Chapter 6

# Immune System Involvement in the Degeneration, Neuroprotection, and Restoration after Stroke

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Additional information is available at the end of the chapter

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**Abstract**

Cerebrovascular diseases are currently among the three primary causes of death worldwide and are the first cause of disability in adults. Nevertheless, there are no neuroprotective or neurorestorative therapies that have shown considerable beneficial effects, except for the FDA-approved recombinant tissue plasminogen activator (rtPA), which has been used for decades for the treatment of stroke and its effectiveness is still controversial. This is why it is very important to develop effective therapeutic options. In order to achieve this objective, it is essential to recognize the secondary mechanisms involved in the pathological development. The immunological system is one of these mechanisms that participate during the acute and chronic phases of disease, both in deleterious and beneficial manners. It is known that the immune system's duality contributes to the ischemic injury through proinflammatory cytokine (tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-1 (IL-1), interleukin-6 (IL-6)), and oxygen reactive species production, etc. Nevertheless, it also provides protection and even restoration through anti-inflammatory cytokine (interleukin-4 (IL-4), interleukin-10 (IL-10), transforming growth factor-$\beta$ (TGF-$\beta$)), and growth factor (brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4)) production. This states that innovative therapeutic options must be proposed with the goal of protecting and restoring the tissue after the ischemic event. Such therapies are exposed in the present chapter.

**Keywords:** cerebral ischemia, neuroprotection, immunomodulation, inflammation protective autoimmunity, neurorestoration

## 1. Introduction

Cerebrovascular diseases (CVD) include hemorrhagic and ischemic brain injuries, the latter being the most common since 85% of cases arise from atherothrombotic (artery stretching) and atheroembolic etiologies. Both of these diseases are the first cause of permanent disabilities in adults, primarily in developed countries, where 30% of patients that have suffered from stroke become incapable of performing their daily routines [1]. Stroke is, in addition, among the first three causes of premature death worldwide; according to the World Health Organization (WHO), 6.7 million deaths were caused by cerebral ischemia in 2012, as well as 46% of deaths being caused by stroke and ischemic heart disease altogether, with mayor incidence in low-income countries, with 80% of cases [2]. As for the United States (which has the most available information), the American Heart Association (AHA) reported in an updated statistics report that the death rates associated with stroke have been dropping over the years, and from 2009 to 2012, the prevalence is estimated in 2.6% with an incidence of almost 800,000 cases a year, one every 40 s [3]. And according to the Center for Disease Control (CDC), there is one death out of every 20 stroke cases, or one every 4 min [4]. On the other hand, European countries experience dramatic differences in disease burden. The EuroHOPE study performed on data from 2007 observed higher incidence of ischemic stroke in Hungary, and Finland and less in Scotland and Sweden and different mortality rates among regions in different countries [5].

The incidence and prevalence of stroke and its recurrence continue to be high due to the low attention and awareness to symptoms which create delay in the seeking of medical attention, allowing damage to progress. People are not aware that time is critical for stroke treatment due to the short therapeutic window of available treatments. Disabilities and dependence among patients tend to get worse in the 6–12 months following stroke; mobility and functionality related to dressing and toileting are the most affected, and deterioration is related to prior-to-stroke comorbidities [6]. Depression is one of the major outcomes and is associated with stroke recurrence [7].

There are a vast array of risk factors associated with cerebrovascular diseases and specifically to cerebral ischemia. The most common are chronic diseases that could be modified through behavioral and lifestyle changes or pharmacological treatment such as diabetes, hypertension, obesity, and atherosclerosis; besides, alcoholism and tabaquism, high salt consumption, and sedentarism are behaviors that are associated with a greater risk of developing stroke [2]. On the other hand, there are a series of risk factors that cannot be controlled and predispose a person to cerebral ischemia: they are age, gender, and ethnicity. For instance, postmenopausal women have greater risk of developing stroke than men the same age, but premenopausal women are protected by estrogenic hormones [8, 9]. It has also been reported in 2010 by the Global Burden of Disease (GBD) that 31% of strokes are among young adults (20–64 years of age) and strokes are common in people below 45 years of age [10]. Non-Caucasians are also at greater risk of stroke than Caucasians [8].

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## 2. Pathophysiology

Usually, when speaking about stroke, two stages of damage to the integrity of the neural tissue are considered. The first stage is the lesion *per se*, caused by the restriction of blood flow from an obstruction in a major vessel and presents the characteristic physiopathology that ends in neural death. This area of the lesion, which is almost immediately damaged at this stage is called the "infarct core." The second stage of damage is that of secondary degeneration that further injures tissue not originally damaged by the restricted blood flow, but that is adjacent to and surrounds the infarct core. This area of lesion is called "ischemic penumbra," it preserves some energy metabolism, and its degeneration is caused primarily by excitotoxicity and inflammation.

Nonetheless, inflammation previously contributes to the development of stroke, since people who suffer from chronic proinflammatory state diseases like hypertension, dyslipidemia, atherosclerosis, and type-two diabetes have endothelial alterations, as well as irregularities in rheology and hemodynamics [11]. Galectin-3 (GAL-3) concentration is increased in these patients; this protein favors atherosclerotic plaque formation and might participate in the development of cerebrovascular disease [12] GAL-3 is also a very important inflammatory and fibrogenic mediator [13, 14]. Interleukin-1β (IL-1β) is a proinflammatory cytokine that has been related to atherosclerotic plaque formation and vascular inflammation [15]; other factors have also been associated with it, such as Von Willebrand coagulation factor, selectin E, and others [16].

Atherosclerotic plaque is characterized by accumulation of molecules of cholesterol and low-density lipoproteins (LDL) in the vessel walls, after being oxidized they chemo-attract monocytes to the site, and they phagocytose these oxidized LDL, which in turn causes them to become foam cells. Foam cells loaded with high amounts of LDL stay trapped in the endothelium and suffer from apoptosis and necrosis. This situation generates a lipidic plaque covered by connective tissue and are infiltrated by activated T cells, macrophages and mastocytes that will chronically produce inflammatory mediators in the endothelial wall [17]. Oxidized cell and molecule accumulation generate endothelial wall activation, thus promoting adhesion molecule expression and easing immune cell aggregation.

Several investigators consider that the severity of endothelial inflammation can imply differences in atherosclerotic plaque rupture vulnerability, which will contribute to the development of ischemia in the surrounding tissue. For this reason, the use of imaging technology such as Computerized axial tomography Computerized axial tomography(CAT);Positron emission tomography (PET) scan, MRI, and Positron emission tomography (PET), has been considered in order to identify the degree of endothelial inflammation and to assess risk of developing an ischemic event [18].

Stroke originates from either a reduction in the arterial lumen, or the release of a thrombus that becomes trapped in a major artery, most commonly the middle cerebral artery (MCA). This occlusion causes diminished blood flow to the site irrigated specifically by that vessel and so glucose and oxygen supply will stop, triggering metabolic insufficiency. The incapacity for

glucose to reach the cells causes a decrease in adenosine triphosphate (ATP) production which interferes with $Na^+$ and $K^+$ pump function; in light of this, intracellular $K^+$ decreases dramatically causing membrane depolarization [19], and thus, further voltage-dependent $Ca^{2+}$ channel dysfunction and opening, unlocking of some $CA^{2++}$ receptor-dependent channels, $Na^+/Ca^{2+}$ channels from cellular and mitochondrial membrane, and $CA^{2+}$ pump deterioration in cell membrane, and endoplasmic reticulum [20].

All of these events triggered by membrane depolarization drive a secretion of excitatory neurotransmitters, especially glutamate that upon binding to its receptors induces greater depolarization and glutamate release, giving rise to the excitotoxicity phenomenon [21]. The massive amounts of calcium will activate a series of enzymes (e.g., calpaines, phospholipases, and endonucleases), and free radicals that in turn lead to neuronal death.

On the other hand, the proinflammatory milieu that is present in the occluded vessel endothelium is lacking in oxygen and altogether with the changes in vascular pressure generate a major reactive oxygen species (ROS) production [22] that promotes higher expression of: Matrix metalloproteinases 2 and 9 (MMP 2 and 9) that digest the basal endothelial sheet [23] and cyclooxygenase 2 (COX-2) and subsequent prostanoid production [24]. Increased ROS production also cause complement and endothelial cell activation that promotes the secretion of proinflammatory mediators such as IL-1 and IL-6, and increased expression of intercellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM), and leukocyte adhesion receptors such as selectins P, E, and L; all this promotes leukocyte adherence and extravasation [25].

When, or if the occlusion is not permanent, the vessel experiences reperfusion (spontaneously or after treatment). During this process blood flow is restored, thus once again providing glucose and oxygen to the already injured tissue. This situation worsens neural tissue damage as a result of an increase in substrate availability that causes an increment of free radical production, lipoperoxidation, and a rise in cell death protein activation, as well as adhesion molecules [26] and metabolic detriment [27].

As free radicals such as nitric oxide (NO) and ROS increase, they interact with their target molecules and activate mechanisms such as apoptosis, arachidonic acid metabolism, and respiratory chain inhibition that, as a consequence, increase inflammatory mediators [26, 27] that contribute to the secondary degeneration.

Although it is worth mentioning that the amount of neural damage depends on how long the vessel is occluded, since it has been observed in several studies that early reperfusion reduces infarct sizes [27].

## 3. Secondary degeneration due to inflammation

Microglia is specialized macrophages that live in the cerebral parenchyma, when at rest or quiescent, these normally exhibit a phenotype characterized by thin processes. These cells are also very sensitive to changes in the cerebral milieu since their primary functions are to

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eliminate cell debris from apoptosis [28], regulate neural synapses [29], neurogenesis [30], trophic factor production [30], inflammatory process [31], damaged cell phagocytosis, and the repair and remodeling processes of the central nervous system (CNS) [32].

During early stages of ischemia, when there is a progressive decrease in oxygen and ATP in the cerebral parenchyma, glial cells release molecules such as lipocalin 2 (LCN2) [33] and IL-4 [34] secreted by neurons as an immediate response to injury. These molecules are capable of activating microglia and induce a protective M2 phenotype characterized by the production of anti-inflammatory cytokines IL-10, IL-4, and increased phagocytosis [33]. This phenotype has been observed during the first 7 days post-ischemia; it reaches it max peak at 3–5 days, and decreases by day 14, suggesting that microglia promotes neuronal survival during this first stage by attempting to reduce inflammatory mediator release by synthesizing transforming growth factor-β (TGF-β), arg-1, and CD206 [35, 36], apart from producing growth factors such as insulin-like growth factor-1 (IGF-1) and ciliary neurotrophic factor (CNTF) that facilitate mechanisms of repair [34].

While the milieu changes from day 3 through day 14 post ischemia toward high concentrations of $Ca^{2+}$, free radicals, glutamate, and debris from neuronal necrosis, microglial phenotype gradually changes from M2 to M1, and begins to express genes such as nitric oxide synthase (INOS), CD16 and CD32 as well surface markers such as CD11b and MCHII [36]. M1 phenotype is distinguished by a decrease in phagocytosis activity and an increase in the production of proinflammatory mediators: IL-1β, IL-6, and tumor necrosis factor $\alpha$ (TNF-$\alpha$) and an increase in NO, $H_2O_2$, ROS, MMPs, and chemokines such as CXCL10, CCL2, MCP-1, CXCL1, and CCL5 [35, 37]; through the Notch pathway signaling [38], all of which propitiate the support for a proinflammatory milieu.

This polarization of microglial activation gives rise to the opportunity to search for ways to modulate it in order to induce an M2 phenotype and through it, be able to get the beneficial effects of an anti-inflammatory milieu, accompanied by trophic factors that ease cellular repair.

On the other hand, macrophages and mast cells dwell around the cerebral parenchyma and the perivascular spaces, also called Virchow-Robin spaces, these cells activate in presence of inflammatory mediators secreted as a result of ischemia/reperfusion [39, 40]. These produce high concentrations of histamine, catepsines, matrix metalloproteinases that further contribute to endothelial damage, blood-brain barrier (BBB), hyperpermeability, and the vasogenic edema formation as well increased production of TNF-$\alpha$ and CXCR, CXCL1/2/3 chemokines that will promote massive leukocyte recruitment to the perivascular region [39, 41], specifically neutrophils monocytes and T lymphocytes.

Neutrophil arrival at the injured perivascular space depends on time and type of occlusion, Nina Vindegaard Groberg et al. published in 2013 that when the occlusion lasts 120 min, there is an important number of neutrophils that arrive 12 h post-ischemia, reaching a peak concentration at 24 h; when the occlusion lasts 60 min concentration peak is observed as far as 3 days post-ischemia [42]. Notwithstanding, Isabel Pérez de Puig et al. results published in 2015 point out neutrophil presence as early as 6 h post-ischemia in permanent MCA occlusion (MCAo), which opens to consideration the fact that neutrophil quantity and distribution are

different among patients [43]. Nevertheless, postmortem tissue analysis from people who suffered from cerebral ischemia in various vessels yielded no difference in neutrophil amount in perivascular zones, leptomeninges, and cerebral parenchyma around the lesion site.

As neutrophils arrive to the injury site, they react almost immediately to damage-associated molecular patterns (DAMPS), TNF-$\alpha$ and Interferon gamma (INF-$\gamma$) which are found widely distributed around the perivascular zone and cerebral parenchyma. This promotes their activation, and thus, they acquire the ability to secrete cytokines, primarily IL-1$\beta$, IL-6, also lytic enzymes, free radicals, and angiogenic factors, as well as chemokines such as CXCL9 and CXCL10 which influence Th1 and Th17 lymphocyte migration [44, 45], triggering an increased amount of cells and a proinflammatory milieu.

In the clinical field, it has been observed that patients who suffered from cognitive deterioration after an ischemic event have high concentrations of neutrophils, showing a high correlation between the degree of tissue damage secondary to inflammation and functional recovery [46]. Even patients that have been treated with recombinant tissue plasminogen activator (rtPA) but that previously presented high neutrophil amount have had the worst results associated with neuroprotection exerted by rtPA [47].

Large efforts are being made to conduct scientific investigations oriented toward the decrease of secondary damage through the inhibition neutrophil recruitment, the adherence of these to endothelial cells through cannabinoid 2 (CB2) receptor activation [48], or through Neurogenin1 (NRG1) growth factor that reduces response to endothelial inflammation causing a decrease in ICAM-1, VCAM-1, and selectin E [49] or by the use of competitive antagonist CXR2/CXR3 [50] all of which have demonstrated to have beneficial effects in the decrease of infarct size in animals subjected to these treatments.

Nonetheless, in the clinical setting, the use of some molecules such as Enlimomab, which reduces leukocyte adhesion, have had negative effects in stroke patients because it made them more prone to suffer from secondary infections that increased complications during their recovery [47].

In response to CCL2, MCP-1, and CXCL1 chemokines, to mention a few, monocytes infiltrate into the perivascular and brain tissue, and as thought up to a few years ago, they differentiated in macrophages indistinguishable from activated microglia, stimulating and exacerbating brain injury [51]; nonetheless, thanks to the identification of different monocyte subtypes investigators have been able to identify some of their roles in the injured tissue.

Recently, two different monocyte population types that express different markers have been identified in mice. Classical or proinflammatory monocytes expressing Ly-6C$^{high}$, CCR2$^{high}$, and CX3CR1$^{low}$ markers have short half-lives and are actively recruited into inflamed tissues, boosting inflammation. The other types, expressing Ly-6C$^{low}$, CCR2$^{low}$, and CX3CR1$^{high}$ markers, have longer half-lives and are found inspecting vessel integrity, aiding its maintenance [52]. Trying to identify the precise roles of each type of monocyte subpopulation is an essential task, since in 2015, Ritzel and his team conducted an experiment in which they demonstrate that 90 min after ischemia; there is a large forfeiture of microglia and a very high

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rise in monocytes coming from the periphery and reach up to 90% of monocytes in the ischemic brain at 72 h post-ischemia, making evident their very important role in injured tissue [53].

Several studies conducted in mice have also demonstrated that the rise of monocytes in blood and cerebral tissue express pro inflammatory markers, from subpopulation Ly-6C$^{high}$ during the acute phase of ischemia [52, 53]. The rise of this subpopulation is correlated with the infarct size and neurological deficit in mice subjected to ischemia/reperfusion [54]. Also, a rise in TNF-$\alpha$ and IL-1$\beta$ production, characteristic of this subtype, is observed during the first 72 h post-ischemia [53]. Nonetheless, it has also been observed that there is a change of phenotype during monocyte differentiation into macrophages, acquiring anti-inflammatory characteristics along with the synthesis of TGF-$\beta$ around the sixth day post-ischemia [55, 56]; but it is still not clear how such differentiation occurs, or what characteristics induce the process.

Experimentally, it has been observed that T lymphocytes reach the cerebral parenchyma later in time, between 24 and 96 h post-ischemia, reaching a max peak at 3–7 days post-ischemia [57, 58]. The increase of monocyte differentiation into macrophage infiltration, the expression of major histocompatibility complex II (MHCII), and costimulatory molecules in the activated microglia and the presence of CNS antigens such as myelin basic protein (MBP), NR2A/2B subtype of the N-methyl-D-aspartate receptor) and the human neuron-specific enolase (NSE) to mention a few, all products of necrosis and neural cell rupture found in systemic circulation and brain parenchyma [59, 60] stimulate antigen presentation. It is worth mentioning that at clinical level, concentration of these proteins has been related to the severity of neurological damage and extent of brain lesion in humans [61].

The characteristics of the immune response to these antigens that have modified their nature due to the degree of necrosis resulting from ischemia, differ depending on the presented epitope [62]. Different from other CNS pathologies, in ischemia, Th1-type immune response to antigens like MBP is infrequent, but exacerbated when exposed in combination to lipopolysaccharides (LPS), since secondary-to-stroke infections are very common [62]. Nonetheless, it has not been possible to clearly establish which mechanism of autoantigens is involved in damage exacerbation.

There are a series of experiments that show the harmful role of T lymphocytes, among which are those performed by Gokhan [26] and his team in 2006 where they observed that lymphocytes are the primary producers of INF-$\gamma$ and other proinflammatory cytokines, that promotes an increment in infarct size [26]; and those performed by Liesz in 2011 [63], in which they observed that by eliminating lymphocytes, infarct size was reduced in animals subjected to cerebral ischemia, all of which matches with Xiong et al.'s results in 2013 [64], where they observed that T lymphocyte deficiency significantly reduces infarct volume in a transient cerebral ischemia model, but not in distal permanent occlusion, which highlights that the model and level of reperfusion used are essential and differential to evaluate damage.

Thanks to new arrangement of more specific cellular markers, some new functions and mechanisms have been identified during stroke for the different T cell subtypes. INF-$\gamma$ production, primarily by T CD4$^+$ cells, is what fundamentally compromises injury exacerbation [63]. T CD8$^+$ cell activation conducts to neural cell death through perforin-granzyme

pathway. Natural killer (NK) cells have a less noxious effect. T γδ cells show an injurious effect at the experimental level through the production of IL-17, IL-23 [38, 63, 64] and IL-6 at the clinical level [65]. Treg lymphocytes have been implicated mostly in neural tissue protection, preventing autoimmunity and inflammation through IL-10 [66].

Immune tolerance to autoantigens is based on the regulation of autoreactive T lymphocytes through various mechanisms involving: elimination, anergy, or suppression via Treg cells, even though several studies have not found benefit from them, since after being eliminated, injured tissue did not present further damage [65].

Recent studies have observed that autoreactive T cells have the ability to promote neuroprotection. This physiological mechanism appears when the CNS suffers from damage and can be potentiated or modulated through active immunization with neural-derived peptides. Such has been demonstrated in several models, like: spinal cord injury [66], multiple sclerosis [67], partial injury to the optic nerve [68], among others. Using T lymphocytes for the bone morphogenic protein (BMP) autoantigen neuroprotection is observed, under morphological, anatomical and functional criteria. Through this immunomodulation mechanism, a major production of anti-inflammatory cytokines and trophic factors has been observed, which is a crucial event to look for in neuroprotection and even neurorestoration.

Each comprised mechanism of immune system participation in cerebral ischemia represents an opportunity to explore immunomodulation and contention that shall not be wasted, in order to look for tissue neuroprotection and neurorestoration.

## 4. Inhibition of immune response as a neuroprotective mechanism

The cytokine accumulation and cellular infiltration increase mentioned above drive an expansion in damage, even though molecules that try to limit it are released. This prejudicial effect increases in relation to passing time and ischemia intensity, which provides a relatively small therapeutic window to look for protection alternatives. Initially, because the immune system has always been considered as one of the responsible mechanisms for damage increase, most neuroprotective therapies are being investigated toward its inhibition, looking to eliminate proinflammatory cytokine production and cell recruitment.

For this reason, strategies to try and delay or stop the biochemical and molecular damaging process are being investigated since over four decades ago in preclinical phases [69, 70] using different compounds that exert neuroprotective mechanisms.

Neuroprotection is a term that refers to the use of different therapies, alone or in combination that protect the brain or the neural cells against damage from immune degeneration, apoptosis, and dysfunction [70, 71].

Neuroprotection is aimed at not only protecting neurons, but also other brain constituents, such as microglia and endothelial cells of the penumbra region [72], and can be achieved through different mechanisms such as: anti-excitotoxic agents, anti-inflammatory agents, antioxidants [71], but our main focus will be in those that are involved with the immune system.

Studies have been conducted in different settings and performed in animals in order to prove the existence neuroprotective characteristics of several molecules through all of these different mechanisms, focusing on those with immunomodulatory and immune inhibition activity [73].

Among those studied, the most recent substances that have demonstrated to have neuroprotective mechanisms through anti-inflammatory activity in the preclinical field, the following are included.

Lycium barbarum polysaccharides are derived from a traditional Chinese plant that when used in a stroke model in mice, the investigators observed a reduced number of apoptotic cells in the peri-infarct zone, as well as a reduction in neurological deficit. This extract has neuroprotective effects through the inhibition of the ERK and JRK pathways, it inhibits MMP-9 and thus protect the BBB integrity, and it also regulates aquaporin-4 in order to reduce brain edema [74].

Piperine (1-peperoylpiperidine) is an extract from pepper usually used in folk medicine to treat different ailments since it appears to be anti-inflammatory. A group led by F. Islam investigated its effect in ischemic brain injury. They pretreated Wistar rats and investigated its neuroprotective role in a period of 24 h after the MCAo and observed a down regulation of COX-2, nitric oxide synthase (NOS-2), and nuclear factor κβ (NF-κβ) in the penumbra region, thus reducing the secretion of proinflammatory cytokines. A decrease in infarct size and less neuronal loss was also observed in the pretreated group [75].

Simvastatin is a pharmacological agent used in the treatment of atherosclerosis and high blood cholesterol, it has shown neuroprotective effects in ischemic brain injury through the upregulation of Nitric Oxide synthase, decrease in ROS production, the fibrinolysis activation through the upregulation of tissue plasminogen activator (tPA), and downregulation of plasminogen activator inhibitor-1 (PAI-1), as well as the recruitment of inflammatory components of the ischemic cascade from monocytes, macrophages, and T lymphocytes [76].

Neuro-erythropoietin (EPO) has proven to be neuroprotective in ischemic models. It decreases susceptibility to glutamate toxicity and nitric oxide, thus being antioxidant, it also induces the production of ant apoptotic and neurotrophic factors and decreases inflammation. Another proposed mechanism for neuroprotection by EPO is the use of the released iron by the ischemic lesion for erythropoiesis, thus limiting its oxidative effects [77].

Levodopa/benserazide is a pharmacological agent that during an investigation was given to rats 2 days after experimental stroke, and at day 7, T cells and chemokines were analyzed. It was discovered that CD3 and CD8 T cell population was diminished in the treated group, as well as lower levels of ICAM-1 in the ischemic hemisphere [78].

Fingolimod modulates the activity of the membrane receptor (S1PR) responsible for the reduction in lymphocyte migration into the brain tissue and the microvasculature; this increases cerebral blood flow by attenuation of adhesion and thrombus formation and protects the brain indirectly [79].

Other molecules occurring naturally, such as fatty acids, have neuroprotective effects through immunomodulation and antioxidation. Omega-3 fatty acids are essential for human consumption since humans lack the ability to synthesize them. *In vivo* and *in vitro* experiments per-

formed by Zhang et al. using fish oils and/or omega-3 fatty acids demonstrate the importance of their consumption since the animals treated and subjected to MCAo showed lesser infarct size and neurological deficit. The mechanisms proposed by these authors are antioxidation through the enhancement of the expression of hemeoxigenase-1 (HO-1) and nuclear translocation of Nrf 2 in the *in vitro* model. The *in vitro* experiment showed increased levels of HO-1 in microglia and astrocytes, and they later proved its involvement in neuroprotection after stroke. Other studies have also shown that they exhibit anti-inflammatory properties, reduce microglial activation, and inhibit neutrophil activation. Altogether, they found that fish oil and omega-3 enriched diets have neuroprotective effects since treated animals showed less infarct size and neurological deficit, and treated cultures showed increased cellular viability [80].

## 5. Modulation of immune response as a therapy for stroke

Immunomodulation refers to the therapeutic approach to alter or modify the immune response for the benefit of the patient. Cytokine production, e change in cellular phenotype, and the complement are manipulated to modify the milieu to which the immune system shall react [81]. Immunomodulation can also be achieved through induction of anti-inflammatory milieu (L-4, TGF-β, other cytokines) in which Tregs and microglia can be induced toward a beneficial response.

The immunomodulation area of study is being increasingly explored, having already found a great diversity in pharmacological proposals that induce this type of response, among which we can mention: *Ganoderma lucidum*, another traditional Chinese plant extract has been used too in ischemic models with neuroprotective effects. This extract has shown these beneficial effects through the decrease of TNF-$\alpha$ and IL-8 and IL-6, as well as MDA levels in the hippocampus, and increases levels of IL-2, IL-4, and IL-10, all of which reduces neuronal loss. On the other side, it has antioxidant effects through the increase of superoxide dismutase activity. Overall, it reduces neurological deficit and the infarct size [82].

Yang et al. demonstrate that by treating animals with minocycline previous to an ischemic event increases blood flow, increases tight junction protein concentration in the ischemic cortex, maintained levels of MMP 2, 9, and 3 needed for repair. It also decreased microglial/macrophage activation, compared to the non-treated group, and activation was alternate at 4 weeks, meaning that microglia/macrophage expressed phenotype M2. This supports the observed decrease in TNF-$\alpha$ and IL-1β and increase in TGF-β and IL-10. Animals treated with minocycline Chad lesser infarct sizes assessed by MRI and (2,3,5-Triphenyltetrazolium chloride) TTC staining [83].

IL-4 is a naturally occurring cytokine produced mainly by Th2 cells, mast cells, eosinophils, and basophils. It is thought to be essential for the promotion of macrophage phenotype differentiation toward an M2 response, rather than the classical M1. IL-4 production reduces over time, and this is associated with neurodegenerative diseases. Liu and his team proved the importance of IL-4 after acute ischemic stroke, since IL-4 Knockout (KO) mice exhibited greater tissue loss at day 5 and functional deficit including memory impairment and spatial

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learning decrease. Overall, they suggest that immunomodulation IL-4 plays a key role in recovery after stroke [84].

Another cytokine involved in immune modulation and anti-inflammation is INF-β. It has been already approved by the FDA for MS treatment and Kuo et al. studied it for experimental stroke, demonstrating a protective effect, since animals treated had less infarct volume and neurological scores. The authors suggest that this is mediated through the INF-β receptor, since animals lacking this receptor (Ifnar1$^{-/-}$ MCAO/R mice) showed no protective effect from the treatment. The mechanisms involved in INF-β neuroprotection are: decrease in inflammatory cytokine expression (IL-1β, IL?6, IL-23p19, and TNF-*α*), reduction in microglial activation and soma size (suggestive of resting state), decrease in macrophage/monocyte, CD4$^{+}$ and γδ T cell and neutrophil infiltration, inhibits TNF-*α* induced ICAM-1 (but not VCAM-1) and E selectin upregulation and inhibition of MMP-9, CCL3 and CXCL3 [85].

Under the bases of the new concept conceived by Dr. Michal Schwartz "protective autoimmunity," various non-encephalitogenic peptides have been tested. These have shown to potentiate neuroprotective effects of the immune system itself, such as Cop-1 is a modified neural peptide used in the treatment of multiple sclerosis that has shown beneficial effects in previous stroke models. Cop-1 competes for binding to MHCII since it has a high, fast, and efficient binding to several MHC molecules in several murine and human antigen-presenting cells without the need of being processed. It is also a MBP epitope 82-100 antagonist, which present a high cross-reaction with this molecule, thus competing with it for the MHC binding site. This copolymer helps modify the milieu since immunization with COP-1 after stroke has shown to induce a Th2 response [86]. Overall, these changes provide an anti-inflammatory milieu (cytokine production: IL-4, IL-5, IL-10, and TGF-β). Under this background effect of Cop-1 in MCAo model where rats were immunized with this neuropeptide after being subjected to the occlusion. Results analyzed 7 days post-ischemia yielded a decreased infarct size and lesser neurological deficit in animals treated with Cop-1, results consisting with neuroprotective benefits [87].

Poly-YE is a high molecular weight copolymer that has proven to exert immunomodulatory effects through the downregulation of Treg, modulation of microglial and macrophage response in the thalamus and an increase in production of insulin-like growth factor-1 (IGF-1) by Nestin$^{+}$ cells. After subjecting rats to experimental stroke, those treated with poly-YE presented diminished infarct size and neurological deficit [88].

Myelin oligodendrocyte glycoprotein (MOG) administered nasally demonstrated reduction in infarct size through the induction of IL-10-secreting CD4$^{+}$ T regulatory cells and reduction of CD11b$^{+}$ cells which contribute to the NO synthesis. Overall, infarct size and neurological deficit were reduced by the nasal MOG administration in a MCAo stroke model [69].

A different mechanism that has also been explored is ischemic tolerance; such consists in bring about a pre-conditioning of the tissue, in order to promote neuroprotection [89]. Among the activated mechanisms are an increase in anti-inflammatory cytokines such as IL-4 and IL-13 that ease hippocampal pyramidal neuron survival after an ischemic event in gerbils [90]. Tu

XK and his team also demonstrated that neuroprotection can be originated by pre-conditioning through modulation of the phosphatidyl inositol 3-kinase (PI3K/Akt) and ERK1/2 pathway modulation [91] (**Table 1**).

| **Therapy** | **Mechanism of neuroprotection**<br>**↑Increase**<br>**↓Decrease** | **Treatment outcome** | **Reference** |
|---|---|---|---|
| *Lycium barbarum* | • Inhibition of proinflammatory pathway<br>• →↓MMP-9<br>• Regulation of aquaporin-4 | Reduction in the number of apoptotic cells in the peri-infarct zone<br>Reduction of edema<br>Decreased neurological deficit | Yang et al. 2012 |
| Piperine (1-peperoylpiperidine) | • Immunomodulation: ↓COX-2, NOS-2, and NF-kB | Decrease in infarct size and neuronal loss | Vaibhav et al. 2012 |
| Simvastatin | • Immunomodulation: recruitment and modulation of macrophage, monocyte, and T lymphocyte activity<br>Antioxidation: ↑NO synthase, ↓ROS<br>• ↑Blood flow: ↑tPA →PAI-1 | Reduce changes in BBB and protect brain against cell stress<br>Reduced amounts of inflammatory proteins in the brain | Campos-Martorell et al. 2014 |
| Neuro-EPO | • Neurotrophic effects<br>• ↓Glutamate NO, use of released Iron<br>• Anti-Inflammation | Higher survival in treated animals, reduced neurological deficit.<br>Increased histological protection | Lagarto Parra et al. 2012 |
| Levodopa/ Benserazide | • Immunomodulation: ↓CD3, CD8 T cells.<br>• ↓ICAM-1. | Attenuation of inflammation, reduced number to T cells, reduced ICAM-1, and T cell-associated IL-5. | Kuric et al. 2014 |
| Fingolimod (FYT720) | • Immunomodulation: ↓lymphocyte migration through ↓S1PR<br>• ↑Blood flow: ↓adhesion molecules, ↓thrombi | Reduction of infarct size<br>Reduction of lymphocytes in cerebral vasculature→increased blood flow | Kraft et al 2012 |

| Therapy | Mechanism of neuroprotection<br>↑**Increase**<br>↓**Decrease** | Treatment outcome | Reference |
|---|---|---|---|
| Omega-3 | • Anti-inflammation: proinflammatory cytokines<br>• Antioxidation: ↑Hemeoxigenase-1, Nrf2<br>• ↓Neutrophil infiltration, microglial activation | →Reduction of infarct size<br>Reduced neurological deficit<br>Increased cell viability (*in vitro*) | Zhang et al. 2014 |
| Ganoderma lucidum | • Immunomodulation: ↓TNF-$\alpha$, IL-8, IL-6. ↑IL-2, IL-4, IL-10<br>• Antioxidation: ↑superoxide dismutase activity | Reduction of infarct size<br>Reduced neurological deficit | Zhang et al. 2014 |
| Minocycline | • ↑Blood flow<br>• Immunomodulation: M2 phenotype, maintenance of MMP's, ↓TNF-$\alpha$, IL-1$\beta$, ↑TGF-$\beta$, IL-10 | Reduction of infarct size | Yang et al. 2015 |
| IL-4 | • Immunomodulation: ↑M2 phenotype ↓M1 phenotype | Decreased tissue loss<br>Better spatial learning and memory | Zhao X, et al. (2015) |
| INF-$\beta$ | • Immunomodulation: ↓MMP-9<br>• ↓Iba1 cells, ↓ TNF-$\alpha$, IL-1b, IL-6<br>• ↓Adhesion molecules, selectin E<br>• ↓Monocyte, macrophages | Reduction of infarct size<br>Decreased neurological deficit | Kuo PC, et al. (2016) |
| Cop-1 | • Immunomodulation: ↑Th2 reg response. ↑IL-4, IL-5, IL-10, ↑M2 microglial phenotype | Reduction of infarct size<br>Decreased neurological deficit | A. Ibarra et al. 2007 |
| Poly-YE | • Immunomodulation: ↓Treg, modulation of microglial and macrophage response<br>• ↑IGF-1 | Reduction of infarct size<br>Decreased neurological deficit | Ziv et al. 2007 |

| Therapy | Mechanism of neuroprotection<br>↑Increase<br>↓Decrease | Treatment outcome | Reference |
|---|---|---|---|
| MOG | • Immunomodulation: ↑IL-10-secreting $CD4^+$ Treg<br>• ↓$CD11b^+$, ↓NO | Reduction of infarct size<br>Decreased neurological deficit | Frenkel et al. 2004 |
| IL-10 | • ↑Stem cell proliferation | Neurogenesis | Wang et al 2015 |
| Noggin | • Modify activated microglial phenotype from M1 to M2 | Neurogenesis and angiogenesis | Shin et al, 2014 |
| Antibodies | • Inhibit signaling pathways that limit axonal growth | Increased neuroplasticity and neurological recovery | Weissner et al, 2003. |
| ALA | • Anti-inflammaton: ↓: IL-1 β, TNF-α, MIP1, Iba-1<br>• ↑SOX2 | Reduction of infarct size<br>Increased neurological recovery | Choi et al, 2015 |
| Tetrmetylpyrazine | • Anti-inflammatory<br>• Antioxidant | Induce dendritic plasticity<br>Greater neurological recovery | Lin et al, 2015 |
| Ischemic pre-conditioning | • Anti-inflammation: ↑IL-4, IL-13<br>• Pathways: PI3K/Akt) and ERK1/2 | Reduction of infarct size | Schaller et al. 2003 |
| | | Increased neurological recovery<br>Pyramidal neuron survival | Tu XK et al 2015<br>Kim DW et al 2015 |

**Table 1.** Neuroprotective mechanisms exerted by diverse therapies.

## 6. Immune response as a neurorestorative mechanism

Even though neuroprotection is a targeted treatment that might be useful in a variety of ailments above mentioned, it does not restore tissue to its original anatomical state. In order to achieve "anatomical normality," alternative and promising therapies are being studied for

achieving neurorestoration through different mechanisms [71] involving the immune system, given that these new strategies have shown that immune cells are able to secrete factors that intervene in neurorestoration processes like neurogenesis.

Different studies have demonstrated that autoreactive T lymphocytes support neurogenesis in young and old animals, and are essential for memory development and spatial learning [92]. This was observed before by studies where the circulating T lymphocyte depletion drives a cognitive deficit from neurogenesis decrease [93].

Active immunization with Cop-1 has demonstrated to be able to increase trophic factor production, such as: IGF-1 in retinal ganglion cells [94] experimental autoimmune encephalitis (EAE) [95], as well as in combination neurotrophin-3 and neurotrophin-4 (NT-3 and NT-4) in EAE [96].

Both brain-derived neurotrophic factor (BDNF) and NT-3/NT-4 have been implicated in neurogenesis regulation mechanisms, differentiation, and neuron survival through its receptors Trks or p75 [97], also, BDNF has been implicated in neuroblast migration processes through the rostral migratory stream [98]. In healthy conditions, neuroblasts are conducted to the olphactory bulb where they mature and contribute to site plasticity [99], or in pathological conditions such as ischemia, they can be conducted toward periphery of the damage zone where they incorporate.

IGF also promotes neural cell proliferation by interacting with its receptor IGF-IR in the sub-ventricular zone as well as the hippocampal dentate gyrus (neurogenic niches in adults) in adult rats [100]. Furthermore, it participates in oligodendrogenesis after being stimulated by Cop-1 in a multiple sclerosis model [95].

Immunization with Cop-1 has demonstrated to induce an increment in neurogenesis and neuron survival during acute and chronic phases of an ischemic event [101]. In the same way, Poli-Y immunomodulator has also shown an increment in cortical and hippocampal neurogenesis, as well as reduction of neural loss [88].

IL-10 use has also shown to have a positive effect on neurogenesis after cerebral ischemia, in 2015 Wang J and his work team observed that Treg cells are capable of increasing stem cell proliferation in the sub ventricular zone through IL-10 production [102].

Noggin is a bone morphogenic protein (BMP) antagonist that has also been tested in a MCAo model and has had neuroprotective as well as neurorestorative results through its ability to modify activated microglial response from M1 to M2 phenotype and induce an increase in several molecule production such as: vascular endothelial growth factor IL-10, Growth Associated Protein-43 (GAP-43), and vascular endothelial growth factor (VEGF) which intervene in neurogenesis and angiogenesis [103].

Antibodies have also been used successfully to inhibit signaling pathways that limit axonal and neurite growth and remodeling, thus allowing an increment neuronal plasticity and neurological recovery in ischemic rats [104].

On the other hand, the use of cell-based therapies is being studied for their neurorestorative properties; for instance, it has been demonstrated that microglia participates in neuronal

precursor cell (NPC) migration and differentiation [105], as well as in neurogenesis, synaptogenesis, and tissue remodeling increase through the release of IGF-1 and neurotrophic growth factor (NGF), among others, in animals subjected to experimental stroke [106].

Other animal models, such as traumatic brain injury (TBI), have had success in the use of combined therapies composed of stem cell co-transplants and pharmacological a or immunomodulatory agents that modify neural tissue milieu in order to favor recovery and restoration. For example, the use of granulocyte-colony stimulating factor (GCSF) and human umbilical cord blood cell (hUCB) transplantation has demonstrated to reduce proinflammatory cytokine expression, increases trophic factor production, and promotes synaptic circuit reestablishment. For this reasons, it has been proposed as a therapy for stroke models [107].

Another mechanism through which brain tissue restoration is pursued is neuroplasticity or synapse plasticity, which is an inherent neurophysiological adaptive trait in which preexisting connections between two neurons can gain or lose strength during neural activity [108], as well as change in structure, function and organization [109]. It responds to different experiences and emphaticism and has been observed in different sections of the CNS [109].

Treatment with tetramethylpyrazine, which has anti-inflammatory and antioxidant effects, has shown to be able to induce dendritic plasticity, observing maintenance of neuroarchitecture through microtubule-associated protein 2 (MAP-2), which has been observed in greater density in peri-infarct zone found dendrites, causing a greater neurological recovery in rats with cerebral ischemia [110].

Alpha-lipoic acid (aLA) has yielded very good results in preclinical investigation since it has shown that its anti-inflammatory capacity through a decrease in proinflammatory cytokine expression such as: IL-1$\beta$, TNF-$\alpha$, MIP1, Iba-1, and the increase in expression of transcription factor SOX2, which is essential for maintenance of auto regeneration properties, as well as an increase in neuron precursor cell proliferation accompanied by a significant reduction in infarct volume and better functional recovery. For all these reasons, aLA is a great candidate to start clinical trials as neurorestorative of brain tissue [111].

## 7. Advances in the clinical field

Even though preclinical trials have yielded promising results, translation into clinical human stroke trials has been unsuccessful. Clinical trials have been conducted very scarcely and have shown very little results [72, 112]. Some agents have been used in the clinical setting after having been observed beneficial in animal models. By 2008, Ginsberg had reported the existence of 160 clinical trials for neuroprotection after stroke and one-third of the by-then-finished 120 trials included more than 200 subjects; nonetheless, most of them failed to prove any benefit [70]. As of March 2016, a search in "www.strokecenter.org" for clinical trials involving neuroprotection yields 25 results of which 12 involve neuroprotection for acute ischemic stroke and only one of them is already in already phase 4. A different search in involving the word "immune" yielded another 25 matches, of which only two are related to immunomodulation in stroke.

One of them, Nasal Selectin E administration is being studied by Hellenbeck, M.D. at the National Institute of Neurological Disorders and Stroke (NINDS) in patients that have suffered from stroke, seeking induction of mucosal tolerance to this adhesion molecule through low-dose nasal administration, in order to promote a response shift from Th1 toward Th2 or Th reg at inflammation sites. This trial is currently at phase I and has not yet published results [113].

Fingolimod, which has been mentioned earlier, is also being used in an ongoing clinical trial with the goal of analyzing neurofunctional effects in stroke patients at different time points after being orally administered. The secondary purpose is to identify if there are any cellular and structural brain modifications through the use of flow cytometry and MRI [114].

In accordance, a pilot trial was conducted combining the use of rtPA and Fingolimod in a randomized multicenter pilot trial that included 47 patients in China. Treatment was provided within 3 and 3.1 h from symptom onset. Whole blood was used to assess lymphocyte and mononuclear cells at day 1, 7, and 90. After day 1, $CD4^+$ T cells, $CD8^+$ T cells, $CD19B^+$ cells, and NK cells had significant decreases in the fingolimod + alteplase group, as opposed to the alteplase only group. At day 7, this trend continued and normalized by day 90th. Other results included lesser infarct volume expansion, smaller hemorrhage, and greater functional recovery in the short and long term in the combined treatment group. Safety was assured during this trial, and further investigation needs to be considered [115].

On the other hand, stem cell therapies are also under clinical scrutiny, their use has proven to be feasible, but not necessarily practical, and it is safe. Most clinical trials have proven that stem cell therapy improves functional recovery but other factors have to be taken into account too, such as cost-effectiveness, comparison to other stroke treatments, time, and type of stroke. According to Young, there are currently nine ongoing stem cell clinical trials for stroke, testing safety, and efficacy as well as most accurate patient selection [116].

Knowledge about the molecular dynamics of cerebral ischemia pathophysiology and the study of neuroprotective mechanisms has promoted the use of combined therapies [89].

The use of combined therapies has also been tried in the clinical field in different diseases. For example, two quadriplegic patients were transplanted with differentiated neural stem cells (NSC) and autoreactive autologous T lymphocytes. These patients regained motor and sensitive functions without adverse effects [117], all the more reason to try these therapies in stroke.

Some stem cell trials have shown to have some beneficial effects on stroke patients, such as the use of human placenta derived adherent (PDA001) cells, isolated from *postpartum* placenta, and were studied for their neurorestorative effects after stroke. Animals were injected with these cells 4 h after being subjected to stroke. Results show an increase in functional recovery 7- and 14 days post-ischemia, as well as an increase level of BDNF, vascular endothelial growth factor (VEGF) which is an angiogenic factor, increases axonal outgrowth, stop apoptosis and increases neurogenesis, and hepatocyte growth factor (HGF) which is also angiogenic, and decreased TUNEL and cleaved caspase 3, showing a decreased infarct volume. Although not many cells survived until day 14, beneficial effects were still observed [118] (**Table 2**).

| Treatment | Outcome | Reference |
|---|---|---|
| Nasal selectin E | Promote a response shift from Th1→Th2 or Th reg<br>Ongoing | [113] |
| Fingolimod | No outcome, still ongoing | [114] |
| rtPA + Fingolimod | Reduced infarct volume<br>Greater neurological recovery | [115] |
| NSC + Autoreactive Autologous T cells | Recovery of motor and sensory functions<br>No adverse effects | [117] |
| PDA001 | Increased functional recovery | [118] |

**Table 2.** Results of some clinical trials.

Most preclinical investigations focus on delivering treatment in the first hour after reperfusion and happen in strictly controlled environments, which is why they have shown beneficial effects. Lack of results in clinical trials is attributed to uncontrolled real life settings, different populations, comorbidity existence, different ischemic territories, duration of occlusion before reperfusion, and a single target for treatment, leaving behind other neural components that might aid recovery. Also, patients are selected after arrival to hospitals and thus, other environmental variables and time window are not accounted for in results [72, 112].

The study and application of new therapies that will aid the ischemic patient recover more effectively needs to continue to be worked on in the basic and preclinical fields, specially through the exploration of immune system characteristics that might be beneficial for stroke therapy and thus achieve a decrease in mortality and an increase in functional recovery after hemiplegia (one-sided paralysis) hemi-hypoesthesia (one-sided decrease in sensory perception) hemianopsia (one eyed decreased vision), paresia (partial paralysis), aphasia (inability to comprehend language), and memory alterations; favorably increases stroke patients quality of life.

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