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, EToxicosis 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...
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 \(\alpha\)-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

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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.)
Table 1
Diverse cell death modalities in stroke.

| Therapeutic inhibition | Necrotic Cell death | Experimental model | Outcome | References |
|------------------------|---------------------|--------------------|---------|------------|
| Pharmacological therapies | 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) |
| Necrostatin (Nec-1) | Necroptosis | Mouse model of MCAO | Reduced infarct size, improved neurological score | Degterev et al. (2005) |
| 7-Cl-Nec-1 | Necroptosis | Mouse model of MCAO | Reduced infarct size, decrease of RIPK1 | Zhang et al. (2019a,b) |
| Ligustroflavone | Necroptosis | Rat model of MCAO | Reduced infarct size, decrease of RIPK1 | Chen et al. (2018a,b) |
| GSK'872 | Necroptosis | Rat model of SAH | Attenuated brain edema, improved neurological function, decrease of RIPK3 and MLKL | Lule et al. (2017) |
| GSK'963 | Necroptosis | Mouse model of ICH | Reduced neuronal death | Xu et al. (2010) |
| Nec-1 | Necroptosis | OGD in mouse primary cortical neurons | Monotherapy protected against OGD-induced cell death. | |
| Gly(14)-humanin | Apoptosis | Mouse model of MCAO | Combination therapy increased neuroprotection in vivo | |
| Ferrostatin-1 (Fer-1) | Ferroptosis | OHSCs | 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) |
| Nee 1 | Necroptosis | Stem cell-derived neurons | More protective compared to necrostatins | Delehouze et al. (2017) |
| CASP3 inhibitor 6E11 | Necroptosis | Hypoxia/reoxygenation injury in human aortic endothelial cells | Reduced infarct volume, attenuated TNF-a expression | Cruz et al. (2018) |
| Dabrafenib | Necroptosis | Phototoxicity-induced focal ischemic injury | Combination therapy increased infarct volume | Hanson et al. (2009) |
| Deferoxamine | Ferroptosis | Rat model of MCAO | Internal delivery decreased infarct volume | |
| 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 mMCAO | 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)
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 (GDSD) 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; OHSs, 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 astroglisis, 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, Nec1 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 RIPK3 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 TNFα 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 lipooxygenases, 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 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 Berge 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 (Müro-Mur et al., 2016; Rajan et al., 2018). Although the control of microglia/macrophage polarization has not been completely characterized in ischemia,
Besides the relevance of marrow-derived Tregs and enhances its mobilization via sympathetic nervous system mediates the increase in bone tissue recovery and immune homeostasis. Also in this experimental para-

which enhances the expansion of Tregs and therefore contributes to tis-


demia (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) (Oleszek, 2002). Besides the applicability of saponins as natural surfac-

tants and emulsifiers, they have demonstrated several additional phar-

macological activities, such as immunostimulating, antimicrobial, hypcholesterolaemic and anti-cancer properties (Ks giel et al., 2017).

In the context of stroke, triterpenoid saponins are the most exten-

sively 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 Aral-

cae. Ginsenosides are also thought to be the main active compounds in ginseng, which has shown some efficacy against pathologies of the cardio-

vascular system, immune system and CNS (Christensen, 2009). In the tMCAO model of ischemia reperfusion, the treatment with ginsenoside Rg1, a major bioactive panaxastrial 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 reper-

fusion models is the ocottillo-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+ immigrunoregulatory 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 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+/γδT cells function. Furthermore, the efficiency of dendritic cells to induce Tregs depends on antibiotic sensibility of the intestinal microbiota in ischemic mice (Benakki et al., 2016). Thus, a complex crosstalk between the CNS and the immune system occurs after stroke and represents a promising target for immu-

nomodulatory 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 surfac-

ants and emulsifiers, they have demonstrated several additional phar-

macological activities, such as immunostimulating, antimicrobial, hypcholesterolaemic and anti-cancer properties (Ks giel et al., 2017).
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/GSK3α (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 (Wang 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 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 herb Huangqi, 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 (Pastoš 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 pantothenate (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 neuroprotectors and immunomodulators in stroke preclinical models.
Ruscogenin is a major component in the traditional Chinese herb *Ophiopogon japonicas* and when administered before MCAO 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-sterogenic 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, terpenoid 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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