Cellular and Molecular Neurobiology (2022) 42:545–556
https://doi.org/10.1007/s10571-020-00966-4

REVIEW PAPER

Role of DAMPs and of Leukocytes Infiltration in Ischemic Stroke: Insights from Animal Models and Translation to the Human Disease

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Received: 28 April 2020 / Accepted: 22 September 2020 / Published online: 29 September 2020

Abstract
Stroke is a leading cause of death and disability worldwide. Several mechanisms are involved in the pathogenesis of ischemic stroke (IS). The contributory role of the inflammatory and immunity processes was demonstrated both in vitro and in animal models, and was confirmed in humans. IS evokes an immediate inflammatory response that involves complex cellular and molecular mechanisms. All components of the innate and adaptive immunity systems are involved in several steps of the ischemic cascade. In the early phase, inflammatory and immune mechanisms contribute to the brain tissue damage, whereas, in the late phase, they participate to the tissue repair processes. In particular, damage-associated molecular patterns (DAMPs) appear critical for the promotion of altered blood brain barrier permeability, leukocytes infiltration, tissue edema and brain injury. Conversely, the activation of regulatory T lymphocytes (Tregs) plays protective effects. The identification of specific cellular/molecular elements belonging to the inflammatory and immune responses, contributing to the brain ischemic injury and tissue remodeling, offers the advantage to design adequate therapeutic strategies. In this article, we will present an overview of the knowledge on inflammatory and immunity processes in IS, with a particular focus on the role of DAMPs and leukocytes infiltration. We will discuss evidence obtained in preclinical models of IS and in humans. The main molecular mechanisms useful for the development of novel therapeutic approaches will be highlighted. The translation of experimental findings to the human disease is still a difficult step to pursue. Further investigations are required to fill up the existing gaps.

Keywords Ischemic stroke · Inflammation · Immunity · MCAO · Damps · Leukocytes

Introduction

Stroke is the second-leading cause of mortality after ischemic heart disease and one of the main causes of disability worldwide (Favate and Younger 2016; Benjamin et al. 2019). In most cases (85–87%), stroke is of ischemic type (ischemic stroke, IS) and is due to cerebral vessel occlusion by an embolus or a thrombus. The remaining type of stroke is hemorrhagic and occurs following the rupture of a cerebral vessel. In both situations, the blood flow is reduced or completely interrupted causing a failure of oxygen and nutrient supply to the tissue.

Several studies performed over the last decades highlighted the key role of inflammatory and immunity responses in the pathogenesis of IS in animal models (Schmidt-Pogoda et al. 2019; Iadecola and Anrather 2011).

The activation of both inflammatory and immunity responses occurs simultaneously to the ischemic brain damage and it amplifies the neuronal injury. All components of the innate immunity system are involved in several steps of the ischemic cascade including resident cells such as glia, microglia, endothelial cells and circulating inflammatory cells such as monocytes, macrophages and leukocytes. The latter cells, once activated, cross the blood brain barrier (BBB) and accumulate in the damaged brain tissue where they can exert either positive or negative effects (Kamel and Iadecola 2012). Numerous studies showed that the adaptive

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immunity is also activated in IS by the action of T and B lymphocytes and of the antigen presenting cells (Chamorro et al. 2012).

Studies in human populations analyzed the role of inflammatory markers, such as damage-associated molecular patterns (DAMPs), fibrinogen, white blood cell count and their correlation with stroke occurrence (Emerging Risk Factors et al. 2010; Goldstein et al. 2006). This correlation becomes more pronounced in vulnerable stroke patients such as those with atherosclerosis, obesity, infections, autoimmune diseases or in patients undergoing surgery. Numerous anti-inflammatory strategies were tested in recent years to reduce brain infarct volume, vascular injury and subsequent stroke in animal models. These anti-inflammatory strategies may have beneficial effects also for the treatment of human stroke (Muir et al. 2007).

In this article, we provide an overview of the current stand of knowledge on the role of immune and inflammatory responses in IS, with major emphasis on the involvement of DAMPs and of leukocytes infiltration. We discuss studies performed in commonly used animal models of IS and novel therapeutic approaches targeting DAMPs and leukocytes infiltration for the treatment of human IS.

Overview of Innate and Adaptive Immunity During IS

Innate immunity represents the first defense of the organism against pathogens and includes all mechanisms already existing before the encounter with the antigen. These mechanisms are unable to discriminate specific antigens and to activate an immunologic memory (Albiger et al. 2007). Inflammation acts as the integral part of the innate immune system and is triggered by several chemical mediators. Some of them directly derive from pathogens, like the pathogen-associated molecular patterns (PAMPs). Other mediators, such as DAMPs, are released from damaged cells and tissues. Both PAMPs and DAMPs activate the innate immune response (Newton and Dixit 2012). DAMPs are passively released from neuronal and non-neuronal dying brain cells and, through the binding with the Toll-like receptors (TLRs), they trigger a cascade of downstream signaling pathways that result in transcriptional changes and post-translational modifications (Gulke et al. 2018). DAMPs release induces a significant increase of other proinflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, reactive oxygen species (ROS) and inducible nitric oxide syntase (iNOS), mainly secreted by activated microglia (Ritzel et al. 2015). These molecules exert detrimental effects contributing to the development of several diseases, such as autoimmune, neurodegenerative and cardiovascular diseases including IS (Scrivo et al. 2011).

During cerebral ischemia, oxygen and glucose deprivation may induce an irreversible brain injury, which depends from the duration of ischemia and from the interested brain area. IS evokes an immediate inflammatory response that involves complex cellular and molecular mechanisms (Schmidt-Pogoda et al. 2019).

Platelets aggregation and leukocytes infiltration represent important cellular mechanisms involved in the response of innate immunity during IS. They increase during brain ischemia and exacerbate blood flow occlusion and brain damage (Kamel and Iadecola 2012). Platelets activation generates pro-inflammatory signals whereas activated leukocytes interact with the endothelium by the binding with adhesion molecules [intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and selectins (Iadecola and Anrather 2011)]. Moreover, leukocytes may also migrate and infiltrate brain tissue. The latter occurs when the permeability of BBB is altered. BBB damage represents a key element of brain injury. Different mechanisms are involved in the BBB breakdown during an ischemic insult. Brain ischemia induces the upregulation of hypoxia-inducible factor 1 (HIF-1) and vascular-endothelial growth factor (VEGF), which in turn contribute to the degradation of tight junction proteins between brain endothelial cells (claudin, occludin and junctional adhesion molecules) (Engelhardt et al. 2014). Increased oxidative stress and DNA damage also increase endothelial cell inflammation leading to upregulation of ICAM-1, pro-inflammatory chemokines and metalloproteinases (MMPs), in particular MMP-2 and MMP-9. Alterations of the permeability of endothelial channels for Na^+, K^+ and Ca^{2+} and the upregulation of aquaporin 4 also contribute to worse cerebral edema (Vella et al. 2015). The structural changes of BBB components facilitate the infiltration of peripheral immune cells, such as T cells, macrophages and neutrophils to the brain with a further worsening of BBB damage (Yang and Rosenberg 2011).

Apart from innate immunity, many evidence suggests that adaptive immunity also plays a contributory role during IS. Generally, the adaptive or acquired immune system is activated to protect the organism by exposure to pathogens or toxins and has immunologic memory. Adaptive immunity is mediated by B and T cells. B lymphocytes produce and release antibodies against all possible antigens. On the other hand, T lymphocytes exert an effector role (Cytotoxic T cells, T_C, that removes pathogens and infected host cells), a helper role (Helper T cells, T_H, that collaborates with B cells and other immune cells), or a regulatory role (regulatory T cells, Tregs).

Several studies suggest that T cell subtypes and their derived cytokines exhibit both adaptive and maladaptive effects in stroke (Papiernik et al. 1992; Selvaraj and Stowe 2017). Other T cell subtypes such as T_H1 and T_H17 cells, worse ischemic brain damage (Yilmaz et al. 2006;

Kleinschnitz et al. 2010; Luo et al. 2015), whereas other subtypes like T_H2 and Tregs may activate tissue repair mechanisms or induce anti-inflammatory cytokines release, such as TNF-β and IL-10. In addition, they may also inhibit T helper cells response and promote Tregs activation (Vignali et al. 2008; Liesz et al. 2009).

In middle cerebral artery occlusion (MCAO) murine models, $CD8^+$cytotoxic T lymphocytes exacerbate ischemic brain damage through the release of different molecules secreted by their cytotoxic granules, such as perforin and the granzymes (Yilmaz et al. 2006; Kleinschnitz et al. 2010). They also produce pro-inflammatory cytokines, such as TNF-α and interferon (IFN)-γ, leading to a further increase of inflammation, impairment of brain injury and neurological deficits (Li et al. 2001; Yilmaz et al. 2006).

Differently from $CD8^+$ T cells, Tregs act as suppressors of the activity of other T cells (Sakaguchi 2000; Bettelli et al. 2006) by releasing soluble inhibitory cytokines, such as transforming growth factor beta (TGF-β), interleukin 35 and interleukin 10 (Schmidt et al. 2012). Tregs can be distinguished from the other T cell subtypes by the expression of specific markers: CD4, CD25 and the transcription factor forkhead box (FoxP3) (Yan et al. 2009; Curiel 2007). Genetic depletion of Tregs profoundly increased brain damage and deteriorated functional outcome in MCAO mice, along with the post-ischemic activation of resident inflammatory cells, such as microglia and T cells. The latter represent the main sources of TNF-α and of cerebral IFN-γ, respectively. Early antagonization of TNF-α and IFN-γ prevented the infarct growth in Treg cells-depleted mice (Liesz et al. 2009).

Role of DAMPs in IS: Pre-clinical Evidence

In the presence of ischemic injury, innate immunity is activated in the early phase (Gelderblom et al. 2009). This process begins with the release of DAMPs. As previously mentioned, following ischemic damage DAMPs are released from various cell types including microglia and brain macrophages, brain endothelial cells and neurons (Chiba and Umegaki 2013; Gulke et al. 2018; Yang and Tracey 2010; Schmidt-Pogoda et al. 2019). Subsequently, by binding TLR4 and the receptor for advanced glycation end product

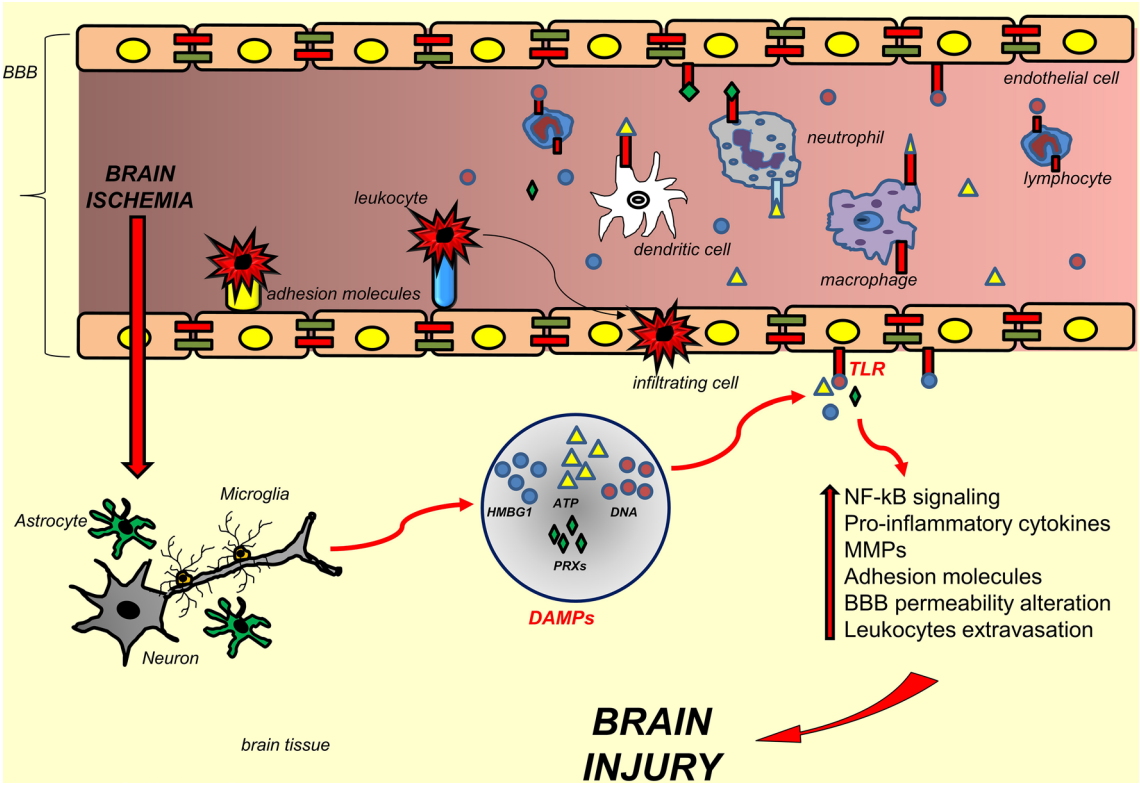

Fig. 1 Summary of the main inflammatory mechanisms involved in the brain injury associated with IS. Schematic representation of the events related to DAMPs-induced inflammation. DAMPs are released from brain cells following an ischemic insult and they interact with TLRs. TLRs activation leads to inflammation, MMPs activation, BBB breakdown and leukocytes extravasation, all factors that belong to the innate immunity and exacerbate the brain injury. See text for further details. Abbreviations legend: *ATP* adenosine triphosphate, *BBB* blood brain barrier, *DAMPs* damaged-associated molecular patterns, *HBMG1* high mobility protein B 1, *MMPs* metalloproteases, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells, *PRXs* peroxiredoxins, *TLRs* toll-like receptors

Table 1 Therapeutics strategies targeting DAMPs and leukocytes infiltration in the middle cerebral artery occlusion (MCAO) murine model

Therapeutic agent	Cell/molecular target	Mechanisms of action	References
Amlexanox (anti-HMGB1 antibody)	DAMPs	*HMGB1 inhibition* Neuroprotective effect	(Halder and Ueda 2018)
Anti-HMGB1 and anti-Prxs antibodies	DAMPs	*HMGB1 and Prxs inhibition* ↓IL-23, IL-12, Il-6, Il-1β, TNFα ↓brain damage	(Halder and Ueda 2018; Shichita et al. 2012b)
Phthalide derivative (CD21)	DAMPs	*Prx-1 inhibition* DAMPs/TLR4 binding inhibition ↓IL-6, TNFα, IL-1β	(Zou et al. 2020; Li et al. 2020)
Anti-selectins antibody	Leukocytes infiltration	*E-Selectins monoclonal antibody* Neurological outcomes improvement BBB injury reduction	(Huang et al. 2000)
TBC-1269	Leukocytes infiltration	*P-Selectin inhibitor* ↓brain myeloperoxidase and ischemic neurons ↑survival rate	(Anaya-Prado et al. 2008)
ICAM-1 gene deletion	Leukocytes infiltration	*ICAM-1 inhibition* Infarct size reduction Neurological functional improvement	(Connolly et al. 1996)
Xanthotoxol	Leukocytes infiltration	*Anti-oxidant and anti-inflammatory natural compound* ↓ICAM-1 and E-Selectin ↓brain edema, BBB damage ↓IL-1β, TNFα and IL-8	(He et al. 2013)
VCAM-1 gene deletion	Leukocytes infiltration	*VCAM-1 inhibition* Infiltrating cells reduction Infarct size reduction	(Liesz et al. 2011b)
Natalizumab	Leukocytes infiltration	*Anti-Integrin α4 monoclonal antibody* Anti-inflammatory effect Functional and neurological outcomes improvement	(Becker et al. 2001)
Fingolimod	Leukocytes infiltration	*Sphinogosine-1-phosphate receptor modulator* Infarct size reduction Functional outcomes improvement	(Liu et al. 2013)

(RAGE), DAMPs activate different pathways including TLRs and inflammasomes (Schaefer 2014; Zhang and Mosser 2008), thus exacerbating ischemic damage (Shichita et al. 2012a; Piccinini and Midwood 2010).

DAMPs include several molecules of different origin: high mobility group protein B1 (HMGB1), histones, DNA, RNA (nuclear compartment) (Goldstein et al. 2006), uric acid (Kono et al. 2010), ATP (Bours et al. 2006), heat shock proteins (cytosol) (Quintana and Cohen 2005), extracellular proteins (fibronectin, biglycan and tenascin C) and mitochondrial components (mtDNA). Once released, DAMPs induce a cascade of detrimental effects in the ischemic brain parenchyma (Kleinschnitz et al. 2010, 2013) (Fig. 1).

Apart from IS, DAMPs were shown to play a crucial role in the pathogenesis of different human diseases by their ability to induce inflammation (Land 2015). DAMPs may also represent a promising target for the development of new therapeutic strategies (Table 1), as it will be discussed later (Richardson et al. 1986; Land 2020).

High Mobility Group Box 1

HMGB1 is a 215 amino-acid nuclear protein that in normal condition binds and modifies the transcription of DNA enhancing genes. In the presence of injury or infection, HMGB1 promotes inflammation (Lotze and Tracey 2005; Yang and Tracey 2005). High levels of systemic HMGB1 were detected in serum of animal models of stroke (Nakano et al. 2019; Muhammad et al. 2008).

Apart from microglia, HMGB1 is secreted by immune cells, such as macrophages, monocytes and dendritic cells (Yilmaz and Granger 2010) and also by non-immune cells. HMGB1 binds different receptors such as TLRs, particularly TLR4 and TLR2, and RAGE that are expressed in the plasma membrane of different cell types (Yang et al. 2010; Qiu et al. 2008; Klune et al. 2008) (Fig. 1). The binding between HMGB1 and TLR4 is mediated by different adaptors including TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TRIF-related adaptor molecule

(TRAM) and myeloid differentiation factor 88 (My88) (Akira and Takeda 2004).The downstream effectors of HMGB1 include nuclear factor (NF)-kB, MAPK and type 1 INF-1, which contribute to a consequent release of TNF-α, IL-6, IL-8 and iNOS with further increase of inflammation (Takeuchi and Akira 2010).

In vitro experiments performed in microglia of MCAO rats and in primary neuronal and glial cell co-cultures confirmed that HMGB1 induced release of inflammatory mediators such as TNF-α, IL-1α, IL-1β, IL-6 and of other inflammatory proteins, increasing excitotoxicity and ischemic neuronal death (Kim et al. 2006).

In addition, in microglia derived from MCAO mouse model and treated with HMGB1 recombinant protein (rhHMGB1), the interaction between HMGB1 and TLR4 led to MAPK activation and NF-kB upregulation, increased production and release of cytokines, including TNF-α, IL-1β and IL-6. Conversely, these events were not found in the TLR4/MCAO-deficient mouse ($TLR4^{-/-}$), demonstrating that TLR4 is essential to mediate the detrimental effects of rhHMGB1. Consistently, studies performed in transgenic mice lacking either TLR2 or TLR4 reported a reduction of the inflammatory response and of the brain injury (Caso et al. 2007; Sansing et al. 2011).

Similarly, when HMGB1 or TLR4 were neutralized with specific antibodies, a protective effect was revealed. In these conditions, levels of inflammatory mediators, including TNF-α, IL-1β and Cyclooxygenase-2 (Cox2), were significantly downregulated as expected (Caso et al. 2007; Yang et al. 2011).

Muhammad et al. demonstrated that the pro-inflammatory effect of HMGB1 was also due to the binding with RAGE receptor in a permanent MCAO mouse model. In this model, the authors found that anti-HMGB1 intraperitoneal administration significantly reduced the infarct volume, but it failed to do so when administered to RAGE deficient mice ($RAGE^{-/-}$) (Liu et al. 2007; Muhammad et al. 2008). The effect of amlexanox, a HMGB1 inhibitor, was also evaluated in the MCAO model. Intracerebroventricular administration of amlexanox significantly protected the brain from ischemic damage (Halder and Ueda 2018). In the same model, treatment with an anti-HMGB1 antibody reduced levels of inflammatory cytokines (IL-23, IL-12, TNF-α, IL-6 and IL-1β) along with an improvement of ischemic brain damage (Halder and Ueda 2018).

Heat Shock Proteins

Heat shock proteins (HSPs) are molecules that normally act as chaperons and are involved in many biosynthetic pathways (Schaefer 2014). Some HSPs also induce inflammation through the activation of TLR2, TLR4 and CD91 (Zhou and Binder 2014; Schaefer 2014). Hsp70 is the most characterized HSP in IS. Depending on its localization, Hsp70 may exert either protective or detrimental effects. When intracellularly expressed, Hsp70 inhibits the expression of pro-inflammatory mediators such as NF-kB, MMPs and ROS. When expressed by the extracellular matrix, Hsp70 activates NF-kB signaling by its binding with TLRs on macrophages, dendritic cells and microglia (Gulke et al. 2018; Bartoletti-Stella et al. 2018; Guo et al. 2018).

Overexpression of Hsp70 by means of transgenes, viral constructs or fusion proteins was reported to exert neuroprotective effects in neurons and glia in preclinical models of stroke, to reduce cerebral infarct size in rats (Shevtsov et al. 2014; Zhan et al. 2010; Sharp et al. 2013) and to associate with the reduction of inflammatory mediators (TNF-α and IL-1β) in cerebral and endothelial cells (Kim and Yenari 2013).

Peroxiredoxins

Peroxiredoxins (Prxs) are a group of cytosolic DAMPs released in the extracellular space by necrotic brain cells (Fig. 1). Once released, Prxs interact with macrophages TLR2 and TLR4 leading to the production of pro-inflammatory cytokines such as IL-23 (Shichita et al. 2012b). IL-23 induces the release of IL-17 from T lymphocytes (γδ-T subtype), which in turn contributes to neuronal cell death (Shichita et al. 2009; Konoeda et al. 2010). In addition, Prxs promote a more pronounced macrophages tissue infiltration compared to HMGB1 (Shichita et al. 2012b). Interestingly, administration of a gastrodin derivative, a phenolic glycoside extract from tuber of *Gastrodiae rhizome,* was reported to reduce post-ischemic expression of Prxs (mainly Prx-1, Prx-2 and Prx-4) and of inflammation in the MCAO model (Mao et al. 2017). Other studies showed the neuroprotective and anti-inflammatory effects of the phthalide derivative CD21, a natural compound isolated from the rhizome of *L. porteri*. This molecule acts as a DAMPs/TLR4 pathway inhibitor in the MCAO model. In the specific, administration of CD21 reduced Prx-1 expression and TLR4/NF-kB activity and suppressed the inflammatory responses mediated by IL-6, TNF-α and IL-1β. These effects were associated with an overall reduction of infarct volume and of neurological deficits (Zou et al. 2020; Li et al. 2020).In the MCAO mouse, Shichita et al. reported that anti-Prxs antibody administration 12 h after stroke was able to attenuate ischemic brain damage and to improve neurological score, along with a reduction of inflammatory cytokines expression (Shichita et al. 2012b). Interestingly, the authors demonstrated that Prxs were produced in the acute phase of IS (from 12 to 24 h after stroke onset) whereas HMGB1 production was observed in the hyperacute phase (within

6 h following stroke). The latter evidence suggests that Prxs display a longer therapeutic time window compared to HMGB1.

Leukocytes Infiltration in IS: Preclinical Evidence

Evidence obtained in MCAO models demonstrated that both cytotoxic T lymphocytes and Tregs are involved in leukocytes infiltration following IS. In the MCAO rat model, cytotoxic T lymphocytes were strongly implicated in the neutrophils infiltration that occurs after reperfusion by producing and releasing pro-inflammatory cytokines, mainly IFN-γ, with an increase of ICAM-1 expression (Schroeter et al. 1994; Jander et al. 1995). Autocrine production of IFN-γ improved also both motility and function of $CD8^+$ cytotoxic cells whereas inhibition of IFN-γ markedly reduced cytotoxic function, motility and cell survival of $CD8^+$ cells (Bhat et al. 2017).

Conversely, systemic administration of purified Tregs derived from donor mice in both MCAO mouse and rat models reduced leukocytes infiltration and peripheral inflammatory cells (dendritic cells, macrophages and neutrophils) in the brain lesions and attenuated BBB damage. In fact, both in vivo and in vitro studies demonstrated that Tregs treatment after ischemia improved BBB integrity, by preserving the ultrastructure of tight junctions and of basement membranes, through the suppression of MMP-9 production (Abdulkareem et al. 2013; Li et al. 2013).

Endothelial selectins and integrins also participate to the process of leukocytes infiltration during IS. When activated, P-selectin and E-selectin translocate to the endothelial surface where they interact with leukocytes. On the other hand, L-selectin is expressed on leukocytes surface and is essential for leukocytes recruitment to the site of injury (Bargatze et al. 1994). Additional proteins, such as integrins and adhesion molecules, including ICAM-1 and VCAM-1, are involved in leukocytes rolling (Edwards and Bix 2019).

Both genetic and pharmacological inhibition of E-selectin led to an improvement of neurological outcome in the MCAO mouse model (Huang et al. 2000), whereas P-selectin-deficient mice showed decreased BBB breakdown (Jin et al. 2010). Administration of the selectin inhibitor TBC-1269 reduced brain myeloperoxidase level, number of ischemic neurons and improved survival rates (80% in TBC-1269 treated rats vs. 40% in controls rats) in a rat model of global cerebral ischemia (Anaya-Prado et al. 2008).

The adhesion molecules ICAM-1 and VCAM-1 were widely investigated in IS (Stanimirovic et al. 1997). Inhibition of ICAM-1 associated with decreased brain damage and leukocytes infiltration (Kitagawa et al. 1998; Kanemoto et al. 2002; Vemuganti et al. 2004). ICAM-1 knockout mice also displayed reduced infarct size, as well as an improvement of neurological function (Connolly et al. 1996). Xanthotoxol is a biologically active linear furocoumarin that occurs in a large number of plants and is mainly extracted from the fruit of *Cnidium monnieri cusson* (He et al. 2007). It exerts several pharmacological activities such as anti-inflammatory, anti-oxidant and neuroprotective effects (Ng et al. 2000). In a rat model of focal cerebral ischemia/reperfusion, xanthotoxol administration correlated with reduction of brain edema, decreased ICAM-1 and E-selectin levels and inhibition of neutrophils infiltration. Xanthotoxol treatment also attenuated BBB disruption and reduced levels of pro-inflammatory cytokines (IL-1β, TNF-α, IL-8) (He et al. 2013).

The downregulation of VCAM-1 improved stroke outcomes in pre-clinical models (Zhang and Wei 2003; Cervera et al. 2004). MCAO mice carrying genetic knockdown of VCAM-1 showed reduced T lymphocytes infiltration and reduced infarct volume (Liesz et al. 2011b). However, intravenous injection of anti-VCAM-1 antibody failed to show a neuroprotective effect in other experimental conditions (Justicia et al. 2006).

Different integrins were targeted for potential pharmacological therapies, such as lymphocyte function-associated antigen 1 (LFA-1), macrophage-1 antigen (Mac-1) and, more recently, antigen-4 (VLA-4). Studies performed in animal models of stroke demonstrated that the administration of monoclonal antibody against VLA-4 was able to reduce leukocytes adhesion to the activated endothelium and to prevent lymphocytes migration to the inflamed tissue (Becker et al. 2001). In addition, integrins downregulation reduced infarct volume and neurological deficits (Arumugam et al. 2004; Chen et al. 1994; Prestigiacomo et al. 1999).

Finally, administration of fingolimod, a sphingosine-1-phosphate receptor modulator widely used for the treatment of multiple sclerosis, was found to inhibit extravascular migration of leukocytes in animal models of cerebral ischemia (Massberg and von Andrian 2006; Liesz et al. 2011a; Liu et al. 2013). The main therapeutic strategies targeting leukocytes infiltration in the MCAO model are reported in Table 1.

DAMPs and Leukocytes Infiltration in Human IS

Recently, Malone et al. highlighted the need to develop novel therapeutic treatments in addition to thrombolysis and thrombectomy, particularly for those patients who, for different reasons, cannot receive these therapies (Malone et al. 2019b). The novel therapies should reduce post-ischemic brain damage and, at the same time, activate the mechanisms of tissue remodelling. Therefore, they could improve the functional outcomes by increasing patients' survival.

Due to the role played by immunity in each stroke phase, a wide range of immune-targeted therapies was developed and is currently being tested (Malone et al. 2019a; Neuhaus et al. 2017) (Table 2).

The pre-clinical evidence described above suggests that DAMPs release and leukocytes infiltration are pivotal mechanisms of the immune response during IS, and they also represent interesting therapeutic targets for the treatment of human stroke. On the other hand, they may also represent useful predictors of both stroke onset and progression.

In this section, we discuss relevant human studies that corroborate the findings obtained in preclinical models of IS.

DAMPs

In patients with cerebral ischemia, elevated HMGB1 levels were found to correlate with severe stroke size (Huang et al. 2013; Schulze et al. 2013; Sapojnikova et al. 2014; Goldstein et al. 2006). In a study performed in 338 patients with IS, plasma level of HMGB1 was reported to correlate with elevated mortality and unfavourable outcome after 1-year of follow-up. This evidence suggests that plasma HMGB1 level may represent an important biomarker for predicting the clinical outcomes of IS after 1-year (Huang et al. 2013).

High plasma level of Prx-1 and low level of Prx-5 were found in patients with IS soon after the onset of symptoms (Kunze et al. 2014; Richard et al. 2016; Mao et al. 2017). Interestingly, in a study of 98 patients the level of Prx-5 was lower in the presence of severe stroke and was inversely correlated with markers of inflammation. The observed reduction of circulating Prx-5 in severe stroke may be explained by its premature degradation or reduced synthesis. Further studies are needed to define the potential role of Prx-5 in stroke. The role of other DAMPs, such as HSPs, should also be evaluated in humans (Kunze et al. 2014).

Some studies reported that DAMPs inhibition represents an efficacious strategy also in human IS. Tada et al. demonstrated that administration of olmesartan, an Angiotensin II type I receptor blocker, showed antioxidant and anti-inflammatory properties, with a significant reduction of plasma levels of Prxs and of the oxidized low-density lipoprotein/β-2-glycoprotein-I complex (oxLDL/β2GPI) after 12 weeks of treatment in stroke patients (Tada et al. 2015). To the best of our knowledge, specific inhibitors of DAMPs have not yet been tested in humans.

Leukocytes Infiltration

Leukocytes infiltration was observed in specimens of stroke patients. In this regard, T lymphocytes, mainly CD8+ cytotoxic cells, and macrophages accumulation were observed in human stroke lesions (Zrzavy et al. 2018). Increased blood levels of $CD4^{+}CD28^{null}$T cells were found to correlate

Table 2 Therapeutics strategies targeting DAMPs and leukocytes infiltration in human IS

Type of study	Therapeutic agent	Cell/molecular target	Mechanisms of action	References
IS patients	Olmesartan	DAMPs	*Angiotensin II type 1 receptor blocker* ↓Prxs	(Tada et al. 2015)
Enlimomab Acute Stroke Phase III clinical trial	Enliomab	Leukocytes infiltration	*ICAM-1 antibody* No beneficial effects in humans were found	(del Zoppo 2010; Enlimomab Acute Stroke Trial 2001)
LeukArrest Phase III and ASTIN clinical trials	Rovelzumab (Hu23F2G)	Leukocytes infiltration	*Anti-Integrin Mac-1 monoclonal antibody* No beneficial effects in humans were found	(Liesz et al. 2011b; Becker 2002)
ACTION Phase II clinical trial	Natalizumab	Leukocytes infiltration	*Anti-Integrin α4 monoclonal antibody* No beneficial effects in humans were found	(Becker et al. 2001; Elkins et al. 2017)
IS patients	Fingolimod	Leukocytes infiltration	*Sphinogosine-1-phosphate receptor modulator* Improvement of neurological functions Decreased microvascular permeability Lower circulating lymphocytes; Smaller lesion volumes and hemorrhage	(Fu et al. 2014; Zhu et al. 2015)

IS ischemic stroke, *ASTIN* acute stroke therapy by inhibition of neutrophils

with stroke severity and serum levels of proinflammatory cytokines in IS patients (Tuttolomondo et al. 2015).

As described above, selectins, adhesion molecules and integrins, expressed on the endothelial cell surface play a pivotal role in leukocytes extravasation. Their suppression represents a potential strategy for conferring protection against IS, as shown in preclinical models.

Fu et al. reported that the oral administration of fingolimod showed beneficial effects on cerebral infarct size, rates of haemorrhagic transformation and neurological function in patients with IS (Fu et al. 2014).The protective effects of fingolimod, administered in combination with alteplase, a fibrinolytic agent, were also observed in a separate trial by Zhu et al. (2015). At the molecular level, the fingolimod prevented normal egress of lymphocytes from lymphoid organs and also inhibited leukocytes and NK cells infiltration into the central nervous system (Chun and Hartung 2010).The efficacy of natalizumab has been also evaluated in the phase II "ACTION" clinical trial in patients with acute IS. Although natalizumab administration did not affect infarct volume, some neurological improvements were reported (Elkins et al. 2017).

Despite the promising evidence that inhibition of leukocytes infiltration could exert protective effects in humans, no other positive results were obtained in this regard. For instance, the inhibition of ICAM-1 by using a specific antibody, enlimomab, did not show beneficial effects in a phase III clinical trial. In fact, patients treated with enlimomab reported higher mortality, increased incidence of serious adverse events and more frequent infections (Enlimomab Acute Stroke Trial 2001; del Zoppo 2010). Rovelzumab (also known as Hu23F2G), a monoclonal antibody targeting the integrin Mac1, also failed to show neuroprotective effects in the human phase III clinical trial "LeukArrest" (Becker 2002). The same negative result was reported by the "Acute Stroke Therapy by Inhibition of Neutrophils" (ASTIN) trial (Krams et al. 2003).

Conclusions

The evidence discussed in our review suggests that DAMPs-induced inflammation and innate immunity mechanisms represent key elements in the early stage of IS. In fact, studies performed in animal models of IS revealed that BBB disruption and the consequent leukocytes extravasation exacerbated ischemic injury. DAMPs also enhanced leukocytes extravasation, thus creating synergistic deleterious effects in the cerebral ischemic area. Therefore, the inhibition of DAMPs and of leukocytes infiltration appears as a promising therapeutic approach for IS. In this regard, we reviewed studies demonstrating that specific antibodies or inhibitors able to target these mechanisms improved neurological outcomes and reduced infarct size both in preclinical models and in IS patients. However, further efforts should be performed in this field. First of all, additional drugs targeting DAMPs and leukocytes infiltration should be characterized in order to achieve a better site-specific action. Some compounds that have been shown to be efficacious in pre-clinical models of stroke failed to exert the same beneficial effects in clinical trials. To overcome this issue, pre-clinical models which best mimic the human immune and inflammatory responses during stroke should be developed. The therapeutic time window for drug administration should also be better defined in humans.

Overall, further human studies are needed to solve the current controversial findings and uncertainties.

Author Contributions Conceptualization: [RS, SR]; Writing—original draft preparation: [RS]; Writing—review and editing: [RS, MF, SR]; Literature search: [RS, MC, FB, SM]; Critical revision: [SR]. All authors read and approved the final manuscript.

Funding This work was supported by a Grant from the Italian Ministry of Health.

Compliance with Ethical Standards

Conflict of interest None to declare.

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