Establishment of Embryonic Stem Cell Derived Microglial Precursors and Application in an Animal Model of Alzheimer's Disease

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Für meine Eltern

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Abbreviations

AD - Alzheimer's disease
ANOVA - analysis of variance
APP - amyloid precursor protein
ATP - adenosine triphosphate
Aβ - amyloid β
BACE - β-site APP-cleaving enzyme
bFGF - basic fibroblast growth factor
BSA - bovine serum albumine
C - carboxy
CCR - chemokine receptor
CD - cluster of differentiation
DNA - deoxyribonucleic acid
CFSE - carboxyfluorescein diacetate succinimidyl ester
CNS - Central nervous system
CX3CR1 - fractalkine receptor
CX3CL1 - fractalkine
DAPI - 4',6-diamidino-2-phenylindole
E - embryonic
EB - embryoid bodies
EGF - epidermal growth factor
eNOS - endothelial NOS
ESdM - embryonic stem cell derived mircoglial precursor
FACS - fluorescence activated cell sorting
FBS - fetal bovine serum
FITC - fluoro-isothiocyanate
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GFP - green fluorescence protein
GM-CSF - granulocyte-macrophage colony stimulating factor
hAPP - human APP
ICAM - intercellular adhesion molecules
ICM - inner cell mass
IFN - Interferon
Ig - immunoglobulin
IL - Interleukin
iNOS - inducible NOS
ITSFn - insulin transferring selenit fibronectin
Kg - kilogram
LFA - leukocyte function-associated molecule
LIF - leukemia inhibitory factor
LPS - lipopolysaccharides
MCP - monocyte chemoattractant protein
MEF - mouse embryonic fibroblast
mES - murine embryonic stem
Mg - miligram
MHC - major histocompatibility complex
MIP - macrophage-inflammatory protein
mm - millimeter
NFκB - nuclear factor-kappa B
NFT - neurofibrillary tangles
nGS - normal goat serum
nNOS - neuronal NOS
NO - nitric oxide
NOS - nitric oxide synthase
P - postnatal
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PE - phycoerytin

PFA - paraformaldehyd
PGK - phosphoglycerate kinase
PLL - poly-L-lysine
PS - presenilin
PtdSer - phosphatidylserine
RA - retinoic acid
RNA - ribonucleic acid
RT - reverse transcriptase
sAPPα - soluble APPα
SEM - standard error of the mean
SSEA - specific cell surface antigen
TGF - transforming growth factor
TLR - toll-like receptors
TNF - tumor necrosis factor
UDP - uridine triphosphate
µm - micrometer
Ψ - retroviral packaging signal

1 Introduction

1.1 Microglia

The central nervous system (CNS) consists of two main cell types: neurons and glial cells. Neurons constitute about half the volume of the CNS and glial cells make up the rest. Glial cells provide support and protection for neurons. They are thus known as the "supporting cells" of the nervous system. The four main functions of glial cells are: (1) to surround neurons and hold them in place, (2) to supply nutrients and oxygen to neurons, (3) to insulate one neuron from another, and (4) to destroy and remove debris of dead neurons. In the vertebrate CNS, glial cells are divided into two major classes: microglia and macroglia. Commonly, astrocytes and oligodendrocytes are referred as macroglia.

Astrocytes are a subtype of glial cells and characteristically star-shaped. They perform many functions, including the formation of the blood-brain barrier, the provision of nutrients to the nervous tissue, and they play a principal role in the repair and scarring process in the brain. Therefore, astrocytes exhibit mainly a supportive function in the CNS.

In contrast, the main function of oligodendrocytes is the myelination of axons exclusively in the CNS of higher vertebrates, a function performed by Schwann cells in the peripheral nervous system. Each oligodendrocyte can wrap numerous axons, and each axon is ensheathed by periodically spaced processes extending from multiple oligodendrocytes. Rapid conduction of nerve impulses in vertebrates requires that most large-diameter axons be wrapped by myelin. When oligodendrocytes are damaged and myelin is disrupted, the neurons cannot communicate resulting in paralysis or motor dysfunction.

Microglia are currently accepted as immune cells in the CNS that respond to injury and brain disease. The main function of microglia is believed to be brain defense, as they are known to scavenge invading microorganisms and dead cells, and also to act as immune cells. However, microglia are also thought to contribute to the onset of or to exacerbate neuronal degeneration as well as inflammation in many brain diseases by producing deleterious factors including superoxide anions, nitric oxide and inflammatory cytokines. Nonetheless, there is accumulating evidence that microglia produce neurotrophic and/or neuroprotective molecules; in particular, it has been suggested that they promote neuronal survival in cases of brain injury. The fact that microglia act like

a ‘double-edged sword’, either in a neurotoxic or in a neuroprotective way, has gained much recent attention.

1.1.1 Origin of microglia

In 1913, Santiago Ramón y Cajal described the ‘third element’ of the nervous system, a cell population distinct from neurons and neuroglia (otherwise termed *astroglia*). Microglia make up one-fifth of the CNS glial population and are believed to be of mesodermal origin as it was originally suggested. However, the origin of microglia remains still a matter of intense debate (Chan *et al.*, 2007). For a long time, mainly due to the lack of cell type specific markers, the origin of microglia was unclear. In the 1980s, cell staining techniques assumed the myeloid origin of microglia, whereas macroglia and neurons arise from neuroectoderm. However, there was never the formal proof that microglia cannot arise from the neuroectodermal lineage as well.

Microglia are widely regarded as the resident mononuclear phagocytes of the nervous system. Apart from the lack of concrete evidence, nowadays, there is good evidence that two types of microglia coexist within the brain. First, it is assumed that resident microglia, which are derived from the mesoderm, colonize the nervous system primarily during embryonic and fetal development (Barron, 1995; Cuadros and Navascues, 1998). Second, it is believed that in the adulthood, bone marrow derived microglia are recruited from the blood and/or bone marrow into the CNS in response to an appropriate stimulus as seen under pathological conditions like in Alzheimer’s disease (Simard and Rivest, 2004; Priller *et al.*, 2006; Simard *et al.*, 2006).

1.1.2 Microglial markers

Characterization of microglial cells with regard to surface expression markers is difficult, as these cells share several antigens with macrophages making them undistinguishable to those. However, several studies using flow cytometry or classic immunostaining defined a profile of microglia corresponding to the following selected antigens: $CD68^+$, CD45 low, $CD11b^+$, CD11c high, MHC class II^+, $IBa1^+$ and $F4/80^+$. The lack of a defined marker for the distinction of microglia and macrogphages is still a major problem in neurobiology (Guillemin and Brew, 2004).

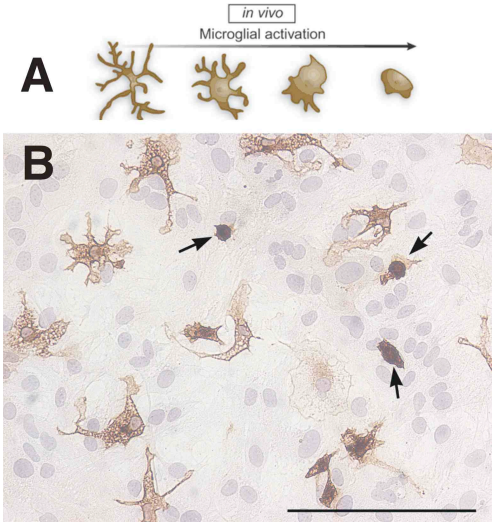

Figure 1-1. Microglia in a murine mixed glial culture. A. Upon activation microglia change from a ramified phenotype into an reactive round-shaped cell (Kettenmann, 2006) (modified). **B.** Bright field image of a murine primary cortical mixed glial culture stained with the microglial marker Tomato lectin (brown) and counterstained with hematoxylin (blue). Three of them, identified with arrows, are round-shaped microglial cells with a strong lectin staining (modified from Saura, 2007).

1.1.3 Microglial phenotypes and motility

Since microglia are the immune effector cells of the CNS, they exist in three distinct forms known as amoeboid, ramified and reactive/activated microglia, which serve different functional roles as outlined below (Fig.1-1).

1.1.3.1 Amoeboid microglia

Amoeboid microglia are associated with the developing CNS. In rats, it has been shown that amoeboid microglia appear late in gestation and disappear soon after birth (Ling *et al.*, 1980; Dalmau *et al.*, 1997). These cells exhibit a round cell body, possess pseudopodia and thin filopodia-like processes and contain numerous lysosomes indicating a motile phagocytic phenotype. During the post-natal period amoeboid microglia are believed to play a role in tissue homeostasis through the removal of inappropriate and unwanted axons (Innocenti *et al.*, 1983; Marin-Teva *et al.*, 2004; Stevens *et al.*, 2007) and through the promotion of axonal migration and growth (Polazzi and Contestabile, 2002). Ultimately, amoeboid microglia develop long crenulated processes and transform into ramified microglia found in the adult CNS (Ling, 1979; Kaur and Ling, 1991).

1.1.3.2 Ramified microglia

Ramified microglia are abundantly present in the brain parenchyma and constitute approximately 10-20% of the total glial cell population in the adult (Vaughan and Peters, 1974; Banati, 2003). These small round cells consist of numerous branching processes and possess little cytoplasm. In the adult brain the resident population of ramified microglia is maintained through local cell division (Lawson *et al.*, 1992). Classically, ramified microglia were considered to be inactive under physiological conditions. However, it is now known that microglia exhibit pinocytotic activity and localized motility (Booth and Thomas, 1991; Glenn *et al.*, 1991). *In vivo* two-photon microscopy studies in living mice showed that microglial processes are substantially motile, and survey their local surroundings through formation of random filopodia-like protrusions, extension and withdrawal of bulbous endings (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Microglial processes directly contact neuronal cell bodies, astrocytes and blood vessels (Nimmerjahn *et al.*, 2005). Therefore it seems likely that microglia monitor the well-being of brain cells and also clean the extracellular fluid in order to maintain central homeostasis (Booth and Thomas, 1991; Thomas, 1992; Fetler and Amigorena, 2005). This behavior of microglia is in agreement with a recent observation made for tissue macrophages, namely that their filopodia act as phagocytic tentacles that engulf debris and retracts them towards the cell body through a force-driven mechanism (Kress *et al.*, 2007).

1.1.3.3 Reactive microglia

In response to injury or pathogen invasion, quiescent ramified microglia proliferate and transform into active 'brain macrophages' also known as reactive microglia (Kreutzberg, 1996; Stence *et al.*, 2001). Reactive microglia, which are involved in brain injury and neuroinflammation, are rod-like shaped, devoid of branching processes and contain numerous lysosomes and phagosomes. Under pathological conditions microglial cells polarize and converge their processes within hours towards the lesion site followed by migration towards the lesion site, where they play a neuroprotective role by phagocytosing damaged cells and debris (Davalos *et al.*, 2005; Haynes *et al.*, 2006). Nucleotides such as adenosine triphosphate (ATP) are released by injured neurons and attract microglia via activation of purinoreceptors (Davalos *et al.*, 2005;

Haynes *et al.*, 2006). Within days after acute lesion microglia proliferate, a process that was termed reactive microgliosis.

1.1.4 Immune function of microglia

The brain's immune system is different from most other organs. Neurons are fragile cells unable to withstand long-lasting exposure to toxic molecules released by activated peripheral immune cells that defend against invading microorganisms. The blood brain barrier prevents unlimited entry of blood-borne leukocytes into the brain parenchyma and protects the CNS through this mechanism from a potential damage as a consequence of a uncontrolled immunological response (Streit, 2002).

Microglial cells represent the innate immunity of the CNS and are responsible for the first line of immune defense. To monitor the entire CNS parenchyma microglial processes extend over non-overlapping territories (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Under even minor pathological conditions including various acute and chronic neurodegenerative diseases microglia are transformed into reactive microglia.

The transformation of microglia into potentially cytotoxic cells occurs in response to numerous triggers, but only a few signals and their receptive structures are clearly identified. Known triggering signals include viral envelopes, bacterial cell wall components, and other infectious agents like prion proteins. *In vitro*, lipopolysaccharide (LPS), which is found in the cell wall of gram-negative bacteria and is known to be a TLR (Toll-like receptor) 4 ligand, serves as standard agent in mimicking infections to induce microglial transformation (Hanisch, 2002).

Recently, chemotactic signals (CCL21 and nucleotides) released by dying or damaged neurons were identified to recruit phagocytic active microglia. Microglia were shown to express receptors like $P2Y_6$ and CXCR3 that function as a sensor after injury, and activation of microglia by chemotactic signals leads to the removal of dying cells or their debris (de Jong *et al.*, 2005; Koizumi *et al.*, 2007). Whether apoptotic cells recruit microglia by the release of single or a combination of chemotactic signals remains still unclear.

Subsequent to chemotactic stimuli, microglia migrate to the lesion site and often serve there as scavenger cells. Microglial phagocytosis is a highly coordinated process, which is mainly regulated by signals that microglia receive from their environment. Microglia express distinct types of receptors which are either involved in the phagocytosis of pathogenic organisms such as TLRs or scavenger receptors for clearance of apoptotic

cellular debris such as receptors recognizing phosphatidylserine (PtdSer) residues (Napoli and Neumann, 2008).

Besides their phagocytic activity microglia have also been shown to promote neuroprotection by secretion of neurotrophic factors like nerve growth factor (Elkabes *et al.*, 1996). In addition, microglia are proposed to play a role in protecting neurons against glutamate-mediated neurotoxicity. Under pathological conditions, astrocytic glutamate uptake is impaired and LPS-stimulated microglia upregulate the glutamate uptake protein GLT1 which might be involved in the clearance of excess glutamate (Hanisch and Kettenmann, 2007).

Upon activation, reactive microglia have many functions similar to tissue macrophages, including phagocytosis, and production of cytokines, chemokines, complement components, matrix metalloproteinases, oxidative radicals, and nitric oxide (Benveniste *et al.*, 2001) (Table 1-1).

Molecule	Function
IL-1	Proinflammatory cytokine
IL-3	Hematopoietic growth factor
TNF-α	Proinflammatory cytokine; apoptosis; neurotoxic
Lymphotoxin (LT)	Apoptosis
IL-6	Pro/Anti-inflammatory cytokine
IL-12	Promotion of Th1 cell development
IL-1 receptor antagonist (IL-1RA)	Anti-inflammatory molecule
TGF-β	Anti-inflammatory cytokine
MIP-1α, MIP-1β, RANTES, MCP-1, IL-8	Chemoattractants
Platelet activating factor	Neurotoxic
Nitric oxide (NO)	Neurotoxic
Prostaglandin E_2 (PGE2)	Anti-inflammatory
Matrix metalloproteinase (MMP)-2, MMP-9	Matrix metalloproteinases
ClqB, C3, C4	Complement components
Class II MHC	Antigen presentation
B7-1, B7-2	Co-stimulation of antigen presentation
ICAM-1	Antigen presentation/adhesion
CD40	Co-stimulation of antigen presentation; cytokine/chemokine induction; neurotoxin production

Table 1-1. Functional properties of microglia (Benveniste *et al.*, 2001)**.**

1.1.4.1 Inflammatory mediators

Resting microglia constitutively synthesize neurotrophins, whereas cytokines are mostly produced upon appropriate stimulation (Elkabes *et al.*, 1996). Signals like LPS or interferon γ (IFNγ) rapidly induce cytokine/chemokine gene transcription and effective release. However, initial release of certain cytokines, such as tumor necrosis factor α (TNFα), has an autocrine impact on further release activities. Similarly, cytokines regulate the expression of cell surface molecules as a prerequisite for chemotactic movements and cellular interactions, including antigen presentation or induction of apoptosis (Hanisch, 2002). Essential cytokine systems produced by microglia include the IL1/IL18 (interleukin) family members, IL2/IL15, anti-inflammatory cytokines like TGFβ (transforming growth factor) or IL10, IL6, type I and type II IFN, TNF and relatives, and chemokines. Some members of these systems are discussed in detail below.

IL1 is a well-characterized pro-inflammatory cytokine and classically consists of a 17 kilodalton (kDa) polypeptide existing in two distinct isoforms, IL1α and IL1β. Although IL1α and IL1β are encoded by separate genes sharing some sequence homology, they elicit similar biological actions. In addition, IL1 is a crucial microglial effector cytokine having a strategic position in innate defense and immune response. Cells of lymphoid and myeloid lineage are the main producers of IL1. The isoform IL1α is mainly cell-associated, while the isoform IL1β is mostly found in soluble form. IL1 is released upon inflammation, but it also functions in cell proliferation and differentiation during CNS development (O'Connor and Coogan, 1999; Shaftel *et al.*, 2008). Especially IL1β were shown to inhibit hippocampal synaptic transmission and long-term potentiation (O'Connor and Coogan, 1999).

TGFβ is an anti-inflammatory growth factor with multiple biological activities on proliferation, tissue development, migration, immune responses, apoptosis, and extracellular matrix remodeling (Blobe *et al.*, 2000). TGFβ exerts strong immunosuppressive effects by inhibiting expression of pro-inflammatory cytokines such as IL1 and TNFα in microglial cells (Benveniste *et al.*, 2001). Most notably and further discussed in the following section, TGFβ was recently shown to be involved in Alzheimer's disease (AD) pathology.

IFNs are a class of widely expressed cytokines and are known to have effects on cell growth and immunomodulation. The IFN family consists of two main classes: type I and type II. Type I IFNs are mainly involved in the resistance of mammalian cells

against viral infections and are secreted by most cells upon stimulation. In contrast, only certain cells of the immune system produce type II IFNs, including natural killer cells and T cells. IFNγ is the only type II IFN and it exhibits no marked structural homology with type I IFN. In microglia, IFNγ causes induction and upregulation of many cell surface molecules, namely MHC class I and II, intercellular adhesion molecules (ICAM-I), costimulatory molecules B7 (CD80/86), leukocyte function-associated molecule 1 (LFA-1), LPS receptor (CD14), Fc and complement receptors, changes in the proteasome composition, as well as release of cytokines, complement and nitric oxide (NO) (Hanisch, 2002).

TNFα is a pro-inflammatory cytokine that is produced by monocytes/macrophages, dendritic cells, or lymphocytes regulating growth and differentiation of cells. In the CNS, TNFα is produced by neurons, astrocytes, and microglia. In particular microglia produce TNFα that acts in an autocrine and paracrine manner to activate the population of immune cells across the brain parenchyma (Nadeau and Rivest, 2000). In CNS insults, rapid release of TNFα by microglial cells could critically influence subsequent events. Upon TNFα release, activated microglial cells and recruited immune cells from the periphery effectively and specifically phagocytose the initially damaged tissue. This early phagocytic activity may prevent subsequent secondary damages, since the absence of TNFα may delay activation of microglial cells, which leads to an exaggerated and non-specific activation of microglia by the accumulation of necrotic debris. However, this microglial activation seems to be TNFα specific, since these effects were not observed with other pro-inflammatory cytokines like IL1 (Blais and Rivest, 2004; Glezer *et al.*, 2007).

NO is a unique biological messenger molecule which mediates diverse physiologic roles. NO is involved in blood vessel relaxation, activation of macrophages and in neurotransmission by central and peripheral neurons. NO is produced from three NO synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). In the CNS, NO plays important roles in the regulation of neurotransmitter release, neurotransmitter reuptake, neurodevelopment, synaptic plasticity, and regulation of gene expression. However, excessive production of NO following a pathologic insult leads to neurotoxicity. Microglia induce membrane-bound iNOS that may be important in host defense function. Moreover, after initial stimulation by cytokines, large amounts of NO produced by iNOS in the microglia may cause cellular damage. NO plays a role in mediating neurotoxicity associated with a variety of

neurologic disorders, including stroke, Parkinson's Disease, and HIV dementia (Dawson and Dawson, 1998).

1.2 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder leading to cognitive, memory and behavioral impairments. It is the most common form of dementia in people over 65 years of age affecting nowadays 20 to 30 million people worldwide (Selkoe, 2005). It will become an increasing health problem due to extended life expectancies in developed countries.

AD often begins with subtle, intermittent deficits in remembering minor events of everyday life, such as forgetfulness and difficulties recalling new names. These mild memory deficits, primarily affecting short-term memory, gradually process to severe dementia and stupor. Late stage symptoms include multiple cognitive and behavioral spheres frequently accompanied by additional neurological symptoms such as extrapyramidal motor signs, slowed movements and hampered motor coordination. Death occurs, on average, 9 years after the initial clinical diagnosis and is usually caused by respiratory complications such as aspiration or pneumonia (Heneka and O'Banion, 2007). No cure or preventative therapy is yet available and the definitive diagnosis of AD is still based on post-mortem histopathological examinations of the patient's brains.

1.2.1 Neuropathological aspects of AD

The pathological hallmark of AD consists of cortical atrophy with accumulation of extracellular deposits of amyloid β (Aβ) peptide in senile plaques and intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau found in neurons in the cerebral cortex (De Strooper and Annaert, 2000).

While the majority of AD cases are sporadic, about 5% are caused by mutations (familiar AD). The discovery of mutations involved in AD led to substantial progress with regard to possible mechanisms underlying the disease. Indeed, several mutations have been found in a gene encoding the amyloid precursor protein (APP). APP is a transmembrane protein which is known to be processed by two different cleavage pathways *in vivo*, the non-amyloidogenic and the amyloidogenic pathway (Fig.1-2). Usually about 90% of APP enters the non-amyloidogenic pathway, and only 10% the

amyloidogenic one, but these ratios may change due to mutations, environmental factors, as well as the age of the individual.

In the prevalent non-plaque-forming pathway (non-amyloidogenic), APP is cleaved by α-secretase within the Aβ domain, at a position 83 amino acids away from the carboxy (C) terminus. A soluble extracellular fragment (sAPPα) and a C-terminal fragment (C83) are produced. The latter can be further cleaved intramembranously by γ-secretase to produce a small peptide termed P3. Importantly, cleavage by the α-secretase occurs within the Aβ region, thereby preventing formation of Aβ (LaFerla *et al.*, 2007).

In the amyloidogenic pathway APP is cleaved by β-secretase, leading to the generation of a soluble extracellular fragment (sAPPβ) and a C-terminal fragment (C99) within the membrane. Subsequent cleavage of C99 (between residues 38 and 43) by the γ-secretase liberates an intact hydrophobic Aβ peptide, which is prone to fibril formation. The γ-secretase has been identified as a complex of enzymes composed of presenilin 1 or 2, (PS1 and PS2), and others. Most of the full-length Aβ peptide produced is 40 residues in length ($A\beta_{40}$), whereas a small proportion (approximately 10%) is the 42 residues variant ($A\beta_{42}$). The longer and more hydrophobic $A\beta_{42}$ is much more prone to fibril formation than is $A\beta_{40}$ and even though $A\beta_{42}$ is a minor form of Aβ, it is the major Aβ species found in cerebral plaques (Esler and Wolfe, 2001).

Mutations in genes encoding the two homologous presenilins, PS1 and PS2, and βAPP itself, have been shown to influence amyloid production and plaque formations (Haass, 1997; Selkoe, 1998). These conditions are the K595M and N596L (Swedish) double APP mutation at the β-site APP-cleaving enzyme (BACE) cleavage site, and a duplication of a small region within the APP gene (Citron *et al.*, 1992; Cai *et al.*, 1993; Rovelet-Lecrux *et al.*, 2006). Taken together, studies on the human genetics of AD points to 'wrong' processing of APP being directly involved in the pathology of the disease. However, neither the physiological function of the fragments (related or not related to Aβ) generated by the proteolysis of APP, nor the physiological function of APP is known at this point.

In AD pathology two types of plaques are distinguished. First, there are neuritic plaques consisting primarily of neuronal processes surrounding a fibrillar Aβ core, and secondly diffuse plaques. The neuritic plaques are also the foci of local inflammatory responses, which are discussed in detail below. In contrast, these inflammatory responses are not found within diffuse plaques, which are considered as clinically benign Aβ deposits (Benveniste *et al.*, 2001).

NFTs are composed of intraneuronal cytoplasmic accumulations of non-membrane-bound bundles of paired helical filaments, consisting of hyperphosphorylated tau. Tau is also found in dystrophic neurites. It is present in aggregates conjugated with ubiquitin comparable to other aggregating intraneuronal proteins, such as α-synuclein predominantly found in Parkinson's disease (Lee *et al.*, 2001).

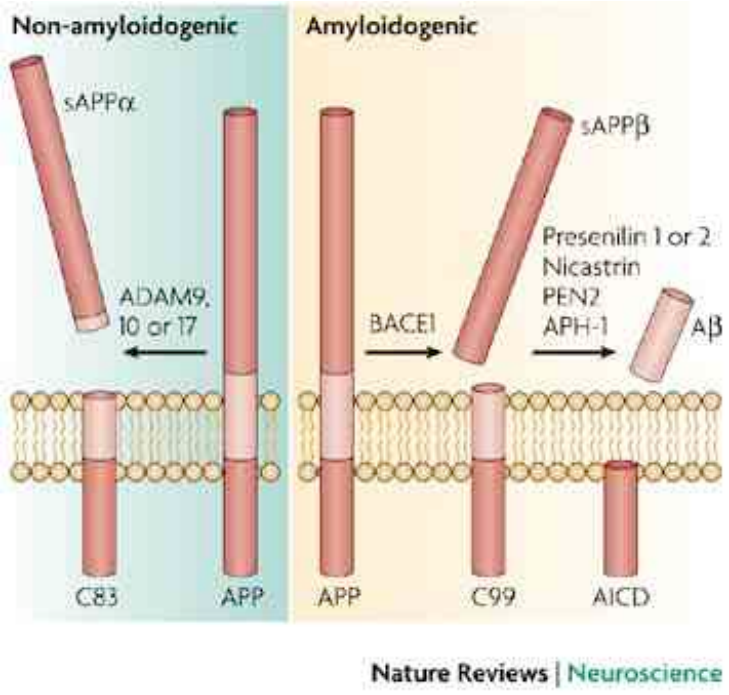

Figure 1-2. APP proteolysis. The Aβ peptide is derived via proteolysis from a larger precursor molecule called the amyloid precursor protein (APP). APP can undergo proteolytic processing by one of two pathways (LaFerla *et al.*, 2007).

1.2.2 Transgenic mouse models of AD

Transgenic mice expressing pathogenic mutations of human genes have become a powerful tool for biomedical research and drug discovery. Based on the discovery of familiar AD linked mutations in APP, different transgenic mouse models overexpressing human mutant and non-mutant APP were generated. Depending on their mutations these mice exhibit various phenotypes allowing the analysis of diverse aspects of AD related pathogenesis.

In 1995, the first APP transgenic mouse model was described that progressively develops many of the pathological hallmarks of AD, including numerous extracellular thioflavin S-positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis and microgliosis (Games *et al.*, 1995). Today, there are more than 40 different types of

APP-transgenic mice available and more than 100 transgenic mice expressing mutations in other genes related to AD (i.e. BACE, PS1, PS2, and tau). In 1997, Sturchler-Pierrat and colleagues generated another APP mouse model, the APP23 mouse. APP23 mice express the 751 amino acid isoform of hAPP with the Swedish mutation (K670N, M671L) (sweAPP) driven by the neuron specific promoter Thy1. Transgene derived APP levels are 7-fold higher than endogenous APP (Sturchler-Pierrat *et al.*, 1997). Histopathologically, these mice are characterized by progressive Aβ deposition, which first appears at 5-6 months of age. In these mice activated microglia and astrocytes are associated with Aβ plaques, and dystrophic neurites and tau protein hyperphosphorylation has also been described (Sturchler-Pierrat *et al.*, 1997). APP23 mice show an age-dependent decline of spatial memory capacities (Kelly *et al.*, 2003).
Due to the fact that single transgenic mice like the APP23 do not allow the analysis of the full spectrum of AD like pathology, double and triple transgenic mice have been generated. The APP/PS1 mouse model is a double transgenic mouse expressing a double mutation in APP (Swedish APP mutation $APP_{K670N, M671L}$ and APP_{V717I} under the mouse Thy-1 promoter) and a mutation in presenilin-1 (PS-1 M146L under the pHMG promoter), and develops large numbers of fibrillar Aβ deposits in cerebral cortex and hippocampus far earlier than their single transgenic littermates (Holcomb *et al.*, 1998). In 2003, Oddo and colleagues created a triple-transgenic model (3xTg-AD) harboring $PS1_{M146V}$, sweAPP, and tau_{P301L} transgenes. 3xTg-AD mice progressively develop plaques and tangles. Prior to visible plaque and tangle pathology, synaptic dysfunction, including long-term potentiation deficits, are developed in an age-related manner. Both Aβ-deposition and tau-aggregates exhibit a very similar expansion pattern as described in AD patients (Oddo *et al.*, 2003). Therefore, 3xTg-AD mice might reflect the neuropathology of AD best and became a powerful model in AD research. However, the simultaneous expression of three mutant proteins does not allow the investigation, how one of these proteins contributes to pathological effects itself.

1.2.3 Neuroimmunological aspects of AD

Aβ plaques and NFTs are the key pathological hallmarks in AD, but may not account for all clinical symptoms of AD. Epidemiological studies on the treatment of arthritis with anti-inflammatory agents showed that anti-inflammatory drugs may have a protective effect against AD. These clinical studies substantiated that the immune system is involved in the pathogenesis of AD (McGeer *et al.*, 1996). Indeed, the earliest

clinical symptoms arise before neuronal degeneration is apparent. Nowadays, there is compelling evidence that cellular and molecular components of the immune system represent a third player in AD pathogenesis. Inflammation in AD appears only to arise within the CNS, with little or no involvement of lymphocytes and monocytes beyond their normal surveillance of the brain (Wekerle *et al.*, 1987). How and when inflammation arises in the course of AD has not yet been fully resolved. Throughout the years, AD inflammation research has been focused on various subjects including cytokines, complement, chemokines, growth factors, oxidative stress, microglial activation, and astrocyte reactivity. However, it is important to realize that inflammatory reactions are highly interactive and almost never occur in isolation from each other. Indeed, it is still widely discussed what stimulators provoke the inflammatory reaction found in AD brain.

1.2.3.1 Complement system in AD

The complement system is a sophisticated system evolved to destroy pathogens and to assist in the phagocytosis of waste material. Four main functions are carried out by complement: recognition, opsonization, activation of inflammation and killing of the pathogen (Boche and Nicoll, 2008). Cumulative evidence suggests that Aβ plaques play a pivotal role as inducers of neuroinflammation. *In vitro* studies proposed that fibrillar Aβ can activate the classical complement cytolytic pathway by binding complement factor C1q, the first component of the C1 complex, in the absence of antibodies (Rogers *et al.*, 1992). Such activated early complement factors could be responsible for the recruitment and activation of microglial cells expressing complement receptors like CR3. Studies in the late nineteen eighties demonstrated that reactive microglia are closely associated with neuritic and amyloid plaques, but not with diffuse plaques (Rozemuller *et al.*, 1989; Sasaki *et al.*, 1997). In AD, it is assumed that the complement system is strongly activated and could participate either in exacerbation or amelioration of the pathology. Recent studies investigating the role of the complement system in AD are controversial. The most recent report showed that C3-deficient APP transgenic mice had accelerated Aβ plaque deposition and neurodegeneration compared to their controls suggesting a beneficial role for C3 in plaque clearance (Maier *et al.*, 2008). However, APP transgenic mice lacking C1q had no change in Aβ plaque load compared to control mice but showed decreased glial activation, surrounding plaques and a slowing of neuronal pathogenesis, suggesting a detrimental effect of C1q on neuronal activity

(Fonseca *et al.*, 2004). Taken together many studies underline the hypothesis that the complement system is involved in the neuroinflammation found in AD. However the beneficial or detrimental role of the complement system is still controversial discussed.

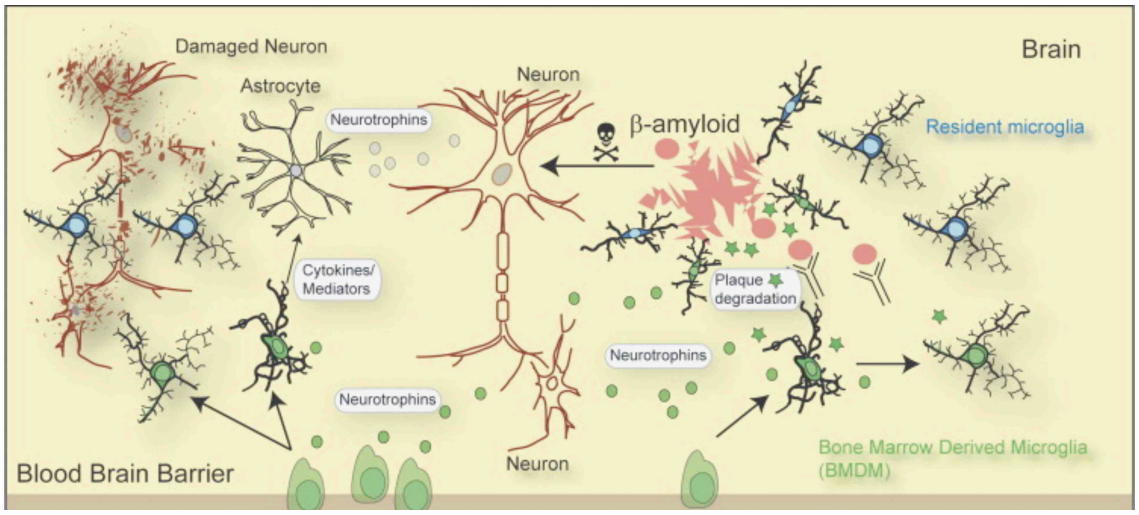

Figure 1-3. Aβ induced immune response leading to neurotoxicity in AD (modified from Glezer *et al.*, 2007)**.**

1.2.3.2 Microglia in AD

The role of microglia in the pathogenesis of AD is not elucidated yet. Some groups have performed a lot of effort in trying to unravel the role of microglia in the pathogenesis of AD. On one hand, there is literature documenting the fact that Aβ serves as a potent microglial activator. Stimulation of microglia with Aβ results in the production of various inflammatory mediators such as IL1β, TNFα, IL6, IL8, MIP (macrophage-inflammatory protein)-1α, MCP (monocyte chemoattractant protein)-1, the complement component C3 and its receptor, NO and glutamate (Benveniste *et al.*, 2001) which might lead to a detrimental effect in the tissue. However, there are also studies that show a neuroprotective role of microglia in AD. The occurrence of activated microglia surrounding Aβ plaques suggests their phagocytic attempt to remove Aβ plaques (Frautschy *et al.*, 1998). Microglia secrete proteolytic enzymes like metalloproteinases that degrade Aβ (Qiu *et al.*, 1997) and express receptors that are involved in the clearance and phagocytosis of Aβ and tissue debris (Napoli and Neumann, 2008).

In a APP transgenic mouse model of AD, bone marrow-derived cells were recruited to senile plaques and eliminated Aβ plaques by a cell-specific phagocytic mechanism (Simard *et al.*, 2006). Recruitment of bone marrow-derived myeloid cells to senile

plaques and differentiation into microglial-like cells after irradiation and bone marrow transplantation have also been observed by several other groups (Malm *et al.*, 2005; Stalder *et al.*, 2005). In all studies the invaded bone marrow-derived cells obtained morphologies and lineage markers very similar to resident microglia. However, two recent reports raised the question whether CNS invasion of bone marrow-derived cells is really a consequence of the disease process or rather induced by irradiation damage generally observed upon the generation of bone marrow chimeric mice (Ajami *et al.*, 2007; Mildner *et al.*, 2007).

Recently the dynamics of microglial cells in an animal model of AD was monitored by *in vivo* two-photon microscopy (Bolmont *et al.*, 2008; Meyer-Luehmann *et al.*, 2008). Plaque formation was followed by microglial activation and recruitment to the plaque within 1-2 days (Meyer-Luehmann et al., 2008). After microglial recruitment dysmorphic neurites appeared over the next days to weeks (Meyer-Luehmann et al., 2008). The microglial cell body stayed at the plaque, while the processes showed permanent movements at the plaque/glia interface (Bolmont *et al.*, 2008). The number of plaque-associated microglia increased at a rate of almost three per plaque per month, independent of plaque volume (Bolmont *et al.*, 2008). Although the microglia appeared to restrict the plaque growth, it is unclear why they failed to remove the plaque by phagocytosis.

The chemokine receptor CCR2, a major chemokine receptor for microglia and blood derived monocytes, contributed to the accumulation of these immune cells in the CNS of a transgenic mouse model of AD, and CCR2 deficient AD mice exhibited increased Aβ deposits in the perivascular areas of the CNS (El Khoury *et al.*, 2007). Therefore some of these recruited cells might limit Aβ plaque load probably through phagocytosis and it is suggested that clearance by resident microglia seems to be limited, maybe due to desensitization of microglial CCR2 that renders them non-responsive to injury (Britschgi and Wyss-Coray, 2007).

1.2.3.3 Cytokines in AD

The major classes of cytokines are the IFNs, ILs, TNFs, and colony-stimulating factors. Four main cytokines have been extensively investigated in AD: IL1, IL6, TNFα and TGFβ. Studies on cytokine production revealed that Aβ stimulates a NFκB-dependent pathway that is required for the production of cytokines (Heneka and O'Banion, 2007).

Increased IL1 expression in reactive microglia surrounding amyloid plaques initially indicated that IL1 may be associated with AD pathogenesis. Since that time, many groups reported on the regulation of APP processing and Aβ production by IL1 *in vitro* (Shaftel *et al.*, 2008). Furthermore, Aβ plaques are positively stained for IL1, IL6 and TGFβ1, and elevated TNFα and TGFβ1 levels have been detected in the serum and cerebrospinal fluid of AD patients (Boche and Nicoll, 2008). The production of interleukins and other cytokines or chemokines may also lead to microglial activation, astrogliosis, and further secretion of pro-inflammatory molecules. However, a second function of cytokine action is manifested by elevated levels of inhibitory, anti-inflammatory cytokines such as IL1 receptor antagonist (IL1Ra), IL4, IL10 and TGFβ. In particular TGFβ signaling moved into the focus of many studies. Tesseur and colleagues reported that the TGFβ type II receptor (TGFβRII) is mainly expressed by neurons, and that TGFβRII levels are reduced in human AD brain and correlate with pathological hallmarks of the disease. *In vivo*, reducing neuronal TGFβ signaling resulted in age-dependent neurodegeneration and promoted Aβ accumulation and dendritic loss in a mouse model of AD (Tesseur *et al.*, 2006). In another recent study, mice lacking functional TGFβ and downstream Smad2/3 signaling were crossed with transgenic APP mice. Aged double-transgenic mice showed complete mitigation of defective spatial working memory accompanied by attenuated brain parenchymal and cerebrovascular Aβ deposits and Aβ abundance (Town *et al.*, 2008). Taken together both studies point out that dysfunction of TGFβ/TGFβRII signaling may accelerate Aβ deposition, CNS inflammation and injury suggesting that intact TGFβ signaling may reduce neurodegeneration, thus having a beneficial role in AD immunopathology.

1.3 Embryonic stem cells

Embryogenesis is the process by which the embryo is formed and develops. It starts with the fertilization of the egg which has the ability to generate an entire organism. This capacity, defined as totipotency, is retained by early progeny of the zygote up to the eight-cell stage of morula. Subsequently, cell differentiation results in the formation of a blastocyst composed of outer trophoblast cells and undifferentiated inner cells (also called inner cell mass). Cells of the inner cell mass (ICM) are no longer totipotent but pluripotent. Pluripotent cells retain the ability to develop into all cell types of the embryo (Fig. 1-3) (Wobus and Boheler, 2005).

In 1981, Evans and Kaufman succeeded in cultivating pluripotent cell lines from mouse blastocysts (Evans and Kaufman, 1981). These cell lines, termed embryonic stem (ES) cells, originated from the ICM and could be maintained *in vitro* on a feeder layer of mouse embryonic fibroblasts (MEF) without any apparent loss of differentiation potential (Fig.1-4). Once established, murine ES (mES) cell lines displayed an almost unlimited proliferation capacity *in vitro* and retained the ability to contribute to all cell lineages. *In vitro*, mES cells maintained a relatively normal and stable karyotype, even during continuous passaging (Smith, 2001).

Since the culture of mES cell lines initially required a monolayer of inactivated MEFs, it was suggested that fibroblasts supplied some important factors either to promote self-renewal or to suppress differentiation. In 1988, two independent groups identified leukemia inhibitory factor (LIF) as the trophic factor responsible for both activities (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF, an IL-6 class cytokine, is a soluble glycoprotein of myeloid origin and induces differentiation in M1 myeloid leukemia cells (Williams *et al.*, 1988). Other members of the IL-6 family show similar properties, especially with respect to maintenance of mES cell pluripotency (Niwa *et al.*, 1998; Burdon *et al.*, 1999). The absence of IL-6 family members, the removal of MEFs, or the inactivation of STAT3, a downstream signaling molecule of the gp130 signaling complex, promote ES cells to spontaneously differentiate *in vitro* (Boeuf *et al.*, 1997; Wobus and Boheler, 2005).

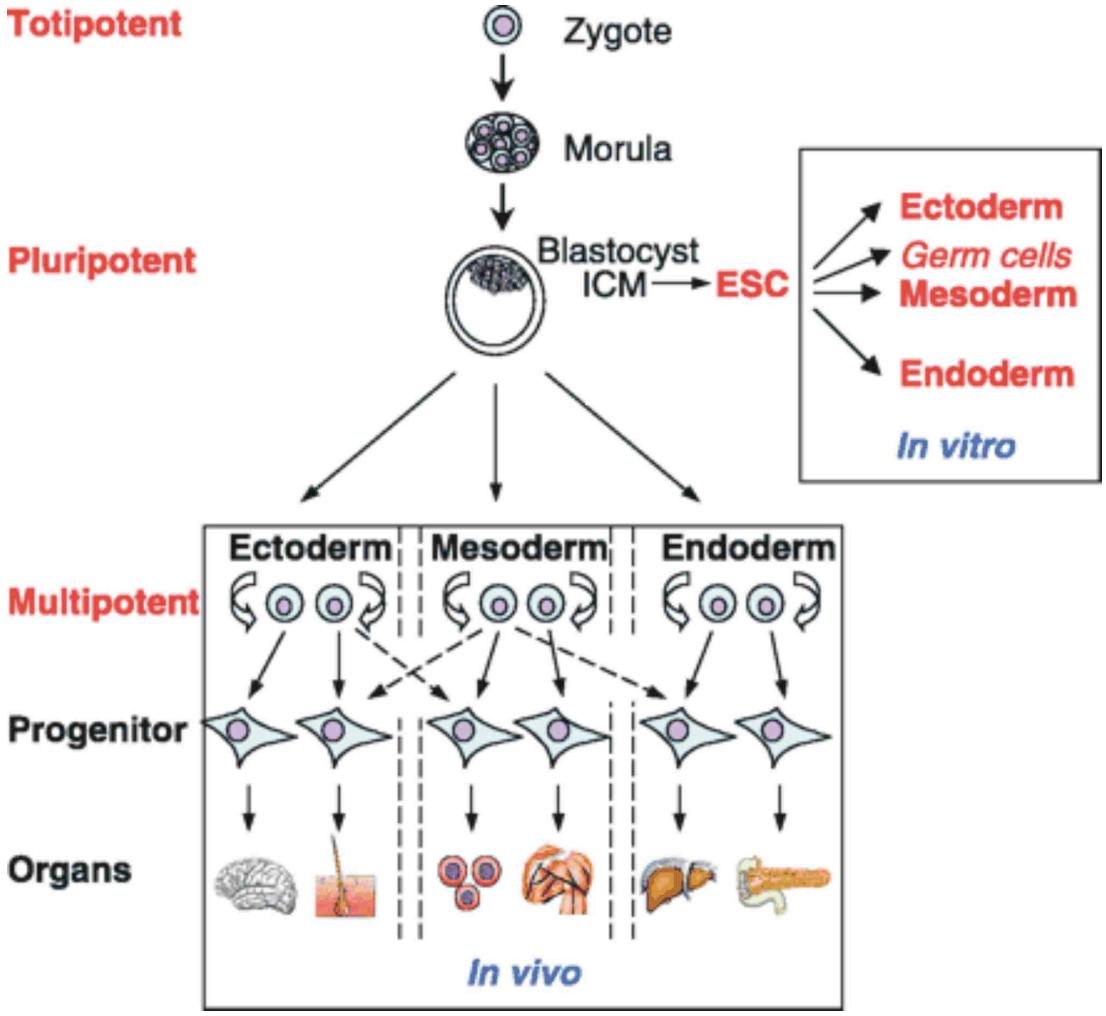

Figure 1-3. Stem cell hierarchy. Zygote and early division stages (blastomeres) to the morula stage are defined as totipotent, since they can generate a complex organism. At the blastocyst stage, only the cells of the ICM retain the capacity to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as germ cells, the founder of male and female gametes. In adult tissue, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also transdifferentiate into cells of other lineages or what factors could enhance their differentiation capability (dashed lines) (modified from Wobus and Boheler, 2005).

Undifferentiated mES cells express specific cell surface and molecular markers that define the basis of stem cell identity or "stemness". The specific cell surface antigen, SSEA-1 (Solter and Knowles, 1978), the membrane-bound receptor, gp130 (Niwa *et al.*, 1998; Burdon *et al.*, 1999), and the enzyme activity of alkaline phophatase (Wobus *et al.*, 1984), but also various signaling molecules like Oct3/4 (Scholer *et al.*, 1989; Pesce *et al.*, 1999), Nanog (Chambers *et al.*, 2003; Mitsui *et al.*, 2003), Rex1 (Rogers *et al.*, 1991; Hosler *et al.*, 1993) or Sox2 (Avilion *et al.*, 2003), define the stemness of mES cells. The ES cell property of self-renewal is therefore a balance of many signaling

molecules. An imbalance in any of these molecules can cause the loss of ES cell stemness.

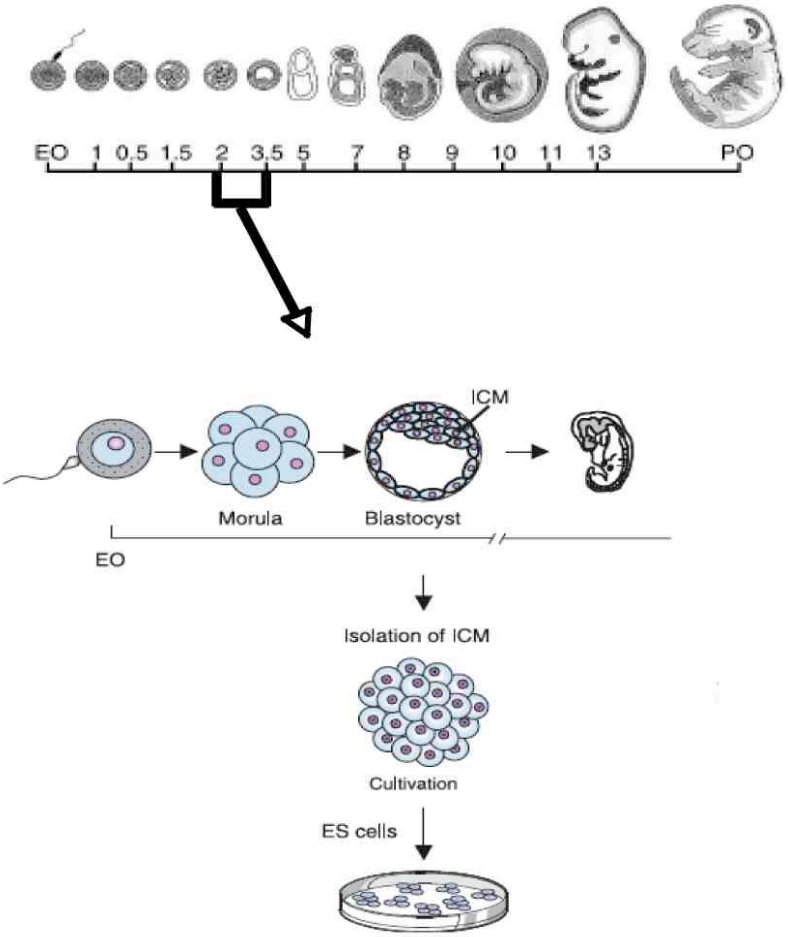

Figure 1-4. Devolopmental origin of pluripotent embryonic stem cell lines of the mouse. The scheme demonstrates the derivation of embryonic stem cells between embryonic day E2 to E3.5. Cells have been derived from the ICM of blastocysts (modified from Wobus and Boheler, 2005).

1.3.1 *In vitro* differentiation potential of ES cells

The *in vitro* differentiation potential of mES cells facilitated the examination of mouse embryogenesis. During mouse embryogenesis, three germ layers are derived from the primitive ectoderm of the epiblast: endoderm, mesoderm, and ectoderm. All tissues and organs of the developing embryo are established from these germ layers.

Differentiation is induced by culturing ES cells as aggregates (embryoid bodies) in the absence of the self-renewal signals provided by feeder cells or LIF. Once differentiation has begun, cells representing primary germ layers spontaneously develop *in vitro*. First,

an outer layer of endoderm-like cells forms within the embryoid bodies (EBs), followed by the development of an ectodermal zone and subsequent specification of mesodermal cells. EB's develop in suspension and their transfer to tissue culture plates allows continued differentiation into a range of specialized cell types (Wobus and Boheler, 2005).

1.3.1.1 Endodermal differentiation

Pancreas and liver are derivatives of the definitive endoderm. The generation of insulin-producing pancreatic cells from an unlimited source as ES cells is especially critical in the treatment of diabetes. Hepatic cells derived from ES cells show hepatic-restricted transcripts and proteins (Hamazaki *et al.*, 2001; Jones *et al.*, 2002) and can successfully integrate and function in a host liver upon transplantation (Chinzei *et al.*, 2002; Choi *et al.*, 2002). Due to these findings, it can be suggested that mES differentiate into all three lineages of the liver (hepatocytes, bile duct epithelial and oval cells). In terms of pancreatic differentiation, the capacity of mES seems to be more restricted. *In vitro*, spontaneous differentiation of mES cells into insulin-producing pancreatic endocrine cells results in only a small fraction (~ 0.1%) (Shiroi *et al.*, 2002). Initially, the first successful induction of pancreatic differentiation of ES cells was obtained by stable transfection with a vector expressing a neomycin-resistance gene under the control of the insulin promoter. This enabled lineage selection and maturation of insulin-expressing cells which resulted in the normalization of glycemia in streptozotocin-induced diabetic mice (Soria *et al.*, 2000). Different modification of the protocol allowed the generation of insulin-producing clusters from ES cells, but further improvements are necessary for the transplantation of functional cells.

1.3.1.2 Mesodermal differentiation

Mesoderm is the germ layer that develops into muscle, bone, cartilage, blood, and connective tissue. Around embryonic day E6.5, blood and endothelial cells are among the first differentiated mesodermal cell types. In these days, the yolk sac, an extraembryonic membrane composed of adjacent mesodermal and primitive (visceral) endodermal cell layers, is formed. The yolk sac gives rise to blood and endothelial cells (Baron, 2001). Hematopoietic cells and blood vessels are believed to arise from a common progenitor cell, the "hemangioblast". mES cells have been successfully used to recapitulate these mesodermal developmental processes in vitro. Differentiation of ES

cells in complex cystic EB's permits the generation of blood islands containing erythrocytes and macrophages (Doetschman *et al.*, 1985), whereas differentiation in semisolid medium is efficient for the formation of neutrophils, mast cells, macrophages, and erythroid lineages (Wiles and Keller, 1991). Application of FCS and cytokines such as IL-3, IL-1, and granulocyte-macrophage colony stimulating factor (GM-CSF) to ES cells generates early hematopoietic precursor cells. The development of all hematopoietic cell types of the erythroid, myeloid, and lymphoid lineages (Nakano *et al.*, 1994) and of natural killer cells was achieved using OP9 cells (Hole, 1999; Wobus and Boheler, 2005). Most of the cellular phenotypes of ES derived hematopoietic cells have been characterized by specific gene expression patterns and by cell surface antigens (Wiles and Keller, 1991; Wang *et al.*, 1992). However the most important issue of these cells is functionality. Differentiated cells must show long-term multilineage hematopoietic repopulating properties to be considered true hematopoietic stem cells. Initially, it was reported that the repopulating ability of ES cell derived hematopoietic progenitors may be restricted to the lymphoid system (Muller and Dzierzak, 1993), but subsequent studies showed a long-term multilineage hematopoietic repopulating potential of ES derived cells (Palacios *et al.*, 1995; Hole *et al.*, 1996).

Besides hematopoetic lineage commitment, mES cells efficiently differentiate into several other mesodermal cell types, including adipogenic (Dani *et al.*, 1997), chondrogenic (Kramer *et al.*, 2000), osteoblast (Buttery *et al.*, 2001), and myogenic (Rohwedel *et al.*, 1994) cells.

1.3.1.3 Ectodermal differentiation

The neuroectodermal lineage gives rise to the peripheral and CNS and to the epithelial lineage, which is committed to becoming epidermal tissue. Epithelial cell differentiation from ES cells displays morphological patterns similar to normal embryonic skin. Compared to native skin, the cells express late differentiation markers of epidermis and markers of fibroblasts which suggest that ES cells have the capacity to reconstitute *in vitro* fully differentiated skin (Coraux *et al.*, 2003). Neuronal and glial differentiation of ES cells became of specific importance with respect to cell based therapies of neurodegenerative disorders. In 1995, three independent groups showed the neuronal differentiation of mES cells (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strubing *et al.*, 1995). Different strategies were used to improve the differentiation of ES cells to neurons, involving the use of retinoic acid (RA) (Rohwedel *et al.*, 1999), lineage selection (Li *et al.*, 1998; Ying *et al.*, 2003), and stromal cell-derived inducing activity

(Kawasaki *et al.*, 2000). Since RA is a teratogenic compound alternative protocols have been established which involve multiple steps of differentiation and selection of neural progenitors. In this protocol, EBs are generated, and subsequently cells are cultured in serum-free conditions to inhibit mesodermal differentiation. The proliferation of neural precursors is then induced by the supplementation of bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor). The withdrawal of these factors induces the final differentiation of neurons (Okabe *et al.*, 1996), (Lee *et al.*, 2000). Gene expression and electrophysiological studies of cell derivatives indicate the presence of all three major cell types of the brain: neurons [dopaminergic and serotonergic (Lee *et al.*, 2000), GABAergic and glutamatergic (Finley *et al.*, 1996) and cholinergic (Fraichard *et al.*, 1995) neurons], astrocytes and oligodendrocytes (Brustle *et al.*, 1999).

Microglia, as already discussed, are believed to origin from the mesoderm, although a contribution from the neuroectoderm can so far not completely excluded. It is assumed that microglial precursor invade the CNS from the yolk sac during embryonic days. Thus, differentiation of mES cells into microglial-like cells was described the first time during a differentiation protocol that normally generates dopaminergic neurons (Lee *et al.*, 2000). In 2005, Tsuchiya and colleagues showed the first time the induction of a large number of mature microglial-like cells from mES. During neuronal differentiation they found a population positively stained for $IBa1^+$ and $CD45^+$, which are common microglial markers, and isolated those cells with a gradient method. After intravenous application of GFP^+ ES cell derived microglial-like cells, they showed microglial migration into the brain parenchyma and detected GFP^+ cells in the corpus callosum, hippocampus and cerebral cortex, but not in other peripheral organs (Tsuchiya *et al.*, 2005).

1.4 Aims of the study

Microglia are resident immune cells of the CNS. They are involved in the homeostasis of the healthy CNS, but they are also found to be involved in many pathological conditions of the CNS. In experimental research, primary microglia are directly isolated from the brain or from mixed glial cultures derived from early postnatal brain tissue, however isolation of primary microglia is time consumable and inefficient due to low cell yield. Alternatively, the microglia-derived oncogenically transformed cell line BV2 is used as substitute for primary microglia, but many studies showed that BV2 cells are limited in several microglial functions and are therefore not suitable as replacement for primary microglia.

Therefore, the first aim of this study is to establish a standard protocol for the differentiation of mES cells into microglial (precursors) cell lines. These lines are characterized according to their immunological and functional properties *in vitro* as well as *in vivo* and compared to primary microglia.

The role of microglia in AD is still under investigation. Some findings point to a protective role of microglia and discuss their diminished phagocytosis of Aβ as a key element in the pathogenesis of AD. According to that hypothesis, we aim to use ES cell derived microglia precursors as a tool in a therapy approach in a mouse model of AD. For that reason, ES cell derived microglial precursors are genetically modified using lentiviral technology. ES cell derived microglial precursors overexpressing GFP under a ubiquitous promoter are injected locally to APP transgenic mice (APP23) to observe their impact on plaque size and distribution.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers and solutions

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

Components	Concentration	Company
$NaH_2PO_4*H_2O$	0.007M	Roth, Germany
$NaH_2PO_4*7H_2O$	0.034M	Roth, Germany
NaCl	0.6M	Roth, Germany
ddH_2O	up to 1 liter	Roth, Germany

• 4% Paraformaldehyd (PFA), pH 7.3

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

• 10X TBE buffer

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

- **6X Loading buffer**

Components	Concentration	Company
EDTA	0.5M	Roth, Germany
Sucrose	60%	Roth, Germany
Bromphenol Blue	0.04%	Sigma, Germany
Xylene Cyanole	0.04%	Sigma, Germany
Ficol-400	2%	Bio-Rad, Germany

- **1% Agarose gel**

Components	Amount	Company
Agarose	0.5g	Biozym, Germany
Ethidium Bromide	1.25µl	Roth, Germany
TBE (1X)	50ml	

- **Reverse transcription (RT) mix**

Components	Amount	Company
Total RNA	5µg	
Hexanucleotide Mix (10X)	1µl	Roche, Germany
dNTP mix (10mM)	1µl	Sigma, Germany
DTT mix (10mM)	2µl	Invitrogen, Germany
5X RT 1st Strand Buffer	4µl	Invitrogen, Germany
RT enzyme (200U/ml)	1µl	Invitrogen, Germany
ddH_2O	up to 20µl	Roth, Germany

- **Real time RT-PCR mix**

Components	Amount	Company
cDNA (200ng/µl)	3µl	
SYBR Green Master Mix (2x)	1µl	Applied Biosystems
Primer mix (10pmol/µl)	1µl	
ddH_2O	10.5µl	Roth, Germany

• Cell lines

Name	Common name	Source
ES-C57BL/6-ATCC	BL6	ATCC, Germany
ES-129-MPI	MPI	MPI Göttingen
293FT	293FT	Invitrogen, Germany
BV2	BV2	

2.1.2 Cell culture media and reagents

• Embryonic stem cell (ES) medium

Components	Concentration	Company
DMEM, high glucose		Gibco, Germany
Fetal bovine serum	15%	Gibco, Germany
Na-pyruvate	0.1mM	Gibco, Germany
L-glutamine	4mM	Gibco, Germany
Non-essential amino acids	0.1mM	Gibco, Germany
L-alanin-L-glutamine	2mM	Gibco, Germany
β-mercaptoethanol	0.1mM	Millipore, Germany
Leukemia inhibitory factor	1000U/ml	Millipore, Germany

• Differentiation medium

Components	Concentration	Company
DMEM, high glucose		Gibco, Germany
Fetal bovine serum	15%	Gibco, Germany
Na-pyruvate	0.1mM	Gibco, Germany
L-glutamine	4mM	Gibco, Germany
Non-essential amino acids	0.1mM	Gibco, Germany
L-alanin-L-glutamine	2mM	Gibco, Germany
β-mercaptoethanol	0.1mM	Millipore, Germany

- **ITSFn medium**

Components	Concentration	Company
DMEM/F12 (1:1)		Gibco, Germany
Insulin	5 µg/ml	Sigma, Germany
Na-selenit	30nM	Sigma, Germany
Transferrin	50µg/ml	Millipore, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany
Fibronectin	5µg/ml	Sigma, Germany

- **N2 medium**

Components	Concentration	Company
DMEM/F12 (1:1)		Gibco, Germany
L-glutamine	0.048mM	Sigma, Germany
D-glucose (45%)	15.3µg/ml	Sigma, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany
Laminin	1µg/ml	Sigma, Germany
bFGF	5ng/ml	R&D Systems, Germany

- **Basal medium**

Components	Concentration	Company
BME		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany
L-glutamine	1%	Sigma, Germany
D-glucose (45%)	1%	Sigma, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany

• 293FT medium

Components	Concentration	Company
DMEM, low glucose		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany
L-glutamine	1%	Sigma, Germany
D-glucose (45%)	1%	Sigma, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany

• Other cell culture reagents

Opti-MEM	Gibco, Germany
Trypsin-EDTA (0.05%)	Gibco, Germany
Gelantine (0.1%)	Sigma, Germany
Poly-L-lysine	Sigma, Germany
PBS (1X)	Gibco, Germany
Tryptan blue	Gibco, Germany
Human beta amyloid 1-42 biotinylated	Bachem, Germany
Normal goat serum	Sigma, Germany
LPS	Sigma, Germany
Recombinant mouse Interferon γ	HyCult Biotechnology,
Fluoresbrite Polychromatic Red Microspheres	Polysciences, Germany
CX3CL1 (Fractalkine)	R&D Systems, Germany
G418 (neomycin)	Sigma, Germany

2.1.3 Antibodies

• Primary antibodies for flow cytometry

Antibody	Host	Conjugation	Company
CD16/CD32 (FC-Block)	rat	-	BD Biosciences, Germany
CD45	rat	biotin	BD Biosciences, Germany
CD11b	rat	biotin	BD Biosciences, Germany
CD34	rat	biotin	Serotec Laboratories, Germany
CD86 (B7.2)	rat	biotin	BD Biosciences, Germany
cKit	rat	biotin	BD Biosciences, Germany

I-A/I-E (MHCII)	rat	biotin	BD Biosciences, Germany
F4/80	rat	biotin	Serotec Laboratories, Germany
CD49d (α4 integrin)	rat	biotin	Abcam, Germany
CD29 (β1 integrin)	hamster	biotin	Abcam, Germany
CX3CR1	rabbit	-	ProSCI, USA
IgG2A isotype	rat	biotin	BD Biosciences, Germany
IgG2B isotype	rat	biotin	BD Biosciences, Germany
Ig isotype	hamster	biotin	Abcam, Germany
Whole rabbit serum			Dianova, Germany

- **Secondary antibodies for flow cytometry**

Antibody	Reactivity	Host	Conjugation	Company
PE	biotin	-	streptavidin	Dianova, Germany
Alexa 488	rabbit	goat	-	Invitrogen, Germany

- **Primary antibodies for immunostaining**

Antibody	Reactivity	Host	Company
IBa1	mouse	rabbit	Wako, Germany
GFP	-	rabbit	Abcam, Germany
4G8 (β amyloid)	human	mouse	Signet, Germany
Nestin	mouse	mouse	Millipore, Germany
βIII tubulin	mouse	mouse	Sigma, Germany
GFAP	mouse	rabbit	Dako Cytomation, Germany

- **Secondary antibodies for immunostaining**

Antibody	Reactivity	Host	Conjugation	Company
Cy3	mouse	goat	-	Dianova, Germany
Cy3	rabbit	goat	-	Dianova, Germany
Alexa 488	rabbit	goat	-	Invitrogen, Germany
Alexa 488	mouse	goat	-	Invitrogen, Germany

- **Other staining reagents**

DAPI	Sigma, Germany
Propidium iodide	Sigma, Germany
CFSE	Sigma, Germany

2.1.4 Primer sequences

(all primers were purchased from MWG, Germany)

Target	Orientation	Sequence
GAPDH	forward	5'- AACTTTGGCATTGTGGAAGG -3'
	reverse	5'- GGATGCAGGGATGATGTTCT -3'
iNOS	forward	5'- AAGCCCCGCTACTACTCCAT -3'
	reverse	5'- GCTTCAGGTTCCTGATCCAA -3'
TNFα	forward	5'- TCTTCTCATTCCTGCTTGTGG -3'
	reverse	5'- AGGGTCTGGGCCATAGAACT -3'
IL1β	forward	5'- CTTCCTTGTGCAAGTGTCTG -3'
	reverse	5'- CAGGTCATTCTCATCACTGTC -3'
TGFβ1	forward	5'- CAATTCCTGGCGTTACCTTG -3'
	reverse	5'- GCTGAATCGAAAGCCCTGTA -3'

2.1.5 Consumables

6-well culture plates	Cellstar, VWR International, Germany
15ml tubes	Cellstar, VWR International, Germany
50ml tubes	Sarstedt, Germany
5ml, 10ml, 25ml pipets	Sarstedt, Germany
Chamber slides	Nunc, Germany
Cryovials	VWR International, Germany
75cm^2, 175 cm^2 culture flasks	Sarstedt, Germany
5ml polystyrene round-bottom tubes	BD Falcon, Germany
3cm, 5cm, 10cm culture dishes	Sarstedt, Germany
500µl, 1000 µl plastic tube	Eppendorf, Germany
PCR tubes	Biozym Diagnostics, Germany
10µl, 100µl, 1000µl tips	Eppendorf, Germany
5ml, 10ml syringes	Braun,Germany
Needles	Braun, Germany
Glass slides for cryosectioning	Menzel, Germany

Bottle top filters (0.25µm pore)	Millipore, Germany
Filters (0.45µm, 0.2µm pore)	Sarstedt, Germany
Transwell (8µm pore filter)	Millipore, Germany

2.1.6 Equipment

Centrifuges	Sorvall Discovery 90SE, Hitachi, Germany
	Megafuge, 1.OR. Heraeus, Germany
	Biofuge Fresco, Heraeus, Germany
Cryostat	Microtom HM560, Microm Int., Germany
Flow cytometer	FACSCalibur, BD Bioscience, Germany
Electrophoresis gel chambers	Blomed Analytik GmbH, Germany
Power supply	Amersham Bioscience, Germany
Heating block	Stuart Scientific, Germany
Incubators	Heracell240, Heraeus, Germany
Laminar air flow workbench	Herasafe, Heraeus, Germany
Microscopes	Axiovert40CFL, Zeiss, Germany
	Axiovert200M, Zeiss, Germnay
	Fluoview1000 Confocal Microscope, Olympus, Germany
pH Meter	Hanna Instruments, Germany
Photometer	Eppendorf, Germany
Real time thermocycler	ABI Prism 5700 Seqeunce Detection System, Applied Biosystems, UK
Thermocycler	T3, Biometra, Germany
Vortex	$2X^2$, VelpScientifica, Germany
Transplantationsequipment	Fine Science Tools, Germany

2.1.7 Software

Openlab4.0.1	Improvision, Germany
CorelDRAW	Graphics Suite 11, Germany
EndNote X1	Thomson ISI ResearchSoft, USA
Microsoft Office	Microsoft USA, 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
SPSS 16.0	SPSS, USA
Axiovision 4.6.3	Carl Zeiss Imaging Solutions, Germany
ImageJ 1.39u	NIH, USA

2.1.8 Kits and additional reagents

• RNA and DNA isolation kit

RNeasy Mini	Qiagen, Germany
RNAse free DNAse Kit	Qiagen, Germany
Endofree Plasmid Maxiprep	Qiagen, Germany

• Additional reagents

Lipofectamine2000 reagent	Invitrogen, Germany
Ampicilin	Sigma, Germany
LB agar and LB media	Fluke Biochemika
DMSO	Sigma, Germany
Glycerol	Sigma, Germany
Tissue tek O.C.T. compound	Sciences Services, Germany
Ethanol	Sigma, Germany
Vectashield	Vector Laboratories, USA
Bovine serum albumin	Sigma, Germany
Hexamer random primers	Roche, Germany
Ploybrene	Sigma, Germany

2.1.9 Anesthethics

Before transplantation, animals were anesthetized with Ketamin/Xylazin intraperitoneal (i.p.) (Ketamin 100 mg per kg body weight and Xylazine 5 mg per kg body weight).

2.2 Mice

Normal C57/BL6 mice were obtained from Charles River (Sulzfeld, Germany) and bred under pathogen free conditions in the animal house. For transplantation experiments either neonatal mice C57/BL6 mice or APP23 mice (Novartis, Basel, Switzerland) were used. APP23 transgenice mice express human APP_{751} in the brain. Mutations in the APP gene of humans cause early-onset familial AD by affecting the formation of the Aβ peptide, the major constituent of AD plaques. These transgenic mice carried the Swedish double mutation at positions 670/671 and developed pathological features reminiscent of AD (Sturchler-Pierrat *et al.*, 1997).

2.3 Generation of ES cell derived microglial precursors (ESdM)

The murine ES cell lines ES-C57BL/6-ATCC (BL6) and ES-129-MPI (MPI) were used for the differentiation into microglial precursors. *In vitro* differentiation was carried out using a modified five-step method originally designed to obtain neurons (Lee *et al.*, 2000). Undifferentiated ES cells (stage 1) were trypsinized and cultured for 2 days on gelantine-coated tissue culture plates in the presence of LIF containing ES medium. To induce EB formation (stage 2), the cells were dissociated into a single-cell suspension and plated onto non-adherent bacterial culture dishes at a density of 2–2.5 $\times 10^4$cells/cm^2 in differentiation medium. After 3-4 days, the resulting EBs were plated onto gelatine-coated dishes. After 24-48 hours, selection and expansion of nestin-positive cells were initiated with ITSFn medium supplemented with 5µg/ml fibronectin (stage 3). After 6 days of selection, cell expansion was initiated in N2 medium supplemented with 1µg/ml laminin and 5ng/ml bFGF (stage 4). After 6 days, differentiation was induced by removing bFGF and culturing for 21 days in N2 medium and with laminin (stage 5) (Tsuchiya *et al.*, 2005).

2.4 Generation and maintenance of ESdM lines

Proliferation of ESdM was observed 21 days after removal of growth factors. ESdM were mechanically isolated and seeded in N2 medium containing 5% FBS to generate individual ESdM lines. For further passaging, cells were detached with a cell scraper or with 0.05% Trypsin/EDTA. Cells were splitted twice a week and kept in 5% CO_2 at 37°C. Phenotype of each ESdM line was confirmed by flow cytometry. Repeated freezing and thawing of cells did not result in loss of phenotype.

2.5 Primary microglia and BV2 cell cultures

Primary microglia were prepared from brains of postnatal day 3 or 4 (P3 or P4) of C57BL/6 mice. In brief, meninges were removed mechanically, and cells were dissociated by trituration and cultured in basal medium for 14 days to form a confluent mixed glial monolayer. To collect microglial cells, the cultures were shaken on a rotary shaker (350 rpm) for 3 hours. The detached microglial cells were seeded on PLL coated culture dishes. Purity of the isolated microglia was determined by flow cytometry analysis with antibody directed against CD11b (data not shown).

The murine BV2 cell line (kindly provided by Dr. Patrick Wunderlich, University of Bonn), which was originally immortalized after infection with a v-*raf*/v-*myc* recombinant retrovirus (Blasi *et al.*, 1990), was maintained at 37°C at 5% CO_2 in basal medium.

2.6 Immunocytochemistry of cultured cells

Cells were fixed in 4% paraformaldehyde (PFA) for 10 min, blocked by 10X bovine serum albumin (BSA) and 5% normal goat serum (nGS) for 30 min, and then immunostained with a polyclonal rabbit antibody directed against IBa1 (1µg/ml) or monoclonal rat-anti-CD45, overnight followed by goat-anti-rabbit-Cy3 antibody (3µg/ml) or goat-anti-rat Alexa488 antibody (2.5µg/ml). Double-labelling was performed by mouse-anti-βIII-tubulin (6.8µg/ml), rabbit-anti-GFAP (3µg/ml) or mouse-anti-nestin (1µg/ml) followed by goat-anti-mouse-FITC antibody (3µg/ml) or goat-anti-rabbit-Cy3 (3µg/ml). Nuclei of immunostained cells were subsequently labeled with DAPI (0.1µg/ml). Images were collected by confocal laser scanning microscopy (Fluoview 1000, Olympus) or fluorescence microscopy (Axioskop2, Zeiss).

2.7 Flow cytometry analysis

Cells were collected from the culture dishes by a cell scraper. Cells were first incubated for Fc-receptor blockade with a rat monoclonal antibody directed against CD16/CD32 (5µg/ml), Fc-block, BD Biosciences) and then stained with either biotin-conjugated anti-CD45, anti-CD11b, anti-CD86, anti-cKit, anti-I-A/I-E (all 5µg/ml), anti-CD34, anti-F4/80, or anti-CD49d (α4 integrin) and CD29 (β1 integrin) (all 10µg/ml) followed by PE-conjugated streptavidin (2.5µg/ml). For staining of CX3CR1, cells were blocked with 1X BSA, then stained with rabbit anti-CX3CR1 (10 µg/ml) and followed by Alexa 488 conjugated goat anti rabbit IgG (2.5µg/ml). Isotype-matched control antibodies or whole rabbit serum were used as negative controls in the same concentration as the responding primary antibody. Data acquisition was performed with a FACSCalibur flow cytometer and analysis was done using FlowJo Software.

2.8 Analysis of cytokine gene transcripts by real-time RT-PCR

RNA was isolated with the RNeasy Mini Kit from 0.5 x 10^5 ESdM, primary microglia and BV2 after stimulation for 24 hours by addition of 500ng/ml LPS or murine IFNγ (500U/ml). Reverse transcription of RNA was performed with SuperScript III reverse transcriptase and hexamer random primers. Quantitative RT-PCR with specific oligonucleotides was performed with SYBR Green PCR Master Mix using the ABI 5700 Sequence Detection System and amplification protocol for the ABI 5700 Sequence Detection System. Amplification specificity was confirmed by the analysis of the melting curves. Results were analyzed with the ABI 5700 Sequence Detection System v.1.3 after establishing the reaction efficiency for each primer pair. Quantification using the delta-CT method was carried out.

2.9 Aβ phagocytosis assay

For *in vitro* Aβ phagocytosis, cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE). Briefly, for CFSE labeling, cells were washed once in PBS and incubated for 5 min at 37°C in 5µM CFSE in 250µl PBS. Then, 5ml PBS was added, and cells were further incubated for 5 minutes at 37°C before being washed in PBS. Primary microglia, ESdM and BV2 were seeded into PLL coated 4 well chamber culture dishes at a density of 2 x 10^4 cells per well. 24h later, cells were treated with biotinylated Aβ42 peptide (10 µg/ml) for additional 24h. To analyze the phagocytosis capacity, cells were fixed in 4% PFA and then permeabilized with 0.1% Triton X-100. Fixed cells were stained with Cy3-conjugated streptavidin. Analysis was made using fluorescence microscopy. 5 photographs were taken of each well and cells emitting a yellow signal were classified as phagocytosing and counted accordingly.

2.10 Chemotaxis assay

Before seeding, primary microglia, ESdM and BV2 were labeled with CFSE. For CFSE labeling, cells were washed once in PBS and incubated for 5 minutes at 37°C in 5µM CFSE in 250 µl PBS followed by washing in PBS. Then, 1 x 10^5 cells were transferred into the upper chamber of a transwell system containing 600µl medium with different concentrations of CX3CL1 in the lower chamber. After 3 hours incubation period, the

number of microglia that had migrated to the lower chamber and on the backside of the membrane was counted in three independent areas by fluorescence microscopy.

2.11 Lentiviral transduction of ESdM

Lentiviral vectors of the third generation, PLL3.7 (provided by L. van Parijs, MIT, Cambridge, MA) were used for the transduction of ESdM. The GFP (green fluorescence protein) gene was cloned under the pgk-promoter in the PLL3.7 backbone. To allow selection of transduced cells the neomycin resistance gene was additionally cloned in this vector under the pgk-promoter. For lentiviral particle production the PLL3.7 plasmid containing the gene of interest was co-transfected together with packaging vectors into 293FT cells. Supernatant was collected after 48-72h, and viral particles in the supernatant were concentrated by ultracentrifugation for 90 min at 25,000 rpm and recovered by suspension in PBS. For ESdM transduction, cells were seeded at 0.5 x 10^6 cells/ml into 6-well plates. 50µl of lentiviral particles and 10µg/ml polybrene were added to the culture and incubated overnight at 37°C. Medium was changed the next day after infection and replaced with normal culture medium. 4 days after transduction cells were selected with 250 µg/ml G418.

2.12 Transplantation of ESdM into neonatal brain

In total, 4 x 10^6 GFP^+ ESdM were transplanted into the two hemispheres of the midbrain of 2 days old C57BL/6 mice. Mice were sacrified for analysis 28 days after transplantation. Animals were perfused transcardially with PBS followed by 4% PFA. After post-fixation in fresh fixative, tissue was cryoprotected at 4°C in 30% Sucrose in PBS. Tissue was embedded in TissueTek and stored at -80°C before cryosectioning on a Micron HM560 cryostat at -20°C.

For GFP and IBa1 staining, sections were treated for 20 min in 90°C citrate buffer followed by blocking with 10X BSA and 5% nGS and 0.1% TritonX for 3 hours. Afterwards sections were immunostained overnight with polyclonal rabbit-anti-mouse IBa1 (1µg/ml) and Alexa 488-conjugated anti-mouse GFP (4µg/ml). For visualization, sections were incubated for 3 hours with secondary fluorescence Cy3-conjugated antibody directed against rabbit IgG (3µg/ml). Images were collected by fluorescence microscopy (Axioskop2, Zeiss).

2.13 Transplantation of ESdM in APP23 mice

Before transplantation, 24 months old APP23 mice were anesthetized with Ketamin/Xylazin (see 3.1.9). Under narcosis, animals were fixed in the stereotactic frame, sculls were shaved and a borehole was prepared at 0.52mm to the front and 2.1mm to the right side. In total, 2 x 10^5 GFP^+ ESdM were transplanted into the right cortex (1mm deep). Cell suspension was applied over 2 min. Afterwards the borehole was fixed with bone wax and was washed with antiseptic solution. Finally, the wound was closed with the help of a stapler. To reduce posttraumatic pain, animals received Rimadyl (5 mg per kg body weight). After 14 days animals were perfused transcardially with PBS followed by 4% PFA. After post-fixation in fresh fixative, tissue was cryoprotected at 4°C in 30% Sucrose in PBS. Tissue was embedded in TissueTek and stored at -80°C before cryosectioning on a Micron HM560 cryostat at -20°C.

For detection of aggregated Aβ, sections were treated for 20 min in 90°C citrate buffer followed by blocking with 10X BSA and 0.1% TritonX for 3 hours. Afterwards sections were immunostained overnight with monoclonal mouse anti human β amyloid (0.5µg/ml).). For visualization, sections were incubated for 3 hours with secondary-fluorescence Cy3-conjugated antibody directed against mouse IgG (3µg/ml). Images were collected by fluorescence microscopy (Axioskop2, Zeiss) and analyzed with ImageJ.

2.14 Analysis of amyloid β plaque load

The amyloid β plaque burden was analyzed with Image J Software. Two representative sections of the transplanted as well as of the contralateral site of each mouse brain were imaged under constant light and filter settings. Color pictures were converted to grayscale to obtain the best contrast between positive immunoreactivity and background. A constant threshold was chosen for all images to detect immunoreactive staining. The total number and size of plaques was related to the total area analyzed.

2.15 Statistical analysis

Data are presented as mean +/- SEM of at least 3 independent experiments. Data were analyzed by ANOVA using SPSS computer software.

3 Results

3.1 Microglial precursors are efficiently derived from mES cells under standard conditions

Here we aim to obtain microglial precursors from mES cells according to a modified standard protocol (Tsuchiya *et al.*, 2005). mES cells (BL6 and MPI) were cultured in LIF containing medium. ES cells formed typical cellular colonies on a layer of irradiated MEFs (Fig. 3-1A). EBs were induced from the ES cells after dissociation and withdrawal of LIF. EBs were kept in suspension culture for 3-4 days (Fig. 3-1B). Next, cells that grow out of EBs were expanded in ITSFn containing culture medium. Under this adherent culture condition cells proliferated and resulted in a mixed cell population, containing a high number of cells immunostained with specific antibodies directed against the intermediate filament protein nestin (Fig. 3-1C). In the next stage, nestin positive cells were expanded in the presence of bFGF and laminin. After 7 days, bFGF was withdrawn and nestin positive cells differentiated into neuronal precursor cells having processes that formed a neuronal network. After 21 days, most of the neuronal precursors were immunolabelled with specific antibodies directed against β-III-tubulin (Fig. 3-1D). Within these β-III-tubulin positive cells, approximately 25% ± 10% cells were detected, which had a microglial-like morphology and were immunostained with antibodies directed against the microglial protein IBa1 (Fig. 3-1D). Typically, these IBa1 positive cells were forming small cellular colonies, indicating proliferative growth of the microglial-like precursor cells (Fig. 3-1D). A subpopulation of cells immunolabelled with antibodies directed against GFAP were also detected in these cultures. The GFAP+ cells showed typical morphology of astrocytes and lacked co-immunostaining with specific antibodies directed against CD45 (Fig. 3-1E,F,G; 3-1H,I,J).

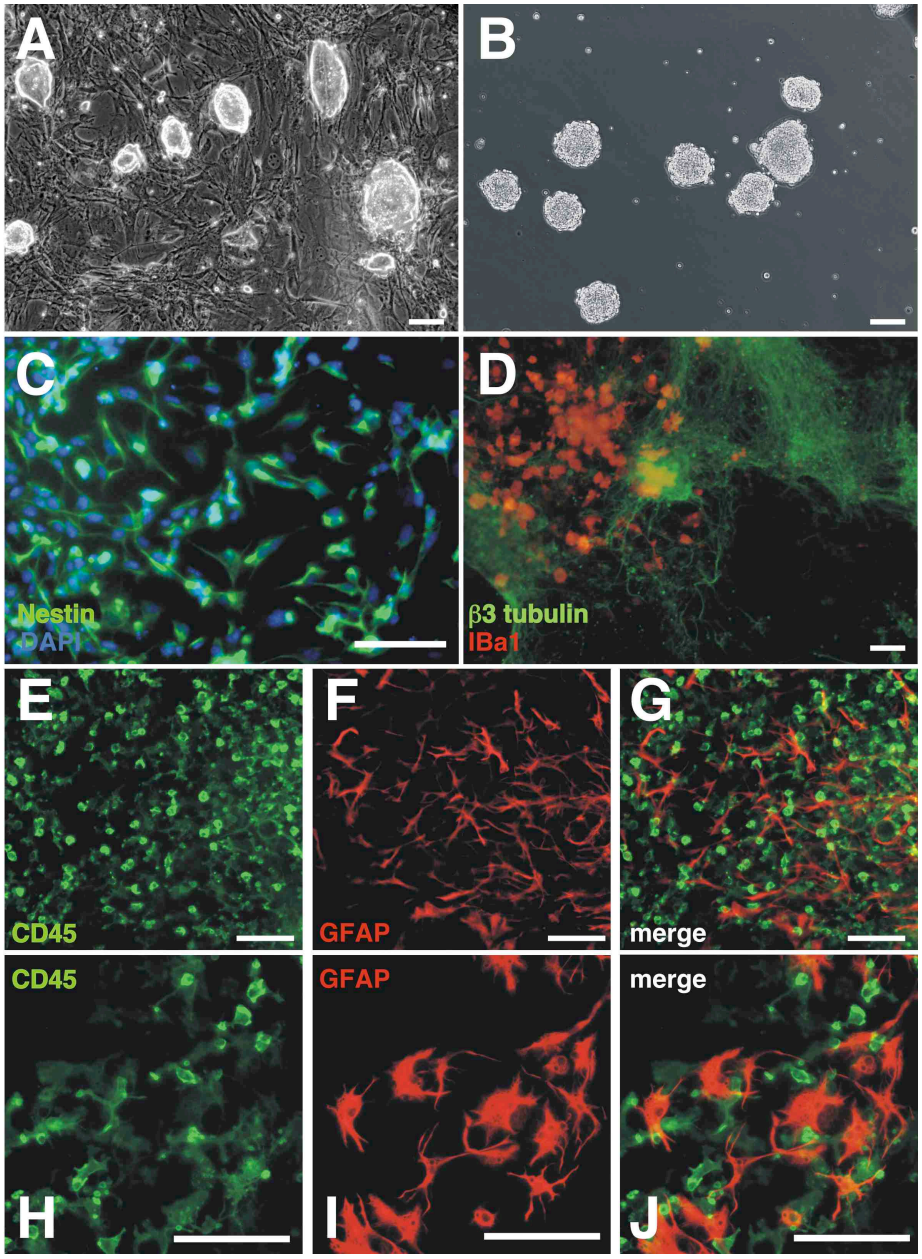

Figure 3-1. Differentiation of mES cells into microglial precursors. A. Phase contrast image of cultured ES cell (ES-C57BL/6) clusters on a layer of irradiated MEFs. **B.** Phase contrast image of EBs derived from ES cells (ES-C57BL/6). **C.** Neural precursor cells after 6 days of selection in ITSFn containing medium. Cells were immunostained with antibodies directed against nestin (green) and nuclei were counter-labelled with DAPI (blue). **D.** Microglial-like cells within a mixed neuronal culture. Microglial precursors were immunostained with antibodies directed against IBa1 (red), whereas neurons were positive for β-III-tubulin (green). Clusters of microglial cells were growing within the neuronal network. **E-G.** After 21 days of culture in the final step of the protocol high numbers of CD45 positive cells were observed. Co-immunostaining with an antibody specific for astrocytes (GFAP, red) showed no colocalization with CD45 (green) positive microglial precursors. **H-J.** Higher magnification of co-immunostaining of astrocytes (red) and microglial precursors (green). Scale bar: 100 µm.

3.2 Establishment of independent ESdM lines

The first microglial-like cells appeared approximately 3 weeks after growth factor withdrawal (Fig. 3-2A) and started to proliferate forming small cellular colonies between neural cells (Fig.3-2B). The cellular colonies were mechanically collected and seeded in culture dishes containing culture medium supplemented with 5% FCS. Cells grew adherent on the culture dishes and expanded under these culture conditions (Fig. 3-2C,D). The expanded ESdM lines homogenously showed immunolabelling by antibodies directed against Iba1 (Fig. 3-2E,F). Several independent ESdM lines were generated from the murine ES cell lines ES-C57BL/6-ATCC and ES-129-MPI (Table 3-1). The genetic background of the ES cell lines did not interfere with the efficiency to generate stably proliferating ESdM lines.

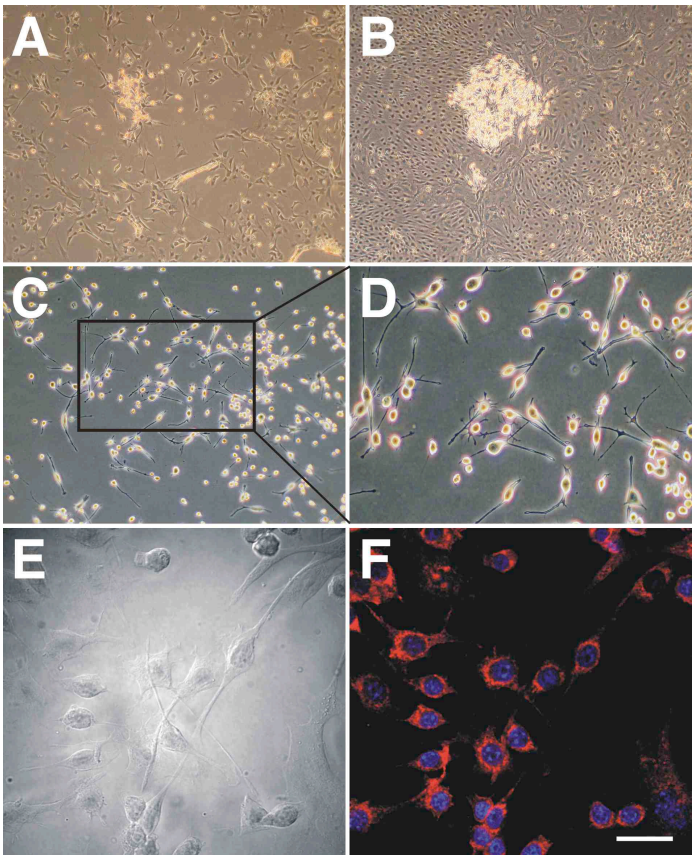

Figure 3-2. Establishment of ESdM lines. A. Phase contrast image of a few bright microglia-like cells next to dark neural precursor cells. Magnification: 10X. **B.** Phase contrast image of a bright shining microglial precursor colony within a dense layer of neural precursor cells. Magnification: 10X. **C.** Culture of mechanically isolated microglial precursors in culture medium containing FCS. Cells showed constant proliferation. Magnification: 10X. **D.** Higher magnification (20X) of insert in C. **E-F.** Phase contrast image of ESdM and immunostaining with antibodies directed against IBa1 (red). All cells of ESdM lines expressed IBa1. Nuclei were counterstained with DAPI. Scale bar 30µm.

Table 3-1. Summary and origin of established ESdM lines.

Label of the cell line established	Origin of the cell line
ESDM-1	ES-C57BL/6-ATCC
ESDM-3	ES-C57BL/6-ATCC
ESDM-4	ES-C57BL/6-ATCC
ESDM-5	ES-C57BL/6-ATCC
ESDM-6	ES-C57BL/6-ATCC
ESDM-7	ES-C57BL/6-ATCC
ESDM-8	ES-C57BL/6-ATCC
ESDM-9	ES-C57BL/6-ATCC
ESDM-10	ES-C57BL/6-ATCC
ESDM-11	ES-129-MPI
ESDM-12	ES-129-MPI

3.3 ESdM lines show exponential growth rate behavior

To determine the growth rate and proliferation ability of ESdM lines, a fixed number of cells were seeded and counted 24h, 48h, 72h and 96h later. Experiments were performed for three individual ESdM lines and summarized in table 3-2. Taken together, ESdM follow an exponential growth rate behavior (Fig. 3-3).

Table 3-2. Growth rate of three individual ESdM lines.

	Number of cells after			
Name	**24h**	**48h**	**72h**	**96h**
ESdM-4 P9	1.6×10^5	3.5×10^5	7.65×10^5	3.4×10^6
ESdM-5 P19	3.2×10^5	6.75×10^5	2.4×10^6	6.4×10^6
ESdM-9 P17	4×10^5	9.8×10^5	2.3×10^6	6.2×10^6
Mean	2.9×10^5	6.7×10^5	1.8×10^6	5.3×10^6
SEM	0.6×10^5	1.5×10^5	4.3×10^5	7.9×10^5

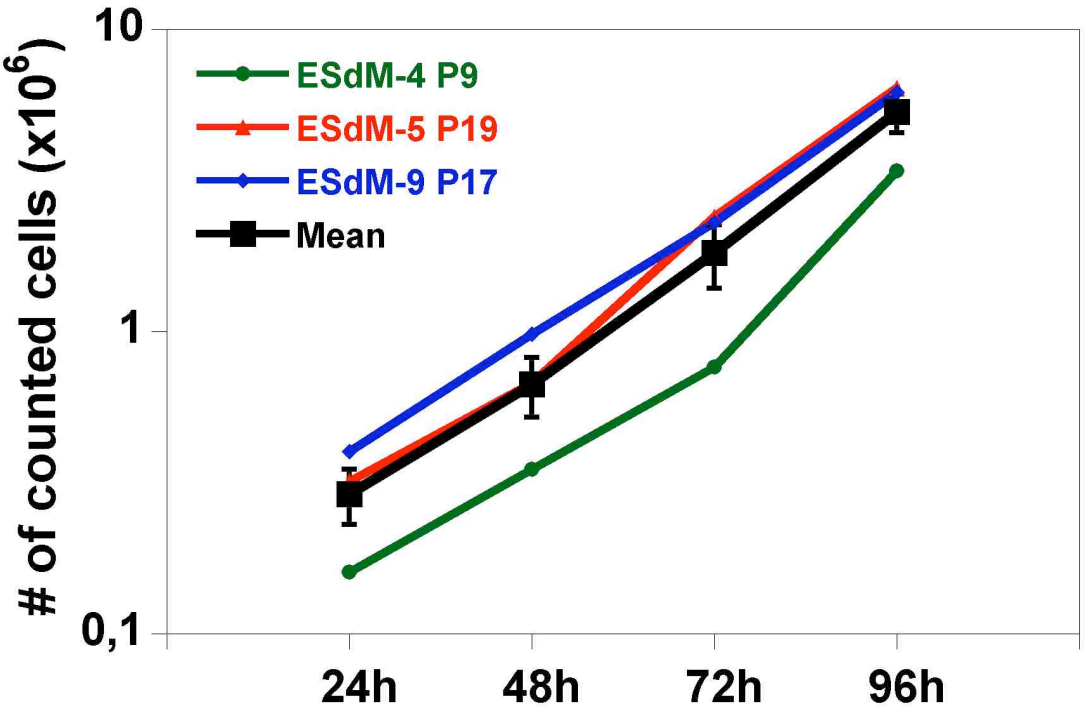

Figure 3-3. Exponential growth rate behavior of three independent ESdM lines and their mean.

3.4 ESdM lines have an immunological surface marker profile similar to primary microglia

Flow cytometry analysis was performed from seven individual ESdM lines to assess the surface marker profile and to confirm microglial identity. Cell surface molecule expression was compared to primary microglial cells as well as to the oncogenically transformed microglial cell line BV2. Figure 3-4 shows examples representing the surface marker profile of ESdM, BV2 and primary microglia. Each isolated ESdM line was marked for nine different epitopes. Detailed expression profile of each line is summarized in table 3-3A+B.

Similar to primary microglia, all ESdM lines showed high expression levels of CD11b and CD45. The cell line BV2 showed similar expression of CD11b and CD45 in respect to ESdM and primary microglia. F4/80 is an antigen that is exclusively expressed on microglia/macrophages. ESdM and the BV2 cell line showed high expression of F4/80, whereas primary microglia had lower, but still clear positive, expression of F4/80. In contrast to the typical microglial marker proteins none of the three tested cell populations were positive for cKit or CD34. Both surface markers are expressed on

certain stem cell populations, indicating that ESdM mainly consisted of differentiated cells, but not of stem cells. Primary microglia displayed little or very low constitutive major histocompatibility complex II (MHC class II) expression. However, expression of MHC class II was detected on 4 out of 7 ESdM lines at very low levels (Table 3-3A+B), which was comparable to MHC class II levels on BV2. As the principal immune effector cells resident within the CNS, microglia form adhesive interactions with other cell types and CNS substrates via members of the integrin superfamily. These molecules are expressed in the cell surface as heterodimers. As examples of the integrin superfamily, expression of two subunits (α4 integrin and β1 integrin) forming a heterodimer were analyzed. ESdM, BV2 as well as primary microglia showed high levels of α4 integrin and β1 integrin. Interactions between the growing family of B7 co-stimulatory ligands and their receptors are increasingly recognized as important pathways for co-stimulation and/or inhibition of immune responses. ESdM showed in 6 out of 7 lines high expression of B7.2 in line with BV2, whereas primary microglia showed a moderate expression of B7.2 compared to ESdM.

In summary, expression profile of cell surface receptors detected by flow cytometry on ESdM was very similar to primary microglia. ESdM showed undetectable levels of stem cell markers, but showed expression of markers identifying a population belonging to the myeloid lineage.

Table 3-3A. Surface marker expression profile of seven individual ESdM lines. ESdM lines were labeled for nine epitopes and the corresponding isotype controls. Values are expressed as mean fluorescent intensity (MFI) of each marker. Bold typed values indicate that the expression of the marker was regarded as positive (MFI ≥ 15).

Line Passage #	**ESDM-1 P11**	**ESDM-3 P2**	**ESDM-4 P2**	**ESDM-5 P2**	**ESDM-6 P2**	**ESDM-8 P2**	**ESDM-9 P2**
rat IgG2b	7.5	6.2	4.7	6.0	8.1	7.6	5.0
rat IgG2A	13	8.1	6.7	7.2	8.5	7.0	6.3
hamster IgG	7.2	6.7	5.3	6.3	10.1	6.4	4.9
cKit	12.5	8.5	7.0	13	10.5	7.9	7.2
CD11b	**176**	**226**	**153**	**109**	**211**	**130**	**222**
CD45	**750**	**184**	**249**	**40.7**	**709**	**99.5**	**210**
F4/80	**70.2**	**47.4**	**235**	**281**	**48.3**	**25.3**	**42.3**
CD34	10.9	6.77	9.7	8.3	10.1	6.2	4.9
B7.2	**88.9**	**57.7**	**106**	**15.7**	**60.7**	**62.3**	**66.6**
α4 integrin	**103**	**176**	**261**	**244**	**40.4**	**41.5**	**52.1**
β1 integrin	**93.7**	**103**	**240**	**172**	**237**	**49**	**70.6**
MHC class II	**22.6**	12.5	4.9	**15.9**	**15**	12.3	**10.9**

Table 3-3B. Surface marker expression profile of BV2 and primary microglia. Both cell types were labeled for nine epitopes and the corresponding isotype controls. Values are expressed as mean fluorescent intensity (MFI) of each marker. Bold typed values indicate that the expression of the marker was regarded as positive (MFI ≥ 15).

Cell type	**BV2**	**Primary microglia**
rat IgG2b	6.9	14.9
rat IgG2A	8.8	10.2
hamster IgG	6.4	9.47
cKit	9.6	14.9
CD11b	**503**	**532**
CD45	**538**	**238**
F4/80	**285**	**48.8**
CD34	14.4	**15.3**
B7.2	**430**	**29.4**
α4 integrin	**561**	**97.8**
β1 integrin	**449**	**89.9**
MHC class II	**19.5**	12.2

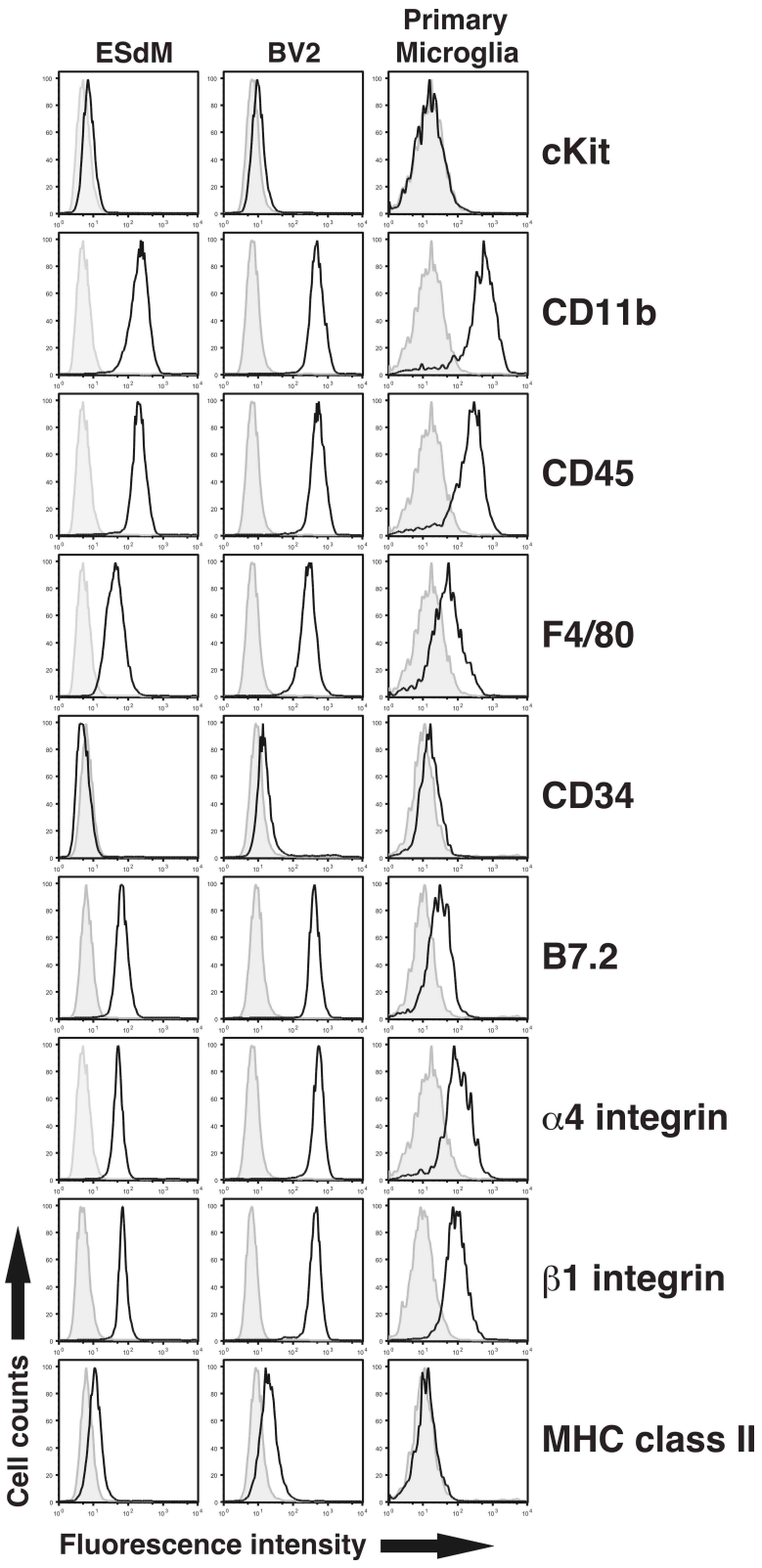

Figure 3-4. Representative flow cytometry analysis of ESdM, primary microglia and BV2. ESdM, BV2 and primary microglia are similar in the expression pattern of cKit, CD11b, CD45, CD34, β1 integrin and MHC class II. Primary microglia show decreased levels of F4/80, α4 integrin and B7.2 compared to ESdM and BV2. Individual isotype control antibodies are represented as filled grey histogram.

3.5 ESdM keep immunological profile of microglia over long-term culture

To prove the stability of microglial markers expressed on ESdM, ESdM were cultured over time. Every 5th passage ESdM were analyzed by flow cytometry for some typical microglial markers. No relevant change of cell surface molecule expression of CD11b, CD45, F4/80, B7.2, α4 integrin, β1 integrin and MHC class II was observed over 20 passages (Table 3-4). After 25 passages the analyzed ESdM line showed decreased expression of all seven checked markers indicating that ESdM phenotype is not stable over long culture time since ESdM are not oncogenically transformed.

Table 3-4. Microglial surface marker profile of ESdM over 25 passages. ESdM were cultured as described. Every 5th passage surface marker profile of ESdM was analyzed. Values are expressed as MFI of each marker.

Line Passage #	**ESDM-5 P10**	**ESDM-5 P15**	**ESDM-5 P20**	**ESDM-5 P25**
rat IgG2b	9.36	4.16	6.3	3.47
rat IgG2A	7.51	4.97	7.64	3.57
hamster IgG	8.35	4.33	6.24	3.7
CD11b	193	145	327	13.3
CD45	276	274	474	39
F4/80	230	138	363	20.6
B7.2	73.8	60.1	83.7	7.14
α4 integrin	286	127	186	26.4
β1 integrin	389	208	429	29.3
MHC class II	16.6	12.3	15.4	3.77

3.6 Functional characterization of ESdM *in vitro*

3.6.1 Pro-inflammatory stimuli influence the transcription of pro- and anti-inflammatory mediators in ESdM

One typical feature of microglia is the inducibility of proinflammatory cytokines and of reactive oxygen species gene transcripts after immune stimulation. Therefore, we determined the gene transcript levels for TNF-α, IL-1β, iNOS and TGF-β1 by real-time RT-PCR in ESdM lines after stimulation with IFN-γ or LPS, and compared them to primary microglia and the cell line BV2.

LPS stimulation significantly increased mRNA transcripts of iNOS in ESdM (49.6 fold +/- 15.9) as well as in primary microglia (55.8 fold +/- 34.5), whereas BV2 showed less upregulation of NOS2 gene transcripts (14 fold +/- 1.1)). TNFα was strongly upregulated after LPS stimulation in primary microglia (14.2 fold +/- 2.3), but only slightly, but still significantly, in ESdM as well as in BV2 (both 2.1 fold +/- 0.2). LPS stimulation had almost no effect on IL1β gene transcripts in BV2 (2.1 fold +/- 0.4), but showed clear upregulation of IL1β gene transcripts in ESdM (11.9 fold +/- 2.5) and in primary microglia (68 fold +/- 10.1). Downregulation of anti-inflammatory cytokines like TGFβ was observed after LPS stimulation in primary microglia (0.5 fold +/- 0.1) and in ESdM (0.7 fold +/- 0.1), but was absent in BV2 (no significant change to non stimulated) (Fig. 3-5A).

After IFN-γ stimulation, ESdM showed significant upregulation of iNOS gene transcripts (461.4 fold +/- 97.5), whereas TNFα was only slightly upregulated (1.5 fold +/- 0.2). Primary microglia showed significant upregulation of iNOS transcripts (~ 1770 fold) and upregulation of TNFα (~ 4.3 fold). BV2 showed also upregulation of iNOS (~ 1770 fold) and TNFα (2.5 fold +/- 0.4) gene transcripts. Downregulation of TGFβ was observed in all three cell types after IFNγ stimulation. IL1β gene transcription was almost unchanged in all three cell types after IFNγ stimulation (Fig. 3-5B).

Taken together, ESdM have a similar response in gene transcription to pro-inflammatory stimuli compared to primary microglia.

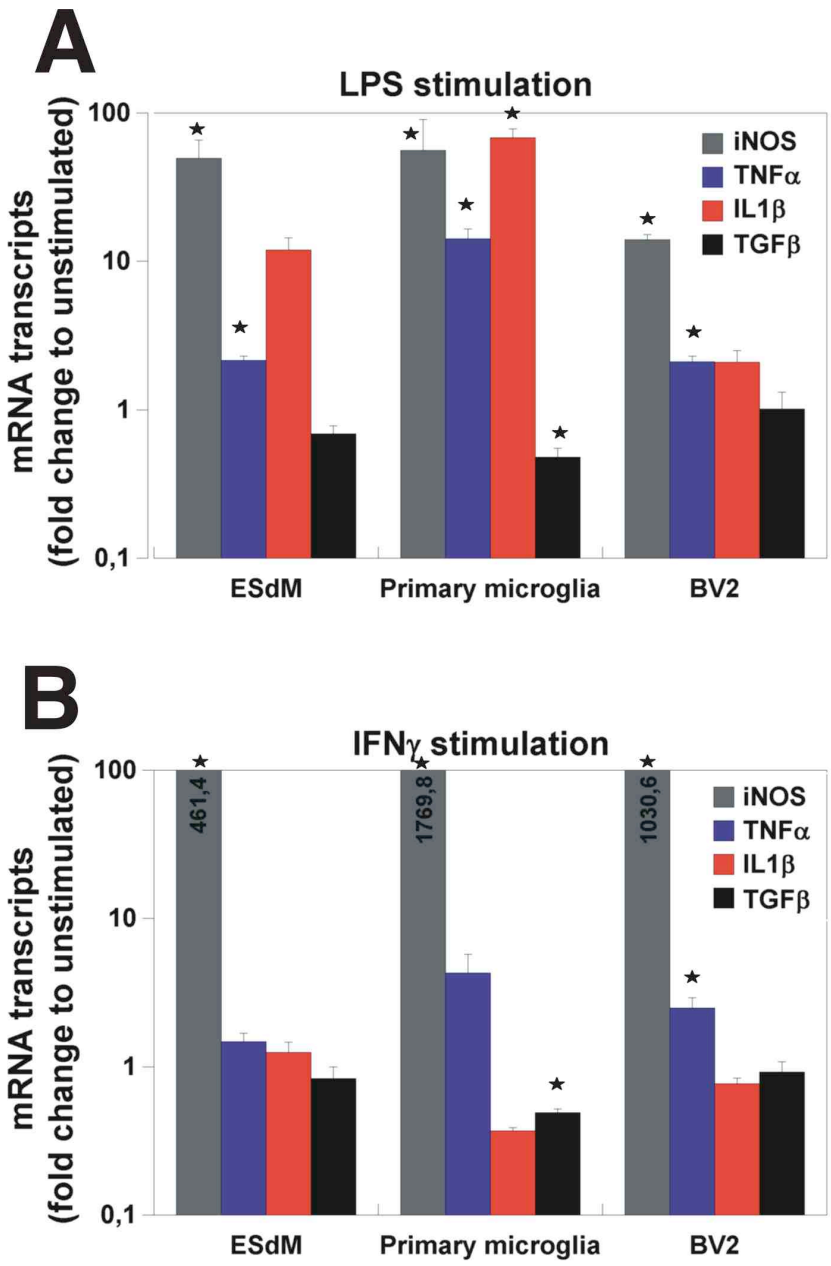

Figure 3-5. Cytokine gene transcripts analysis of ESdM, BV2 and primary microglia after LPS (A) and IFNγ (B) stimulation. A. LPS stimulation upregulated significantly iNOS and TNFα gene transcripts in all three cell types. However, LPS stimulation increased IL1β gene transcripts in ESdM and primary microglia and but showed almost no effect in BV2. Downregulation of anti-inflammatory cytokines like TGFβ was observed after LPS stimulation in primary microglia and in ESdM, but was absent in BV2 (no significant change to non stimulated). **B.** After IFNγ stimulation all three cell types showed dramatic upregulation of iNOS gene transcripts, whereas IL1β gene transcription was almost unchanged. TNFα gene transcripts were only slightly upregulated in ESdM, but moderately increased in primary microglia and BV2. Downregulation of TGFβ was observed in all three cell types after IFNγ stimulation. Data are represented as means +/- SEM of three individual experiments and were analyzed using one-way ANOVA (*$p<0.05$; $n=3$).

3.6.2 Migratory potential of ESdM towards fractalkine

The major role of chemokines is to guide the migration of cells. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine. The biological effects of all chemokines are mediated by specific receptors that belong to the large family of G protein-coupled receptors with seven transmembrane regions. In the CNS, fractalkine (CX3CL1) is the most prominent chemokine and is constitutively expressed in neurons. It has several singular features that make it particularly appealing in the context of neuroinflammation and neuroprotection. Its receptor CX3CR1 (fractalkine receptor) is also highly expressed in the CNS, primarily by microglia. Thus, we checked the migratory capacity of ESdM towards fractalkine and compared it with primary microglia as well as with BV2. However, before performing the chemotaxis assay, ESdM were analyzed for the presence of the fractalkine receptor. Flow cytometry analysis showed that ESdM were clearly positive for CX3CR1 (Fig. 3-6A).

Next, to determine optimal concentration of fractalkine for *in vitro* migration, CFSE labeled ESdM cells were seeded at a density of 1 x10^5 cells in the upper chamber of the transwell. Increasing concentrations of fractalkine (0-20ng/ml) were added to the lower chamber of the transwell system. The cells were kept for 3 hours at 37°C. Migrated cells into the medium as well as under the transwell were counted under UV microscope and regarded as migrated. As demonstrated in Figure 3-5B, ESdM were attracted to the chemokine stimulus in a concentration dependent manner. Optimal concentration for the migration of ESdM was assessed at 15ng/ml fractalkine. At higher concentrations (20ng/ml), ESdM showed even less migration (Fig. 3-6B).

To compare the *in vitro* migration of ESdM with primary microglia and BV2, all three cell types were CFSE labeled and assessed to 15ng/ml fractalkine in the transwell migration system. Under basal conditions, there was low migration of primary microglia (44 counted cells +/- 7.4), however under the CX3CL1 gradient migration was increased about 3 fold (137 counted cells +/- 26.3). Migration capacity of ESdM was very similar to primary microglia under basal conditions. In line with primary microglia ESdM showed about 3 fold increased migration (113.7 counted cells +/- 13.7) under the CX3CL1 gradient. In contrast, BV2 already showed high basal migration (95.8 counted cells +/- 19.2), and migration under CX3CL1 condition was only increased about 1.4 fold (131 counted cells +/- 22.8) (Fig. 3-6C).

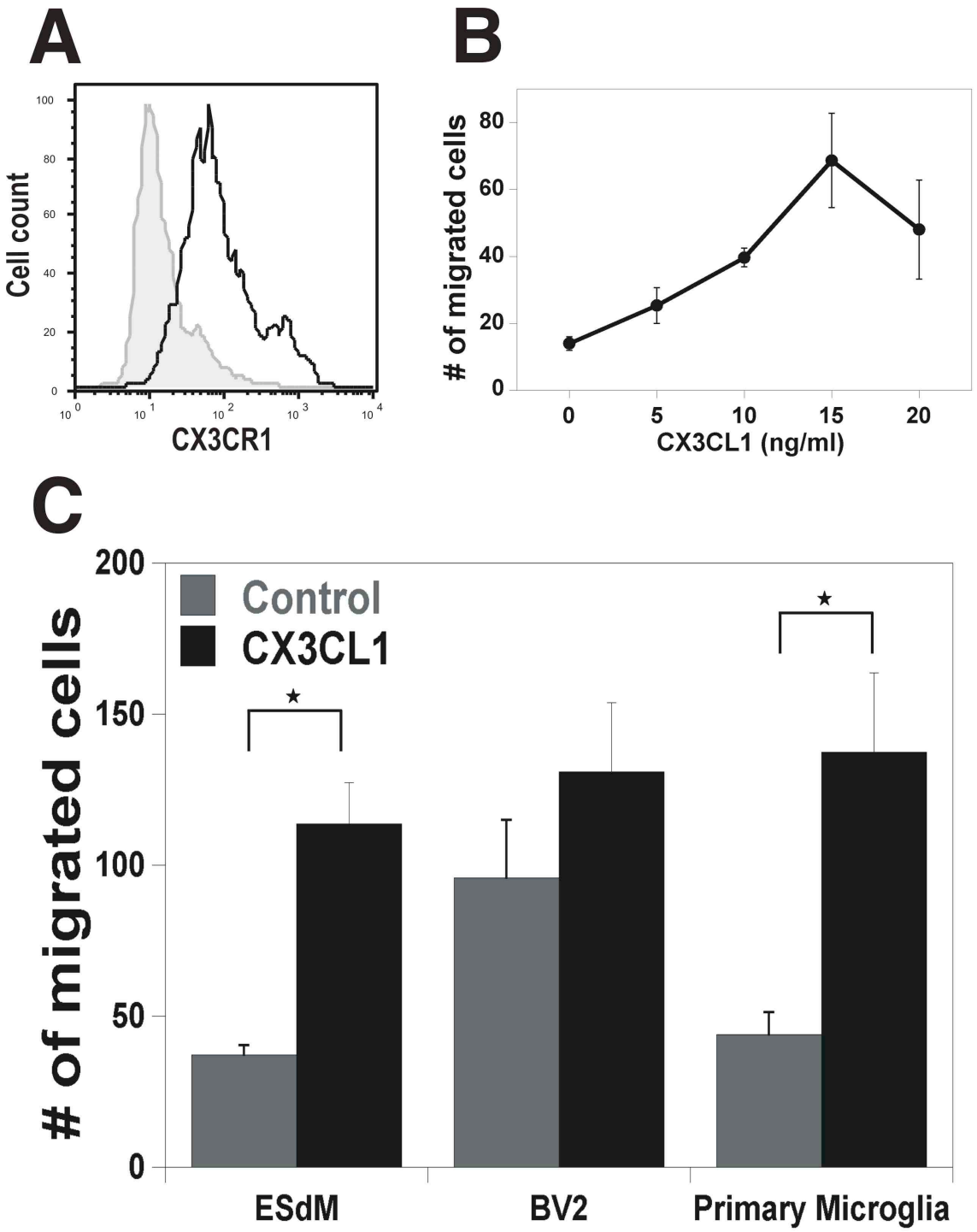

Figure 3-6. Migratory capacity of ESdM. A. Flow cytometry analysis of ESdM with an antibody directed against CX3CR1 (fractalkine receptor). Isotype control antibody is represented as filled grey histogram. **B.** Migration of ESdM towards the ligand CX3CL1 (fractalkine) was concentration-dependent. **C.** Migratory capacity towards fractalkine of ESdM was compared to primary microglia or BV2 cell line. ESdM showed directed migration comparable to primary microglia, whereas BV2 showed almost no specific migration. Data are presented as means +/- SEM of three independent experiments and were analyzed using one-way ANOVA (*$p<0.05$; n=3).

3.6.3 Aβ uptake of ESdM *in vitro*

Aggregation of Aβ is one of the key pathological hallmarks of AD. In addition, phagocytic capacity is an important functional aspect of primary microglia. *In vitro*, microglial cells have been demonstrated to have the ability to clear Aβ efficiently. Therefore we aimed to compare the phagocytic potential of ESdM with primary microglia and the microglial cell line BV2. For that, CFSE labeled cells were seeded on chamber slides and stimulated for 24h with the pro-inflammatory agent LPS. Afterwards, biotinylated human $A\beta_{1-42}$ was applied to the cells for 24h. After fixation and staining of $A\beta_{1-42}$ with Cy3 conjugated streptavidin, cells emitting a yellow signal were counted as having phagocytosed (Fig. 3-7A).

Compared to primary microglia ESdM showed slightly less Aβ phagocytosis under basal conditions. However, after LPS stimulation ESdM increased Aβ phagocytosis from 29% +/- 5.2% to 52.1% +/- 6.3%. Primary microglia showed highest capacity in Aβ phagocytosis after stimulation with 500ng/ml LPS (from 47.6% +/- 5.5% to 83.9% +/- 4.3%). The microglial cell line BV2 had compared to the both other cell types the lowest Aβ phagocytosis. Under basal conditions, only 21.9% +/- 6.9% of cell were phagocytosing and upon stimulation, BV2 increased phagocytosis to only 43.4 +/- 6.8% (Fig. 3-7B).

Taken together these data show that ESdM efficiently take up aggregated Aβ *in vitro* at a comparable level to primary microglia, and superior to BV2 cell line.

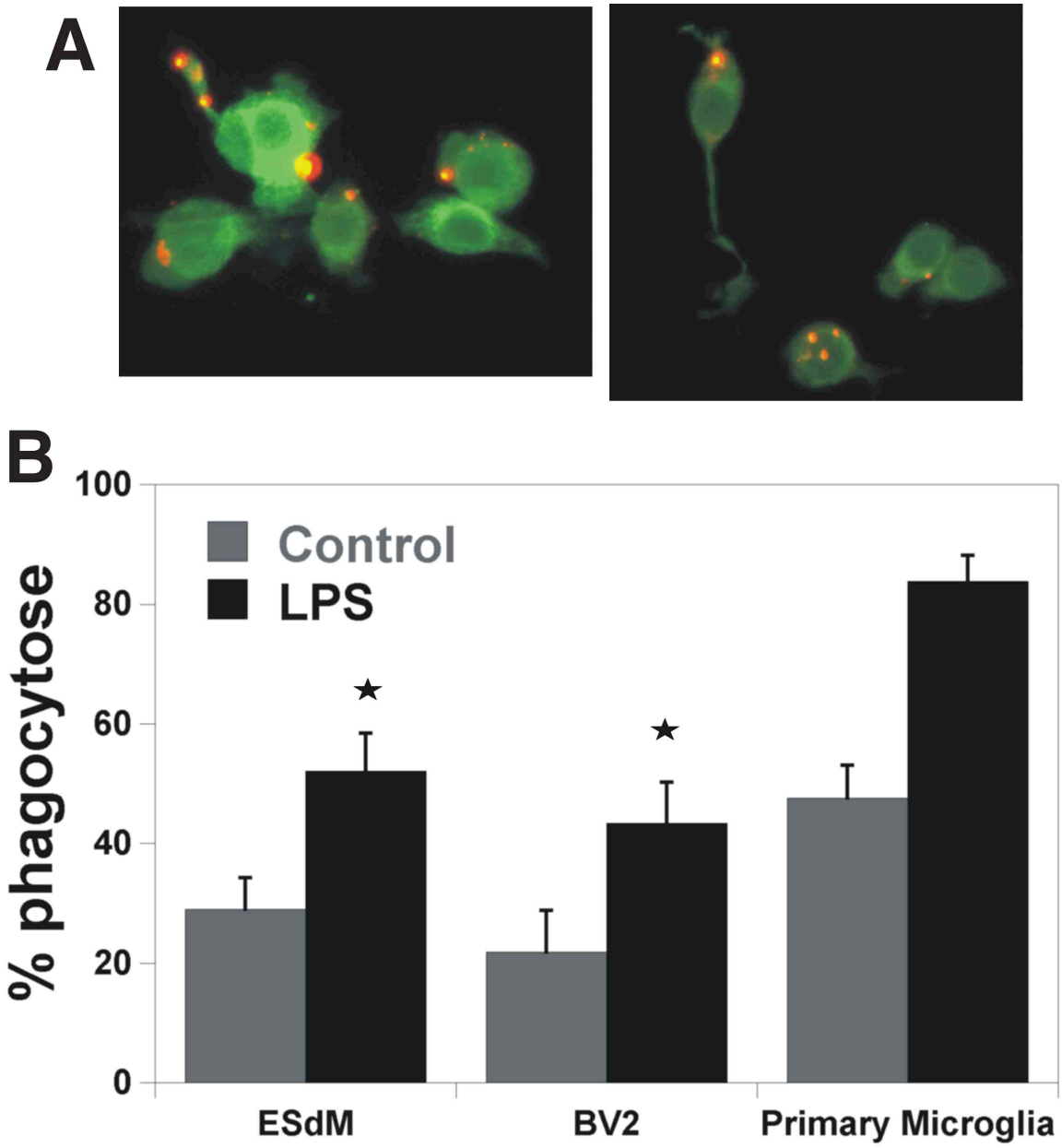

Figure 3-7. Aβ phagocytosis of ESdM. A. Representative pictures of CFSE labeled ESdM having phagocytosed Cy3 labeled $A\beta_{1-42}$. Cells emitting a yellow signal were counted as phagocytosing. **B.** Aβ phagocytosis of ESdM, BV2 and primary microglia under basal conditions and after treatment with LPS for 24h. All three cell types showed increased phagocytosis after LPS stimulation. However primary microglia showed highest, but not significant, Aβ phagocytosis under both conditions. Data are presented as means +/- SEM of three independent experiments and were analyzed using one-way ANOVA (*$p<0.05$; n=3).

3.6.4 ESdM exhibit microglial identity *in vivo*

Under non pathological conditions, microglia exhibit a ramified morphology *in vivo*. For *in vivo* detection, ESdM were genetically modified with lentiviral particles produced from a vector overexpressing GFP and the antibiotic selection marker neomycin under ubiquitous promoters pgk (phosphoglycerate kinase) (Fig. 3-9A). ESdM were lentivirally transduced with particles leading to GFP expression in 8% +/- 3.2 % of cells at 3 days after transduction. Then an antibiotic selection procedure was applied over 7 days. After transduction and selection, ESdM were homogenously expressing GFP (Fig. 3-9B). To analyze the engraftment potential of ESdM *in vivo*, GFP^+ ESdM were transplanted into both hemispheres of postnatal day 2 (P2) mice. In total, $2x10^6$ cells were transplanted by injection in each hemisphere. After 28 days animals were sacrificed and analyzed by immunohistochemistry. GFP^+ cells were found engrafted within the brain parenchyma having a ramified morphology (Fig. 3-9C). Immunofluorescence labeling with specific antibodies directed against IBa1 was performed. Many (82.9% +/- 7.3%) of IBa1+ cells showed immunoreactivity for GFP. Data indicate that upon transplantation ESdM can integrate into the normal CNS and might function there as microglia.

3.7 ESdM as a tool for a cell therapy approach in APP23 mice

After *in vitro* characterization, ESdM seem to be a good tool in a cellular therapy approach of AD since ESdM have been shown to take up Aβ. At first, ESdM were transduced with lentiviral particles to overexpress GFP followed by antibiotic selection to enhance GFP expression (Fig. 3.9A+B).

Systemic transplantation (into tail vein) of GFP^+ ESdM was not successful since ESdM were not detected in the target region (data not shown). It seemed that ESdM were not able to overcome the blood brain barrier to enter the region of interests.

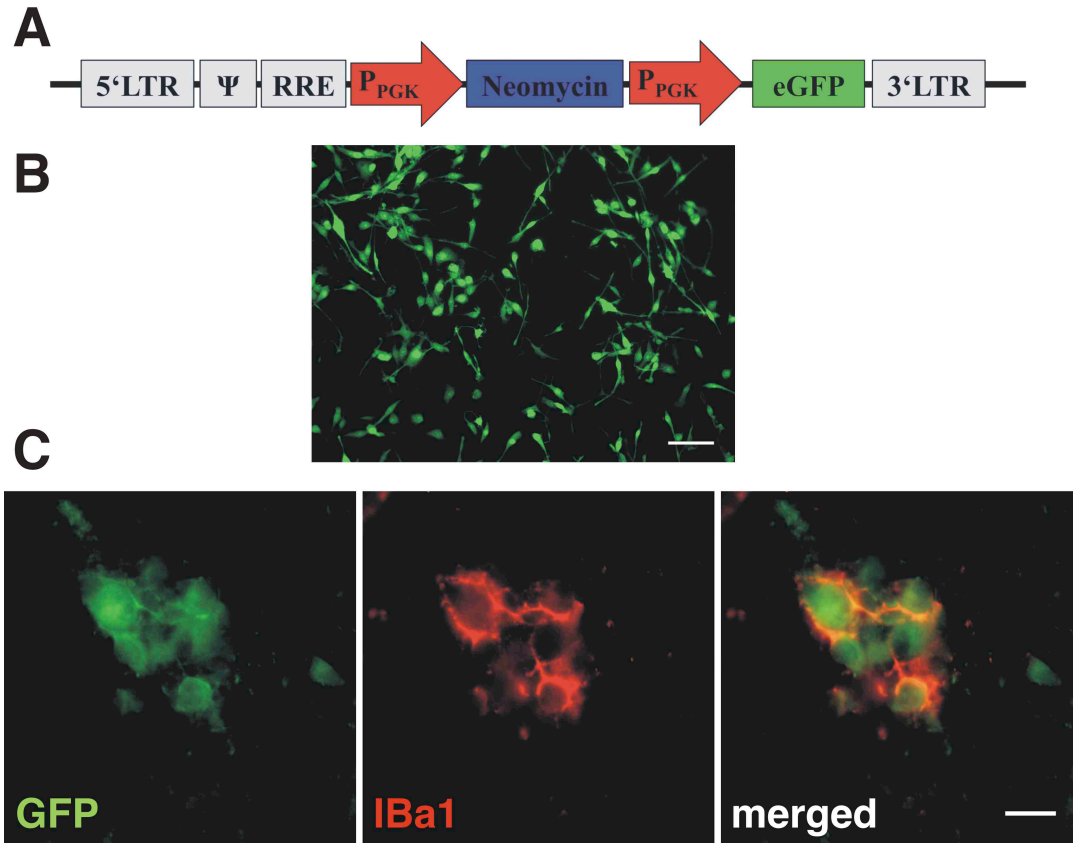

Figure 3-9. ESdM exhibit a ramified morphology after transplantation into neonatal mice. A. Schematic representation of the lentiviral construct to genetically modify ESdM. **B.** After transduction and selection ESdM express homogenously GFP. Scale bar 100µm. **C.** Lentiviral transduced GFP^+ cells were transplanted into P2 mice. After 28 days brains were analyzed for the presence of GFP^+ cells which also co-immunostained with the microglial marker IBa1. GFP^+ cell were found in a ramified morphology and were also reactive for IBa1. Scale bar 20µm.

To overcome this inhibitory condition, GFP^+ ESdM were stereotactically injected to the region of interest, here the cortex of APP23 mice. In total, 4 APP23 mice were injected with $2x10^5$ GFP^+ ESdM and analyzed for the presence of Aβ at the transplantation site. As control, the exact cortical area found on the contralateral site of each transplanted brain was analyzed in the same manner. Upon transplantation, GFP^+ ESdM were detected in close proximity of the transplantation site and stayed there in circle-like area (Fig. 3-10A+D). Migration to other areas except the transplantation site was not observed. Immunostaining for aggregated Aβ showed that at the transplantation site significant less positive staining was present. Thus, ESdM seemed to efficiently clear Aβ after *in vivo* transplantation in their close proximity (Fig. 3-10A+B). Some Aβ aggregates were still found in the area of transplantation, and in between these plaques co-localization of GFP^+ ESdM with Aβ immunostaining was detected indicating that ESdM were phagocytosing Aβ (Fig. 3-10B-F, white arrows).

Quantification of the Aβ plaque burden in the transplanted region and in the corresponding contralateral site (Fig. 3-11A) showed that in the transplanted area the mean plaque size was significantly decreased to 17,690µm^2 +/- 112µm^2 compared to 65,266µm^2 +/- 108µm^2 measured in the contralateral site (Fig. 3-11C). In addition, transplantation of ESdM resulted in more but smaller plaques (190 counted plaques with a size between 20-400µm^2) and only 2 counted plaques with a size above 400 µm^2. In contrast, the contralateral site had 79 counted plaques with a size between 20-400µm^2 and 28 counted plaques with a size above 400µm^2 reaching a size up to 6900µm^2. This plaque size distribution indicates that ESdM were able to disrupt Aβ aggregates (Fig. 3-11B). Taken together, the results of the cellular therapy approach of ESdM in an animal model of Alzheimer's disease indicate that ESdM are capable to remove plaques after stereotactic injection.

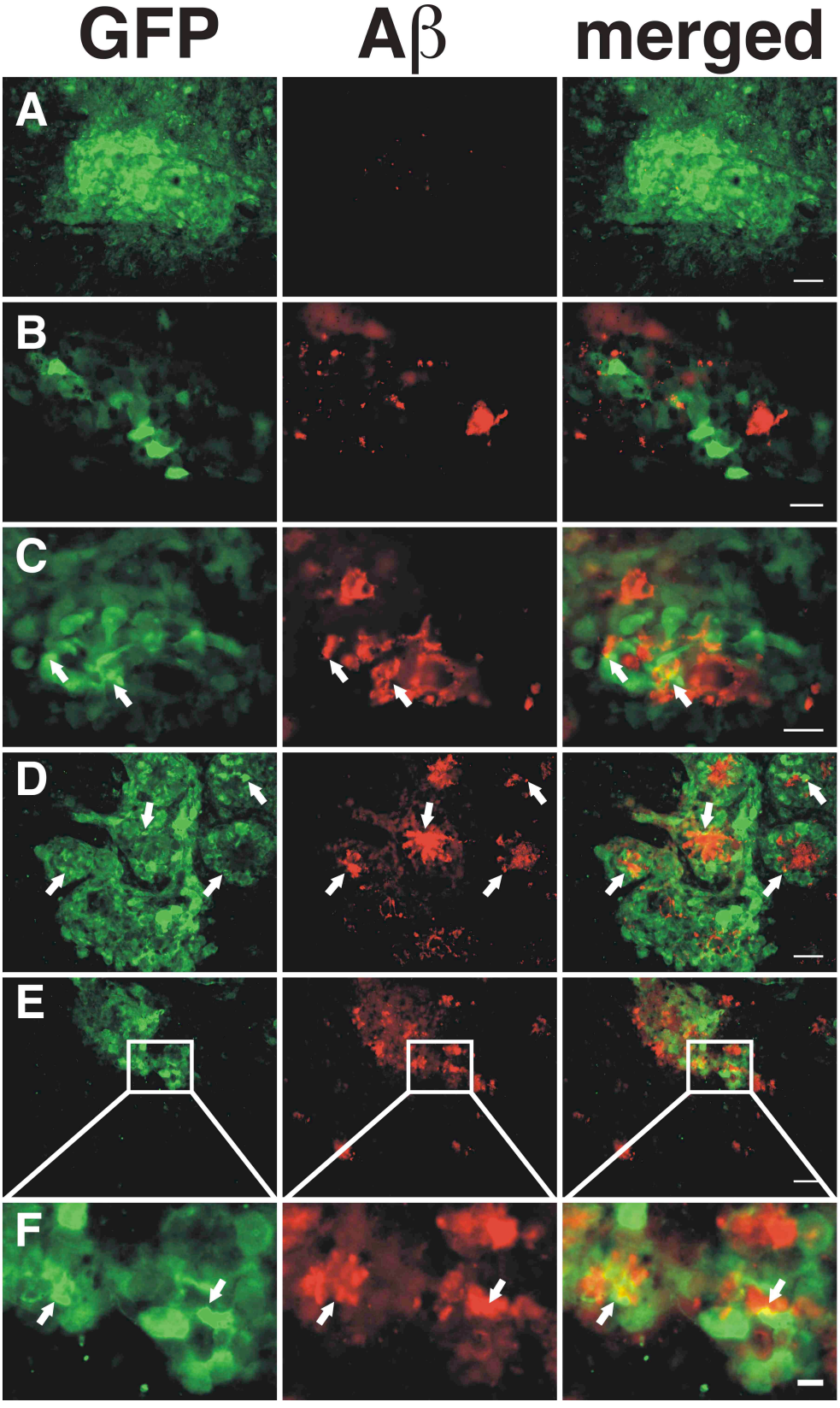

Figure 3-10. ESdM efficiently remove Aβ aggregates after stereotactic injection. A.+B. At the site of transplantation ESdM removed most of all Aβ aggregates. **C.-F.** GFP+ ESdM co-localize with Aβ immunostaining indicating the presence of active phagocytosis of Aβ. Scale bar 50µm, except in F 10µm.

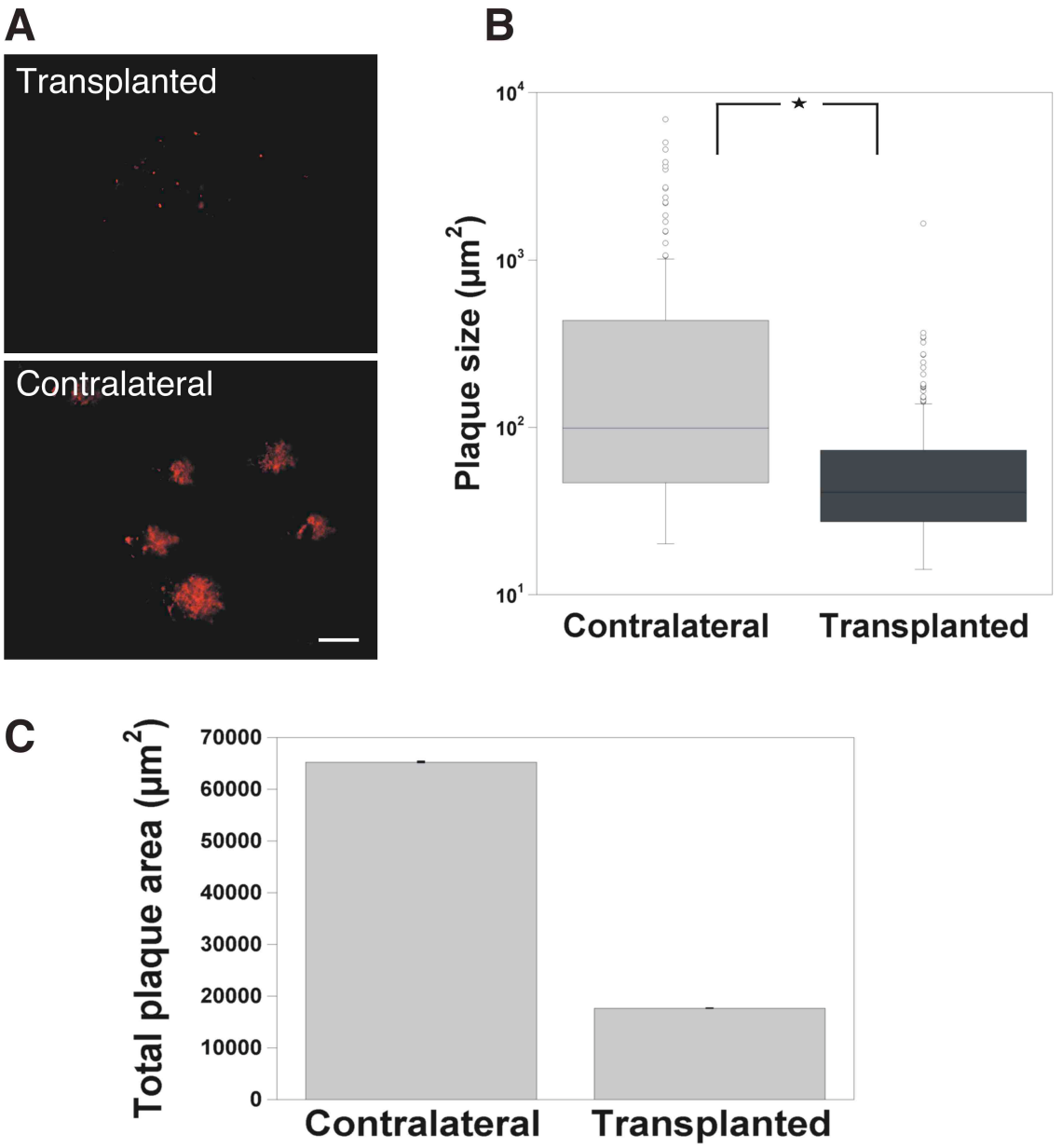

Figure 3-11. Quantification of Aβ plaque burden after transplantation. A. Representative images of the transplanted and the contralateral site used for quantification of Aβ plaque burden. **B.** In the transplanted area significantly more but smaller plaques are detected. In addition, the mean plaque size is smaller in the transplanted hemisphere compared to the contralateral site indicating a beneficial effect of the cell therapy. **C.** Looking at the total plaque area, transplanted regions had significantly less positive staining for aggregated Aβ compared to the contralateral site. Data are presented as box plot (B) or as means +/- SEM (C) of four individual animals and were analyzed using one-way ANOVA (*$p<0.05$; n=4).

4 Discussion

4.1 ESdM – an alternative to primary microglia?

Our study was initiated by a pioneering work from Tsuchiya and colleagues, who suggested that a small population of microglial-like cells can be found next to neural cells which differentiated out of mouse EBs (Tsuchiya *et al.*, 2005). Their differentiation protocol was based on a protocol for the generation of dopaminergic neurons (Lee *et al.*, 2000). During neuronal differentiation they found a 'contaminating' population positively stained for IBa1 and CD45, which they isolated out of the mixed neuronal culture with a gradient method for further investigations (Tsuchiya *et al.*, 2005). However, they did not report any generation of stable proliferating microglial cell lines and do not provide an extensive characterization of their ES cell derived microglial-like cells. In this work a population of microglial precursors was differentiated from two different strains of mES cells. According to our modified differentiation protocol we were now able to induce microglial precursors from mES cells in a consistent and efficient manner. In contrast to the previous report (Tsuchiya *et al.*, 2005) our differentiation resulted in a high amount of proliferative microglial precursors. Upon mechanical isolation we were even able to keep ESdM lines in culture over 25 passages, sufficient to generate high cell amounts for biochemical analysis, cell screening assays or experimental cell therapy experiments.

4.1.1 Phenotype of ESdM

Immunostaining during the differentiation protocol revealed an IBa1$^+$ and CD45$^+$ cell population within a neuronal network of β-III-tubulin$^+$ cells and next to a subpopulation of GFAP$^+$ cells, a classical marker for astrocytes. Interestingly, after isolation we were able to further propagate these IBa1/CD45$^+$ cells under defined culture conditions leading to a homogenous population of cells comparable in their phenotype to primary microglia. Cells did not show any changes in cell surface protein expression after freezing and thawing procedure allowing straightforward handling.

The origin of microglia is still widely discussed. The most widely accepted hypothesis is that microglia arise from the mesoderm and colonize the CNS in two waves at early embryonic and fetal development, although it is still questioned whether a subpopulation is derived from the neuroectoderm lineage (Chan *et al.*, 2007). Findings

suggest that there is a developmental window during embryogenesis where microglial cells colonize the nervous system. There is evidence that two separate 'populations' of microglia one originating from an undefined precursor during embryonic development the other from fetal myeloid precursors contribute to the colonization of the CNS. Indeed it is unclear whether microglia may derive from more than one type of progenitor cells during embryonic and fetal development (Chan *et al.*, 2007). In the healthy adult brain it is assumed that microglia are replenished intrinsically and do not require significant turnover from circulating blood progenitors, whereas it is widely believed that under detrimental pathologic conditions and inflammatory brain diseases bone marrow derived inflammatory monocytes are recruited from the blood into the brain (Simard and Rivest, 2004; Simard *et al.*, 2006). However, recent findings strongly suggested that maintenance and local expansion of microglia are solely dependent on the self-renewal of CNS resident cells even in denervation or CNS neurodegenerative disease (Ajami *et al.*, 2007). Our differentiation procedure to derive microglial precursors from ES cells is based on a classical protocol for obtaining neurons via neuroectodermal differentiation. Thus, data favor the view that microglia might derive from a neuroectodermal progenitor cell. However, we cannot exclude that a few mesodermal cells from the stage of EB formation survive in the culture during the following steps of neuroectodermal differentiation and give rise to microglia at later stages of neuronal differentiation. Clonal analysis of single cells during differentiation of ES cells to microglia would bring light to the question of microglia's origin and whether microglia originating from neuroepithelia could provide a source for microglia turnover in the brain.

Since being first described, several markers to define the phenotype of microglia have been proposed although there is no single marker to definitely distinguish between microglia and tissue macrophages. In literature, ramified microglia are described to constitutively express CD11b/CD18 complex, as well as CD45 (Guillemin and Brew, 2004). CD45 is a membrane tyrosine phosphatase that is used to distinguish cells of the hematopoietic lineage from the endothelial lineage. All ESdM lines analyzed in this work were highly immunoreactive for CD11b, CD45 and F4/80. F4/80 is exclusively expressed on microglia/macrophages (Austyn and Gordon, 1981). Although ESdM were generated from mES cells, almost no detectable levels of cKit, a stem cell marker, were expressed on all analyzed ESdM lines indicating that all cells committed to differentiation. CD34, which is expressed on hematopoetic stem cells, was also absent

on all analyzed ESdM lines. Expression and/or induction of immune effector molecules is a prerequisite of microglia for antigen presentation, co-stimulation of bystander cells, as well as adhesion. Antigen presentation depends on MHC class I and II molecules. Although it is reported that microglia can successfully present antigen, it is known that under non-activated conditions, microglia do not express high levels of MHC class II (Benveniste *et al.*, 2001). This is in harmony with our findings, since ESdM displayed only very low amount of MHC class II and primary microglia do not display any MHC class II. The co-stimulatory molecule B7.2 as well as two members of the integrin family were found to be expressed on ESdM indicating that they can exhibit immunoregulatory functions. Taken together the analyzed surface antigen expression of all ESdM lines was almost indistinguishable to primary microglia.

Long-term propagation up to passage number 25 did not result in any change of the surface marker profile. Nevertheless there was a reduction of fluorescence intensity of several cell surface protein in the flow cytometry analysis after passage 25. Changes in cell surface proteins after 25 passages could be explained by a physiological limit of doublings of differentiated cells since ESdM lines were not manipulated with oncogenic genes.

To analyze the engraftment potential of ESdM *in vivo*, ESdM were lentivirally transduced with GFP and transplanted into both hemispheres of P2 mice. The GFP^+ cells were found engrafted within the brain parenchyma and had a ramified morphology. Microglial identity was confirmed by colocalization with the microglial marker IBa1. Thus, from our observation we can conclude that upon transplantation ESdM can integrate into the normal CNS and might function there as microglia.

4.1.2 Functionality of ESdM

The inducibility of proinflammatory cytokines and reactive oxygen species after pro-inflammatory triggers is a typical feature of microglia (Hanisch, 2002). In our experimental paradigm we used two different inflammatory triggers (LPS and IFNγ) to study cytokine gene transcription by real-time PCR. In general, ESdM always showed a similar response in gene transcription compared to primary microglia, but real-time PCR analysis also manifested slight differences between primary microglia, ESdM and BV2. In detail, ESdM upregulated IL1β gene transcription about 12 fold, whereas primary microglia showed 68 fold increase after LPS stimulation. Interestingly, BV2 was not able to upregulate IL1β gene transcription at all, which might be a consequence

of oncogenic transformation. One should keep in mind that ESdM are generated completely *in vitro* meaning that any microglia coming out of this differentiation procedure never received any stimulus from the CNS environment. This lack of intrinsic and/or extrinsic factors might be the reason for the slightly different behavior of ESdM after inflammatory stimulus. Taking this into consideration it is reasonable to define the isolated population rather as microglial precursors than microglia.

Fractalkine is constitutively expressed in neurons, as soluble and a membrane-bound form, both promoting chemotaxis (Re and Przedborski, 2006). A transgenic mice having a reporter gene encoding GFP under the CX3CR1 locus clearly demonstrated that microglia are the only cell type expressing receptors for fractalkine in the CNS (Jung *et al.*, 2000). Accordingly, ESdM expressed the fractalkine receptor and displayed migration towards fractalkine very similar to primary microglia in an *in vitro* transwell migration system. Interestingly, BV2 lacked chemokine-directed migration towards fractalkine, although they displayed undirected constitutive high migratory activity. Taking these observations together it seems that ESdM had the same strength in responding to fractalkine compared to primary microglia, whereas BV2 showed already a high undirected migratory activity under basal conditions. The answer if oncogenic transformation brings the cell to a pre-activated status and so leading to non-specific migration of these cells remains open.

Many cells of the immune system are able to phagocytose particles via a set of phagocytic receptors and digest the taken-up material by a professional lysosomal machinery. Microglial phagocytosis is a highly coordinated process, which is mainly regulated by signals that microglia receive from their environment (Napoli and Neumann, 2008). Standard *in vitro* assays to determine the phagocytic capacity of cells include the phagocytosis of microsphere beads, apoptotic neurons and/or aggregated Aβ. Because of the functional relevance in an animal model of AD, we focused here on the phagocytosis of aggregated Aβ to demonstrate a functional phagocytic capacity of ESdM *in vitro* (Bard *et al.*, 2000). We showed that ESdM are able to phagocytose Aβ peptides and that this effect is significantly increased after LPS stimulation. Levels of Aβ uptake of ESdM under LPS stimulated were comparable to primary microglia and superior to BV2. The capability of ESdM to take Aβ aggregates up makes them an interesting tool to use them in a cell therapy approach of an animal model of AD (discussed in detail later).

Microglia differentiated from ES cells might be a future tool to have sufficient amounts of cells for further investigations. Our results show that ES cells can differentiate into functional microglial precursors. In minor points ESdM looked less mature compared to primary microglia, but it has to be proven if any treatment, i.e. long-term application of GMCSF, would improve ESdM maturity. In summary, we were able to prove that the phenotype and the functionality of ESdM lines were very similar to primary microglia and in several aspects clearly superior to the oncogenic transformed microglial cell line BV2. Therefore, ESdM are a novel optimized tool to study microglial function *in vitro* and to apply them for cell therapy approaches.

Except from biopsied or post-mortem tissue, the derivation of functional microglial cells from the human's brain is almost impossible. The analysis of mouse ESdM showed in a convincing manner that cells derived from ES cells are able to substitute to a certain degree primary cells. Therefore, the differentiation of microglial precursors from human ES cells would overcome the obstacle always coming along with human tissue. Human ESdM would give the advantage to study microglial functions in the human brain. Our future aim will be to adapt the protocol for the differentiation of human ES cells to microglial precursors.

4.2 ESdM – a tool in a therapeutic approach of an animal model of Alzheimer's disease

After the detailed characterization of ESdM, it becomes striking to analyze their potential in a mouse model of AD. Until now there is low experimental evidence whether the phagocytic function of exogenously applied microglia effectively contributes to Aβ clearance in the CNS. Indeed, there is only one group that reported the administration of exogenously applied microglia for a cell therapeutic approach in a rat animal model of AD. In 2007, Takata and coworkers showed that microglial transplantation increased Aβ clearance (Takata *et al.*, 2007). However, the rat model of AD was not reflecting the situation of AD as good as common mouse models. In this animal model plaques are not formed due to genetic mutations, but due to exogenously applied $A\beta_{42}$ aggregates. Thus the rat AD model cannot really reflect the pathoimmunological aspects of AD and accordingly findings have to be critically evaluated.

The route of injection for cell application in CNS therapy needs to be accurately chosen. The less invading cell application is the intravenous (i.v.) injection into the tail vein of the host. After entering the circulation homing of cells to the target tissue can be achieved. In CNS cell therapy approach, one prerequisite is that cells have the ability to overcome the blood brain barrier. In our experimental paradigm, we were not able to detect GFP^+ cells in the brain parenchyma after i.v. injection into the tail vein of APP23 mice. It cannot be excluded that ESdM were not able to overcome the blood brain barrier and therefore were homed to other tissues like spleen or liver. Since we did not achieve to target cells to the CNS, we did not follow up any further investigations about the destination of ESdM after i.v. injection.

Several groups have demonstrated that bone marrow-derived cells are able to become brain macrophages, and a major role of bone marrow-derived brain macrophages in Aβ clearance is also suggested (Simard and Rivest, 2004; Malm *et al.*, 2005; Simard *et al.*, 2006). Thus, bone marrow cells may be a suitable source of microglial-like progenitors in clinical application. However, recent reports provided the evidence that irradiation, which is used to create bone-marrow chimerism, had influence on the permeability of the blood brain barrier leading to migration of bone marrow derived cells to the CNS, which is normally not achieved under physiologic conditions (Ajami *et al.*, 2007; Mildner *et al.*, 2007). According to this premise and in regard with our difficulties to overcome the blood brain barrier we challenged our APP transgenic mice by irradiation before we applied i.v. GFP^+ ESdM. However, we were again not able to detect GFP^+ cells in the CNS after i.v. injection of ESdM in irradiated APP transgenic mice.

Alternatively, there are studies describing the migration of exogenously applied microglia into the brain of gerbils after intra-arterial injection (Imai *et al.*, 1997; Suzuki *et al.*, 2001). Intra-arterial application into the subclavian artery avoids the circulation of cells through the body and facilitates the direct entry of cells into the brain. This application route may be a promising alternative, however practical realization is very difficult in mice.

To overcome the limitation of the blood brain barrier and to introduce our cells next to Aβ plaques, we switched our experimental setup to stereotactic local injection of cells. GFP^+ ESdM were locally injected to the cortex of APP23 mice. Careful analysis revealed that ESdM showed limited migration towards surrounding plaques after transplantion. Interestingly the area that was covered by ESdM after local injection showed a significantly decreased total plaque area as well as smaller plaques. The

observations suggest that ESdM were restricted in their migration behavior after local injection, however their capacity to phagocytose Aβ contributed to beneficial clearance of their microenvironment. The question whether ESdM transplantation might influence residential cells around the Aβ deposits, such as neurons, astrocytes, and endogenous microglia, is still outstanding. Careful morphological analysis of the surrounding tissue is in progress.

The lack of migration of ESdM after transplantation is in contrast to our results we obtained in the *in vitro* migration assay, where ESdM follow a precise gradient of fractalkine. One could speculate that after cell transplantation into brains of animals with progressed pathology, cells are overwhelmed by signals from the environment leading to a desensitization of migration, which renders them non-responsive to their environment and holds them at their transplantation origin.

The protective function of microglia in Aβ clearance is not negligible, but careful interpretation of literature raises important questions. Why does Aβ continue to accumulate, and why does AD pathology progress despite continued microglial recruitment? There are two main theories, which could explain the failure of microglia to hinder AD progression, (1) microglial cells become overwhelmed by the excess amount of Aβ produced and cannot keep up with the pace of Aβ accumulation, (2) the phenotype of accumulating microglia changes and these cells become more pro-inflammatory and lose their ability to clear Aβ, resulting in reduced Aβ phagocytosis and degradation, and increased Aβ generation/accumulation. Recently, Hickman and coworkers provided evidence that the inflammatory response in AD is a 'double-edged sword'. Early recruitment of microglia to the sites of Aβ accumulation as part of the attempt to clear these neurotoxic aggregates delays AD progression. However, with increasing age, recruited microglia become dysfunctional and show reduced ability to clear Aβ, but maintain their ability to produce pro-inflammatory cytokines leading to disease progression (Hickman *et al.*, 2008).

In summary, we demonstrated the possible usefulness of exogenously transplanted ESdM for the clearance of Aβ in the brain of APP23 mice. Our data confirm that microglia, which were not influenced in their clearance capacity due to ongoing inflammatory disease progression, retain their ability to efficiently phagocytose aggregated Aβ. Limiting factors in our model were the lack of migration of ESdM after transplantation. Thus, the beneficial effect of exogenously applied ESdM was very restricted to the local area where cells were transplanted. However, further

investigations on microglia and their application in APP transgenic mice could provide a novel approach for AD therapy.

5 Summary

The role of microglia in Alzheimer's disease (AD) is controversial, as it remains unclear if the microglia form the amyloid fibrils or react to them in a phagocytic role. However, there has been recognition that microglia may also play a neuroprotective role through phagocytosis of Aβ. To study microglial function, primary microglia are typically derived from mixed glial cultures from early postnatal brain tissue. Isolation of such primary microglia is time consumable and inefficient due to low cell yield. To overcome these limitations we established a reproducible procedure to differentiate mouse ES cells into the desired microglial cells.

In this work, two distinct ES cell lines were differentiated into microglial precursor cells by a method allowing differentiation of embryoid bodies to microglia within a mixed brain culture. Several independent microglial precursor (ESdM) lines were generated from the ES cells and characterized by flow cytometry, immunocytochemistry and functional assays. All ESdM showed expression of IBa1, CD11b, CD45, F4/80, α4 integrin and β1 integrin, but were negative for cKit and CD34. Furthermore, stimulation with interferon-γ (IFNγ) or lipopolysaccharides (LPS) demonstrated up-regulation of pro-inflammatory mediator gene transcription including nitric oxide synthase-2 (iNOS), interleukin-1β (IL1β) and tumor necrosis factor-α (TNFα) at levels comparable to primary microglia. ESdM showed efficient and rapid phagocytosis of amyloid-β (Aβ) peptide, which was increased after stimulation with LPS. ESdM expressed the chemokine receptor CX3CR1 and demonstrated concentration dependent migration towards its ligand CX3CL1. After *in vivo* transplantation into postnatal brain tissue ESdM showed engraftment as cells with a microglial phenotype and morphology.

The therapeutic application of ESdM was assessed in an animal model of AD (APP23). To overexpress GFP, ESdM were lentivirally transduced and locally injected to the cortex of APP23 mice. Immunohistochemistry showed that ESdM were able to contribute beneficially to a clearance of Aβ at the transplantation site resulting in a significant decreased total plaque area as well as in smaller plaques.

In summary, ESdM are stable proliferating cells substantially having most characteristics of primary microglia and therefore being a suitable tool to study microglial function and to use them as a source for cell therapy applications.

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8 Erklärung/Declaration

Hiermit versichere ich, dass diese Dissertation von mir persönlich, selbständig und ohne jede unerlaubte Hilfe angefertigt wurde. Die vorliegende Arbeit wurde an keiner anderen Hochschule als Dissertation eingereicht. Ich habe früher noch keinen Promotionsversuch unternommen.

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

Bonn, September 2008

Isabella Napoli