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Review

Emerging immune and cell death mechanisms in stroke: Saponins as therapeutic candidates

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ABSTRACT

The complexity of the ischemic cascade is based on the integrated crosstalk of every cell type in the neurovascular unit. Depending on the features of the ischemic insult, several cell death mechanisms are triggered, such as apoptosis, necroptosis, ferroptosis/oxytosis, ETosis or pyroptosis, leading to reactive astrogliosis. However, emerging evidence demonstrates a dual role for the immune system in stroke pathophysiology, where it exerts both detrimental and also beneficial functions. In this review, we discuss the relevance of several cell death modalities and the dual role of the immune system in stroke pathophysiology. We also provide an overview of some emerging immunomodulatory therapeutic strategies, amongst which saponins, which are promising candidates that exert multiple pharmacological effects.

1. Ischemic stroke: a complex and still challenging disease

The term "stroke" classically characterizes a neurological deficit referred to an acute focal injury of the central nervous system (CNS) by a vascular cause. Modern neuroimaging techniques and clinical observations have shown that the duration and reversibility of brain ischemia are variable, leading to a plethora of different clinical scenarios. Therefore, the term stroke includes several subtypes of ischemic insults as well as cerebral hemorrhages (Arsava et al., 2017). In general, ischemic stroke is the consequence of a transient or permanent focal vascular occlusion in the brain and accounts for more than 68% of all subtypes of strokes worldwide (Langhorne et al., 2018). Although stroke mortality rates and mortality-to-incidence ratios are decreasing, the absolute number of people affected annually and the disability-adjusted life-years are increasing, with most of the burden in low-income and middle-income countries and a high lifetime risk in East Asia, Central and Eastern Europe (Collaborators et al., 2018). Despite the hundreds of clinical trials evaluating neuroprotective compounds for ischemic stroke, not one treatment has been shown to be effective in ischemic stroke functional recovery, and yet all current treatment strategies are based on re-establishing perfusion using pharmacological and mechanical thrombolysis (Muir et al., 2017). In this review, we provide an overview of the main mechanisms of stroke pathophysiology, with a special focus on the post-stroke cell death mechanisms and inflammatory responses. Finally, we elaborate on emerging immunomodulatory therapeutic approaches, such as saponins, as promising compounds for regulating immune and cell death homeostasis.

2. Pathomechanisms of stroke

During an ischemic stroke, the interruption of blood flow to a brain region deprives its surrounding tissue of oxygen and glucose, leading to disrupted ATP synthesis and energy failure, which impairs ion homeostasis and acid-base balance (Fig. 1) (Li et al., 2016a,b). The inhibition of

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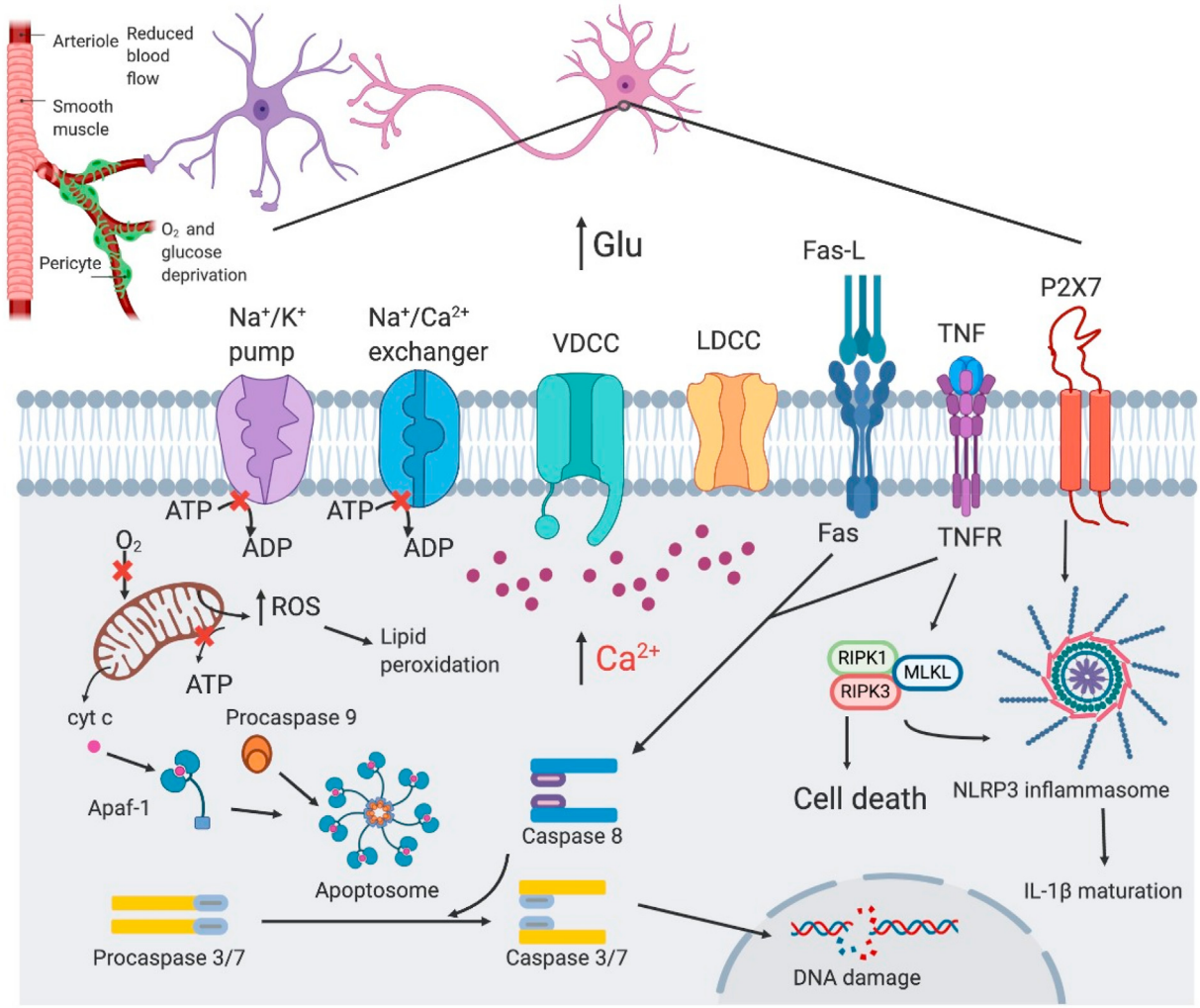

Fig. 1. Overview of cell death mechanisms involved in stroke-induced neuronal damage. During an ischemic stroke, a reduction in blood supply deprives the surrounding brain tissue of glucose and oxygen, which impairs the mitochondrial production of ATP needed for ionic pumps. Then, the transmembrane potassium gradient dissipates and the intracellular levels of sodium and calcium rise, leading to spreading depolarizations in astrocytes (depicted in purple) and neurons (represented in pink). Sustained depolarization causes the increase in extracellular glutamate which activates neuronal post-synaptic ligand-dependent calcium channels (LDCC), such as NMDA receptor, contributing to the high intracellular calcium levels, also supported by voltage-dependent calcium channels (VDCC). This excess in calcium triggers several mechanisms of regulated necrosis in neurons, such as caspase (CASP) 3-dependent intrinsic apoptosis, TNF/Fas-related extrinsic apoptosis, CASP-independent receptor-interacting protein kinase (RIPK)-regulated necroptosis, NLRP3 inflammasome-induced pyroptosis and ferroptosis, which is associated with oxidative stress and lipid peroxidation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

oxidative phosphorylation also induces more free radical production by the mitochondrial chain, increases intracellular Na^+ and ultimately leads to membrane depolarization after the loss of ATP substrate for the Na^+-K^+ pump (Caplan and Liebeskind, 2016). The abrupt and almost complete breakdown of transmembrane ion gradients, together with neuronal edema and mitochondrial disruption, are all hallmarks of spreading depolarizations contributing to excitotoxicity and neuronal cell death (Hartings et al., 2017; Luckl et al., 2018). These waves of sustained depolarization, also known as cortical spreading depression, correlate with the synaptic release of glutamate, one of the major excitatory neurotransmitters, and its electrogenic transport from depolarized astrocytes. The consequential increase in extracellular glutamate results in overstimulation of several receptors, such as α-amino-3--hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate, members of the acid-sensing ion channel (ASIC), metabotropic and NMDA-type glutamate receptors. Consequently, an influx of Na^+ and Ca^{2+} ions through the channels gated by these receptors occurs (Mayor and Tymianski, 2018). Ultimately, the increase in intracellular Ca^{2+} triggers the activation of secondary signal cascades comprising several proteases, lipases and kinases, which leads to organelle dysfunction and finally to several cell death pathways, including apoptosis (Chen et al., 2017; Wu et al., 2018), necrosis (Xu et al., 2016), autophagy (Liu et al., 2013; Song et al., 2017), necroptosis (Yang et al., 2017; Zhan et al., 2019) and ferroptosis (Tuo et al., 2017).

2.1. A diverse landscape of cell death modalities involved in brain stroke

Already in the 19th century, Rudolf Virchow essentially described the basis for two prototype types of cell death, known today as apoptosis and necrosis (Virchow, 1860). Apoptosis is considered as the typical form of programmed cell death during development and homeostasis, whereas necrosis is typically more linked to pathophysiological conditions. An increasing amount of evidence has radically changed this view and revealed the existence of multiple molecular pathways of necrosis (Galluzzi et al., 2018). Cellular necrosis is defined by rounding, swelling, cytoplasmic granulation and plasma membrane rupture, with consequent

Table 1
Diverse cell death modalities in stroke.

Therapeutic inhibition	Necrotic Cell death	Experimental model	Outcome	References
Pharmacological therapies				
Necrostatin (Nec-1)	Necroptosis	Mouse and rat model of MCAO, neonatal mouse HI, mouse model of BCAS, mouse and rat model of ICH, I/R injury in rats	Inhibition of neuronal cell death, reduced infarct size, improved neurological outcome, decrease of RIPK1, RIPK3 and MLKL, improved recovery, mitoprotection, reduced inflammatory response, neuroprotection	(Chavez-Valdez et al., 2012; Chang et al., 2014; King et al., 2014; Yin et al., 2015; Shen et al., 2017; Chen et al., 2018b)
7-Cl-Nec-1	Necroptosis	Mouse model of MCAO	Reduced infarct size, improved neurological score	Degterev et al. (2005)
Ligustroflavone	Necroptosis	Rat model of MCAO	Reduced infarct size, decrease of RIPK1	Zhang et al. (2019a,b)
GSK′872	Necroptosis	Rat model of SAH	Attenuated brain edema, improved neurological function, decrease of RIPK3 and MLKL	Chen et al. (2018a,b)
GSK′963	Necroptosis	Mouse model of ICH	Reduced neuronal death	Lule et al. (2017)
Nec-1	Necroptosis	OGD in mouse primary cortical neurons	Monotherapy protected against OGD-induced cell death.	Xu et al. (2010)
Gly(14)-humanin	Apoptosis	Mouse model of MCAO	Combination therapy increased neuroprotection *in vivo*	
Ferrostatin-1 (Fer-1)	Ferroptosis	OHSCs Collagenase-induced ICH in mouse	Reduced iron deposits, neuroprotection, improved neurological outcome, reduced lipid ROS, prevent neuronal death, reduced infarct size	Li et al. (2017)
Fer-1, Liproxstatin-1 (Lip-1)	Ferroptosis	Hb-induced cell death in OHSCs and human induced pluripotent stem cell-derived neurons	Increased neuronal rescue	Li et al. (2017)
Nec-1	Necroptosis			
CASP3 inhibitor	Apoptosis			
6E11	Necroptosis	Hypoxia/reoxygenation injury in human aortic endothelial cells	More protective compared to necrostatins	Delehouze et al. (2017)
Dabrafenib	Necroptosis	Photothrombosis-induced focal ischemic injury	Reduced infarct volume, attenuated TNF-α expression	Cruz et al. (2018)
Deferoxamine	Ferroptosis	Rat model of MCAO	Internasal delivery decreased infarct volume	Hanson et al. (2009)
Fer-1, Lip-1	Ferroptosis	Mouse model of MCAO	Improved neurological outcome, decreased infarct volume	Tuo et al. (2017)
NSA	Necroptosis	Mouse model of MCAO	Reduced infarct size and improved neurological outcome	Zhou et al. (2014)
Ibrutinib	Pyroptosis	Mouse model of MCAO	Suppression of infarct growth and less neurological damage	Ito et al. (2015)
NLRP1-antibody	Pyroptosis	Mouse model of thromboembolic stroke	Reduced brain edema	Abulafia et al. (2009)
MCC950	Pyroptosis	Mouse model of tMCAO	Reduction of infarct volume and edema, improved neurological outcome	Ismael et al. (2018)
Brilliant blue G	Pyroptosis	Rat model of SAH and ICH	Improved neurological deficits, decreased brain edema, repressed CASP1 activation, decreased neuronal cell death, decreased expression of P2X7R, less infiltrating neutrophils, iNOS and NOX2 expression	(Chen et al., 2013; Feng et al., 2015)
DHA	Parthanatos	Rat model of MCAO	Neuroprotectin D1 (NPD1) synthesis, increased Iduna,	Belayev et al. (2017)

(*continued on next page*)

Table 1 (*continued*)

Therapeutic inhibition	Necrotic Cell death	Experimental model	Outcome	References
			improved neurological effects, decreased edema and lesion volumes	
Genetic therapies				
RIPK1	Necroptosis	Autologous blood-induced ICH in mouse and rat	Attenuated brain injury, less permeability of plasma membrane, decreased neuronal death, less weight loss, improved neurological score	(Lule et al., 2017; Shen et al., 2017)
RIPK3	Necroptosis	OGD-induced neuronal death	RIPK3 knock down inhibited OGD-induced necrotic death	Vieira et al. (2014)
		Collagenase-induced ICH in mice	Less necrotic cells	Zhu et al. (2012)
MLKL	Necroptosis	H/I in neonatal rats	Improved neurological score and decreased infarct volume	Qu et al. (2016)
NLRP3	Necroptosis	Mouse model of MCAO	Reduced infarct size, less damage to blood-brain barrier	Yang et al. (2014a,b)

leakage of cellular contents into the extracellular space. Multiple modes of necrosis share these morphological hallmarks, and they have been examined for common or distinct underlying signaling pathways (Vanden Berghe et al., 2014). The recent discovery of phylogenetically preserved mechanisms *viz.*protease-mediated cleavage of the pore-forming effector proteins gasdermins (GSDM**)** in for example apoptosis, netosis and pyroptosis, challenges the generally accepted dichotomy between non-leaky, immune-silent apoptosis and leaky, immunogenic necrosis. This view implies that apoptosis might be classified as a mode of necrosis, with the notion that the leaky stage after apoptosis is normally not reached *in vivo* due to quick phagocytosis by neighboring cells or phagocytes (Vanden Berghe and Hoste, 2018). There is increasing evidence that also multiple modes of necrosis are simultaneously present in the ischemic core and penumbra area after ischemic or hemorrhagic stroke (Table 1). The predominance of a particular mechanism of ischemic neuronal cell death depends on brain maturity and region, the gender of the organism and the ischemic area, either ischemic core or penumbra (Puyal et al., 2013). Additionally, all human strokes and time to hospital admissions differ from each another, showing high inter-individual variability.

MCAO, middle cerebral artery occlusion; HI, hypoxia-ischemia; BCAS, bilateral carotid artery stenosis; ICH, intracerebral hemorrhage; I/R, ischemia/reperfusion; SAH, subarachnoid hemorrhage; OGD, oxygen and glucose deprivation; OHSCs, organotypic hippocampal slice cultures; Hb, hemoglobin; RIPK, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like; ROS, reactive oxygen species; TNF, tumor necrosis factor; P2X7R, P2X purinoceptor 7; iNOS, inducible nitric oxide synthase; NOX, NADPH oxidase.

Many aspects, such as endoplasmatic reticulum (ER) stress, mitochondrial integrity, disrupted blood-brain barrier, gliosis and astrogliosis, increase of oxidative stress and release of free radicals, play an important role in the progression of cell death during brain stroke. However, a main trigger for initiation of neuronal cell death after stroke injury is the exaggerated increase of intercellular calcium (Fig. 1).

Neuronal apoptotic cell death is mainly regulated by CASP3 in the context of brain cell death (Kuida et al., 1996). In case of intrinsic apoptosis, cytochrome c (cyt c) is released by depolarization and dysfunction of the mitochondria. Once in the cytosol, cyt c will complex with apoptotic protease-activating factor 1 (Apaf-1), CASP9 and executor caspases (CASP3 and -7), finally leading to DNA fragmentation and cell death (Sekerdag et al., 2018). The extrinsic pathway, in the context of neurological damage, is mainly initiated upon interaction with TNF, Fas and TNF related apoptosis inducing ligand (TRAIL) surface receptors. This interaction activates CASP8 and 10, followed by activation of CASP3. Inhibition of Bcl-2 family members have already been shown to improve neurological outcome and decrease behavioral abnormalities after MCAO (Wei et al., 2016). Furthermore, pre- and post-treatment with SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), reduced FasL expression, attenuated cyt c release by mitochondria and suppressed CASP3 activation during I/R injury in rats (Guan et al., 2006a,b).

It is clear that both intrinsic and extrinsic apoptotic pathways are involved in brain stroke. Beside apoptosis, which occurs mainly in the penumbra within few hours to days after brain injury, necrosis starts in the first hours in the ischemic core.

In contrast to apoptosis, which requires activation of CASP3 and -8, necroptosis is CASP independent. Upon CASP8 inhibition or disruption, necroptosis depends on the formation and activation of the necrosome, a complex composed of RIPK 3 and MLKL (Li et al., 2012; Vanden Berghe et al., 2014).

Cell specificity for necroptosis was shown in a model of I/R induced hippocampal CA1 neuronal death, in which detection of CASP3 and -8 was absent. Hereby, expression of CASP8 was only observed in astrocytes and microglia, but not in neurons, indicating the vulnerability for neurons to necroptosis. Furthermore, Nec-1 pretreatment in a global ischemia model, blocked the upregulation of RIPK3 and the neuroprotective effect was correlated to translocation of RIPK3 and apoptosis-

inducing factor (AIF) (Xu et al., 2016). In fact, Nec-1 treatment decreased RIPK1 and RIPK3 proteins in the hippocampus, as well as several inflammatory cytokines such as IL-1β, TNFα and IFNγ, in chronic brain hypoperfusion with adult mice, who also showed improved cognitive function upon treatment (Zhang et al., 2016a,b,c). Also the outcome of intracerebral hemorrhage (ICH), induced by collagenase injection in mice, was ameliorated upon Nec-1 treatment (King et al., 2014). Similarly, Nec-1 treatment or genetic knockdown of RIPK1 in autologous blood-induced ICH in mice as well as in rats, ameliorated the neurological outcome and decreased edema volume (Lule et al., 2017; Shen et al., 2017).

Not only inhibition of RIPK1 activity, but also other molecular targets of the necroptotic cell death pathway have shown promising results in the context of stroke. Similarly to RIPK1, RIPK3 overexpression in hippocampal neurons significantly increased injury upon OGD, while significant protection was evident after RIPK3 knockdown (Vieira et al., 2014). Dabrafenib, a RIPK3 inhibitor (Li et al., 2014), decreased the infarct size in a model of permanent focal ischemia with a concomitant reduction of TNF mRNA expression levels (Cruz et al., 2018). Thereby, similarly to RIPK1, RIPK3 is not exclusively linked to necroptosis as it is also able to activate the NLRP3 inflammasome needed for pyroptosis, this in the absence of inhibitor of apoptosis proteins (IAPs) (Lawlor et al., 2015). The downstream target of RIPK3, MLKL pseudokinase, has also recently been proposed as a therapeutic target for stroke. Experiments based on hypoxia/ischemia in neonatal rats, lacking MLKL by siRNA-induced inhibition, revealed an improved neurological score and a decrease in infarct size (Qu et al., 2016).

On the other hand, ferroptosis is characterized by the generation of redox-active iron that promotes the formation of phospholipid peroxyl radicals through Fenton-type reactions and/or activation of lipoxygenases, which drives the process of lipid peroxidation and ultimately cell death (Angeli et al., 2017). Differences between oxidative glutamate toxicity, oxytosis and excitotoxicity, such as the involvement of calcium influx, were used to argue for coining a novel form of regulated necrosis *viz*.ferroptosis. To date, it has been proposed, and more generally accepted, that ferroptosis, glutamate toxicity, oxytosis and excitotoxicity represent very similar, or even the same forms of regulated necrosis (Lewerenz et al., 2018). Cell death by ferroptosis is counteracted genetically by the phospholipid repair enzyme Glutathione Peroxidase 4 (GPX4) (Yang et al., 2014a,b), and pharmacologically by iron chelators, lipophilic natural radical traps such as vitamin E and synthetic radical traps such as Fer-1 and Lip-1 (Friedmann Angeli et al., 2014; Skouta et al., 2014; Zilka et al., 2017). Hemorrhagic brain stroke is characterized by activated microglia metabolizing hemoglobin (Hb) to ferrous/ferric iron, inducing ROS, which create highly reactive hydroxyl radicals able to attack lipid membranes. This mechanism of action might explain why hemin typically induces ferroptosis in many cell lines (Hassannia et al., 2018; Imoto et al., 2018). Recently, it was demonstrated that intracerebroventricular treatment with Fer-1 after ICH in mice, exhibited marked brain protection and improved neurological functioning (Li et al., 2017). Similarly, Zille et al., verified the contribution of both necroptosis and ferroptosis in experimental ICH in vitro and *in vivo* (Zille et al., 2017). A known major risk factor for stroke is age, which relates to an increase of brain iron in humans as well as in rodents (Tuo et al., 2017). Interestingly, tau-iron interactions were identified as a pleiotropic modulator of ferroptosis and ischemic stroke outcome in a mouse MCAO model. Consequently, intranasal delivery of Fer-1 and Lip-1, was recently shown to attenuate neurological deficits (Tuo et al., 2017).

Besides the evidence regarding the role of ferroptosis/oxytosis in stroke pathophysiology (recently reviewed in (Lewerenz et al., 2018)), several other cell death mechanisms have been associated with brain ischemia reperfusion injury. When LPS, some chemokines, and other damage-associated molecular patterns (DAMPs) stimulate neutrophils, mast cells, eosinophils and basophils, the NADPH-oxidase dependent release of ROS initiates a signaling cascade that leads to the degradation of nuclear and cellular membranes and the formation of extracellular traps (ETs), which contain decondensed DNA with embedded histones and antimicrobial granule proteins. The formation of ETs is a relevant mechanism for innate immune responses and constitutes the hallmark of a cell death pathway in leukocytes, known as ETosis (Vanden Berghe et al., 2014; Galluzzi et al., 2018). As will be discussed in forthcoming sections, infiltrating neutrophils play a major role in the stroke-induced innate immune response (Frieler et al., 2017) and display signs of ETs formation, such as histone-3 citrullination, chromatin decondensation, and extracellular projection of DNA and histones (Perez-de-Puig et al., 2015). The release of neutrophil ETs has been found to induce the activation of platelets and coagulation factors, promoting thrombus formation (Laridan et al., 2017). Interestingly, the presence of H3Cit, a biomarker of neutrophil ETs, was shown in isolated thrombi of ischemic stroke patients (Laridan et al., 2017). Administration of RIPK1 or MLKL inhibitors (i.e., Nec-1 or necrosulfamide (NSA), respectively) as well as genetic knockdown of RIPK3, was able to prevent ETs formation and lysis of neutrophils (Desai et al., 2016), indicating the dependency of ETosis to components of the necroptotic cell death pathway. Thereby, it has been demonstrated that MLKL expression increases in a time-dependent manner after ischemia reperfusion and such increments can be prevented by NSA (Zhou et al., 2017). Furthermore, these effects of NSA on MLKL expression levels were positively correlated with better neurological function and reduced infarct sizes (Zhou et al., 2017). However, it was recently shown that NSA also functions as an inhibitor of gasdermin D (GSDMD), the pore-forming protein essential in pyroptosis (Rathkey et al., 2018).

Molecularly, pyroptotic cell death is dependent on the activation of the inflammatory caspases, CASP1, CASP4 and -5 in human and CASP11 in mice, which are activated within inflammasomes for example upon triggering of NOD-like receptors (NLRs) in response to intracellular PAMPs. Then cleaved and oligomerized GSDMD forms a membrane pore, leading to cell leakage and death. Pyroptosis is also characterized by the release of two major inflammatory cytokines, IL-1β and IL-18 (Galluzzi et al., 2018). Some evidence suggests that pyroptosis might be a major cell death mechanism of neurons within the ischemic core during stroke. In this sense, the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib interferes with IL-1β maturation by suppressing CASP1 activation, leading to reduced infarct sizes and better neurological outcomes (Ito et al., 2015). Also, genetic knock out of NLRP3 in mice decreased lesion volume and neurological damage after tMCAO induction (Yang et al., 2014a,b). In the same model, pharmacological inhibition of NLRP3 by MCC950 reduced infarct size, edema and ameliorated neurological outcome (Ismael et al., 2018). In hemorrhagic stroke it has been shown that NLRP3 activation is dependent on activation of the purinergic 2 × 7 receptor, and its antagonist brilliant blue G seems to be protective in ICH (Feng et al., 2015).

Considering the multiplicity of the necrotic cell death pathways involved, and their dynamic mutual interplay with inflammatory processes in the pathophysiology of stroke, better molecular profiling of patients will be needed to stratify and determine the most optimal combinational treatment. This approach could pave the way for precision medicine in neurological disorders or strokes (Vanden Berghe and Hoste, 2018).

3. Dual role of the immune response in damage and repair

The phenotypic heterogeneity of microglia has been shown to represent a dynamic continuum which depends on age, brain region localization and pathological conditions in mice (Eggen et al., 2019) and also in humans (Böttcher et al., 2019). Microglia are among the first non-neuronal cells on the scene during the innate immune response to ischemic stroke. Microglia/macrophages respond to acute brain injury by becoming activated and developing classic M1-like (pro-inflammatory) or alternative M2-like (anti-inflammatory) phenotypes (Miro-Mur et al., 2016; Rajan et al., 2018). Although the control of microglia/macrophage polarization has not been completely characterized in ischemia,

experimental evidence suggests the involvement of IL-4 in the induction of the neuroprotective M2 phenotype after 5 and 14 days post-injury (Liu et al., 2016). Also, the blockade of the mammalian target of rapamycin prevents microglia polarization toward the M1 phenotype (Li et al., 2016a,b) and T-LAK-cell-originated protein kinase positively regulates M2 skewing by inhibiting histone deacetylases 1 and 2 activity (Han et al., 2018). Similarly to macrophages, neutrophils have been proposed to acquire an N2 alternative anti-inflammatory phenotype, which can also be pharmacologically induced by rosiglitazone, an activator of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ), potentially contributing to the resolution of brain inflammation after stroke (Cuartero et al., 2013).

The landscape of the adaptive immune response after ischemic stroke is still an active field of research and debate. Leukocytes not only infiltrate the infarct core and peri-infarct regions, but they also infiltrate to sites of secondary neurodegeneration, remotely but synaptically connected to the primary infarct (Jones et al., 2018). Also, recent studies have revealed the choroid plexus as a key route for post-stroke brain T cell invasion, both in experimental and human ischemia. T lymphocyte cytotoxic effector activities have been proposed to rely on innate antigen-independent functions. After 24 h of tMCAO, conventional type 2 IRF4$^+$/CD172a$^+$ dendritic cells constitute the major source of IL-23 in the ischemic brain, inducing the production of IL-17 in γδT cells and therefore promoting neutrophil infiltration (Gelderblom et al., 2018). Besides the relevance of γδ T cells in the early acute ischemic injury, a role for other T lymphocyte subsets should not be ruled out. In fact, it has been demonstrated that accumulating CD4$^+$ T cells produce IL-21 and contribute to early brain damage after focal ischemia in mice and patients (Clarkson et al., 2014). In addition, natural regulatory T cells (Tregs) contribute to an increased infarct size 24 h post-insult, due to their enhanced interaction with activated endothelial cells, which causes microvascular dysfunction, augmented thrombus formation and deficient reperfusion (Kleinschnitz et al., 2013). An interaction of Tregs and IL-17$^+$γδ T cells has recently been recognized in a novel gut-brain axis. As a consequence of microbial dysbiosis, intestinal dendritic cells induce local Tregs, which suppress IL-17$^+$γδ T cells function. After brain ischemia, infiltration of these intestinal γδ T cells to the meninges is reduced, which decreases chemokine production and leukocyte recruitment and therefore improves stroke outcome (Benakis et al., 2016).

In a delayed stage of ischemia, mechanisms of damage and protection apparently coexist. A progressive brain accumulation of proliferating and activated CD4$^+$ and CD8$^+$ T cells has been demonstrated, up to one month after transient MCAO ischemia. These cells also showed a close interaction with reactive astrocytes and a progressive production of pro-inflammatory cytokines such as IL-17, TNFα and perforin (Xie et al., 2018). Simultaneously, activated microglia and astrocytes release TGF-β, which enhances the expansion of Tregs and therefore contributes to tissue recovery and immune homeostasis. Also in this experimental paradigm of ischemia reperfusion, it has been shown that the activation of the sympathetic nervous system mediates the increase in bone marrow-derived Tregs and enhances its mobilization via β_2 and β_3 adrenergic receptors (Wang et al., 2015a,b). One of the most prominent mechanisms of Tregs function, in the context of stroke and intracerebral hemorrhage, involves the secretion of IL-10, a cytokine which mediates microglia polarization towards the neuroprotective M2 phenotype, by increasing the expression of the glycogen synthase kinase 3 beta (GSK3β) (Zhou et al., 2017) and sustaining Tregs-mediated neurogenesis in the subventricular zone after stroke (Wang et al., 2015a,b).

In the clinical scenario, immunosuppression after the acute post-stroke stage correlates with a high incidence of infections (Chamorro et al., 2007). The suppression of systemic immunity has also been associated with a post-stroke microbiota dysbiosis, usually detected in stroke patients (Yamashiro et al., 2017). In the experimental setting, depletion of the intestinal microbiome in mice receiving broad spectrum antibiotics before stroke induction, increased post-stroke mortality unrelated to ischemic lesion size, whereas the survival rate was improved by re-colonization of the ischemic mice with complex gut microbiota (Winek et al., 2016). In ischemic mice, bacterial priming of intestinal dendritic cells leads to the proliferation of local Tregs in the small intestine and inhibition of effector IL-17$^+$γδ T cells function. Furthermore, the efficiency of dendritic cells to induce Tregs depends on antibiotic sensibility of the intestinal microbiota in ischemic mice (Benakis et al., 2016). Thus, a complex crosstalk between the CNS and the immune system occurs after stroke and represents a promising target for immunomodulatory therapies.

4. Saponins as immunomodulators and regulators of cell death in stroke

Saponins are known as surface-active compounds that are widely distributed in the plant kingdom. They comprise a non-polar aglycone or non-saccharide moiety coupled with polar mono or oligosaccharides, which explains their detergent-like properties in aqueous solutions (Oleszek, 2002). Besides the applicability of saponins as natural surfactants and emulsifiers, they have demonstrated several additional pharmacological activities, such as immunostimulating, antimicrobial, hypocholesterolaemic and anti-cancer properties (Ks giel et al., 2017).

In the context of stroke, triterpenoid saponins are the most extensively studied and specifically amongst these are the ginsenosides. This special group of triterpenoid saponins is nearly exclusively found in plant species of the genus *Panax* (ginseng), which belongs to the family Araliaceae. Ginsenosides are also thought to be the main active compounds in ginseng, which has shown some efficacy against pathologies of the cardiovascular system, immune system and CNS (Christensen, 2009). In the tMCAO model of ischemia reperfusion, the treatment with ginsenoside Rg1, a major bioactive panaxatriol triterpenoid saponin in *P. ginseng* (Kiefer and Pantuso, 2003), has been shown to reduce the infarct volume and the neurological deficit of ischemic rats (Lin et al., 2015). Other studies have demonstrated that Rg1 inhibits the activity of miR-144 which induces the Nrf2/ARE signaling pathway, thereby enhancing an antioxidant response (Chu et al., 2019). Also when administered several days before tMCAO in mice, the neuroprotective effects of Rg1 are related to an increase in the expression of brain-derived neurotrophic factor (BDNF) in the hippocampal CA1 region and a reduction in serum TNFα and IL-6 (Wang et al., 2018). In a permanent MCAO model, the treatment with Rg1 improves the impaired motor coordination of ischemic animals and reduces the infarct volumes. These results also demonstrated a mechanism involving enhanced angiogenesis by a PI3K/Akt/mTOR signaling-mediated increase in the expression of VEGF (Chen et al., 2019).

Another ginsenoside with effects on neurogenesis in ischemia reperfusion models is the ocotillol-type saponin pseudoginsenoside F11 (pF11). Repeated doses of pF11 before and after the onset of tMCAO in mice, improved the long-term behavioral outcome regarding cognitive and sensorimotor dysfunction, and also promoted neurogenesis by increasing the number of cortical NeuN$^+$ cells and BDNF expression levels (Yuan et al., 2020). Concerning immunomodulation, pF11 was shown to shift neutrophils and macrophages in vitro from an OGD-induced M1-like phenotype towards a CD206$^+$immunoregulatory functional state (Hou et al., 2020). However, one of the most interesting pharmacological activities of pF11 is the regulation of calcium overload, which is an early pathological event in neuronal cell death mechanisms in stroke (Caplan and Liebeskind, 2016). In fact, the administration of pF11 to rats exposed to tMCAO improved the neurological dysfunction and decreased infarct volumes with a wide therapeutic window of 4h after reperfusion. Moreover, it was demonstrated that pF11 repressed the sustained calcium overload by the decrease in the autolysis of μ-calpain, the cleavage of α-Fodrin and the increase in expression levels of Ca(2+)/calmodulin –dependent protein kinase (CaMKII) (Zhang et al., 2019a,b).

Similarly, the dammarane-type triterpenoid saponin ginsenoside Rd also modulates calcium homeostasis, specifically by the inhibition of

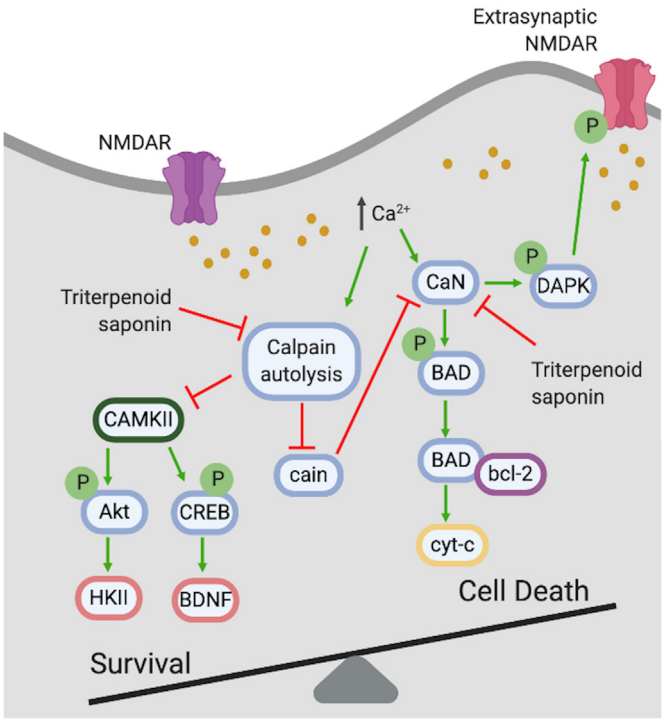

Fig. 2. Proposed neuroprotective mechanism of triterpenoid saponins in ischemic stroke. Ischemia/reperfusion-induced over-activation of NMDA receptor promotes the increase of intracellular calcium levels, which then activates calcium-sensing proteins such as calcineurin (CaN), calpain and CAMKII. Triterpenoid saponins (TS), such as ginsenosides, inhibit CaN, therefore preventing the phosphorylation and activation of death-associated protein kinase 1 (DAPK1) and the subsequent activation of extrasynaptic NMDA receptor. The inhibition of CaN by TS also prevents the cell death mediated by cyt c release. Calpain autolysis and activation is also inhibited by TS, hence avoiding the proteolysis-mediated inhibition of calcineurin-inhibitor (cain) and CAMKII. Then, activated cain is able to inhibit CaN and Ca^{2+}-activated CAMKII promotes BDNF production and fully functioning of hexokinase II (HKII). Overall, neuronal survival is sustained over cell death.

receptor-operated Ca^{2+} channels without any effects on the voltage-dependent inward Ca2+ current in vascular smooth cells (Guan et al., 2006a,b). This evidence sparked an interest in the stroke research community, because of the disappointing results obtained from several modulators of neuronal voltage-gated cation channels and calcium blockers for the treatment of stroke (Gribkoff and Winquist, 2005). Recent whole-cell patch-clamp experiments in cultured rat primary cortical neurons, revealed that ginsenoside Rd reduced NMDA receptor currents and related excitotoxicity. These results suggested that Rd attenuated calcineurin activity, thereby mitigating the phosphorylation of the NR2b subunit mediated by death-associated protein kinase 1 (DAPK1) and hence preventing ischemic neuronal cell death (Wang et al., 2017a,b; Zhang et al., 2020). Considering that calpain is involved in the proteolytical regulation of calcineurin, in response to excessive glutamate-induced neuronal cell death (Kim et al., 2002; Wu et al., 2004) it would be reasonable to suppose that Rd regulates calpain activation as well as pseudoginsenoside F11. Regarding the inflammatory response after tMCAO, the treatment with Rd 4h after reperfusion decreased the phosphorylation of IκBα and p65 nuclear translocation in the ischemic penumbra region, while it diminished proteosome activity in rat primary microglia (Zhang et al., 2016a,b,c).

Besides the genus *Panax,* triterpenoid saponins are also abundant bioactive compounds in other species of the family Araliaceae, such as *Aralia taibaiensis*. Among them, total triterpenoid saponins from A. taibaiensis (sAT) decrease infarct volumes and neurological dysfunction when administered seven days before tMCAO in rats, by a mechanism involving the reduction of oxidative stress. Moreover, sAT reduced cleaved CASP3 and Bax, while it increased the levels of phosphorylated Akt (Duan et al., 2019), which is known to protect against stroke-induced cell death (Xie et al., 2013). Interestingly, sAT also up-regulated the silent information regulator2 homologue 1 (SIRT1), which is involved in glucose metabolism homeostasis (Koronowski et al., 2017). Similar antioxidant and anti-apoptotic properties were demonstrated in diabetic mice exposed to chikusetsu saponin IV (a major component of sAT), several days before tMCAO. In addition, chikusetsu also decreased IL-6 and TNFα levels in ischemic diabetic mice, while it increased serum and brain adiponectin, receptor AdipoR1 and the ratio of phosphorylated GSK3β/GSK-3β (Duan et al., 2016). Other studies have shown that oral daily doses of chikusetsu, concomitantly given with a high fat diet to mice, reduced the high levels of cholesterol and triglycerides induced by the diet. Furthermore, chikusetsu reduced pro-inflammatory mediators, such as IL-6, TNFα, MCP-1, CCL-5 and serum amyloid A3, also polarized adipose tissue macrophages from an M1-like inflammatory phenotype ($CD11c^{+}$ and $iNOS^{+}$) towards an immunoregulatory M2-like functional state (Yuan et al., 2017). Considering that adiponectin is involved in the switch of macrophages to an M2-like immunomodulatory phenotype (Ohashi et al., 2010), treatment with chikusetsu could also increase adiponectin levels in ischemic non-diabetic mice.

In addition to triterpenoid saponins from plant species of the family Araliaceae, clematichinenoside saponin is found in species of the genus Clematis (family Ranunculaceae) and has been suggested to have neuroprotective and anti-inflammatory effects. In conditions of LPS-induced systemic inflammation, before the onset of I/R by tMCAO, the repeated administration of clematichinenoside decreased infarct size and improved the neurological deficit, protected the BBB and reduced neutrophil infiltration and the expression of TNFα and IL-1β in serum and brain (Han et al., 2016). Moreover, the treatment with clematichinenoside in ischemic rats exposed to tMCAO diminished neurological dysfunction, infarct volumes and brain edema and neuronal apoptosis. The compound reduced the expression ratio bax/bcl-2 by a mechanism involving CREB phosphorylation, then promoting ERK1/2 and cPKC-mediated up-regulation of bcl-2, thereby preventing apoptosis (Bluwstein et al., 2013; Liu et al., 2015).

Another anti-apoptotic saponin is astragaloside IV, a lanolin-alcohol shaped tetracyclic triterpenoid saponin found in the traditional Chinese herbHuangqi, obtained from the dried roots of the plant *Astragalus membranaceus* (Li et al., 2014). A previous systemic review analyzed the experimental evidence regarding the neuroprotective potential of astragaloside IV associated with a reduction in BBB permeability, through its antioxidant, anti-inflammatory and anti-apoptotic effects (Wang et al., 2017a,b). More recently, in vitro and *in vivo* results suggested Akt as the molecular target for the neuroprotective activity of astragaloside IV and the involvement of hexokinase II (HKII) interaction with mitochondria (Li et al., 2019). At the physiological state, the interaction of HKII with voltage-gated anion channels in the mitochondrial outer membrane supports the efficiency of oxidative metabolism and prevents the opening of the mitochondrial permeability transition pore (Tait and Green, 2010). In contrast, when glutamate levels become excessive due to ischemic insult, oxidative stress increases and HKII detaches from mitochondria, thereby causing the opening of the mitochondrial permeability transition pore and promoting the release of pro-apoptotic proteins (Pasdois et al., 2012). The treatment with astragaloside IV increased the levels of phosphorylated Akt, which then interacted with HKII to protect hexokinase activity and improved the efficiency of glycolysis. In this context, the mitochondrial release of pro-apoptotic proteins was decreased as well as neuronal apoptosis and parthanatos (Li et al., 2019). Altogether, the experimental evidence suggest calpain and calcineurin as common molecular targets for ginsenosides, and possibly other triterpenoid saponins, in their neuroprotective mechanism of action (Fig. 2).

In addition to glycosylated triterpenoid saponins, their aglycone forms such as ruscogenin and diosgenin, have also been described as neuroprotectants and immunomodulators in stroke preclinical models.

Ruscogenin is a major component in the traditional Chinese herb *Ophiopogon japonicas* and when administered before tMCAO in mice, decreases infarct size and neurological deficits by interfering with the NF-κB signaling pathway. Specifically, ruscogenin inhibited the I/R-induced up-regulation, phosphorylation and nuclear translocation of p65, thereby suppressing the expression of NF-κB-regulated proteins such as ICAM-1, iNOS, COX-2, TNFα and IL-1β (Guan et al., 2013). Interestingly, it has been demonstrated that diosgenin in a prophylactic scheme, decreases the infarct volume and neurological dysfunction in the MCAO ischemia reperfusion injury, through inhibition of the NF-κB-mediated inflammatory response and apoptosis (Zhang et al., 2016a,b,c). Contrarily, the authors commented on the inefficacy of diosgenin when administered shortly after the reperfusion in this model and hypothesized a mechanism of action involving the regulation of the peripheral immune response (Zhang et al., 2016a,b,c). Considering that diosgenin induces IL-10-producing Treg cells and exerts a probiotic effect in a mouse model of food allergy (Huang et al. 2012, 2017), these could be relevant mechanisms by which diosgenin improves stroke outcome and regulates ischemic neuroinflammation.

Considering the pharmacological potential of steroidal sapogenins, we used diosgenin as a chemical scaffold for the design and synthesis of new molecular entities. In this sense, we recently studied a steroidal sapogenin derivative (S15) which exerts neuroprotective effects in stroke-related models. In vitro, the non-estrogenic S15 compound counteracts glutamate-induced excitotoxicity while diosgenin did not improve the viability of damaged cells (Garcia-Pupo et al. 2016, 2017). In addition, S15 regulates the transcriptome of the ischemic brain towards an inflammatory homeostasis, involving the enhanced gene expression of Treg cell related cytokines, such as IL-10 and TGFβ (Garcia-Pupo et al., 2017).

The disease and target-directed design of new chemical leads represents a promising strategy for enhancing the pharmacological activity of plant-derived compounds, while potentially decreasing undesired side effects.

5. Concluding remarks

Taking these findings together, there is evidence sustaining the involvement of several neuronal cell death pathways in brain ischemic injury, at least in experimental animal models. The expanding repertoire of cell death pathways and the discovery of new alternatives for inhibiting them, requires the precise identification of unique and relevant markers of each cell death mechanism. In addition, more reliable imaging techniques are required to detect specific forms of neuronal death in patients. Regulated necrosis, such as necroptosis and pyroptosis, results in cell lysis and release of intracellular contents which elicits a robust inflammatory response. The post-stroke central and peripheral immune response involves both innate and adaptive immune mechanisms. The dual role of the immune system in post-stroke inflammation, repair and immune homeostasis has become increasingly evident. However, the biphasic function of myeloid cells and T lymphocytes, and their mutual modulation with the gut microbiome in stroke patients, entails further studies. The dynamic interplay between the immune response and the diversity of necrotic cell death pathways involved in brain ischemic damage, imposes the need for precise molecular profiling of patients, paving the way for personalized medicine. Considering the potentially wider therapeutic window for strategies targeting the post-stroke inflammation, several emerging therapeutic candidates have shown efficacy in stroke pre-clinical models and are currently being evaluated in the clinical scenario. However, the challenge of designing a therapeutic compound with immunoregulatory functions, without completely abrogating the post-stroke immune response still exists. In this context triterpenoid saponins and their aglycone forms, such as diosgenin and its analogs, are polypharmacological compounds with neuroprotective and immunomodulatory functionalities which could represent interesting and promising drug candidates for future stroke therapy and prevention.

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