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A Role for Potassium Permeability in the Recognition, Clearance, and Anti-inflammatory Effects of Apoptotic Cells

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Abstract The benefits of programmed cell death by apoptosis are the safe and efficient clearance of damaged, infected, or surplus cells, primarily mediated by tissue-resident macrophages or tissue-infiltrating blood monocytes that differentiate into macrophages. Microglial cells are macrophages of the brain parenchyma, important immune surveillance cells that respond to various injuries and diseases of the brain. It is often stated that how a macrophage interacts with an apoptotic cell defines subsequent inflammatory responses, i.e., will engulfment be beneficial or detrimental for tissue repair, regeneration, and immunity. Our focus has been to better understand how macrophages discriminate between living and dying cells. Following our initial findings with platelet endothelial cell adhesion molecule (PECAM)-1, our studies have revealed a key role for potassium ion permeability in regulating integrin-dependent binding of apoptotic cells by macrophages and their subsequent response to proinflammatory stimuli. Specifically, apoptotic cells represent a depolarizing stimulus for macrophages where PECAM-1-mediated cell–cell interactions delay subsequent membrane repolarization. It is salient that potassium leak represents an early feature of cells destined to die by apoptosis that could trigger depolarization of macrophages that lie in close apposition. We speculate that how a tissue-resident macrophage responds to strong depolarizing stimuli has wider implications for inflammation and autoimmunity.

Keyword Apoptosis · Phagocytosis · Potassium permeability · Voltage-gated potassium channels · Immune regulation

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The Importance of Cell Death in the Phagocytic Removal of Effete Cells

Apoptosis, a physiological form of programmed cell death, is often invoked as a prerequisite for the phagocytic removal of unwanted, damaged, or infected cells by the stroma of complex metazoans [1–3]. Although tissue-resident macrophages such as microglia are not the only cells capable of engulfing apoptotic cells, they are considered the more efficient phagocyte [4]. Apoptosis is often defined as a caspase-dependent cell death program leading to characteristic morphological and phenotypic changes that include cell shrinkage, nuclear condensation, and DNA fragmentation [5–7]. Caspases are a family of cysteinyl proteases that cleave to the carboxy-side of aspartyl residues in key structural, signaling, and anabolic proteins that can lead to either their activation or deactivation and the ordered dismantlement of a dying cell [8, 9]. Apoptosis and caspase-dependent events are further implicated in revealing novel or altered cell surface ligands that mark a dying cell for phagocytic removal [1–3]. The purpose of this review, however, is not to list or describe the various

receptor–ligand interactions implicated in the recognition and clearance of apoptotic cells [1, 10, 11]. Instead, we seek to explore an alternative hypothesis that events preceding caspase activation may better mark a cell as being ripe for removal [12]. We propose that this initiating event, which can be uncoupled from the execution of an apoptotic cell death program, is the sustained loss of intracellular potassium that serves to depolarize cells that lie in close proximity. While potassium leak from dying cells will undoubtedly influence the electrical properties of neurons and the functional responsiveness of both glia and astrocytes within the brain, our focus is the effect increased extracellular potassium will have on macrophage, or microglial, behavior. We further speculate that how a tissue-resident macrophage responds to membrane depolarizing stimuli will also influence their proinflammatory and anti-inflammatory responses with potential consequences for the integrity and function of neuronal networks.

The Role of Ion Fluxes in Maintaining Cell Viability

A useful indicator of cell viability is the ability of a cell to maintain ionic gradients across lipid bilayers. Establishment of the various ion gradients in mammalian cells is primarily driven by the Na^+/K^+-ATPase pump which realizes the energy of ATP hydrolysis to expel three sodium ions for every two potassium ions sequestered [13]. As a consequence, cells typically have an intracellular Na^+ concentration of around 12 mM while accumulating K^+ to a concentration of 155 mM. In contrast, the extracellular milieu may reach a concentration as high as 145 mM for Na^+ and as low as 4 mM for K^+. The overall viability of the cell is very much dependent on maintaining these gradients that can then be coupled to the movement of essential ions and metabolites via a complex interplay of cotransporters and exchangers.

The movement of essential ions and metabolites across a membrane also has important implications for both osmotic, or turgor, pressure and a transmembrane potential. Osmotic pressure derives from the solvation sphere that surrounds all water-soluble molecules and is a pressure that lipoprotein membranes are unable to bear without undergoing rupture, a feature of cell death by necrosis [13]. A transmembrane potential occurs where there is selective ion permeability across that membrane [14, 15]. Under "resting" conditions, the membrane potential (ΔVrest) of all cells is determined predominantly by K^+ permeability. It is relevant that dynamic changes in membrane potential remain the most energy-efficient way for both excitable and nonexcitable cells to spatiotemporally regulate intracellular signaling events [16].

Cell Shrinkage and Potassium Flux in Dying Cells

Cell shrinkage associated with cell death has recently been termed apoptotic volume decrease, or AVD, to distinguish it from dynamic changes that occur in viable cells exposed to hypertonic or hypotonic conditions (reviewed in [17]). An essential feature of cell shrinkage in AVD is the simultaneous leakage of K^+, Na^+, and Cl^- from a dying cell that dictates the concomitant loss of water. It has been shown, however, that it is the movement of the ions, rather than water, which is prerequisite for the initiation and execution of the apoptotic cell death program [18]. Specifically, studies suggest that the loss of intracellular K^+ is ubiquitous and a prerequisite for the activation of caspases, where physiological levels of intracellular K^+ are inhibitory [17–26].

Apoptosis, a Default Program in the Absence of Phagocytosis

The suggestion that cell shrinkage and K^+ efflux can precede apoptosis is a semantic argument based on a definition in which apoptosis requires caspase activation. Nevertheless, there are cell death programs that are caspase-independent, where caspase activation is blocked [27] or nonapoptotic, such as autophagy [28]. Regardless, it remains our contention that early K^+ efflux may identify effete or damaged cells as being ready for phagocytic removal before they have engaged cell death machinery. There are two important corollaries worth considering from the perspective of apoptosis. The first is that caspase-dependent events associated with apoptosis may not necessarily be the trigger for the recognition and clearance of effete cells. Second, dynamic changes in intercellular ion gradients may influence receptor–ligand interactions that promote the swift recognition and removal of the dying cell [15]. We speculate that what distinguishes healthy from dying cells is that dying cells have lost their ability to re-establish ion gradients while undergoing an irreversible AVD.

In support and given the central importance of *Caenorhabditis elegans* in informing us as to the mechanism of both cell death and phagocytosis [3], it seems a peculiar oversight that we often ignore the observation that, in development, surplus cells are removed before they exhibit any overt morphological evidence of having engaged a cell death program [29]. And although it is difficult to extrapolate in vitro findings to what actually happens in vivo, there is direct and inferential evidence from in vitro studies that cell shrinkage and membrane depolarization can precede caspase activation by anywhere up to 1–2 h [30–34]. Furthermore, the caspase-mediated execution phase can last for several hours [35, 36]. Given that

engulfment is a rapid process that typically occurs on the order of 2–5 min, including apoptotic cells [37], this suggests an alternative hypothesis that apoptosis may be a default program in the absence of phagocytosis [12]. In humans, this would be particularly advantageous in pathological settings where phagocytic capacity may be overloaded. Under these circumstances, apoptosis may be better viewed as protecting neighboring tissue from the intracellular release of "danger" signals and the inadvertent activation of a proinflammatory response while also promoting scavenger backup clearance mechanisms, especially by tissue-resident macrophages [4, 38].

The Sensing and Binding of Effete Cells by Tissue-Resident Macrophages

Macrophages will respond to an increase in extracellular K^+ by typically activating voltage-gated K^+ channels in an attempt to re-establish a resting membrane potential [39]. The speed with which the cell repolarizes, however, will critically depend on both the repertoire of voltage-gated potassium channels expressed and their respective gating properties.

We recently reported that the voltage-gated potassium channel Kv11.1 (KCNH2) was a downstream effector of PECAM-1-dependent signaling in regulating the binding of apoptotic leukocytes by an erythroleukemic K562 cell line [39]. We extended those studies to show that not only do human monocyte-derived macrophages (hMDMΦ) express Kv11.1, but dofetilide, a selective inhibitor of Kv11.1, could also augment the binding of apoptotic leukocytes [39]. Our studies suggested that Kv11.1 functioned to help re-establish a resting membrane potential following macrophage membrane depolarization. Depolarization occurred when macrophages came into contact with apoptotic cells, and repolarization was delayed when macrophage PECAM-1 was ligated by apoptotic cell PECAM-1. Although we have not as yet determined the magnitude of the depolarizing effect on macrophages, apoptotic cells were found to depolarize K562 cells by as much as 22.6±2.4 mV. Thus, inhibiting macrophage membrane repolarization influenced receptor–ligand interactions and promoted a more efficient binding of the apoptotic cell [15, 39].

Our contention that macrophages express Kv11.1 is supported by quantitative polymerase chain reaction (PCR), Western blot with three different polyclonal antibodies, and characteristic K_i values for known inhibitors as determined by competitive displacement binding studies using homologous and heterologous ligands (Table 1; Fig. 1). Although these data are consistent with each other, indicating that the cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ are best at inducing Kv11.1 expression, we have experienced our own difficulty in isolating a Kv11.1 current in hMDMΦ by patch-clamp techniques. Detection of such currents is exceedingly difficult due to (1) the unusual gating properties of Kv11.1 where under normal extracellular K^+ concentrations there is rapid inactivation following cellular depolarization and (2) high electrical resistance which makes them susceptible to leak-induced depolarization. Thus, revelation of an endogenous Kv11.1 current in a macrophage remains challenging except where such currents have been identified in microglia [40, 41].

Table 1 The effect of macrophage phenotype on the relative expression of selected potassium channels

Potassium channel	Blood monocytes	d5 hMDMΦ			
		M-CSF	M-CSF + IL-4	GM-CSF	GM-CSF + IFN-γ
KCNA3 (Kv1.3)	18,100±3,500	4,700±2,300	3,600±1,200	3,300±1,800	3,400±2,200
KCNN1 (KCa2.1)	0	4±5	10±6	2±2	1±3
KCNMA1 (KCa1.1)	18±7	7,200±300	16,200±6,400	1,100±1,500	1,300±1,400
KCNH1 (Kv10.1)	0	0	0	1	1
KCNH2 (Kv11.1)	4±2	24±3	97±88	303±64	971±107
KCNH6 (Kv11.2)	0	1	2	2	2
KCNQ1 (KvLQT1)	1,000	1,000	1,000	1,000	1,000

Potassium channel expression as assessed by quantitative reverse transcription PCR (qPCR) of human monocyte-derived macrophages (hMDMΦ) cultured in the presence of M-CSF (40 ng/ml) or GM-CSF (40 ng/ml) for 4 days before adding either IFN-γ (10 ng/ml) or IL-4 (10 ng/ml) for a further 24 h. Results are corrected for 18S and expressed relative to KCNQ1, arbitrarily set at 1,000, which remained consistent across treatments ($\Delta Ct = 16.4 \pm 0.2$; $n \geq 3$ for each treatment). Template cDNA for RT-PCR was generated using the Transcriptor First-Strand cDNA Synthesis kit (Roche). Standard PCR reactions were carried out for 35 cycles using Promega's PCR master mix (M7502) with primers at a final concentration of 20 nM each. qPCR was carried out using Applied Biosystems TaqMan reaction master mix on an Applied Biosystems ABI 7900 machine with the following TaqMan primer sets: KCNA3 (Hs00704943_s1), KCNH1 (Hs00608142_m1), KCNH2 (Hs00542479_g1), KCNH6 (Hs00229215_m1), KCNMA1 (Hs001119498_m1), KCNN1 (Hs00158457_m1), and KCNQ1 (Hs 00165003_m1)

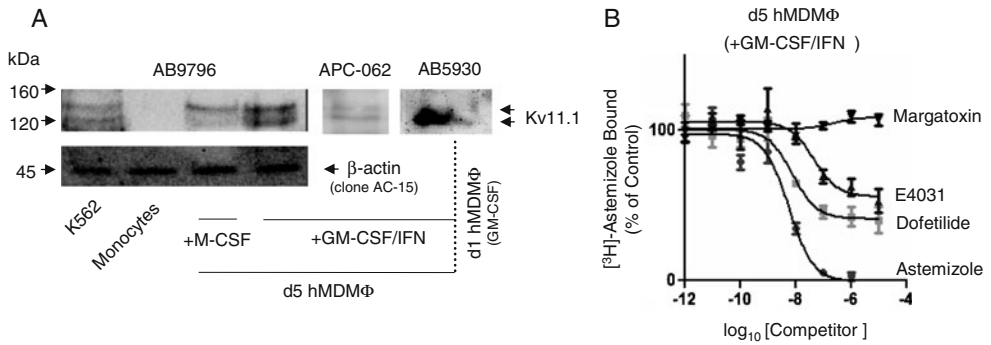

Fig. 1 Human monocyte-derived macrophages express Kv11.1. **a** Peripheral blood mononuclear cells were isolated from freshly drawn venous blood following dextran sedimentation and Percoll gradients [4, 52]. Monocytes were further enriched following adhesion to 48-well culture plates with unbound lymphocytes removed by repeated washing of the adherent monolayer. Western blot analyses were performed with an equivalent number of cells following Triton-X100 extraction of a hypotonic lysed and washed cell pellet. Separate transfer blots were probed with one of three pAbs: AB5930 (Chemicon), APC-062 (Alomone), and AB9796 (Chemicon). K562 erythroleukemic cells are a positive control for Kv11.1 expression. **b** Competitive displacement of ^{3}H-astemizole (1.2 nM) bound by hMDMΦ with unlabeled astemizole, dofetilide, E4031, and margatoxin. Binding studies were performed with intact cells in PBS containing 0.1% BSA and 50 mM KCl. K_i values for astemizole (4.8 nM), dofetilide (6.4 nM), and E4031 (37.6 nM) are consistent with selective inhibition of Kv11.1 [67]. Margatoxin, a selective inhibitor of Kv1.3, is not known to inhibit Kv11.1. Specific binding for astemizole was estimated at 15 fmol per million cells in which 500,000 cells were used per assay. Freshly isolated blood monocytes were cultured with GM-CSF and IFN-γ as in Table 1. No specific binding of ^{3}H-astemizole was apparent for monocytes differentiated in the presence of M-CSF. Each point represents the average of two different blood donors, each performed in triplicate

Why Do Some Macrophages Phagocytose Better than Others

The differential expression of Kv11.1 by monocytes differentiated in the presence of M-CSF vs. GM-CSF highlights an important facet of macrophage cell biology. The M1–M2 paradigm used to describe classically and alternatively activated macrophages is a convenient conceptual framework to stereotype macrophages as being either proinflammatory or anti-inflammatory [42–44] (Fig. 2). The main proponents for classifying macrophages thus have been Siamon Gordon and Alberto Mantovani, who both recognize that this is an oversimplification of a functional continuum. Classically activated M1 macrophages (M1Φ) arise following exposure of mononuclear phagocytes to IFN-γ, either alone or in combination with the bacterial endotoxin lipopolysaccharide (LPS) or cytokines such as tumor necrosis factor (TNF)-α and GM-CSF. M1Φ are efficient at producing the proinflammatory cytokines interleukin (IL)-1β, TNF-α, and IL-6 where IL-1β/TNF-α and IL-6 represent primary and secondary response genes, respectively [45]. In contrast, M2 macrophages (M2Φ) is a generic term for mononuclear phagocytes exposed to other stimuli such as IL-4 or IL-13 and are more efficient at producing the anti-inflammatory cytokines transforming growth factor (TGF)-β and IL-10 (Fig. 2). Although M2Φ are often considered reparative in the resolution of inflammation, gene expression profiling studies suggest that they are also homeostatic, a phenotype associated with exposure to M-CSF [44].

It is therefore salient that M1Φ are characterized by a predominance of outwardly rectifying potassium currents, i.e., prefer to conduct K^+ out of a cell, especially in response to a strong depolarizing stimulus [46, 47] (Fig. 2). In contrast, M2Φ are characterized by inwardly rectifying potassium currents and thus would be predicted to exhibit a much delayed repolarization relative to M1Φ in response to strong depolarizing stimuli. Given that M2Φ are more efficient than M1Φ in the phagocytosis of apoptotic cells [48–51], it is perhaps not so surprising that PECAM-1 can

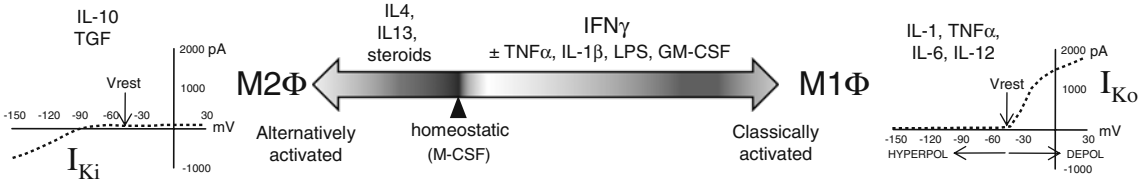

Fig. 2 The M1–M2 paradigm describes classically and alternatively activated macrophages. Classically activated M1 macrophages (M1Φ) can be defined by their expression of proinflammatory cytokines as well as outwardly rectifying currents (I_{Ko}) following depolarization. In contrast, M2 macrophages (M2Φ) are characterized by their expression of anti-inflammatory cytokines and inwardly rectifying currents (I_{Ki})

promote engulfment by delaying repolarization [39, 52]. These data suggest the possibility of a dynamic role for membrane potential in the engulfment of apoptotic cells.

The Immunomodulatory Effects of Apoptotic Cells on Macrophage Behavior

There is a general awareness that apoptotic cells can modify the proinflammatory responses of monocytes, macrophages, and immature dendritic cells, both in vitro and in vivo [1]. These effects are likely to be multifactorial since various reports have described either a requirement for engulfment, cell–cell contact, or exposure to soluble factors released by apoptotic cells [1, 53, 54]. Regardless of the precise mechanism by which apoptotic cells exert their immunomodulatory effects, macrophages respond to apoptotic cells with either increased secretion of TGF-β and IL-10 or by suppressed expression of TNF-α, IL-6, and IL-12 in response to additional activating stimuli. Thus, apoptotic cells have the apparent ability to promote the interconversion of a macrophage from an M1 to M2 phenotype [1, 55].

We have observed LPS to be a depolarizing stimulus for macrophages. It is therefore of more than a passing interest that selective inhibition of Kv11.1 with the inhibitors dofetilide, E4031, or astemizole can all inhibit IL-6 expression in response to LPS when using both tissue-resident murine peritoneal macrophages and hMDMΦ (unpublished observations). In contrast, macrophages maintained their ability to make TNF-α, IL-1β, and CXC chemokines (primary response genes). Thus, activation of Kv11.1 following LPS-induced depolarization favored, whereas inhibition suppressed, IL-6 expression.

Our studies complement those of Seydel and colleagues who demonstrate that LPS can activate the large conductance calcium- and voltage-activated K^+ channel ($K_{Ca}1.1$). Furthermore, paxilline, a selective inhibitor of $K_{Ca}1.1$, can suppress the ability of hMDMΦ to express IL-6 in response to LPS while having no effect on the secretion of the CXC chemokine IL-8 [56–58]. In contrast with dofetilide, paxilline was also found to inhibit LPS-induced TNF-α expression. This subtle difference in the ability of $K_{Ca}1.1$ and Kv11.1 to differentially regulate TNF-α and IL-6 expression without affecting CXC chemokines suggests that macrophage responses to LPS, and perhaps other TLR/IL-1R ligands, can be fine-tuned by the activity of individual voltage-gated potassium channels that would otherwise function to re-establish a resting membrane potential. Thus, pharmacological inhibition of two outward rectifiers, a feature of M1Φ, can promote an M2-like response in hMDMΦ exposed to a proinflammatory M1 stimulus.

Similar observations have also been made with microglia. Inhibition of unspecified outwardly rectifying K^+ currents with 4-aminopyridine or inhibiting Kv1.1 (KCNA1) expression and function inhibited proinflammatory cytokine expression [59, 60]. It is of interest that PGE_2 can also suppress LPS-induced activation and expression of outwardly rectifying K^+ currents in microglia with a concomitant suppression of IL-1β [60]. PGE_2 has been implicated previously in mediating the inhibitory effects of apoptotic

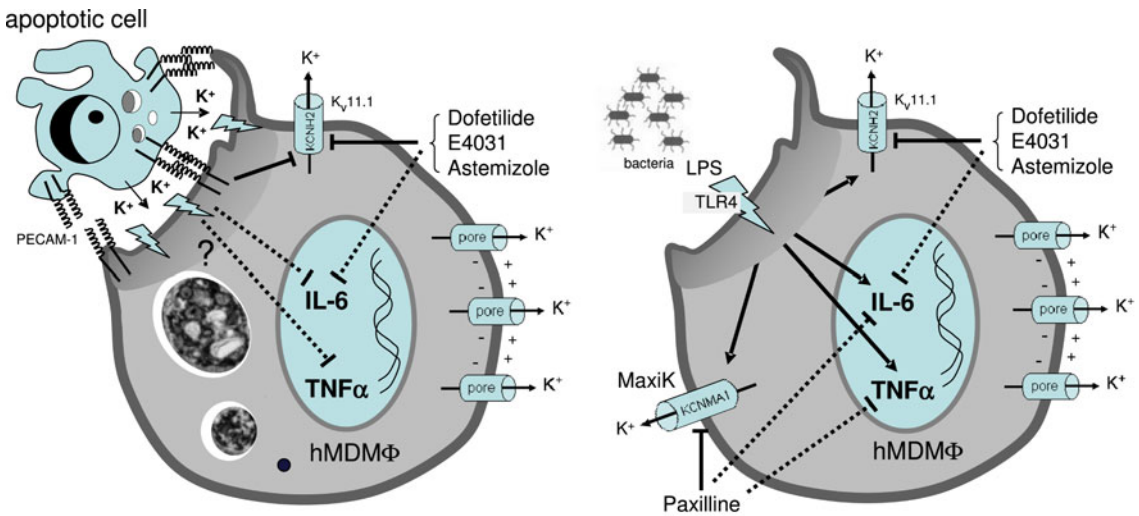

Fig. 3 Activation of outwardly rectifying voltage-gated potassium channels following depolarization supports proinflammatory cytokine expression. The binding of apoptotic cells (**a**) or LPS (**b**) results in the activation of both Kv11.1 (KCNH2) [39] and $K_{Ca}1.1$ (KCNMA1) [58]. In the case of apoptotic cells, homophilic ligation of macrophage PECAM-1 delays macrophage repolarization [39] where apoptotic cells suppress LPS-induced IL-6 expression [55]. Although inhibition of Kv11.1 with dofetilide, E4031, or astemizole and $K_{Ca}1.1$ with paxilline can all inhibit IL-6 expression, only paxilline inhibits TNF-α. The data suggest that activation of $K_{Ca}1.1$ and Kv11.1 is permissive for TNF-α and IL-6 expression, respectively

cells on LPS-induced expression of proinflammatory cytokines by hMDMΦ [61].

It is therefore tempting to speculate that inhibition of outwardly rectifying K^+ currents in macrophages that are induced following the binding of apoptotic cells may modulate intracellular signaling events to suppress proinflammatory cytokine expression (Fig. 3). Precisely how K^+ currents mediated by voltage-gated channels affect transcription is not known although MAPK pathways have been implicated previously [58, 62, 63]. A role for calcium is excluded on the basis that macrophages do not express voltage-gated calcium channels and where depolarization would reduce the driving force for calcium entry. Nevertheless, it is implicit in our argument that the anti-inflammatory effect of apoptotic cells on transcription is, perhaps, an epiphenomenon due to changes in membrane electrophysiology rather than as a consequence of specific receptor–ligand interactions. It also follows that, by inhibiting repolarization, apoptotic cells have the ability to convert the apparent electrical responses of an M1 macrophage to those of an M2, an anti-inflammatory phenotype more efficient in the engulfment of apoptotic cells [48–51].

Concluding Remarks

Microglial activation within the central nervous system is an underlying feature in the progression of a diverse range of neurodegenerative diseases and their associated neuropathies. Precisely how the stromal environment of the brain can influence the resting and activated states of microglia remains poorly defined. That said, evidence exists to suggest that how tissue-resident macrophages detect dying or damaged cells can have a profound effect on their subsequent inflammatory responses. We have argued that potassium efflux by dying cells can serve as a depolarizing stimulus for juxtaposed macrophages that then influences cell–cell binding and engulfment. Furthermore, by inhibiting the repolarizing currents of a depolarized macrophage, we suggest that apoptotic cells can further suppress the expression of proinflammatory cytokines (Fig. 3). In support, we make the point that (1) macrophages primed for proinflammatory responses predominantly express outwardly rectifying potassium currents while those primed for anti-inflammatory responses predominantly express inwardly rectifying potassium currents and (2) inhibition of outwardly rectifying potassium currents can suppress the expression of proinflammatory cytokines. Thus, macrophage phenotype, including microglia, can be defined by both their functional potassium channel and cytokine expression profile [46, 64] (Fig. 2). Whether selective inhibition of specific potassium currents can ever form the basis for treatment of inflammatory conditions remains to be seen, especially as many of the potassium channels expressed by macrophages have multifarious roles in cells other than immune cells. This is perhaps best illustrated with Kv11.1, a potassium channel better known for its involvement in the repolarization of the cardiac action potential and in spike frequency adaptation of neuroendocrine cells [65, 66].

Pertinent questions in cell death signaling: an outward perspective

- How do tissue-resident macrophages discriminate between living and dying cells?
- What chemical signals are released or presented by dying or dead cells to promote their phagocytic removal by tissue-resident macrophages?
- What is the mechanism(s) by which apoptotic cells promote an anti-inflammatory phenotype in classically activated macrophages?
- Can cation and anion loss from dying cells influence the function and behavior of the stroma, including macrophages?

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