Published in final edited form as:

Trends Mol Med. 2011 October ; 17(10): 541–547. doi:10.1016/j.molmed.2011.05.012.

Regulatory T cells in CNS injury: the simple, the complex, and the confused

James T. Walsh and Jonathan Kipnis*

Neuroscience Graduate Program and Medical Scientist Training Program, Department of Neuroscience, University of Virginia, Charlottesville, VA 22908, USA

Abstract

Regulatory CD4⁺CD25⁺Foxp3⁺ T cells (Treg) have received significant attention for their role in controlling immune responses. Although knowledge of Treg biology has burgeoned, wide gaps remain in our understanding of Treg function under both normal and pathological conditions. Pioneering studies demonstrated roles for Treg in cancer and autoimmune diseases, including experimental autoimmune encephalitis, and this knowledge is often applied to other pathologies including neurodegenerative conditions. However, differences between immunity in neurodegeneration and in malignancy or autoimmunity are often neglected. Thus, Treg manipulations in CNS neurodegenerative conditions often yield unexpected outcomes. Here, we explore how the immunology of neurodegeneration differs from that of cancer and autoimmunity and how these differences create confusion about the role of Treg in neurodegenerative conditions.

CNS injury and the immune system

Traumatic injuries in the central nervous system (CNS) are associated with an immune response. The regulation of this immune response by regulatory T cells has been intensively studied recently, but the results are equivocal, leading to great confusion in the field. We first provide here an introduction on CNS injury and describe a role for the immune system in CNS pathology and then discuss the regulation of CNS immunity and the associated confusion.

CNS injuries

Research on injuries to the CNS has yielded a vast amount of basic knowledge in recent years, but development of effective therapies in this area is still desperately needed. Acute mechanical or biochemical injury to the mammalian CNS often results in irreversible functional deficit [1] because of the poor ability of injured axons to regrow and a destructive cascade of events that causes the damage to spread to neurons that had escaped the primary injury [2]. This spread of damage, or secondary degeneration, is attributed to devastating changes in metabolic, oxidative, and trophic factors that inevitably follow the primary cellular damage [3]. Attempts to promote CNS recovery have focused on two goals: neuroprotection (the arrest of self-perpetuating degeneration) and stimulation of regrowth [2, 4]. In the absence of intervention, the extent of recovery is a function of the amount of tissue that escapes the initial injury minus the loss associated with the subsequent

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*Corresponding author: Kipnis, J. (kipnis@virginia.edu), Tel: 434-982-3858; Fax: (434)-982-4380.

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neurodegenerative process. This secondary loss of tissue makes the outcome significantly worse than could have been predicted by the severity of an initial injury and is shared across different CNS injury models (Box 1).

Box 1

Commonly used CNS injury models in rodents

- **Optic nerve injury** [39, 65]
 - Primary damage is due to direct mechanical injury
 - Large area of secondary degeneration, killing previously uninjured neurons
 - Displays both apoptotic and necrotic cell death
 - Pure white matter injury; secondary degeneration of cell bodies (retinal ganglion cells) in the retina
 - Blood-brain barrier breakdown
 - Moderate T cell infiltrate
- **Spinal cord injury** [66, 67]
 - Primary damage is due to direct mechanical injury
 - Large area of secondary degeneration, killing previously uninjured neurons
 - Displays both apoptotic and necrotic cell death
 - Mixed grey and white matter degeneration in both primary and secondary insults
 - Blood-brain barrier breakdown
 - Moderate T cell infiltrate
- **Stroke** [68, 69]
 - Primary injury is due to loss of blood flow to CNS
 - Large area of secondary degeneration, killing previously uninjured neurons
 - Displays both apoptotic and necrotic cell death
 - Can affect grey or white matter, depending on the location of infarct: most common experimental stroke affects both grey and white matter
 - Blood-brain barrier breakdown
 - Moderate T cell infiltrate
- **Blast injuries** [70, 71]
 - Primary injury is due to pressure wave from explosion
 - Extent of secondary degeneration is largely unknown
 - Displays both apoptotic and necrotic cell death
 - Complete pathogenesis unknown, thought to be a diffuse axonal injury initially

- Blood-brain barrier breakdown
- Moderate T cell infiltrate

Lack of regeneration in the mammalian CNS

Unlike mammals, lower vertebrates are able to undergo extensive recovery after CNS injuries, even reconstituting the entire caudal end of a transected spinal cord [5]. The most obvious reason for this difference is that lower vertebrates are capable of neurogenesis, in which new neurons are produced from radial glia [6]. Other factors causing loss of neuroregenerative capacity in the mammalian CNS are mainly extrinsic to neurons, such as the production of inhibitory molecules by astrocytes and oligodendrocytes [7, 8] including Nogo, myelin associated glycoprotein (MAG), and oligodendrocytes myelin glycoprotein (OMgp) families, whose neutralization leads to an increase in axonal outgrowth [9].

Innate immune response to CNS injury

The brain has long been considered an “immune-privileged” site, protected by a blood-brain barrier (BBB) that is impenetrable to immune mediators, and hence seemingly unaffected by the immune system (other than resident microglia) [10]. It is now recognized, however, that activated immune cells can access the intact brain [11] and when the BBB breaks down after CNS trauma, a massive infiltration of immune cells to the CNS parenchyma is evident. Mechanisms contributing to the breakdown of the BBB include injury-induced immediate damage to the vasculature, as well as later damage due to inflammatory, apoptotic, and metabolic causes [12]. As soon as a few hours after injury, the pro-inflammatory cytokines interleukin (IL)-1 β , tumor necrosis factor alpha (TNF α) and IL-6, which are secreted copiously into the injured tissue, bind to endothelial, neuronal, and glial cell receptors and upregulate the production of inflammatory mediators [13, 14], leading in turn to neutrophil influx [15]. As a consequence, the normally low expression of class I and II major histocompatibility complex (MHC) proteins in astrocytes and microglia is increased [16], enabling the injured tissue to initiate and sustain an immune response from the adaptive immune system. Adhesion molecules, also upregulated after injury, regulate the temporal segregation of infiltration by innate and adaptive immune cells, so that neutrophils enter the CNS shortly after injury but by 48 hours have greatly decreased in number [17]. In contrast, macrophages infiltrate 2 to 5 days post-injury and lymphocytic infiltration peaks on day 7 [18].

Mechanisms of peripheral and central tolerance

Adaptive immune responses are controlled at several levels throughout the body. First, during T-cell development a process of central tolerance occurs in the thymus, which results in deletion of many self-antigen specific T cells. In this process, thymocytes are positively selected for either CD4 or CD8 expression, then undergo negative selection in which cells with high avidity are deleted by apoptosis upon recognition of peptide–MHC complexes (including self-peptide–MHC complexes) expressed on dendritic cells or on thymic medullary epithelial cells [19].

Not all self-reactive T cells are deleted through central tolerance, however, as some receptors recognize low-avidity self-peptide–MHC complexes [20]. Control by naturally occurring regulatory CD4⁺CD25⁺Foxp3⁺ T cells (Treg), via prevention of peripheral T cell activation, is the most intensively studied mechanism for regulating the response of these self-reactive cells that have survived thymic selection. These cells have received a great deal of new interest due to their absence in the scurfy mouse mutant, which dies of devastating autoimmune disease by the age of 3 weeks. The mutation was traced to the X chromosome

and mapped to the gene encoding the transcription factor forkhead box P3 (*Foxp3*) [21]. Expression of *Foxp3* is sufficient for CD4⁺ lymphocytes to acquire a suppressive phenotype in mice [21], and conditional ablation of *Foxp3* in lymphocytes confers a multisystem autoimmune disease with massive lymphoproliferation which, as in the scurfy mouse, kills the animal at about 3 weeks of age [21].

Regulatory and effector T cells

Regulatory T cells

Although Treg display the same T cell receptors as CD4⁺*Foxp3*⁺ effector T cells (Teff), unlike Teff they do not proliferate in response to antigen presentation *in vitro*. Instead, they are stimulated to divide in response to IL-2, whose production is a hallmark of activated Teff, *in vitro*; in fact, some Tregs proliferate in response to IL-2 *in vivo* [22]. Although Treg constitutively express the high-affinity IL-2 receptor CD25, they are unable to produce IL-2 on their own [22]. Indeed, it was surprising to find that half of all *Il2* knock-out mice died of autoimmune disease by 9 weeks of age [23] until it was realized that Treg require IL-2 to survive [22]. This constitutive expression of CD25 is thought to be one of the ways by which Treg suppress proliferation of Teff, that is, by acting as a sink for IL-2, which is also needed for Teff proliferation. Treg do require antigen stimulation to become suppressive, but once activated their suppressive ability seems not to be antigen-specific [24]. Whereas Treg can apparently utilize various mechanisms to suppress Teff, the most strongly suppressive mechanisms are contact-dependent [25]. Treg utilize the anti-inflammatory signaling molecule transforming growth factor (TGF)- β both by secreting it and expressing it on their surface [25]. One mechanism of TGF β -mediated suppression is its ability to affect the differentiation of naïve T cells [26]. TGF β , in the absence of other inflammatory signals, is able to induce *Foxp3* expression and confer a suppressive phenotype to these cells. This action can be a double-edged sword because TGF β in combination with the inflammatory cytokines IL-6 and IL-22 is able to induce IL-17 expression in T cells, a phenotype that has been associated with autoimmune disease and inflammation [27].

The ability of Treg to suppress immune responses is not constant, but can be modulated by signals from their extracellular environment. For example, after burn injuries the suppressive ability of Treg is decreased because of changes in both their secretion of anti-inflammatory cytokines and their surface expression of TGF- β . What kinds of signals can induce this change in suppression? There are a few examples of signals that increase Treg suppressive ability; the best studied is IL-2 (also known for its role in Treg development) [28]. By contrast, a multitude of factors are known to decrease the suppressive capacity of these cells. They include pro-inflammatory cytokines produced by lipopolysaccharide-activated APCs [29], TNF [30], B cells [31], glucocorticoid-induced TNF receptor ligands [32], IL-4 [33], Toll-like receptor (TLR)-2 signaling through Treg [34], and two factors suggested to be important in CNS trauma, namely, unmethylated CpG-island rich DNA segment signaling through TLR9 [35] and the neurotransmitter dopamine [36].

Autoimmune T cells are beneficial after CNS injury

Self-antigen-specific T cells are not exclusive to patients with autoimmune disease; T cells reactive against self-antigens also are commonly found in healthy controls [37]. Because of the toll taken by autoimmune diseases, it would appear to make sense biologically if such self-reactivity provided an evolutionary advantage. Whereas the role of self-reactive cells in fighting cancer has been known for some time [38], the role of the adaptive immune system in other sterile situations did not emerge until later. One of the first clues that self-reactivity by the adaptive immune system could be beneficial came from CNS injury models: passive immunization with T cells specific for myelin basic protein (MBP) increased neuronal

survival after optic nerve crush while those without specificity to antigen that is presented in the CNS did not show benefit [39]. This seminal finding suggested that MBP-specific T cells, which cause experimental autoimmune encephalomyelitis (EAE) in susceptible strains, can actually be beneficial after CNS injury in both EAE-susceptible and resistant strains, provided that the immune response is well controlled. In fact, it seemed that this injury-attenuating response was not exclusive to MBP. Indeed, the proteolipid protein (PLP) and the myelin oligodendrocyte glycoprotein (MOG), both associated with myelin, could also exert beneficial effects in mice actively immunized with epitopes from these proteins prior to CNS injury [40]. The beneficial effects of CNS-directed adaptive immune responses to injury have by now been tested using several different experimental paradigms [41]. Further illustrating the importance of the adaptive immune system, neuronal survival after CNS injury in nude mice (which lack mature T cells) was significantly decreased relative to neuronal survival in similarly injured normal controls. The decrease in survival was reversed, however, upon adoptive transfer of T cells from naïve mice [42].

The identity of the cell type that mediates this beneficial effect after CNS injury is not yet known, and literature reports are conflicting. Some studies have indicated that T_H1 cells have the greatest potential for such neuroprotection [43], whereas others suggest that the vital part of recovery from CNS injury derives from soluble molecules from T_H2 cells [44]. In the absence of additional *in vivo* studies that corroborate either of these claims, this remains an area requiring further investigation.

The role of Treg and autoimmune T cells in autoimmune diseases and in cancer has been intensively studied. The best evidence regarding Treg and autoimmune Teff cell function in the CNS is available through extensive research with the EAE model, a commonly used model of multiple sclerosis, [45, 46], as well as from studies on experimental autoimmune uveitis (EAU) [47]. Although there is a neurodegenerative component in human multiple sclerosis and in some models of EAE, here we refer to EAE as an “autoimmune” and not as a “neurodegenerative” disorder. The autoimmune components in the animal models are crucial for disease initiation (we do not discuss here the EAE model induced by Theiler’s virus [48]) and therefore, it is plausible that induction of Treg would benefit the disease pathology, as has been previously described [49, 50]. In this manuscript, however, we propose that the differences between the nature of immunity in neurodegenerative conditions, both acute (physical injury) and chronic (e.g., Alzheimer’s, and Parkinson’s), and in malignancy or autoimmunity (including EAE) are often neglected. The basic principles of Treg biology are “borrowed” from other fields and applied to injury or chronic neurodegenerative conditions, which often results in unexpected outcomes and overall confusion. Below, we discuss the “confusion” in more details.

The simple, the complex, and the confused

Treg in CNS injury: the simple

Early experiments, though lacking the sophisticated methods available today, hinted that the presence of Treg limited the beneficial potential of autoimmune Teff [42]. The techniques used in those studies did not rely on the targeting of specific molecules, given that they were performed at a time when the molecular characteristics of Treg were just beginning to emerge. Although these techniques could not provide unequivocal answers, they were able to demonstrate a drop in Treg numbers and an increase in the proportion of Teff to be associated with increased neuronal survival after CNS injury. As an example, a three-day postnatal thymectomy resulted in a decrease in Treg relative to Teff in adult mice, permitting increased post-injury neuroprotection compared to non-thymectomized controls [42]. Similarly, low-dose irradiation, which triggers apoptosis preferentially in Treg and promotes proliferation of Teff, increased neuronal survival after injury to either the rodent

optic nerve or spinal cord and following glutamate-mediated toxicity of retinal ganglion cells [51]. Moreover, several compounds that decrease the number or activity of Treg have also shown neuroprotective effects. Dopamine, which can decrease the suppressive ability of Treg through ERK1/2 signaling, increases neuroprotection when administered systemically [36]. In a more immune-relevant paradigm, the synthetic, bacterial-DNA mimetic CpG modulated the regulatory capacity of Treg, presumably by acting through Toll-like receptors, and increased neuronal survival by decreasing immune suppression [35]. The results of these experiments collectively support the simple hypothesis that Teff are required for neuroprotection after CNS injury and that this ongoing response is limited by Treg. In line with this hypothesis, the limiting of the beneficial Teff response by Treg can be viewed as “the evolutionary compromise between a need and a risk” [52].

Treg in CNS injury: the complex

The real situation, however, is not always that simple and cannot always be explained in terms of Treg-imposed restraint on a beneficial, but potentially risky, autoimmune response to injury. In Balb/c mice, transfer of splenocytes depleted of CD25⁺ cells into nude mice increased the beneficial effect on neuronal survival beyond the benefit that could be achieved by transferring whole splenocytes. In line with this, transfer of isolated CD4⁺CD25⁺ cells (i.e., Treg) from naive mice into injured, immune-competent Balb/c mice partially abolished the beneficial neuroprotective effect [53]. However, transfer of the same CD4⁺CD25⁺ population resulted in the opposite phenotype when the recipient mice were of the C57/B6 background; that is, neuroprotection in the C57/B6 background was increased following injection of exogenous Treg [53]. This finding was surprising because when the same two strains were subjected to immunization experiments with retina-specific antigens, neuronal survival in both strains was increased [53]. These results suggest that the nature of the endogenous response to injury in C57/B6 mice differs from that in Balb/c mice and that Treg manipulation might lead to neurodestruction or neuroprotection based on the genetic background of the recipient mouse. Interestingly, when Treg were transferred into injured, immune-compromised mice, the treatment was beneficial in mice of both genetic backgrounds. Moreover, upon injection into an immune-deficient host these Treg not only lose their suppressive activity [54] but also undergo proliferation and acquire effector function. It is, therefore, questionable whether these studies measure the effect of the injected CD4⁺CD25⁺ Tregs directly or the effect of the cells they differentiate into within immune-deficient hosts. Clearly, the nature of this “Treg” population requires further experimental clarification.

The findings described above raise concern with regard to the development of Treg-based therapies for clinical use. At this point it is obvious that we do not completely understand the complexity of Teff/Treg interactions in situations involving CNS injury or neurodegenerative disorders. Therefore, instead of a rush to examine the protective activity of Treg under different neurodegenerative conditions, what is needed is a thorough study and systematic analysis of the interactions between the cellular participants to understand the complexity.

Treg in CNS injury: the confused I (strains and immunity)

As noted above, confusion still reigns with regard to Treg function, and particularly to the protective properties of these cells under acute and chronic neurodegenerative conditions. Because most of the present knowledge in neuroimmunology comes from early studies on animal models of multiple sclerosis, both the commonly used cell lines and the acquired data are dominated by strains susceptible to autoimmune disease. Furthermore, because most existing transgenic and knockout mice are on a B6 background, this is the most intensively studied strain in neuroimmunological investigations of CNS injury and chronic

neurodegeneration. As described above, Treg have opposite effects when transferred into Balb/c and C57Bl/6J mice, and their effects after injury in other strains are as yet unknown. This finding alone should raise questions among experimenters as to the most relevant animal model of CNS neurodegeneration for use in studies of the immune response. The use of several mouse strains in any study carried out to support the claims for a neuroprotective therapy or agent is probably necessary. The failure to transfer most of the proposed therapies from the laboratory to the clinic might be due in no small measure to a failure to follow this practice. Moreover, mice that are lymphopenic or otherwise immune-compromised might not be reliable models for addressing the role of Treg in neurodegeneration because the basic properties of Treg change in immune-compromised hosts. Efforts at this stage should be aimed at understanding the immunobiology of Treg under neurodegenerative conditions (both in immune-competent and immune-compromised hosts), before translation into human therapeutics is considered. At some point in the future we might then be justified in shifting the focus to clinical trials of Treg-based neuroprotective therapies.

Treg in CNS injury: the confused II (anti-CD25 antibody)

The discovery that CD25 is highly expressed on Treg led researchers to realize that the use of antibodies against this molecule would result in Treg clearance via the FcγRIII-mediated mechanism [55]. Use of anti-CD25 antibody resulted in exacerbation of the infarct zone and functional deterioration in a stroke model of CNS injury [56], leading to the conclusion that depletion of Treg results in impaired recovery and that Treg are needed for neuroprotection after stroke. However, CNS injury results in presentation of self-antigens, leading to Teff activation and proliferation. Activated effector T cells express CD25, therefore, while CD25 serves as a marker for naturally occurring Treg in naive animals, it cannot distinguish between Treg from activated Teff in “challenged” mice, such as after CNS injury. Thus, mice that are treated with anti-CD25, which targets and depletes Treg in the initial stages of treatment will exhibit a faster Teff response (Teff are activated and proliferate more efficiently when Treg are removed). However, the anti-CD25 antibody is cleared slowly and will thus eventually target the emerging, activated, CD25-expressing Teff and result in their depletion as well. Therefore, exacerbation of CNS injury using anti-CD25 antibody does not necessarily imply that Treg are needed for a better recovery; it might also suggest that activated Teff, also eliminated by the antibody, are needed for neuroprotection. With the development of alternative tools to study the effect of Treg depletion, such as the expression of the diphtheria toxin receptor controlled by the *Foxp3* promoter [57], many of the experiments whose results have been equivocal because of this dual targeting of the anti-CD25 antibody [56, 58, 59] need to be re-evaluated to determine the effect of each target individually.

Concluding remarks

A population of suppressor T cells were known to exist for many years, but the markers with which they could be recognized had not been identified [60]. This population was rediscovered as regulatory T cells approximately a decade ago. Treg express both CD25 (also expressed by activated Teff) and the transcription factor *Foxp3* (uniquely expressed by Treg) [61, 62]. Early studies suggest opposite activities for Treg function in autoimmune disease and cancer: an enhanced functioning of Treg would be needed in autoimmune diseases to suppress the ongoing inflammatory response, whereas in cancer Treg function would need to be attenuated, allowing effector T cells to be easily activated to fight off the malignancy (Figure 1). Since that early period, a role for Treg has been proposed in nearly every clinical condition. In the field of neurodegeneration, there is some consensus that Treg regulate the response to CNS injury [63, 64]. The original studies on Treg in CNS injuries [42] suggested that Treg activity serves as a restraining self-checking mechanism to prevent excessive reaction on the part of the beneficial immunity activated spontaneously in

response to CNS injury. Subsequent studies assigned a more direct neuroprotective role to Treg, bypassing the immune response. It was proposed that Treg, based on their suppressive function in the periphery, also suppress the characteristic glial response to injury in the CNS, assumed to be destructive to neuronal survival [53]. Although the data obtained from animal models in these later studies is intriguing, it is important to remember that after an injury to the CNS, the spontaneous immune response itself plays a role in the overall effect that injected, activated Treg will exert on neuronal survival. The answer, as is typical in biology, depends on the timing and strength of the response and how the response-related signals integrate with the signals of their downstream target cells, the Teff, to achieve an immune response that is robust and properly controlled (Figure 1). Because these parameters have yet to be verified, they preclude, at least at this point, any application of potential Treg therapies in clinical situations. The good news is that after a decade of largely inconclusive findings on the precise role of Treg in CNS injury, we are reaching a point where newly available molecular tools should facilitate more elegant studies of these cells and help to clear up the remaining confusion and controversy (Box 2).

Box 2

Outstanding questions in the field

- Is the adaptive immune system playing different roles in grey matter injury and white matter injuries?
- Is the adaptive immune system playing different roles in traumatic and ischemic injuries?
- What are the roles of adaptive vs. innate immune responses in CNS injuries?
- Do Tregs have any direct effect on neuroprotection, or do they only work via suppressing Teff and what is the mechanism of T cell mediated neuroprotection?
- Is the timing of Treg depletion vital for neuroprotection?
- Are certain CNS antigens more neuroprotective than others?
- Are T cells initially activated in the tissue or in the draining lymph nodes?

Acknowledgments

We thank Shirley Smith for editing the manuscript. This work was supported in part by an award to J. Kipnis from the National Institute of Neurological Disorders and Stroke (R01NS061973).

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Glossary

- White matter – areas in the brain containing myelinated axons in the CNS. The most prominent in the brain is the corpus callosum, which connects the two hemispheres
- Grey Matter – areas of the brain containing cell bodies and unmyelinated axons
- Ischemia – a decrease in blood flow, as seen in stroke
- Infarct zone – area damaged by an ischemic event
- FCγRIII – a receptor on innate immune cells that recognizes antibody and which mediates phagocytosis of material that is bound to an antibody
- Lymphopenic – having a decreased number of circulating lymphocytes
- Retinal ganglion cells – the retinal neurons whose axons make up the optic nerve
- Experimental autoimmune encephalomyelitis – experimental model of multiple sclerosis induced by passive transfer of CNS-antigen specific T cells or immunization with CNS antigens
- Experimental autoimmune uveitis – autoimmune model of the eye induced by immunization with retinal antigens or passive transfer of T cells specific to retinal antigens
- Thymectomy – removal of the thymus
- Lymphoproliferation – an expansion of lymphocytes
- Thymic medullary epithelial cells – cells in the thymus that are important in maintaining self-tolerance. These cells express AIRE, and therefore can display an expanded repertoire of self-antigens
- Parenchyma – The area of the brain inside the BBB, consisting of astrocytes, oligodendrocytes, microglia, and neurons
- Foxp3 – a transcription factor that regulates a set of genes, including CTLA4 and TGFβ, conferring suppressive ability to CD4⁺ lymphocytes
- CD25 – alpha chain of IL-2 receptor. Expressed on all Treg and on activated Teff cells (as well as some other leukocytes)

Figure 1. Model of proposed Treg-based therapeutic interventions in different pathological conditions

While the role of Treg is well studied in cancer and autoimmune diseases (green shaded areas), its role in CNS acute and chronic neurodegenerative conditions (grey shaded) is understudied. In autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), increasing suppression of Tregs would ameliorate symptoms of the disease, whereas inhibiting the Treg suppression would be the therapeutic aim in cancer. We propose that in acute CNS injury Treg suppression needs to be inhibited immediately after injury to allow a beneficial autoimmune response, but then in the late stage of injury this suppression needs to be reinstated to prevent destructive autoimmune attack from developing.