

● PERSPECTIVE

Targeting acute inflammation to complement spinal cord repair

Immune effector mechanisms play key roles in the progressive (secondary) neurodegenerative changes that follow spinal cord injury (SCI). In our recent paper (Brennan et al., 2015), we showed that the inflammatory response to SCI includes rapid and robust activation of the innate immune complement system, with tissue levels of complement component 5a (C5a – an activation product generated by the proteolysis of complement factor 5 (C5)) peaking 12 to 24 hours post-injury.

Complement system activation normally forms the front-line of host defense to microbial challenges. It is now widely recognized, however, that the activation of complement can also modify disease course and/or outcomes in sterile inflammatory conditions, including those that affect the nervous system like SCI. Complement activation in such conditions has been mostly thought of as detrimental, but several reports have emerged in recent years ascribing positive roles in tissue regeneration and repair to at least some components of the complement cascade (e.g., Haynes et al., 2013). In studying the role of C5a in SCI, we generated novel insights regarding how this particular complement activation product also appears involved in endogenous repair processes. Specifically, we showed that engagement of the primary receptor for C5a, C5aR1 (also known as C5aR or CD88), regulates astrocyte proliferation during the post-acute phase of SCI (Brennan et al., 2015). Here, we overview these newly identified mechanisms and highlight outstanding questions that remain to be addressed when considering therapeutic targeting of the C5a-C5aR1 axis to treat central nervous system (CNS) injury.

Breakdown of the blood-spinal cord barrier (BSB) following a neurotraumatic event permits the entry of circulating blood serum proteins, including complement factors, into the injured parenchyma. Permeability to larger macromolecules such as the parent protein of C5a, *i.e.*, C5 (~180 kDa), is likely restricted to the first hours to days after SCI (e.g., Lee et al., 2014), but may be longer for smaller molecules like C5a (12–14.5 kDa). Although the liver is a major source of complement proteins in the body, many of these can also be synthesized by cells that are intrinsic to the CNS. The pathophysiological role of the complement system in SCI is therefore not necessarily restricted to the (sub-) acute phase when the BSB is most compromised.

Proteolytic cleavage (*i.e.*, activation) of C5 following neurotrauma is a key event that is thought to contribute significantly to secondary neuroinflammatory pathology *via* 1) activation of the terminal pathway (which causes cell death through formation of the lytic membrane attack complex (MAC) on target cells), and 2) injurious pro-inflammatory C5a signaling *via* C5aR1. Neutralizing either C5 or its bioactive cleavage products, C5a and C5b, is therefore a promising therapeutic strategy to attenuate secondary immune-mediated pathology in SCI and other forms of acquired CNS injury. Intriguingly, interference with C5a-C5aR1 signaling in the more chronic phase of SCI (14 days post-injury on-

wards) does not yield a therapeutic benefit. In fact, injury outcomes under these conditions are worsened in both rat and mouse models of contusive SCI (Beck et al., 2010; Brennan et al., 2015). These findings suggest more complex roles for C5a signaling in neurotrauma, with some aspects appearing neuroprotective and/or aiding repair. A key focus of our laboratory over the past few years has been to better understand these roles of C5a in the context of SCI.

It is now firmly established by our own work (Brennan et al., 2015) and that of others (Li et al., 2014) that a lack of C5aR1 signaling in the very acute phase of injury (12 hours post-SCI) leads to significant reductions in pro-inflammatory cytokine levels (e.g., interleukin-1 beta (IL-1 β), IL-6 and chemokine (C-X-C motif) ligand 1 (CXCL1)) at the lesion site, which correlate with improved outcomes, at least when generic targeting of C5aR1 is restricted to the (sub-) acute phase. CNS cells, in particular astrocytes but also microglia, most likely mediate these beneficial effects of disarming the C5a-C5aR1 axis as they are known to be the primary source of pro-inflammatory cytokines early after SCI (Pineau and Lacroix, 2007) and express C5aR1. The fact that expression of key pro-inflammatory cytokines is at least in part regulated *via* C5aR1 is of significance because elevated levels of e.g., IL-1 β have been directly associated with neuronal death and axon retraction *in vivo* and *in vitro*. Improved outcomes in absence of C5aR1 signaling therefore likely involve neuroprotection due to an abrogation of the acute inflammatory response to SCI.

Pro-inflammatory cytokines like IL-1 β and IL-6 also favor polarization of activated microglia and macrophages towards a classical pro-inflammatory M1 phenotype, which is generally considered injurious and not supportive of regeneration. Specifically, M1-polarized macrophages are an additional source of pro-inflammatory cytokines, and they exacerbate secondary SCI pathology by direct neurotoxicity and axonal retraction (Gensel et al., 2009). Whether the polarization of activated microglia and macrophages following SCI is, at least transiently, more biased towards a regeneration-favoring M2 phenotype in absence of C5aR1 signaling due to associated reductions in pro-inflammatory cytokine levels is still to be determined. It also remains to be seen whether or not C5aR1 is directly involved in recruitment of pro-inflammatory Ly6C^{hi} monocytes/macrophages, which normally peaks between 4 and 7 days post-SCI. Although C5aR1 appears largely dispensable for neutrophil recruitment in SCI, significantly fewer Ly6C^{hi} monocytes/macrophages are present at the lesion site following inhibition of the C5a-C5aR1 axis (Beck et al., 2010; Brennan et al., 2015). Such a chemotactic role for C5aR1 may, however, be difficult to entangle from e.g., diminished recruitment of inflammatory monocytes as a result of reduced pro-inflammatory cytokine levels in absence of C5a-C5aR1 signaling or, alternatively, attenuated secondary injury; these two possibilities are also not mutually exclusive. Specific targeting of C5aR1 in blood monocytes may shed some light on this issue and potentially unlock new C5aR1-based therapeutic strategies to reduce recruitment of these cells to the inflamed CNS.

As SCI is hallmarked by chronic, non-resolving and M1-biased inflammation (e.g., Kroner et al., 2014), the finding that continued or prolonged interference with the pro-inflammatory C5a-C5aR1 axis leads to a worsening

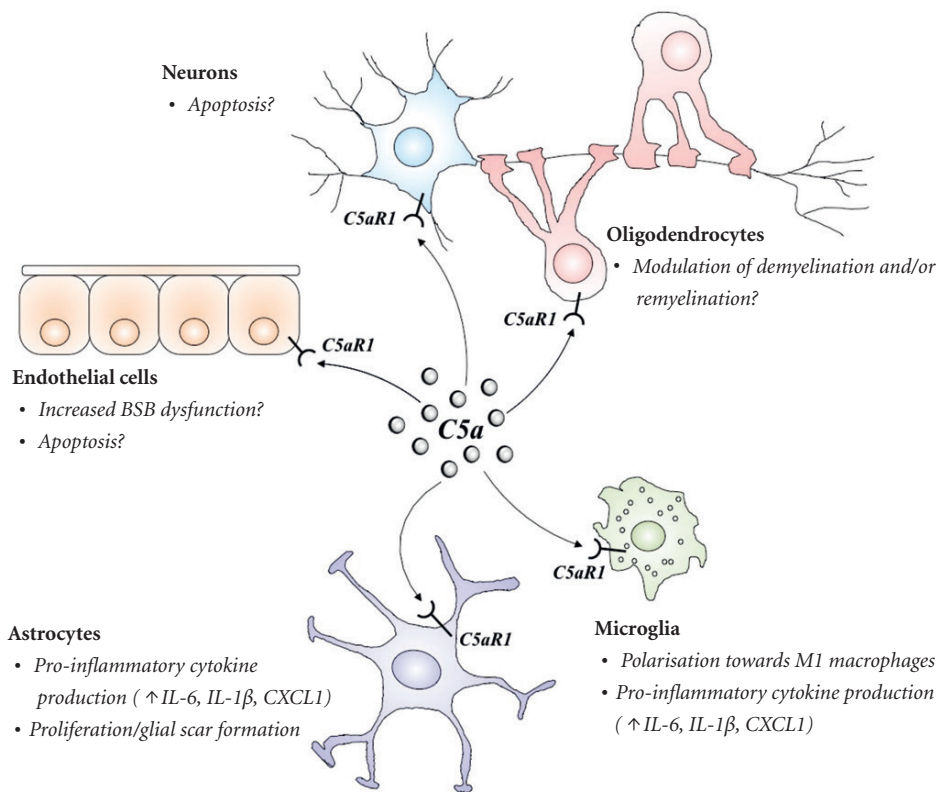


Figure 1 Overview of established and possible roles for C5a-C5aR1 signaling in traumatic spinal cord injury (SCI). Complement activation and C5a generation after SCI contributes to secondary inflammatory pathology through C5aR1 *via* the induction of pro-inflammatory cytokine expression, neuronal apoptosis and/or endothelial cell dysfunction, which increases leakiness of the blood-spinal cord barrier. Reparative aspects of C5a-C5aR1 signaling in the post-acute phase of SCI likely include its role in astrocyte proliferation, which is required for the effective formation of a glial barrier (scar) between the necrotic lesion core and spared spinal cord tissue. BSB: Blood-spinal cord barrier; C5a: complement component 5a; C5aR1: complement component 5a receptor 1; CXCL1: chemokine (C-X-C motif) ligand 1; IL-6: interleukin-6; IL-1 β : interleukin-1 beta.

rather than improvement in SCI outcomes seems, at least at first sight, somewhat surprising. When investigating the possible neuroprotective and/or reparative mechanisms that are driven by C5aR1 signaling in the post-acute phase of SCI, we discovered that astrocytes depend on the C5a-C5aR1 axis for proliferation; this dependency is likely key to the dual and opposing roles for C5aR1 signaling in SCI.

Various molecular signals, including pro-inflammatory cytokines, have previously been implicated in promoting astrocyte hypertrophy, proliferation and/or migration toward sites of inflammation. This proliferation and accumulation of reactive astrocytes along the margins of the necrotic lesion core is part of a critical, barrier-forming process that limits the spread of secondary pathology into neighboring neural tissue that is vulnerable to degeneration but not directly affected by the initiating neurotraumatic event (Okada et al., 2006). Our latest findings add C5a to the repertoire of molecules that regulate this beneficial aspect of the glial ‘scar’ by showing that C5a can directly induce astrocyte proliferation, most likely through activating the STAT-3 pathway downstream of C5aR1. In addition to the direct mitogenic effect of C5a, C5aR1 signaling may also influence astrocyte proliferation *via* its regulation of IL-6 production (Okada et al., 2004), either in a direct autocrine fashion or indirectly *via* activated microglia and macrophages; these possibilities are again not mutually exclusive and may act simultaneously.

Consistent with the view that prolonged interference with C5a-C5aR1 signaling negatively impacts on endogenous wound healing responses, a chronic lack of C5aR1 signaling not only resulted in reduced astrogliosis at and around the lesion epicenter, it also significantly increased lesion size and the spread of infiltrating immune cells across the injured

spinal cord segment. These histopathological changes directly correlated with worsened SCI outcomes. If impairments in the endogenous proliferative response of astrocytes following SCI were indeed responsible for the late worsening in outcomes under C5aR1-deficient conditions, then the number of newly generated astrocytes at and around the lesion site should negatively correlate with lesion volume, which was indeed the case as revealed by pulse-chase studies with 5-bromo-2'-deoxyuridine (BrdU). Furthermore, as short-term (up to 7 days post-injury) antagonism of C5aR1 led to sustained improvements in SCI outcomes (*i.e.*, no dual phenotype with early improvements but a late worsening), it also stands to reason that the astroglial response at and around the lesion epicenter in this scenario then, at a minimum, should be similar to that of vehicle-treated controls, as was observed. Thus, abnormal astrocytic responses with sustained absence of C5aR1 signaling are likely a key contributing factor to a worsening of outcomes in the more chronic phase of SCI due to a failure to effectively segregate the necrotic lesion core from neighboring intact tissue. Specific ablation of C5aR1 in astrocytes (and microglia) *via* conditional gene targeting approaches in future experiments could provide more direct and definitive evidence for this premise. Whether or not exogenous stimulation of C5aR1 in the post-acute phase of injury could in fact aid recovery by augmenting astrocyte proliferation, and in doing so, accelerate sealing of the injury site, also remains to be addressed. The adverse pro-inflammatory consequences of C5aR1 agonism are, however, likely to outweigh any such benefits. Furthermore, augmenting the astroglial response to SCI would likely also increase the non-permissive/repulsive nature of the scar and thus have a negative bearing on (complementary)

strategies aimed at promoting axonal regeneration; these issues should be carefully contemplated and balanced when designing C5aR1-based interventions.

In summary, our recent work has identified a critical time window to which therapeutic inhibition of the C5a-C5aR1 axis in SCI should be restricted in order to avoid adverse outcomes. The more delayed beneficial aspects of C5a-C5aR1 signaling (> 7 days post-injury) likely involve facilitating astrocyte proliferation as part of the natural wound healing response to SCI. Although acute targeting of the C5a-C5aR1 pathway remains a credible and promising therapeutic avenue to promote SCI recovery, a number of significant questions remain. Specifically, the exact mechanism(s) via which pharmacological blockade of C5aR1 signaling during the acute phase improves SCI outcomes remain largely unknown and require further investigation (Figure 1).

Although we did not find a role for C5aR1 signaling in the generation and survival of oligodendrocytes after SCI, a possible requirement for functional maturation of these cells cannot be excluded at present; specific deletion of the *C5ar1* gene in the oligodendrocyte lineage could resolve this outstanding issue via detailed histological studies. Apart from CNS glia, various neuronal populations are also thought to express C5aR1 and reportedly upregulate this receptor in neurodegenerative disease. Activation of C5aR1 has been directly implicated in neuronal apoptosis *in vitro*. Specific targeting of C5aR1 expression in neurons could again reveal a direct role in neuronal death *in vivo*; conditional ablation in other cell populations may reveal indirect neuroprotective effects. A vascular mechanism in relation to C5a-C5aR1 signaling in SCI pathophysiology should also be explored. Endothelial cells are known to express C5aR1 (Jacob et al., 2011), and its activation by C5a causes nuclear translocation of nuclear factor kappaB and endothelial cell apoptosis. *In vivo*, C5aR1 antagonism preserved the integrity of endothelial cells in a mouse model of lupus cerebritis, which coincided with reduced neuronal apoptosis. Conditional knockout approaches would again be required to specifically demonstrate whether the targeting of C5aR1 directly ameliorates endothelial cell dysfunction and BBB breakdown in SCI, or whether this is an epiphenomenon of attenuated inflammation. In order to fully appreciate the complex roles of C5a in SCI, the role of its alternate receptor, complement 5a receptor-like protein 2 (C5L2 – now renamed as C5aR2) must also be investigated, for example through the use of *C5L2*^{-/-} mice (Chen et al., 2007). Lastly, and perhaps the most pertinent question from the viewpoint of clinical translation, is how long C5aR1 antagonism can be delayed before losing therapeutic efficacy. As tissue levels of C5a do not peak until at least 12 hours post-SCI, it seems reasonable to assume that initiation of C5aR1 antagonism could likely be delayed for at least several hours but this remains to be tested. Until then, the future seems bright for C5aR1-based therapeutics as a putative immunomodulatory intervention to treat acute SCI.

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Faith H. Brennan, Marc J. Ruitenber^{*}

School of Biomedical Sciences, The University of Queensland,
Brisbane, Australia (Brennan FH, Ruitenber MJ)
Queensland Brain Institute, The University of Queensland, Brisbane,
Australia (Ruitenber MJ)
Trauma, Critical Care and Recovery, Brisbane Diamantina Health
Partners, Brisbane, Australia (Ruitenber MJ)

**Correspondence to:* Marc J. Ruitenber, Ph.D.,
m.ruitenber@uq.edu.au.

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