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Invited review

From blood to brain: amoeboid microglial cell, a nascent macrophage and its functions in developing brain[1]

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Key words

developing brain; amoeboid microglial cells; functions

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Abstract

Amoeboid microglial cells (AMC) in the developing brain are active macrophages. The macrophagic nature of these cells has been demonstrated by many methods, such as the localization of various hydrolytic enzymes and the presence of complement type 3 surface receptors in them. More importantly is the direct visualization of these cells engaged in the phagocytosis of degenerating cells at the ultrastructural level. Further evidence of them being active macrophages is the avid internalization of tracers administered by the intravenous or intraperitoneal routes in developing rats. The potential involvement of AMC in immune functions is supported by the induced expression of major histocompatibility complex class I and II antigens on them when challenged by lipopolysaccharide or interferon-γ. Immunosuppressive drugs, such as glucocorticoids and immune function-enhancing drugs like melatonin, affect the expression of surface receptors and antigens and the release of cytokines by AMC. Recent studies in our laboratory have shown the expression of insulin-like growth factors, endothelins, 2',3'-cyclic nucleotide 3'-phosphodiesterase, and *N*-methyl-*D*-asparate receptors. This along with the release of chemokines, such as stromal derived factor-1a and monocyte chemoattractant protein-1, suggests multiple functional roles of AMC in early brain development.

Introduction

Amoeboid microglial cells (AMC), the nascent form of ramified microglia that persist in the adult brain, are present ubiquitously in the brain during fetal and early postnatal development. They are present transiently until 10–14 d of age (Figure 1A) when all of them transform into the adult, ramified microglial cells (Figure 1B). There is recent evidence that the ramification of AMC may be regulated by cytoskeletal elements as transfection of the cells with a cytoskeleton-related gene, in particular, juxtanodin has resulted in the branching of the cells assuming a ramified external morphology (Figure 2). AMC exist singly or in clusters in the subventricular white matter. Other areas in which AMC are preponderant include the cavum septum pellucidum and subependymal cysts closely associated with the third and fourth ventricles and the cerebral aqueduct. In the brain tissue, the cells are widely distributed; some of them may be spatially associated with neurons, blood vessels, or dispersed freely. The close association of AMC to blood vessels has been reported in many areas of the brain[1–3].

The origin and mode of the formation of AMC has been a contentious issue for decades, and many theories had been proposed since the first description of a cellular "third element" other than neurons and neuroglia[4] and identification of microglia by del Rio-Hortega[4,5]. Three hypotheses have been put forward in relation to the origin of microglia: (i) mesodermal[6,7]; (ii) neuroectodermal[8–10]; and (iii) monocytic[11–13] . Investigations carried out in our laboratory over the past 3 decades tend to support the monocytic origin of AMC, although the possibility that these cells may be derived by direct invasion of fetal macrophages cannot be excluded[14]. We have shown that circulating monocytes invade the brain during embryonic and early postnatal life and then transform into AMC; hence, they are monocyte-derived brain macrophages like other tissue macrophages.

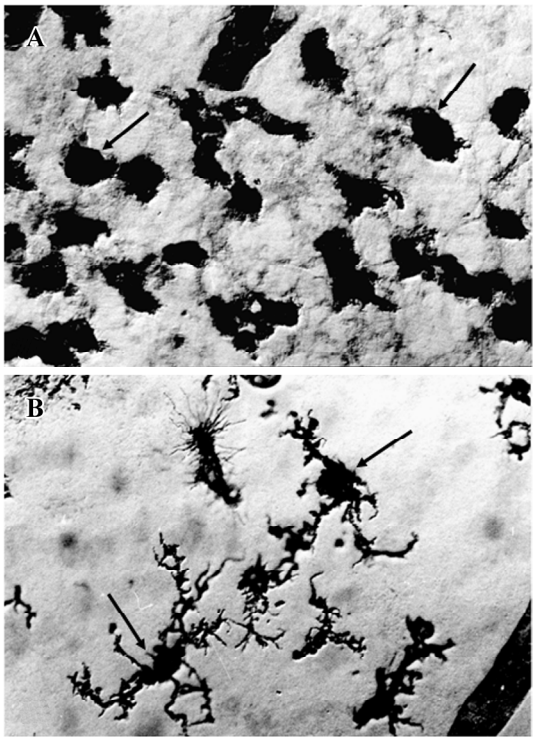

Figure 1. (A) Lectin-labeled amoeboid microglial cells (arrows) in the corpus callosum of a 5-d-old rat showing a round morphology with some thick processes. (B) Ramified lectin-labeled microglial cells (arrows) are seen in the adult corpus callosum. Scale bar=10 μm.

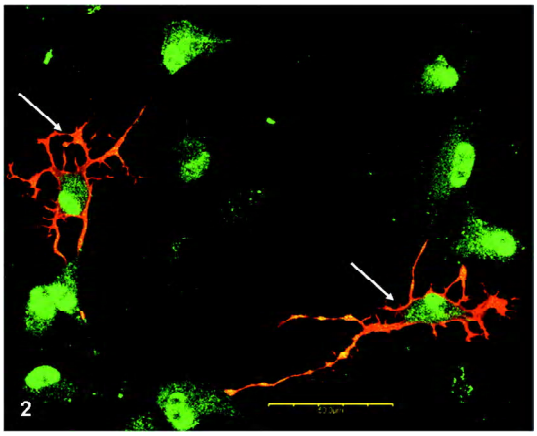

Figure 2. Ramification of AMC (arrows) can be induced by transfection with a cytoskeleton related gene-juxtanodin (orange). Scale bar=50 μm.

Nature of AMC

AMC are multifunctional immune cells in the developing central nervous system (CNS) that play an important role in the defense of neural parenchyma. Early studies have shown AMC to be active macrophages in the developing brain[13,15], removing cellular debris during normal development as well as in pathological conditions. Besides their scavenging function, AMC may also exert a cytotoxic effect through the secretion of toxic factors, such as nitric oxide[16]. Recent studies in our laboratory have greatly amplified the functional roles of these cells during development, in addition to their primary role in phagocytosis.

AMC function as phagocytes The phagocytic nature of AMC has been shown by various methods and observations, including the localization of hydrolytic enzymes, ultrastructural features shared by tissue macrophages, uptake of exogenous substances, and the activation of surface receptors and antigens related to phagocytosis.

Hydrolytic enzymes Our early cytochemical studies have shown the presence of hydrolytic enzymes, including acid phosphatase, aryl phosphatase, non-specific esterase and 5'-nucleotidase in the lysosomes in AMC[12,17,18]. The high contents of these hydrolytic enzymes in AMC suggested that these cells were active macrophages. This observation is supported by recent studies which have reported the localization of hydrolytic enzymes, such as acid phosphatase in macrophages, in the pineal gland[19]. A study investigating the ability of peritoneal, alveolar, and splenic macrophages and Kupffer cells to kill pathogens[20] reported that acid phosphatase activity was significantly increased in macrophages which ingested the pathogens and killed them. *In vitro* studies have shown that AMC were phagocytic and possessed non-specific esterase activity[21]. With time, these cells transform into ramified microglia-like cells and lose their phagocytic property as well as non-specific esterase content[21].

Ultrastructural observations Ultrastructural studies carried out in our laboratory in the normal postnatal brain and in the brains of rats subjected to hypoxia or *Escheria coli(E coli)* treatment have supported the phagocytic property of AMC. The macrophagic nature of AMC was evidenced by their engagement in the phagocytosis of degenerating axons and cells in the normal developing brain[22]. Under the electron microscope, some AMC in the corpus callosum of postnatal rats were observed to extrude portions of their cytoplasm which were phagocytosed by neighbouring AMC[22].

In the fetal and postnatal rat brains, AMC were found to phagocytose dead cells in the brain after transient maternal hypoxia[23]. Neonatal rats exposed to hypoxia also showed AMC in the corpus callosum engaged in the phagocytosis of apoptotic cells[24] and degenerating axons[16]. Further evi-

dence of the phagocytic nature of AMC comes from their involvement in the removal of *E coli* introduced directly into the neonatal brain. Many of the injected *E. coli* were sequestered by AMC in less than 3 h[25]. It was concluded from these observations that AMC form a protective barrier which is deemed to be necessary in the early developmental period when the blood-brain barrier (BBB) is deficient.

Tracer studies It is well established that the BBB in the developing brain is immature. Administration of tracers, such as rhodamine isothiocyanate and horseradish peroxidase (HRP), intraperitoneally or intravenously, resulted in the labeling of AMC in the corpus callosum and other regions[26,27]. This suggested that substances in circulation leaked through the immature BBB and were phagocytosed by AMC. Another tracer, biotinylated dextran, when injected in various areas of the brain far removed from the corpus callosum, also resulted in the labeling of AMC[28]. It was concluded that AMC become labeled by ingesting the tracer which diffused slowly through the extracellular spaces from the injection site. Injection of HRP in the lumbosacral region of the spinal cord also resulted in the labeling of AMC in the corpus callosum[29]. The results suggested an ascending diffusion of the injected HRP in the spinal cord via wide interstitial spaces to reach the cerebrum where it was engulfed by the AMC.

ED1 antigens Further support to the macrophagic nature of AMC was lent by the expression of ED1 antigens on these cells. ED1 antigens are expressed by cells of monocyte/macrophage lineage[30]. These antigens were expressed by AMC in the fetal[31] and postnatal period[32], but not in adult rats.

Complement type 3 receptors Complement type 3 receptors (CR3) were detected on the cell membranes of AMC by using the antibody OX-42. It was proposed that these receptors are related to the active role of AMC in endocytosis[33]. This was supported by reports that CR3 receptors on monocytes and their derivative macrophages mediate endocytosis[34–37].

Lectin histochemistry Microglial cells engulfing pyknotic and fragmented nuclei of cells undergoing programmed cell death have also been visualized by use of lectin histochemical staining in conjunction with cresyl violet counterstaining[38].

Antigen presentation Although the CNS, under normal conditions, has been considered an immunologically privileged site for a long time, our studies showed for the first time that major histocompatibility class (MHC) I antigens were expressed by AMC[39]. MHC antigens are surface molecules required for the participation of macrophages in the activation of T lymphocytes by presenting certain antigens to them. MHC I antigens serve as restriction elements for cytotoxic/suppressor lymphocytes[40,41]. The expression of these antigens on AMC was related to their phagocytic activity[39]. The presence of MHC I antigens on AMC also suggests that these cells are ready to interact with infiltrating lymphocytes as the BBB is immature in the developing brain and the danger of a potential immune threat in early development may be present.

MHC II antigens, required for interaction with helper/inducer T lymphocytes, on the other hand, are not expressed by AMC under normal conditions. The expression of MHC II is induced under pathological and experimental conditions, for example, these antigens are expressed when the cells are challenged with lipopolysaccharide (LPS)[42] or with interferon-γ (IFN-γ)[43]. MHC II expression on AMC was also induced on the introduction of live *E coli* in their vicinity[25]. The expression of these antigens on AMC under pathological conditions suggests that they have the capability of interacting with helper/inducer cells to mount a potential immune response.

Other protective functions AMC in the developing brain express transferrin receptors[32] which facilitate the acquisition of iron needed for various functions of cells. As transferrin receptors are also important for the proliferation of cells, they may be involved in the differentiation and maturation of AMC. Additionally, the presence of these receptors on AMC may serve a protective function to sequester excess iron for storage in pathological conditions where there is an excessive influx of iron into the brain. In support of this, the increased expression of transferrin receptors and iron was reported[44] in AMC in newborn rats subjected to hypoxia. Hypoxia/reoxygenation is known to increase the iron content of the brain in newborn animals[45]. Excess iron not safely sequestered in storage is hazardous as it promotes the formation of free radicals[46], resulting in oxidative tissue damage.

AMC in the developing brain, especially in the white matter tracts, are also thought to promote axonal growth[47]. Cultured microglial cells derived from neonatal rat brains and transplanted into injured spinal cords enhanced the regeneration and elongation of spinal cord axons[48] indicating their involvement in axonal growth. In the developing white matter, AMC have also been reported to function as guides for developing axons, perhaps through the manufacture of extracellular matrix molecules, such as thrombospondin[49,50].

Insulin-like growth factors Insulin-like growth factor (IGF) I and II are known to regulate the development of the nervous system[51]. IGF-I plays an important role in promoting cell proliferation and differentiation[52] in the devel-

oping brain. The role of IGF II during postnatal development of the brain is less clear. Our recent study[53] has shown the expression of IGF-I and IGF-II in AMC. The expression of IGF-I and IGF-II was markedly enhanced in the cells by LPS, but was significantly suppressed with all-trans retinoic acid (RA). From these findings, it was suggested that IGF-I expression in AMC may be linked to the state of cell activation. IGF-I has been shown to enhance phagocytic activity of neutrophils *in vitro* when they were challenged with *E coli*[54]. It was demonstrated by Inoue *et al*[55] that IGF-I increased the killing capacity and phagocytosis of peritoneal macrophages when they were activated by *E coli*. IGF-I expression in AMC may also have paracrine functions, such as the modulation of the proliferation and development of the oligodendrocytes and myelination, as it has been considered as an important factor for oligodendrocyte survival and myelination[56,57]. Although the exact function of IGF-II in the developing brain is not clear, its pattern of association with oligodendrocytes and myelin suggests that it may also play a role in myelination[58,59] or in the phagocytic activity of AMC. IGF-I may also be related to the antigen-presenting function of AMC as it is known to stimulate the proliferation of immunocompetent cells and modulate the cellular immune functions of neutrophils and macrophages[60].

Nitric oxide Nitric oxide (NO) is synthesized from *L*-arginine by the family of NO synthase (NOS) enzymes. NOS from neurons and endothelial cells are constitutively expressed enzymes, the activities of which are calcium depend-ent. Inducible NOS (iNOS), which is calcium-independent and NO-generated from this isoform, is known to mediate immune functions. NO production in macrophages has been described to have protective or destructive functions. NO production by macrophages is stimulated by various pathogens, such as bacteria, viruses, and parasites[61–63] and has a role in their phagocytic activity[64]. The overproduction of NO however has toxic effects as it leads to the formation of toxic reactive nitrogen intermediates which can have deleterious effects[65].

It has been reported that activated microglial cells in the white matter may contribute to perinatal brain injury[66] through the secretion or production of noxious substances. NO production in the corpus callosum in response to hypoxia was found to increase in the neonatal brain along with iNOS expression in AMC[16,67]. *In vitro* studies have shown that NO produced by AMC is highly damaging to the oligodendrocytes resulting in their lysis[68]. The induction of iNOS in the activated microglia contributed to tissue injury through NO overproduction in fetal brain injury caused by umbilical cord occlusion[69]. The sustained production of NO endows macrophages with cytotoxic activity against viruses, bacteria, and fungi[70]. On the other hand, NO has suppressive effects on lymphocyte proliferation and can damage normal host cells[70] which can be deleterious.

Glutamate receptors The weak expression of NMDA (*N*-methyl-*D*-asparate) receptor subtype 1 (NMDAR1) was localized in AMC in the corpus callosum in the neonatal brain. This may facilitate the cells to be responsive to the release of glutamate from the neighboring callosal axons undergoing degeneration in the remodeling of the developing brain. The expression of NMDAR1 on AMC was enhanced in response to hypoxia[16]. This may be linked to the elevation of extracellular glutamate in the white matter in hypoxia/ischemia, which has been described to be possibly due to the release from damaged axons[71]. Excess glutamate leads to toxicity and death of oligodendrocyte progenitors[72]. Immunostimulated microglia have been reported to enhance the NMDA receptor-mediated excitotoxicity in part through the expression of iNOS[73]. The expression of NMDA receptors on microglial cells has also been reported in excitotoxic lesions in mice[74]. Although the expression of NMDAR1 receptors on AMC has been reported to have detrimental effects in the developing brain, they may have a protective role by sequestering excess glutamate released by degenerating axons.

Inflammatory response Microglial cells play an important role in the development of an inflammatory response in the developing brain[75]. They are thought to cause damage to axons and the developing oligodendrocytes by releasing inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α in many pathological conditions, such as hypoxic–ischemic conditions, and have been shown to express both p55TNFαR1 and p75TNFαR2 receptors[76]. Aberrant TNF-α/p55TNFαR1 signaling in the CNS can have a potentially major role in CNS pathologies in which oligodendrocyte death and demyelination is a primary pathological feature[77]. Besides their inflammatory actions, cytokines IL-1β and TNF-α may also be involved in the transcriptional activation of the iNOS gene[78,79]. Microglia in the developing brain have also been shown to express the chemokine receptor CCR5 until 2 weeks of age[80,81]. It was proposed that CCR5 were involved in microglial recruitment and activation during brain development and after neonatal brain injury, such as hypoxic-ischemic injury.

Endothelins Endothelins (ET), consisting of 3 subtypes, ET-1, ET-2, and ET-3, are multifunctional peptides produced by a wide variety of cells under normal and pathological conditions. Besides basal vasoconstriction, they are known to exert mitogenic and anti-apoptotic actions[82,83], as well as

act as growth-promoting factors involved in embryonic and fetal development[84–86]. It has been reported that macrophages and monocytes act as a source of ET-1 production during infection and inflammation[87,88].

We have recently reported the expression of ET, especially ET-1 in AMC[89]. It was further demonstrated that the expression decreased in response to hypoxia. The stimulation of AMC with LPS enhanced the expression of ET-1. It was suggested that the expression of ET-1 may have autocrine functions, such as synthesis and secretion of chemokines, including stromal derived factor-1α (SDF-1α) and monocyte chemoattractant protein-1 (MCP-1), or paracrine actions on the developing glial cells, neurons, and blood vessels bearing the ET receptors.

2',3'-cyclic nucleotide 3'-phosphodiesterase The expression of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), a myelin-associated enzyme commonly regarded as a selective marker for immature oligodendrocytes, was localized in AMC in the developing rat brain from prenatal d 18 to postnatal d 10[90]. Although the function of CNPase in AMC is not known, it may serve a cytoskeletal role to change the shape of the cells for migration or for the transportation of cytoplasmic materials. It may also be involved in the phagocytic function of AMC or in the secretion of pro-inflammatory cytokines and growth factors by them. CNPase may also be involved in transformation of the cells from the early round and amoeboid shape to a ramified form with growth.

Response to drugs

Glucocorticoids Glucocorticoids, which are potent anti-inflammatory and immunosuppressive drugs, have been reported to suppress the number and the phagocytic activity of macrophages[91]. The administration of glucorticoids, such as cortisone or dexamethasone, in postnatal rats resulted in a substantial reduction in the number of AMC in the corpus callosum[11,92]. The decrease in the number of AMC was attributed to the suppression of the number of circulating monocytes, as glucocorticoids are known to reduce their numbers[93]. Another effect of glucocorticoids on AMC was their premature differentiation or maturation into ramified microglia. However, the phagocytic activity or proliferation of AMC did not appear to be affected by glucocorticoid treatment[92]. Dexamethasone also inhibited microglial activation by downregulating the neurotoxic and pro-inflammatory mediators such as NO, TNF-α, and IL-6[94–96]. Preliminary results in our laboratory (unpublished data) demonstrated that dexamethasone inhibited the migration of microglial cells by suppressing the release of MCP-1, a chemokine which regulates the migration of activated microglial cells to the inflammatory sites in the CNS. It has been further shown that the downregulation of MCP-1 expression in activated microglial cells by dexamethasone was mediated via the MKP-1-dependent inhibition of the JNK and p38 mitogen-activated protein kinase pathways.

Chloroquine Chloroquine, an antimalarial drug with anti-inflammatory properties, has proven to be a beneficial therapeutic agent in certain inflammatory disorders[97]. Chloroquine is known to exert its anti-inflammatory effect by downregulating the synthesis of pro-inflammatory cytokines, such as TNF-α and IL-1β[98]. It also reduces the expression of MHC II on Kupffer cells[99]. Besides its anti-inflammatory actions, chloroquine inhibits lysosomal degradation and produces lysosomal changes resembling that of lysosomal storage disease in a variety of cells, including macrophages[100,101]. Increased vacuolation and the accumulation of lysosomes were observed in AMC in response to the intraperitoneal administration of chloroquine in 1-d-old rats. This was attributed to the failure of digestion of internalized substances. The phagocytic activity and antigen-presenting function of AMC, however, was enhanced in response to chloroquine as evidenced by the upregulation of CR3 receptors and MHC I antigens[102].

Colchicine The number of AMC in the corpus callosum of postnatal rats was reduced following colchicine treatment[103]. It also brought about an early differentiation of AMC into the ramified form. Colchicine is a microtubule-disrupting drug[104,105] and is known to induce apoptosis in different cell types. *In vitro* studies have shown that macrophages assume irregular profiles following depolymerization of microtubules by colchicine[106]. It has been hypothesized that microtubules are important for the secretion of lysosomal enzymes and the intracellular degradation of materials phagocytosed by macrophages[107]. It has also been suggested that microtubules are necessary for the fusion of lysosomes with endosomes which must precede intracellular digestion of phagocytosed materials[108], whereas others have reported that intact microtubules are not required for the lysosomal-endosomal fusion[109]. We found that the mitotic activity of AMC was suppressed by colchicine, but the phagocytic activity remained unaffected[103].

All-trans retinoic acid (RA) Recently, we have shown that the RA, a vitamin A metabolite, can suppress the LPS/β-amyloid-induced activation of microglial cells in primary culture by inhibiting the expression and production of TNF-α and NO[110]. It has been further shown that RA enhances the mRNA expression of TGF-β1, which acts as an

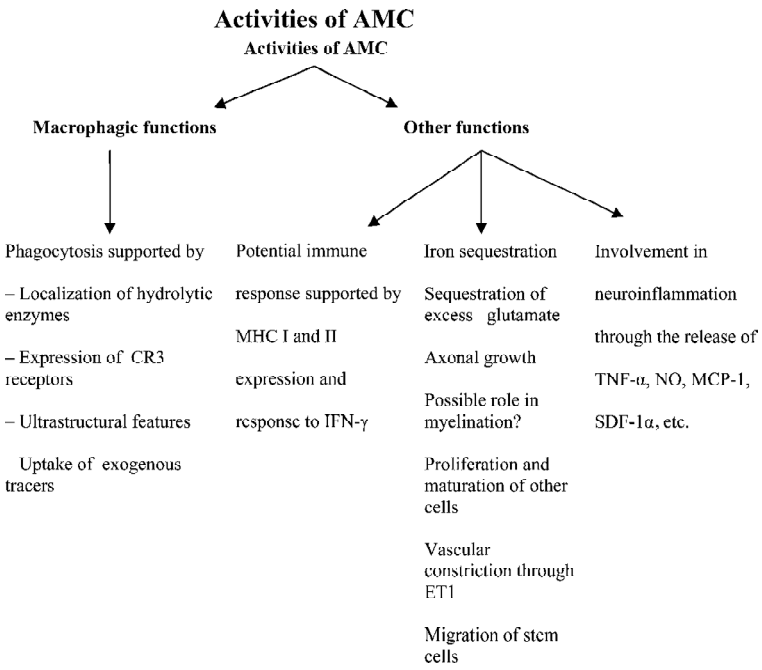

Figure 3. Activities of AMC.

immunosuppressor, and RA receptor (RAR) β1, and attenuates NF-κB translocation from the cytoplasm to the nucleus in activated microglial cells. It has been suggested that the inhibition of TNF-α and NO synthesis by RA in the activated microglia is mediated via the inhibition of NF-κB translocation, which could be caused by the upregulation of RAR and TGF-β1 gene expression. RA has also been shown to suppress the expression of IGF-I and IGF-II in activated microglia, indicating that RA is effective in inhibiting the action of a wide array of molecules specific for activated microglia[41]. In view of these results, it was suggested that RA could be considered a potential therapeutic agent that may inhibit the inflammatory response of microglia in neurodegenerative diseases.

Melatonin Melatonin, a neurohormone synthesized by the pineal gland, is known to have immunomodulatory actions[111,112]. In addition to its immunomodulatory actions, melatonin has also been reported to be important for phagocytosis under physiological conditions[113]. AMC showed a significant increase in cell numbers and upregulation of CR3, MHC I and MHC II, and CD4 antigens in response to melatonin administration[114], indicating enhanced endocytic and antigen-presenting capacity. The expression of these receptors and antigens returned to control levels on cessation of melatonin administration, suggesting that increased immune potentiality of the microglial cells and its maintenance requires the continuous action of the drug.

Toxic effects of AMC

It has been reported that microglial cells may be involved in causing apoptosis[47] in the developing brain. Although there is no direct evidence for this observation, microglial cells have been identified as the source of nerve growth factor, a pro-apoptotic agent responsible for neuronal death in the developing eye[115].

Conclusion

Early descriptions of the function of AMC focused on their primary role, that is, phagocytosis in the developing brain. In light of recent voluminous findings based on the expression of a plethora of molecules and growth factors in these cells, multiple functional roles of these cells, such as antigen presentation, vascular regulation, chemokine release, modulation of proliferation, and the development of other cells in the developing brain are proposed as summarized in Figure 3.

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