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Debris clearance by microglia: an essential link between degeneration and regeneration

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Microglia are cells of myeloid origin that populate the CNS during early development and form the brain's innate immune cell type. They perform homoeostatic activity in the normal CNS, a function associated with high motility of their ramified processes and their constant phagocytic clearance of cell debris. This debris clearance role is amplified in CNS injury, where there is frank loss of tissue and recruitment of microglia to the injured area. Recent evidence suggests that this phagocytic clearance following injury is more than simply tidying up, but instead plays a fundamental role in facilitating the reorganization of neuronal circuits and triggering repair. Insufficient clearance by microglia, prevalent in several neurodegenerative diseases and declining with ageing, is associated with an inadequate regenerative response. Thus, understanding the mechanism and functional significance of microglial-mediated clearance of tissue debris following injury may open up exciting new therapeutic avenues.

Keywords: neuroinflammation; microglia; neurodegeneration; regeneration; phagocytosis; multiple sclerosis, Alzheimer disease

Abbreviations: Aβ = amyloid-β; BDNF = brain derived neurotrophic factor; CR3 = complement receptor type 3; EAE = experimental autoimmune encephalomyelitis; IGF-1 = insulin-like growth factor-1; IL-4 = interleukin-4; TLRs = toll like receptors; TNF-α = tumour necrosis factor-α; TREM-2 = triggering receptor expressed on myeloid cells-2

Introduction

Over several decades the question of whether microglia and brain macrophages play harmful or beneficial roles in CNS injury and disease has been widely debated and reviewed (Streit, 2005, 2006; Block *et al*., 2007; Hanisch and Kettenmann, 2007). In our view it is clear that they can fulfil both roles and we do not intend to argue for one position against the other. Instead, we start with the premise that microglia perform many beneficial roles in diseases and review how recent advances in our understanding of microglial biology relate to these, highlighting how phagocytic removal of tissue debris has an important function in creating a pro-regenerative environment within the CNS. Microglia engaged in phagocytosis generally assume a macrophage phenotype, which is indistinguishable from the macrophage phenotype assumed by monocyte-derived cells. Recent evidence demonstrates that transition of monocyte-derived cells into microglia is a very rare event that only occurs under very defined host conditions (Mildner *et al*., 2007). In general microgliosis arises as a result of a proliferative response of resident microglia, present within the CNS due to invasion by myeloid precursors during development (Ajami *et al*., 2007; Ransohoff, 2007). Therefore, in this article the term

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macrophage will be used to infer a cell of predominantly CNS origin, if not explicitly defined as blood- or monocyte-derived macrophage.

Microglial motility

Under pathological conditions such as infectious diseases, stroke or neurodegenerative processes, microglia become activated, migrate to and within the lesion site, release a wide range of soluble factors that include cytotoxins, neurotrophins and immunomodulary factors and clear cellular debris by phagocytosis. Until recently it was thought that, in contrast to their frenzied activity in pathology, microglial cells under normal conditions are quiescent and non-motile cells. However, *in vivo* imaging on living mice has revealed that their highly ramified processes are remarkably motile, continuously and randomly undergoing cycles of filopodia-like protrusion formation, extension and withdrawal of bulbous tips (Davalos *et al*., 2005; Nimmerjahn *et al*., 2005). This high motility of the processes enables microglia to effectively monitor the status of the local surroundings and possibly to endocytose small cellular debris or budded vesicular structures, including that from apoptotic cells, from the microenvironment. Thus microglia might behave like monocyte-derived macrophages, whose filopodia can act as phagocytic or endocytic tentacles, efficiently pulling engulfed vesicular material towards the cell body (Kress *et al*., 2007). In addition to the high motility of their processes under normal conditions, they polarize and converge their processes at sites of brain injury attracted by extracellular ATP and mediated via microglial purinoreceptors (Davalos *et al*., 2005; Haynes *et al*., 2006). Recently, it was shown by *in vivo* two-photon microscopy that not only the processes, but also the cell bodies of microglial cells are activated and recruited to newly formed amyloid-β (Aβ) plaques within 1–2 days in an animal model of Alzheimer's disease (Meyer-Luehmann *et al*., 2008).

An important function of microglial cells responding and migrating towards the chemokine ligand of CX3CR1 appears to be the support of endangered neurons since deficiency in the chemokine receptor CX3CR1 resulted in increased neuronal death in animal models of amyotrophic lateral sclerosis and Parkinson's disease (Cardona *et al*., 2006). The precise mechanisms by which CX3CR1-positive microglia might assist compromised neurons have yet to be determined, although it seems likely that it will relate in part to the release of neuroprotective and trophic factors.

Microglial production of trophic factors and protective cytokines

Microglial cells are able to produce and release a plethora of soluble mediators ranging from cytotoxic mediators to trophic factors, which can exert deleterious as well as beneficial effects on the surrounding tissue. Important insights into this dual nature are derived from *in vitro* experiments using organotypic hippocampal slice cultures, where it has been shown that microglia become neurotoxic following treatment with lipopolysaccharides (LPS) but become neuroprotective when pre-activated with interleukin-4 (IL-4) (Butovsky *et al*., 2006). The protective effect of IL-4 conditioned microglia is associated with a downregulation of tumour necrosis factor-α (TNF-α) and an upregulation of insulin-like growth factor-1 (IGF-1) gene transcripts. IGF-1 has neuroprotective effects but also exerts survival and pro-regenerative activities on oligodendrocyte-lineage cells, preventing acute glutamate-mediated toxicity and promoting oligodendrocyte differentiation from precursor cells *in vitro* (Ness and Wood, 2002; Hsieh *et al*., 2004; Butovsky *et al*., 2006). Brain derived neurotrophic factor (BDNF), having both protective and growth promoting effects on neurons, was suggested to be produced by microglial cells and to stimulate axonal sprouting towards a wound edge (Batchelor *et al*., 2002). Recent data also indicate that microglial-derived BDNF is implicated in neuropathic pain by causing a shift in the neuronal anion gradient of spinal lamina I neurons, thus contributing to tactile allodynia (Coull *et al*., 2005). However, it is unclear whether microglia *in vivo* produce and release sufficient amount of BDNF for these effects on neurons.

Under certain conditions microglial cells are able to produce anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β), which have neuroprotective effects in experimental animal models of traumatic injury and stroke (Streit, 2005; Hanisch and Kettenmann, 2007). Often, a clear distinction between cytokines that are either harmful or beneficial cannot be made since the primarily cytotoxic pro-inflammatory cytokines IL-1β and TNF-α released from activated microglia can directly or indirectly evoke a neuroprotective or pro-myelin regenerative response. For example, TNF-α has been shown to protect neurons against Aβ mediated toxicity (Barger, 1995) under pathological conditions and the absence of glial derived TNF-α revealed a role in homoeostatic synaptic scaling under physiological conditions (Stellwagen and Malenka, 2006).

The release of cytokines, chemokines and other soluble mediators is the first step for successful repair and contributes to the creation of an environment conducive for regeneration. The factors secreted attract phagocytic and repair-promoting effector and precursor cells, which are able to replace damaged tissue. This process is especially evident during remyelination, the regenerative event in which new myelin sheaths are restored to demyelinated axons and that can occur with impressive efficiency in experimental models and clinical disease (Ludwin, 1978, 1980; Woodruff and Franklin, 1999; Sim *et al*., 2002; Patrikios *et al*., 2006; Patani *et al*., 2007). Studies of remyelination in animals lacking pro-inflammatory cytokines such as TNF-α (Arnett *et al*., 2001, 2003) and IL-1β (Mason *et al*., 2001) have suggested that inflammatory cytokines and, as will be discussed later, the inflammatory response to demyelination are required to trigger efficient remyelination. The remyelination-enhancing effects of IL-1β and TNF-α could be due to direct effects or indirectly mediated via the induction of IGF-1 (Arnett *et al*., 2001, 2003), although IGF-I is likely to be a redundant component of environmental factors governing remyelination (O'Leary *et al*., 2002). In addition to trophic and pro-regenerative effects of secretory

factors directly or indirectly derived from microglia, the phagocytic clearance of debris is also instrumental for repair as discussed in the following sections.

Microglial phagocytic receptors

There are two distinct functional types of phagocytic receptors. First, receptors recognizing microbes such as toll like receptors (TLRs) which support removal of pathogens and simultaneously stimulates a pro-inflammatory response in the phagocytes (Ravichandran, 2003), and second, receptors recognizing apoptotic cellular material such as receptors that recognize phosphatidylserine (PS) and which are important for ingesting apoptotic cell corpses and stimulate an anti-inflammatory response in phagocytes (Ravichandran, 2003). This silent phagocytosis that takes place without inducing inflammation is one of the major beneficial functions of phagocytes (Fig. 1).

The specificity of these phagocytic receptors and their respective ligands are often unknown but are gradually beginning to emerge. For example, T-cell immunoglobulin- and mucin-domain-containing molecule-4 (Tim4) has recently been shown to recognize phosphatidylserine residues (Miyanishi *et al*., 2007). In addition, a number of microglia-specific phagocytic receptors have been observed recently, including microglial metabotropic P2Y6 receptor that recognizes the nucleotide UDP released from injured neurons and stimulates microglial phagocytosis (Koizumi *et al*., 2007). Furthermore, triggering receptor expressed on myeloid cells-2 (TREM2)-mediated signalling in microglia has been shown *in vitro* to facilitate debris clearance in the absence of inflammation (Takahashi *et al*., 2005). The paramount importance of these receptors has recently become clear as patients with a loss of function mutation of either TREM2 or DAP12 develop an inflammatory neurodegenerative disease leading to death at the fourth or fifth decade of life. Thus, even though the ligand of TREM2 is unknown, microglial TREM2/DAP12-mediated phagocytosis appears to be an essential function for CNS tissue homoeostasis (Neumann and Takahashi, 2007).

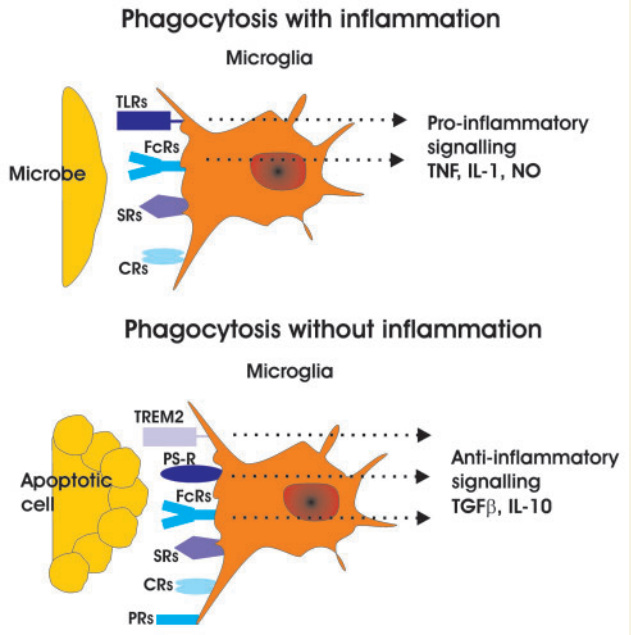

Fig. 1 Microglial phagocytic receptors. Phagocytosis is associated with inflammation during uptake of microbes, while phagocytosis of apoptotic cells is executed without inflammation. Recognition of microbes induces a microglial phagocytic response, which is associated with release of pro-inflammatory mediators such as TNF and NO. Particularly, TLRs recognize microbial patterns leading to pro-inflammatory activity release in microglial cells. Furthermore, Fc-receptor (FcR) engagement antibodies binding induces pro-inflammatory activity dependent of the Fc-receptor subtyp and antibody isotype. Recognition and phagocytosis of apoptotic cells induces an anti-inflammatory cytokine profile in microglia. Phosphatidylserine receptors (PRs) recognizing phosphatidylserine residues in apoptotic membranes stimulated microglial production and release of TGF-β and IL-10. Triggering receptor expressed on myeloid cells-2 (TREM2) induces anti-inflammatory activity of microglia. Purine receptors (PRs) such as P2Y6 are recognizing UDP. Phagocytic receptors: PRs = purine receptors; PSRs = phosphatidylserine receptors; CRs = complement receptors; TLRs = toll like receptors; FcR = Fc-receptors; SRs = scavenger receptors; TREM2 = triggering receptor expressed on myeloid cells-2. Soluble mediators: TNF = tumour necrosis factor-α; IL-1 = interleukin-1β; NO = nitric oxide; TGF-β = transforming growth factor-β; IL-10 = interleukin-10.

Microglial phagocytosis during restructuring of neuronal connections

Selective synapse elimination and axon pruning are vital late-stage refinements in the formation of functional neural circuits. In the brain of the adult fruitfly Drosophila a program involving glia acts to achieve pruning of the axonal connection of the mushroom body γ neuron (Broadie, 2004). Phagocytic glial cells actively invade the mushroom body lobes and engulf axonal varicosities prior to the axonal degeneration and accumulate acidic degradative organelles at the time of axonal pruning (Awasaki and Ito, 2004; Awasaki *et al*., 2006). Insect glial cells appear to be active phagocytes, which engulf the axons and synapses by an extrinsic mechanism (Awasaki and Ito, 2004; Awasaki *et al*., 2006). While microglial cells in mammals are the principal phagocytes in the CNS, Schwann cells of the peripheral nervous system (PNS) can assist blood-derived macrophages in removing myelin debris and have been recognized as playing a key role in synapse removal during development of the PNS (Bishop *et al*., 2004). During development of the murine CNS, apoptotic neurons and their connections, which have been established in excess, are actively and rapidly removed by microglia (Frade and Barde, 1998; Marin-Teva *et al*., 2004). At the initial but still reversible stages of programmed cell death of developing Purkinje neurons, caspase-3-mediated activation of Ca-independent phospholipase A2 results

in the production of pysophosphatidylcholine and exposure of phosphatidylserine on the cell membrane, leading to active cell death and removal by microglial cells (Marin-Teva *et al*., 2004).

Recent data indicate that microglia via its complement receptor C3 might be involved in synapse removal of unwanted synapses that have been tagged by complement for elimination during development (Stevens *et al*., 2007). Complement C1q and C3, both components of the classical complement cascade, are expressed by distinct synapses throughout the postnatal CNS. Mice deficient in C1q or C3 exhibited large sustained defects in CNS synapse elimination, as shown by the failure of anatomical refinement of retinogeniculate connections and the retention of excess retinal innervation by lateral geniculate neurons. Our knowledge of the removal mechanism of synapses and axons during reorganization of the normal and injured adult mammalian CNS is still incomplete, nevertheless it is becoming increasingly clear that microglia play a central role.

Microglial phagocytosis in acute CNS injury

In acute injury, microglia has been shown to react within a few hours with a migratory response towards the lesion. For example, in an *in vitro* model of entorhinal cortex injury microglia migrated towards the zone of axonal degeneration where loss of the denervated dendrites of interneurons occurred (Rappert *et al*., 2004). This migration is functionally significant since in chemokine receptor CXCR3 deficient mice, where microglia do not migrate, no loss of dendrites was observed (Rappert *et al*., 2004). Thus, denervated neuronal dendrites are not retracted autonomously, but require a trigger signal or active removal by microglia. Similarly, in response to an experimental axonal lesion to facial nerve motoneurons in rats, a glial cell mediated removal or 'stripping' of synapses from the perikaryon and dendrites of affected cells has been reported (Streit, 2005). It was recently suggested that microglia and major histocompatibility complex (MHC) class I related receptors take up a main role in the process of synapse removal after motoneuron injury (Cullheim and Thams, 2007).

In most cases of acute injury deposition of tissue debris is observed due to cell death. In general tissue debris does not linger for long periods after tissue damage due to efficient removal by macrophages. However, in the CNS the myelin debris associated with Wallerian degeneration can persist for very long time periods (Miklossy and Van der Loos, 1991; Vargas and Barres, 2007). In the CNS microglia are the first cell type engaged in phagocytosis. However, their phagocytic capacity as compared to blood-borne macrophages might be limited (Mosley and Cuzner, 1996; Popovich *et al*., 1999). In the second instance, blood-borne macrophages assist and could significantly contribute to the removal of debris (Amat *et al*., 1996; Stoll and Jander, 1999). The capacity of macrophages to phagocytose myelin can be altered by environmental mediators. After treatment with TNF-α a massive reduction of the amount of myelin ingested by macrophages via their complement receptor type 3 (CR3) occurred *in vitro* (Bruck *et al*., 1992). Immunofluorescence analysis indicated that TNF-α caused a reduction of CR3. Similarly, *in vivo* experiments have demonstrated that pre-activation of macrophages transplanted into transected optic nerve has profound effects on the rate of myelin clearance (Lazarov-Spiegler *et al*., 1998). Myelin contains several growth inhibitory molecules such as Nogo A, which exhibit inhibitory effects on axonal regrowth (Schwab, 2004). Thus, the rapid removal of myelin-associated inhibitors is important for establishing an environment beneficial for axon regeneration. A number of observations suggest that insufficient myelin clearance in the CNS after acute injury may contribute to the failure of axonal regeneration, while efficient myelin clearance in the PNS during Wallerian degeneration by Schwann cells and invading and resident macrophages facilitates axonal regeneration (David and Lacroix, 2003; Vargas and Barres, 2007). In support of this notion, it was observed that the transected optic nerve of amphibians exhibits a rapid phagocytic response, which leads to an effective clearance of myelin debris and finally, successful axonal regeneration (Battisti *et al*., 1995; Perry *et al*., 1995).

Recent evidence indicates that the presence of myelin molecules not only inhibits axonal outgrowth but also affects the differentiation of oligodendrocyte precursor cells into mature oligodendrocytes during remyelination (Kotter *et al*., 2005). Thus, the myelin debris generated during demyelination needs to be rapidly removed by phagocytic cells, for remyelination to proceed efficiently. This is reflected in the strong correlation between the efficiency of remyelination and the effectiveness of myelin debris removal, both occurring in young animals more effectively than in older adult animals or young animals in which additional myelin was experimentally added (Shields *et al*., 1999; Kotter *et al*., 2005, 2006; Dubois-Dalcq *et al*., 2005).

Microglial phagocytosis in multiple sclerosis

Phagocytically active macrophages, identified by staining against myelin degradation products or lysosomal lipids, have been extensively described in multiple sclerosis lesions (Li *et al*., 1993; Bruck *et al*., 1995). Most of these myelin-laden macrophages are localized in the perivascular areas in active inflammatory lesions. Phagocytic cells have also been analysed in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. During the first attack of acute and chronic relapsing EAE, immunostaining with an antibody against lysosomal membranes of phagocytes demonstrated at the ultrastructural level that these phagocytes were seen to contain degraded myelin products in lysosomes (Bauer *et al*., 1994). However, the functional role of phagocytosis in EAE is unclear. Recently, a beneficial role of the microglial phagocytic TREM2 receptors has also been demonstrated in EAE. Antibody-mediated blockade of TREM2 during the effector phase of EAE results in disease exacerbation with more diffuse CNS inflammatory infiltrates and demyelination in the brain parenchyma (Piccio *et al*., 2007). In another study, intravenous transplantation of myeloid precursor cells genetically engineered to over-express TREM2 at the clinical peak of EAE

improved myelin removal in the lesioned spinal cord, created an anti-inflammatory cytokine profile in the lesions and facilitated recovery (Takahashi *et al*., 2007). Thus, debris clearance by resident endogenous microglia appears to be insufficient and it is possible to promote recovery in EAE by the transplantation of phagocytic TREM2 positive cells that contribute to the clearance of debris.

Microglial phagocytosis in Alzheimer disease

In Alzheimer disease microglia can be beneficial by phagocytosing Aβ or harmful by secretion of neurotoxins. Recently it was shown in an animal model of Alzheimer disease plaque formation that microglia accumulation is associated with rapid appearance and local toxicity of Aβ plaques (Meyer-Luehmann *et al*., 2008) (Fig. 2). Using *in vivo* multiphoton microscopy plaques were revealed to form over 24 h followed within 1–2 days by microglial activation and recruitment to the plaque, and finally the appearance of dysmorphic neurites over the next days to weeks. In an animal model of tauopathy, exhibiting certain aspects of neurofibrillary tangle formation of Alzheimer disease, early microglia activation was associated with loss of synapses preceding tangle formation (Yoshiyama *et al*., 2007). In APP/PS1 and APP23 transgenic mice, bone marrow-derived myeloid cells were recruited to senile plaques and differentiated into microglial-like cells after irradiation and bone marrow transplantation (Malm *et al*., 2005; Stalder *et al*., 2005). Invading bone marrow-derived cells gradually obtained morphologies and lineage markers very similar to resident microglia. However, CNS invasion of bone marrow derived cells might be induced by irradiation performed for the transplantation of bone marrow cells, and not a consequence of the disease process (Mildner *et al*., 2007). Furthermore, it is unclear whether bone marrow-derived cells develop a real microglia phenotype, since resident microglia underwent a distinct developmental program coming from the yolk sac and invaded the CNS during early stages of development. Recently, it was shown that the chemokine receptor CCR2 expressed on microglia, invading blood-derived macrophages and circulating monocytes is required for accumulation of these CD11b+ cells in the CNS in a transgenic mouse model of Alzheimer disease (El Khoury *et al*., 2007). Diseased mice deficient in CCR2 demonstrated increased perivascular Aβ deposits and died prematurely possibly due to amyloid angiopathy, indicating that CCR2 function on circulating monocytes or microglia is required for prevention or clearance of perivascular Aβ deposits (El Khoury *et al*., 2007). Thus, there is certain evidence that either local microglia or invading blood-derived macrophages restrict Aβ deposits in an animal model of Alzheimer disease.

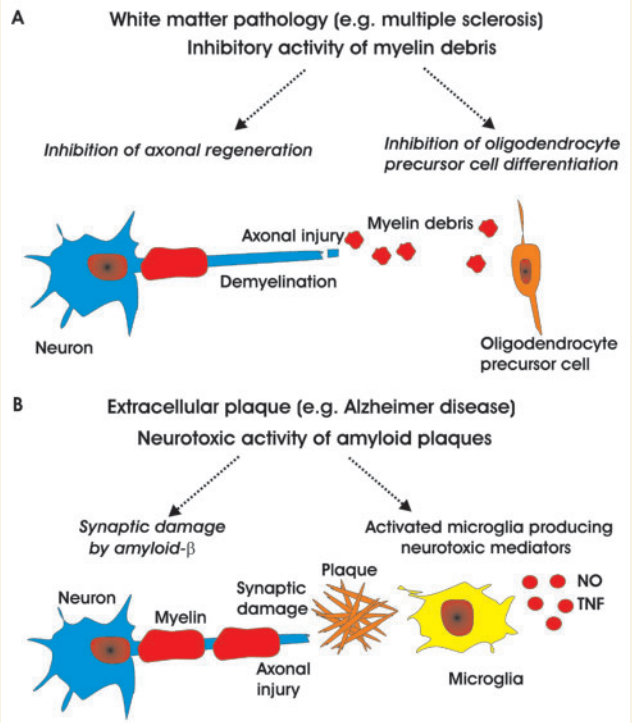

Fig. 2 Detrimental effects of myelin debris and extracellular aggregates (**A**) Inhibitory activity of myelin debris in multiple sclerosis. Immune mediated demyelination and oligodendrocyte injury liberates myelin. Myelin debris inhibits axonal re-growth and regeneration. Furthermore, myelin debris inhibits oligodendrocyte precursor cell differentiation. (**B**) Neurotoxic activity of Aβ plaques in Alzheimer disease. Extracellular Aβ directly damages synapses and stimulates microglial to release neurotoxic mediators such as TNF-α and nitric oxide (NO). Microglial cytotoxic mediators induces synaptic and axonal injury.

Microglial phagocytosis in ageing

Ageing is associated with senescence of microglia and impaired microglial clearance functions. In particular, data indicate that microglia in aged rodent and human brains show a replicative senescence with a reduced self-renewal capacity (Streit, 2006). Microglia in aged animals were characterized by the presence of lipofuscin granules, decreased processes complexity, altered granularity and increased mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-1β (Sierra *et al*., 2007). Furthermore, older rats compared to young rats showed delayed recruitment of phagocytic cells and less clearance of myelin after a toxin-induced demyelination lesion (Zhao *et al*., 2006), which correlates with the slower remyelination in older animals (Sim *et al*., 2002). Thus, microglia dysfunction occurring as a result of ageing might contribute to the exacerbation of chronic neurodegenerative diseases and Aβ plaque load in Alzheimer disease and a reduced repair capacity in aged individuals (Fig. 3).

Conclusion

The removal of non-functional or degenerated tissue is an essential role of microglia. This response is most strikingly seen following injury in adulthood and can be viewed as an exaggerated version of a normal physiological task performed by microglia to

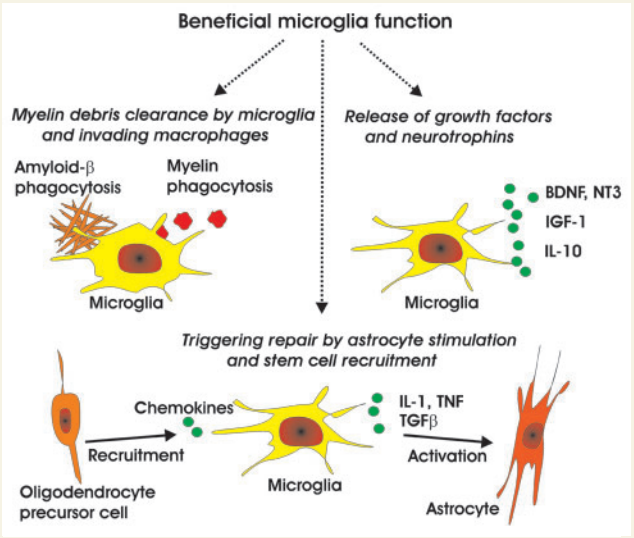

Fig. 3 Beneficial microglia function. Microglial beneficial function is mediated via several effector mechanisms. Firstly, microglia and invading macrophages clear myelin debris or extracellular aggregates. Secondly, microglia initiate the repair process by stimulating neighbouring astrocytes to produce trophic support factors and by recruiting stem and precursor cells. Thirdly, microglial cells produce a variety of neurotrophins, growth factors and anti-inflammatory cytokines stimulating sprouting of axons and myelin repair. Soluble mediators: BDNF = brain derived neurotrophic factor; NT3 = neurotrophin-3; IGF-1 = insulin-like growth factor-1; IL-10 = interleukin-10; IL-1 = interleukin-1; TNF = tumour necrosis factor-α; TGF-β = transforming growth factor-β.

remove superfluous cells undergoing apoptosis in development and adulthood. If phagocytosis is compromised as it is evident in loss-of-function mutations of either TREM2 or DAP12, this results in a chronic degenerative CNS disease. In the context of a homoeostatic role for microglial phagocytosis, the clearance function fits comfortably with a pro-regenerative contribution to the complex events occurring in the damaged CNS. At present this is most clearly evident in the inefficient remyelination associated with inhibition of precursor differentiation and in impaired axon regeneration in the presence by uncleared myelin debris. Similarly, limited clearance of affected tissue or dysfunction of microglia are features of several neurodegenerative diseases and are exacerbated with ageing. These relatively diverse lines of evidence point to the generic importance of the microglia-mediated phagocytic removal of debris in creating environments most conducive to intrinsic regenerative processes. Allowing these to occur will require a deeper understanding of the mechanisms and functional significance of microglia and macrophage-mediated clearance of tissue debris following injury from which new CNS regenerative medicines may emerge.

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