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Atypical Antiinflammatory Activation of Microglia Induced by Apoptotic Neurons

Possible Role of Phosphatidylserine–Phosphatidylserine Receptor Interaction

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Abstract

In the central nervous system (CNS), apoptosis plays an important role during development and is a primary pathogenic mechanism in several adult neurodegenerative diseases. A main feature of apoptotic cell death is the efficient and fast removal of dying cells by macrophages and nonprofessional phagocytes, without eliciting inflammation in the surrounding tissue. Apoptotic cells undergo several membrane changes, including the externalization of so-called "eat me" signals whose cognate receptors are present on professional phagocytes. Among these signals, the aminophospholipid phosphatidylserine (PS) appears to have a crucial and unique role in preventing the classical pro-inflammatory activation of macrophages, thus ensuring the silent and safe removal of apoptotic cells. Although extensively studied in the peripheral organs, the process of recognition and removal of apoptotic cells in the brain has only recently begun to be unraveled. Here, we summarize the evidence suggesting that upon interaction with PS-expressing apoptotic neurons, microglia may no longer promote the inflammatory cascade, but rather facilitate the elimination of damaged neurons through antiinflammatory and neuroprotective functions. We propose that the anti-inflammatory microglial phenotype induced through the activation of the specific PS receptor (PtdSerR), expressed by resting and activated microglial cells, could be relevant to the final outcome of neurodegenerative diseases, in which apoptosis seems to play a crucial role.

Index Entries: Apoptosis; brain macrophages; cytokine; inflammation; nerve growth factor; neurodegeneration; nitric oxide; microglial activation; prostaglandin E_2; transforming growth factor-β.

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Introduction

Microglia represents the largest population of phagocytes associated with the CNS and accounts for about 10% of the non-neuronal cells in brain parenchyma. It is now widely accepted that they derive from cells of the mononuclear phagocyte lineage that enter the developing CNS. During embryogenesis, these cells are highly activated and contribute to the removal of dead cells and debris naturally occurring in the shaping of the developing brain. In the postnatal period, microglia acquires a highly differentiated phenotype, characterized by a ramified morphology and the absence of cell-surface and cytoplasmic molecules typically associated with other tissue macrophages *(1–3)*. The quiescent or "resting" microglia, found throughout the brain under nonpathological conditions, rapidly reacts in response to a number of different acute and chronic insults, and the presence of activated microglial cells is an early and common feature of most brain pathologies *(4)*.

In response to acute conditions, such as traumatic injury, ischemia or infection, microglial cells proliferate, migrate to the site of injury, and display a repertoire of reactive changes including the synthesis of pro-inflammatory and cytotoxic molecules such as free radicals and cytokines. In vitro and in vivo studies have shown that pro-inflammatory molecules, for example interferon-γ, released by infiltrating hematogenous cells or by resident cells, could promote the exacerbation of microglial reaction and tissue damage. On the contrary, other factors such as transforming growth factor-β (TGF-β) and some prostaglandins (PGs), released either by the activated microglia or by other brain resident cells, may limit microglial activation favoring the resolution of traumatic events *(5,6)*.

The role of microglia in chronic neurodegenerative diseases remains, to date, not fully understood. Increasing evidence indicates that in these chronic pathologies activated microglia could sustain a local inflammatory response in the absence of neutrophil infiltration and mononuclear cell perivascular cuffing. In these diseases, microglial activation is an early sign that often precedes neuronal death, and in some cases, astrocyte reaction *(7)*, suggesting an important role for these cells as "sensors" of subtle environmental changes, but leaving open the question as to whether microglial activation is a major cause of neuronal damage.

In spite of the extensive studies performed in the last decade *(4)*, the concept of "activated" microglia still awaits a rigorous definition. From the neuropathologist's point of view, it is generally accepted that activated microglia has upregulated cell-surface and/or cytoplasmic antigens and a morphology characterized by cell body enlargement and loss of ramified processes. The fulfillment of these criteria, however, does not imply *per se* anything about the functional state of microglia and its ability to promote or limit brain inflammation and tissue damage. On the other hand, the in vitro models extensively used to characterize the functional properties of microglia, are often criticized for some drawbacks. Among these are the morphological and antigenic features of purified microglial cultures, which suggest a partial activation even in the absence of any additional stimulation. Furthermore, there are some experimental constrains, such as the relatively short time of stimulation and the abrupt addition of fibrillogenic peptides or other activators, which bear little resemblance to the slow build-up of amyloid occurring in vivo. Recent studies on a prion animal model (*8* and references therein) failed to demonstrate increased syntheses of pro-inflammatory cytokines and nitric oxide, but showed an upregulation of immunoregulatory molecules, such as PGE_2 and TGF-β in microglia. These results, which are consistent with the elevated levels of PGE_2 measured in cerebrospinal fluid of Creutzfeldt–Jakob patients *(9,10)*, support the hypothesis that the pro-inflammatory phenotype that is often attributed to "morphologically" activated microglia may not always occur in chronic neurodegenerative diseases.

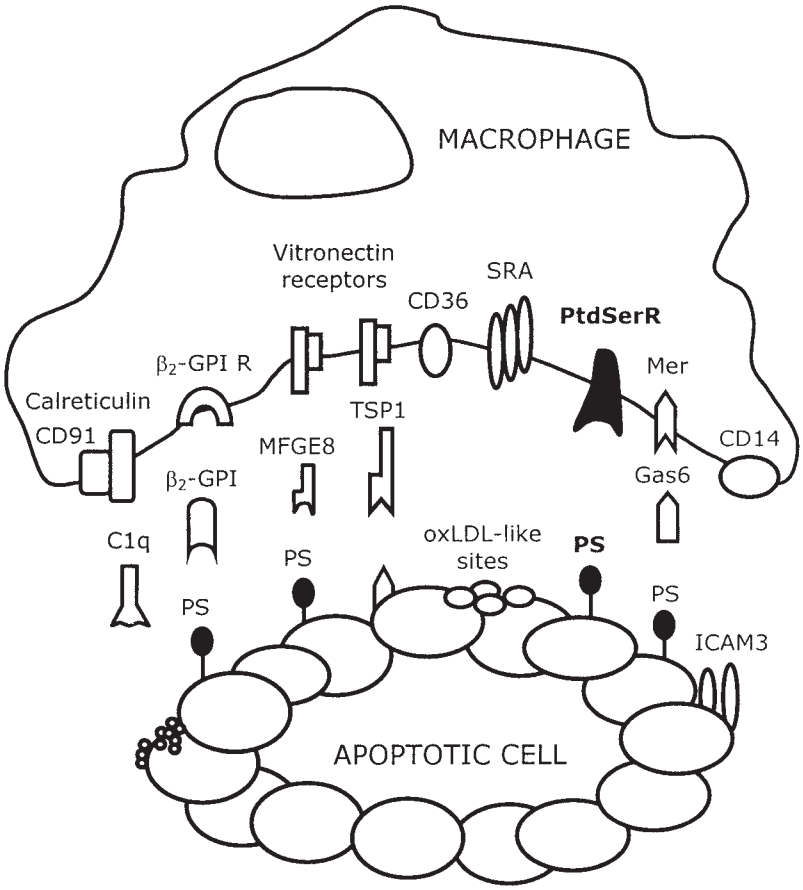

Fig. 1. Schematic representation of a multitude of "eat-me" signals on apoptotic cells and their cognate receptors on macrophages. *See* text for details.

On the basis of the above considerations, a new concept of "atypical" microglial activation can be proposed, by which activated microglia is no longer envisaged as effector cells that promote inflammation. On the contrary, microglia could control or even prevent the spreading of an inflammatory reaction, by acquiring "antiinflammatory" functions. The development of experimental models on which perform further functional studies is necessary to elucidate the final outcome of microglial "antiinflammatory" functions in the pathogenesis of chronic diseases.

In this review article, we summarize the current studies supporting a role for the interaction between apoptotic neurons and microglia in the induction of atypical, antiinflammatory microglial activation and discuss the possible neuroprotective and neurotrophic functions of activated microglia in neurodegenerative diseases.

Recognition and Removal of Apoptotic Cells in Periphery

Apoptosis is a process of programmed or "active" cell death, that is essential in the shaping of organs during development and in the maintenance of tissue homeostasis in adult life

(11). During apoptosis, biochemical cascades activate proteases that destroy molecules required for cell survival and execute the program of suicide cell death.

Morphologically, apoptosis is characterized by surface membrane changes, shrinkage of the cell body, nuclear condensation, and DNA fragmentation.

A main feature of apoptotic cell death is the efficient and fast removal of dying cells by macrophages and nonprofessional phagocytes. This process, highly conserved among nematodes, insects, and mammals ensures the uptake of death-destined cells from the surrounding tissue before their lysis and the release of intracellular contents that can disrupt the tissue homeostasis.

As mentioned earlier, apoptotic cells undergo several membrane changes, including the externalization of so-called "eat me" signals, whose cognate receptors are present on professional phagocytes. Additional soluble molecules, such as complement components, the plasma protein β2 glycoprotein I (β2-GPI), the milk-fat globule epidermal growth factor 8 (MFGE8), and thrombospondin-1 (TSP1), contribute to the process by bridging phagocytes and apoptotic-cell surface (*see* Fig. 1).

The mechanisms of specific recognition and removal of apoptotic cells by phagocytes have been well documented in the peripheral immune system (for review, *see* ref. *12* and Fig. 1). Many of the macrophage receptors that contribute to apoptotic cell recognition are also key components of the innate immune system. An example is given by the scavenger receptors, a family of three classes of cell surface proteins (class A, B, and mucin-like) that mediate the binding and internalization of modified proteins, such as acetylated or oxidized low-density lipoproteins, or other molecules including polynucleotides, TSP1, anionic phospholipids, and various bacteria *(13).* CD36, a class B scavenger receptor, is one of the first macrophage receptors that have been implicated in the apoptotic cell recognition through the binding of oxidized sites on the surface of the apoptotic cells *(14).* Another system of recognition involves the CD91-calreticulin complex which binds apoptotic cells through the complement protein C1q. Recent studies reported the specific binding of apoptotic cells by C1q and pentraxins, a group of acute-phase proteins normally involved in the innate response to invading pathogens *(15,16).* CD14, better known as lipopolysaccharide (LPS) receptor, besides its role in innate immunity to invading organisms, is also involved in the phagocytosis of apoptotic cells, particularly leukocytes, through ligands exposed on the apoptotic-cell surface *(17).* Other molecules, such as intercellular adhesion molecule 3 (ICAM3), which normally mediate adhesion of healthy leukocytes, can promote phagocytosis of dying leukocytes. Similarly, the integrin $\alpha_V\beta_3$, (the vitronectin receptor), through the binding of TSP1, can either stimulate the motility of healthy leukocytes or promote the phagocytosis of apoptotic leukocytes *(12).* It has been proposed that vitronectin receptor and CD36 cooperate to form a high affinity-binding site for TSP1 bound to the apoptotic cells *(18).*

The multiplicity of receptors used by phagocytes in the recognition of apoptotic cells underlines the importance of the clearance process. However, the contribution of each single receptor to the clearance process remains poorly defined.

In recent years, the aminophospholipid phosphatidylserine (PS) has been recognized as a new "eat me" signal and a key mediator for the clearance of apoptotic cells *(19).* PS is predominantly found in the inner leaflet of plasma membrane of virtually every cell of the body. The asymmetric distribution of PS is maintained through an ATP-dependent activity and several mechanisms, including scramblase activation and flippase inhibition, may contribute to PS exposure (for review, *see* ref. *20*). As a consequence of such mechanisms, PS becomes permanently exposed on the external surface during the early phase of apoptosis *(21).* PS on apoptotic cells is recognized by a specific phosphatidylserine receptor (PtdSerR), which has been recently cloned. This receptor, widely expressed in different peripheral tis-

sues and cells, is predicted to be a type II glycoprotein of 48 kDa, and displays at least one tyrosine phosphorylation site and several potential protein kinase (PK) C phosphorylation sites in its intracellular domain that could account for signaling properties *(22).*

It has recently been proposed a model in which several ligands or bridging molecules, including PS, "tether" the apoptotic cells to the macrophages through high avidity/low affinity interactions with phagocyte receptors. In addition, the low avidity/high affinity interaction of PS with PtdSerR seems to have a unique role in "tickling" the engulfment process *(23).*

The ability of macrophages to phagocytize dying cells without acquiring an overt inflammatory phenotype is another hallmark of apoptosis. Increasing evidence indicates that this atypical macrophage phenotype is the result of an active process, in which some antiinflammatory functions are prompted, whereas other pro-inflammatory genes are kept silent *(12).* Seminal studies by Fadok and colleagues *(24)* have shown that the specific interaction of PS with its receptor has a unique role in the induction of such active process. These studies demonstrated that apoptotic cells inhibit the release of the pro-inflammatory cytokine TNF-α in elicited human macrophages, whereas the productions of antiinflammatory TGF-β and the lipid mediator PGE_2 are increased, suggesting that specific PS-dependent mechanisms can prevent the inflammatory reaction and promote a silent and safe removal of apoptotic cells.

Microglial Receptors Involved in the Recognition of Apoptotic Neurons

In the CNS, apoptosis plays a fundamental role during development when a large number of immature neurons compete for a limited supply of target-derived trophic factors *(11).* In the adult, apoptosis has been proposed as a primary pathogenic mechanism in several chronic neurodegenerative diseases, including Alzheimer (AD), Parkinson, and Creutzfeldt-Jakob (CJD) diseases *(25,26).* Apoptosis is also a feature of acute neurological diseases such as stroke or brain trauma. For example, after an ischemic insult, neurons die by necrosis in the core of the lesion where hypoxia is more severe, and by apoptosis in the penumbra where collateral blood flow reduces the degree of hypoxia *(27).*

Most of the phagocytic receptors that are found in the peripheral macrophages have also been described in microglial cells, and many of these are upregulated in response to microglial activation (*see* Table 1). These receptors include the Fc receptors (FcR) and the complement receptors (CR1, CR3, and CR4), which mediate phagocytosis of specific targets through the binding of the constant fragment of immunoglobulins and the complement protein C3bi, respectively *(28).* The presence of the C1q receptor (C1qRp) has been demonstrated in rat microglial cultures, where through the interaction with its ligand, enhances the phagocytosis promoted by FcR and complement receptors *(29).* C1qRp is also expressed by human microglia *in situ (30).* Members of the class A and class B of scavenger receptors have been implicated in the endocytosis of cellular debris and pathogenic peptides, such as myelin fragments and fibrillar β amyloid *(31),* as well as in the clearance of apoptotic neurons *(32,33).* Furthermore, the mannose receptor—another mediator of phagocytosis that binds and internalizes mannosylated and fucosylated residues present in a variety of pathogens and proteins—has been detected in cultured microglia and brain perivascular macrophages *(34–36).*

Little is known about the "eat me" signals induced by apoptosis in neurons. Several in vitro studies have shown that hippocampal neurons, neuronal cell line HN2–5, and differentiated PC12 cells redistribute PS from the inner to the outer leaflet of the plasma membrane during early phases of the apoptotic process *(37–40).* This event seems crucial for the recognition and phagocytosis of apoptotic neurons by microglial cells *(41).* More recently, in an animal model of retinal degeneration, it has been demonstrated that apoptotic photoreceptors expose PS on their membrane and are

Table 1
Microglial Receptors Involved in Phagocytosis

Receptor	Function	References
Fc receptors (FcR I, II and III)	Absorption of immunoglobulins	*28,29*
Complement receptors (CR1, CR3 and CR4)	Absorption of complement components	*28,29*
Scavengers receptors (Class A, Class B, mucin-like)	Uptake of cellular debris, pathogen peptides, polyanionic molecules, oxidized molecules, apoptotic cells	*28,31–33*
Vitronectin receptor ($\alpha_V\beta_3$ integrin)	Adhesion to other cells, extracellular matrix and other proteins Uptake of apoptotic neurons	*28,33*
Phosphatidylserine receptor	Uptake of apoptotic neurons, photoreceptors and PC12 cells	*33,42,44,46*
Mannose receptor	Not yet identified	*34–36*

phagocytized by macrophages through the PtdSerR and the vitronectin receptor *(42).*

Witting et al. *(33),* have shown that at least three classes of receptors are involved in apoptotic cerebellar granule recognition by microglial cells. These authors used a co-culture model of primary microglia and cerebellar granule neurons to examine the receptor systems involved in the recognition/uptake of apoptotic neurons. They found that phagocytosis of apoptotic neurons by microglial cells was reduced by the aminosugars *N*-acetylglucosamine or galactose, suggesting the involvement of asialoglycoprotein-like lectins. A partial inhibition of microglial binding/uptake of apoptotic neurons was also seen with the tetrapeptide Arg-Gly-Aps-Ser (RGDS) interfering with $\alpha_V\beta3$/CD36/TSP pathway. Interestingly, they found a time-dependent inhibition of the uptake of apoptotic neurons, suggesting that, as for the peripheral macrophages, the membrane changes necessary for the vitronectin receptor recognition develop later in the apoptotic process. Finally, microglial phagocytosis of apoptotic neurons was specifically inhibited by PS-containing lipid vesicles, but not by phosphatidylcoline (PC) vesicles, suggesting a specific interaction with receptors for PS *(33).* A PS-dependent mechanism was also suggested by Chang et al. *(43),* who studied the uptake of apoptotic glioma cells by human microglia, normal human astrocytes, and glioma cells.

Expression of PtdSerR on Microglial Cells

The existence of a specific receptor for PS on microglia suggested by Witting et al. *(33)* was later proved in our laboratory *(44).* Using the sequence available for PtdSerR identified in peripheral macrophages *(45),* we demonstrated the expression of PtdSerR mRNA in microglial cells. The transcript for PtdSerR was detectable in unstimulated cultures after 8 h. Its expression increased in the presence of LPS, suggesting that the process of activation upregulates the expression of PtdSerR. A correlation between the presence of PtdSerR and the state of cell activation was previously reported in human monocyte-derived macrophages *(45).* We were unable to detect the receptor at the protein level by using a specific antibody

Table 2
Effects of PS-Liposomes on Activated Microglial Cell Functions

	Nitrite (μ*M*)	TNF-α (pg/mL)	IL-1β (pg/mL)
Unstimulated	0.2 ± 0.1	32 ± 8	11 ± 5
LPS	14.5 ± 2.2	3479 ± 639	480 ± 134
LPS + PS-liposomes	5.6 ± 1.7*	895 ± 209*	68 ± 22*

Microglial cells were cultured for 24 h in 10% FCS-containing medium, in the presence or absence of 10 ng/mL LPS and 10 μM PS-liposomes. Supernatants were collected after 24 h and analyzed for nitrite accumulation and TNF-α and IL-1β production. *$p < 0.05$ vs LPS (for further details see ref. 44).

raised against human macrophage PtdSerR, which is at the moment the only available antibody *(45).* This was probably due to a poor ability to react with the rat receptor, since recently the group of Chan et al. *(46)* reported the expression of PtdSerR protein in human microglia.

Effects of PS-PtdSerR Interaction on Microglial Activation

Given the presence of PtdSerR in microglial cells, we then studied the effects of PS-PtdSerR interaction on microglial functions. With this objective, we exposed purified rat microglial cultures to PS-containing liposomes to mimic the interaction with apoptotic neurons. We found that in LPS-activated microglia, PS liposomes specifically inhibited the production of NO, IL-1β, and TNF-α, known as potent pro-inflammatory substances (*see* Table 2, and ref. *44*). The inhibitory activity was specific for PS, as it was mimicked by PS head group *O*-phospho-L-serine but not by PC-containing liposomes, supporting the hypothesis that PS, through the interaction with its receptor, may affect the functional state of microglia and prevent the acquisition of pro-inflammatory properties *(44).* To investigate whether PS, when exposed on apoptotic cells rather than in artificial vesicles, could affect microglial activation, we devised an experimental system in which microglial cells were co-cultured with healthy, apoptotic, or necrotic PC12 cells *(47).* The extent of apoptotic process in PC12 cells was assessed by evaluating PS externalization by the use of annexin-V, which binds specifically to PS *(48).* Similar to the experiments with PS-liposomes, the synthesis of NO and TNF-α by LPS-activated microglia was significantly decreased upon interaction with apoptotic PC12 cells, but not with healthy or necrotic cells. Furthermore, after interacting with apoptotic PC12 cells, activated microglial cells secreted a remarkable amount of PGE_2 (*see* Fig. 2A and ref. *47*). Increased levels of PGE_2 were found in co-cultures of resting microglia and apoptotic cerebellar granule cells (De Berardinis, Polazzi, and Minghetti, unpublished observation). An increased synthesis of PGE_2 in parenchymal microglia may have several functional consequences that are far from being completely understood. Despite its classical pro-inflammatory role on vascular permeability and leukocyte extravasation, PGE_2 may be immunosuppressive and downregulate the process of microglial activation *(6,49).* In several in vitro systems, exogenous PGE_2 has been found to protect neurons against glutamate- or LPS-induced cytotoxicity *(50–52),* or at very high concentrations, to stimulate the release of glutamate and to induce neuronal apoptosis *(53–55).* Elevated PGE_2 levels have been found in several human neurological diseases and in their animal models. PGE_2 levels were elevated during the recovery phase in a murine model of multiple sclerosis, suggesting a protective effect of PGE_2 in this pathology *(56).* The levels of PGE_2 have also been found increased in the cerebrospinal fluid (CSF) of patients affected by CJD, in both sporadic and variant forms *(9,10),* consistently with the high expression of cyclooxygenase-2 (COX-2), the inducible isoform of the first limiting enzyme in PGE_2 synthesis, found selectively localized to microglial cells in a murine model of prion disease *(57).*

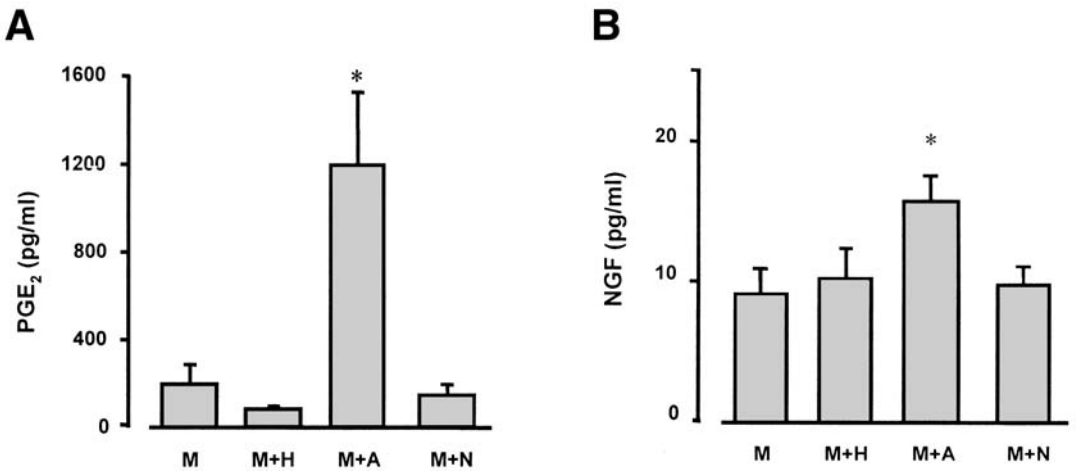

Fig. 2. PGE_2 and NGF synthesis in co-cultures of microglial cells and healthy, apoptotic or necrotic PC12. **(A)** Microglial cells (M) were subcultured for 24 h in 10% FCS-containing medium, which was replaced with 1% FCS-containing medium before addition of healthy (H), apoptotic (A), or necrotic (N) PC12 cells, in the presence or in the absence (not shown) of 10 ng/mL LPS. Supernatants were collected after 24 h and analyzed for PGE_2 accumulation. *$p < 0.05$ vs M, M + H and M + N ($n = 4$ independent experiments). **(B)** Microglial cells (M) cultured as above were maintained with healthy (H), apoptotic (A) or necrotic (N) PC12 cells for 24 h. Supernatants were then collected and analyzed for NGF production. *$p < 0.005$ vs M, M + H, and M + N ($n = 5$ independent experiments).

Another metabolite increased in the co-cultures of microglia and apoptotic PC12 cells is TGF-β1, a pleiotropic molecule that, similar to PGE_2, is involved in the modulation of inflammatory and immune responses *(58).* In the CNS, TGF-β1 exerts many biological effects on different cell types, including microglia, astrocytes, and neurons *(59).* In normal brain, TGF-β1 is barely detectable, but its expression strongly increases after acute or chronic diseases such as ischemia, AD, and prion diseases *(60–62).* Recently, a high expression of TGF-β1 has been described in an animal model of prion disease with a pattern closely resembling that of COX-2 *(63).* As neuronal apoptosis has been described in prion and other chronic neurodegenerative diseases *(25–27),* it is tempting to speculate that PS exposure on apoptotic neurons and PS interaction with the specific receptor on microglia contribute to the molecular mechanisms leading to the increased expression of TGF-β1 and COX-2 in microglia.

Finally, we investigated whether the interaction with apoptotic cells could stimulate the release of neurotrophic factors, known to be produced by microglial cells *(64).* We found a significant increase in the levels of the neurotrophin nerve growth factor (NGF), only when microglial cells were cultured with apoptotic PC12 cells (Fig. 2B and ref. *47*). NGF, one of the best characterized neurotrophins, is crucial for the survival or death of several neuronal populations, as well as for the regulation of neuronal phenotype and functions *(65).* During the course of acute as well as chronic neurodegenerative diseases, elevated levels of NGF are present at the site of injury where inflammatory cells and dying neurons are present *(66,67).* Several studies reported that microglia in co-cultures can either potentiate the survival of cortical neurons or protect apoptotic neurons via soluble factors *(65,68).* It has been hypothesized that microglial cells, once in contact with degenerating neurons, are

capable of releasing trophic factors to facilitate the functional recovery of the surrounding compromised neurons *(69).* Our findings extend this hypothesis, suggesting that NGF release is specifically induced in microglia upon interaction with apoptotic, but not necrotic, neurons.

Signaling Pathways Elicited by PS in Microglial Cells

As described above, the recognition of apoptotic cells recruits a multitude of receptors (*see* Table 1) that may activate intracellular signaling cascades leading to the expression of inflammatory molecules and to the generation of reactive oxygen species in macrophages and microglial cells *(32,70–72).*

In apparent conflict with these observations, apoptotic cell clearance is an anti-inflammatory and nonimmunogenic process. This finding led to the hypothesis that the existence of regulatory mechanisms is able to break down the link between the engagement of the mentioned receptors and the deriving pro-inflammatory signals. An intriguing possibility is that PtdSerR delivers a powerful dominant anti-inflammatory signal by its own or in association with other membrane molecules, as predicted by the presence of charged residues in the putative transmembrane domain of the receptor *(22).*

The signaling pathways evoked by PtdSerR have just begun to be characterized. Hoffmann et al. *(23)* showed that PS liposomes or anti-PtdSerR antibodies induce membrane ruffling in 3T3 fibroblasts and human monocytes through activation of the low-molecular-weight GTPases Rac1 and Cdc42, in line with the described role of these two molecules in the uptake of apoptotic cells *(73).* In FcγR-mediated phagocytosis, Rac1 and Cdc42, besides regulating actin polymerization, activate signaling pathways associated with inflammation, such as NF-κB, JnK and p38 MAPK, and ROS production *(74).* As suggested by Leverrier and Ridley *(73),* a different duration of Rac and/or Cdc42 activation during apoptotic cell uptake may determine the lack of production of pro-inflammatory mediators. However, other peculiar or predominant signals are likely to regulate this event. Todt et al. *(75)* recently reported that PtdSerR induces the activation of several PKC isoforms in murine peripheral macrophages. In particular, the βII isoform was shown to be necessary for the engulfment of apoptotic thymocytes. Since PKCβII requirement is unique for phagocytosis of apoptotic cells but not for other types of particles, the authors suggested that PKCβII could be relevant to the signaling pathways mediating the suppression of pro-inflammatory molecules. Whether this is a general feature of PtdSerR engagement in all cell types remains to be clarified.

We recently investigated the signal transduction elicited in microglial cells by the interaction PS-PtdSerR (*see* Fig. 3 and ref. *76*). In this cell type, PS-liposomes did not affect the activation of the nuclear transcription factor NF-κB, a crucial regulator of microglial synthesis of pro-inflammatory products *(75,76),* consistent with the observation that phagocytosis of apoptotic cells does not trigger NF-κB activation in peripheral macrophages *(79,80).*

On the other hand, PS-liposomes affected CREB, another important transcription factor controlling microglial activation. CREB is phosphorylated and activated in response to cAMP or other stimuli *(81),* and cAMP elevating agents are known to regulate the production of both pro-inflammatory and antiinflammatory molecules in microglial cells *(49).* The broad range of cellular functions regulated by CREB is due to the presence of CREB consensus sequences in several genes, including iNOS and TNF-α, and to the existence of different CREB isoforms acting either as repressors or activators of gene expression *(81).* In resting microglial cultures, PS liposomes induced a significant phosphorylation of CREB after 15 min of incubation. However, the presence of PS-liposomes delayed the LPS-induced phosphorylation of CREB *(76).* Given the multiple actions of CREB *(81),* an alteration of its kinetics

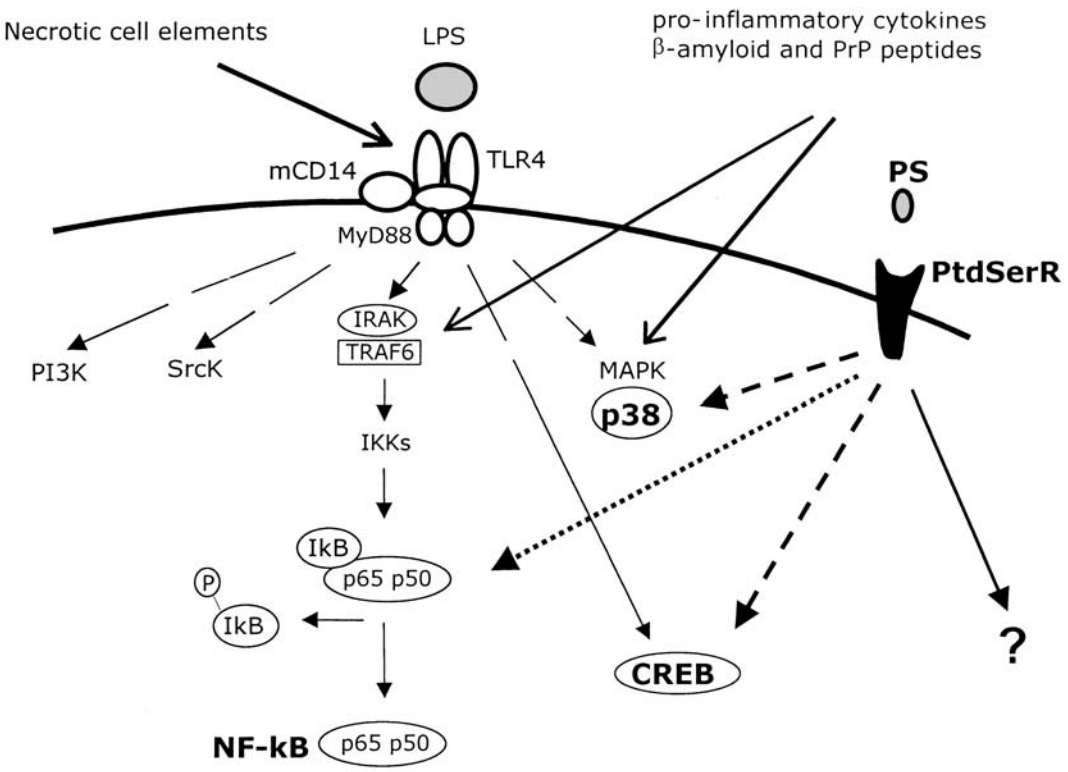

Fig. 3. Cell signaling evoked by PS-PtdSerR interaction. The PS-PtdSerR interaction elicits intracellular signals, here schematically represented, which may interfere with the transduction pathways triggered by pro-inflammatory agents such as LPS or necrotic cell elements recognized by the Toll-Like receptor 4 (TLR4) complex. Some of these signaling pathways are also evoked by other activating agents (cytokines, fibrillogenic peptides) acting on specific receptors. IRAK, IL-1 receptor associated kinase; TRAF6, TNF-α receptor-associated kinase; IKKs, IκB kinase; PI3K, phosphatidylinositol 3 kinase; SrcK, Src tyrosine kinase.
– – → inhibition; ·····→ no effects; ——→ activation.

of activation could be sufficient to alter the balance between the effectors of LPS signaling cascade, and significantly affect the resulting microglial functions.

The MAP kinase p38 is a central regulator of microglial pro-inflammatory activities in response to LPS or other stimuli, including the β-amyloid peptide and the PrP-fragment 106–126 *(82)*. PS-liposomes mildly enhanced the levels of p38 phosphorylation, and hence its activation, in resting microglial cultures but significantly reduced the levels of p38 phosphorylation induced by LPS. In view of the amplifying feature of the p38-dependent signaling cascade, involving a multitude of intracellular targets such as protein kinases and transcription factors *(83)*, even a partial inhibition of p38 may significantly affect the synthesis of several pro-inflammatory molecules and the overall activation state of the cell.

In addition to CREB and p38, other molecules are likely to play a relevant role in the signaling pathway elicited by PtdSerR in microglial cells. Further investigation is needed to sort out the upstream and downstream transduction events triggered by the PS-PtdSerR interaction and their possible interplay with pathways dependent on other

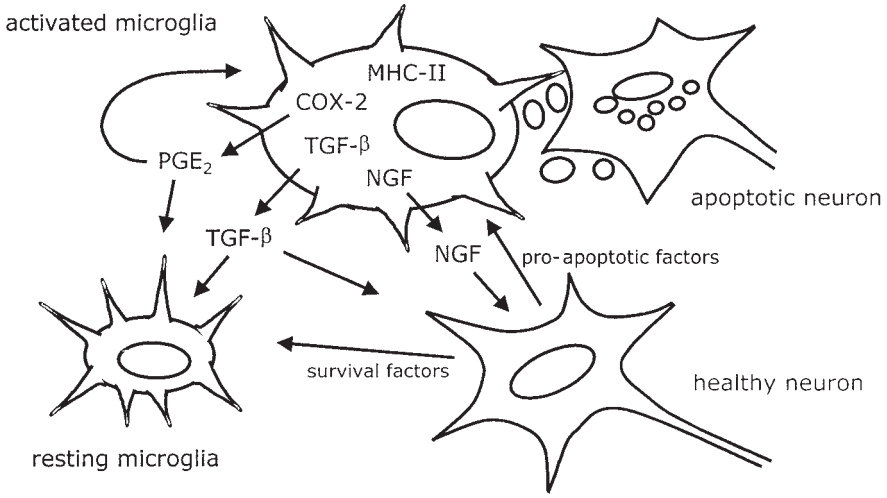

Fig. 4. Atypical activation of microglial cells in chronic degenerative diseases. Microglial cells, upon interaction with PS-expressing apoptotic neurons, become stimulated to produce molecules such as TGF-β and NGF that may exert neuroprotective and neurotrophic activities on healthy neurons and/or on neurons not yet committed to suicide. In addition, TGF-β1, as well as PGE_2, could act on microglia preventing their further activation. Healthy neurons are also important for controlling activation and survival of microglia, through the production of factors that may promote the survival of resting microglia or induce the apoptotic death of activated microglia, as recently reviewed by Polazzi and Contestabile *(92).* MHC, major histocompatibility complex antigens.

receptor systems known to be involved in the recognition of apoptotic cells. In particular, it will be important to analyze, in microglial cells, the expression and the role of the Mer receptor—a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family—that has been shown to mediate the binding of PS to peripheral macrophages through the recognition of the bridging molecule growth arrest-specific gene 6 (Gas6) *(84,85).* Similar to PtdSerR, Mer could play a dual role in triggering the uptake of tethered cells and in down-regulating the production of pro-inflammatory molecules such as TNF-α and IL-1β. Indeed, peritoneal macrophages obtained from knockout mice expressing an isoform of Mer with a truncated cytoplasmic tail (mer^{kd}) can bind, but not ingest, apoptotic thymocytes, though fully competent in the phagocytosis of other particles *(86).* Compared to wild-type, mer^{kd} macrophages showed elevated and prolonged NF-κB activation and abnormal Jun-*N*-terminal kinase activity upon LPS-stimulation, whereas p38 activation was unaltered, suggesting that Mer exerts regulatory functions on selected intracellular pathways *(87).* It would be of particular interest to study whether Mer can interact or cooperate with PtdSerR in mediating the microglial anti-inflammatory functions.

Conclusions

In the present review, we have summarized the evidence supporting that, upon interaction with PS-expressing apoptotic neurons, microglia may no longer promote the inflammatory cascade, but rather exert neuroprotective and neurotrophic functions (Fig. 4). We propose that the specific microglial functional state induced by the signaling cascade evoked by

PtdSerR occupancy could be relevant to the final outcome of neurodegenerative diseases in which apoptosis is a primary pathogenic mechanism.

An extensive comprehension of the pathways taking place in vivo during the recognition of apoptotic cells is complicated by the dynamic changes in the surface of cells undergoing apoptosis, and by the fact that the expression of all the receptors so far identified is developmental regulated and affected by environmental cues *(88).* Recently, it has been suggested that oxidation of PS is an intrinsic feature of the apoptotic program and that oxidized-PS, in addition to non-oxidized-PS, could constitute an important "eat me" signal for macrophages *(89,90).* The possible contribution of oxidized-PS vs non-oxidized PS to the modulation of the functional responses of macrophages and microglial cells needs to be investigated. In addition, several studies indicate that PS exposure by neurons undergoing apoptosis is reversible and precedes genomic DNA destruction and loss of membrane integrity, suggesting a "window of opportunity" for rescuing neurons not yet committed to suicide *(38,40,91).* A detailed understanding of the molecular mechanisms regulating expression and functions of PtdSerR could provide additional tools to promote the microglial neuroprotective and neurotrophic functions and to favor the reversal of the apoptotic process by appropriate therapeutic interventions.

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