

# Role of immune responses for extracellular matrix remodeling in the ischemic brain

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**Abstract:** Neuroinflammation is one of the key components contributing to the devastating outcome of ischemic stroke. Starting with stroke onset, inflammatory processes contribute both to cell damage and tissue remodeling. The early release of alarmins triggers the upregulation of multiple proinflammatory cytokines, resulting in the compromised integrity of the blood–brain barrier. From this moment on, the infiltration of peripheral immune cells, reactive gliosis and extracellular matrix (ECM) alterations become intricately intertwined and act as one unit during the tissue remodeling. While the mechanisms of leukocyte and glia activation are amply reviewed, the field of ECM modification remains as yet under explored. In this review, we focus on the interplay between neuroinflammatory cascades and ECM in the ischemic brain. By summarizing the currently available evidence obtained by *in vitro* research, animal experimentation and human studies, we aim to propose a new direction for the future investigation of stroke recovery.

**Keywords:** ischemic stroke, extracellular matrix, neuroinflammation, neuroplasticity

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## Introduction

Ischemic stroke remains a leading cause of death and disability in the adult population. Despite plenty of efforts devoted to understanding and treating the disease, most novel approaches have only a discouragingly limited impact on patients' wellbeing.<sup>1</sup> We suggest that to improve the translation of scientific advances from bench to bedside, the pathophysiology of ischemic stroke should be investigated from complementary points of view. Today, most studies of stroke put the major focus on neuronal plasticity and repair,<sup>2–4</sup> blood–brain barrier (BBB) dysfunction,<sup>5</sup> and neuroinflammation.<sup>6</sup> In this review, we will address the relationship between the immune response and the reorganization of the extracellular matrix (ECM) during the acute and chronic phases of ischemic stroke. Although both aspects have been studied individually, their interaction is rarely considered in both experimental and clinical settings. We propose that the brain's immune response and ECM regulation should be considered as a functional unit, as first proposed by Schönherr and Hausser,<sup>7</sup> opening new perspectives in stroke treatment.

The ECM is a congregation of multiple adhesion molecules, polysaccharides, proteins and proteoglycans arranged three-dimensionally in the extracellular space. During development and adulthood, this complex fulfils various functions, such as regulating cell migration, proliferation, adhesion, differentiation,<sup>8</sup> synaptic plasticity,<sup>9</sup> maintenance of the BBB<sup>10</sup> and tissue architecture, integrity and homeostasis.<sup>11</sup> In the central nervous system (CNS), ECM can be divided into two compartments, the interstitial matrices and basement membranes (BMs).<sup>12</sup> The interstitial matrix is based on diffuse meshworks of hyaluronic acid (HA), which incorporate mainly collagens and proteoglycans.<sup>13</sup> The BMs are associated with the basal portion of cerebral endothelial cells and consist mainly of laminins, collagen IV, nidogens and heparan sulfate proteoglycans.<sup>14</sup>

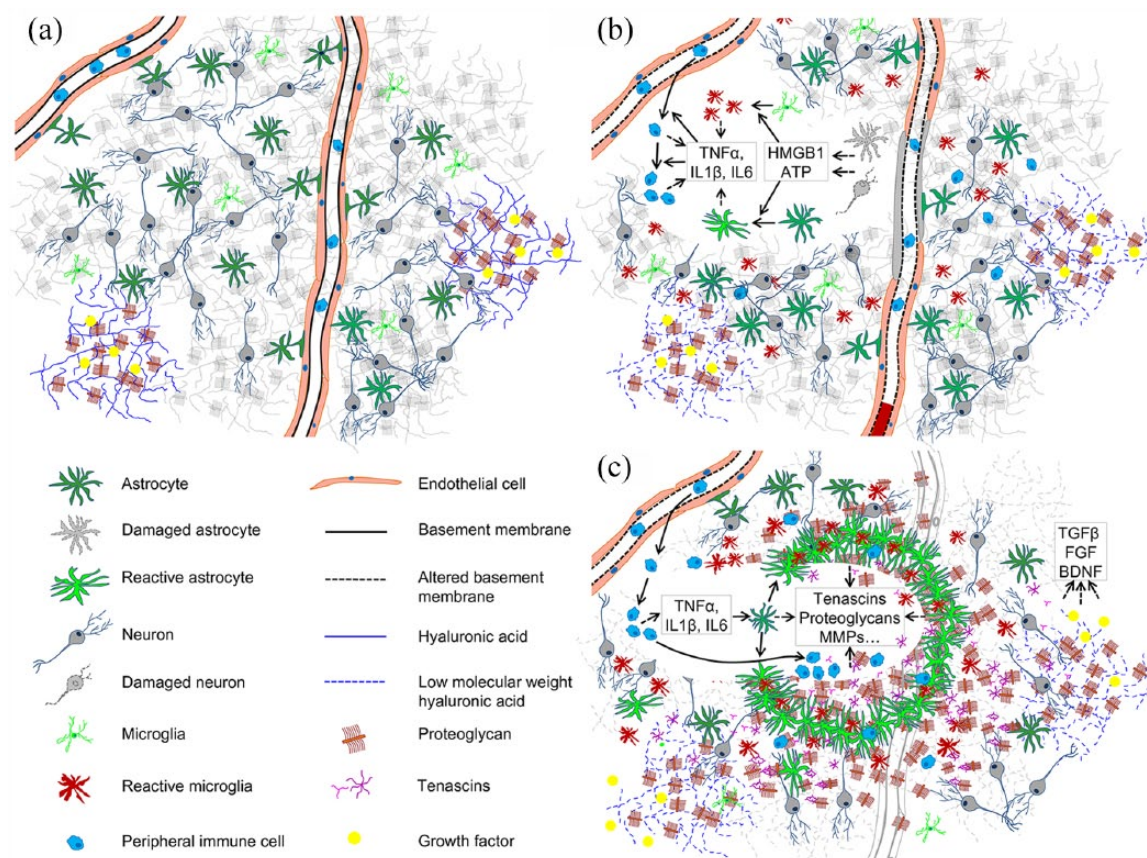
The interplay between the immune activation and the ECM is involved in different tissue compartments during the three overlapping phases of ischemic stroke: cell death and inflammation, tissue remodeling and neural plasticity and rewiring.<sup>15</sup> Within this review, we will focus on the three main

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**Figure 1.** ECM and cellular organization in healthy tissue (a), during initial neuroinflammation after stroke (b) and during subsequent tissue remodeling and matrix deposition (c). ECM, extracellular matrix.

aspects of this interplay: (a) interaction between infiltrating leukocytes and endothelial BMs during the initial neuroinflammatory response; (b) reactive gliosis and ECM remodeling; (c) potential of ECM modifications for restorative therapy. For convenience, the key highlights of initial immune cell infiltration and reactive gliosis following ischemic stroke and subsequent tissue replacement are schematically summarized in Figure 1.

### Multicellular release of alarmins and early neuroinflammatory response

In cerebral ischemia, the cessation of blood flow induces severe metabolic stress causing cell death in the hypoperfused territories. Within the early phase, initial cell death causes the disruption of the BBB<sup>16</sup> and activates the innate immune system.<sup>17</sup> The compromised supply of oxygen and nutrients triggers the release of household molecules from the damaged cells. Heat shock protein

70 (HSP70), adenosine triphosphate (ATP) and high mobility group box 1 protein (HMGB1) are among these. In ischemic tissue, these molecules are recognized as danger signals, or alarmins. The term ‘alarmins’ was proposed to differentiate these molecular signals from the exogenous stimuli associated with pathogens.<sup>18</sup> One of the most studied alarmins in the context of brain injury is HMGB1. In healthy tissue, this nuclear protein organizes the DNA and regulates transcription.<sup>19</sup> Starting in the first hour after ischemia, HMGB1 translocates into the cytoplasm, undergoes post-translational modification, and its acetylated form is released into the extracellular space.<sup>17,20</sup> Several studies demonstrated that the released HMGB1 binds to the receptor for advanced glycation end products (RAGE) and Toll-like receptors 2 and 4 (TLR2 and TLR4) on leukocytes, neurons, astrocytes and microglia,<sup>17,21–23</sup> inducing the multicellular release of the proinflammatory cytokines. On microglia, HMGB1 can also bind

with macrophage antigen complex 1 (MAC1), driving the proinflammatory phenotype of these cells.<sup>24</sup> Also, the early HMGB1 release can directly induce astrocyte reactivity<sup>25</sup> and stimulate astrocytic glutamate exocytosis.<sup>25</sup> The reactive gliosis profile is further reinforced by extracellular ATP. In fact, ATP itself acts as an important alarmin in ischemic tissue and can be secreted by multiple cell types. In the CNS, extracellular ATP binds to purinergic P2X and P2Y receptors on the plasma membrane of endothelial cells, leukocytes, neurons and glial cells, allowing the nonselective diffusion of small monovalent and bivalent cations.<sup>26</sup> On one hand, the abundant release of ATP from reactive microglia is suggested to induce astrocyte reactivity and excitatory neurotransmission.<sup>27</sup> But on the other hand, the reactive astrocytes may trigger the microglial inflammatory response *via* the release of astrocytic ATP.<sup>28,29</sup> Apparently, extracellular ATP acts as a feed-forward element during the early stage of the induction of reactive gliosis. Although the exact interplay between the glia activation cascades remains to be clarified, current data suggest that the multicellular production of alarmins converges on the elevated extracellular levels of proinflammatory cytokines. Under both experimental and clinical conditions, these initial responses can be further amplified by infiltrating peripheral immune cells following the BBB disruption.

In addition to alarmins of intracellular origin, similar signaling properties are attributed to several ECM components, including low molecular weight HA,<sup>30,31</sup> fibronectin and heparan sulfate.<sup>32</sup> Similar to pathogen-associated molecular patterns (PAMPs), these molecules bind to pattern recognition receptors, leading to the activation of the immune response. It is hypothesized that the cleavage of ECM alarmins from the intact matrix can be mediated by the overproduction of reactive oxygen species.<sup>33</sup> Under normal circumstances, high molecular weight HA serves as an ECM backbone, being essential to prevent neuronal overexcitation and epileptiform activity.<sup>34</sup> As a result of injury, high molecular weight HA is broken down to smaller fragments, which amplify inflammatory responses by binding to TLR2 and TLR4 receptors on leukocytes.<sup>35</sup> Interestingly, the CD44 glycoprotein, which is widely expressed by multiple brain cells (including microglia, astrocytes, lymphocytes and vascular epithelium), can sequester the low molecular weight HA, thereby limiting its inflammatory effect.<sup>36,37</sup>

### Early neuroinflammatory response and peripheral immune cell infiltration

In the healthy brain, the BBB is a semipermeable barrier formed by endothelial cells, astrocytes and pericytes that allows the selective entry of secreted regulatory molecules to the brain parenchyma.<sup>38</sup> In ischemic stroke, the loss of tight junctions between endothelial cells allows the entry of plasma proteins and peripheral immune cells.<sup>39</sup> The BBB breakdown is triggered within the first few hours<sup>40</sup> by the initial upregulation of proinflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ .<sup>41–43</sup> This early inflammatory response prepares the vascular surface to interact with the first leukocytes infiltrating the CNS, namely with neutrophils and monocytes. The released TNF- $\alpha$  and IL1 $\beta$  interact with p55-p75 and IL1ra receptors on the surface of endothelial cells,<sup>42,44</sup> inducing the upregulation of endothelial adhesion molecules such as P- and E-selectins, PCAM1, ICAM1 and integrins.<sup>45,46</sup> In turn, the enhanced adhesion slows down rolling leukocytes, leading to their immobilization on the luminal surface of capillaries and venules and facilitating migration of neutrophils and monocytes into the brain parenchyma.<sup>47</sup> Although these mechanisms have widely been explored, more recent evidence suggests that extravasation of peripheral immune cells does not solely depend on endothelial adhesion molecules, but that ECM mediated interactions are crucially involved in this process. In fact, leukocyte infiltration is largely regulated by laminin-8 and -10 of the endothelial BMs. Following the intravital application of TNF- $\alpha$  in mice lacking laminin-10, BBB integrity is compromised *via* the destabilization of junctional cadherin,<sup>48</sup> resulting in facilitated transmigration of leukocytes. On the other hand, laminin-8 stimulates leukocyte migration, and knockout mice lacking this BM molecule exhibit reduced recruitment of immune cells to inflammatory loci.<sup>49</sup> Intriguingly, the composition of capillary and venular basement membranes is heterogeneous, and peripheral neutrophils tend to associate with low expression regions which contain less laminin-10, collagen IV and nidogen-2.<sup>50</sup> In IL $\beta$  stimulated tissue, neutrophils can use these regions to enter brain parenchyma. During the initial neuroinflammatory response, the infiltrating leukocytes are able to modify the BM matrix by secreting neutrophil elastase<sup>51</sup> and macrophage metalloproteases.<sup>52</sup> Although the exact mechanisms of leukocyte–endothelium interactions still need further investigation, they clearly result in



BBB permeability and transmigration of peripheral neutrophils and macrophages during the first 24h after stroke in rodent models.<sup>53</sup> At later time points, BBB leakage persists, and T-cells enter the perilesional brain tissue. In different animal models of ischemic stroke, the presence of blood macrophages and T-cells in the brain parenchyma typically peaks between 7 and 30 days after stroke.<sup>53–55</sup> Both human and mouse models show that the infiltration of regulatory T-cells has a positive effect on neurological recovery by the secretion of anti-inflammatory cytokines, IL10 and tumor growth factor (TGF)- $\beta$ .<sup>56,57</sup> In addition, regulatory T-cells can facilitate neurological recovery by producing neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF).<sup>58,59</sup> Notably, elevated levels of pro- and anti-inflammatory cytokines, compromised BBB function, activated macrophages, regulatory T-cells and reactive glia can be observed several weeks after ischemia, playing an important role in tissue remodeling.<sup>15</sup> The role of ECM in further inflammatory cascades is closely related to the modification of the interstitial matrix by reactive glia. During the recovery phase, the altered expression of glycoproteins and proteoglycans has an important effect on the behavior of immune cells in the brain parenchyma.<sup>12</sup>

### **Glial scar formation and extracellular matrix deposition**

After initial cell death during the first hours after stroke, the inflammatory response triggers the proliferation of glial cells. Stimulated by TNF- $\alpha$  and IL6 cytokines, resident astrocytes become reactive [this process is usually defined by the upregulation of glial acidic protein (GFAP) expression], and form a compact glial scar around the lesion core.<sup>60,61</sup> Glial scar astrocytes contribute to the recruitment of peripheral macrophages into the injury site by secreting chemokine (C-C motif) ligand 3 (CCL3) and chemokine (C-X-C motif) ligands 1 and 2 (CXCL1 and CXCL2).<sup>62</sup> The enrolled macrophages remove damaged tissue components, enabling tissue remodeling. Inside the lesion core, tissue remodeling is characterized by non-neural cell proliferation and ECM deposition. Fibrocytes, fibroblasts, immune cells and surrounding glia produce massive amounts of collagens, proteoglycans and tenascins, building up the fibrotic scar. It is important to distinguish the fibrotic scar from the astrocytic

scar. While the astrocytic scar surrounds the lesion site and may promote neural remodeling and rewiring,<sup>63</sup> the fibrotic replacement of damaged tissue provides rapid wound repair at the cost of inhibiting axonal regeneration.<sup>64</sup>

The astrocytic scar limits secondary damage to brain tissue surrounding the ischemic area by restricting propagating cell death. The compromised formation of glial scars in transgenic animals leads to increased neuronal death, demyelination and worsens neurological recovery in traumatic or ischemic brain injury.<sup>65–68</sup> These beneficial aspects of glial scar formation can partly be explained by the regulatory properties of ECM. For instance, astrocytic scar can limit the migration of reactive immune cells into the perilesional tissue by promoting chondroitin sulfate proteoglycan (CSPG)-mediated cell adhesion.<sup>69</sup> In addition, reactive astrocytes that express tenascin C (TnC) can suppress the production of cytokines by infiltrating T-cells.<sup>70,71</sup> The tenascin gene family comprises four genes in mammals,<sup>72</sup> of which TnC and tenascin-R (TnR) are expressed in the CNS. TnC is a major constituent of the neural stem cell compartment<sup>73</sup> and implicated in axon growth and guidance, synaptic plasticity and regeneration of the CNS.<sup>74,75</sup> TnR is exclusively expressed in the CNS and associated with perineuronal nets.<sup>76</sup>

Although the glial scar is crucial for lesion demarcation, the majority of ECM molecules synthesized by reactive astrocytes limits beneficial neural plasticity in the injury recovery phase. Besides creating a compact layer in close proximity to the lesion core, reactive glia express keratan sulfate proteoglycans (KSPGs)<sup>77</sup> and CSPGs.<sup>78,79</sup> In a number of studies, these molecules were described to limit postinjury neuronal regeneration and axonal sprouting (Jin et al, 2018). Based on ample evidence supporting the inhibitory properties of the glial scar, several treatment strategies were proposed that counteract astrocyte proliferation and ECM accumulation (for review see Rolls and colleagues<sup>80</sup>). The suggested approaches included enzymatic ECM degradation,<sup>81,82</sup> ECM blockade by anti-CSPG antibodies,<sup>83</sup> inhibition of ECM formation<sup>84</sup> and inhibition of astrocyte proliferation.<sup>85</sup> Despite their certain relevance, these concepts are based on the perception that the glial scar is an obstacle that has to be circumvented to improve stroke recovery. However, a recent study demonstrated that after spinal cord injury, a subpopulation of scar-forming astrocytes releases a

set of CSPGs that aid axon regeneration.<sup>63</sup> Another ECM component of the glial scar, TnC,<sup>86</sup> was shown to promote the outgrowth of retinal axons<sup>87</sup> and sensory axon regeneration following spinal cord injury.<sup>88</sup>

In healthy nervous tissue, ECM incorporates many regulatory molecules which play pivotal roles during brain development. For example, several members of the heparan sulfate proteoglycan (HSPG) family are necessary for cell positioning, lineage specification and neural wiring guidance.<sup>89,90</sup> During development, the CSPG/HSPG expression ratio provides a bidirectional control over axon pathfinding, with HSPGs promoting and CSPGs repelling axonal outgrowth.<sup>91</sup> Upon ischemia, the dynamics of the CSPG/HSPG ratio closely resembles the developmental profile,<sup>92</sup> providing a potential mechanism of targeted neural rewiring.

Taken together, the altered expression of ECM components in reactive astrocytes can shape the extracellular space in a polarized way, preventing axonal outgrowth into the lesion core, but enabling neural rewiring under certain conditions in perilesional areas.<sup>93</sup> Considering that reactive glia and leukocytes persist in the brain parenchyma during long periods after stroke onset, one could predict that both infiltrating immune cells and glia are central for long term ECM remodeling. Unfortunately, our current understanding of how ECM changes can influence neurological recovery is very limited. Also, there are no therapeutic approaches to manipulate these alterations, and thus further research is critically needed. Since the crude depletion of ECM components induces explicit dysregulation of neural networks,<sup>94–96</sup> we propose that a beneficial stroke recovery requires a reversible and delicate modulation of ECM composition and structure. With this scope, matrix proteases may be promising targets for future investigation, as they comprise an intrinsic regulatory system within ECM.<sup>97</sup>

### ECM remodeling and neurological recovery

Albeit the main scaffold of interstitial brain matrix is composed by HA and proteoglycans, many regulatory functions of the ECM rely on associated signaling molecules.<sup>76</sup> Originating from different cell types, the vast majority of short range signaling factors become embedded in the extracellular meshwork. Unlike the constitutive ECM elements,

the expression of these components may dynamically change, promptly responding to the augmenting cellular environment. Growth factors, ILs and molecular guidance signals can either directly bind the core protein of proteoglycans or interact with polysaccharide side chains.<sup>98</sup> Importantly, both regulatory molecules like BDNF, fibroblast growth factor (FGF), TNF- $\alpha$  and TGF- $\beta$  can be immersed in the same mesh as their cleavage factors. Within ECM, the two major families of neural endogenous proteases are matrix metalloproteases (MMPs) and A disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs). Both MMPs and ADAMTSs can specifically degrade proteoglycans and glycoproteins, locally modifying the ECM structure and composition.<sup>99</sup> However, they also modulate neural plasticity by cleaving growth factors such as BDNF.<sup>100</sup> ADAMTSs and MMPs are upregulated in perilesional areas following ischemic stroke and brain trauma, where they can promote neural recovery *via* ECM remodeling.<sup>101</sup> In particular, proteoglycan digestion by ADAMTS4 was shown to enhance axonal regeneration and sprouting after spinal cord injury similar to chondroitin sulfate degradation.<sup>102</sup> Upon ischemia, MMP2 and MMP9 are suggested to promote leukocyte infiltration, and to remove ECM barriers for neural plasticity.<sup>103</sup> In stroke, the modulation of MMP9 activity using synthetic compounds, endogenous inhibitors and hypothermia, was recently proposed as a part of restorative therapy.<sup>104</sup> It was also reported that in the subacute phase of ischemia MMP9 is expressed by proliferating neural progenitors in the subventricular zone, proposing a new role of MMPs in restorative neurogenesis.<sup>105</sup>

### ECM remodeling as new target for stroke therapy

Taken in view of the coherent activation of brain innate immunity, peripheral immune cell infiltration and brain ECM changes, it is tempting to suggest that the available immunotherapies can support neurological recovery after stroke. However, to the best of our knowledge, there are currently no reports describing a successful usage of cytokine-directed or lymphocyte-targeted therapy in stroke, in contrast with cancer and autoimmune diseases.<sup>106</sup> Recently, low doses of colchicine was proposed as a novel treatment to mitigate atherosclerotic plaque inflammation, which reduces recurrent stroke rates in patients.<sup>107</sup> The beneficial effect of colchicine is presumed to be mediated by

**Table 1.** Summary of the most important findings discussed in this review.

HMGB1 protein is released early after the onset of brain ischemia from the degenerating neurons. HMGB1 promotes the upregulation of matrix proteases and stimulates proinflammatory cytokine synthesis.	Andersson and colleagues <sup>22</sup> ; Qiu and colleagues <sup>20</sup> ; Qiu and colleagues <sup>21</sup>
Low molecular weight hyaluronan acts as an endogenous danger signal.	Scheibner and colleagues <sup>30</sup>
Microglia activation triggers an astrocyte-driven upregulation of excitatory neurotransmission.	Pascual and colleagues <sup>27</sup>
Astrocytes trigger rapid microglial response to brain injury via ATP release.	Bianco and colleagues <sup>29</sup> ; Davalos and colleagues <sup>28</sup>
Endothelial basement membrane matrix regulates immune cell recruitment to inflammatory loci.	Kenne and colleagues <sup>49</sup> ; Song and colleagues <sup>48</sup> ; Wang and colleagues <sup>51</sup> ; Wang and colleagues <sup>50</sup>
Astrocyte scar formation is central for axon regeneration after spinal cord injury.	Anderson and colleagues <sup>63</sup>
The disturbed formation of astrocytic scar is detrimental for the recovery after traumatic brain injury.	Bush and colleagues <sup>65</sup>
Chondroitin sulfate proteoglycans inhibit neurite outgrowth.	Jin and colleagues 2018 <sup>112</sup>
The depletion of inhibitory chondroitin sulfate proteoglycans improves neuronal regeneration and functional recovery after spinal cord injury.	Bradbury and colleagues <sup>82</sup> ; Monnier and colleagues <sup>84</sup> ; Moon and colleagues <sup>81</sup> ; Tan and colleagues <sup>83</sup>
The integrity of brain extracellular matrix is essential for retaining synaptic connectivity.	Bikbaev and colleagues <sup>94</sup>
During development, the bidirectional control over axon pathfinding is determined by CSPG/HSPG expression ratio, with HSPGs promoting and CSPGs repelling axonal outgrowth. Upon ischemia, the dynamics of the CSPG/HSPG expression closely resembles the developmental profile.	Coles and colleagues <sup>91</sup>
Ultrastructural modifications of perineuronal ECM are proposed as a mechanism to support neural plasticity after stroke.	Dzyubenko and colleagues <sup>111</sup>
ATP, adenosine triphosphate; CSPG, chondroitin sulfate proteoglycan; ECM, extracellular matrix; HMGB1, high mobility group box 1; HSPG, heparan sulfate proteoglycan.	

inhibition of neutrophil activation and chemotaxis.<sup>108</sup> In rheumatoid arthritis, anti-TNF- $\alpha$  treatments reduced plasma levels of sulfated glycosaminoglycans (GAGs),<sup>109</sup> suggesting a possibility to modulate ECM metabolism by cytokine targeting. But despite these perspectives, immunotherapy has to be evaluated with great care. Firstly, the rough depletion of inflammatory cascades may interfere with glial scar formation, deteriorating stroke outcome. Secondly, the nonlocalized inhibition of proinflammatory cytokines has significant bystander effects and may result in adverse events, such as an increased susceptibility to infections.<sup>110</sup>

From our point of view, the most promising strategy to improve neurological recovery after stroke is to combine the general supportive care with a spatially confined modulation of brain ECM. In our recent work,<sup>111</sup> we proposed that the remodeling of perineuronal ECM may promote neural rewiring in the stroke brain. To our knowledge, there are currently no treatments which enable to alter ECM ultrastructure and composition in a controlled and localized way. However, the existing evidence (Table 1), although admittedly fragmented, allows proposing two innovative directions in the search for ECM-modifying

agents: (a) a delicate modification of proteoglycan complexes and (b) a subtle modulation of matrix protease activity. Based on these strategies, we believe that in the next years the combined efforts of neuroscientists and translational neurologists may allow developing new encouraging therapies that improve stroke recovery by a mode of action that is spatially confined to ECM.

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The authors declare that there is no conflict of interest.

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