\ ^{x}10^{4}$ cells, the difference is not significant. The overexpression of DAP12 in ESdM led to an even stronger decrease of GL261 cell

number. GL261 cells in presence of DAP12 overexpressing cells expanded to $47.4^{x}10^{4}$ +/- $15.2^{x}10^{4}$ cells (Fig.3.23-A, B).

The phagocytosis experiments resulted in a matching result, as the ESdM lacking DAP12 ITAM domain only showed low uptake levels (17.5% +/- 9.3% for SMA560 and 22.7% +/- 7.7% for GL261 co-culture) of PKH26+ particles. The overexpressing cells showed a higher uptake level (76.1% +/- 12.4% for SMA560 and 77.3% +/- 6.2% for GL261 co-culture) in comparison to the co-culture with control ESdM (53.9% +/- 9.1% for SMA560 and 58.7% +/- 9.6% for GL261 co-culture) (Fig. 3.23-C).

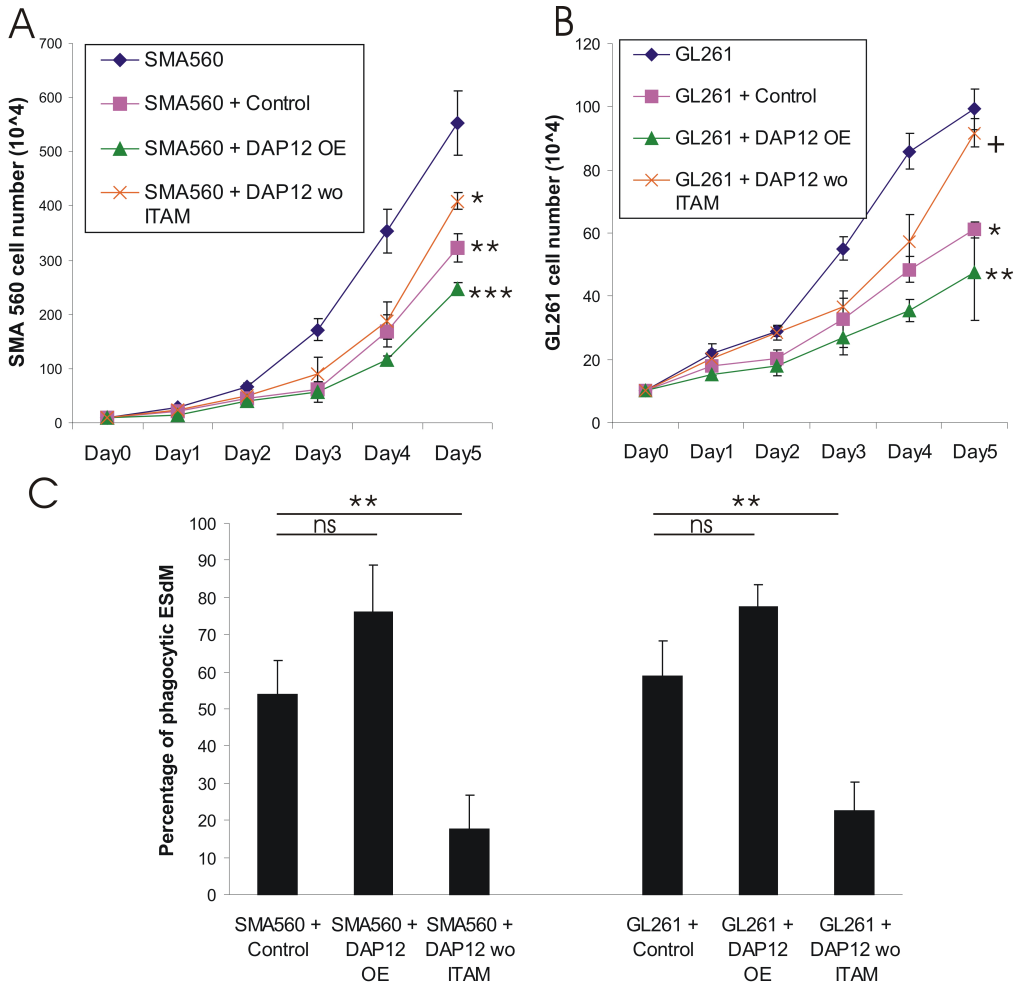

Fig. 3.23: Effects of DAP12 on glioma proliferation and phagocytosis through ESdM. A) Quantification of SMA560 cell number in co-culture with ESdM, DAP12 overexpressing ESdM (DAP12 OE) and DAP12 lacking ITAM ESdM (DAP12 wo ITAM). Lacking ITAM resulted in a smaller effect of ESdM on glioma cell number in comparison to the control ESdM. Overexpression of DAP12 increased the reducing effect of ESdM on SMA560 cells. B) Quantification of the co-culture of ESdM, DAP12 overexpressing ESdM and DAP12 lacking ITAM ESdM with GL261 cells. Overexpression of DAP12 decreased the GL261 cell number, whereas lacking ITAM domain in the DAP12 resulted in a weaker effect of ESdM. Data is presented as mean +/- SEM. Statistical analysis via ANOVA followed by Bonferroni, */+ $p<0.05$; **$p<0.01$; ***$p<0.0001$. + indicates the significance in comparison to the wildtype ESdM, * in comparison to the mono-culture. C) Quantification of uptake of PKH26+ particles by ESdM, DAP12 overexpressing ESdM and DAP12 lacking ITAM ESdM after co-culture with SMA560 and GL261 glioma cells. Data is presented as mean + SEM. Statistical analysis via ANOVA followed by Bonferroni, **$p<0.01$.

3.4.5. Effects of SiglecH on glioma proliferation and phagocytosis through ESdM

To evaluate the role of SiglecH in uptake of glioma cells through ESdM and the effect of glioma cell growth rate, SiglecH was knocked down using a lentiviral vector approach. Confirmation of the knock-down was performed via qRT-PCR. Treatment of ESdM with the knock-down plasmid aimed at SiglecH led to a 10-fold decrease of gene transcript in comparison to ESdM treated with a control scramble RNAi plasmid (Fig.3.24).

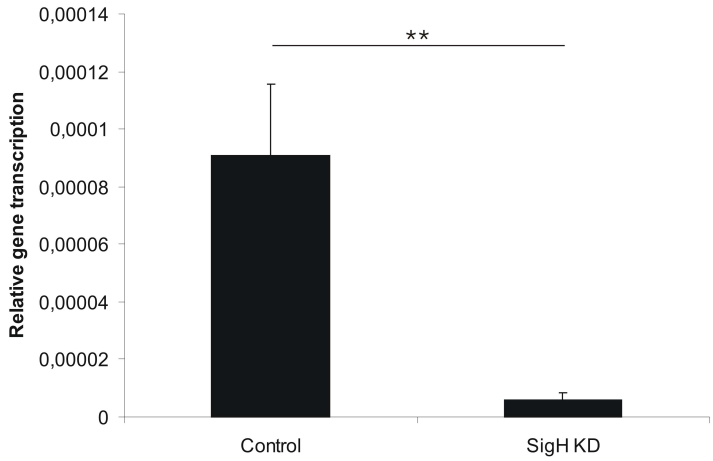

Fig. 3.24: Lentiviral knock-down of SiglecH (SigH KD) resulted in a 10-fold lower gene transcript level than in corresponding control ESdM. Data is presented as mean +SEM, statistical analysis via students t-test, **p<0.005

The SiglecH knock-down ESdM polarized into M1 subtype were used for phagocytosis assay and in the co-culture system with glioma cells to determine if the knock-down altered the behaviour of ESdM or glioma cells.

In regard to cell expansion of the SMA560 or GL261, the SiglecH knock-down cells had a smaller reducing effect than the control. SMA560 cells alone reached 504.4 $^{x}10^{4}$ +/- 65.8 $^{x}10^{4}$ cells after 5 days, in presence of ESdM that number decreased to 172.7 $^{x}10^{4}$ +/- 30.4 $^{x}10^{4}$ cells. Knock-down of SiglecH lowered the effect of ESdM, SMA560 expanded to 357.0 $^{x}10^{4}$ +/- 11.8 $^{x}10^{4}$ cells (Fig.3.25-A).

In GL261 culture, cells grew to a cell number of 171.3 $^{x}10^{4}$ +/- 34.3 $^{x}10^{4}$ cells, ESdM decreased that cell number to 66.9 $^{x}10^{4}$ +/- 4.3 $^{x}10^{4}$ cells. The knock-down of SiglecH in ESdM resulted in a milder effect of ESdM, GL261 expanded to 142.9 $^{x}10^{4}$ +/- 28.5 $^{x}10^{4}$ cells (Fig.3.25-B).

The phagocytosis assay showed that knock-down of SiglecH altered the uptake of glioma

cells through ESdM. In co-culture with SMA560, control ESdM showed an uptake of 39.2% +/- 11.3%, whereas the SiglecH knock-down only showed an uptake rate of 18.2% +/- 5.4%. In co-culture with GL261 cells, 63.0% +/- 8.0% of ESdM showed uptake of PKH26+ particles. The knock-down of SiglecH reduced the uptake to 19.2% +/- 8.0% (Fig. 3.25-C).

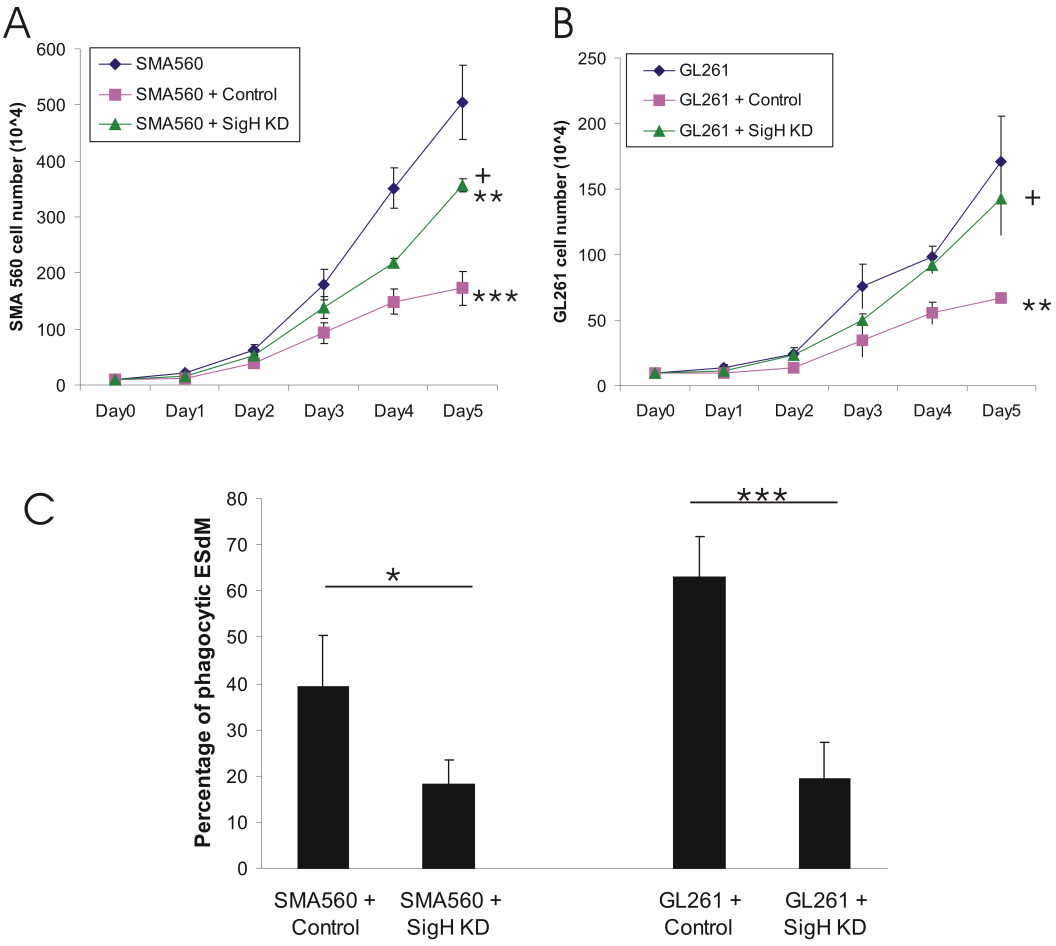

Fig. 3.25: Effects of SiglecH knock-down on glioma proliferation and phagocytosis through ESdM. A) Quantification of SMA560 cell number in co-culture with control ESdM and SiglecH knock-down ESdM (SigH KD). B) Quantification of the co-culture of ESdM and SiglecH knock-down ESdM with GL261 cells. Data of A and B is presented as mean +/- SEM. Statistical analysis via ANOVA followed by Bonferroni, */+ $p<0.05$; **$p<0.01$; + indicates the significance in comparison to the wildtype ESdM, * in comparison to the mono-culture. C) Quantification of uptake of PKH26+ particles by ESdM and SiglecH knock-down ESdM in co-culture with SMA560 or GL261 glioma cells. Data is presented as mean + SEM. Statistical analysis via students t-test, *$p<0.05$, ***$p<0.0001$.

3.4.6. Role of Trem2 in glioma proliferation and phagocytosis through ESdM

As a second phagocytic receptor in microglia-glioma-interaction, Trem2 is a suitable candidate. Thus, Trem2 was knocked down in ESdM using the lentiviral system. FACS analysis showed that the knock-down was successful, as lower levels of Trem2 were found after treatment with knock-down plasmid targeted for Trem2 (Fig.3.26).

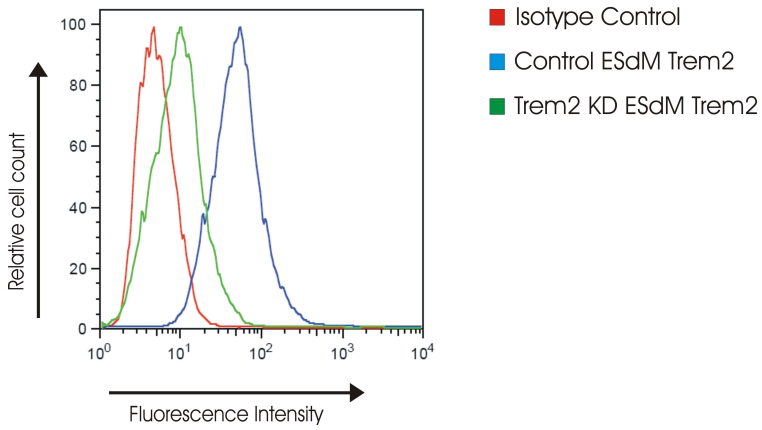

Fig. 3.26: Validation of Trem2 knockdown via FACS. Control ESdM showed an intense signal for Trem2 expression, whereas the signal of the ESdM lentivirally transducted with Trem2 shRNA expressed lower levels of Trem2.

The Trem2 knock-down cells were used for proliferation assay and phagocytosis experiments to analyze the effect of the knock-down.

During the proliferation assay, SMA560 cells expanded to 504.3 $^x10^4$ +/- 50.5 $^x10^4$ cells in 5 days. Co-culture with ESdM reduced glioma cell number to 184.3 $^x10^4$ +/- 25.7 $^x10^4$, knock-down of Trem2 in ESdM in co-culture resulted in 299.6 $^x10^4$ +/- 25.5 $^x10^4$ SMA560 glioma cells (Fig.3.27-A). GL261 glioma cells reached 182.7 $^x10^4$ +/- 31.2 $^x10^4$ cells, in presence of ESdM they reached 71.0 $^x10^4$ +/- 6.7 $^x10^4$. In co-culture with Trem2 knock-down ESdM, GL261 cells expanded to 106.0 $^x10^4$ +/- 9.0 $^x10^4$ cells (Fig.3.27-B).

In regard to phagocytosis of glioma cells, Trem2 knock-down led to a decrease in phagocytosis capacity of ESdM in co-culture with glioma cells. Co-cultured with SMA560 glioma cells, control ESdM showed an uptake rate of 46.5% +/- 6.7%, knock-down of Trem2 decreased the phagocytosis activity to 31.9% +/- 5.4%. In case of the GL261 glioma cells, 59.4% +/- 9.2% of control ESdM showed uptake of PKH26+

particles. The knock-down of Trem2 resulted in a trend to lower uptake levels with 46.0% +/- 4.3% (Fig.3.27-C).

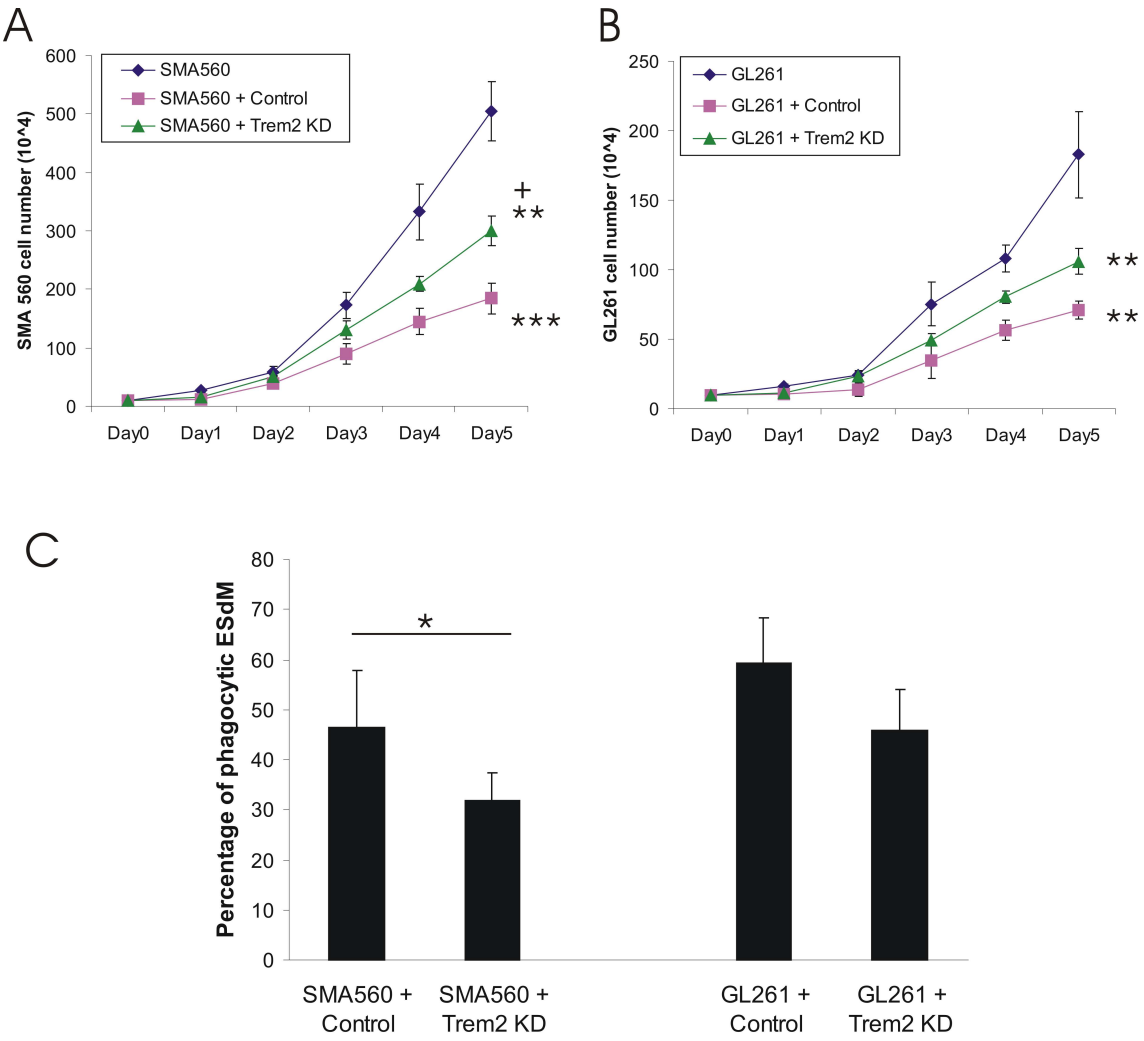

Fig. 3.27: Effects of Trem2 knock-down on glioma proliferation and phagocytosis through ESdM. A) Quantification of SMA560 cell number in co-culture with control ESdM and Trem2 knock-down ESdM (Trem2 KD). B) Quantification of the co-culture of ESdM and Trem2 knock-down ESdM with GL261 cells. Data of A and B is presented as mean +/- SEM. Statistical analysis via ANOVA followed by Bonferroni, + $p<0.05$; ** $p<0.01$; *** $p<0.005$; + indicates the significance in comparison to the wildtype ESdM, * in comparison to the mono-culture. C) Quantification of PKH26+ particle-uptake by ESdM and Trem2 knock-down ESdM in co-culture with SMA560 or GL261 glioma cells. Data is presented as mean + SEM. Statistical analysis via students t-test, * $p<0.05$.

4. Discussion

4.1. Derivation of embryonic stem cell lines

The establishment of pluripotent cells from mouse embryos was firstly described in 1981 (Evans and Kaufman, 1981) and is still considered to be a breakthrough in cell biology. The ICM of a blastocyst represents a pluripotent cell population which gives rise to all embryonic tissue including germline cells (Bradley *et al.*, 1984). As ESCs are derived from that unique cell population, they share the capacity of differentiating into all cell types. Defined culture conditions including LIF result in ESC lines with self-renewing and indefinite growth capacities without senescing (Williams, 1988; Miura *et al.*, 2004).
Germline competent ESCs can be used for gene targeting and furthermore to generate genetically modified mice strains (Schoonjans *et al.*, 2003).
During the last years, ESCs have also been used for drug-assays and for the development of stem cell based therapy approaches and are therefore considered to be a powerful tool in disease modeling (Prelle *et al.*, 2002). The protocols for establishing ESC lines have been revised and improved in the last decade and result in a high yield of lines which are suitable for screening experiments (Cheng *et al.*, 2004; Bryja *et al.*, 2006).
Seeding of blastocysts to a MEF layer resulted in primary ESC lines, which could be kept in culture for several passages. All derived ESC lines were positive for Sox2, Oct4 and SSEA1 and stained positive for AP.
The transcriptions factors Sox2 and Oct4 are essential in developmental processes in mammals through the regulation of other essential genes involved in embryonic development (Kiefer, 2007; Nichols *et al.*, 1998). Their main function is to maintain self-renewal and to keep cells from differentiation. It has been demonstrated that knock-down of Sox2 or Oct4 gene leads to embryonic lethality and promotes the differentiation of ESCs in culture (Niwa *et al.*, 2000; Chew *et al.*, 2005).
SSEA-1 is another well known marker for murine pluripotent stem cells, its main function lies in regulation of adhesion and migration of cells during embryonic implantation (Kerr *et al.*, 1992). Elevated levels of AP are typically found on the

membrane of ESCs and can therefore be used as a ESC marker (Kuegler *et al.*, 2010). The generated ESCs could successfully be differentiated into neuronal precursors and finally into microglial cells. Nevertheless, to fully ensure ESC properties, lines have to be tested for the ability to differentiate into cells of all three germ-layers.

The generation of ESC and the subsequent differentiation into ESdM bears a high potential for studying the effects of genetic diseases especially in regard to microglial function.

4.2. Characterization of embryonic stem cell derived microglia

4.2.1. Generation of ESdM

Microglia functions are often studied on primary microglia cells, which can be isolated and enriched from mixed glial cell cultures derived from the brains of postnatal mice. As an alternative, primary microglia can be directly isolated from adult brain tissue by density gradients and flow cytometry sorting (Havenith *et al*., 1998; Ford *et al.*, 1995). Cell yield obtained by these protocols is rather low and not sufficient for biochemistry of systematic screening methods. Therefore, oncogenically transformed microglia cell lines such as BV2 have often been used (Blasi *et al.*, 1990; Bocchini *et al.*, 1992). But, oncogenic transformation might lead to a limited reflection of microglial properties, likely making microglial cell lines a poor substitute for primary microglia.

In contrary to primary microglia, murine ESCs provide unlimited self-renewal capacity and can differentiate into cell types of all three germ layers. Recently, a protocol for differentiation of microglia-like cells from murine ESC was described (Tsuchiya *et al.*, 2005).

To overcome the problems mentioned above, a protocol for differentiation of mouse ESCs into microglia precursor cells was established (Napoli *et al.*, 2009; Beutner *et al.*, 2010). The developed protocol is based on a protocol for neuronal differentiation (Lee *et al.*, 2000) and was initiated by the finding that microglia-like cells can be found next to neural cells differentiated from EBs (Tsuchiya *et al.*, 2005).

During development, primitive yolk sac macrophages start to populate the neuroepithelium as microglia precursor cells around embryonic day 7.5 (Ginhoux *et al.*, 2010). Microglia precursor cells start to proliferate locally until date of birth to finally differentiate into the resident pool of adult microglia (Alliot *et al.*, 1999). ESdM were derived via a protocol which mimics brain development from EBs. EBs show both neurogenesis and yolk-sac like hematopoieses (Keller *et al.*, 1993). Therefore, ESdM are likely differentiated through a yolk-sac like primitive macrophage precursor cell and reflect *in vivo* microglia development.

ESCs were differentiated into ESdM and characterized. ESdM provide the possibilities to study the microglia functions without oncogenic transformation and an insufficient cell number. Additionally, the protocol could be applied to a broad range of ESC derived from transgenic mice, resulting in genetically modified ESdM. The differentiation resulted in high cell amounts of proliferative ESdM, which could be kept in culture up to 25 passages without losing their properties.

4.2.2. Phenotype and genotype of ESdM

In the protocol applied to the ESCs, ESdM are generated via EB formation which are plated. In the plated EBs, a variety of cell types appeared, e.g. contracting heart muscle cells. In the neural differentiation step, immunostaining revealed a mixed cell population positive for neural precursors, glial and microglial cell markers.

The Iba1+/CD45+ cells could be further cultured in defined medium to a homogenous population of ESdM. ESdM could be stained for the classical microglia markers Iba1 and CD68, with no negative cells present. Hence, the differentiation protocol resulted in a pure ESdM population.

Several markers assigned to the microglial phenotype were found. Microglia are described as CD68+, CD45 low, CD11b+, CD11c high, MHC class II+, IBa1+ and F4/80+, CD18+ cells (Guillemin and Brew, 2004; Ford *et al.*, 1995).

To determine the phenotype of ESdM in regard to surface marker expression, FACS analysis was performed. The ESdM showed expression of CD11b, CD11c, CD29 (β1 integrin), CD45, CD49, CD68, CD80, CD86 (B7.2) and CD115. Additionally, expression

of Trem2, F4/80, MHCII and CX3CR1 could be shown. All these markers are membrane proteins assigned to monocytes, microglia and macrophages (Austyn and Gordon, 1981; Ford *et al.*, 1995; Napoli *et al.*, 2009, Ulvestad *et al.*, 1994; Jung *et al.*, 2000). The surface expression of these markers is comparable to primary microglia. The presence of β1 integrin and the co-stimulatory molecule CD86 indicate that the ESdM are capable of immunoregulatory functions.

ESdM showed no expression of Ly6G and CCR2, which is a hallmark of regulatory monocytes which home to non-inflamed tissue (Geissmann *et al.* 2003). This pool of resident monocytes patrols blood vessel walls and also replaces tissue macrophages. In comparison, Ly6G+/ CCR2+ monocytes are referred to as inflammatory monocytes, which are present in the circulating blood and migrate to inflamed tissues (Ransohoff, 2011).

ESdM were virtually negative for cKit and CD34, which are markers for stem cells. The lack of these stem cell population markers indicates that the cells are fully commited to differentiation. Thus, ESdM display microglial precursor cells identity.

In addition to the analysis of the phenotype, a RNA microarray was carried out to further validate the ESdM identity. The array confirmed the previous findings and showed a comparable gene expression of primary microglia and ESdM. Of the 44 000 genes analyzed, about 20 000 genes were found to be expressed in both primary microglia and ESdM. Of those expressed genes, more than 19 000 genes were coexpressed in both cell types, whereas less than 1 000 were expressed selectively. Furthermore, the genes coexpressed were mostly found in comparable amounts in ESdM and primary microglia. Of the genes coexpressed, genes assigned to microglia such as CD206, CD63, TREM2 or MHCI were found in high amounts. However, genes assigned to other cell types like GFAP and Olig2 (astrocyte genes), β-III-tubulin and nestin (neuronal genes) or Nanog and Sox2 (ESC genes) were only weakly expressed. Thus, ESdM express a similar genetic profile like primary microglia. Additionally, the microarray data mirror the full commitment of ESdM to differentiation and their microglial identity.

4.2.3. Functionality of ESdM

To further reveal the properties of the ESdM, a variety of functional assays was performed. One of the typical properties of microglia is the production of pro-inflammatory cytokines and ROS upon pro-inflammatory stimuli (Hanisch, 2002). To mimic the *in vivo* situation in the cell culture, the inflammatory stimuli LPS and IFN-γ were used to induce cytokine production by ESdM. Data were obtained via qRT-PCR with primers against NOS2, TNF-α and TGF-β. Treatment of ESdM with IFN-γ resulted in an increase of NOS2 by a factor of 1000 and a 20-fold increase of TNF-α transcripts. Treatment with LPS increased the expression of NOS2 and TNF-α, but to a lower extent than the treatment with IFN- γ. The transcripts of TGF-β remained almost unaffected by both treatments. This might be due to the fact that ESdM were not exposed to intrinsic and extrinsic factors present in the brain.

NOS2 is a member of a family of enzymes that contributes to neuronal transmission and the immune system. NOS2 synthesizes nitric oxide from the terminal nitrogen atom of L-arginine (Ignarro *et al.,* 2001). The nitric oxide can react with superoxide leading to peroxynitrite formation and cell toxicity. Thus, NOS2 is believed to play a role in host immunity, anti-microbial and anti-tumor responses via the oxidative burst of macrophages (Chinje *et al.*,1997).

TNF-α is cytokine involved in the acute phase reaction or systemic inflammation. The major function of TNF-α is the regulation of immune cells, but it is known to be involved in inflammation, apoptosis and inhibition of tumorigenesis (Old *et al.*, 1985; Gaur *et al.*, 2003). It is known that macrophages and microglia release large amounts of TNF-α in response to LPS or other bacterial products (Gosselin and Rivest, 2007). The inducibility of NOS2 and TNF-α indicates that IFN- γ and LPS are immunostimulatory substances for ESdM *in vitro* and that ESdM react to inflammatory stimuli comparable to primary microglia.

The also analyzed TGF-β is one of the cytokines of the TGF-family. TGF-β has multifunctional properties and is, among others, involved in cell proliferation and differentiation. Furthermore, it has a direct anti-inflammatory impact on Th1 cells signaling (Massagué *et al.*, 1992). Due to its anti-inflammatory properties, TGF-β is also involved in tumor progression and immune suppression (Michiel *et al.*, 1992).

Another feature of microglial cells is the capability of migration. To study the migratory potential of ESdM, a migration assay using CX3CL1 was performed. CX3CL1 is constitutively expressed by neurons in a soluble and a membrane-bound form, which both promotes chemotaxis (Re and Przedborski, 2006). It was previously shown that microglia express the corresponding receptor CX3CR1 in a GFP-CX3CR1 transgenic mouse (Jung *et al.*, 2000). Napoli *et al.* showed in 2009 that ESdM express CX3CR1 to a similar extent as primary microglia (Napoli *et al.*, 2009). Therefore, one could expect that ESdM migrate towards CX3CL1 in a microglia-like fashion. Using a transwell migration system, the migration of ESdM towards different concentrations of CX3CL1 was analyzed. The outcome clearly showed that ESdM are capable of migration in a dose-dependant manner. Migration rate increased up to a concentration of 20 ng/ml CX3CL1 and almost doubled in comparison to non-directed migration. Thus, ESdM respond to the CX3CL1 by migration along a concentration gradient towards the source of the chemokine.

Activated microglia are known to perform phagocytosis, which is a highly coordinated process and mainly regulated by environmental signals (Napoli and Neumann, 2008). One of the standard assays to determine the phagocytosis capacity of cells is the uptake of fluorescent microsphere beads (Steinkamp *et al.*, 1982). Therefore, ESdM were incubated with fluorescent microsphere beads and number of positive cells was determined via flow cytometry. Unfortunately, it is not possible to distinguish between cells that have ingested beads and cells with beads attaching at the cell surface. Thus, only cells with a higher fluorescence signal than from one bead were taken into account. The assay revealed that over 20% of the ESdM were phagocytically active. In order to activate the ESdM, they were treated with LPS, which led to an increase in microsphere bead uptake. To further improve this method, a threshold for unspecific fluorescence of beads on the cell surface could be obtained by using cytochalasin-D, which blocks phagocytosis (Robinson, 1978).

4.2.4. Subdifferentiation of ESdM

It is known for several years that cells from the monocyte-macrophage lineage are heterogenous in regard to the response to microenvironmental signals and can be distinguished into several alternative polarized activation modes (Gordon *et al.*, 2003; Mosser *et al.*, 2003). The classical activation of macrophages via microbial molecules of pro-inflammatory cytokines results in a phenotype called M1, which produces pro-inflammatory cytokines and NO. The alternative activation leads to a phenotype called M2, which is considered an anti-inflammatory subtype (Mantovani *et al.*, 2004). The M1 and M2 subtype represent the most extreme forms of the polarization continuum, as macrophages can undergo a highly differentiated modulation of properties in response to their environment.

So far, little is known about the capacity of microglia to undergo a similar subpolarization. However, some evidence for microglia subdifferentiation arouse during the last years. Butovsky and colleagues showed in 2006 that microglia stimulated with IL-4 are able to support neuronal survival, oligodendrogensis and neurogenesis (Butovsky *et al.*, 2006). Most recently, some pioneer work was accomplished regarding the differentiation spectrum of M1 and M2 microglia (Michelucci *et al.*, 2009). The publication nicely showed that upon stimuli with IFN-γ cells expressed the inflammatory cytokines IL-6 and TNF-α, whereas stimulation with IL-10 resulted in expression of anti-inflammatory cytokines.

To evaluate whether the generated ESdM would react in a similar fashion, they were treated with IFN-γ and LPS to trigger the M1 (neurotoxic) or with IL-4 for the M2 (neuroprotective) subdifferentiation. Treatment with IFN- γ and LPS resulted in an upregulation of genes assigned to the M1 subtype, namely iNOS, CXCL10 and TNF-α. Immunocytochemistry revealed that ESdM treated for neurotoxic subdifferentiation expressed CD86, iNOS and CD16/32, which goes along with the findings in macrophages (Mantovani *et al.*, 2004). Treatment of ESdM with IL-4 on the other hand resulted in gene expression of anti-inflammatory molecules such as arginase, FizzI, IL-10 and YM1. Staining against arginase and CD206 showed that cells treated with IL-4 expressed slightly more of these M2 markers than ESdM treated with IFN-γ and LPS.

These findings indicate that the ESdM are capable to subdifferentiate into different

subtypes, but additional experiments have to be performed to further validate the outcome of the experiments. If later experiments will proof the successful subdifferentiation of ESdM, it would be of prosper interest to apply the subdifferentiated cells into mouse models of neurodegenerative diseases to evaluate the neurotoxic versus the neuroprotective properties of the ESdM subtypes.

4.3. Therapy of experimental autoimmune encephalomyelitis by NT3-GFP-ESdM

MS is the most prevalent cause of disability caused by neurological impairment in young adults. The hallmarks of MS are immunce cell infiltration, demyelination and axonal damage (Hemmer *et al.*, 2002). As the CNS fails to remyelinate the axons in the lesion sites, disease course results in irreversible functional losses of patients (Ferguson *et al.*, 1997).

Recent studies have shown that transplantation of neural precursor cells in EAE afflicted mice attenuated the inflammatory processes and clinical severity of disease (Einstein *et al.*, 2003; Pluchino *et al.*, 2005). The role of microglia implemented in MS disease makes them a potential target for therapeutical approaches. In MS, they not only attack the myelin sheeth of neurons, they also secrete inflammatory cytokines. Thus, altering microglia properties in favor of neuro-protective functions could resolve inflammation in MS lesions and lead to beneficial effects in regard to disease course. As NT3 is one of the most potent neurotrophins and has been shown to enhance neuronal regeneration, it was used to genetically modiefy ESdM used for therapeutic experiments in EAE afflicted mice.

4.3.1. Beneficial effects of NT3-GFP-ESdM supernatant on neurons *in vitro*

ESdM were lentivirally transduced with vectors expressing GFP or GFP and NT3 under the constitutively active PGK-promotor to evaluate the effects of NT3 in *in vitro* experiments.

NT3 was shown to exert a neuroregenerative effect in axonal injury (Guo *et al.*, 2006) and to play a pivotal role in cortical reorganization after spinal cord injury (Ramu *et al.*,

2007). Furthermore, NT3 was demonstrated to enhance axonal plasticity in injured spinal cords of rats (Zhou *et al.*, 2003). It is closely linked to survival and maintenance of a variety of neural populations and stimulates axonal outgrowth and neurogenesis (Chen et al., 1996; Memberge et al., 1995).

Thus, one would expect a positive effect of the supernatant from NT3-GFP-ESdM on neurons, as the supernatant likely contains a sufficient amount of NT3 to trigger neuronal regeneration. To validate this hypothesis, primary cultures were exposed to either water, GFP-ESdM supernatant, NT3-GFP-ESdM-supernatant or recombinant mouse NT3. After 72 hours, the neuronal cultures were stained for GAP43, which is highly expressed in neuronal growth cones. Therefore, GAP43 can be used as a marker for neuronal growth and plasticity (Benowitz *et al.*, 1997). Staining for GAP43 revealed a growth promoting effect of the NT3-GFP-ESdM supernatant on the neuronal culture.
Neurons cultured with medium supplemented with either supernatant of NT3-GFP-ESdM or 10 ng/ml recombinant NT3 showed a strong staining intensity for GAP43. Furthermore, neurons cultured under these two conditions appeared to be more viable and more axons were found. Taking together, these findings indicate that the secretion of NT3 by NT3-GFP-ESdM is sufficient to trigger GAP43 expression and therefore promote neuronal outgrowth in primary neuronal cultures.

4.3.2. Effect of NT3-GFP-ESdM on experimental autoimmune encephalomyelitis afflicted mice *in vivo*

To reveal the effect of transduced ESdM *in vivo*, EAE-afflicted mice were treated with PBS, GFP-ESdM or NT3-GFP-ESdM. EAE was induced by injection of activated MOG-emulsion into both inguinal lymph node regions of female C57BL/6J mice. About 12 days post induction, the first clinical symptoms could be seen. The clinical score of the mice rose to a clinical score of 3 at day 19.
To determine if the ESdM migrate to the EAE lesion sites in the spinal cord, a migration analysis was performed. Thus, spinal cord samples were collected and analyzed at different time points after intravenous GFP-ESdM cell injection. Already 24 hours post injection, GFP-positive cells could be found in the lumbal part of the spinal cord in close

vicinity to the inflamed regions. As the number of GFP positive cells increased over time, one could assume that the ESdM stably engraft in the spinal cord tissue. It is most likely that the migration of ESdM is due to the attraction to the inflammatory sites via chemokines. One of the candidates to orchestrate this migration is CX3CL1, as it is reported to be highly expressed in EAE (Sunnemark *et al.*, 2005). Though, the question remains if the grade of inflammation plays a role in the migration process of ESdM to the lesion sites, as ESdM did not home to the CNS in healthy mice. Apparantly, an inflammatory stimulus derived from the spinal cord lesions is necessary for the homing process. The co-localization of GFP with the microglial marker Iba1 additionally indicates the microglia fate of the ESdM upon engraftment in the spinal cord tissue. Taken together, these findings show that ESdM are a suitable tool to locally deliver trophic substances to the lesion sites as physiological vehicles.

Until now, several studies aiming at various therapeutical approaches for EAE as a model for MS have been carried out. Previous to our work, it has been shown that human neural precursors derived from ESCs have a therapeutic effect on EAE-afflicted mice (Aharonowiz *et al.*, 2008). Human neural precursors had a similar beneficial effect on the clinical course of EAE like ESdM, but had to be transplanted into the lateral brain ventricles due to lacking migratory capacities.

In addition to NT3, a variety of other neurotrophic factors have been shown to abate the disease course of EAE. For example, the administration of NGF as well as ciliary neurotrophic factor (CNTF) was shown to reduce inflammation in EAE afflicted rats (Flügel *et al.*, 2001; Linker *et al.*, 2002).

It was postulated by Caggiula *et al.* that neurotrophins are promising candidates for MS due to the link between neurotrophin levels and complete remission of MS in the post-relapse phase (Caggiula *et al.*, 2005). Neurotrophins have been shown to be expressed endogeneously by T cells and other immune cells in human MS lesion sites (Hohlfeld *et al.*, 2006). In EAE, T cells and NK cells infiltrating the CNS produced NT3 and BDNF and potentially promoted neuronal protection during disease course (Hammarberg *et al.*, 2000). Furthermore, injection of NT3, but not of BDNF was shown to enhance regenerative sprouting in the corticospinal tract and enhanced neuronal repair (Schnell *et*

al., 1994). Lentiviral introduction of a NT3 gradient in the spinal cord lesion was also shown to increase the number of ascending sensory axons extending into the lesion sites (Taylor *et al.*, 2006). Thus, NT3 was chosen as a representative candidate for gene transfer in EAE.

Still, neuroregeneration remains a major challenge and is, among other issues, addressed in the here presented experiments.

To analyze the clinical course of the EAE after injection of 4 million GFP-ESdM or NT3-GFP-ESdM in comparison to PBS, the clinical score of the mice was measured on a daily basis. After administration of the cells or PBS on the clinical peak (score 3) of the disease, a beneficial effect could be observed upon cell treatment. Treatment with GFP-ESdM resulted in a mild improvement of clinical scores in comparison to the control group. However, the group treated with NT3-GFP-ESdM showed a highly significant decrease of clinical scores compared to both the PBS and the GFP-ESdM treated mice. This positive effect of the NT3-GFP-ESdM is most likely due to the local administration of NT3 to the spinal cord lesions, which promotes neurite outgrowth and helps to attenuate the inflammatory properties of the lesion sites. The following discussion will reveal further insights into the mechanisms underlying the beneficial effect of NT3-GFP-ESdM on EAE afflicted mice.

4.3.3. Evaluation of axonal injury and demyelination

One of the major hallmarks of MS and the murine model EAE is the demyelination of neurons followed subsequently by axonal injury and degeneration. Thus, the grade of demyelination and axonal injury in the spinal cord was obtained 40 days post induction of EAE after treatment with PBS, GFP-ESdM or NT3-GFP-ESdM, respectively.

Staining for myelin with Luxol Fast Blue showed smaller demyelinated areas in NT3-GFP-ESdM treated animals in comparison to the control group. This decresased LFB staining represents either demyelination itself or loss of myelin due to axonal destruction.

These data indicate that the treatment with NT3-GFP-ESdM scotches the inflammatory mechanisms underlying the demyelination process and/or leads to a higher remyelination rate in the inflammatory lesions.

In case of axonal damage, APP accumulates in the axons and becomes detectable through immunological staining. Axonal damage was significantly decreased in mice treated with NT3-GFP-ESdM.

It is known that microglia and macrophage cell activation contributes to MS and EAE pathology by antigen presentation to T cells and secretion of pro-inflammatory cytokines (Benveniste, 1997). The antigen presentation of microglia is based on the expression of molecules such as MHC class II, CD83 or CD40, which are essential for interaction of microglia with T and B cells (Aravalli *et al.*, 2007; Raivich and Banati, 2004). Furthermore, the expression of TLR is crucial for generation of neuroimmune responses (Jack *et al.*, 2007; Lee and Lee, 2002).

Rasmussen and colleagues demonstrated the persistent activation of microglia in the chronic phase of relapsing-remitting EAE, which leads to loss of neuronal synapses (Rasmussen *et al.*, 2007). More directly, activated microglia are not only involved in antigen presentation and inflammatory signaling, but contribute to the degradation of myelin through phagocytosis (Bauer *et al.*, 1994).

On the other hand, microglia were shown to also have a beneficial role in MS and EAE pathology. To give an example, microglia can secrete anti-inflammatory cytokines like IL-10 and TGF-β depending on the milieu in the CNS (Napoli and Neumann, 2009). This could explain the positive effect of GFP-ESdM on EAE afflicted mice, as secretion of these cytokines would decrease the inflammatory response and therefore weaken disease severity. Furthermore, microglia are capable of secretion of neurotrophic factors such as insulin-like growth factor-1 (IGF-1), BDNF or NT3, which might contribute to promoting neurogenesis (Napoli and Neumann, 2009). The stronger therapeutical effect of NT3-GFP-ESdM compared to GFP-ESdM nicely fits into these findings.

4.3.4. Immune cell infiltrates

As mentioned in chapter 4.2.1., microglia as well as macrophages play a pivotal role in MS and EAE pathology. Thus, the severity of EAE can be determined via the number of invading macrophages into the spinal cord. Staining for MAC-3, a macrophage marker, revealed that the number of macrophages does not differ significantly in the case of PBS and NT3-GFP-ESdM treated animals.

Another cell type which can be used to evaluate EAE disease is the T cell. In the EAE model used in this study, autoreactive myelin antigen-specific CD4+ T cells are activated and expanded within the peripheral lymphoid tissue (Wekerle *et al.*, 1994). Approximately 10 days post immunization with MOG, activated T cells start to cross the BBB in massive amounts, whereas non-activated T cells are unable to infiltrate into the CNS (Flügel *et al.*, 2001). The invasion of activated T cells subsequently leads to further recruitment and activation of T cells and production of effector cytokines. These cytokines attract immune cells like granulocytes, microglia and macrophages which further mediate inflammation and demyelination (Dijkstra *et al.*, 1992). Thus, EAE pathogenesis can be considered to be mostly T cell mediated as they orchestrate the autoimmune demyelinating inflammation in the CNS.

The analysis of T cell invasion in the EAE mice treated with either PBS, GFP-ESdM or NT3-GFP-ESdM revealed that the T cell number does not differ significantly in these groups. This finding indicates that the severity of disease was the same in all cases, therefore the therapeutic effect was due to the cell treatment.

4.3.5. Cytokine milieu and neural regeneration

In order to further analyze the underlying mechanisms of the beneficial effect of NT3-GFP-ESdM on EAE disease course, analysis of cytokine expression levels of the lumbal spinal cord tissue was performed 4 days after treatment via qRT-PCR. The results clearly showed a significant down-regulation of the pro-inflammatory cytokines iNOS, TNF-α, and IL-1β. Additionally, expression of anti-inflammatory cytokines NT3, TGF-β and IL-10 were found to be increased upon treatment with NT3-GFP-ESdM. Administration of GFP-ESdM resulted in a similar trend which mirrors the outcome of the analysis of the clinical score. Thus, GFP-ESdM and especially NT3-GFP-ESdM are able to promote an anti-inflammatory milieu in EAE lesions.

This finding might be the main cause for the therapeutic effect of the injected cells, as an interruption of the inflammatory signaling would abandon the mechanisms resulting in demyelination and neuronal injury. The cytokine micromilieu established by microglia was furthermore shown to determine the course of inflammatory processes in EAE, thus

is one of the important features to be addressed in therapeutical approaches (Carson, 2002; Heppner *et al.*, 2005).

iNOS is involved in the production of NO, a soluble cytotoxic mediator of macrophages which induces a reversible conduction block in demyelinated axons and axonal degeneration (Redford *et al.*, 1997). TNF-α as well as IL-1β are the most prominent cytokines which can be detected at the lesion sites of the CNS. They are released by activated microglia and inhibit neural outgrowth (Neuman *et al.*, 2002). Furthermore, they are known to induce neuronal cell death (Merrill *et al.*, 1992).

IL-10 and TGF-β both down-regulate the immune response and are closely linked to benign course of MS symptoms (Link, 1998; Navikas *et al.*, 1995). TGF-β is additionally known to be involved in T cell response inhibition (Navikas *et al.*, 1996). The observed upregulation of NT3 is clearly due to the presence of the NT3-GFP-ESdM, its beneficial effects have already been discussed.

Staining for the neurite outgrowth marker GAP43 revealed that in presence of NT3-GFP-ESdM, neurons regenerate in the EAE lesions in the spinal cord. This regenerative effect could be seen as the most important outcome of the study, as neural regeneration still remains a major problem in MS research.

Taken together, the anti-inflammatory milieu created by the administration of GFP-NT3-ESdM might be the key to the therapeutic effect and might play a crucial role for further therapeutical investigations.

4.3.6. NT3-GFP-ESdM as a tool for multiple sclerosis therapy

The results presented here revealed a promising possibility for NT3-GFP-ESdM as therapeutical tool for EAE. Using ESdM yields a couple of advantages in comparison to bone marrow derived myeloid precursor cells in respect to homing and engraftment properties.

ESdM are derived from a protocol mimicking the *in vivo* development of microglia including a yolk sac intermediate stage. They are able to cross the BBB after intravenous application in response to a lesion-associated trigger in the adult CNS without any physical or pharmacological manipulation of the organism. Furthermore, ESdM could be detected in the CNS up to 21 days post injection in the EAE spinal cord lesions, whereas

bone marrow derived cells disappear after four days (Takahashi *et al.*, 2007). Another advantage is the phenotype of the ESdM, which shows similarities to regulatory monocytes and M2 polarized macrophages (Rae *et al.*, 2007; Geissmann *et al.*, 2003). ESdM without genetic modification did not have any effect on the clinical score of EAE but also stably engrafted into the lesion sites.
Another advantage of ESdM is their availability in high numbers and pure cultures. Therefore, ESdM appear to be a safe and well suited tool for CNS gene therapy (Neumann, 2006).

Some problems and concerns still remain to be overcome to further validate this potential in regard to MS therapy.
Evaluating the results, one has to keep in mind that EAE cannot be equaled to MS, although EAE is used as a common model for MS. To give an example, the mechanisms underlying MS are still unclear (Degenhardt *et al.*, 2009), therefore it is difficult to compare MS to EAE. Nevertheless, EAE and MS share some disease features like the inflammatory lesions, immune cell infiltration, demyelination and axonal regeneration (Kornek *et al.*, 2000). It is therefore likely that at least part of the here discussed results found in murine EAE can be transferred to human MS.
The usage of NT3 as neurotrophic factor in this study might arise some further objections. It was reported that the systemic administration of high doses of NT3 in animal models evokes pronounced pain and gastrointestinal symptoms (Thoenen *et al.*, 2002). Something similar could be observed in humans in a clinical trial after intracerebroventricular infusion of another neurotrophic factor, NGF, which resulted in a constant pain sensation (Eriksdotter Jönhagen *et al.*, 1998). Anyway, it is not known if the delivery of NT3 used in this study does result in similar complications. If so, one would need alternatives to neurotrophic factors in general. It has been discussed previously that high dosages of neurotrophins might result in severe side effects such as epileptic activity (Binder *et al.*, 2001). In this here presented approach, these effects can be overcome due to the local administration of the NT3, which is effective in lower dosages and does not involve systemic side-effects. No visible side-effects in the mice

treated with NT3-GFP-ESdM could be seen, thus one would not expect major complications in treatment of patients with MS.

For further perspectives, especially for clinical trials in humans, the differentiation protocol presented here was modiefied to differentiate human induced pluripotent stem (iPS) cells into microglial cells. This might clear the way for broad application fields for the therapeutical usage of ESdM in neurodegenerative research. ESdM appear as a suitable vehicle and a promising tool to be used in therapeutical approaches for MS.

4.4. Phagocytosis of glioma cells through ESdM

Due to the highly invasive growth pattern of glioma cells, glioma are barely curable by standard therapies such as surgical resection, radio- and chemotherapy. In most cases, infiltrating glioma cells remain in the healthy tissue and cause tumor reformation. The role of microglia in tumor progression has been broadly discussed as they are known to be capable of acting in favor or against the tumor. Altering microglia properties could therefore be used in a therapeutical approach for glioma.

4.4.1. The cell lines GL261 and SMA560

In order to evaluate the effect of ESdM on glioma cells co-culture experiments with GL261 and SMA560 glioma cell lines were performed.

The GL261 glioma cell line is a commonly used mouse model for human glioblastoma. The original GL261 tumor was induced by intracranial injection of 3-methylcholantrene into C57BL/6 mice and maintained through serial transplantations of tumor pieces into syngeneic mice (Ausman *et al.*, 1970). Thus, GL261 thus were derived through artificial induction, which might not fully portray spontaneously arising human tumors.

Nevertheless, tumors derived from GL261 cells have similar growth characteristics and angiogenic properties like human glioblastoma. Mouse *in vivo* experiments have shown the invasive growth property of GL261, that is the main reason for therapy failure in human patients (Newcomb and Zagzag, 2009). Furthermore, GL261 cells carry point mutations in the *K-ras* and *p53* gene, which are also frequently found in cells derived

from human glioblastoma (Szatmári *et al.*, 2006). Thus, GL261 cells are used as a valid model for glioblastoma therapy. As the GL261 line is derived from C57BL/6 mice, it represents the syngeneic situation in the experiments, as ESdM are derived from the same mouse strain.

To further test the effectiveness of ESdM, a non-syngeneic set-up was also performed. For this purpose, the SMA560 cell line was used. In contrast to the artificially derived GL261 line, SMA560 cell line is derived from a spontaneously developing astrocytic tumor in VM/Dk mice. This mouse strain has a high incidence of spontaneous astrocytoma development (Fraser *et al.*, 1971). It has been demonstrated that the cell line is highly tumorigenic, retains glial differentiation features and produces high levels of TGF-β (Sampson *et al.*, 1997).

Both cell lines fulfill the requested properties of a valid animal glioma model as postulated by Wilson. They are of glial origin, capable of intraparenchymal growth, uniformly fatal in reasonable time, transplantable intracranially and subcoutaneously, correspond with therapeutic sensivity of human glioblastoma and can be cultured *in vitro* (Wilson, 1978).

Thus, these cell lines were used to assess data about interaction of ESdM with glioma cells to evaluate the therapeutical potential of ESdM. Both glioma cell lines were influenced at a similar level by the ESdM, thus the genetic background of the cell appears to have no effect on ESdM interaction with glioma cells.

4.4.2. The effects of ESdM on glioma cells

In proliferation assays it was shown that the presence of ESdM reduced the cell number of GL261 and SMA560, whereas control mono-cultures of glioma cells showed exponential growth rates. Therefore, the reduction of growth was not due to the N2 medium lacking FCS in which the experiments were performed. Glioma cells showed a similar growth behaviour in glioma medium containing FCS. It is also quite unlikely that the reduced cell number is caused by lack of space or nutrition, as glioma cells are able to grow in hypoxic and ischemic condition and grow in three-dimensional cell agglomerates upon space restriction (Gorin *et al.*, 2004). Nevertheless, one has to keep in mind that lacking FCS or splitting procedure before co-seeding might influence glioma cell

susceptibility to ESdM attack. The observed effect might also be more severe *in vitro* than *in vivo*, as tumor supporting cells like lymphocytes or endothelial cells are absent (Watters *et al.*, 2005). Interestingly, data obtained by the diploma student Julia Reinhardt also showed that the timing of seeding cells is of importance. Pre-seeded glioma cells showed less susceptibility to ESdM growth inhibition. This might be caused by the pre-established micromilieu of the glioma cells which overrides the immune response of microglia cells (Wiendl *et al.*, 2002).

The experiment did not reveal the cause of reduced glioma numbers, which could be due to decreased proliferation, enhanced apoptosis or phagocytosis of glioma cells by microglia. To further determine this aspect, Annexin V staining was performed to assess number of apoptotic cells in the co-culture system. The experiment showed that apoptosis levels were comparable in pure glioma cultures and co-cultures with ESdM. Thus, the reduced cell number in the co-culture is not due to an increase in apoptosis. Mora *et al.* could show that activated microglia decreased proliferation and migration of glioma cells and furthermore triggered autophagy-dependent glioma cell death (Mora *et al.*, 2009). The processes underlying the microglial anti-tumor properties are most likely mediated by chemokines produced by the ESdM. A study with primary microglia and microglia cell line BV-2 has previously shown that CathepsinB secretion by microglia might be involved in anti-tumor effects (Hwang *et al.*, 2009).

An analysis of the chemokine profile of the ESdM after co-culture with glioma cells performed by Kristian Welle revealed an upregulation of CX3CL1, CXCL9 and CXCL10. The soluble form of CX3CL1 is known to potently attract T cells and monocytes via interaction with the receptor CX3CR1 (Imai *et al.*, 1997).

CXCL9, also known as monokine induced by gamma interferon (MIG) is also a T cell chemoattractant, which is induced by IFN-γ. This chemokine interacts with the chemokine receptor CX3CR1 (Farber *et al.*, 1997). The third upregulated chemokine CXCL10 (also known as interferon inducible protein 10, IP-10) is also secreted in response to IFN-γ. This chemokine plays a role in several mechanisms, including chemoattraction of monocytes/macrophages, T cells, NK cells and dendritic cells, promotion of T cell adhesion to endothelial cells, anti-tumor activity and inhibition of angiogenesis (Sarris *et al.*, 1995; Angiolillo *et al.*, 1995; Farber *et al.*, 1997).

The induction of these cytokines is likely to mediate an anti-tumor milieu which subsequently leads to a decrease in tumor cell growth.

One of the major functions of microglia and macrophages is phagocytosis of microbes, dead cells or debris in the CNS. Experiments could show that the ESdM indeed phagocytosed glioma cells as particles of PKH26+ glioma cells were found inside ESdM after co-culture for 24 hours with SMA560 or GL261 cells. Microglia are furthermore capable of triggering glioma apoptosis through production of NO and cytotoxic molecules (Hwang *et al.,* 2009; Mora and Régnier-Vigouroux, 2009). In contrast, a mechanism which is called silent phagocytosis is taking place without inducing inflammatory processes (Kurosaka *et al.,* 2003). It has been postulated by Fadeel *et al.* that apoptosis is not neccessary for phagocytosis of glioma cells, but that the cell surface expression of molecules engaging phagocytic cells termed “eat me signal” is sufficient (Fadeel *et al.,* 2004). The equal level of apoptosis in the co-culture hints towards this phagocytosis mechanism, which can be referred to as “buried alive”.

During the last years, evidence arose that the accumulation of microglia in glioma is due to a local production of various chemoattractants and growth factors by the glioma cells. For example, monocyte chemotactic protein 1 (MCP-1, also known as CCL2) is secreted by glioma cells and interacts with the microglial receptor CCR2 (Galasso *et al.*, 2000). Additionally, various gliomas secrete growth factors which are known to induce macrophage/microglia proliferation such as CSF-1 or granulocyte-colony stimulating factor (G-CSF) (Alterman *et al.*, 1994). Despite the fact that microglia are recruited in high numbers to the tumor site, phagocytosis of glioma cells or debris by those microglia was not observed (Hao *et al.*, 2002). Furthermore, even a tumor promoting role has been proposed for microglia, as they might facilitate immunosuppression of the tumor microenvironment (Badie *et al.,* 2001). The secretion of MMP by microglia is one example for a proces which can help to increase the tumor proliferation rate (Rao *et al.*, 2003). Umemura *et al.* demonstrated that tumor-infiltrating microglial cells obtain an immuno-suppressive phenotype and promote TGF-β production via an autocrine loop (Umemura *et al.*, 2008). This immuno-suppressive role of microglia and the promoted

anti-inflammatory cytokine mileu play a crucial role in tumor tolerance, as both leads to CD4+ T cell tolerance and inactivation of cytotoxic CD8+ T cell mediated tumor targeted immune responses (Carpentier and Meng, 2006). It was nevertheless shown that the immuno-suppression of the glioma can be conquered. One attempt to overcome the immunosuppression was performed by Carpentier and colleagues, who demonstrated long-term survival of rats with glioma after single-dose administration of CpG oligodeoxynucleotide, an immuno-stimulatory molecule that enhances microglial production of IFN-α, IFN-β, IL-12 and TNF-α via stimulation of TLR9 (Carpentier *et al.*, 2000; Auf *et al.*, 2001). Furthermore, IL-12 restores anti-cancer properties of microglia and enhances elimination of cancerous cells (Chiu *et al.*, 2011) and could be thus used to further increase anti-tumor properties of ESdM.

Apparantly, ESdM were able to overcome the immunosuppression at least in the *in vitro* co-culture and diminished glioma cell number through phagocytosis.

4.4.3. Effect of ESdM polarization

It was demonstrated that especially the classical activated M1 subtype is known for killing tumor cells. On the other hand, the M2 subtype shows pro-tumoral properties by suppressing immune responses (Mantovani *et al.*, 2004).

Thus, this paradigm was investigated in the ESdM-glioma co-culture. ESdM were polarized into M1 or M2 subtype before using them for co-culture experiments. M1 polarization led to a slightly enhanced anti-tumor behavior of ESdM, whereas M2 treatment only had little effect in comparison to control ESdM. M2 microglia are assigned to tumor promoting functions, furthermore M2 microglia strongly resemble TAMs (Mantovani *et al.*, 2004b).

Increased anti-tumor properties in the M1 subtyped are owed to a variety of changes in microglia functions. M1 microglia secrete pro-inflammatory and cytotoxic factors such as IL-1β, NO and superoxide anions which might play a role in reducing glioma cell number (Goerdt and Orfanos, 1999). M1 polarization leads to enhanced production of inflammatory CC motif chemokines and IFN-γ responsive chemokines, which recruit NK cells and Th1 cells to the tumor site and result in higher tumor resistance (Mantovani *et al.*, 2004). It has been shown that inflammation triggers an anti-glioma immune response,

higher phagocytic activity and also leads to killing of tumor cells (Auf *et al.,* 2001; Nickles *et al.,* 2008). M1 activated microglia are also known to secrete enhanced levels of CXCL10, which has an anti-tumor effect and inhibits angiogenesis (Angiolillo *et al.,* 1995; Farber *et al.,* 1997).

FACS analysis revealed that M1 polarization also led to an increase in expression of phagocytic receptors Trem2 and SiglecH, which will be discussed in detail later on.

Nevertheless, one has to keep in mind that the subpolarization of microglia is a highly sensitive process. Thus, the tumor micromilieu is quite likely to reverse the effects of *in vitro* subpolarization into M1 subtype. This objective could be overcome via usage of protein tyrosine phosphatase SHP-1 knock-down cells. SHP-1 is a negative regulator of pro-inflammatory gene transcription, thus a lack of SHP-1 results in increased inflammation in the CNS (Christophi *et al.,* 2009). ESdM lacking SHP-1 are likely to be immune to the tumor micromilieu and could lead to higher clearance of tumor cells *in vivo*.

4.4.4. Function of DAP12 in microglia-glioma interaction

DAP12 is a short polypeptide consisting of an extracellular tail, a single transmembrane domain and a cytoplasmic ITAM (Turnbull and Colonna, 2007). DAP12 is a signaling molecule found in macrophages and microglia that functions via the tyrosine kinase SYK and protein kinase ZAP-70 which bind to the ITAM (Turnbull and Colonna, 2007). The signaling cascade triggered through protein tyrosine phosphorylation leads via various down-stream pathways such as extracellular-signal-regulated kinases (ERKs) to effector function and cell activation. Functions include cytokine secrection by macrophages and enhanced phagocytosis (Colonna, 2003). It has previously been demonstrated that DAP12 controls the phagocytic properties of microglia and the inflammatory response followed by neuronal death (Takahashi *et al.,* 2005).

Furthermore, DAP12 intersects with the TLR-pathway and alters the release of TNF-α, IL-6 and IL-12, a set of cytokines which is crucial to innate and adaptive immune response and pathogen clearance (Hamerman *et al.,* 2009). In regard to tumor response it is of interest that mice lacking proper DAP12 function show impaired immune responses including non-functional NK cell activation and disabled Th1 response (Tomasello *et al.,*

2000; Bakker *et al.*, 2000). Both of these features are well known to be involved in anti-tumor responses in the CNS. In addition to the pro-inflammatory and phagocytosis-enhancinc features, DAP12 was also described to mediate inhibitory signals. Primary microglia lacking functional DAP12 express higher levels of iNOS, IL-1β and IL-6 (Roumier *et al.*, 2008).

In the here presented experiments, the overexpression of DAP12 led to an enhanced anti-tumor effect of ESdM, which is most likely due to the enhanced phagocytosis. To be more precise, the anti-tumor effect appears to be mediated via the ITAM of the DAP12, as a dominant negative DAP12 form lacking that motif displays only little anti-tumor properties. Without a phosphorylated functional ITAM, kinases are unable to bind and downstream signal transduction is blocked.

In summary, the obtained results underline the discussed role of DAP12 in microglia-glioma interaction as described in the literature. DAP12 signaling could be a promising target for immune therapy in glioma, although one has to keep in mind that DAP12 signaling affects a broad variety of receptors and functions.

4.4.5. The role of Trem2 and SiglecH

Phagocytosis has been shown to involve ITAM signaling, which in mammal microglia is mainly mediated via DAP12 (Ziegenfuss *et al.*, 2008). Trem2, complement receptor 3, signal-regulatory-protein (SIRP) β-1 and SiglecH belong to the DAP12-associated receptors (Ivashkiv, 2009; Lanier 2009). To investigate the receptors involved in glioma phagocytosis, Trem2 and SiglecH were chosen as representative candidates.

In both cases, knock-down of the receptors led to a lower phagocytosis rate of glioma cells through ESdM and higher proliferation rates of glioma cells in the co-culture. Thus, data imply that Trem2 and SiglecH play a role in microglia-glioma interaction and anti-tumor properties.

SiglecH is a DAP12-associated CD33-related Siglec which is found specifically in mice (Crocker *et al.*, 2007). SiglecH signals through the ITAM of DAP12, which is in contrast to most siglecs signaling via immunoreceptor tyrosine-based inhibition motif (ITIM). Until now, function and ligand of SiglecH remain unclear. However, its involvement in

the uptake of antigens and in the modulation of cellular functions via DAP12 signaling has been demonstrated (Blasius and Colonna, 2006).

It could be shown that SiglecH Fc fusion protein binds to glioma cells, but not to astrocytes (Beutner and Kopatz, unpublished data). Stimulation of ESdM into M1 subtype led to an increase in SiglecH expression and phagocytosis rate, thus data implies an involvement of SiglecH in phagocytosis activation. This hypothesis is also underpinned as knock-down of SiglecH resulted in lower phagocytosis rates. Preliminary data gave first clues that sialylation of surface glycoproteins might play a role in SiglecH-mediated microglia-glioma interaction. Siglecs have also been mentioned in regard to cell-targeted therapies. They can be used for the "Trojan horse" strategy in which therapeutic agents conjugated to an antibody bind to Siglecs and are efficiently carried into the cell (O´Reilly and Paulson, 2009). This property could be used to "load" ESdM with therapeutic agents and deliver them into the tumor residuum via tumor-initiated microglia attraction.

One has to bear in mind that SiglecH is exclusively found in mice. Nevertheless, the human Siglec16 was shown to be a DAP12-associated receptor and has a similar structure like SiglecH (Cao *et al.*, 2008). It would be therefore of interest to investigate the role of Siglec16 in human induced pluripotent stem cell derived microglia in interaction with human glioma lines such as U87.

Trem2 is a DAP12-associated receptor expressed on myeloid cells such as macrophages and microglia. Trem2 was described to be involved in CNS immune homeostasis (Neumann and Takahashi, 2007). Alike SiglecH, the ligands of Trem2 are not known so far. Its role in phagocytosis has been demonstrated in several studies. Expression of Trem2/DAP12 in Chinese hamster ovary cells was shown to enhance immune answer and phagocytosis of bacteria (N`Diaye *et al.*, 2009). Takahashi *et al.* have shown that Trem2-transduced myeloid precursor cells mediated clearance of tissue debris via phagocytosis in EAE (Takahashi *et al.*, 2007).

In contrast to the before discussed beneficial effects of inflammation in regard to anti-tumor responsed, Trem2 is a negative regulator of inflammatory responses (Takahashi *et al.*, 2007) and its expression on macrophages is decreased after treatment with LPS

(Turnbull *et al.*, 2006).

Stimulation of ESdM towards M1 subtype led to a slight increase in Trem2 expression, which might also be responsible for enhanced phagocytosis after stimulation. Nevertheless, in comparison to knock-down of DAP12 and SiglecH the effects of the Trem2 knock-down were less potent. One might thus assume that Trem2 is not the main receptor involved in glioma-microglia interaction.

4.4.6. Evaluation of ESdM as therapeutic tool for glioma

Although microglia are known to be involved in tumor progression, there is accumulating evidence that they might be a suitable tool for therapeutic application.

Clearly, microglia are situated ideally within the CNS to confront migrating and resident tumor cells. Another beneficial property of microglia is their ability to migrate from the blood stream to the CNS, which opens up interesting possibilities for therapeutic gene transfer into mature CNS tissue and the tumor residuum (Flügel *et al.*, 2001). As microglia are closing the gap between the immune-privileged CNS and the peripheral immune system, they are an attractive cell type in immunotherapy for glioma (Yang *et al.*, 2010). Innate microglia associated with gliomas appear to be incapable of inducing effective anti-tumor responses. But if this problem could be overcome by administration of ESdM, CNS immunity against tumors could be significantly enhanced. Additionally, treatment with ESdM yields less problems than treatment of tumors with neural or mesenchymal stem cells (Herrlinger *et al.*, 2000; Aboody *et al.*, 2000; Uhl *et al.*, 2005), which all include genetic modifications of the cells. Furthermore, ESdM can be obtained in virtually unlimited numbers by differentiation of ESC or iPS obtained from the patients, respectively.

It would be of interest if a beforehand subpolarization of ESdM into the M1 anti-tumor subtype would result in an even stronger therapeutic effect. Additionally, chemo-attractants for cytotoxic immune cells could be added to the ESdM treatment in order to maximize the beneficial effect of the therapy.

To conclude, ESdM might be a suitable and powerful tool for immunobased cell-therapy of glioma, though further studies are needed to fully evaluate their therapeutic properties.

5. Summary

Microglia are the resident immune cells of the central nervous system (CNS). They are known to have detrimental as well as beneficial effects. To overcome the limitations of primary microglia, embryonic stem cell derived microglia precursor cells (ESdM) were differentiated out of mouse embryonic stem cells. ESdM showed expression of microglial markers such as Iba1, CD45 or CD68, but lacked stem cell markers. Stimulation of ESdM with Interferon-γ (IFN- γ) or lipopolysaccharides leads to an up-regulation of inflammatory cytokines and to increased phagocytosis. Furthermore, ESdM migrate in a dose-dependent manner towards fractalkine CX3CL1. Gene expression of ESdM resembles that of primary microglia and preliminary data indicate that they can be subpolarized into a neuro-toxic or a neuro-protective subtype by IFN-γ or interleukin-4.

After lentiviral transduction of ESdM with Neurotrophin 3 (NT3), they were applied to experimental autoimmune encephalomyelitis (EAE) afflicted mice to reveal possible therapeutic chances for the treatment of multiple sclerosis (MS). EAE mice treated with NT3-green fluorescent protein (GFP)-ESdM showed stable recovery of clinical symptoms, accompanied by less demyelination and less axonal damage in the spinal cord tissue. ESdM migrated to the lesions and promoted an anti-inflammatory cytokine profile. Furthermore, in close proximity to the NT3-GFP-ESdM, the axonal growth protein GAP-43 could be found, indicating neural regeneration due to the presence of NT3. To summarize, NT3-GFP-ESdM can be considered a promising tool for therapeutic approaches to EAE as a model of MS.

ESdM were also applied in co-culture systems with glioma cells to determine their potential for therapeutical approaches. ESdM phagocytosed glioma cells and reduced glioma cell number *in vitro*. Phagocytosis and proliferation inhibition were enhanced through subpolarization of ESdM into M1 subtype. The anti-tumor effects were most likely mediated via DAP12 and the DAP12-associated receptors SiglecH and Trem2. Knockdown of these molecules decreased the anti-tumor activity of ESdM, while overexpression of DAP12 resulted in stronger anti-tumor effects. Thus, ESdM could provide functions to also fight glioma *in vivo*.

In summary, ESdM might provide a broad range of possible applications in therapeutical approaches of CNS diseases.

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8. Declaration

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

Hiermit versichere ich, dass die vorgelegte Arbeit – abgesehen von den ausdrücklich bezeichneten Hilfsmitteln – persönlich, selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde. Aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle kenntlich gemacht worden.
Die vorliegende Arbeit wurde an keiner anderen Hochschule als Dissertation eingereicht. Ich habe früher noch keinen Promotionsversuch unternommen.

Clara Beutner Bonn, January 2012