Invited Focus Article

A Tale of Two Cells: the crosstalk of regulatory T cells and Microglia in the ischemic brain

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One-sentence abstract

Treg exert a beneficial immunomodulatory role on post-stroke neuroinflammation that is amplified by microglial cells.

Manuscript

Our immune system is a highly intricate and remarkable defense mechanism that protects our body against infections and internal threats such as tumors. However, it is crucial to tightly regulate the power of the immune system to prevent it from going astray. This is where regulatory T cells (Treg) come into play as they have a central role in controlling the immune response.

Treg are part of the adaptive immune system and exert their suppressive function by secreting or activating immunoregulatory cytokines, such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β). In addition, Treg can inhibit the activation of effector T cells through surface molecules such as CTLA-4. CTLA-4 captures activating molecules on antigen-presenting cells, preventing them from interacting with effector T cells. Treg are crucial for dampening the immune reaction once the initial threat has been eliminated, and they also play a vital role in maintaining immune tolerance. Treg are specifically selected during thymic development to recognize autoantigens, allowing them to selectively inhibit other immune cells from mounting a response against our own body. When immune tolerance is disrupted, autoimmune diseases can occur. Multiple sclerosis is an example of such an autoimmune disorder. In this case the immune system attacks oligodendrocytes, the cells that produce the insulating sheaths of nerve fibers called myelin.

Beyond infections and tumors, the immune system is involved in diseases that may not initially appear related to immunity. One such example is stroke, a common cerebrovascular condition and a leading cause for morbidity and mortality worldwide. Stroke occurs when a brain blood vessel is occluded, leading to irreversible damage to brain tissue due to the lack of sufficient oxygen and glucose supply. Surprisingly, the inflammatory response to the ischemic brain injury has a substantial impact on stroke outcome. Under healthy conditions, the brain contains only very few circulating immune cells. Microglial cells, the resident macrophage-like myeloid cells of the brain, constitute the main population of immunocompetent cells in healthy conditions. Microglia are crucial for normal brain development and function and are involved in various neurological diseases. In stroke, microglial cells respond quickly to ischemia-associated danger signals by changing morphology, proliferating and becoming immunologically activated. Microglia are

crucial in the acute phase and they were shown to promote BBB integrity, clear debris and contain excitotoxicity. Microglia are also an important source of secreted factors: on one hand, they produce proinflammatory cytokines and contribute to the neuroinflammatory environment, on the other hand they secrete growth and pro-angiogenic factors, which promote tissue repair[1]. This dual role reflects the heterogeneity of microglia function and is line with the fact that microglia can adopt different "polarized" states. Finally, microglia are involved in synaptic pruning and network remodeling, however how exactly this process affects chronic stroke recovery remains to be elucidated.

Neuroinflammation associated with stroke also attracts immune cells from the circulation. During the acute phase within hours to few days after stroke, innate immune cells like neutrophils and monocytes invade the brain in large numbers. On the other hands, adaptive immune cells like T cells invade with a delayed kinetic and increase in number mainly during the chronic phase weeks to months after stroke[2].

Among adaptive immune cells, Treg also enter the brain after stroke. Given their important function in modulating inflammation, their role has been the subject of numerous studies over the past two decades. The initial study addressing the function of Treg cells in stroke demonstrated that their depletion resulted in exacerbation of post-stroke neuroinflammation and worsened stroke outcome[3]. In this context, the beneficial effect of Treg was predominantly mediated by the anti-inflammatory cytokine IL-10, one of the main immunomodulatory molecules secreted by Treg[3]. Based on the finding of a neuroprotective function of Treg in stroke, numerous studies have tested various therapeutic strategies to increase Treg cell number or their immunosuppressive capacity to improve stroke outcome, namely adoptive Treg transfer, mucosal immunization with cerebrovascular antigens and Treg expansion with a CD28 superagonist. A meta-analysis summarizing these different studies revealed an overall benefit of Treg expansion in stroke outcome[4].

Beyond their function in modulating the acute inflammatory response to ischemic brain injury, Treg have also been shown to play an important role for functional recovery during the chronic phase after stroke[5,7]. Interestingly, Treg counts in the recovering brain increases significantly during the chronic phase from 2 weeks after stroke induction onwards. As for the acute phase, Treg depletion results in heightened neuroinflammation and a worse functional outcome during chronic recovery. Mechanistically, Treg secrete amphiregulin (AREG), which in turn keeps at bay excessive reactivity of glial cells, namely microglia and astrocytes[5].

An interesting aspect emerging from these studies is that Treg play an essential role in modulating stroke-induced inflammation, despite the relatively low number in the brain. This led to the hypothesis that their action might be further "amplified" by other more abundant immunocompetent cells such as brain-resident microglia. Recently, Benakis and colleagues studied in details the direct influence of Treg on microglia phenotype acutely after stroke[6]. They showed that adoptive transfer of Treg into the cerebrospinal fluid dampens post-stroke microglia activation. Among the pathways affected, Treg repress synaptic pruning by microglia, which was most likely mediated by Treg' IL-10 effect on microglial reactivity. In another study, the authors observed that depletion of Treg results in compromised white matter integrity, which was accompanied by impaired signal transmission between different brain regions. After stroke, Treg contribute to preserve white matter function by secreting osteopontin (OPN). Interestingly, Treg produce OPN and in turn stimulate microglia themselves to produce it, giving a clear example of how

microglial cells can amplify Treg action. Overall, this mechanism promotes the formation of new oligodendrocytes resulting in improved (re-)myelination[7]. These studies highlight how microglia-amplified Treg function can affect brain-wide connectivity, since both synapses and white matter tracts are influenced by these processes. Despite the potent effect of Treg on post-stroke recovery, it is evident that inflammation persists even months after brain ischemia, indicating that complete resolution is not achieved. Recent research from multiple laboratories has revealed that microglia fail to regain a homeostatic phenotype after stroke and instead remain in a chronically activated state. The underlying reasons why Treg are unable to reinstate a homeostatic environment are not yet clear. Nevertheless, these findings suggest that enhancing Treg-microglia crosstalk following stroke holds promise as a therapeutic intervention.

Several studies have already developed strategies to increase the number of Treg in the brain. In normal conditions, the number of Treg in the brain is extremely low due to the lack of interleukin 2 (IL-2), which is crucial for Treg survival. Potentially, other currently unknown reasons could also contribute to insufficient Treg expansion in the injured brain. Nevertheless, increasing the availability of IL-2 can lead to expansion of the Treg population. IL-2 has a very short half-life, which can be overcome by administering it in combination with a specific IL-2 antibody. This IL-2:IL-2Ab treatment significantly increases the number of peripheral Treg and, in the context of stroke, also brain-invading Treg. Functionally, this approach improves stroke outcome in terms of behavioral improvement and white matter integrity[7].

Treg express a high affinity receptor for IL-2 called CD25, making them capable of effectively utilizing circulating IL-2. However, conventional T cells can also respond to IL-2 and increase their pro-inflammatory potential. While administering IL-2 at a low dose can selectively target Treg without affecting conventional T cells, determining the optimal dosage of IL-2 to prevent potential side effects remains a concern when translating to clinical applications. To address this issue, mutated forms of IL-2 known as IL-2 muteins have been developed. These engineered compounds maintain the Treg-expanding capacity of IL-2 while sparing conventional T cells. Furthermore, some mutations improve the half-life of IL-2, making it a more feasible potential drug. Although IL-2 muteins have been tested in various disease conditions, including autoimmunity, their potential use in the context of stroke requires further investigation.

One limitation of simply boosting the number of Treg is the potential compromise of the physiological immune response to infections. To prevent global immunosuppression, targeted approaches that specifically expand Treg in a particular organ are crucial. Yshii and colleagues developed a triple-lock delivery system that enables astrocytes to produce IL-2 in an inducible manner. This treatment significantly increases the number of brain Treg while leaving the peripheral compartment unaffected. Preceding stroke induction, Treg expansion was shown to be neuroprotective, resulting in smaller infarcts. However, expanding Treg after stroke did not affect the lesion size[8]. Nonetheless, further investigation is warranted to determine if such a treatment could still improve brain connectivity and behavioral outcome. Additionally, this method could be highly versatile and used to deliver other molecules to enhance functional recovery after stroke. In addition to therapeutically increasing Treg cell counts, alternative therapeutic approaches can be designed to boost the production of Treg-derived immunomodulatory molecules at the site of inflammation. Among these molecules, IL-10 has been shown to have a strong effect on microglial function. In a model of peripheral infection, microglia respond to the stimulus by upregulating pro-inflammatory markers, but they return to a homeostatic state within a week through IL-10 sensing. Impaired IL-10 sensing in microglia leads to a hyperactive state and the death of experimental mice[9]. Based on this observation, it is tempting to speculate that insufficient IL-10 stimulation contributes to the chronic reactivity of microglia after stroke. Thus, directly administering IL-10 or modifying the triple-

lock system mentioned earlier for IL-10 delivery could be viable approaches to investigate the impact of this cytokine on stroke outcome.

While the role of Treg, microglia, and their interactions in the context of stroke has been the subject of several studies, many aspects still require elucidation. As mentioned before, other molecules such as TGF-β and CTLA-4 contribute to the immunomodulatory function of Treg. However, their role has not been thoroughly investigated in the context of stroke and could provide valuable insights for new therapeutic interventions. Furthermore, although some studies have focused on the effect of Treg on microglia, the involvement of microglia themselves in maintaining the phenotypic changes of Treg in the brain remains largely unaddressed. Advancements in single-cell technologies now enable the study of molecular partners that mediate the crosstalk between different cell types. Applying these technologies will likely uncover additional candidates governing the bidirectional Treg-microglia interactions after stroke. Lastly, it should be noted that Treg have the capacity to recognize specific antigens. In stroke, it has been observed that T cell receptor (TCR) signaling is crucial for Treg accumulation in the brain[5]. A recent study showed that microglia-Treg interaction is crucial to maintain functionality of Treg, in a model of relapsing-remitting neuroinflammation. This interaction was dependent of MHC class II-mediated antigen presentation, suggesting that this mechanism might be important not only for Treg accumulation in the brain, but also for their phenotypic stability. The authors suggest that IL-27 could play an important role in microglia-driven Treg stability[10], but other signaling pathways yet to be elucidated are likely involved. Post-stroke brain Treg show a strong bias in TCR sequence, suggesting that Treg might clonally expand in response to antigen presentation[5]. However, whether the post-stroke Treg response is truly specific to a set of antigens is yet to be determined. Ischemic brain damage results in the release of numerous brain antigens, which theoretically could stimulate a specific immune response. If brain Treg indeed mount an antigen-specific response, it would be possible to develop an approach to target them specifically. Specificity remains a significant hurdle when modulating Treg function, as this could easily result in broad immunosuppression and potential opportunistic infections. Leveraging on antigen-specificity could be a way to overcome such an issue. Alternatively, a temporally and spatially controlled approach such as the triple-lock system would be fitting[8], even though viral vectors suitable for use in humans need be further improved. Finally, it is crucial that Treg maintain their immunosuppressive capacity upon expansion and do not acquire a proinflammatory phenotype. For this, dissecting how exactly microglia can modulate T cell phenotype will be fundamental. Overall, understanding the crosstalk between Treg and microglia appears very promising to enhance functional recovery after stroke. Further exploration of this cellular dialogue will provide valuable insights into improving stroke outcomes.

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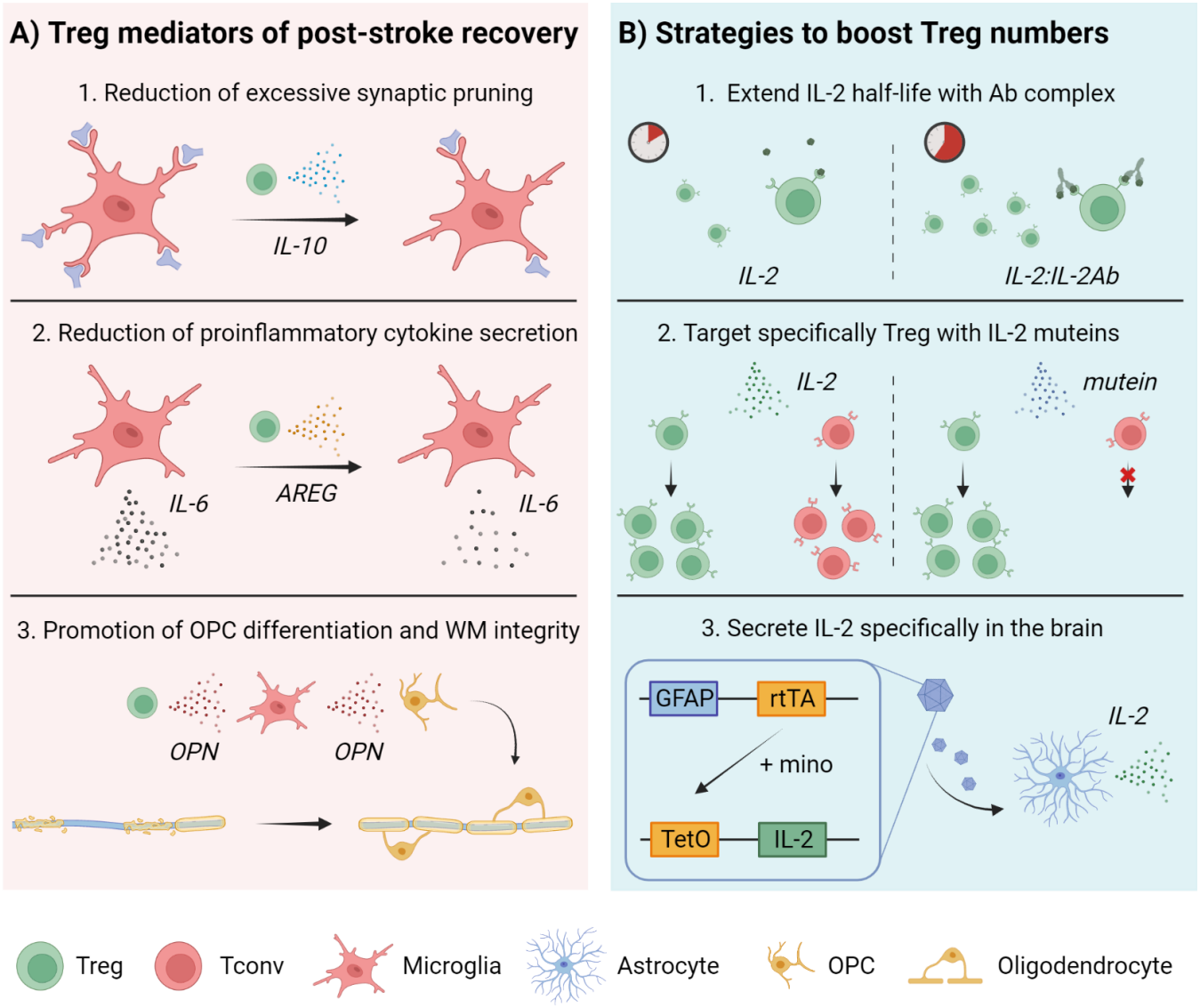

Figure 1. **(A)** Treg-secreted factors that affect microglia and improve stroke outcome. (1) Treg secrete interleukin 10 (IL-10) and decrease the aberrant synaptic pruning mediated by microglia acutely after stroke. (2) Treg secrete amphiregulin (AREG) and suppress the release of proinflammatory cytokines like IL-6 by microglia. (3) Treg produce osteopontin (OPN), which induces microglia themselves to produce it. OPN in turn favor the differentiation of oligodendrocyte progenitor cells (OPC) into mature oligodendrocytes, therefore promoting white matter (WM) integrity. **(B)** Approaches to increase Treg numbers by supplementation of IL-2, a crucial survival factors for Treg. (1) IL-2 has a very short half-life when injected *in vivo*, however combining it with specific antibodies (Ab) increases its bioavailability and induces a significant Treg expansion. (2) IL-2 can potentially expand conventional T cells (Tconv) and promote inflammation. Engineered forms of IL-2 known as "muteins" specifically expand Treg but not Tconv. (3) A triple-lock viral vector system allows IL-2 secretion by astrocytes directly in the brain. The triple control consists of a viral particle with astrocytic tropism, an astrocyte-specific promoter (GFAP) and an inducible system (rtTA-TetO) which is active only when the drug minocycline is administered.