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Role of purinergic signalling in neuro-immune cells and adult neural progenitors

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1. ABSTRACT

Inflammation has a key role in a vast range of central nervous system diseases. Under acute and chronic neurodegenerative conditions, resident microglia and astrocytes and blood-borne immune cells concur to neuroinflammation and remodeling/repair at the inflammed site. Distinct inflammatory cell states characterized by either a beneficial or a detrimental phenotype have been identified, depending upon the timing after the initial insult and activation by specific pro- or anti-inflammatory molecules. Of note, quiescent adult neuroprogenitor cells located in both brain's neurogenic areas and parenchyma have been recently shown to interact with immune cells and actively participate to restore function. Among other systems, extracellular nucleotides and their receptors have emerged as early alerting signals in inflammation and as key players in orchestrating the release of inflammatory molecules and the interaction between different cell types. Here, we revise the state of the art in this expanding field with the final aim of unveiling whether new purinergic-based therapies may be useful to halt excessive inflammation and foster endogenous repair in neuroinflammatory disorders.

2. INTRODUCTION: THE PURINERGIC THEORY OF NEUROINFLAMMATION

Inflammation starts after tissue injury or infection as a host defense against foreign or altered endogenous substances. It is recognized as a fundamental mechanism to protect the integrity of the organism, regain homeostasis and enable repair that becomes detrimental only when it is innaturally prolonged or amplified. No vascularized tissue, organ or apparatus, is free from this response (1).

Players of inflammation are *cells* and *soluble factors*. Concerning *cells*, besides typical inflammatory cells such as circulating and resident leukocytes and tissue mast cells, the family of inflammatory cells has been growing during the years to also include fibroblasts, endothelial cells, adipocytes and, more recently, brain cells. While the central nervous system (CNS) was initially looked at as an immunoprivileged organ where no inflammation could occur thanks to the existence of the blood brain barrier (BBB), there is now strong evidence that this is not true. CNS host resident immune cells (typically, microglia, the brain immunocytes, and astrocytes) react to pathogens or damage, migrate to the site

of injury, phagocytose invading microorganisms or cell debris and elaborate and secrete inflammatory mediators. Furthermore, after acute insults (stroke, trauma) or during chronic diseases (e.g., multiple sclerosis or Alzheimer's disease), blood-borne T and B lymphocytes invade the CNS and activate resident or infiltrating inflammatory cells, thus resulting in inflammation. Interestingly, blood-borne cells have been also recently involved in the stimulation of quiescent, residential adult brain's precursor cells (2) that become activated as a result of this interaction and might contribute to the healing process via the generation of newborn cells to substitute neurons and glia that have died during the inflammatory response.

Concerning *soluble factors*, starting from arachidonic acid metabolites, the family of inflammatory molecules has followed an exponential growth to include cytokines, chemokines, nitric oxide and hundreds of other systems, among which the purinergic system constituted by extracellular nucleotides and nucleosides, their metabolic enzymes and receptors, the P1 and the P2 receptors (3). Apart from the demonstration that P2 receptors are expressed in all the cell types involved in inflammatory reactions (ibidem), a major seminal study at the basis of the *"purinergic theory of neuroinflammation"* is the article published by Wang and colleagues in 2004 using an experimental model of mechanical spinal cord injury (4). Low ATP concentrations were detected within the lesioned area that was found to be surrounded by a peri-traumatic area, which was, instead, characterized by sustained ATP release. This phenomenon persisted for several hour after the initial insult; ATP levels returned back to basal value 10 min after cardiac arrest, demonstrating that release was not simply due to leakage from dying cells but indeed relied on a metabolically active mechanism. One year later, Davalos and colleagues showed that a mild focal mechanical injury to the brain triggers ATP release from astrocytes, which, in turn, results in rapid activation of microglia (5). Under ATP stimulation, microglia converged to the site of damage to form a barrier between healthy and injured tissue. To further support their conclusions, Davalos and colleagues showed that injury-induced microglia migration was inhibited by inoculation of wide-range P2 receptor antagonists or the ATP-degrading enzyme apyrase; conversely, direct ATP injection into the brain elicited a potent chemotactic effect on microglia. These papers introduced the concept that ATP, which is rapidly released from brain cells as a result of cell perturbations, may act as a *"danger signal"* for the surrounding tissue to activate response to injury and repair. Moreover, the Davalos et al paper postulated a protective beneficial role for activated microglial cells at the site of injury and highlighted ATP as one of the main molecules eliciting such activation.

Based on this evidence, the aim of this review is two-fold. First, we summarize some recent literature data highlighting the existence of different glial cell states characterized by either a beneficial or a detrimental phenotype, depending upon the timing after the initial inflammatory insult and the inflammatory agents involved. A better understanding of these different phenotypes will greatly aid in finding new ways to attenuate the detrimental effects associated to neuroinflammation, while maintaining the beneficial ones. Second, we revise recent data highlighting a pivotal role for the purinergic system in alerting and tuning immune and inflammatory reactions to aversive influences in the CNS during both acute and chronic neurodegenerative diseases. Special emphasis will be given to recent developments implicating purinergic signalling in the cross-talk between infiltrating blood circulating cells and adult brain's precursors, and to the role of P2 receptors (for P1 receptors in neuroinflammation, the reader is referred to some other recent authoritative review in the fields, see: (6, 7).

Our final aim is to unveil if new purinergic-based therapies may be useful to halt excessive inflammation and foster endogenous repair in neuroinflammatory disorders.

3. PURINERGIC SIGNALLING IN RESIDENT AND BLOOD-BORNE IMMUNE CELLS

3.1. The double-edged actions mediated by microglia and macrophages during CNS repair

Microglial cells are recognized to play a central role in brain inflammation, and it is, thus, a lucky coincidence that experiments performed in this cell type have set the seminal information for our understanding of purinergic signalling in inflammation (8-12). Expression of several P1 and P2 receptors and the ability of purinergic molecules to profoundly affect multiple microglia responses (such as proliferation, process motility, migration, phagocytosis, cytokine and chemokine release) make this cell type a paradigm for purinergic studies in inflammation (13). While the surveillance properties of microglia are essential for the maintenance of CNS integrity, excessive or uncontrolled microglial activation has severe and deleterious consequences (14). In addition to residential microglia, blood-derived macrophages which infiltrate damaged areas due to blood-brain-barrier (BBB) breakdown, have been so far considered as detrimental pro-inflammatory key players. However, in the last years, data from many groups suggest that responses following CNS injury are not only to be minimized, but also represent reparative mechanisms to be exploited for the treatment of diseases characterized by neuroinflammatory events. One of the key issues is now to dissect the mechanism determining the shift between a beneficial and a detrimental immune phenotype; the answer is likely to be found in the finely regulated balance between released molecules activating/repressing the expression of specific membrane receptors and specific timing after the insult.

The most convincing evidence on the existence of distinct beneficial/detrimental immune cell phenotypes has been reported for macrophages. The classical activation of these cells by lipopolysaccharide (LPS) or interferon-gamma (IFN-gamma) indeed leads to the pro-inflammatory phenotype called M1, characterized by the release of pro-inflammatory cytokines, free radicals and prostaglandins. M1 cells act as potent effectors that kill microorganisms and tumor cells but may also mediate detrimental effects on neural cells. Instead, M2 macrophages acquire an anti-

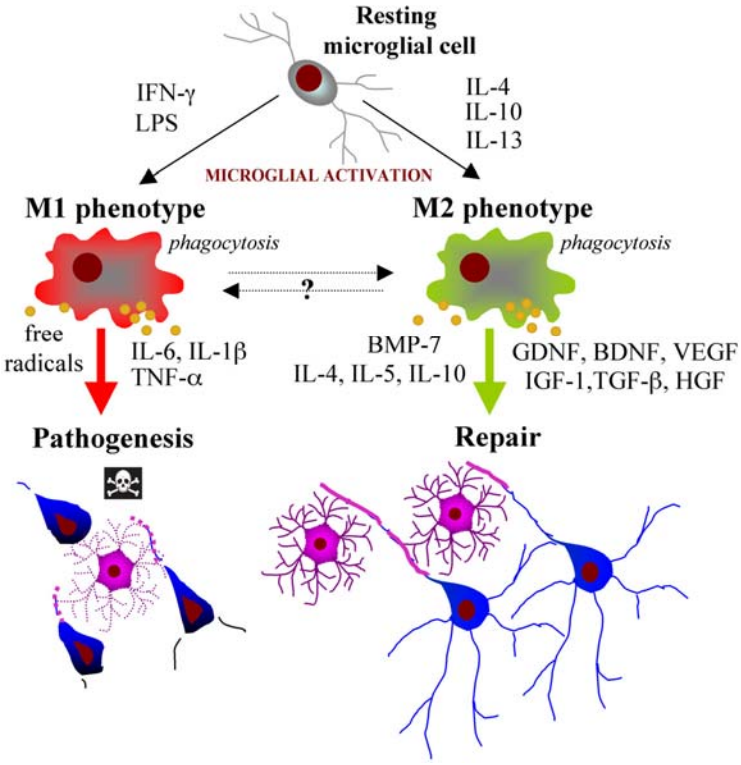

Figure 1. The two opposite immune phenotypes of microglial cells during brain inflammation. A detrimental/pro-inflammatory immune cell M1 phenotype is acquired upon classical activation of resting microglia by either LPS or INF-gamma, while IL-4, IL-10 or IL-13 typically induce an anti-inflammatory M2 phenotype representing an alternative, apparently beneficial, activation state more related to a fine tuning of inflammation, scavenging of debris, promotion of angiogenesis, tissue remodeling and repair. M1 cells release pro-inflammatory cytokines such as IL-6, TNF-alpha, IL-1 beta and free radicals, that contribute to amplifying the neuroinflammatory response. Pro-inflammatory cytokines exert cytotoxic effects on oligodendrocytes and neurons leading to demyelination and axonal damage. M2 cells release neurotrophic factors such as GDNF, BDNF, bFGF, IGF-1, TGF-beta, HGF that provide trophic support to neurons in the injured area, in part by potentiating the recruitment, proliferation and differentiation of oligodendrocyte precursor cells. Autoregulatory processes, including phagocytosis of cellular debris and apoptotic cells, can down-regulate the activated M1 phenotype by a negative feed-back mechanism (20). This may result in the shift of M1 towards the M2 phenotype. Presently, it is not known whether the opposite (i.e., the shift from a detrimental M2 to a beneficial M1 phenotype) is possible, nor are known the endogenous signals involved in this shift. Purinergic signalling is involved in these changes at various stages (see text). A similar activation state had been previously reported for blood-borne macrophages (see text and (17))

inflammatory phenotype, typically induced by interleukin-4 (IL-4) or interleukin-13 (IL-13) (15, 16). Anti-inflammatory macrophages represent a beneficial activation state more related to a fine tuning of homeostatic processes, such as scavenging of debris, promotion of angiogenesis, wound healing and tissue repair after injury. Specific environmental signals are able to induce these different polarization states (17). The subclassification of macrophages into M1 and M2 cells is an oversimplification to highlight that monocyte-derived cells are a plastic lineage and may assume different phenotypes depending upon a specific stimulation.

A similar possibility has been also recently raised for microglia, by showing that these cells, under certain conditions, can be pushed to both extremes of the M1 and M2 differentiation spectrum (18) (Figure 1). While inflammatory microglia release a number of cytotoxic molecules such as IL-6, TNF-alpha, IL-1beta and free radicals, an anti-inflammatory phenotype of microglia has been shown to produce neurotrophic factors such as glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), hepatic growth factor (HGF), insulin growth factor-1 (IGF-1), transforming growth factor-beta (TGF-beta) and vascular endothelial growth factor (VEGF), potentially providing trophic support to neurons in distress (19, 20). Human microglial cells (HMO6), transplanted in ischemic rats significantly up-regulated the transcripts for GDNF, BDNF, VEGF, bone morphogenetic protein-7 (BMP-7), and cytokines such as IL-4, IL-5. This behaviour demonstrates that, at variance from what has been initially thought, the ischemic lesion with its microenvironment instructs infiltrating cells favouring a beneficial phenotype (see also section 4). As a confirm, HMO6 cells were found in the core of the lesion starting from day 5 after the

transplantation, fostering tissue repair and functional recovery at later times (10-14 days) after the transplantation) (19).

A strong immune response mediated by macrophages has been also found in all forms of CNS trauma. Supplementing an injured spinal cord with activated macrophages has been shown to promote axon regeneration, but at the same time induced neurotoxicity (21). In models of traumatic CNS injuries, activators such as zymosan and LPS act through the activation of Toll-like receptors on macrophages/microglia and produce a wide array of both deleterious and reparative responses (22). It is difficult to separate the injury/repair effects of activated macrophages from those initiated by other cells affected by trauma, mainly due to the complex cell-to-cell interactions and to the different downstream pathways following damage.

Several data suggest that activated microglia and macrophages have different function. Spatial organization of infiltrating myeloid progenitor cells around the lesion site has a direct impact on the functional parameters of recovery following spinal cord injury (SCI) (23, 24). Moreover, infiltrating cells mediate a function essential for repair that cannot be provided by resident microglia. This has been demonstrated by the replacement of bone marrow cells of naive mice with donor bone marrow containing myeloid cells reporter for green fluorescent protein (GFP). In these chimeric mice, most of the blood-derived macrophages were fluorescently labelled. Three days after SCI, fluorescent macrophages spontaneously migrate at the borders of the lesion in the region of the glial scar, whereas activated microglia is not compartmentalized. The characterization of these cells demonstrated that the anti-inflammatory mechanism involved the release of IL-10 from the alternatively activated macrophages, since no functional recovery has been observed in knock-out mice for this cytokine (23).

The distribution of the resident activated microglia differed from that of labelled macrophages, since it was present both in the core and at the margins of the lesion. Selective ablation of macrophages led to an increase in the number of activated microglia, but with an impairment in the recovery of motor function. It's likely that, after SCI, microglia become locally activated to remove cell debris immediately after injury. However, to start tissue repair, macrophages seem to be needed, since they release trophic factors and cytokines such as IL-10 responsible for functional recovery. Their characteristic distribution at the borders of the lesion may be due to the microenvironment generated by the glial scar, with which they interact to correctly perform their action.

Resident microglia are embedded in the CNS prior to injury and are immediately activated by the insult; these cells are likely very important for alerting surrounding cells and start reactions, but are not probably directly involved in tissue repair. A subpopulation of infiltrating macrophages seems to be specifically needed for acquiring this non-classical reparative phenotype and has to be instructed and locally activated (25).

It has also been demonstrated that after SCI, depletion or inhibition of CNS macrophages confers neuroprotection and promotes functional recovery (26, 27). However, the controlled activation or even augmentation of this response can enhance tissue repair (e.g., axon growth, remyelination) (28). The different final effects are strictly dependent on the balance between the different subpopulations of macrophages. The lesion is a dynamic environment that can address macrophage differentiation to favour either an inflammatory or a reparative phenotype.

After SCI, M1 macrophages infiltrate the lesion site and the adjacent tissues even chronically after injury and this could exacerbate secondary neurodegenerative processes. M1 macrophages release oxidative metabolites and proteases that can kill neurons and glia (29), whereas M2 macrophages play a reparative role within 3 and 7 days after injury.

The most relevant hallmarks of M2 macrophages in CNS repair are represented by arginase 1 (Arg1) and mannose receptor (CD206), both increased early after injury and returned to the baseline 14 days after injury. Similarly, inducible nitric oxide synthase (iNOS), an enzyme preferentially induced in M1 macrophages, was maximally increased 1-3 days after injury, whereas the expression of CD86, CD16 and CD32, surface receptors associated to the M1 phenotype, increases as a function of time after injury (29). These data suggest that after injury the majority of macrophages assume a M1 phenotype. Differentiation to the M2 phenotype is restricted to a low number of macrophages, which are essential for a correct reparative response, but probably not sufficient to a full recovery of the damaged tissue. Importantly, recent data also suggest that a fully differentiated macrophage subpopulation can reversibly change its phenotype and function in response to signals in the microenvironment (30). Therefore, it is likely that M1 can shift to M2 macrophages in the wound site, which opens interesting perspectives in the modulation of local reparative responses.

3.2. Purinergic regulation of microglia and macrophages

Microglial activation is heavily implicated in the pathogenesis of virtually all CNS diseases, including brain and spinal cord injury, stroke, Alzheimer's (AD) and Parkinson's disease (PD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (32). As stated before, ATP is released in large amounts at the sites of CNS injury and contributes to microglial activation through both ionotropic P2X and metabotropic P2Y receptors (4, 5, 31). On this basis, modulation of microglial activation by pharmacological agents acting on P2 receptors may represent a new strategy for therapeutic intervention in inflammatory human diseases (see below).

Among P2X receptors, one of the most studied candidates for inflammatory modulation is $P2X_7$. It has been known for years that the $P2X_7$ receptor is highly expressed by both microglia and macrophages. Its activation stimulates the formation of a pore allowing the

entry of non-selective large hydrophilic cations (33) and is also associated to increases in interleukin-1beta release and other cellular inflammatory responses (10, 34).

Regarding microglia, expression of $P2X_7$ is up-regulated in many brain diseases, in which the presence of microglia is a crucial player (see also below). For these reasons, in the last years, $P2X_7$ has attracted increasing interest as a possible target for new anti-inflammatory approaches (11, 35, 36). However, this receptor has been reported to mediate both beneficial and detrimental actions, ranging from induction of cell death, to regulation of phagocytosis, to production of neurotrophins. For example, the short-term exposure of microglial cells to ATP reduced phagocytosis, even after activation with LPS or other nucleotides. Also benzoyl-benzoyl-ATP (BzATP), acting as an agonist at $P2X_7$ was able to induce this effect and could slow down clearance of dead cells at the site of injury and impair recovery. In line with these findings, oxidized ATP (OxATP) or Brilliant-blue G, which antagonize $P2X_7$, enhanced microglial phagocytosis and facilitated CNS tissue repair (37). The systemic administration of OxATP has been also proposed for the treatment of SCI, in which prolonged release of ATP was toxic for neurons (4). In the same model, administration of Brilliant-blue G reduced local activation of astrocytes and microglia and neutrophil infiltration. These observations suggest that blockade of $P2X_7$ not only protected spinal cord neurons from purinergic excitotoxicity, but also reduced local inflammatory responses sustained by glial and blood-borne cells (38).

However, other authors have demonstrated that the in vitro stimulation of microglial $P2X_7$ also induced the production of neurotrophic factors and counteracted glutamate-induced neuronal death (39). In line with these findings, in a rat model of middle cerebral artery occlusion (MCAO), one day after injury, the i.c.v. administration of the $P2X_7$ agonist BzATP enhanced the performance of rats in the rota-rod test compared to non-treated animals. Moreover, three days after MCAO, the massive loss of MAP2, the major component of neuronal dendrites, was significantly inhibited in the ischemic core compared to untreated animals (40). Finally, administration of the $P2X_7$ antagonist OxATP led to progressive neuronal death, to the formation of a thinner glial scar and to diminished functional recovery. These results are not necessarily in contradiction; neuroinflammation itself is a complex mechanism, and phagocytosis could not be labelled as a positive or a negative effect of microglial activation. It is possible that ATP initially induces neuroprotection via activation of microglial $P2X_7$, and that this may be later turned into a detrimental effect. It would be very interesting to assess if these opposite action of $P2X_7$ may be related to the acquisition of different microglial M1 and M2 phenotypes. In a recent study, overexpression of $P2X_7$ receptor, in the absence of pathological insults, was sufficient to drive the activation and proliferation of microglia in rat primary hippocampal cultures (14). It has been also suggested that $P2X_7$ overexpression is causative of microglial activation after injury, rather than a consequence (14). This hypothesis is in line with the proposed role of ATP as a danger signal. In this context, $P2X_7$ may act as a "sensor" and as an early player in initiating the downstream cascade, whereas many other different receptors and mechanisms contribute to the final outcome. However, it has to be mentioned that both BzATP, OxATP and Brilliant-blue G are non-selective agonist and antagonists at $P2X_7$, respectively (41-43). Moreover, OxATP also demonstrated off-target effects, promoting anti-inflammatory actions independent of the expression of any P2 receptors (44). For this reason, effects other than stimulation/blockade of $P2X_7$ cannot be completely ruled out.

Besides P2X receptors, G-protein coupled P2Y receptors are also crucially involved in microglial function. UDP is a potent inductor of phagocytosis through the $P2Y_6$ receptor (45). Exposure of microglial cells to 2-methylthioADP (2-MeSADP) (a $P2Y_1$ agonist), ADP (a $P2Y_{12}$ agonist) and UTP ($P2Y_{2/4}$ agonist) also increased microglial phagocytotic activity when compared to control (37). The $P2Y_{12}$ receptor seems to be particularly important, since it has been also involved in microglial chemotaxis (46), and in the phenotypic changes associated to microglial activation. Microglial cultures obtained from wild-type mice showed a robust membrane ruffling after in vitro exposure to ADP or ATP, whereas this was not observed in microglia from $P2Y_{12}$ knock-out mice. Adenine nucleotides were also able to induce a strong chemotaxis of microglia and process extension, both in culture and *in vivo*, as demonstrated by two-photon microscopy (47). After injury, induced with focal laser ablation or ATP injection, microglia showed immediate responses characterized by the extension of branches toward the site of damage. The same experiment performed on microglia lacking $P2Y_{12}$ showed a significant delay but did not abolish the ability to respond to local damage, suggesting that other signalling mechanisms compensate the loss of $P2Y_{12}$. Resting microglia exhibit a basal nucleotide-induced homeostatic activity, even in the absence of an injury, not mediated by $P2Y_{12}$. This receptor is expressed by resting microglia and is progressively down-regulated during microglial activation. Interestingly, the transcript for $P2Y_{12}$ was not observed in peripheral macrophages, suggesting that this receptor may be a molecular marker for differentiating between these two closely related cell types (47).

Much fewer information is available regarding the role of specific P2 receptors in the macrophages that infiltrate the diseased brain during inflammation. In experimental autoimmune encephalomyelitis (EAE), infiltrating macrophages within the local inflammatory lesion were prominently $P2X_4$-positive. This receptor was strongly up-regulated in macrophages in perivascular areas and, to a lesser extent, in brain and spinal parenchyma (48). The number of $P2X_4$-positive macrophages perfectly correlated with disease progression, starting early after EAE induction at a pre-symptomatic stage with a peak 14 days. Infiltrating macrophages are responsible for both the production of several harmful factors for myelin and for the phagocytosis of myelin sheaths. Thus, $P2X_4$ has been proposed as a marker of EAE progression and may also

represent a possible target for the therapy of autoimmune diseases such as multiple sclerosis.

Monocyte-derived macrophages express various purinergic receptors such as $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$ (49). Although their pathophysiological roles in inflammation are well characterized (50), brain macrophages are virtually indistinguishable from phagocytic microglia, and for this reason they are often put together and studied as "microglia/macrophages". However, as previously mentioned in Section 3.1, macrophages are likely to play a unique role in CNS repair, since infiltrating immune cells seem to follow different patterns/kinetics of activation compared to resident cells (25).

In this respect, notable advancements can derive from the use of fluorescence reporter animals where either residential microglia or blood-borne macrophages are selectively labelled, and whose migration and final destiny in the CNS can be followed overtime after various kinds of injury. A transgenic mouse line in which microglia is fluorescently labelled through the expression of the GFP under the fractalkine promoter has been recently become available ($Cx3cr1^{eGFP/+}$ mice). Two-photon imaging on these transgenic mice has demonstrated that, in a model of Alzheimer's disease, this chemokine is critical for microglia recruitment, leading to progressive neuronal cell loss (51). $Cx3cr1^{eGFP/+}$ mice have been also used to study the involvement of microglial purinergic signalling in a model of status epilepticus induced by intraperitoneal kainate injections. In vivo electrophysiological recordings have demonstrated that activation of microglia in situ involves an increase of all types of purinergic responses, including those mediated by $P2X_7$, $P2Y_6$ and $P2Y_{12}$, with the appearance of new voltage-activated potassium currents (52). By applying two-photon microscopy to these mice, it will be also possible to draw a well-defined time course of microglia/macrophages morphological changes, rate of proliferation, phagocytic activity and motility after acute ischemic or traumatic damage, and dissect the role of the purinergic system in these changes.

4. PURINERGIC REGULATION OF INFLAMMATORY ASTROGLIOSIS AND SCAR FORMATION: AN EMERGING BEHAVIOUR OF ASTROCYTES AS MULTIPOTENT STEM-LIKE CELLS

It has been known for a long time that astrocytes are key actors in the long-term inflammatory response to acute and chronic injuries, undergoing reactive astrogliosis (53, 54). During this process, these cells become hypertrophic, elongate glial fibrillary acidic protein (GFAP)-positive processes and continue to divide and migrate to form the glial scar. Among many mediators contributing to astroglial cell activation, ATP and other nucleotides released from the cytoplasm of "stressed" cells, are able to trigger and sustain astrogliosis both in vivo (55) and in vitro, as demonstrated in primary rat cortical astrocytes (56, 57).

In the past years many reports have described the co-expression of multiple P2X and P2Y receptors in astrocytes (58), but the specific contribution of each single P2 receptor to astrogliosis has not been clearly defined yet, in part due to the lack of selective agonists and antagonists (58). Recent data reported that $P2Y_2$ stimulation can promote astrocyte migration and cell proliferation by mediating cytoskeletal rearrangements through a direct interaction with integrins (59) and by the trans-activation of growth factors receptors respectively (60-62). Moreover, via $P2Y_4$ receptors, ATP and UTP can induce astrocyte expression and release of thrombospondin-1, a large multidomain matrix glycoprotein participating in cell-to-cell interactions such as those occurring during repair (63). Finally, activation of $P2Y_1$ receptors *in vivo* enhances astrogliosis after traumatic injury by the involvement of the phosphoinositide 3 kinase (PI3-K/Akt) signaling pathway (64). Considering the specific roles played by some P2Y receptor subtypes in astroglial reactivity, it is likely that the interaction between different P2 receptors participates in the signalling cascade culminating in reactive astrogliosis (see also below). Among P2X receptors, the $P2X_7$ subtype deserves special attention due to its multifunctional roles in the regulation of astrocytic reactivity. During pathological conditions, this receptor is activated by high concentrations of ATP and through pore formation modulates both the synthesis of cytokines (e.g. IL-1 beta; TNF-alpha, (65-67)), gliotrasmitters such as glutamate, GABA, ATP and other purines (68), and the expression of other purinergic receptors (e.g.$P2Y_2$, (69)). Furthermore, stimulation of $P2X_7$ increases the production of lipid inflammatory mediators such as cysteinyl-leukotrienes (70), decreases glutamate uptake via Na^+-dependent transporter and reduces the expression and activity of glutamine synthase (71). All these data suggest that astrocytic $P2X_7$ might be considered as a potential therapeutic target to modulate neuroinflammation.

The final outcome of reactive astroglia on neuronal cells is quite complex, with dual detrimental or beneficial effects depending on the extent and type of injury, and the timing after the initial insult (54). Major well-known detrimental features of astrogliosis are represented by the formation of scar tissue, which may inhibit neurite regrowth and circuitry remodelling, the release of pro-inflammatory cytokines (e.g. TNF-alpha), nitric oxide, arachidonic acid metabolites, and reactive oxygen species that can damage recovering neurons and enhance axon demyelination by promoting inflammation (72, 73). Moreover, reactive astrocytes can impede remyelination from oligodendrocytes precursor cells (OPCs) by increasing secretion of bone morphogenetic proteins (BMPs) that inhibits oligodendrocyte differentiation (74), see also below).

The concept that astroglial reactivity can also be neuroprotective has only recently been established (75). Reactive astrocytes secrete glucose nutrients and growth factors that give trophic and metabolic support to damaged neurons at the injury site. These molecules can contribute to the generation of an appropriate microenvironment able to trigger remodeling and repair through the recruitment and the instruction of microglia/macrophages, as reported

in Section 3.1. Moreover, the scar-forming astrocytes separate the damaged tissue from its surroundings, stimulate neurovascularization, restore the BBB and, due to their scavenging activity, regulate excessive levels of glutamate, K^+ and other ions. Interestingly, the purinergic system seems to promote beneficial properties in reactive astrocytes by increasing N-cadherin protein expression when P2X/P2Y and A3 adenosine receptors are activated. The increase in N-cadherin plays a part in stabilizing cell-to-cell contacts between astrocytes in the glial scar to help isolate damaged areas and prevent secondary cell death (76).

However, one of the most intriguing findings on the beneficial role of reactive astrocytes relates to the recent demonstration that the glial scar and its components (e.g chondroitinsulphate proteoglycan, CSPG) crucially control multiple steps of adult neurogenesis, from proliferation of neural progenitors to migration and integration into pre-existing neuronal circuits in the adult brain (77). The emerging reparative potential of astroglial cells has been strengthened by the discovery that, in some injury conditions, parenchymal astroglial subsets can also behave as plastic multipotent stem-like cells ((78), see also below). During their reaction to damage, quiescent parenchymal astrocytes change their phenotype, displaying many molecular traits of progenitor/stem cells of germinal sites (54, 78, 79). Moreover, different signals such as BMPs, Notch signaling, Sonic Hedgehog Homologues, Wnt pathway, bFGF and epidermal growth factor (EGF), known to be crucially involved in germinal cells signaling, are to some extent activated upon injury and mostly produced by reactive astrocytes, confirming the acquisition of progenitor features by these cells (80). However, while *in vivo* reactive astrocytes produce only other astrocytes, likely due to local inhibitory signals, when maintained *ex vivo,* these cells disclose their ability to self-renew and to generate all the three cell types that are present in the adult CNS, i.e., neurons, astrocytes and oligodendrocytes. This suggests that, even in the adult nervous system, multipotency is retained within the astroglia lineage and can be specifically revealed after injury when astrocytes become reactive and "dedifferentiate" to multipotent stem-like cells (54, 78). Interestingly, it has been demonstrated that also extracellular nucleotides regulate neural stem/precursor properties in vitro and in germinal territories ((81, 82), see below) suggesting that they could be potential factors for the acquisition of stem potentials in astroglial cells.

5. PURINERGIC SIGNALLING IN ADULT NEURAL PRECURSOR CELLS

As mentioned above, it has been recently demonstrated that neural progenitor cells (NPCs) from the two adult neurogenic areas (the subventricular zone, SVZ, of the lateral ventricles and the hippocampus), take part in the neuroinflammation response upon CNS injuries. A variety of insults, including ischemia and mechanical injury, stimulate NPCs from the SVZ to move towards damaged areas in an attempt to re-establish neuronal connections and to replace damaged neurons (83). Indeed, these cells constitute a heterogeneous cell population with the capability of self-renewal and differentiation into neurons, astrocytes and oligodendrocytes. Furthermore, endogenous NPCs are recruited during chronic neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis and migrate into areas of demyelination, where they differentiate into glial cells (84).

Progenitor cells have been also detected in non-neurogenic areas. Besides subsets of parenchymal astrocytes endowed with potential stem/progenitor properties (see above), another type of glia, with progenitor properties, has been recently identified based on expression of the membrane chondroitin sulphate proteoglycan NG2 in various CNS regions in addition to typical neurogenic niches (80, 85). NG2 positive cells ($NG2^+$; also known as polydendrocytes) are oligodendrocyte precursor cells (OPCs) that generate oligodendrocytes in the developing and mature CNS and serve as the primary source of remyelinating cells in demyelinated lesions (85). Recent data indicate that these cells also form synapses and generate action potentials (86). Moreover, under specific conditions they can also give rise to neurons and astrocytes (85), indicating their multipotential capability, even if this concept is currently a highly debated topic (see also below).

High relevant is that $NG2^+$ cells also react to many pathological conditions by active proliferation, hypertrophy, NG2 upregulation and contribution to glial scar formation, suggesting that they actively participate in the neuroinflammatory events of the injured nervous system. Recently, it has been shown that only those lesions that open the BBB can cause an OPC reaction. In particular, this reaction is triggered by platelets, macrophages and inflammation-associated cytokines (87), suggesting a direct cross-talk between infiltrating activated blood-borne cells and adult parenchymal precursors. Interestingly, some studies have showed that, in response to injury, in addition to oligodendrocytes, $NG2^+$ progenitors may generate astrocytes (88) and their choice versus the astrocytic lineage is associated with translocation of the oligodendrocyte-specific transcription factor olig2 from the nucleus to the cytoplasm (89). BMPs, known to repress oligodendrogenesis while enhancing the development of astrocytes (90) have been recently implicated in the lineage choice of adult $NG2^+$ cells via an ID4, olig1/olig2-mediated interaction (74). In particular, BMP signaling upregulates the expression of ID4, which, in turn, blocks the translocation of olig1/2 to the nucleus, thus inhibiting oligodendrocyte differentiation and favouring astrocyte formation. These data suggest that BMP signaling may play a crucial role in the regulation of remyelination in the adult CNS following traumatic or demyelinating injuries (74).

Of note, the P2Y-like receptor GPR17, which specifically responds to both uracil nucleotides and cysteinyl-leukotrienes (91), has been found to be expressed by $NG2^+$ OPCs that take part in the remodelling and repair after ischemia. GPR17 was found to be up-regulated in rodent models of demyelination, as well as in multiple sclerosis (MS) patients (92, 93). Moreover, *in vitro* data showed that activation of GPR17 by its endogenous

agonists can promote the differentiation of OPCs to mature oligodendrocytes (92). This suggests that, besides being an intrinsic regulator of oligodendrogenesis, as it has been recently proposed (93), GPR17 can also be extrinsically regulated by endogenous ligands that accumulate in the extracellular milieu both under physiological and pathological conditions (Fumagalli et al., under revision). In line with this hypothesis, both cysteinyl-leukotrienes and nucleotides accumulate at the site of injury in the damaged CNS (31, 94) suggesting that they may activate repair by specifically stimulating GPR17. On this basis, the pharmacological modulation of GPR17 on adult NPCs may foster the brain's response to damage, thus favouring functional recovery.

It is however important to highlight that neuronal or glial replacement from endogenous progenitor cells in degenerative diseases is very limited, likely due local unfavourable inflammatory environment (see also below). Neural progenitor cell-based therapy has been studied as a promising tool for inducing regeneration of damaged brain tissue and it has been developed in many experimental models of neurological disorders such as multiple sclerosis, Huntington's disease, stroke injury and Alzheimer's disease (95, 96). However, in all cases, low terminal differentiation of transplanted NPCs has been observed, together with the propensity of these cells of maintaining an undifferentiated phenotype within the host tissue. In line with these results, despite demonstrated mobilization of endogenous NPCs from adult neurogenic niches towards damaged brain areas (97), migrating precursor cells do not seem to be able to change their intrinsic differentiation potential (98) suggesting that only a minimal percentage of these cells can effectively substitute damaged or dying neurons. These data suggest that stem cells may confer neuroprotection by a "bystander" mechanism that is alternative to cell replacement (95). This includes the *in vivo* enhancement of neurotrophic factors (e.g., nerve growth factor, NGF, BDNF, ciliary neurotrophic factor, CNTF and GDNF (99-101) which increase survival of endogenous NPCs, and/or inhibit inflammatory and neurotoxic factors (102, 103). In stroke, the pro-survival and remodelling effects mediated by NPCs have been associated to scavenging of inflammatory molecules, toxic metabolites and free radicals. Additionally, adult NPCs have immunomodulatory actions, determining down-regulation of inflammatory T cells and macrophages in the inflamed area (104).

It is in general believed that an uncontrolled local inflammatory response exacerbates neuronal loss and blocks repair processes, (105), whereas a local immune response that is properly controlled can support survival and promote tissue remodeling after injury (106, 107). Such a control is strictly orchestrated by a timely and specific cross-talk between local CNS-resident cells reacting to injury (astrocytes, microglia and activated NPCs) and inflammatory blood-derived cells. This concept is supported by the observation that remyelination requires T cells and is indeed impaired in demyelinated mice that are devoid of macrophages or leukocyte-derived proteases such as the matrix metalloproteinases (MMPs) (108, 109). Experiments in models of CNS injury have shown that the neuroprotective effect of CNS-specific T cells is mediated by modulation of microglia and macrophages at the site of injury (28, 110). Activation of these cells has been associated to the local production of chemo-attractant proteins, such as monocyte chemo-attractant protein-1 (MCP-1). The latter is known to be necessary for NPC migration, because NPCs lacking chemokine (C-C motif) receptor 2 (CCR2, the receptor for MCP-1) exhibited reduced migration towards the site of IFN-gamma/TNF-alpha injection (111). Interactions between $NG2^+$ glial progenitors and microglia/macrophages have been also recently reported in an experimental model of spinal cord contusion (112). On this basis, unveiling the timing and molecular factors involved in the interaction between NPCs and infiltrating blood circulating cells may help identifying crucial signals providing a permissive enviroment for brain repair by endogenous NPCs.

In addition to ectonucleotidases and to GPR17, other functional P2X and P2Y receptors, have been found to be expressed by adult NPCs. Activation of $P2Y_1$ and $P2Y_2$ receptor subtypes in SVZ-derived adult NPCs triggers cytosolic calcium increases and augments cell proliferation in the presence of growth factors, supporting the hypothesis that extracellular nucleotides participate in the neurogenesis occurring during inflammatory CNS disorders (113). Interestingly, $P2X_7$ receptor has been recently implicated in NPCs death induced by extracellular ATP. Thus, excessively high levels of extracellular ATP in inflammatory CNS injury may limit endogenous repair or the efficiency of NPC-based cell therapy by promoting progenitor cell death via $P2X_7$ receptor subtype activation (114). Moreover, ATP acts as an antagonist at the P2Y-like receptor GPR17 (115), thus preventing the beneficial trophic/differentiating effects that are normally mediated by this receptor on NPCs.

6. TARGETING SPECIFIC PURINOCEPTORS IN NEUROINFLAMMATORY DISEASES

Being P2 receptors expressed on all CNS cells including the cell types that are directly involved in inflammatory and immune reactions, it is not surprising that dysfunctions of these receptors have been found in several CNS inflammatory diseases, including both acute ischemia/hypoxia, traumatic/mechanical injury to the brain, spinal cord or peripheral nerves, and chronic CNS pathologies, such as multiple sclerosis, Parkinson's and Alzheimer's disease. Many of these findings have been already discussed in the above sections and have been nevertheless summarized in a previous review (3). A summary table reporting changes of specific P2 receptor subtypes in some experimental models of disease is enclosed (Table 1). A schematic model of the events following an acute injury, such as brain ischemia, has been summarized in Figure 2.

In some cases, a causative role for some receptor changes has been shown by the demonstration that pharmacological treatments with receptor antagonists could ameliorate brain damage in various *in vitro* and *in vivo*

Table 1. Changes of specific P2 receptor subtypes in some experimental models of CNS diseases

Disease model	Receptor subtype	Main functions	References
Cerebral ischemia	P2Y-like GPR17	Immature, proliferating GPR17-expressing OPCs accumulate around ischemic brain damage: possible role in post-injury repair	(92)
Cerebral ischemia	$P2X_7$	Following ischemia, the $P2X_7$ receptor is up-regulated on neurons and glial cells in rat cerebral cortex. Little effect on ischemic cell death in knockout mice and after treatment with $P2X_7$ antagonist KN62	(116-118)
Experimental allergic encephalopathy (EAE, a model of multiple sclerosis)	$P2X_7$	Mice deficient in $P2X_7$ receptors are more susceptible to EAE. The $P2X_7$ antagonist BBG has been shown to exhibit beneficial effects	(119, 120)
EAE	$P2X_4$	The number of $P2X_4$-positive infiltrating macrophages perfectly correlated with disease progression: possible marker for EAE progression	(48)
EAE	P2Y-like GPR17	GPR17 is up-regulated in demyelinating lesions in the CNS, as well as in human multiple sclerosis plaques	(93)
Alzheimer's disease	$P2X_7$	The $P2X_7$ receptor is up-regulated in the brain of patients and in animal models of disease	(121, 122)
Alzheimer's disease	$P2Y_1$, $P2Y_2$	$P2Y_1$ and $P2Y_2$ may exert protective effects	(123, 124)
Huntington's disease (HD)	$P2X_7$	*In vivo* administration of the $P2X_7$-antagonist Brilliant-Blue G to HD mice prevented neuronal apoptosis and attenuated motor-coordination deficits.	(125)
Parkinson's disease	$P2X_7$	$P2X_7$ might contribute to cell degeneration	(126, 127)

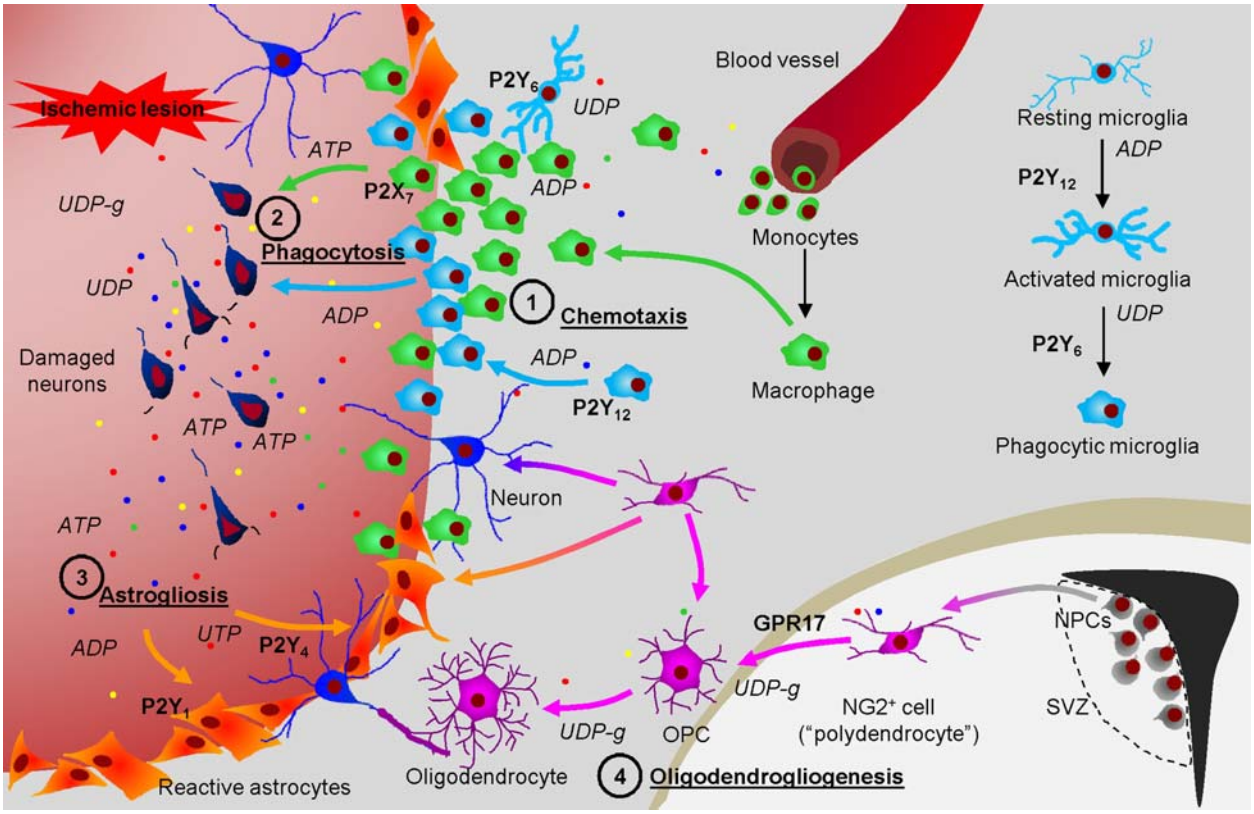

Figure 2. Brain ischemia and purinergic signalling. Extracellular nucleotides (colored circles), which are massively released by damaged neurons at the site of injury act as "danger signals" to activate response and trigger protective events in response to an ischemic injury. (1) Recruitment of microglia and monocyte-derived macrophages to the site of injury (chemotaxis), and (2) phagocytosis of cell debris. (3) Astrocytes accumulate at the borders of the lesion, become reactive and start the formation of a glial scar (astrogliosis). Some of these cells also re-acquire stem cell-like properties, and undergo a sort of "de-differentiation" to multipotent precursor cells, which may generate new neurons and new glial cells. (4) At later times, parenchymal OPCs or neural precursor cells (NPCs) coming from the SVZ differentiate into either neurons or mature oligodendrocytes (oligodendrogliogenesis), that, by producing new myelin sheaths around damaged axons, favour the repair of neuronal circuitries and the re-establishment of cell-to-cell communication. A peculiar role seems to be played by NG2-positive OPCs (polydendrocytes) that, besides generating functional oligodendroglia, can also generate new neurons and new astrocytes under some specific conditions. Activation of both parenchymal and SVZ NPCs seems to require specific contacts with infiltrating blood-borne cells (see text for details). Purinergic signalling and specific purinoceptors, as indicated, have been involved in several of the events described above.

neurodegeneration models. This supports the view that, when released in excessive amount and/or for prolonged periods, endogenous purinergic molecules can contribute to neurodegeneration by either overstimulating P2 receptors on neurons or, indirectly, by triggering release of proinflammatory cytokines from astrocytes or microglia. However, in most cases, the reasons at the basis of the detected P2 receptor dysfunction and their pathophysiological significance are unknown. It is unclear whether altered receptor function just represents an epi-phenomenon consequent to neuronal damage and dysfunction, or whether it reflects an attempt to compensate damage and start repair. Nor is known whether changes of the purinergic system are pivotal to disease onset, and what is their role in the associated functional deficit. Future studies aimed at dissecting the precise meaning of these receptor changes will be of great help in addressing novel purine-based anti-inflammatory strategies.

7. CONCLUSIONS

Neuroinflammation is a complex phenomenon that initiates as a defense and repair mechanism that can, however, be turned into a detrimental event under dysregulated conditions. Excessive or dysregulated inflammation sets the basis for and contributes to neurodegenerative diseases. Recent research has succeeded in identifying the specific cells and soluble factors involved in these events. However, much remains to be known regarding the times at which any given cell is activated, their different phenotypes at distinct stages of inflammation and how cell responses and interactions are timely orchestrated by inflammatory mediators. In this scenario, purinergic molecules have emerged as both alerting danger signals that initiate inflammation and as key players in tuning inflammatory responses at later stages. It is therefore anticipated that the exact elucidation of purinergic signalling in distinct cell types and at distinct stages of inflammation may help identifying new pharmacological strategies to implement beneficial and turn down detrimental inflammation in neurodegenerative diseases.

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Abbreviations: BMP: bone morphogenetic protein; IL: interleukin; LPS: lipopolysaccharide; IFN: interferon; TNF: tumor necrosis factor; NPCs: neural precursor cells; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; GDNF: glial-derived neurotrophic factor; BBB: blood-brain-barrier; CNS: central nervous system; CSPG: chondroitinsulphate proteoglycan; bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; IGF: insulin like growth factor, TGF: transforming growth factor, HGF: hepatic growth

factor; SVZ: subventricular zone, OPCs: oligodendrocyte precursor cells; VEGF: vascular endothelial growth factor; SCI: spinal cord injury; GFP: green fluorescent protein; Arg1: arginase 1; iNOS: inducible nitric oxide synthase; BzATP: benzoyl- benzoyl-ATP; OxATP: oxidized ATP; 2-MeSADP: 2-methylthioADP; MCAO: middle cerebral artery occlusion; EAE: experimental autoimmune encephalomyelitis; GFAP: glial fibrillary acidic protein; MS: Multiple Sclerosis; MMPs: matrix metalloproteinases; MCP-1: monocyte chemo-attractant protein-1

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