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The Immune System and Stroke: From Current Targets to Future Therapy

RUNNING TITLE: The Immune System and Stroke

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ABSTRACT

Stroke is a major cause of morbidity and mortality worldwide. Despite the intensive search for new therapies, hundreds of agents targeting various pathophysiological mechanisms have failed clinical trials, and the thrombolytic agent tissue plasminogen activator is currently the only FDA-approved medication for the treatment of acute ischaemic stroke (AIS). The immune system is involved in all stages of stroke, from the pathogenesis of risk factors to neurotoxicity, to tissue remodelling and repair. There is a bi-directional interaction between the brain and the immune system, with stroke-induced immunosuppression and subsequent infection a principal source of patient mortality. Newer work also points to a role for the gut microbiota in the immune response to stroke, while clinical sequelae such as dementia might now also be explained in immune terms. However, the exact roles of innate and adaptive components have not been fully elucidated, with studies reporting both detrimental and beneficial functions. Time is a key determinant in defining whether immunity and inflammation are neuroprotective or neurotoxic. The local inflammatory milieu also has a clear influence on many proposed treatments. This review examines the individual components of the immune response to stroke, highlighting the most promising future stroke immunotherapies.

INTRODUCTION

Stroke, a cerebrovascular disease of which 87% are ischaemic in nature, remains a major cause of morbidity and mortality worldwide[1, 2]. It stands as the most common cause of acquired disability, with roughly 100 million disability-adjusted life years (DALYs) lost annually by survivors [1]. However, despite the intensive search for new therapeutic strategies, the options for acute ischaemic stroke (AIS) treatment are limited; intravenous recombinant tissue plasminogen activator (rtPA) is the only FDA-approved medication[3]. tPA can restore perfusion to the brain via thrombolysis and thereby prevent cell death in the hypoperfused but potentially salvageable area known as the ischaemic penumbra. However, beyond a therapeutic window of 4.5 hours, the benefits of tPA are outweighed by its risks, with the incidence of haemorrhagic transformation increasing dramatically [2]. Alternatives to thrombolysis include intra-arterial thrombectomy with medical devices, but these are not without their own complications and limitations [3].

Over the last few decades, neuroprotection has been the main focus of proposed treatments in AIS. To date however, the field of brain-protective medicines remains a therapeutic graveyard, with over 1000 different molecules buried across hundreds of failed clinical trials [4]. Yet, despite the poor clinical translation, both animal and human studies show that the immune response plays a pivotal and multi-faceted role in central nervous system (CNS) injury and new candidate therapies have been suggested as a result.

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Several reviews have examined the intricate connection between the immune system and CNS in stroke [5-9]. To the best of our knowledge, however, none have examined the full spectrum of immune targets and potential therapies. The aim of this review, therefore, is to discuss not only the contribution of the immune system to disease initiation, progression and resolution in stroke, but also the most promising avenues for future stroke immunotherapy.

THE IMMUNE RESPONSE TO ACUTE ISCHAEMIC STROKE

Stroke Onset

Following the onset of brain ischaemia, a sequence of events involving the CNS, its vasculature, the blood, and lymphoid organs is invoked. Ischaemic stroke starts in the blood vessels, where arterial occlusion results in hypoxia, reactive oxidative species (ROS) production, and changes in shear stress across the lumen wall [10, 11]. The coagulation cascade is triggered, resulting in microvascular occlusion. Platelet aggregation is exacerbated by the concomitant fall in the bioavailability of nitrous oxide (NO). The combined effect of oxidative stress, inflammatory mediators (e.g. IL-1, TNF-α), downregulated endothelial junction proteins and upregulated leukocyte- or vascular-derived proteases increases blood brain barrier (BBB) permeability. A critical point is reached if these processes causes detachment of endothelial cells from the basement membrane, at which stage the unimpeded entry of free water and serum into the brain leads to haemorrhage [6].

Endothelial cell-derived prostaglandins and chemoattractant also drive leukocyte entry into the infarct site [6]. The infiltration of neutrophils, macrophages, and other leukocytes is further promoted by the activation of high-avidity integrin molecules on the leukocyte surface and the upregulated expression of corresponding ligands on the endothelium e.g. vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1(ICAM-1). Activated leukocytes produce ROS, proteolytic enzymes, leukotrienes, cytokines, platelet-activating factor which promote vasoconstriction, platelet aggregation, and further neurotoxicity [10]. In the perivascular space, activated macrophages secrete a host of pro-inflammatory cytokines, while mast cell degranulation results in the release of histamine, proteases and TNF-α, further degrading BBB integrity.

While all these processes are occurring in the blood vessels and perivascular spaces, ischaemia also impacts the brain parenchyma. Hypoperfusion causes an immediate deprivation of glucose and oxygen, leading to a fall in ATP production A series of interconnected cytoplasmic and nuclear events then begins (bioenergetic failure, acidotoxicity, excitotoxicity, oxidative stress and inflammation), causing neuronal cell death [11]. The release of danger/damage-associated molecular patterns (DAMPs) from dying and dead neurons precipitates a new phase of the inflammatory response [8]. Activation of pattern-recognition receptors (PRRs) leads to the production of IL-1β and TNF-α. An inflammatory environment emerges as a result, containing IL-17, perforin, granzyme, and increasing concentrations of ROS [11].

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The adaptive arm of the immune response is activated as early as 24 hours post-stroke [8]. It is widely accepted that T cells have a damaging effect in this acute phase of stroke, as mice lacking T cells showed smaller infarct size and improved functional outcome [12]. The fact that this neuroprotection is abolished by the re-introduction of T cells further corroborates the view that T lymphocytes are involved in stroke pathophysiology. Natural killer (NK) cells and CD4+ T cell subtypes exacerbate neurotoxicity in the early stages of stroke in an antigen-independent manner, possibly via the secretion of several cytokines, including IFN-γ and IL-17 [6]. Infiltrated γδ and CD8+ T cells mediate further neuronal cytotoxicity. Antigen-specific T cells, on the other hand, only contribute later to stroke pathophysiology, participating in autoimmune or tolerance reactions in a manner largely dependent on whether they are skewed towards a more damaging (T helper 1 (T_H1), T helper 17 (T_H17)) or protective (T helper 2 (T_H2), T regulatory (T_{reg})) phenotype [9]. Lastly, B cells can produce antibodies against brain-derived molecules, resulting in further neuronal damage in the weeks following disease onset and possibly leading to clinical stroke sequelae such as dementia [13].

Initial inflammation is self-limiting and ultimately gives way to structural remodelling and functional reorganisation. The end of the acute phase is characterised by three processes [10]: 1) removal of dead cells/tissue debris, 2) creation of an anti-inflammatory milieu, and 3) production of pro-survival factors.

The processes of repair and reconstruction, which involves neuronal sprouting, angiogenesis, neurogenesis, and alteration to the cellular matrix, are carried out in concert by many cells types, ranging from immune cells to neurons and astrocytes. Together, these cells produce growth factors and proteases, allowing for re-modelling of the ischaemic site [10].

Stroke-induced immunosuppression

The relationship between the immune system and central nervous system operates bidirectionally. In the early stages of stroke, activation of primary and secondary lymphoid organs produces a systemic inflammatory response, a transient 24-hour period in which circulating levels of cytokines, chemokines and pro-inflammatory mediators are elevated. This early inflammatory response is followed two days post-stroke by a second phenomenon termed stroke-induced immunodepression (SIID) [14]. SIID is hallmarked by lymphopenia, splenic atrophy and increased levels of anti-inflammatory cytokines[17]. Mechanistically, the CNS elicits these changes in immune function through complex humoral and neural pathways, which include the sympathetic nervous system (SNS), the vagus nerve, and the hypothalamic-pituitary-adrenal (HPA) axis. It is unclear whether the nature of the immune response after stroke is influenced by brain infarct location, but the extent of brain damage does seem to be a crucial factor in SIID [15].

Large studies have estimated that approximately 30% of stroke patients contract post-stroke infections, the most prevalent of which are pneumonia and urinary tract infections [16]. The most commonly implicated microorganisms include *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*. Roughly half of pneumonia

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cases occur within the first two days after stroke, while almost all others are seen within a week. Indeed, a prospective study of stroke patients showed that 75% of post-stroke infections are diagnosed within three days of hospitalization, indicating a large role for SIID and not poor patient care [16].

Although SIID is detrimental with respect to resulting post-stroke infections, it may teleologically have a protective function, whereby downregulation of the peripheral immune response shields the CNS from autoimmunity. Blood-brain barrier breakdown during the acute stage of stroke allows almost unfettered access of immune cells to the CNS, and antigens normally sequestered in the brain become visible, possibly for the first time. Evidence suggests these antigens can enter the circulation and be presented to lymphocytes in lymph nodes within hours of experimental stroke, thereby inducing autoreactive T and B cells [17]. While the phenomenon of stroke-induced immunosuppression could prevent such autoimmune responses, there are mitigating factors which help to subvert it, including infection. Autoreactive immune responses are promoted in two main ways [8]:

- Bacterial components activate Toll-like Receptors (TLRs) on Antigen-Presenting Cells (APCs) resulting in overall increased antigen presentation and immune stimulation.
- Increased levels of IFN-γ provide an inflammatory milieu which favours the induction of T_H1 type responses

The overall consequences of SIID therefore remains unclear, with the net outcome likely to differ significantly between patients. On the contrary, the deleterious effects of infections at all stages of stroke are beyond question. As such, strategies which aim to reduce their prevalence (including prophylactic antibiotics, hygiene precautions, scoring algorithms for infection diagnosis, suction or modified diets to prevent aspiration, and antiseptic-impregnated catheters) are sure to receive increased attention going forward.

Stroke and the Gut Microbiota

The gastrointestinal (GI) tract contains a large and complex immune subsystem [9]. Several mechanisms allow elements of the gut-associated immune system to relay diverse signals from commensal bacteria to the host. In this way, the gut microbiota plays a key role in the immune system, both in terms of its maturation and subsequent homeostasis. Recently, the relationship between the gut microbiota and the brain has been better elucidated. Studies have shown, for instance, a link between commensal bacteria and autoimmune conditions in the CNS [18]. Importantly, dysbiosis of the gut microbiota could impact acute brain injuries such as stroke. Indeed, antibiotic-induced disturbances of the intestinal flora have been shown preclinically to influence both post-stroke neuroinflammation and overall stroke outcome, with the same effect transferable between mice by faecal transplant [19].

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Stroke can markedly affects the composition of the intestinal microbiota, which may be detrimental to overall disease outcome [20]. A reduction in species diversity and an overgrowth of certain phyla, such as Bacteroidetes, is observed after stroke. This dysbiosis is postulated to influence immune function through an aberration in T cell homeostasis, increased levels of pro-inflammatory cytokines, and migration of intestinal lymphocytes to the ischaemic brain [20]. However, given that our knowledge of the gut microbiota and to some extent stroke itself is still limited, there may be many more mechanisms by which the brain-gut axis affects disease outcome, such as the infiltration of activated intestinal monocytes into the infarct site in the acute phase of stroke.

TARGETS & THERAPIES

The next section considers the key innate and adaptive components that demonstrate potential as drug targets, and from this, highlights an array of plausible future stroke immunotherapies.

Complement

A wealth of evidence indicates a harmful role for the complement pathway in the pathology of acute ischaemic stroke. The complement system, however, also plays an important part in the subacute and chronic post-stroke processes of neurogenesis and repair [21]. Of note, although expression of complement proteins was long thought to be restricted to cells of the immune system, it is now known that astrocytes, microglia and neurons express complement proteins and their receptors. The complex role of complement in stroke has confounded efforts to develop anti-complement stroke therapies. To date, the majority of pre-clinical studies have focused on the contribution of anaphylatoxins C3a and C5a to perpetuating ischaemia-induced neuroinflammation and neuronal death [22]. Mice lacking C3a receptors, for example, showed reduced ischaemic injury and improved functional outcome, while similar results were achieved with use of a C3a receptor antagonist [23]. In a separate study, C3a receptor antagonism suppressed T cell infiltration, promoted neuroblast formation and provided better histologic and functional outcomes [24]. Inhibition of C5a receptor decreased infarct size and demonstrated an improving trend in neurological function [25]. Importantly, stroke patients deficient in mannose-binding lectin have better outcomes [26]. Increased complement protein deposition has also been demonstrated in the infarcted human brain. These findings overall corroborate the view that complement has a deleterious role in the early stages of AIS. However, clinical translation of this effect has been limited by concerns such as the requirement for high dose of inhibitor, low bioavailability, poor efficacy, and increased risk of post-stroke infection [21]. Site-specific inhibitors such as the hybrid molecules sCR1 and sCR1sLex were designed to circumvent these issues. sCR1 ameliorated ischemic injury in mice, while sCR1-sLex further reduced infarct volume, improved neurological outcome, and inhibited neutrophil and platelet recruitment in mice [27]. Nevertheless, sCR1 did not prove neuroprotective in a non-human primate model of stroke, even though it was administered prior to middle cerebral artery occlusion [28]. Similar studies performed with sCR1sLex were terminated after interim analysis showed larger infarct volumes in the treated

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baboons [29]. Other proposed site-targeted complement inhibitors include compounds attached to recombinant portions of complement receptor type 2 (CR2). A single dose of CR2-Crry ameliorated brain injury and improved neurological outcome in mice without increasing the susceptibility to infection [30]. Intravenous immunoglobulin (IVIG) reduced mortality and functional impairment [31]. The future of complement therapy in stroke may require both intravenous immunoglobulin (IVIG) and cocktail of the antagonists and inhibitors described above. Since tPA can activate the complement pathway, these agents may also be indicated as an adjuvant to enhance the therapeutic window for thrombolytic therapy in the acute phase of the disease [32].

Inflammatory mediators

The pathogenic role of IL-1 has been demonstrated in rodent models of cerebral ischaemia, where IL-1β administration at time of reperfusion lead to increased brain oedema, whereas IL-1α/β double knockout mice showed reduced infarct size [33]. Separate studies focused on IL-1R1 or its inhibitor, in which deficiency/inhibition of the former or overexpression/administration of the latter both improved stroke outcomes [34, 35]. The recombinant IL-1 receptor antagonist (IL-1Ra), Anakinra, has been extensively studied in almost all stroke types, with a recent systematic review concluding it provides a clear reduction in infarct size in experimental stroke [36]. Phase-II clinical trials have also proved intravenous Anakinra safe and well-tolerated [37]. However, as the intravenous formulation is no longer manufactured, the effects of a subcutaneous formulation, administered twice daily for 72 hours after acute ischemic stroke was investigated [38]. The results showed IL-1Ra significantly reduced plasma concentrations of IL-6 and C-reactive protein. Mediation analysis, however, also revealed a residual negative effect, possibly due to an interaction between IL-1Ra and tPA. Further experimental studies are therefore required to determine whether therapies targeting IL-1 can be safely used in conjunction with thrombolysis.

TNF-α is produced within the first hour after stroke and the circulating levels of TNF-α are correlated with infarct size and neurological impairment, while inhibition of TNF-α protects against ischaemic brain injury [7]. TNF-α works in synergy with IL-1β, though it seems to mediate neuroprotective as well as neurotoxic functions. TNF-α receptor-deficient mice, for example, showed increased infarct volume, whereas pre-ischaemic treatment with TNF-α appeared to induce tolerance and reduce ischaemic injury [39, 40]. The action of TNF-α may depend on both location and timing; higher levels in the striatum, for instance, led to neurodegeneration, whereas release in the hippocampus led to neuroprotection [41]. Further studies highlight gene polymorphism, receptor subtypes, and cell signalling pathways as determinants of what response TNF-α will elicit [42, 43]. In terms of treatment, several inhibitors of TNF-α and its receptors have been studied, albeit in the setting of post-stroke chronic neurological dysfunction [44]. Notably, Etanercept or its murine analogue, attenuated motor deficits in both human stroke and a rodent model of traumatic brain injury [44]. To date however, no human trials of Etanercept in acute ischaemic stroke have been published. Thus, both the efficacy of TNF-α therapies and the possibility that these agents might exacerbate post-stroke infections have yet to be addressed.

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Chemokine molecules seem to play a detrimental role in the early stages of ischaemia. CCL5 knockout mice, for example, showed smaller infarct size, while inhibition of CXCL8 offered neuroprotection in transient brain ischaemia [45, 46]. Similarly, overexpression of CCL2 exacerbated brain injury, most likely due to increased immune cell recruitment and infiltration at the ischaemic site [47]. However, evidence has emerged which points to a second, neuroprotective function for chemokines, possibly due to the increased recruitment of stem cells after ischaemic insult [48]. Several therapeutic approaches have been proposed in AIS including modified chemokines, small molecule receptor antagonists, neutralising antibodies, and pathogen-derived receptor antagonists [49]. A number of these approaches have demonstrated promise. Use of the pan-chemokine specific inhibitor NR58–3.14.3, for example, reduced infarct size, attenuated the post-ischaemic inflammatory response, and improved neurological outcome in rats when infused over a 72-hour period [50]. Separately, antibodies directed against CXCL8/CCL2 improved experimental stroke outcome by reducing brain oedema and BBB permeability respectively [49]. In comparison to other stroke immunotherapies, the clinical translation of approaches targeting chemokines may be far off, with issues such as timing of administration and drug concentration still yet to be addressed. In particular, the risk of blanket chemokine inhibition on the recruitment of neuroprotective immune cells (e.g. M2 skewed macrophages) has yet to be resolved. Even so, drugs such as JWH-133 have achieved some form of selective response. The synthetic cannabinoid 2 receptor agonist, inhibits CXCL2-mediated neutrophil recruitment to the CNS without compromising response in the periphery [49]. This lack of systemic modulation may be particularly beneficial, given the SIDS-induced mortality from post-stroke infections.

Matrix metalloproteinases (MMPs) become dysregulated in ischaemic stroke, resulting in neurovascular disruption and parenchymal destruction. Several MMPs, including MMP-2, MMP-3, and MMP-9, have been implicated in BBB degradation and infarct growth [6]. In particular MMP-9, which is upregulated 15-48 hours after stroke, has been linked to disease pathology, with higher serum levels a predictor of poorer outcome [51]. Data from knockout mouse models and pharmacological experiments demonstrated the deleterious role of MMPs in acute stroke and the reduction in ischemic damage that can be achieved with MMP-targeted treatments [52]. Further evidence however, suggests that MMPs also contribute to neurovascular remodelling and neurovasculogenesis [53]. Due to this biphasic response, it is possible that future MMP inhibitors (such as a recombinant form of the natural inhibitor, TIMP-1) might only be used short-term post-stroke, so as not to interfere with angiogenesis. MMP inhibition might also be clinically relevant in the context of thrombolysis, as levels of MMP-9 are known to be elevated in response to tPA administration. In fact, it is believed that the interaction between tPA and MMP-9 is responsible for the phenomenon of haemorrhagic transformation; therefore, drugs which inhibit MMP-9 activity (e.g. minocycline) might be used clinically as a combination therapy with tPA in order to improve patient safety and lengthen the therapeutic window for tPA treatment [54].

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Adhesion molecules

E- and P-selectin have been implicated in particular in leukocyte recruitment, promoting neuroinflammatory responses and ischaemic injury in the early phases of the disease [10]. Selective blockade of E- or P-selectin is associated with improved neurological outcomes in experimental stroke [55]. In contrast, an anti-L-selectin antibody did not reduce infarct volume or neutrophil infiltration in a rabbit model of transient focal cerebral ischaemia [56]. At the level of clinical translation, an ongoing clinical trial is investigating the use of intranasal E-selectin in the secondary prevention of stroke. Such a therapy, which relies on the induction of tolerance to the inflammatory response following cerebral ischaemia, may yet offer a way forward for selectin-based treatments [57].

Several *in vitro* and human studies have demonstrated an increased production of ICAM-1 following ischaemia [58]. Expression levels of this adhesion molecule increase within hours after the onset of ischaemia and peak 12-48 hours later, making them a suitable target in the acute phase. Indeed, ICAM-1 knockout mice showed reduced infarct size, decreased neurological deficit, and improved cerebral blood flow [59]. Several other studies which employed ICAM-1 deficiency/blockade confirmed a reduction in ischaemic damage and leukocyte infiltration [60, 61]. Disappointingly however, results of a phase III clinical trial involving anti-ICAM-1 antibody enlimomab showed poorer stroke outcomes and higher mortality, possibly as a result of increased neutrophilic activity [62]. Retrospectively, pro-inflammatory responses seen in enlimomab-treated patients have been attributed to the antigenicity of this murine antibody. As a result, a more definitive verdict on the future of anti-ICAM-1 immunotherapy has been postponed until humanized antibodies are tested.

Future adhesion molecule-based therapies may also target the integrin molecules expressed by leukocytes as well as activated endothelium. Such molecules include leukocyte function-associated antigen-1 (LFA-1), very late adhesion molecule-4 (VLA-4), and Mac-1 [58]. Experimental studies of Mac-1 blockade or Mac-1/LFA-1 deficiency showed benefits such as reductions in infarct volume, neurological deficit, and leukocyte adhesion/infiltration [63]. Natalizumab, an anti-VLA-4 antibody used to attenuate neuroinflammation in multiple sclerosis (MS), reduced brain damage and improved clinical outcomes in experimental stroke [64]. Recent results of the phase II clinical trial "ACTION" however, failed to show a reduction in infarct growth, although improvements in functional outcomes warranted further investigation [65]. Disappointingly, the follow-up phase IIb trial ("ACTION2") did not meet its primary or secondary endpoints. As a result, further development of natalizumab in acute ischaemic stroke will not be pursued.

Immune cells

Typically, neutrophils are thought of as the "first responders" to stroke, with infiltration starting within 30 minutes of the onset of ischaemia and peaking 1-3 days later [5]. However, despite the demonstration of deleterious mechanisms (e.g. capillary sludging, neutrophil extracellular trap formation), an experimental model of neutrophil depletion showed no effect

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on infarct size [66]. Furthermore, treatments which should reduce neutrophil infiltration, such as neutrophil inhibitory factor, failed to improve stroke outcomes in human clinical trials [67]. One possible reason for the discrepancy between the original hypothesis and the negative clinical outcomes is the ability of neutrophils to polarise from a pro-inflammatory "N1" phenotype to an anti-inflammatory "N2" phenotype in response to local cytokine signalling. This phenomenon, similar to the theory of classically (M1) or alternatively-activated (M2) macrophages, can be pharmacologically manipulated with drugs such as rosiglitazone [68]. Therefore, a better understanding of the functional significance of the N1/N2 ratio could increase the viability of neutrophils as a target in the future. It could be argued however, that due to the limited applicability of neutrophil-based therapies to the acute phase, research into stroke immunotherapies should instead focus on targets that contribute to the later stages of stroke pathophysiology.

Resident microglia become activated within minutes of the onset of ischaemia, reaching peak levels within 2 days [7]. Conversely the levels of monocytes/macrophages, which infiltrate from the peripheral circulation 1-2 days later, peak between days 3 and 7. Overall, the importance of local microglia activity supersedes that of monocytes/macrophages in the early phases of acute ischaemic stroke, though the blood-derived cells may mediate further brain injury in the latter stages of post-stroke neuroinflammation [7]. The effect of microglia and monocytes/macrophages in stroke depends on the M1/M2 polarization status. It is believed that the anti-inflammatory M2 gives way to the pro-inflammatory M1 subtype during disease progression [9]. Polarisation to either phenotype is associated with the presence of specific cytokines in the local milieu (i.e. M1: IFN-γ, M2: TGF-β, IL-10) (Figure 1). Treatments which impair microglial activation, such as the free radical scavenger, edaravone, or hyperbaric oxygen, were shown to be neuroprotective, suggesting a detrimental role of microglia/macrophages in stroke [69, 70]. Minocycline, a semisynthetic second-generation tetracycline also known to act via inhibition of microglial activity, proved effective in several experimental stroke models [71]. An open-label placebo-controlled clinical trial found treatment with minocycline significantly improved clinical outcome [72]. A subsequent pilot study however, concluded intravenous minocycline was safe but not efficacious, but pointed to the need for larger studies [73]. Yet, separate pre-clinical studies, in which blocking macrophage infiltration led to undesirable outcomes such as increased haemorrhagic transformation or worsened long-term behavioural recovery, imply a protective effect for these cells [74]. In some studies, inhibition of microglial activation also increased infarct size, again indicating a neuroprotective role [75]. Evidently, a more nuanced understanding of the transitions between M1 and M2 phenotypes in stroke and a greater appreciation of the differences between resident microglia and infiltrating monocytes/macrophages is needed to warrant further human trials of therapies targeting these cells.

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T cells

γδ T cells are predominantly thought to have deleterious effects in stroke. Depletion of γδ T cells significantly reduced cerebral ischaemia-reperfusion (I/R) injury in an experimental model of stroke [76]. The same study found that IL-17 was responsible for the negative impact of γδ T cells on infarct size and neurological function. A separate study demonstrated that, not only are γδ T cells the principal source of IL-17 in the ischaemic brain, but blockade of the IL-17 axis improved stroke outcome through reduced neutrophil invasion [77]. Increased production of IFN-γ and perforin by γδ T cells, on the other hand, correlated positively with clinical improvement, possibly due to increased protection against infection [8]. Thus, future therapies aimed at γδ T cells must counteract the detrimental effects of secreted IL-17 without compromising the ability to prevent infection. Furthermore, the exact crosstalk between stroke and the gut microbiota would have to be further elucidated, considering the role of γδ T cells and IL-17 in the brain-gut axis [19].

Cytotoxic cells such as CD8+ T cells, NK T cells and NK cells are known to exacerbate stroke pathology. CD8+ T cells accumulate in the pan-necrotic area of the ischaemic brain as early as three hours post-stroke, where they mediate many cytotoxic responses, partly through perforin [10]. However, NKT cells have garnered interest not for their neurotoxic roles, but for their importance in host immune function [78]. It has been shown that stroke can induce behavioural changes in hepatic invariant NKT (iNKT) cells to an anti-inflammatory phenotype that facilitates infection [79]. These changes, brought about by noradrenergic neurotransmitters, can be experimentally reversed through administration of propranolol. Similar results can be achieved through the activation of iNKT cells with α-galactosylceramide, demonstrating a possible method of infection control in stroke patients. Such therapies would be an attractive alternative to antibiotics, but concerns remain as to whether the drugs could influence cerebral blood flow, or even promote inflammatory responses at the infarct site. Indeed, in a recent clinical study, beta-blockers did not reduce patient risk for post-stroke pneumonia, but did lead to higher 30-day mortality [80], highlighting the need for further study.

CD4+ T cells are also recruited to the ischaemic hemisphere, in even greater numbers than their CD8+ counterparts [10]. In stroke, infiltrating CD4+ cells are predominantly of the Th1 type, while peripherally Th17 cells may also contribute to ischaemic injury [81]. Adoptive transfer of Th1 or Th17 CD4+ cells worsened behavioural outcomes in an experimental model of ischaemic stroke [82]. Systemic treatments that promoted the Th2 phenotype on the other hand, e.g. IL-33, were beneficial [83]. Autoimmune CD4+ cells are also implicated in stroke damage. The number of myelin basic protein (MBP)-responsive Th1 cells, for example, act as a marker for poor clinical outcome [84]. The fact that many stroke patients display increased concentrations of CNS antigens in lymph nodes, and increased circulating levels of T cells specific for them, strongly indicates the presence of a self-directed immune response [17]. Studies have also shown immunization with myelin antigens worsened outcomes in experimental stroke, while systemic inflammation helps prime such deleterious autoimmune responses [84, 85]. The production of IL-4 or brain-derived neurotrophic factor (BDNF) by Th2 cells, on the other hand, leads to direct neuroprotection and a tissue-healing

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phenotype in myeloid cells [86]. Thus, therapies which could alter the CD4+ phenotype towards a Th2-dominant phenotype could, in theory, be favourable. There has been some success in inducing tolerance to CNS antigens. Intranasal or oral application of MBP weeks before stroke onset reduced infarct volume and improved recovery in rats [87] while separately intranasal MBP administration ameliorated the Th1 response [88]. Further studies however, cast doubt over the long-term involvement of autoreactive T cell responses in stroke, while the safety of induced mucosal tolerance has also been called into question [89]. As a result, therapies which instead target T cell infiltration have received increased interest. A wealth of experimental data now supports the use of fingolimod in ischaemic stroke. In a mouse model of transient ischaemia, fingolimod reduced infarct size, neurological deficit, and inflammation (with fewer activated leukocytes and expression of ICAM-1 on affected blood vessels) [90]. This sphingosine 1-phosphate receptor modulator, used for the treatment of relapsing-remitting MS, may prevent lymphocyte trafficking into the CNS. Further mechanisms of action may include suppression of reperfusion injury, direct neuroprotection and reduced microthrombosis [90]. Recent small-scale clinical trials with ischemic or haemorrhagic stroke patients have shown promising effects on functional recovery [90]; h[94]owever, while fingolimod remains one of the most compelling stroke immunotherapies to date, further large-scale clinical trials are required.

Generally, CD4+ regulatory T cells (Treg), are believed to be neuroprotective in stroke, with the only detrimental effects thought to be a role in secondary microthrombosis [91]. Approximately 20% of all CD4+ T cells found in the ischaemic hemisphere are regulatory T cells (Treg) [91]. Treg cells, whose circulating levels increase following the onset of ischaemia, reduce systemic inflammatory responses but also ameliorate stroke-induced immunosuppression by correcting lymphopenia [92]. In addition to Treg-produced IL-10, secondary mechanisms of Treg-mediated neuroprotection may include TGF-β and IL-35 production, priming of M2 macrophages, reduced inflammatory cytokine secretion, etc [91]. The increased infarct volume seen in experimental models of Treg depletion support a protective function in stroke, although not all studies agree [93, 94]. Several studies which instead focused on Treg expansion (e.g. via adoptive transfer, or using CD28 super agonist) generally demonstrate a beneficial role of Treg in stroke [95, 96]. Reasons for this inconsistency in experimental/clinical models may include differences in infarct size, microthrombosis, and variation of the local inflammatory milieu. Nevertheless, with newer studies pointing to involvement of Treg in both the brain-gut axis and protection against tPA-mediated haemorrhagic transformation, there is scope for future Treg-targeted immunotherapies [19, 97].

B cells

B cells provide both beneficial and detrimental functions in experimental and human stroke. The deleterious effects are suggested to be mediated through B cell derived immunoglobulins, which have been found in the cerebrospinal fluid of stroke patients [13]. These brain-antigen specific antibodies may be involved in late-phase chronic inflammatory responses which impair functional stroke recovery [98]. However, mice deficient in B cells

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alone did not display neuroprotection [12]. A separate study reported that B cell deficiency itself leads to worsened stroke outcomes [13]. The same study highlighted that adoptive transfer of B cells can ameliorate neurological deficits and reduce lesion size. Regulatory B cells (Bregs), which secrete the anti-inflammatory cytokine IL-10, were proposed to be responsible for this neuroprotective effect [99]. In summary, B cells appear to have a conflicting role in stroke, with the positive effects of IL-10 on the local inflammatory milieu counterbalanced by the negative effect of antibodies secreted from differentiated B cells. As in the case of T cells, future stroke immunotherapy will therefore rely on enhancing the regulatory phenotype (fingolimod, glatiramer acetate) [13]. The B-cell depleting antibody, anti-CD20, has received increased interest in stroke research for its apparent ability to ameliorate antibody-induced long-term cognitive impairment. In a mouse study, anti-CD20 IV injection every 2 weeks, beginning 5 days post-stroke, prevented B cell and IgG recruitment to the infarct site, and prevented the appearance of delayed cognitive deficits [98]. As rituximab is already well-characterised and approved for use in another CNS disorder (MS), anti-CD20 may well be the next immunomodulatory therapy to progress to trial in AIS.

Antibiotics/ brain-gut axis

To date, the clinical trial results of various antibacterial agents in stroke have not been encouraging. Despite significant reductions in the risk of overall post-stroke infection (notably, the risk of pneumonia remains unchanged), a recent systematic review concluded preventative antibiotics have no effect on functional outcome or mortality in stroke [100]. Some researchers have argued this may relate to the individual antibiotics tested, as the fluoroquinolone class in particular display neurotoxic properties, whereas ceftriaxone, a cephalosporin, offers neuroprotection. In a small randomized controlled trial, the combined administration of mezlocillin and sulbactam did lead to improved clinical outcomes [100]. Future research may therefore rely on identifying both the correct antibiotic regimen and patient cohort (e.g. through blood-based biomarkers, risk scoring models) most likely to benefit from prophylactic therapy.

Recently, interest in the gut microbiota and the brain-gut axis has extended from a role in stroke pathology to a possible place in stroke treatment. One study considered the use of faecal microbiota transplant (FMT) therapy. Mice which received FMT showed normalised gut flora and improved stroke outcome, suggesting an exciting future for such novel therapies [20].

CONCLUSION

Over the past several decades, a broad picture of the immune response to stroke has emerged. A host of innate and adaptive immune components have been implicated in both ischaemic injury and post-stroke repair (Figure 2). Thus, it is now widely accepted that many elements of the immune system have multi-faceted roles in stroke, often switching between beneficial and detrimental phenotypes. Functional polarisation occurs as a result of local or systemic stimuli, both of which are affected by disease progression. The existence of this dual-edged

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immunity, coupled to the phenomenon of stroke-induced immunosuppression, will have to be taken into consideration to develop effective stroke therapies.

Many of the drugs trialled to date target the innate immune response to stroke. Thus, their use would be restricted to the acute phase of the disease, a caveat which, similar to tPA, would limit widespread clinical use. Conversely, therapies modulating the adaptive immune response to stroke (fingolimod, rituximab, natalizumab, Treg/Breg-based therapies) could feasibly be used across a timescale of days to weeks post-stroke, lending them greater clinical promise. Separately, the development of drugs which could bolster the host defence against post-stroke infection has become increasingly important in the era of antibiotic resistance.

Ultimately, the usefulness of immunomodulatory drugs for stroke treatment will depend on greater knowledge of how the intimate, bi-directional communication between the CNS and the immune system changes with disease progression. Likewise, an increased understanding of the time-sensitive nature of many immune targets should lead to better translation from bench to bedside in the years to come. However, while time is the most obvious factor to take into consideration, patient comorbidities and pre-existing conditions are also likely to be important predictors of immunomodulatory drug efficacy. Hypertension, hyperlipidemia, diabetes (to name a few important stroke risk factors) are all associated with a pro-inflammatory phenotype, but most preclinical studies are performed in healthy young mice. Changes are therefore needed at both the preclinical stage, so that candidate drugs are tested in animal models with the relevant comorbidities, and at the clinical level, where patients may have to be stratified based on their individual inflammatory/immune status as assessed by specific markers. This patient-specific phenotype may ultimately be a more important predictor of efficacy than time alone.

The ever-growing list of immunomodulatory drugs approved for other indications which now show potential as therapies in stroke may also provide an opportunity to fast-track future trials. Care, however, must be taken to avoid the pitfalls of past trials, where candidate therapies were put forward without sufficient experimental evidence in support [4]. Appreciation must also be given to the overall immune response to stroke, and the untoward ramifications which could occur from manipulating even a single target. Despite such limitations, the possible advantages of immunotherapy over other candidate drugs in stroke continues to attract greater interest. With applications ranging from reduced post-stroke infection, to improved functional outcomes, to better cognitive recovery, drugs targeting the immune system could offer unique benefits in an acute disease plagued with long-term consequences.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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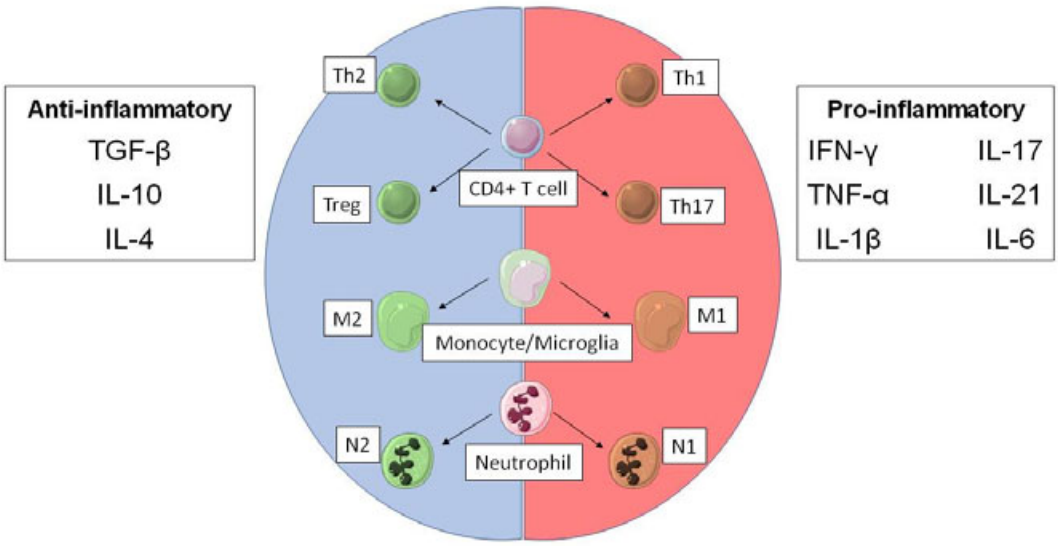
Figure Legends

Figure 1: The effect of the inflammatory milieu on immune cell function in stroke. The balance between pro- and anti-inflammatory cytokines influences the differentiation of activated microglia and leukocytes into neuroprotective (left) or harmful (right) phenotypes. (*Adapted from Servier Medical Art)

Figure 2: A time course of immune targets in stroke. Targets are placed according to the predominant role each plays in either neurotoxicity (red) or tissue remodelling and repair (blue). Potential therapies are highlighted in parentheses. (*Adapted from Servier Medical Art).



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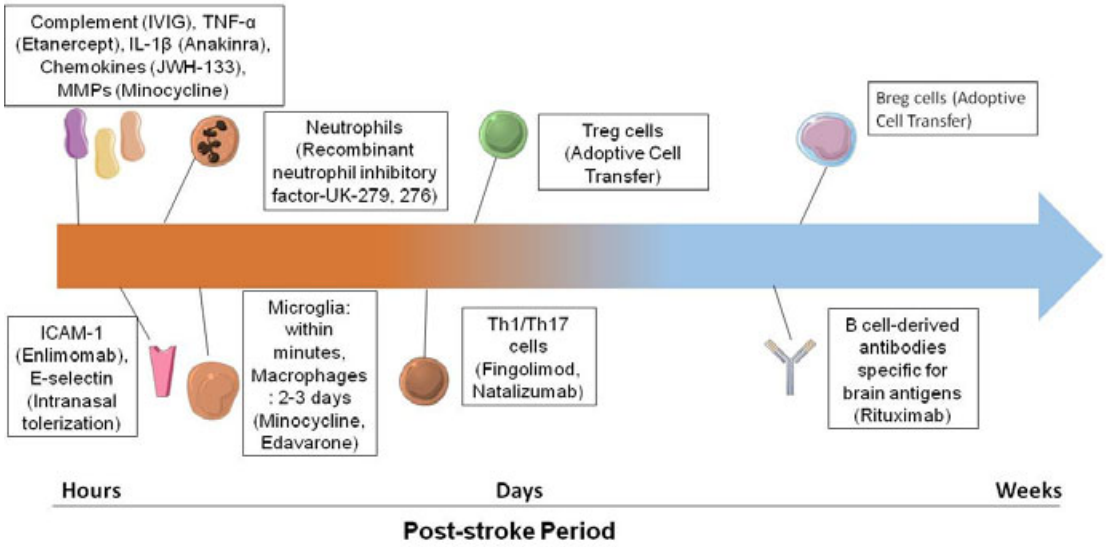

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