

# Anti-inflammatory treatments for stroke: from bench to bedside

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**Abstract:** So far, intravenous tissue-type plasminogen activator (tPA) and mechanical removal of arterial blood clot (thrombectomy) are the only available treatments for acute ischemic stroke. However, the short therapeutic window and the lack of specialized stroke unit care make the overall availability of both treatments limited. Additional agents to combine with tPA administration or thrombectomy to enhance efficacy and improve outcomes associated with stroke are needed. Stroke-induced inflammatory processes are a response to the tissue damage due to the absence of blood supply but have been proposed also as key contributors to all the stages of the ischemic stroke pathophysiology. Despite promising results in experimental studies, inflammation-modulating treatments have not yet been translated successfully into the clinical setting. This review will (a) describe the timing of the stroke immune pathophysiology; (b) detail the immune responses to stroke sift-through cell type; and (c) discuss the pitfalls on the translation from experimental studies to clinical trials testing the therapeutic pertinence of immune modulators.

**Keywords:** clinical trial, immunomodulatory drugs, inflammation, stroke models, translation

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## Introduction

Every year, 15 million people worldwide suffer a stroke. Of these, 5 million die and another 5 million are left permanently disabled, placing a burden on family and community ([http://www.who.int/cardiovascular\\_diseases/en/cvd\\_atlas\\_15\\_burden\\_stroke.pdf?ua=1](http://www.who.int/cardiovascular_diseases/en/cvd_atlas_15_burden_stroke.pdf?ua=1)). In the United States a stroke event happens every 40 seconds, and every 4 minutes, someone dies of stroke (American Stroke Association). Ischemic stroke is provoked by an arterial occlusion in the brain that leads to the rapid death of the brain tissue irrigated by that particular artery. So far, intravenous tissue-type plasminogen activator (tPA) is the only available pharmacological agent for acute ischemic stroke, but this agent is frequently underutilized due to its limited therapeutic window (4.5 h) and increased risk of intracerebral hemorrhage. Recently, mechanical thrombectomy has demonstrated beneficial effects on ischemic stroke in selected patients<sup>1</sup> and has become the standard of care for patients with large-vessel occlusion up to 24 h of stroke onset.<sup>2,3</sup> Additional agents to combine with tPA administration or thrombectomy to

improve efficacy and ameliorate outcomes associated with stroke are needed, in particular, for those patients not eligible for thrombolysis or thrombectomy, or with no access to specialized stroke unit care. Easily administrable agents reducing tissue damage even modestly would drastically decrease the burden of stroke on society and would ameliorate patient outcomes and quality of life.<sup>4</sup>

Stroke-induced inflammatory processes, which include mechanisms of innate and adaptive immunity, are a response to tissue damage due to the absence of blood supply but have also been proposed as key contributors to all the stages of the ischemic stroke pathophysiology.<sup>5</sup> However, despite promising results in experimental studies, inflammation-modulating treatments have not yet been translated successfully into the clinical setting. This review will focus on the innate and adaptive immune responses participating in ischemic brain injury and their impact on tissue damage and repair in both experimental models of stroke and available clinical data. It has been

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structured in three parts: a first part describing the timing of the stroke pathophysiology from an immunological point of view (Figure 1); a second part detailing the immune responses to stroke sift-through cell type; and a third part discussing the pitfalls on the translation from preclinical to clinical stroke research of immunomodulating therapeutic agents.

### Timeline of inflammatory events after stroke

Basically, inflammatory responses after stroke can be decomposed in three phases.<sup>5</sup> The acute phase (first hours after stroke onset) corresponds to the clearing of dead cells mainly by resident phagocytic cells such as microglia/macrophages and a first entry of leukocytes, mainly neutrophils. The subacute phase (first days after stroke) corresponds to resolution of inflammation. The late phase (days and weeks after stroke) corresponds to tissue repair by astrocytes and microglia (glial scar).

#### *The acute phase of stroke: tissue injury, microglial and endothelial activation*

The arterial occlusion at the origin of ischemic stroke leads to deprivation of oxygen and nutrients that are essential for neuronal survival, leading to rapid neuronal death in the core of the lesion site only minutes after stroke onset. The absence of blood supply into the tissue is followed by a cascade of events starting with a reduction in cellular adenosine triphosphate (ATP) that is required to maintain ionic gradients. The disruption of ionic gradients leads to an influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , cellular depolarization and release of neurotransmitters, including the excitatory neurotransmitter glutamate.<sup>6</sup> As energy-dependent removal of glutamate is impaired, glutamate accumulation leads to overactivation and opening of monovalent ion channels followed by water influx, thus resulting in cellular swelling and death.<sup>7</sup> Dying neurons at the core of the lesion express free radicals, damage-associated molecular patterns (DAMPs) and high-mobility group box 1 (HMGB1) protein that lead to inflammatory reactivity by microglia. Edaravone, a free radical scavenger, has shown beneficial properties in stroke patients with large-vessel occlusion, especially when combined with recombinant tissue-type plasminogen activator, but not in combination with thrombectomy.<sup>8</sup> Activated microglia

adopt a pro-inflammatory status, expressing pro-inflammatory cytokines [interleukin 1 (IL-1), IL-6] that are released into blood circulation.<sup>9</sup>

Pro-inflammatory cytokines exacerbate endothelial cell (EC) activation contributing to leukocyte rolling, adhesion and infiltration in brain tissue. Leukocytes start to roll on the vessel wall with the help of selectins expressed by activated EC. After rolling, leukocytes strongly adhere to the vessel wall by strong links with intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1).<sup>10,11</sup> Into the brain, there is almost no way for leukocytes to pass through the blood-brain barrier (BBB) in nonpathological conditions.<sup>12</sup> However, the BBB integrity is disrupted after stroke onset and tight junctions between EC disappear, allowing leukocyte infiltration into the injured brain tissue.<sup>13</sup> Clinical data and experimental models of stroke have shown that neutrophils infiltrate within hours of stroke onset.<sup>14,15</sup>

#### *The subacute phase: resolution of inflammation*

In the first days after stroke, different leukocyte populations, including macrophages and lymphocytes, infiltrate the brain.<sup>14,15</sup> The infiltration of leukocyte subpopulations differs among murine permanent and transient mechanical focal cerebral ischemia models<sup>16</sup> and the inflammatory response seems to be more pronounced after permanent electrocoagulatory middle cerebral artery occlusion (MCAO) compared with 30-min and 90-min transient mechanical vascular occlusion (TMVO).<sup>16</sup> Descriptive data on the timeline inflammatory responses in thrombus-induced experimental stroke models are missing.

Preclinical studies performed on TMVO models of ischemic stroke have shown a secondary injury after reperfusion (i.e. cerebral ischemia/reperfusion injury). While mechanical thrombectomy also promotes rapid reperfusion, it does not seem to provoke such a secondary injury in patients with proximal middle-cerebral-artery or internal-carotid-artery occlusion and a penumbral region of tissue (i.e. a brain region that is ischemic but not infarcted yet, which is therefore salvageable). This is probably due to a better collateral circulation and slower infarct growth in these patients and is associated to a higher proportion of good functional outcomes at 3 months after stroke, compared with patients with little or no penumbra,

even when performed late (16–24 h) after stroke onset.<sup>2,3,17</sup> In the DEFUSE 2 study, patients with little or no penumbra had a greater lesion growth despite reperfusion. This greater lesion growth could be the result of reperfusion-related edema based on the larger infarct core volumes in the no penumbra group.<sup>17</sup>

#### *The late phase: tissue repair and glial scar*

Neuroinflammation is also considered necessary for the reparation phase that persists after the initial brain insult.<sup>18–20</sup> This phase aims at restoring tissue integrity and involves matrix remodeling, neurogenesis, axon sprouting, dendritogenesis and oligodendrogenesis.<sup>21</sup> All these processes are common to acute brain injuries (including ischemia, hemorrhage, and trauma), neuroinflammatory (including multiple sclerosis) and neurodegenerative disorders,<sup>22</sup> that initiate an extensive glial response known as reactive gliosis. Reactive gliosis involves an enhanced expression of specific markers, such as various extracellular matrix molecules (ECM) like chondroitin sulfate proteoglycans (CSPG) and glial fibrillary acidic protein (GFAP) for astrocytes. Glial scar formation is crucial for sealing the lesion site to remodel the injured tissue, in order to spatially and temporally control the local immune response and revascularization of blood capillaries. Nonetheless, the glial scar also acts as an obstacle to axon regeneration and thus avoids the recovery of central nervous system (CNS) function in the chronic phase after stroke. Experimental studies on the glial response to ischemic stroke have been performed in animal models using variations of permanent or focal transient MCAO and photothrombosis.<sup>23–25</sup> The human brain after ischemic injury seems to share similar properties to these experimental models of stroke.<sup>26</sup>

#### **Immunomodulating therapeutic strategies for stroke: sift-through cell type**

Clinical trials on stroke immunology have targeted both innate and adaptive immune responses, by reducing microglial activation, inhibiting leukocyte or lymphocyte migration to the brain parenchyma, and blocking the IL-1 receptor. The increasing number of immunomodulatory treatments that are already established for other indications in humans have provided a good opportunity to fast-track innovative proof-of-concept trials in stroke. However, and despite promising preclinical results,

immunomodulatory drugs have not shown clear benefits in large clinical trials on stroke. Another strategy has been the induction of hypothermia to reduce inflammatory responses and apoptosis triggered after stroke (among other mechanisms). Several trials have shown its safety and feasibility, alone or in combination with thrombolysis;<sup>27–30</sup> however, its efficacy in the treatment of ischemic stroke is still debatable, especially because of increased pneumonia incidence and mortality in the hypothermia group.<sup>31</sup> New clinical trials on the effects of the combination of thrombolysis/thrombectomy plus hypothermia are ongoing.<sup>32</sup> Figure 2 summarizes the results of clinical trials targeting the immune responses triggered after ischemic stroke.

#### *Microglial cells*

*Pathophysiology.* Microglial cells constitute 10–15% of the brain cells and are the resident mononuclear phagocytes of the brain parenchyma.<sup>33</sup> As aforementioned, microglial response is one of the first steps of the innate immune responses triggered after stroke. Microglial cells are in close contact with neurons and constantly survey the environment with their processes.<sup>34</sup> These cells can adopt a large spectrum of phenotypes ranging from pro-inflammatory to anti-inflammatory and neuroprotective. At the acute phase after stroke onset, microglial cells at the core of the lesion detect DAMPs and HMGB1 *via* their receptors mainly belonging to the Toll-like receptor (TLR) family.<sup>13,35–37</sup> This ligand–receptor link leads to internalization of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (I $\kappa$ B) kinase into the nucleus and the subsequent activation of the nuclear factor of kappa light chain enhancer of activated B-cells (NF- $\kappa$ B) pathway and thus to the activation of microglial cells. Also, loss of the constitutively expressed neuronal ligand CX3CL1 (fractalkine) after neuronal death results in enhanced microglial activation through their receptors CX3CR1.<sup>38</sup> Experimental models of both transient and permanent ischemic stroke have shown an increase in the number of microglial cells on the ipsilateral cortex in the first 24 h after stroke onset.<sup>14,16</sup> Once activated, microglial cells express high levels of CD11b, CD45 and CD68 corresponding to a phagocytic phenotype responsible for the clearance of cellular debris,<sup>9</sup> as well as tumor necrosis factor and the pro-inflammatory interleukins IL-1 and IL-6 that are released into blood circulation.<sup>9</sup> IL-1 has been targeted to

reduce further cerebral injury mediated by inflammation. A meta-analysis showed that IL-1 receptor antagonist (IL-1Ra) administration was associated with a 38.2 % reduction in mean infarct volume across 16 published preclinical studies.<sup>39</sup> In a phase II clinical study, recombinant human IL-1Ra (rhIL-1Ra; anakinra) administered at hospital arrival has shown beneficial effects in patients with cortical infarcts, with better clinical outcomes after 3 months in the treated group.<sup>40</sup> However, the recently published results of a second phase II study on the effect of subcutaneous IL-1Ra administration (SCIL-STROKE) has shown that, in spite of a significant reduction in plasma inflammatory markers associated with a worse outcome after ischemic stroke, IL-1Ra treatment was not associated with a favorable outcome on modified Rankin Scale (mRS).<sup>41</sup>

Recently developed techniques of total microglial depletion have shown that microglia seem essential to the limitation of stroke damages, since microglia-depleted mice show larger infarct volumes than nondepleted mice.<sup>42</sup>

*Therapeutic strategies targeting microglia.* Minocycline, an anti-infective agent of the tetracycline family used for the treatment of infections caused by a wide range of organisms, has been tested for the treatment of stroke. It is highly lipophilic and crosses the blood–brain barrier. Minocycline, by acting through different pathways, is able to inhibit microglial activation, decrease migration of T cells, reduce neuronal apoptosis, block free radical production, decrease CNS expression of chemokines and their associated receptors, and inhibit matrix metalloproteinases, particularly matrix metalloproteinase-9.<sup>43–58</sup> In preclinical stroke studies, minocycline ameliorated behavioral function and decreased lesion volume and hemorrhagic transformation.<sup>44–47</sup> It did not alter the fibrinolytic effect of rtPA<sup>46</sup> and could enlarge the time window for thrombolysis.<sup>49</sup> Two phase II clinical trials have shown minocycline to be safe and potentially effective in acute ischemic stroke, alone or in combination with tPA, when administered in the first 24 h after stroke onset and for 5 days.<sup>59,60</sup> However, a third pilot study performed in a small sample of acute stroke patients has shown that minocycline administration in the first 24 h was safe but not efficacious.<sup>43</sup> The authors acknowledged that this third study was not powered to identify reliably or exclude a modest but clinically important treatment effect of minocycline.<sup>43</sup> Larger clinical trials

are thus needed to study the effect of minocycline in stroke patients.

Recently, preclinical studies have focused also on the switch of microglial cell phenotype from M1 to M2 as a potential target to improve stroke outcome, by administering IL-33<sup>61</sup> or IL-4.<sup>62</sup>

#### *Endothelial cells*

*Pathophysiology.* In the brain, the functional unit formed by tightly jointed ECs and astrocytic endfeet constitute the BBB, a strong protective blood–tissue barrier against pathogens. A few minutes after stroke onset, the BBB is disrupted, and local ECs are activated.<sup>63,64</sup> When activated, ECs express cellular adhesion molecules (CAMs) which allow leukocyte rolling and adhesion to the luminal side of the EC and eventually leukocyte transmigration toward the brain parenchyma.<sup>5</sup> After their activation, the first adhesion molecules expressed by ECs are the selectins (E- and P-selectins). They are constitutively produced by ECs and stored in Weibel-Palade bodies until EC activation. The initial adhesive interactions between leukocytes and selectins expressed on the luminal side of the venular endothelium are tethering (capture) and rolling. These low-affinity (weak) interactions are subsequently strengthened as a result of the sequential activation of different families of adhesion molecules that are located on the surface of leukocytes and ECs.<sup>65</sup> A few hours after stroke onset, ECs start to express the VCAM-1 and the ICAM-1,<sup>63,66</sup> allowing leukocytes to adhere and remain stationary on the vessel wall. Finally, ECs express the platelet endothelial cell adhesion molecule (PECAM-1), responsible for leukocyte transmigration into the brain parenchyma.<sup>11</sup>

*Therapeutic strategies targeting selectins.* Strategies for the blockade of CAMs through the intravenous injection of antibodies have failed in ameliorating stroke outcome both in clinical and preclinical trials.

Enlimomab, a murine ICAM-1 antibody, reduces leukocyte adhesion and infarct size in experimental stroke studies.<sup>67</sup> However, the clinical trial performed in stroke patients showed that the administration of enlimomab within 6 h after stroke onset is not an effective treatment for ischemic stroke and, indeed, significantly worsened stroke outcome and increased adverse events.<sup>68</sup> One of the possible explanations for this

failure is the murine origin of the injected antibody to stroke patients.<sup>69</sup>

Induction of mucosal tolerance to E-selectin to minimize inflammation and risk of further cerebral insult has been tested in a phase I clinical trial, but results have not been disclosed.<sup>70</sup>

L-selectin, a CAM located in the surface of leukocytes, has also been targeted in an experimental stroke model in rabbits by using a humanized monoclonal antibody (HuDREG200). This strategy, alone or in combination with alteplase, failed to significantly ameliorate stroke outcome.<sup>71</sup>

### Neutrophils

*Pathophysiology.* The principal role of neutrophils is to enhance leukocyte recruitment by degranulation of their content, rich in cytokines/chemokines, proteolytic enzymes and activated-complement system.<sup>72</sup> The extent of neutrophil infiltration after stroke is still debated, being described either limited to the perivascular space or infiltrating the brain tissue in preclinical stroke models (permanent or mechanical transient occlusion of the MCA) and human postmortem samples.<sup>73–75</sup>

It was postulated that neutrophils could contribute to brain injury after ischemic stroke by obstructing microvessel circulation, damaging endothelial cells and ECM by hydrolytic enzymes and free radicals, promoting intravascular thrombus formation together with platelet activation, and releasing cytokines and chemotactic factors that could promote extension of the inflammatory response.<sup>76</sup>

*Therapeutic strategies targeting neutrophils.* Preclinical and clinical studies aiming at blocking neutrophil infiltration have targeted CD11/CD18 (LFA-1 for lymphocyte function-associated antigen 1), located at the surface of neutrophils, to prevent their adhesion to ICAM-1 (expressed at the luminal surface of ECs). Preclinical studies have shown that CD11/CD18 monoclonal antibodies are not beneficial in permanent stroke models,<sup>77,78</sup> but are beneficial on a transient mechanical ischemic stroke model.<sup>79</sup> In spite of this, two clinical trials have been performed to prevent neutrophil recruitment after stroke onset and have shown no beneficial effect. On the HALT phase III trial, the humanized antibody

Hu23F2G, administered twice daily, appeared to produce an improvement in mRS score in a phase II study. However, the sponsor terminated a subsequent phase III study when a ‘futility analysis’ advised that no benefit of the treatment would occur if the study was completed. No public information regarding the database, outcomes, safety issues, or relative number of adverse events has appeared.<sup>76</sup> On the ASTIN (Acute Stroke Therapy by Inhibition of Neutrophils) study, UK-279,276 (another CD18 antagonist) was administered within 6 h of stroke symptom onset, but, similar to the HALT study, the trial was stopped early due to futility.<sup>80</sup>

### Lymphocytes

*Pathophysiology.* Adaptive immunity also plays an important role during stroke. Lymphocytes consist of distinct subpopulations with diverse functions and can be subdivided into two groups: pro- and anti-inflammatory lymphocytes. The consequences of the adaptive immune response on ischemic stroke are still debated, as both beneficial and deleterious results have been reported depending on the type of T-helper (Th) immune response set in motion after the activation of lymphocytes.<sup>81–83</sup> Both transient (mechanical) and permanent experimental models of stroke have shown that lymphocyte infiltration into the ischemic tissue comes later after stroke onset (starting at 3 days).<sup>14,16</sup> Postmortem human samples have shown that lymphocyte infiltration into the ischemic area occur from day 3 and can be present up to 53 years after stroke.<sup>15</sup>

The choroid plexuses (CP) have recently been proposed as the preferential route for lymphocyte infiltration into the lesion site after experimental stroke. Indeed, CP infarction reduces lymphocyte infiltration after stroke onset; however, the ischemic volume is not modified in these mice, even if lymphocyte infiltration is actually reduced.<sup>84</sup> These intriguing results raise important questions about the role of lymphocytes on the development of the ischemic lesion.

*Therapeutic strategies targeting lymphocytes.* Therapeutic strategies targeting lymphocytes would be desirable because they focus the delayed phase of the injury and thus could have a particularly wide therapeutic window. Several preclinical studies have shown that pro-inflammatory lymphocytes, such as TH1, TH17, and  $\gamma\delta$  T

cells worsen stroke outcome, and that blocking of their brain invasion is neuroprotective.<sup>85–87</sup>

Contrary to pro-inflammatory lymphocytes, regulatory T cells (Treg) and B cells have been characterized as anti-inflammatory and as disease-limiting protective cells. Targeting Tregs as the endogenous orchestrators of the postischemic immune response has been proposed as a more effective therapy than blocking only a particular inflammatory pathway.<sup>5,13</sup> However, because of the complex function of regulatory cells in immune homeostasis and disease, as well as partially divergent findings using different stroke models, the pathophysiologic function of regulatory lymphocytes in stroke remains uncertain. As an example, among nine studies using Treg-depletion paradigms, three of the studies revealed an increase in infarct volume<sup>88–90</sup> whereas five studies did not detect any effect on stroke outcome<sup>88,89,91–93</sup> and one study even observed a reduction of infarct size in Treg-deficient mice.<sup>94</sup> To explain this, the experimental model used (permanent *versus* transient mechanical) and more particularly, the resulting volume of the ischemic lesion, has been proposed as a determinant on the overall effect of Treg depletion that seems to provide a benefit only on small lesion volumes provoked on the permanent stroke model.<sup>95</sup>

Another strategy for Treg modulation has been the administration of enhancers of Treg function by adoptive cell transfer of purified Treg to wild-type animals to increase circulating Treg numbers, or by the administration of a CD28 superagonist (CD28SA), which provokes *in vivo* expansion of Tregs and amplification of their suppressive function. However, the obtained results are again controversial, with some studies describing an improvement of stroke outcome<sup>96</sup> and other studies describing an increase of ischemic volume.<sup>97</sup> Once again, it has been proposed that stroke severity might predict the net biological effect of Tregs.<sup>95</sup>

It has been proposed that Tregs have a deleterious role on ischemic stroke not related to their established immunoregulatory characteristics but to a specific effect on microvascular thrombus formation in a model of transient mechanical occlusion.<sup>94</sup> Secondary microthrombosis arises within minutes after reperfusion in transient mechanical models of stroke once the occluding filament has been removed. It provokes delayed cerebral blood flow (CBF) reduction, secondary ischemia and

lesion growth, which is responsible for ~70% of the final ischemic lesion size.<sup>98</sup> However, clinical studies have shown that secondary ischemia and lesion growth are modest or nonexistent after reperfusion in selected patients,<sup>2,17,99,100</sup> and concerns have been raised about the clinical relevance of transient mechanical vascular occlusion stroke models.<sup>101</sup> For these reasons, it would be interesting to test the role of Tregs in nonmechanical models of ischemic stroke, where secondary microthrombosis is absent.<sup>102</sup>

To increase the complexity of this question, fingolimod (FTY720), an immunomodulatory drug currently approved by the US Food and Drug Administration for the treatment of multiple sclerosis that inhibits lymphocyte circulation and brain immigration, has been tested in models of permanent and transient mechanical ischemia. As in the case of the aforementioned CP infarction,<sup>84</sup> although a reduction of lymphocyte brain invasion was detected using fingolimod, no effect was observed on infarct volumes and behavioral dysfunction in any of the models. This lack of neuroprotection despite effective lymphopenia was attributed to a divergent impact of fingolimod on cytokine expression and possible activation of innate immune cells after brain ischemia.<sup>86</sup> A phase II clinical trial using fingolimod (administered within 6 h after the onset of symptoms) combined with alteplase and mechanical thrombectomy on ischemic stroke (FAMTAIS) is ongoing.<sup>103</sup>

Two clinical pilot studies have tested fingolimod, alone and later than 4.5 h after stroke onset for 3 days,<sup>104</sup> or in combination with tPA (thus before 4.5 h after stroke onset) for 3 days.<sup>105</sup> These two studies have shown a beneficial effect of the oral administration of fingolimod within 72 h of stroke onset, especially when combined with tPA, where patients who received the combination exhibited smaller lesion volumes, less hemorrhage, and a better recovery at day 90.

*Therapeutic strategies targeting global leukocyte infiltration.* The transendothelial migration of leukocytes through the interaction between the molecules VLA-4 (leukocyte very late antigen-4) (present at the surface of neutrophils, monocytes, and T and B lymphocytes among other blood cell types) and VCAM-1 (vascular cell adhesion molecule-1) has been targeted in several studies. Antibodies against the alpha chain of VLA-4 (anti-CD49d antibodies) have shown efficacy in several models of autoimmune diseases, and the

humanized antibody natalizumab is currently one of the most effective therapies for patients with multiple sclerosis.<sup>106</sup> However, preclinical data have shown conflicting results from four positive and one inconclusive studies on the use of anti-CD49d antibodies on ischemic stroke.<sup>86,107–110</sup> Recently, a preclinical randomized controlled multicenter trial highlighted the importance of testing new therapies in different experimental stroke models (discussed further on), since the administration of CD49d-antibodies reduced both leukocyte invasion and infarct volume after the permanent distal occlusion of the MCA (which causes a small cortical infarction), whereas it did not reduce leukocyte invasion or infarct volume after transient proximal mechanical occlusion of the MCA (which induces large lesions).<sup>111</sup> These results could suggest that the benefits of immune-targeted therapies may be dependent on infarct localization and severity.

The recently published results of the administration of one dose of natalizumab in patients with acute ischemic stroke (ACTION trial) have shown that natalizumab administered up to 9 h after stroke onset did not reduce infarct growth (primary endpoint of the study), but more patients in the natalizumab group than in the placebo group had mRS scores of 0 or 1 at day 30, although this beneficial effect disappeared 90 days after stroke.<sup>112</sup> These results reinforce the need of new studies to understand (a) how natalizumab exerts a positive effect on functional outcome without reducing the infarct volume at 30 days, and (b) why this beneficial effect on functional outcome is lost at 90 days. Additionally, the ACTION II trial has been recently completed. Its primary objective was to assess the effects of natalizumab on functional independence and activities of daily living.<sup>113</sup> Results have not been published yet.

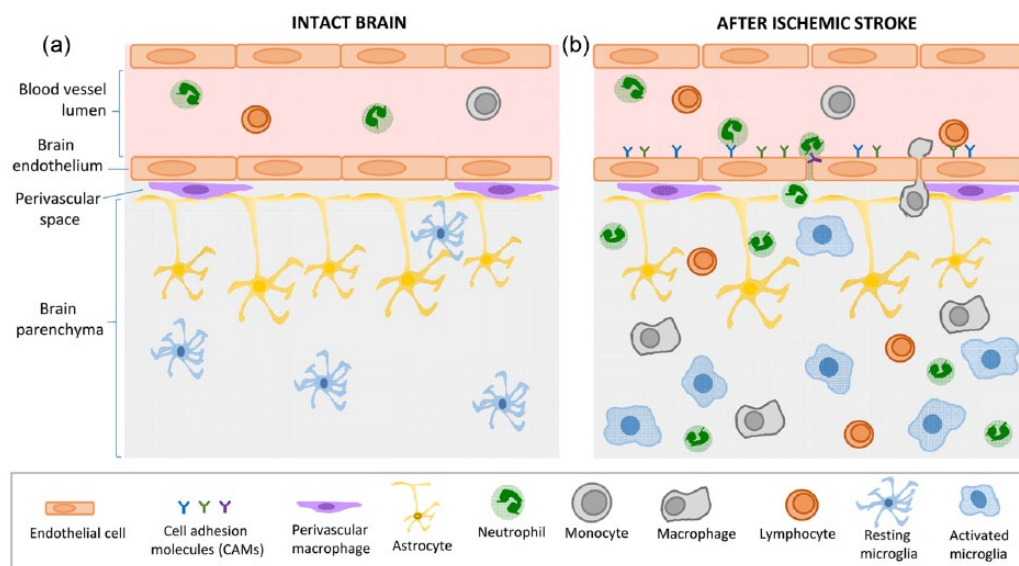
### **Immunomodulating therapies in stroke: a gap between our expectations and reality**

The reasons for the failure of immunomodulatory therapies in clinical trials are multifactorial. As discussed, one of the main issues of the bench-to-bed translation of immunomodulatory therapies for stroke is the huge gap between human pathophysiology and the stroke models that have mainly been used to study stroke immunology. In experimental stroke models, preclinical stroke research nowadays disposes of a wide range of experimental models including permanent and transient arterial

occlusions. In addition to this, the origin of the occlusion may also vary from a transient mechanical occlusion to real blood clots located in the lumen of the artery. In the case of clot-induced stroke models, researchers can even adopt different strategies to determine the nature of clots (fibrin-rich or platelet-rich) and, consequently, clot susceptibility to thrombolytic agents<sup>114–116</sup> to mimic the heterogeneity of human thrombi subtypes. It is important to note that despite the variety of available stroke models, the vast majority of preclinical data on transient models of ischemic stroke has been obtained on mechanical occlusion models that provoke thromboinflammation and secondary microthrombosis. This secondary microthrombosis is a major pathophysiological mechanism leading to brain damage which does not occur in other models based on clot-induced ischemic stroke like the thrombin-induced stroke model.<sup>102,116</sup> As in the aforementioned case of Treg-targeted drugs, immunomodulatory agents that have clearly shown beneficial effects on mechanical occlusion models of stroke could actually be targeting thromboinflammation and secondary microthrombosis. Since it is unclear whether thromboinflammation is a universal pathophysiological mechanism of human stroke, immunomodulatory drugs deserve to be evaluated or re-evaluated in experimental stroke models not inducing secondary thromboinflammation before trying to apply them in clinical practice.

Other reasons for the failure in the translation of immunomodulatory therapies from bench to bedside may include poorly designed preclinical and clinical studies, and underpowered clinical trials with overambitious and pathophysiologically irrelevant therapeutic windows.<sup>117,118</sup> In addition, a biased selection of substances for clinical testing may partially explain the slow progress in developing treatments.<sup>118</sup>

It is also possible that the positive results seen with immunomodulatory drugs in experimental studies are not transposable to the complexity and the variability of the immunologic response in human stroke, which may depend on different parameters including prestroke status of the patient and the natural history of the disease.<sup>63,93</sup> In most animal studies, complete occlusion is induced and is either maintained or followed by complete reperfusion. In human stroke, however, only a few patients are likely to experience both complete occlusion and complete reperfusion (excepting thrombectomized patients). The



**Figure 1.** Immune reactions after ischemic stroke.

(a) In the intact brain, the functional unit formed by tightly jointed ECs and astrocytic endfeet constitute the BBB, a strong protective blood–tissue barrier from pathogens. Immune cells circulate freely in the blood, and in the brain parenchyma, resting microglia survey the environment with their processes. (b) A few minutes after stroke onset, the BBB is disrupted and local ECs are activated. The tight junctions between ECs disappear and activated ECs express CAMs. This allows white cell rolling and adhesion at the luminal side of the blood vessel and then transmigration from the vascular compartment to the brain parenchyma. Once infiltrated in the tissue, neutrophils secrete pro-inflammatory factors that will recruit monocytes/macrophages, and later lymphocytes to the parenchyma. After stroke, microglia switches from a resting form to an activated state, adopting a phagocytic phenotype and secreting pro-inflammatory factors. BBB, blood–brain barrier; CAM, cellular adhesion molecule; EC, endothelial cell.

unlimited variety of outcomes seen in stroke patients suggests that stroke itself, as well as the body's response to ischemic stroke represents a seamless continuum of exigencies rather than the definitive scenario employed in animal studies.<sup>68</sup> In addition, stroke risk factors such as diet, smoking, aging, atherosclerosis, hypertension, alcohol consumption and stress cannot be replicated well in the laboratory; actually, most of the preclinical studies are performed in healthy mice. Moreover, although stroke remains the third cause of death in women,<sup>119</sup> most of the preclinical studies are performed on male animals. Inflammatory cells such as microglia, dendritic cells, neutrophils and lymphocytes show variations between sex, and so may play different roles in the pathophysiology of stroke.<sup>120–123</sup>

The failure of immunomodulatory drugs clinically tested on stroke to date may also indicate that the inflammatory response after stroke is an important aspect of the regenerative process triggered after stroke and only becomes detrimental when exaggerated, perhaps in relation to the severity of ischemic injury (as shown in preclinical stroke models) or to concomitant disease states or risk factors. However, many of the preclinical




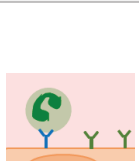
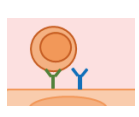
studies examining immunomodulation were targeted at the acute phase, not during the phases where recovery is expected to occur. Thus, this hypothesis needs to be addressed in future studies.

Another important element to be considered when testing immunomodulatory drugs for stroke is that postischemic systemic immunomodulation and infectious complications are some of the main comorbidities after stroke. In experimental models, the systemic immune responses differ substantially among stroke models and infarct volume.<sup>89,124</sup> Translational studies of immunomodulatory therapies for stroke must account for this heterogeneity, especially in the case of drugs with long-term effects such as natalizumab, that blocks the  $\alpha$ -4 integrin for at least 4 weeks.<sup>125</sup>

## Conclusion

Factors contributing to the failure in the translation of therapies targeting stroke-induced immune responses to clinical practice include nonmodifiable factors (differences in the cerebrovascular system in humans and rodents, variability in the immune response in patients depending on their



 <p>IL-1 receptor antagonist IL-1 receptor</p>	<p><b>Therapeutic strategies targeting IL-1-induced inflammation</b></p> <p><b>Recombinant human IL-1 receptor antagonist, rhIL1-Ra</b></p> <p>Positive results in a Phase II clinical trial (intravenous administration) (Emsley <i>et al.</i>, 2005)</p> <p>Not beneficial in a Phase II clinical trial (subcutaneous administration) (<b>SCIL-STROKE</b>) (Smith <i>et al.</i>, 2018)</p>
	<p><b>Therapeutic strategies targeting microglial activation</b></p> <p><b>Minocycline</b></p> <p>Positive results in two Phase II clinical trials (Lampl <i>et al.</i>, 2007; Padma Srivastava <i>et al.</i>, 2012)</p> <p>Phase IV clinical trial stopped for futility (<b>NeuMAST</b>) (Kohler <i>et al.</i>, 2013)</p>
	<p><b>Therapeutic strategies targeting endothelial selectins</b></p> <p><b>Enlimomab</b> (mouse anti-ICAM-1)</p> <p>Worse stroke outcome in a Phase III clinical trial (Enlimomab Acute Stroke Trial Investigators, 2001)</p> <p><b>E-selectin tolerance</b></p> <p>Results from Phase II clinical trial not disclosed (<a href="http://clinicaltrials.gov/show/NCT00012454">http://clinicaltrials.gov/show/NCT00012454</a>)</p>
 	<p><b>Therapeutic strategies targeting leukocyte infiltration</b></p> <p><b>UK-279, 276</b> (Recombinant neutrophil inhibitory factor, blocking its interaction with ICAM-1) (<b>ASTIN</b>)</p> <p>Phase II clinical trial stopped for futility (Krams <i>et al.</i>, 2003)</p> <p><b>Hu23F2G</b> (Humanized monoclonal antibody against neutrophil CD11/CD18, blocking its interaction with ICAM-1) (<b>HALT</b>)</p> <p>Phase III clinical trial stopped for futility (results not disclosed) (del Zoppo, 2010)</p> <p><b>Fingolimod</b> (sphingosine-1-phosphate receptor on lymphocytes)</p> <p>Positive results in two pilot studies (Fu <i>et al.</i>, 2014; Zhu <i>et al.</i>, 2015)</p> <p><b>Natalizumab</b> (humanized antibody against the VCAM-1 leukocyte ligand VLA-4) (<b>ACTION</b>)</p> <p>Not beneficial in a Phase II clinical trial (Elkins <i>et al.</i>, 2017)</p>

**Figure 2.** Summary of clinical trials targeting the immune responses triggered after ischemic stroke. CD11/CD18, LFA-1 for lymphocyte function-associated antigen 1; IL-1, interleukin 1; rhIL1-Ra, recombinant human interleukin 1 receptor antagonist; ICAM-1, intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule; VLA-4, leukocyte very late antigen-4.

prestroke inflammatory status, which is extremely difficult to mimic in animal models of stroke...) that need to be considered when interpreting results. However, most of these factors are modifiable and include, among others, the use of clinically relevant stroke models and therapeutic time windows, the comparison of a same molecule in different stroke models, the improvement in the experimental designs and the inclusion of females and comorbidities in preclinical studies.

Now that we are in the age of acute revascularization, one might wonder if we can do better than thrombectomy for patients with penumbra. If the answer is ‘no’ then there is probably no need for immunomodulatory treatments for these patients. If the answer is ‘yes,’ and also for all the other patients, immunomodulation will retain therapeutic interest. Preclinical studies should be conceived according to the targeted stroke subpopulation. In any case, a strictly linear bench-to-bedside

paradigm is probably not optimal for translating basic scientific findings into clinically effective stroke therapies, and a bedside-back-to-bench paradigm will help in this translation.

In spite of the limitations of experimental stroke models, our knowledge about the inflammatory responses triggered after stroke onset exponentially increases thanks to preclinical and clinical studies, and gives rise to novel therapeutic targets and improved strategies, with the hope of ameliorating stroke outcome in patients.

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### Conflict of interest statement

The authors declare that there is no conflict of interest.

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