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Microglia: Activation and Their Significance in the Central Nervous System

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Microglia are resident monocyte-lineaged cells in the brain. Their characteristic feature is that they react to injury and diseases of the brain and become morphologically and functionally activated. Although some triggar molecules which activate microglia are predicted to be released from injured or affected cells, such molecules have not yet been identified. The main role of activated microglia is believed to be in brain defense, as scavengers of dead cells, and as immune or immunoeffector cells. Recent biochemical and neurobiological studies have further indicated that they significantly affect the pathological state and/or regulate the regenerative state and remodeling of the brain by producing a variety of biologically active molecules including cytotoxic and neurotrophic molecules.

Key words: cytotoxic molecules, microglia, neurotrophic molecules, pathology, phagocytes.

Among glial cell types of the central nervous system (CNS), microglia were found to be the third glial cell type by del Rio Hortega (*1*). This cell type is observed uniformly in a scattered fashion throughout the normal adult brain, comprising approximately 5–20% of all glial cells (*2*).

Microglia in the early stage of brain development show a macrophage-like morphology with a relatively large cell body and short processes and are generally called "ameboid microglia" (*3*) (Fig. 1). The essential function of the ameboid microglia is considered to be to phagocytose dead and dying cells, including neurons which have undergone naturallyoccurring cell death (apoptosis) in late embryonic to early postnatal stages.

As the brain develops, ameboid microglia decrease in number and appear to be replaced by increasing numbers of ramified microglia, which have small cells bodies with long, branched processes (Fig. 1). Although ameboid microglia are generally believed to transform into ramified microglia during brain development, the exact relationship between these two types of microglia is still unclear. Ramified microglia are thought of as being functionally inactive or in a resting state and are called "resting microglia." As long as the brain is maintained in healthy condition, the microglial cell density and ramified-morphology is sustained.

When the brain is injured or affected by brain diseases, ramified microglia morphologically transform into "activated microglia" or "reactive microglia," which show retracted processes and enlarged cell bodies and become proliferative at the affected site (Fig. 1). These transformed microglia are functionally activated and appear to be implicated in many pathological states. In particular, their cytotoxic and inflammatory roles have been determined.

The origin of resident microglia is generally considered to be bone marrow-derived monocytes (*3–5*) At early embryonic stages, monocytes enter the brain parenchyma through the blood brain barrier (BBB), and thereafter settle, transform, and mature into ramified microglia, either through or not through the stage of ameboid microglia (Fig. 1). Actually, microglia are characterized by monocyte/macrophage antigens (described below). In contrast to this monocyte-origin theory, a neuroectodermal-origin theory has also been proposed. According to this theory, microglia are believed to be born in neuroectodermal-derived glioblasts (matrix cells) (*6*, *7*). Thus, the origin of resident microglia is still controversial, although the former theory is predominant

Microglia have been generally believed to act primarily as a defence line of the brain. If the CNS is infected, ramified microglia transform into phagocytes (activated microglia) and phagocytose the infectious microbes. Dead or dying cells in the CNS are also engulfed by ramified microglia-derived macrophages. An immune or immunoeffector role of microglia in the brain has been proposed due to their immunological properties (*5*). Activated microglia can function as antigen-presenting cells in the immune system when the BBB is disrupted. In addition, microglia have been shown to have the ability to produce a variety of biologically-active substances which induce inflammation and cell death, and regulate the regenerative processes. Microglia currently are accepted as "a sensor for pathological events" in the brain (*8*).

One of the characteristic properties of microglia is their activation, which is induced in many pathological conditions. Much attention has been paid to the roles that activated microglia play there. Simultaneously, interest has been expressed in the trigger molecules responsible for microglial activation *in vivo*. Anothers interesting question is whether activated microglia *in vivo* are as a whole benefical

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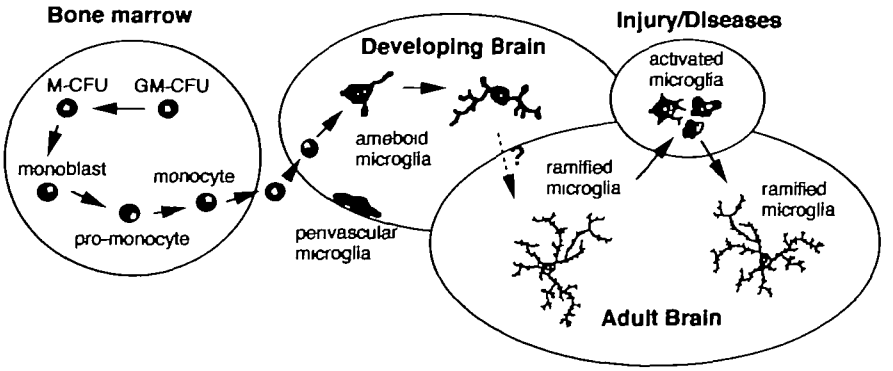

Fig 1 **Microglia in the developing and adult brain.**

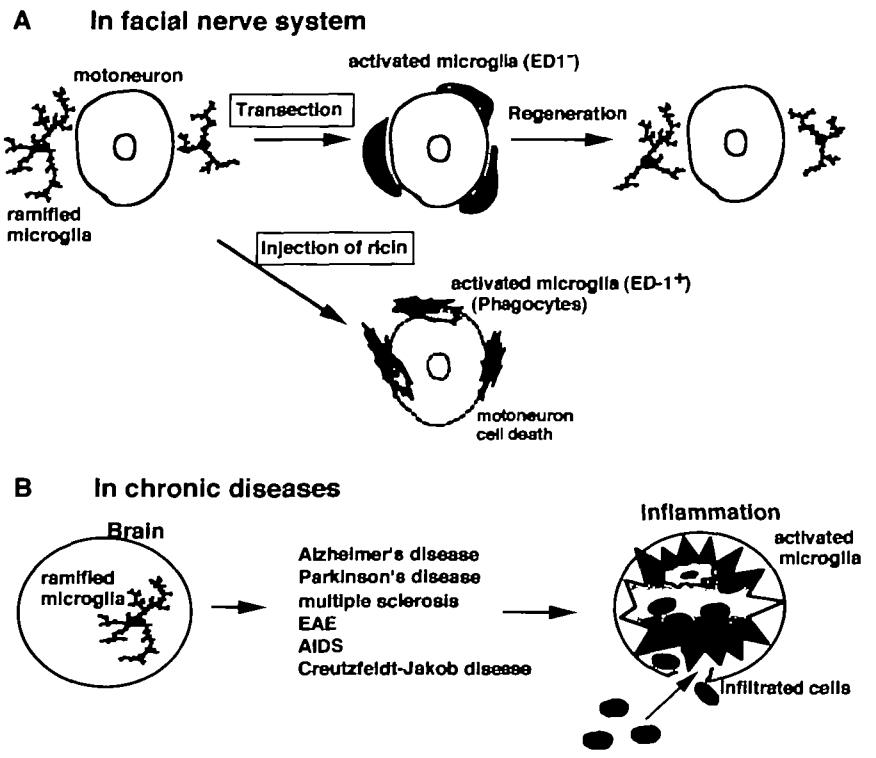

Fig 2 **Microglial reactions in acute injury and chronic diseases.** A. Transection of facial nerve and injection of toxic ricin into facial nerve. B. Activation of microglia in chronic diseases

or harmful. Therefore, in this article, we addressed questions concering the activation and functional significance of microglia.

Microglial activation in acute injury and chronic inflammation

When the brain is injured or affected by brain diseases, the resident ramified microglia transform into "activated microglia." These activated microglia induce or enhance various cellular antigens such as complement receptor (CR) 3, major histocompatibility complex (MHC) class I and MHC class II, and enzyme activities such as 5'-nucleotidase, which are associated with the functional state of microglia.

In acute injury such as axotomy of the facial nerve, microglia in the facial nucleus begin to change morphologically just after transection and proliferate 2–3 days later (Fig. 2A). Subsequently, the activated microglia increase in number over a period of 1–2 weeks and surround injured motoneuron cell bodies (Fig. 2A). After the persistent activation, they gradually regenerate and return to the morphology and cell density of the ramified type. The state of activated microglia in the axotomized facial nucleus is divided into two, based on phagocytic properties (*5*, *9*) (Fig. 2A). One state is activated ($OX42^+$, $Iba1^+$) but non-phagocytic ($ED1^-$). The other is activated ($OX42^+$, $Iba1^+$) and phagocytic ($ED1^+$). Whether or not they express antigen for ED1 antibody depends on the presence or absence of dying cells in the milieu. These activated microglia are derived from resident ramified microglia, because facial nerve transection at the stylomastoid foramaen does not injure the BBB of the axotomized facial nucleus and no peripheral monocyte-lineage cells infiltrate into the brain parenchyma.

Similarly, in the case of chronic inflammatory diseases such as Alzheimer's disease, multiple sclerosis (MS) and acquired immunodeficiency syndrome (AIDS), microglia/macrophages in the activated state are observed in the

affected sites (Fig 2B) However, it is difficult to observe the responses of only resident microglia, because blood-derived cells including monocytes and macrophages infiltrate into the brain parenchyama, and these infiltrated cells and resident microglia can not be distinguished from each other due to their shared immunological markers (Fig. 2B). Presumably, resident microglia as well as the infiltrated cells are activated in these chronic diseases and act as immune and/or inflammatory cells

Trigger molecules for microglial activation

The molecular and cellular mechanisms involved in microglial activation have been analyzed (*10*, *11*). General interest has been shown in what molecule(s) activate microglia *in vivo*. To date, various kinds of stimulators for microglial activation have been predicted (Fig. 3). One category is non-material stimulators. The electrical change resulting from neuronal injury and changes in the ion milieu around injured neurons are candidates. The other category is that of biologically active substances, which may include low molecular weight molecules such as peptides and hormones. Growth factors or cytokines may also be able to activate microglia. Among the cytokines are colony-stimulating factors (CSFs) including macrophage (M)-CSF and granulocyte-macrophage (GM)-CSF (*12*). Activated microglia in the axotomized facial nucleus actually express a specific receptor for M-CSF and GM-CSF with which microglia can proliferate and transform. These CSFs are believed to be produced in the surrounding astrocytes (Fig. 3).

As other activators, calcitonin gene–related peptide (CGRP) and ATP (*13*) are considered as neuron-derived candidates in the facial nerve transection model (Fig. 3). Indeed, CGRP and ATP induced immediate early gene mRNA in microglia Likewise, ATP could cause biological responses such as chemotaxis (*14*), release of plasminogen (PGn) (*15*) and interleukin (IL)-1β, and activation of nuclear factor (NF) κB in cultured microglia (*16*). Therefore, it is likely that some molecules derived from neurons and/or astrocytes trigger the activation of microglia *in vivo* (Fig. 3).

Apart from activation factors, a suppressive factor against activated microglia is also speculated to be present *in vivo*. However, details of its activity remain unclear at present.

Microglial proliferation accompanying activation

Proliferative activity is one of the characteristic properties of activated microglia *in vivo*. The factor(s) necessary for mitogenesis in microglia were clarified by *in vitro* study It is evident that a certain kind of CSF is necessary for the proliferation of microglia. Microglia can proliferate in response to GM-CSF, M-CSF, and multi-CSF (IL-3) (*17*).

The requirement of the proliferating factor for microglia was verified in an axotomy model using osteopetrotic mouse (op/op mouse). The op/op mouse cannot produce active M-CSF due to a frame-shift mutation. The transection of the facial nerve in an op/op mouse results in fewer activated microglia in the axotomized facial nucleus (*18*), implying that M-CSF is a major factor for the activation and proliferation of microglia.

CSF-like proliferating factors for microglia were isolated from the neonatal rat brain as microglial mitogens (MMs) (*19*). MM1, whose molecular weight is 50 kDa and pI is 6 8, showed GM-CSF–like activity On the other hand, MM2, with a molecular weight of 22 kDa and a pI of 5.2, showed non-IL-3–like activity and was proved to be secreted by cultured astrocytes. Both factors stimulate proliferation of ameboid microglia, but not of macroglia, monocytes, or peritoneal macrophages. They are produced in the injured brain, but not in the normal adult brain.

Other than CSFs, IL-2, IL-4, IL-5, and tumor necrosis factor (TNF) α are reported to induce proliferative activity in cultured microglia.

As a whole, CSF or CSF-like factors may be responsible for the proliferation and activation of microglia *in vivo*.

Cytotoxicity of activated microglia

In chronic diseases, inflammatory reactions are long-lasting and accompanied by activation of microglia and infiltrated blood-derived cells, by which cytotoxic molecules and proinflammatory cytokines are suspected to be produced Furthermore, these molecules cause harmful secondary reactions that may lead to the injury or death of weak neurons. Although these complex events make it difficult to distinguish the functions of each cell type, activated microglia have been generally suspected to be harmful

An interesting point is whether activated microglia somehow kill neurons by producing cytotoxic molecules. As suggested by their resemblance to tissue macrophages, activated microglia in culture have been shown to produce

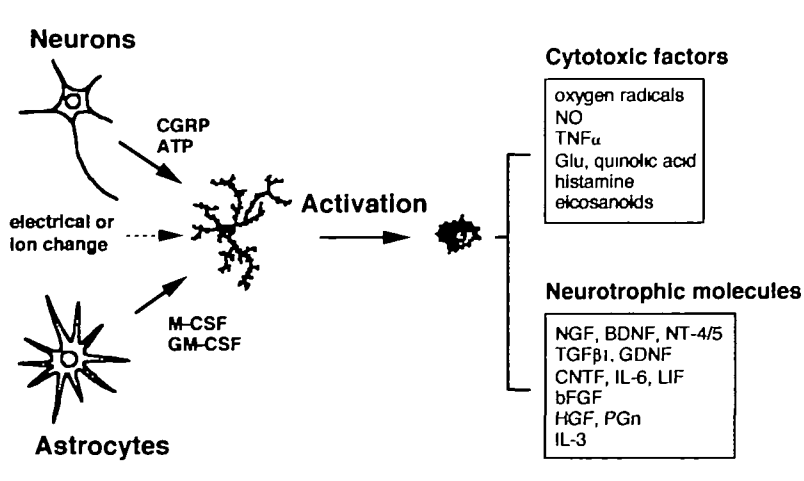

Fig 3 **Putative molecules for microglial activation and activated microglia-derived cytotoxic and neurotrophic molecules.**

several potentially cytotoxic molecules, including superoxide anion (*20*), nitric oxide (*21*), and proinflammatory cytokines (Fig. 3). Lipopolysaccharide (LPS), interferon γ (INFγ), and β-amyloid (*22*) are among the stimulators for the production of harmful factors from microglia.

Reactive oxygen species (ROS) including superoxide anions, hydroxy radicals, and hydrogen peroxide are generally hazardous, particularly to myelin and its forming cells, oligodendrocytes, owing to their capability of inducing lipid peroxidation. LPS and phorbol-12-myristate-13-acetate (PMA) are stimulators of ROS production from cultured microglia.

Nitrogen oxides such as NO are highly reactive free radicals, of which nitrite peroxide is the strongest species. These radicals are believed to inhibit respiratory enzymes, oxidize the SH group of proteins, and enhance DNA injury, finally resulting in neuronal cell death. LPS and β-amyloid are known stimulators of NO production from microglia. In the presence of INFγ, β-amyloid synergistically stimulates the production of NO and TNFα (*23*) in microglia.

The targets of TNFα in the CNS have been reported to be oligodendrocytes and myelin due to their susceptibility to this factor *in vitro*. Some neurons have also been reported to undergo apoptosis by treatment with this factor The producers of TNFα in the CNS are microglia as well as astrocytes. Although activated microglia-derived TNFα has been suspected to cause inflammation in MS and AIDS dementia, recent evaluation of TNFα function by gene knockout has showed that TNFα is not essential for the initiation and/or progression of inflammation (*24*), suggesting that TNFα is not necessarily neurotoxic.

Additionally, microglia-derived eicosanoids, vasoactive histamine or an excitotoxic glutamate may possibly promote the degenerative processes and inflammation

It is easy to imagine that neurons in pathological states can be severely damaged if acted upon cooperatively by potentially cytotoxic molecules (Fig. 3) However, the release of harmful factors from activated microglia and the cytotoxicity of these factors have been reported mainly based on *in vitro* studies. These findings regarding toxicity remain to be confirmed *in vivo*.

Neurotrophic action of activated microglia

We addressed the question of what role activated microglia play in the survival of neurons in the brain. The intercellular interaction between activated microglia and neurons was analyzed by using cultured embryonic neurons and microglia. The microglia in culture are regarded as a type of activated microglia because of their phagocytic properties, although they do not produce IL-1β, TNFα, and NO

As a first trial, the effect of microglia on neuronal survival was examined in a co-culture system. Microglia did not kill healthy neurons *in vitro*, indicating that their phagocytic properties are not necessarily dangerous for neurons. Secondly, the effects of conditioned microglial medium (CMM) on neurons were examined. The results showed the CMM expressed no neurotoxicity Rather, CMM significantly enhanced neuronal survival and the neurite outgrowth of neocortical neurons (*25*). Furthermore, CMM promoted the survival and neurite outgrowth of mesencephalic neurons (*26*). In mesencephalic neurons, dopamine uptake was markedly enhanced, as were the dopamine content and intensity of staining by anti-tyrosine hydroxylase antibody. In addition, CMM showed only a weak effect on other types of neurons such as cholinergic and glutamatergic neurons in our system. Different kinds of neurotrophic molecules were predicted to be released from cultured microglia.

Neurotrophic molecules from microglia

To date, several molecules in the CMM have been identified as neurotrophic substances and classified into various categories as described below (Fig 3).

Neurotrophins. Neurotrophins are a family of protein factors composed of at least five structurally related members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and NT-6. Of these, NGF, BDNF, NT-3, and NT-4/5 are expressed in mammalian tissues

Neurotrophins were originally shown to be produced in target neurons or tissues in both the peripheral nervous system (PNS) and CNS, but later these factors were found to be produced in glial cells as well. In addition to astrocytes, microglia have been found to express the mRNAs and proteins of these neurotrophins. Miwa *et al.* (*27*) showed that the cultured microglia express NGF, BDNF and NT-4/5 mRNAs under non-stimulated conditions, and that expression is increased by stimulation with LPS. BDNF protein has been immunocytochemically recognized in the microglia, and its level was found to be elevated by stimulation with LPS. Other researchers have also reported that NGF, BDNF, and NT-3 (*28*) are expressed and produced in microglia *in vitro* and *in vivo*.

The possibility that microglia secrete of neurotrophins was investigated by detecting neurotrophin proteins in their CMM. Microglia were found to constitutively secrete a limited amount of BDNF and NT-4/5, but NGF and NT-3 were undetectable. Stimulation with LPS on the assumption of activated microglia *in vivo* enhanced the release of BDNF and NT-4/5, and newly induced NGF release.

It is an accepted that neurotrophins regulate neuronal function and support the survival and enhance the growth of various types of neurons in both the PNS and CNS (*29*, *30*). Thus, the neurotrophins released from microglia may influence primarily surrounding neurons in a paracrine fashion. The BDNF and NT-4/5 secreted by non-stimulated or activated microglia may participate in the survival and regeneration of various types of neurons. BDNF may be involved in various functions including synapse modification, neurotransmitter release, long-term potentiation and mechanosensation (*31*). NGF, which is released only in the activated state, seems to facilitate the survival of cholinergic neurons in the regenerative state

TGFβ family. Of the five known TGFβ isoforms, TGFβ1–3 isoforms are expressed in the CNS, while TGFβ1 mRNA is expressed in activated microglia at the axotomized facial nucleus (*32*) and in reactive microglia during experimental allergic neuritis Supporting the results of in situ hybridization, microglia in culture produced and secreted TGFβ1. Generally, the TGFβ family has been seen to promote neuronal survival of various types of neurons (*33*). TGFβ1–3 promote the survival of dopaminergic neurons and prevent MPP^+-dependent neuronal toxicity. TGFβ1 promotes the re-elongation of injured axons of cultured hippocampal neurons. TGFβ1 expressed by activated

microglia may contribute to the recovery and survival of injured motoneurons (*32*).

Glial cell line–derived neurotrophic factor (GDNF) is a member of the TGFβ superfamily, which includes three other structually related factors: neurturin, persephin, and artemin (*34*). GDNF exerts survival effects on dopaminergic neurons and motoneurons *in vivo* and *in vitro* and is an important factor in the development or maturation of both CNS and PNS. Analysis of GDNF-null mice clarified that a lack of GDNF gives rise to abnormalities in some nervous systems. GDNF has been suggested to be produced in non-neuronal cells such as astrocytes, Schwann cells and skeletal muscle, and the GDNF derived from these cells is considered to act as a neurotrophic factor. GDNF was detected in CMM, showing the ability of microglia to produce this cytokine This result indicated that microglia are GDNF suppliers in the CNS. The neurotrophic effect of CMM on dopaminergic neurons may be partially attributed to TGFβ1 and GDNF.

IL-6 family. Interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF) have been grouped as the IL-6 family, since these cytokines share a common receptor component (gp 130) in their signal transduction.

IL-6 is a proinflammatory cytokine which is produced in activated microglia as well as in astrocytes. It was detected in CMM in our cultured microglia, and its secretion was enhanced by stimulation with LPS. IL-6 can act as a neurotrophic factor for septal cholinergic neurons

The major sources of CNTF in the nervous system have been believed to be astrocytes and Schwann cells (*35*). However, this cytokine was also detected in CMM, indicating that microglia have the ability to produce it. CNTF stimulates survival or differentiation in a variety of neuronal cell types, such as sensory, sympathetic, and ciliary cells, and motoneurons, and it arrests the division of neuronal precursor cells. The neuroprotective effects of CNTF have been demonstrated in a number of *in vitro* cell models as well as *in vivo* models which exhibit motor neuron degeneration CNTF and LIF, but not IL-6, enhanced the BDNF-induced promotion of survival of basal forebrain cholinergic neurons in the CNS.

LIF has been suggested to be produced by astrocytes and microglia, as has been reported in lesioned cortex of the adult rat brain (*36*), and it has been detected in CMM. LIF can act as a neurotrophic factor, enhancing neuronal survival, and as a differentiation factor; and it may act as an injury factor in both CNS and PNS.

Fibroblast growth factor 2 (FGF2). Basic fibroblast growth factor (bFGF: FGF2) has been assumed to be contained in CMM, because we have previously found this factor in cultured microglia In fact, bFGF was detected in CMM, indicating that it is a secretory product of microglia. In our system, it exerted neurotrophic effects on cortical and mesencephalic neurons. The neurotrophic effects of bFGF on various types of neurons have been reported (*37*).

Kringle molecules. Plasminogen (PGn), a zymogen of active protease plasmin, was identified in CMM as a neurotrophic factor for dopaminergic neurons. PGn markedly enhanced the neurite outgrowth, dopamine content and dopamine uptake of mesencephalic neurons *in vitro* (*38*). The molecular mechanism of the neurotrophic effect exerted by PGn remains to be clarified, although a receptor-like protein for PGn was identified on the neuronal surface One possible neurotrophic mechanism of PGn is that PGn-derived plasmin causes functional activation of the inactive TGFβ family, which would result in neurotrophic effects.

Hepatocyte growth factor (HGF) was also identified as a neurotrophic molecule in CMM. This factor showed activity for promoting the survival and neurite outgrowth of cortical neurons, and enhancing the survival and maturation of mesencephalic dopaminergic neurons (*39*). The neurotrophic effect of HGF is mediated by phosphorylation of its specific receptor, c-met, and the subsequent activation of MAP kinase. HGF and PGn have a unique common structure called the kringle domain, which is folded into a specific shape by S-S bonds. However, the relationship between the kringle structures of these molecules and their neurotrophic effects is uncertain.

Other factors. Interleukin-2 (IL-2) and IL-3 were found to mimic the neurotrophic effects of CMM. IL-3 promoted the survival and neurite outgrowth of neocortical neurons in our assay system, and it is reported to exert neurotrophic effects on cholinergic neurons. This cytokine is released from non-stimulated microglia, as described previously (*40*), while it remains uncertain whether IL-2 is produced in microglia.

IL-1β and TNFα, while not released in non-stimulated microglia, are induced when activated by LPS. Although IL-1β is not generally described as a neurotrophic factor, it can enhance the biosynthesis of somatostatin in diencephalon neurons. TNFα acts to promote the survival of neurons during glucose-deprivation or in the presence of excitatory amino acid (*41*), and it can attenuate *N*-methyl D-aspartate (NMDA)–dependent neuronal death, acting as a kind of neurotrophic factor. However, since this cytokine can kill oligodendrocytes or myelin *in vitro*, it is generally grouped with the cytotoxic molecules (described above)

Are activated microglia beneficial or harmful?

The potential of activated microglia to produce cytotoxic and neurotrophic molecules *in vitro* allows us to suppose the presence of three different states of microglia: cytotoxic, neurotrophic, and bifunctional states. Non-stimulated cultured microglia which are semi-activated and exhibit phagocytic activity, but do not produce IL-1β, TNFα, and NO, correspond to the neurotrophic state, because some neurotrophic molecules including neurotrophin (BDNF) are released. That this state is neurotrophic was confirmed by assaying the effect of CMM for neurons (*25, 26*). LPS-activated microglia turned out to correspond to a mixture of cytotoxic and neurotrophic states, because they produce not only induced cytotoxic molecules but also enhanced amounts of neurotrophic molecules. Although it has been questioned whether the CMM from LPS-activated microglia is harmful or neurotrophic, this issue remains to be definitively resolved. On the other hand, only the cytotoxic state has not yet been identified *in vitro*. The orientation of microglia into the cytotoxic cells appears to occur under the strict control of a specific trigger molecule.

The molecular mechanism including the signaling pathway by which the neurotrophic and cytotoxic states of activated microglia are distinguished is still only partially known and poorly understood. Further study is necessary to resolve the regulatory mechanism of microglial activation.

Significance of activated microglia

Activated microglia act primarily in the defence of the brain, as brain scavengers, and in tissue remodeling An immune and/or immunoeffector role has also been proposed. Activated microglia have been suggested to be implicated in pathogenesis and inflammation. They have also been shown to produce a variety of biological factors. Recent biochemical and neurobiological studies have revealed that activated microglia produce and secrete not only cytotoxic/harmful molecules but also neurotrophic molecules including neurotrophins and neurotrophic cytokines.

As a whole, activated microglia appear to play a significant role in pathological and regenerative states of the brain, expressing both or either of two potentially opposing functions: cytotoxic and neurotrophic actions

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