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REVIEW ARTICLE

Regulatory T cells in ischemic stroke

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Recent evidence shows that when ischemic stroke (IS) occurs, the BBB would be destructed, thereby promoting the immune cells to migrate into the brain, suggesting that the immune responses can play a vital role in the pathology of IS. As an essential subpopulation of immunosuppressive T cells, regulatory T (Treg) cells are involved in maintaining immune homeostasis and suppressing immune responses in the pathophysiological conditions of IS. During the past decades, the regulatory role of Treg cells has attracted the interest of numerous researchers. However, whether they are beneficial or detrimental to the outcomes of IS remains controversial. Moreover, Treg cells exert distinctive effects in the different stages of IS. Therefore, it is urgent to elucidate how Treg cells modulate the immune responses induced by IS. In this review, we describe how Treg cells fluctuate and play a role in the regulation of immune responses after IS in both experimental animals and humans, and summarize their biological functions and mechanisms in both CNS and periphery. We also discuss how Treg cells participate in poststroke inflammation and immunodepression and the potential of Treg cells as a novel therapeutic approach.

Keywords: regulatory T cells; ischemic stroke; inflammation; crosstalk; stroke-induced immunodepression; ischemic stroke therapy

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INTRODUCTION

Ischemic stroke (IS) is a cerebrovascular disease resulting in high mortality and disability [1], which causes over six million deaths per year worldwide [2]. As thrombolytic therapy (tissue-plasminogen activator) is the only current treatment for IS and is time-limited (4.5–6 h) [3], it is urgent to explore novel treatments that contribute to neurologic protection against IS. Researchers have recently highlighted the important roles of inflammation and the immune response in IS, as inflammation is a fast-reacting and rate-determining step that lasts for several days after IS [1, 4, 5]. For decades, due to blockade by the blood-brain barrier (BBB), it has been mainly accepted that the entry of immune cells into the brain is restricted [6]. However, it has been recently shown that when IS occurs, the BBB is destroyed, thereby allowing immune cells to migrate into the brain, indicating that immune responses can take place in both the central nervous system (CNS) and the periphery [7].

Regulatory T cells (Treg cells) are a subset of T cells that have been demonstrated to be vital for maintaining self-tolerance and limiting inflammatory collateral damage by suppressing active (self-reactive) immunity [8, 9]. Treg cells also consist of several subpopulations, among which natural Treg cells are the most studied. These cells are generally characterized as $CD25^+CD4^+$ and coexpress the transcription factor forkhead box P3 (Foxp3) [10]. The depletion of $CD25^+CD4^+$ Treg cells causes autoimmune diseases, while the reconstitution of Treg cells prevents this process [11]. However, there are several other types of Treg cells known as inducible Treg cells, such as Th3, Tr1, and natural killer Treg cells, that acquire their functions by stimulation with specific antigens [12]. Treg cells have been shown to exist mainly in lymphoid tissues [13]. However, it has been suggested that these cells also reside in various tissues [13]. According to different phenotypically and functionally distinct Treg cell subpopulations, Treg cells show site-specific functions in circulation and tissues [14].

In this article, we review how Treg cells fluctuate and play a role in the regulation of immune responses after IS in both experimental animals and humans and summarize the biological functions and mechanisms of Treg cells in both the CNS and the periphery. We also discuss how Treg cells are involved in poststroke inflammation and immunodepression and the potential of Treg cells as a novel therapeutic approach.

TREG CELLS IN IS

Treg cells play a crucial immunomodulatory role in IS. However, the contribution of Treg cells can be either beneficial or detrimental and remains controversial. Many studies have investigated how Treg cells fluctuate in both human patients and experimental models.

Clinical observation of Treg cells in IS patients

Previous studies have shown that compared to those of controls, the levels of circulating Treg cells declined in stroke patients [15, 16], and the clinical observations of Urra et al. showed that circulating Treg cell levels decreased noticeably in the 2 days after stroke [17]. However, although the decrease in Treg cells was demonstrated to be related to apoptosis, there was no correlation with the outcomes of stroke [17, 18]. In contrast, other studies demonstrated that although the suppressive effects of Treg cells were attenuated, the proportion of Treg cells in the peripheral blood was higher in IS patients than in controls [19, 20]. Yan et al. examined the number of Treg cells, and the results showed that Treg cells were enhanced in the blood of IS patients compared to those in healthy controls on the 7th day after IS onset [20].

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Moreover, Yan et al. discovered that the number of Treg cells increased beginning on the 7th day and lasted until the 3rd week after IS [19]. Interestingly, Pang et al. identified a relationship between infarct size and the trend in Treg cell fluctuations [21]. IS patients were divided into two groups according to infarct size (A group: <28.6 mL; B group: ≥28.6 mL). Treg cells were increased in the A group, while in the B group, cells were decreased in 3 days but increased after the 7th day [21]. Although there were some limitations in these studies, such as the small sample size (46 patients with acute stroke) [17] (67 subjects of 25 with acute IS) [20], this controversial finding led to the hypothesis that the fluctuation in Treg cell numbers could be a type of evolutionarily adaptive response, and these cells may leave the circulation and migrate to target tissues during the time after stroke occurs to protect against the inflammation triggered by IS [12, 16].

Treg cells in experimental models of IS

There is evidence confirming that Treg cells are recruited to the spleen, distal and proximal lymph nodes, and ischemic hemisphere in experimental models [22]. Treg cells located in different sites are heterogeneous, indicating that the function of Treg cells might be dependent on the environment [23].

Changes in Treg cells in the spleen. Treg cells in peripheral tissues have reparative functions in IS. As T cells patrol for cognate antigen in draining lymph nodes, and although T cells do not cross the brain, they significantly affect the severity of IS via immune signaling and the secretion of molecules such as interferon gamma (IFN-γ) [24, 25]. Kleinschnitz et al. discovered that Treg cells in the circulation were markedly decreased at the 24-h time point after IS and returned to a normal level after 3 days [26]. Interestingly, it was also shown that the fluctuation in Treg cells was accompanied by splenic atrophy [27]. The results of Offner et al. showed significant splenic atrophy in MCAO mice compared to sham-operated mice within 96 h, while Treg cells remained stable within 22 h but increased at the 96-h time point [27]. Moreover, Hilary et al. discovered a significant reduction in splenic volume at 24–48 h, accompanied by a rapid decrease in Treg cells in the blood and spleen after MCAO [28]. However, splenic atrophy and the number of splenocytes returned to normal 96 h after stroke [28]. In contrast, another study demonstrated that Treg cells doubled the day after IS onset and continued to increase 3 days after IS, while these cells could not be detected in the cerebral parenchyma until the 5th day [29].

In summary, these findings illustrate that Treg cells fluctuate in different locations in the brain and periphery and accumulate 3 days following the onset of IS [12]. Furthermore, these cells do not cross the BBB until the 5th day after IS [12].

Changes in Treg cells in the brain. As reviewed previously, only a few Treg cells infiltrate the brain during the acute phase; hence, whether Treg cells exert an effect at that time remains questionable [13, 14]. Gelderblom et al. discovered that in the first week after tMCAO, only a small number of $CD25^+Foxp3^+$ Treg cells could be observed [30]. In contrast, in the distal pMCAO model, Llovera et al. detected a large number of T cells and Treg cells in the ischemic hemisphere, and $Foxp3^+$ Treg cells accounted for ~20% of all $CD4^+$ T cells [31], which indicated that the inflammatory reaction induced by pMCAO is stronger than that induced by tMCAO [32]. Moreover, Stubbe et al. discovered that in the acute phase in the tMCAO model, there was only a low level of $Foxp3^+$ Treg cells in the brain with no expression of tissue Treg cell-specific genes, while Ito et al. and Stubbe et al. demonstrated that 14 and 30 days after IS, which is the later stage, substantial numbers of Treg cells could be detected in the ischemic hemisphere and continued to increase thereafter [33], suggesting that there was a delayed response of Treg cells in the adaptive immune response [22, 33].

PARADOXICAL ROLES OF TREG CELLS IN IS

As reviewed previously, the dynamics of Treg cells in IS seem to be controversial. Moreover, the biological activities of Treg cells remain unclear, among which neuroprotective effects remain the mainstream view (Table 1).

Neuroprotective effects of Treg cells

Liesz et al. demonstrated that Treg cells served as neuroprotective modulators of inflammation in the brain after IS, and these cells were further confirmed to protect against secondary infarct growth. In their study, the researchers depleted Treg cells with a CD25-specific antibody. Compared to the controls, Treg cell-depleted mice showed significantly larger infarct sizes on the 7th day after pMCAO, accompanied by worse neurological performance. Moreover, to investigate whether these consequences resulted from the loss of Treg cells, the researchers administered $CD4^+CD25^-$ cells, total $CD4^+$ cells, or Treg cells to lymphocyte-deficient $Rag2^{-/-}$ mice. The mice that received $CD4^+CD25^-$ cells showed larger infarct sizes than the mice that received other cell types, indicating that $CD25^+$ Treg cells play a crucial role in neuroprotection after IS [34]. Zhang et al. illustrated the similar neuroprotective role of Treg cells. First, C57BL/6 mice were intraperitoneally administered interleukin (IL)-2/IL-2 Abs or isotype IgG 3 days before or 2 h after tMCAO. IL-2/IL-2 Abs enhanced the number of Treg cells, further leading to decreased infarct size and improved sensorimotor outcomes compared with those of the controls. Furthermore, the researchers adoptively transferred IL-2/IL-2 Abs to depletion of regulatory T cells (DEREG) mice. The results showed no additional protective effects of IL-2/IL-2 Abs. When Treg cells were administered to IL-2/IL-2 Abs-treated and isotype-treated mice, although the mice all showed improved neuroprotective outcomes, the protection was more robust in the former group, suggesting that Treg cells played a neuroprotective role after IS and that the effects of Treg cells were boosted in the presence of IL-2/IL-2 Abs [35].

Detrimental roles of Treg cells

Although numerous studies have shown beneficial effects of Treg cells after IS, some recent research findings have shown the opposite effects. Kleinschnitz et al. established the Treg cell-depleted DEREG mouse model via gene modification rather than using a CD25 antibody. After 30 or 60 min of tMCAO in DEREG mice, $Rag1^{-/-}$ mice, or C57BL/6 mice, the brain infarct volumes of DEREG mice induced with 60 min of tMCAO were markedly reduced 24 h poststroke compared to those of controls and were enlarged again when Treg cells were reconstituted over 3 weeks. Furthermore, when $CD4^+CD25^+$ Treg cells were transferred into C57BL/6 mice 1 day before tMCAO, the infarct size was enhanced significantly 1 day after IS onset with 30 min of tMCAO. More specifically, the transfer of $FoxP3^+$ Treg cells into $Rag1^{-/-}$ mice resulted in similar infarctions sizes compared to those of the $Rag1^{+/+}$ controls at 1 day after IS onset with 60 min of tMCAO [26]. Consistent with this finding, Schuhmann et al. administered the superagonist anti-CD28 antibody (CD28 SA) to C57BL/6, DEREG or $Rag1^{-/-}$ mice induced with 30 min of tMCAO to expand Treg cells. The results illustrated that in wild-type mice, the infarct volumes in the experimental group were larger than those in the controls 1 day after IS, while the other two groups of mice showed no significant differences, confirming that Treg cells enhanced infarct size and worsened neurological function after IS [36].

Neutral effects

Apart from the results discussed in the former two sections, several findings indicate that Treg cells do not affect poststroke outcomes. Ren et al. inserted the coding sequence of the diphtheria toxin receptor into the Foxp3 allele to specifically target Treg cells. By treating the resulting mice with diphtheria toxin, the researchers achieved almost complete Foxp3 depletion

Table 1. Studies investigating the outcome of Treg cells in murine models.

Study	Role for Treg cells	Model	Infarct volume	Treg depletion	Treg adoption	Outcome
Liesz et al. [34]	Protective	Mice: pMCAO	10–30 mm^3	Anti-CD25 mAb	None	Infarct size was enlarged significantly 3 and 7 days after pMCAO
				None	$CD4^+CD25^-$ cells, total $CD4^+$ cells, or Treg cells to $RAG2^{-/-}$ mice 7 days before MCAO	Larger infarct size in the mice received $CD4^+CD25^-$ cells
Zhang et al. [35]	Protective	Mice: 60 min tMCAO	20–70 mm^3	DEREG mice	IL-2/IL-2Ab	Decreased infarct size and improved sensorimotor outcomes 3 and 7 days after tMCAO
Kleinschnitz et al. [26]	Detrimental	Mice: 30 or 60 min tMCAO	60–90 mm^3	DEREG mice	None	Reduced infarct volumes and better neurological function 1 day after IS
			5–10 mm^3	DEREG mice	$CD4^+$ $CD25^+$ Treg cells, $CD4^+$ $CD25^-$ T cells, $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells, or $CD4^+CD25^-$ $FoxP3^-$ T cells to $RAG2^{-/-}$ mice	Larger infarct size than $RAG^{-/-}$ mice
Schuhmann et al. [36]	Detrimental	Mice: 30 min tMCAO	40–80 mm^3	None	CD28 SA	Enhanced infarct size and worse functional outcomes
			30–40 mm^3	$Rag1^{-/-}$ mice or DEREG mice	CD28 SA	No significant difference in infarct size and behavior texting between treatment groups and controls
Ren et al. [37]	Neutral	Mice: 60 min tMCAO	~50% (Cortex; Hemisphere) ~90% (Striatum)[a]	DEREG mice	None	No significant difference in infarct volumes and behavioral evaluations
Stubbe et al. [22]	Neutral	Mice: 30 min tMCAO	~40 mm^3	Anti-CD25 mAb	None	No significant difference associated with Treg depletion

[a]This study analyzed the outcomes with infarct volume percent of the lesion.

in the mice. The results showed that there was no significant differences in the infarct volumes and behavioral performance between vehicle- and diphtheria toxin-treated mice challenged by 60 min of tMCAO [37]. Interestingly, in the study of Stubbe et al., as previously described, although Treg cells fluctuated after the onset of IS, when $CD4^+CD45RB^{high}$ cells were adoptively transferred into $Rag1^{-/-}$ mice 1 day before MCAO, the mice showed no de novo generation of Treg cells 2 weeks after occlusion. Moreover, to clarify the role of $CD25^+$ Treg cells in the late phase after MCAO, the researchers injected anti-CD25 mAbs 3 and 14 days after tMCAO. Additionally, the researchers measured the infarct size 3 and 27 days after IS and evaluated neural function at 14 and 27 days after IS onset. However, the results showed no significant difference between the Treg depletion group and the controls [22].

Probable reasons for the paradoxical functions of Treg cells

The extent of brain damage. Emerging evidence has shown that infarct size could be a major determinant of changes in Treg cells. Liesz et al. demonstrated that only experimental models with extensive infarcts showed reduced lymphocyte counts; similarly in stroke patients, only severe IS patients went through poststroke peripheral immunosuppression [38, 39]. Moreover, splenic lymphocyte apoptosis and the production of blood cytokines were more significant in extensive infarcts than in small infarcts [38]. Consistent with that finding, Hug et al. indicated that infarct size was the major factor associated with the reduction in T helper cell counts on days 1 and 4 poststroke, which was further associated with infections [40]. The paradoxical findings reviewed here could be explained partly by the different types of IS models. With different infarct volumes, the lesions in the hemisphere could be diverse, thus resulting in the different effects of Treg cells.

The timing of Treg cell analysis. Generally, the neuroprotective effect of Treg cells was described in the later phase of infarct development, focusing on tissue reorganization and repair, while detrimental effects were observed during the acute phase [26, 34]. Moreover, Schumann et al. injected CD28 SA into tMCAO model animals and showed a positive correlation between Treg cell expansion and enhanced stroke size at 24 h after IS [36]. Na et al. also injected CD28 SA into tMCAO model animals and observed decreased infarct sizes and alleviated functional deficits 7 days after stroke onset [41]. However, Schumann et al. injected CD28 SA 3 days before or immediately after occlusion while the injection time in Na et al. was 3 or 6 h after IS onset; thus, the timing of Treg cell expansion is also a factor that cannot be ignored regarding this paradoxical phenomenon [36, 41, 42].

The inflammatory environment. It has been demonstrated that the role of Treg cells is associated with the specific inflammatory environment to maintain homeostasis [43]. Poststroke neuroinflammation is dissimilar among murine pMCAO and tMCAO models; that is, the role of Treg cells may depend on the experimental model [44]. It has been shown that neuroinflammation is more significant in pMCAO mice than in tMCAO mice, as more T-cell infiltration, microglial activation, and the expression of cytokines such as IL-1 and TNF-α could be observed 5 days after pMCAO than tMCAO [44]. Furthermore, compared to pMCAO models, tMCAO mice have been suggested to have secondary microthrombosis and potential endothelial damage within minutes after reperfusion [45, 46]. Secondary microthrombi were demonstrated to be responsible for ~70% of the final ischemic lesion size [47]. Moreover, microthrombosis was demonstrated to be partially independent of the inflammatory mechanism, which might contribute to pathophysiology and thus have a crucial impact on the outcomes of Treg cells [14]. In addition, the occurrence of secondary microthrombosis might affect local tissue-specific pathophysiological cascades associated with

endothelial activation, thromboinflammation, and microvascular dysfunction, resulting in secondary ischemic infarctions and altered neuroinflammatory responses [14]. Ito et al. proved that tissue Treg cells exert tissue-specific effects, contributing to the maintenance of tissue homeostasis and repair by interacting with both inflammatory cells and tissue cells [13]. However, clinical studies have illustrated that secondary microthrombosis is modest or nonexistent after reperfusion [46, 48]. Therefore, the above-mentioned differences between the tMCAO and pMCAO models might directly impact Treg cell function.

MECHANISMS OF TREG CELLS IN IS PROGRESSION

Cytokines-based secretion pathways

Through the secretion of anti-inflammatory cytokines, Treg cells are known to attenuate brain injury after IS [18]. Interleukin-10 (IL-10) is an essential cytokine that has been indicated to have pleiotropic immunosuppressive functions [49]. IL-10 plays a crucial role in cytokine-mediated modulation of neuroinflammation after IS by targeting antigen-presenting cells (APCs), such as targeting macrophages to inhibit the release of proinflammatory mediators [14, 49]. IL-10 is derived from both innate and adaptive immune cells, such as microglia and several subtypes of T cells, including Treg cells and Th2 cells, while T cells are the main sources of IL-10 [49, 50]. Furthermore, it has been verified that IL-10 plays a crucial role in the protective mechanism of Treg cells [50]. Several studies have described improved outcomes with the production of lymphocyte-derived IL-10 or the administration of IL-10 in mouse models [41, 51], indicating the protective effects of IL-10 on IS.

Moreover, interleukin-33 (IL-33), a novel cytokine of the IL-1 family, was reported to activate Treg cells associated with CNS repair and is known to facilitate the recovery of tissue after CNS injury and induce M2 macrophage-related genes [13, 52]. Several studies have shown that immediate intraperitoneal injection of IL-33 after tMCAO resulted in the enhancement of $CD4^+CD25^+Foxp3^+$ Treg cells and a reduction in IFN-γ^+ Treg cells in the spleen and brain compared to those in controls [52, 53]. Moreover, IL-33 upregulated Foxp3 mRNA levels, suggesting a protective role of IL-33 through promoting the Treg response [53]. Treg cell culture supernatant was treated with IL-33, the results showed increased production of IL-10, IL-35, and transforming growth factor-β (TGF-β), all of which are Treg cell cytokines [52], accompanied by decreased infarct sizes after IS [53]. However, the effects of IL-33 are suppressed when suppression of tumorigenicity 2 (ST2) is blocked [54]. The mice that were administered the anti-CD25 antibody showed a reduced protective effect of IL-33 [52]. Thus, IL-33 plays a neuroprotective role after IS onset and enhances Treg cells through the secretion of cytokines and a reduction in apoptosis-related proteins in an ST2-dependent manner [54].

In addition, there are several Treg cell-related mechanisms that impact the outcomes after IS through the secretion of other cytokines such as IL-35 and TGF-β [50].

Perforin- and granzyme-mediated cytolytic pathways

The suppressive activity of Treg cells includes inducing the death of effector cells by cytolysis [55]. Activated human $Foxp3^+$ Treg cells were shown to express granzyme A but little granzyme B [56, 57]. Furthermore, Treg cell-mediated killing occurred in a perforin-dependent, granzyme A-dependent, and Fas-FasL-independent manner to activate certain subtypes of T cells, such as $CD4^+$ and $CD8^+$ T cells, and other kinds of cells [56]. In contrast, the expression of granzyme B was reported to be upregulated after the activation of Treg cells in mice [58]. Gondek et al. claimed that granzyme B-deficient mice showed suppressive Treg cell activity in vitro, while this response seemed to be perforin-independent [59]. Another study by Cao et al. confirmed that Treg cells could kill NK cells and CTLs in a perforin- and granzyme B-dependent manner, leading to the suppression of these cells in vivo [60]. Overall, Treg cell-mediated suppression works through perforin- and granzyme-mediated cytolytic mechanisms.

The PD-1/PD-L1 pathway

Programmed death-1 (PD-1) is an inducible immunomodulatory receptor expressed on B and T cells, as well as myeloid-derived cells, that regulates the adaptive immune response [61]. PD-L1 is a ligand of PD-1, and the interaction between PD-1 and PD-L1 can have an effect on the maintenance of peripheral tolerance [62]. Li et al. demonstrated the neuroprotective role of PD-L1 via the Treg cell-mediated inhibition of neutrophil-derived metalloproteinase-9 [63]. In contrast, Bodhankar et al. showed that PD-L1 enhanced CNS inflammation and infarct size, as evidenced by the decreased expression of CD80 by APCs and increased PD-1 and PD-L2 expression in $PD\text{-}L1^{-/-}$ mice, indicating the presence of an alternative suppressor T-cell signaling pathway [64]. When the researchers further blocked the PD-L1 checkpoint, the infarct volumes were reduced markedly, and $CD8^+$ Treg cells were increased in the lesioned CNS hemisphere [65]. Hence, more work is required to determine how the PD-1/PD-L1 pathway precisely affects Treg cells.

The Htr7-based pathway

Serotonin (5-HT) is an important neurotransmitter that is involved in a wide range of physiological processes in the CNS, including pain, mood, anxiety, cardiovascular function, circadian rhythms, and feeding, and has been shown to participate in almost all neuropsychiatric disorders [66, 67]. 5-HT receptors have been proven to play a crucial role in enabling 5-HT to exert its effects [67]. Htr7 is a kind of G protein-coupled receptor for 5-HT that is generally expressed in the CNS and links to adenylate cyclase, which was further demonstrated to enhance the level of cyclic adenosine monophosphate (cAMP) [68, 69]. Intracellular cAMP was shown to promote the proliferation of Treg cells and enable Treg cells to maintain immune homeostasis by transmitting cAMP through membrane gap junctions to effector T cells, resulting in the suppression of these cells [58, 70]. There were improvements in neurological symptoms and an elevated level of Treg cells after the administration of 5-HT, which was not observed in Htr7-deficient mice [13]. In general, Treg cells can proliferate and be activated via an Htr7-dependent pathway [13].

Treg cell crosstalk with other cells

Treg cell crosstalk with microglia. Microglia are resident macrophage-like neuroimmune cells that are involved in the innate immune response in the CNS [71, 72]. After IS, microglia are activated, accumulate in the brain and differentiate into two subsets: the M1 proinflammatory phenotype and the M2 anti-inflammatory phenotype [73]. A lack of Treg cells induces microglia to transform into the M1 phenotype; in contrast, enhancing Treg cells promotes the transition toward the M2 phenotype [74]. Furthermore, M2 microglia were shown to promote Treg cell polarization by secreting IL-10 and TGF-β, resulting in reductions in infarct sizes after experimental IS [75]. Conversely, Treg cells play an important role in promoting the development of M2 microglia by elevating the IL-10-mediated expression of glycogen synthase kinase 3 beta [74, 76], which indicates that the crosstalk between M2 microglia and Treg cells via the secretion of IL-10 and TGF-β protects against brain damage after IS [73]. Additionally, Shu et al. found that microglia significantly induced hypoxia-inducible factor 1-alpha (HIF-1α) expression in Treg cells, and the inhibition of HIF-1α suppressed microglia-induced Sirt2 upregulation, which was proven to attenuate the anti-inflammatory effects of Treg cells [77].

Treg cell crosstalk with astrocytes. Astrocytes are crucial immune cells that maintain CNS homeostasis through neurotransmitters,

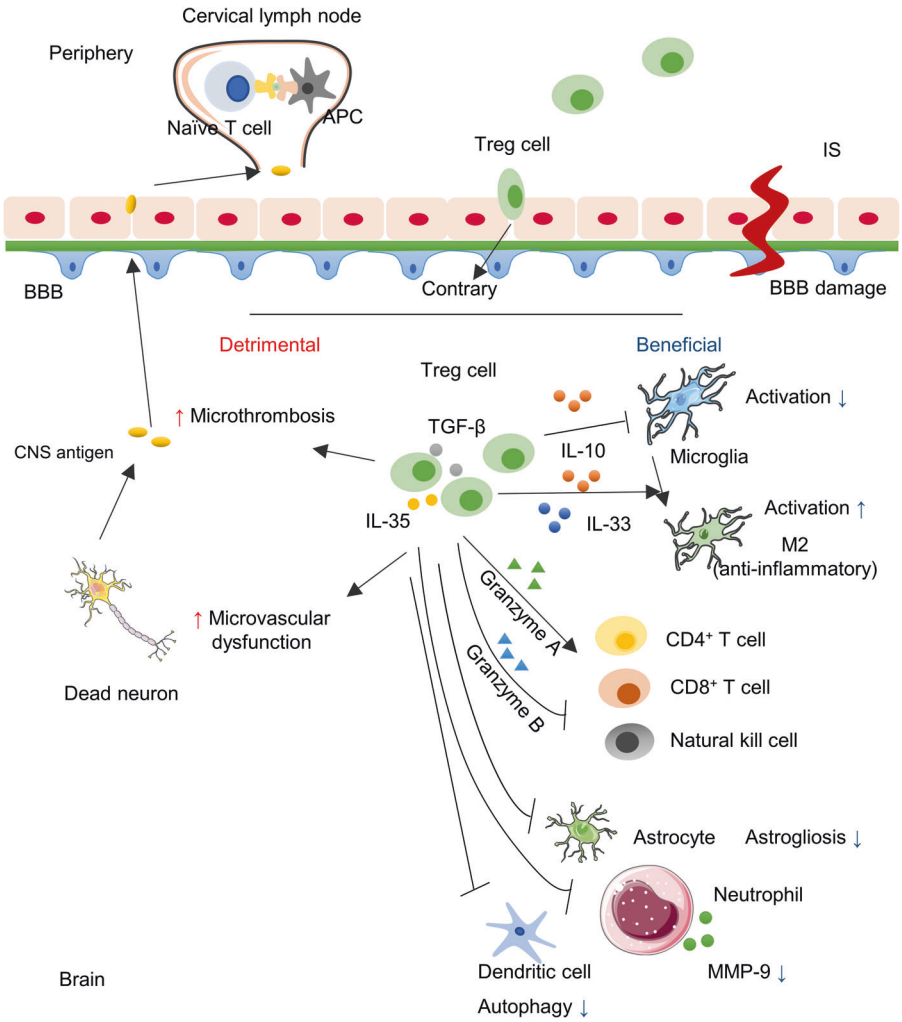

Fig. 1 The role of Treg cells in ischemic stroke. Ischemic stroke leads to the damage of the BBB and neuron cells. The CNS antigen would be generated, pass the BBB and drain to the peripheral lymphoid nodes where they are taken up by APCs and further presented to naïve T cells. Treg cells as crucial immunomodulators, are transferred into the brain and exert a neuroprotective effect through the cytokine-dependent pathway via IL-10, IL-33, etc. Moreover, Treg cells modulate effector cells through the granzyme-dependent pathway, the crosstalk with several other cells, and the inhibition of neutrophil-derived MMP-9. However, Treg cells can be detrimental to cerebral injury with microthrombosis and microvascular dysfunction.

synaptic plasticity, pH, cerebral blood flow, and water balance in pathological conditions such as IS [33, 78]. In recent decades, astrocytes have been shown to play pathogenic roles in disease processes due to their transformation of hyperplastic and hypertrophic cells; however, recent studies have demonstrated that astrocytes might exert neuroprotective effects [33, 78], which are influenced by the context and time of brain or spinal cord injury [33, 78]. Tobias et al. discovered an increase in astrocytes in DEREG mice, which was consistent with the results of Minako et al, indicating that both a reduction in T-cell infiltration into the brain and the depletion of Treg cells significantly elevated reactive astrocyte markers [33, 79]. In summary, Treg cells suppress astrogliosis, and the crosstalk between Treg cells and astrocytes affects neurological recovery.

Treg cell crosstalk with dendritic cells. Dendritic cells (DCs) are a group of specialized APCs with crucial roles in the response to foreign and self-antigens by initiating and regulating the T-cell response [80, 81]. The crosstalk between DCs and Treg cells has been demonstrated to be crucial in immunomodulation. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a molecule in the CD28 family that is secreted by DCs and plays an important role in activating Treg cells [82, 83]. Several studies have shown that through the binding of CTLA-4 to CD80 and CD86 on DCs, Treg cells can modulate DC biology via the phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin (mTOR) pathway, thus exerting tolerogenic effects [84]. Furthermore, indoleamine 2,3-dioxygenase (IDO), a rate-limiting enzyme in tryptophan catabolism, was proven to be induced by the CTLA-4-CD80/86 axis [85]. The metabolites derived by IDO were demonstrated to promote DCs to express ILT3 and ILT4, thus enhancing the induction of Treg cells [86]. In summary, DCs and Treg cells can affect each other to exert effects on the immune response and maintain tolerance [81] (Fig. 1).

TREG CELLS PARTICIPATE IN IMMUNODEPRESSION AND ASSOCIATED INFECTIONS

There is homeostasis between the immune system and the nervous system under physiological conditions [87]. This balance can be interrupted, causing the activation and infiltration of immune cells into the brain [88]. However, although the immune response in the CNS contributes to neuronal injury in the early phase, the subsequent decrease in immune cells in the circulation

causes stroke-induced immunodepression (SIID) as early as 12 h after IS and can last for months and years [89, 90], and SIID enhances host susceptibility to infection [91]. Recent studies have shown that ~30% of stroke patients experience infectious complications, with the most prevalent complications being pneumonia and urinary tract infections [91], which contribute to the ~20% mortality and high morbidity in stroke survivors [92]. Importantly, Treg cells have been proven to have long-term effects on SIID by promoting IL-10 secretion [89, 93]. Interestingly, Pang et al. discovered a relationship between infarct size and infection, and patients with larger infarct sizes showed a higher probability of infection [21].

Treg cells in human patients

Recent preclinical evidence attributed the risk of infection to stroke-induced immunosuppression. Generally, a significantly decreased spleen size has been observed in some patients in clinical studies after stroke [94, 95]. Moreover, Jiang et al. investigated that on days 1, 3, and 7 after stroke onset, the number of B cells, Th cells, NK cells, and cytotoxic T cells decreased, but the results showed an increase in the number of Treg cells, indicating a correlation between the splenic volumes and immune cells; B cells, Th cells, and cytotoxic T cells correlated positively with spleen size, while Treg cells correlated negatively with spleen volumes after stroke onset [95]. Furthermore, the balance between Th cells and Treg cells has attracted attention in the context of immunosuppression. Th cells are a crucial subtype of T cells that participate in the adaptive immune response by initiating and exacerbating the inflammatory response, whereas Treg cells play an immunosuppressive role in the immune response [96]. Compared to those of healthy controls, the levels of TNF-α and IL-10 were enhanced significantly, while IFN-γ was reduced in the serum of stroke patients [95]. IFN-γ secreted by Th cells plays an essential role in host survival, and IL-10 and TGF-β promote the differentiation of Treg cells [97, 98]; thus, changes in these cytokines in the circulation after IS may contribute to functional changes in the immune system and influence poststroke infection [95].

Treg cells in animal models

Splenic atrophy was also observed in an experimental study conducted by Offner et al. and was accompanied by a drastic reduction in splenocyte numbers and an increase in the number and activation of splenic $CD4^+FoxP3^+$ Treg cells secreting IL-10 [27]. As mentioned previously, the imbalance between Th cells and Treg cells can contribute to poststroke infection [96]. Dang et al. showed that the transcription factor HIF-1α exerted a regulatory effect on the balance between Treg cells and Th17 cells by directly binding to the loci encoding RORγt and histone modification enzymes such as p300 affecting the promoter regions of these target genes, thereby inducing their transcription and inhibiting FoxP3 [99, 100]. Furthermore, the sympathetic nervous system is one branch of the autonomic nervous system and has been demonstrated to modulate poststroke immune structures [91]. One of the factors mediating systemic immunosuppression after IS was that the hypothalamic-pituitary-adrenal axis was activated, leading to excessive secretion of glucocorticoids and thus inducing T cells to decrease the production of IFN-γ, further resulting in the enhanced secretion of IL-10 derived from Treg cells and apoptotic cell death [93].

IS THERAPY ASSOCIATED WITH TREG CELLS

Several clinical studies have used the expansion of peripheral Treg cells as a potential treatment for diseases such as Alzheimer's disease (NCT01409915), Parkinson's disease (NCT01882010), and amyotrophic lateral sclerosis (NCT04055623) [25]. Although it remains controversial whether Treg cells are beneficial or detrimental to the brain after IS, the therapeutic potential of Treg cells after IS has attracted the attention of numerous researchers due to their long therapeutic window after the onset of IS [101]. Although several studies have suggested an increased level of Treg cells in the late phase of IS, a reduction in circulating Treg cells soon after IS has been revealed [17, 20], providing a therapeutic strategy to increase the number of Treg cells in the early phase after IS. Li et al. enriched Treg cells in mice or rats at 2, 6, and 24 h after reperfusion [29]. The results illustrated that adoptively transferring Treg cells at all three time points significantly reduced brain infarcts, indicating that treatment with Treg cells might be effective for up to 24 h after IS [29]. In general, the application of Treg cells as a treatment for IS has arisen recently (Table 2).

Sources of Treg cells

As mentioned previously, the most direct approach for Treg cell therapy is adoptive transfer of purified Treg cells. However, as Treg cells are a small subset of T cells and constitute 5% of $CD4^+$ T cells in circulation, it is necessary to expand Treg cells in vivo or in vitro [102]. The most accessible source of Treg cells is peripheral blood [101]. As the rate of the expansion of Treg cells in vitro is limited, Treg cells derived from umbilical cord blood (UCB) were proven to be another often used source [101]. Moreover, Dijke et al. showed that discarded pediatric thymuses could be a novel source of Treg cells with the potential to overcome the limitations of UCB and peripheral blood [103].

Stimulants to expand Treg cells

Direct stimulants. The most frequently used stimulants to expand Treg cells are anti-CD3 and anti-CD28 antibodies [104, 105]. In the presence of IL-2, Treg cells can maintain their suppressive function and phenotype [105]. Moreover, to ensure highly selective Treg cell expression, rapamycin, a mTOR inhibitor, is often added to prevent the proliferation of T effector cells [106, 107]. However, this suppression could affect Treg cells, which could result in requiring a long time for Treg cells to expand. Therefore, manufacturing practice-licensed artificial antigen-presenting cells are frequently used together with anti-CD3/CD28 antibodies and IL-2 to stimulate the pure expansion of Treg cells [102, 108].

Moreover, high concentrations of IL-2 have been used to expand Treg cells, and Ethan noticed that IL-2 coupled to anti-IL-2 mAbs could also inhibit the expansion of other T-cell subsets by blocking the binding site on IL-2, thereby selectively expanding Treg cells [109, 110]. Furthermore, the engagement of DR3, a member of the tumor necrosis factor receptor superfamily, was proven to facilitate the expansion of Treg cells [111, 112]. Therefore, an agonistic antibody against DR3 might be an excellent choice for expanding $FoxP3^+$ Treg cells in vivo [111, 112].

Indirect stimulants. In addition to stimulants that directly act on Treg cells, FMS-like receptor tyrosine kinase ligand (Flt3L) can expand Treg cells in an indirect manner [113, 114]. This strategy has been revealed to work by expanding plasmacytoid dendritic cells [113]. As reviewed previously, DCs crosstalk with Treg cells, further leading to an increased number of Treg cells [101]. However, as Flt3L does not induce Treg cells, Flt3L is generally used in combination with rapamycin [113].

Pathological approaches

Previous studies suggested that Treg cells could be expanded through a pathological approach of "mucosal immunization" by the administration of cerebrovascular antigens [115–117]. E-selectin is a glycoprotein adhesion molecule that plays a role in maintaining immune tolerance, is specifically expressed on endothelial cells and recruits immunomodulatory factors to the vascular tree where thrombosis or hemorrhage occur [115, 116].

Table 2. Studies to increase Treg cells frequency and function as therapeutic approaches in IS.

Study	Treg expansion	Model	Treatment time point	Treatment duration	Effects
Li et al. [29]	Adoptive transfer	Mice: 60 min tMCAO	2, 6, or 24 h after reperfusion	Tail vein injection once	Reduced brain damage, prolonged protection, and improved functional outcomes
		Rats: 120 min tMCAO	2, 6, or 24 h after reperfusion	Tail vein injection once	Decreased infarct size, and improved functional performance
Na et al. [41]	CD28 superagonist	Mice: pMCAO	3 or 6 h after pMCAO	Intraperitoneal administration once	Reduced infarct size and attenuated functional deficit 7 days after IS
		Mice: 60 min tMCAO	3 h after tMCAO	Intraperitoneal administration once	Reduced infarct size and attenuated functional deficit 7 days after IS
Xie et al. [107]	Rapamycin (mTOR inhibitor)	Rats: 90 min tMCAO	6 h after tMCAO	Intracerebroventricular infusion with an osmotic minipump to the left lateral ventricle 7 days for neurological behavioral tests and lesion volume measurement; 3 days for studying the neuroinflammatory response	Decreased lesion volumes, reduced production of proinflammatory cytokines and chemokines, and improved behavioral deficits
Gee et al. [117]	"Mucosal immunization" by Myelin basic protein	Rats: 3 h tMCAO	9, 7, 5, 3, 1 days before tMCAO	Instilled into each nostril every other day for a total of 5 doses	Lower neurological scores (better), induced response of Treg cells, and less likely of autoimmune response
Ishibashi et al. [115]	"Mucosal immunization" by Recombinant human E-selectin	Rats: pMCAO	32, 30, 28, 26, 24, 12, 10, 8, 6, 4 days before tMCAO	Instilled into each nostril every other day for 10 days, and repeated once after 11 days for a total of 10 doses	Decreased infarct volumes, enhanced survival of neural progenitor cells and neurons, and improved functional performance

Researchers have proven that the repeated intranasal administration of recombinant E-selectin could enhance the number of Treg cells and thus induce mucosal tolerance and promote brain repair after stroke [115, 116]. Moreover, Gee et al. showed that intranasal administration of myelin basic protein could induce the response of Treg cells, leading to the prevention of deleterious autoimmunity after IS [117].

CONCLUSION

In summary, accumulating evidence from experimental and clinical studies has demonstrated that Treg cells play a complicated role in IS. As one of the potent immune cells that participate in the immune response after IS, Treg cells are capable of modulating several immunological pathways by secreting cytokines, cytolysis, essential receptor-based pathways, and interacting with other cells. Although there are still some controversial views on the functions of Treg cells, current studies of adoptive transfer or the expansion of Treg cells in vivo and in vitro have suggested that Treg cells might be an innovative therapeutic strategy for the treatment of IS. However, as Treg cells can exert different effects in different phases after IS onset, it is necessary to further evaluate the proper time, dose, and site of injection of the expanded Treg cells. Moreover, as the survival time of Treg cells is limited, it is necessary to investigate the safety of this strategy in humans. Overall, a comprehensive understanding of Treg cells is still urgently needed.

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ADDITIONAL INFORMATION

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