HHS Public Access

Author manuscript

Transl Stroke Res. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Transl Stroke Res. 2016 October ; 7(5): 415–419. doi:10.1007/s12975-016-0476-4.

Regulatory T cell therapy for ischemic stroke: how far from clinical translation?

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Ischemic stroke remains a leading cause of death and disability worldwide. The effective treatment for stroke is very limited [1–3]. Accumulating evidence implicates inflammation and immune responses in the pathophysiology of stroke as well as other brain injuries [4–7]. For ischemic stroke, the sudden occlusion of a cerebral vessel leads to acute ischemic damage, which is followed by immediate activation of local immune cells and prompt mobilization of peripheral immune cells. Initially, innate immune cells function to restrict brain damage by clearing cell debris and neutralizing neurotoxins. However, consequent amplification of the adaptive immune response propels the progress of ischemic brain injury. Immunomodulation (immunotherapy) therefore has become a promising concept for stroke treatment [8–13].

Recent research findings have shown that regulatory immune cells, including regulatory T cells (Treg) [14–16] and regulatory B cells [17–19] may serve as endogenous modulators to control immune responses in the injured brain. Particular interest has arisen regarding the therapeutic potential of Treg in ischemic stroke. Treg are a rare, specialized T lymphocytes characterized by co-expression of the cell surface markers CD4 and CD25 (IL-2Ra) and by expression of the transcription factor forkhead box p3 (Foxp3). Treg can be classified into two subpopulations: naturally-occurring, thymus-derived Treg (nTreg) and induced Treg (iTreg) that are derived from $CD4^{+}CD25^{-}$ T cells in secondary lymphoid organs in response to antigen exposure. The primary function of Treg is to suppress the proliferation and function of other immune cells, especially effector T lymphocytes and to maintain immune homeostasis. The protective effect of Treg in ischemic stroke was first documented by Liesz A et al.[15]. They reported that Treg depletion using a CD25-specific antibody resulted in enhanced tissue loss and worsened neurological functions 7 days after cerebral ischemia. Later studies using a genetic mouse model of inducible Treg depletion, however, led to controversial results, showing either no effect [20] or even a detrimental effect [21] of Treg in the stroke model. Such a striking discrepancy may be attributed to the different

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Conflict of Interest: Authors have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

approaches used to deplete Treg, the dynamic nature of post-stroke immunity and the variance in stroke severity [22, 23].

Despite these controversial results of cell depletion studies, interest in Treg therapy of stroke has piqued, initially due to a series of encouraging reports of early Treg-based clinical trials in autoimmune disease and transplantation [24–30]. In addition, clinical studies have revealed a dramatic decrease in the number of circulating Treg soon after stroke onset, which provides a rationale for Treg augmentation in stroke patients [31–33]. The results from animal models of stroke further suggest that boosting the number and/or function of Treg could protect against ischemic brain injury. A recent meta-analysis of current preclinical studies indicates an overall neuroprotective effect of Treg-targeted therapies in models of stroke. Adoptive transfer of 2 million purified polyclonal Treg, which is the most straightforward approach to Treg augmentation, has been shown to provide acute protection and to promote long-term recovery in a mouse model of stroke [14, 16]. Impressively, the therapeutic window of Treg administration can be delayed until 24h after the onset of ischemia, making it applicable to humans who may not be treated in the clinic for many hours after symptom onset. The Treg-enabled neuroprotection may involve the interplay of multiple cellular and molecular mechanisms, including restricting excessive central nervous system (CNS) and peripheral immune responses, ameliorating acute blood brain barrier damage, and promoting neural stem cell proliferation for brain repair [14–16]. Intriguingly, the early protective effect of adoptively-transferred Treg does not require passage across the blood brain barrier. Rather, these cells may provide CNS protection by ameliorating the deleterious activities of peripheral immune cells [34, 16]. The Treg may infiltrate into the ischemic brain 5 days after stroke and exert further immune modulation or restorative effects in the brain. Collectively, these preclinical results fuel hope for the development of Treg therapy into a clinically feasible treatment for stroke.

The clinical application of Treg as a cell therapy requires the isolation and purification of sufficient numbers of cells from the blood. However, Treg represent only 5–10% of normal circulating T cells [35, 36]. Such a low frequency, as well as the anergic property of Treg apparently restricts their clinical utility as a cell therapy for stroke. This limitation, promisingly, has been overcome with the development of approaches for *ex vivo* or *in vivo* Treg expansion. Several methods have been developed to successfully expand Treg *ex vivo*. When crosslinked with anti-CD3 and anti-CD28 antibodies in the presence of exogenous IL-2, Treg expand robustly *ex vivo,* while retaining their phenotype and suppressive activities [37, 38]. The addition of the serine-threonine protein kinase inhibitor rapamycin prevents the acquisition of T effector cell functions and allows selective expansion of Treg, even if they are not absolutely pure initially [39, 40]. Significantly, repetitive stimulation with good manufacturing practice (GMP)-licensed artificial antigen-presenting cells (aAPC) in the presence of anti-CD3/CD28 Ab, IL-2 and rapamycin can greatly (about 3000-fold after a single re-stimulation and 50 million-fold after 4 rounds of re-stimulation) expand the number of human peripheral blood-derived nTreg with retention of Treg signatures [41]. Such massive *ex vivo* expansion dramatically advances the potential clinical utility of Treg therapy. Accumulating evidence from bench-to-bedside studies demonstrates the safety and preliminary evidence of efficacy of *ex vivo*-expanded Treg in autoimmune disease (type-1

diabetes) [28], organ transplantation [30] and graft-versus-host disease (GVHD) following hematopoietic stem cell transplantation [25, 26].

It is certainly an intriguing idea to expand autologous Treg for stroke treatment. However, the *ex vivo*-expansion of Tres usually takes several weeks to achieve a target number of cells. Considering the abrupt onset and fast progress of most cases of stroke, autologous Treg might not be applicable for stroke patients. In this regard, the non-autologous expansion of nTreg derived from umbilical cord blood (UCB) provides a possibility for advance preparation of large number of third party Treg that could be promising for clinical translation. Indeed, it has been reported that nTreg can be purified more readily from UCB than from peripheral blood due to the relative paucity of $CD25^+$ non-Treg in UCB [42]. Murine studies have shown that adoptive transfer of ex-vivo-expanded UCB-derived Treg could ameliorate GVHD [43, 44]. One clinical trial has confirmed the safety and potential efficacy of *ex vivo*-expanded UCB Treg to prevent acute GVHD [25]. The potential clinical application of third-party UCB-derived Treg in stroke patients merits further investigation.

An alternative approach to increase the number of therapeutic Treg is to expand Treg *in vivo* using different stimulants. For example, complexes of IL-2 and a specific anti-IL-2 Ab JES6-1 can induce selective expansion of Treg by blocking the binding site on IL-2 that is needed for the expansion of other T cells [45]. Injection of IL-2/IL-2Ab complexes for a short period has been shown to expand the number of highly-activated Treg in multiple organs [46, 47]. *In vivo*-expanded Treg are effective in treating experimental autoimmune disease and reducing transplant rejection [46, 47]. Another promising method for *in vivo* Treg expansion is the use of monoclonal Ab to DR3 (aDR3). A single dose of aDR3 in mice selectively expands functional Treg *in vivo* and significantly ameliorates acute GVHD [48]. In addition, pre-treatment with aDR3 for 4 days led to nTreg expansion in recipient mice and prolonged graft survival after allogeneic heart transplantation [49]. Different from IL-2 complexes or aDR3, Flt3 ligand (Flt3L) represents an indirect but effective strategy for *in vivo* Treg expansion. Flt3L is a hematopoietic growth factor that stimulates the development of conventional myeloid dendritic cells (DCs) and non-conventional plasmacytoid DCs [50]. Both preclinical and clinical studies have revealed that Flt3L administration greatly elevates peripheral Treg cell numbers *via* expansion of DCs [51, 52]. Furthermore, Flt3L and rapamycin can synergistically induce antigen-specific Treg via selective expansion of plasmacytoid DCs [53]. All of these approaches for *in vivo* Treg expansion, although still in the initial stages of preclinical exploration, may represent promising agents for immunoregulatory therapies in a variety of clinical settings, including ischemic stroke.

Despite promising evidence of the effectiveness of Treg-targeted immunotherapies in animal models of stroke, there are still concerns about the potential risks of clinical translation of Treg therapy. The first concern is that augmenting Treg could exacerbate post-stroke immunosuppression and therefore increase the risk of infectious comorbidities or cancer. This concern, however, has been partially addressed by animal studies showing that adoptive Treg therapy does not exacerbate post-stroke immunosuppression [54, 16]. Indeed, Treg therapy helps to preserve the lymphocytic populations in blood and spleen after stroke and reduces the risk of post-stroke infection [54, 16]. Nevertheless, the effects of Treg therapy on post-stroke immunity in patients still need to be carefully evaluated. Another justified

concern is that the expanded Treg may convert to effector Th17 cells in an inflammatory milieu, especially in the presence of IL-6. It is reported that when some factors, such as rapamycin, transforming growth factor (TGF)-β and all trans retinoic acid, are added into the cocktail for Treg expansion, they can enhance the suppressive function of expanded Treg and prevent their conversion into Th17 cells [55, 56]. However, the effectiveness of these agents in the expansion of human Treg awaits further evaluation. In addition, for therapies involving *in vivo* Treg expansion, the potential toxicities of Treg stimulants need to be carefully assessed.

It is becoming increasingly clear that the modulation of post-stroke immune responses will be an effective strategy to restrict ischemic brain injury and promote brain recovery [57]. More and more preclinical studies validate Treg as a promising candidate for immune cell therapy for stroke. With the development of techniques that enable Treg expansion while maintaining their function and stability, the road to effective application of Treg to clinical setting of stroke becomes broader. In spite of these unprecedented advances, some concerns and challenges remain. For example, for the treatment of CNS diseases like stroke, there is a requirement for *ex-vivo*-expanded Treg to express appropriate homing (chemokine) receptors so that they can preferentially traffic to the injured brain versus other lymphoid or inflamed tissues. Approaches need to be established to confirm the homing and persistence of adoptively-transferred Treg in the human brain. In addition, the therapeutic dose of polyclonal Treg in human stroke is unclear. Moreover, post-stroke immune response changes occur dynamically during the pathological process and differ with factors such as age, gender or co-morbidities [58–60]. Therefore, we envision that Treg-targeted therapies, like immunotherapies of cancer, need to be personalized according to the patients' immune condition and adjusted accordingly during the course of stroke recovery. A collaborative effort between basic neuroscientists, immunologists and neurologists will be required for the ultimate successful bench-to-bedside translation of Treg therapy for stroke treatment.

Acknowledgments

Funding: Xiaoming Hu was supported by the NIH/National Institute of neurological disorders and stroke (NINDS) grants NS094573 and NS092618.

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