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Review

Glial-Neuronal Interactions in Pathogenesis and Treatment of Spinal Cord Injury

Nadezda Lukacova [1]*, Alexandra Kisucka [1], Katarina Kiss Bimbova [1], Maria Bacova [1], Maria Ileninova [1], Tomas Kuruc [1], Jan Galik [1]

[1] Institute of Neurobiology of Biomedical Research Centre of Slovak Academy of Sciences, Soltesovej 4,6, 040 01 Kosice, Slovak Republic; kisucka@saske.sk (A.K.); bimbova@saske.sk (K.K.B.); bacova@saske.sk (M.B.); ileninova@saske.sk (M.I.); kuruc@saske.sk (T.K.) galik@saske.sk (J.G.)

* Correspondence: lukacova@saske.sk

Abstract: Traumatic spinal cord injury (SCI) elicits an acute inflammatory response which comprises numerous cell populations. It is driven by the immediate response of macrophages and reactive M1 microglia, which triggers activation of genes responsible for the dysregulated microenvironment within the lesion site and in the spinal cord parenchyma immediately adjacent to the lesion. Recently published data indicate that microglia induces astrocyte activation and determines the fate of astrocytes. Conversely, astrocytes have the potency to trigger microglial activation and control their cellular functions. Here we review current information about the release of diverse signaling molecules (pro-inflammatory *vs* anti-inflammatory) in individual cell phenotypes (microglia, astrocytes, blood inflammatory cells) in acute and subacute SCI stages, and how they contribute to delayed neuronal death in a the surrounding spinal cord tissue which is spared and functional but reactive. In addition, temporal correlation in progressive degeneration of neurons and astrocytes and their functional interactions after SCI are discussed. Finally, the review highlight the time-dependent transformation of reactive microglia (M1) and astrocytes (A1) into their neuroprotective phenotypes (M2a, M2c and A2) which are crucial for spontaneous post-SCI locomotor recovery. We also provide suggestions on how to increase functional outcome after SCI and discuss key therapeutic approaches.

Keywords: microglia and astrocytes phenotypes; intercellular crosstalk; lesion microenvironment; neuroinflammation; *in vivo* glia-to neuron reprogramming; subpial delivery; gut dysbiosis; electrostimulation; rehabilitation; neuroprotective strategies

1. Introduction

Spinal cord injury (SCI) is one of the most devastating events leading to serious neurological deficits. The complex pathophysiology of SCI, consisting of primary and secondary mechanisms, may explain the difficulty in finding a suitable therapy [1]. Traumatic SCI is caused by several distinct events, which follow a somewhat overlapping temporal sequence: the acute phase (seconds to minutes after injury), the secondary phase (minutes to weeks after injury), and the chronic phase (months to years after the injury) [2]. After direct mechanical insult, the spinal tissue undergoes a cascade of cellular and molecular events which exacerbate the primary lesion. Secondary injury includes disruption of the vasculature and increased blood-spinal cord permeability, ischemia, local edema, ionic imbalance, inflammation, cell death (necrosis and apoptosis), and activation of inhibitory molecules followed by demyelination and axonal degeneration [3-4]. While neurons and glia die at the lesion site (approximatelly 2-4 mm) within minutes and hours after SCI, cell types, including neurons, astrocytes, microglia and oligodendrocytes surrounding the lesion site are lost in a delayed manner [5-6].

Inflammatory response is one of the key mechanisms of secondary injury. It includes activation of resident cells (microglia, astrocytes) and recruitment of immune cells

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(macrophages and neutrophils) from the bloodstream to the injury site. Resident and immune cells release proinflammatory cytokines, including interleukins (IL1 , IL-6) and tumor necrosis factor-α (TNF-α), all of which increase the extent of the inflammatory response. These events play an important role in secondary tissue damage and cell death. After SCI, many factors with antagonistic pro- and anti-inflammatory properties act simultaneously on macrophages and microglia. Astrocytes begin migrating out of the epicenter, producing molecules, such as proteoglycans and laminin in the extracellular space. Reactive astrocytes invade the region surrounding the lesion center and lead to glial scar formation. While the glial scar limits regeneration and acts pathologically as a physical and biochemical barrier to axonal regeneration [7-8], the transformation of reactive astrocytes into their polarization states in the subacute phase serves some neuroprotective function [9].

To develop appropriately targeted repair strategies, there is a need for detailed understanding of how various cell populations interact with each other within the lesion site and in the surrounding spinal cord tissue in both acute and subacute phases of SCI. This review focuses on microglia-astrocyte crosstalk in an acute inflammatory response which comprises numerous cell populations. We pay special attention to activation of M2a, M2c and A1 phenotypes in the subacute phase after SCI and highlight the neuroprotective effects of microglial and astroglial polarization on the functional outcome. Some other promising strategies for spinal cord repair, such as anti-inflammatory treatment *via* effects on M1/M2 macrophages after atorvastatin treatment, neuronal reprogramming from local glial cells, epidural oscillating field stimulation, and probiotic treatment of SCI-induced gut dysbiosis, all involved in early treatment after SCI, will be discussed separately.

2. Neutrophils and monocytes respond early after SCI

The response to spinal cord trauma is mediated by multiple coordinated molecular pathways, which are activated soon after SCI within the lesion site and spread throughout the spinal cord in spatio-temporal manner. After SCI, neutrophils infiltrate the epicenter of injury and produce neurotoxic effects by promoting the expression of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2), and releasing pro-inflammatory cytokines [10-11]. Since neurons and glia synthesize pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF)-α and interleukin 1β (IL-1β), as part of normal intercellular communication [12], their release after SCI evokes inflammation and causes dysregulation of cytokine release leading to the death of neurons and oligodendrocytes [13]. Recently, a ~12-fold increase in IL-1β level has been reported 4 h after Th9 compression [14]. The level of this cytokine rapidly decreased one day after SCI, however, apoptosis appeared in cell populations all around the lesion site (5 mm from the lesion epicenter). The authors detected caspase-3 expression in neurons, astrocytes and oligodendrocytes, but not in microglial cells.

Blood-derived monocytes are also massively recruited at the lesion site, and they play a dual role, i.e. they remove cell debris and repair injured spinal cord tissue [15] as well as producing neurotoxic factors [16]. After SCI, monocytes differentiate into macrophages and adopt many of the markers and behaviors of microglia. Since the similarities between monocyte-derived macrophages (MDMs) and microglia have complicated the development of efficient prediction tools to discriminate between them, they are sometimes still referred to as microglia/macrophages [17]. A very recent study by Kisucká et al. [9] demonstrated that microglial/macrophage marker Cd11b was markedly expressed at the lesion site (3 mm) and in adjacent spinal cord tissue (3 mm cranially and caudally) one week after Th9 compression.

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3. Microglia rapidly accumulate around the lesion site and influences neurons and astrocytes in the subacute phase after SCI

Traumatic SCI results in a dysregulated microenvironment which is largely driven by the immediate and robust response of resident astrocytes and microglia [18] leading to neuronal death. Min et al. [6] studied the discrete roles of microenvironment- regulating cells after Th9 contusion at the lesion site (approximately 2 mm), where neurons acutely died immediately post-SCI, and in two penumbra regions (P1 and P2). In area P1, immediatelly surrounding the lesion, the neurons underwent death between 12 h and 1d, while in area P2, neurons remained healthy for up to two weeks. The authors found that ramified Iba-1+ cells (resident microglia) died earlier than neurons in area P1 where delayed neuronal death occurred, and that neurons remained healthy in region P2, where microglia were morphologically activated. These findings did not confirm the evident connection between activation of microglia and delayed neural death. Round Iba-1+ cells with strong expression of CD45 (identified as glia and/or infiltrated blood cells) appeared after neurons had died, and expressed phagocytic activity. The authors suggest that Iba-1+ cells, including ramified and round cells, are innocent in delayed neuronal death, and they speculate that loss of the supportive function of astrocytes may contribute to delayed neuronal death. Bellver-Landente et al. [17] studied the response of microglia in a mouse model of SCI (Th10-Th11 contusion) and found extensive microglial proliferation during the first week post-SCI. The authors discovered that microglia formed a dense cellular interface at the border of the lesion between reactive astrocytes and infiltrating MDMs, refered to as the "microglial scar". Depletion of microglia after SCI using PLX5622 (CSF1R inhibitor which crosses the blood–spinal cord barrier) reduced the number of neurons and oligodendrocytes at the injury site, disrupted the organization of the astrocytic scar and impaired functional outcome. Accordingly, the central nervous system (CNS) delivery of microglial proliferation factor M-CSF at the site of contusion boosted microglial proliferation and enhanced locomotor recovery. Similarly, the use of a lentivirus-mediated herpes simplex thymidine kinase/ganciclovir (HSV1tk/GGV) system in which suicide gene expression was regulated by hGFAP protor to selectively ablate reactive proliferating astrocytes in a mouse crush injury model showed impeded glial scar formation, exacerbated neuroinflammation, increased loss of neurons and failure of spontaneous functional recovery [19] .

All these data indicate that proliferating microglia are a key cellular component of the microglial scar which develops during the first week post-SCI to protect neural tissue. In the light of our recently published data, the first week post-injury is critical for modulation of reactive microglia/astrocytes into their neuroprotective phenotypes [9]. The gene expression of microglia/macrophages and M1 microglia was strongly upregulated at the lesion site (3 mm area) and caudally (3 mm) one week after Th9 compression, but attenuated afterwards. The common astrocytes (GFAP and S100B) and reactive A1 astrocytes were profoundly expressed predominantly at the lesion site and cranially (3 mm area) two weeks post-SCI. However, gene expression of anti-inflammatory M2a microglia (playing a role in cell repair and regeneration) and M2c microglia (involved in phagocytic activity and wound healing), as well as A2 astrocytes which are responsible for up-regulation of neurotrophic factors was greatly activated at the lesion site one week post-SCI [9].

4. Microglial and astrocyte polarization and their interactions

Under physiological conditions, astrocytes mediate CNS homeostasis and provide trophic and metabolic support for neurons regulating synaptic signaling or plasticity [20-21], and they control the cerebrovascular tone and modulate local blood flow [22-23]. Recently, Escartin et al. [24] defined reactive astrocyte nomenclature and pointed out that astrocyte phenotypes should be specified by a combination of molecular markers (not only by GFAP alone) and by functional readouts, predominantly in *in vivo* conditions. It is also very well established that astrocytes in the white and gray matter are morphologically distinct. White matter astrocytes are necessary for secure salutary conduction,

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while in the gray matter they are regionally specialized, reflecting for instance the specific neurotransmitter system of that area [25]. Accumulating evidence suggests that GFAP is predominantly expressed in white-matter astrocytes while S100β tends to be expressed in the astrocytes located in the gray matter [26-27].

In response to SCI, astrocytes undergo multiple morphological and functional changes in the process of reactive astrogliosis [28]. They migrate towards the lesion site within the first hours after injury [29-30] and proliferate for 24 hours with a peak after 48 hours [31]. This initial response is necessary to reestablish the blood-brain barrier and restrict further migration or proliferation which causes the lesion site to expand into surrounding healthy tissue [32-35]. Subsequently, astrocytes rapidly increase the expression of intermediate filaments of glial fibrillary acidic protein (GFAP), vimentin [34,36] and other astrocyte-specific markers [9,24], and they release molecules limiting spontaneous axon sprouting and inhibit regeneration [7,37-40]. In the acute phase of SCI, naïve astrocytes became reactive and after further proliferation they invade the region surrounding the lesion center, leading to the formation of cystic cavities surrounded by glial scar [41]. Astrocytic scar in the chronic stages of SCI, as the final form of reactive astrogliosis is widely regarded as a principal cause of axonal re-growth failure and poor functional outcome [42].

For a long time it was not clear whether reactive astrocytes were harmful or beneficial [43]. As mentioned above, multiple activated states of microglia were identified after SCI with more pro-inflammatory and detrimental effects attributed to the M1 phenotype, while more regulatory and protective actions were attributed to M2 phenotypes [9,44]. Liddelow and Barres, [45] and Liddelow et al. [46] previously reported that activation of microglia by classical inflammatory mediators can convert astrocytes into a neurotoxic A1 phenotype in a variety of neurological diseases. These findings pointed to the key role of molecules secreted by activated microglia in the induction of reactive astrocytosis. The application of suitable anti-inflammatory drugs inhibiting the formation of A1 astrocytes induced by activated neuroinflammatory microglia, could be used as a potential therapeutic agent for the injured spinal cord. Similarly, increased presence of M2 microglial phenotypes at the lesion site might represent a promising strategy for tissue regeneration after SCI [47]. At present, the microglia-astrocyte conversation which ensures their tight reciprocal modulation after SCI is an undeniable fact, and as our recently-published experimental data show, time-dependent regulation of M1/M2 polarization (the expression of M2a, M2c markers) and A1/A2 polarization at the lesion site and 3 mm cranially and/or caudally from the injury epicenter is key for functional outcome after SCI [9].

5. In vivo conversion of astrocytes to neurons

The spatial and temporal patterns of astrocyte and neuron death are similar one week post-SCI [6]. Since it is known that the spinal cord lacks the ability to produce new neurons in adulthood, neurons dying at the lesion site cannot be replaced. In recent years, growing attention has been focused on *in vivo* glia-to-neuron reprogramming [48-50]. It has been established that astrocytes are particularly promising candidates for reprogramming into neurons, as they maintain some of the original patterning information from their radial glial ancestors [49,51]. Using exactly defined transcription factors in vitro [52-53], astrocytes have been successfully reprogrammed into different types of functional mature neurons. Su et al. [48] examined the possibility of reprogramming endogenous non-neural cells, such as scar-forming astrocytes into neurons in the adult mouse spinal cord. They indicated that a high-mobility group of DNA-binding domain transcription factor, SOX2, known to be essential for specification and/or maintemance of progenitor identity [54-55] uniquely converted resident astrocytes into DCX+ neuroblasts and MAP2+ mature neurons. Their data suggest that in the adult spinal cord, a threshold of SOX2 expression is required to induce cell fate change. When mice after Th8 hemisection were injected with hGFAP-GFP-T2A-SOX2 lentivirus, all the induced DCX+ cells also expressed GFP, indicating an origin from virus-transduced cells. Approximately 3–6% of GFP+ cells

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surrounding the core viral injection sites were reprogrammed by SOX2 to become DCX+ cells between 4–8 wpi. DCX+ cells were also positive for neuronal marker TUBB3. These data indicate that neurogenesis can be induced by SOX2 in an injured environment of the adult spinal cord. These results also show that SOX2-induced adult neurogenesis can generate mature neurons with features of GABAergic interneurons in injured VPA-treated spinal cords. Although the number of converted neurons was low, the authors found that new neurons were capable of forming synapses with preexisting ChAT+ motor neurons, suggesting potential integration into the local neural network of the injured spinal cord. Recently published data have shown that spinal cord - derived adult astrocytes express a high level of NOTCHI1 signaling which is responsible for neuronal stem cell maintenance and neurogenesis in the embryonic as well as the adult brain [56], and they are not susceptible to neuronal reprogramming [50]. These findings indicate that further in vivo studies are necessary to enhance the reprogramming process and to obtain neurons with appropriate subtype identities and projections which are required for functional recovery after SCI.

6. Efficacy of parenchymal, intrathecal and subpial application in experimental neurobiology

Over the past decade, a direct parenchymal injection of viral vectors (lentivirus, retrovirus, adeno-associated virus) has become frequently used for *in vivo* reprogramming, achieving a broad range of reprogramming efficacy and neuronal survival [49,57-58]. Although effective in delivering these viruses into the spinal parenchyma, the invasive nature of this approach limits the number of injections that can be performed. Similarly, direct parenchymal injection and/or intrathecal delivery of Rho-kinase inhibitors are the most frequently used techniques for promoting post-SCI neuritogenesis [59]. Currently, these delivery techniques are also used for transplantation of grafted fetal or iPSC-derived neural stem cells (NSCs) after SCI [60-61]. Although transplantation-based cell therapies face several major hurdles for treatening SCI in human patients (such as the use of a therapeutically optimal time window, potential risk for tumor formation, large quantity of cells used for transplantation, and direct parenchymal injections of cells), some of them have recently reached the clinical trial stage with several phase I or phase II trials underway.

Miyanohara et al. [62] studied the penetration of AAV9 virus within the spinal cord after intrathecal delivery, and found a lack of transduction in deeper gray matter cells, playing a key role in spinally-mediated motor trafficking. To achieve more effective transgene penetration, Marsala´s lab described a novel subpial delivery technique which allows multisegmental transgene expression in adult pigs, rats and mice [62-63]. This delivery technique permits widespread transgene expression within the spinal parenchyma and does not require direct spinal cord tissue needle penetration. This novel subpial delivery technique can potentially be used in pre-clinical and human clinical studies to regulate genes of interest in specific spinal cord segments and/or in the projection of motor and ascending sensory axons. This novel approach is extremely effective in achieving trans-spinal occupation by grafted cells, particularly in the treatment of spinal cord injury characterized by multisegmental degeneration [64-66].

7. Neuroprotection of microglial phenotypes after SCI

There is growing evidence that mechanisms responsible for the neuroprotective functions of activated microglia include several functional behaviours [67]. One of the most important ways in which microglia could contribute to neuroprotection is synaptic stripping, a process in which microglia selectively remove inhibitory synapses from injured neuronal perikarya [68]. This intimate interaction between microglia and synapses is associated with motoneuron regeneration [69], promotion of neuronal survival [70] and reduction of neuronal cell death [71]. It is also well known that microglia actively promote neurogenesis following CNS injury through producing insulin-like growth factor-1,

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which suppresses apoptosis and increases proliferation and differentiation of neural stem cells [72]. Activated microglia may also boost neurogenesis by means of an unconventional mechanism through provoking non-committed oligodendrocyte progenitor cells to adopt a neuronal phenotype [73]. Another essential mechanism of neuroprotective microglial function is microglial phagocytosis, a process necessary for maintaining CNS homeostasis [74-76]. Moreover, microglia can suppress neuroinflammation, restore homeostasis, and protect nerve tissues by producing anti-inflammatory cytokines and cytoactive factors for repairing tissue [77].

Currently, there is direct evidence, that the neuroprotective environment after SCI is associated with the alternatively-activated, proliferating phenotypes of M2 microglia. These microglial phenotypes play an important role in the healing process by sustaining homeostasis and dampening inflammation, resulting in the release of neurotrophic factors and anti-inflammatory cytokines to promote tissue sparing and functional recovery after SCI, and this effect persists for five weeks after SCI [17]. Microglial M2 phenotypes can be categorized into M2a, M2b, M2c and M2d subtypes. M2a, b and c phenotypes are considered as anti-inflammatory repair microglial cells, and they can be distinguished by observing the changes in expression of the relevant markers. The M2a subtype is responsible for tissue repair and regeneration by expressing anti-inflammatory and immuno-regulatory molecules. This phenotype is activated by interleukin-4 (Il-4) and interleukin-13 (Il-13), which inhibit the production of pro-inflammatory molecules after SCI, resulting in the upregulation of arginase-1 and CD206 [78-79].

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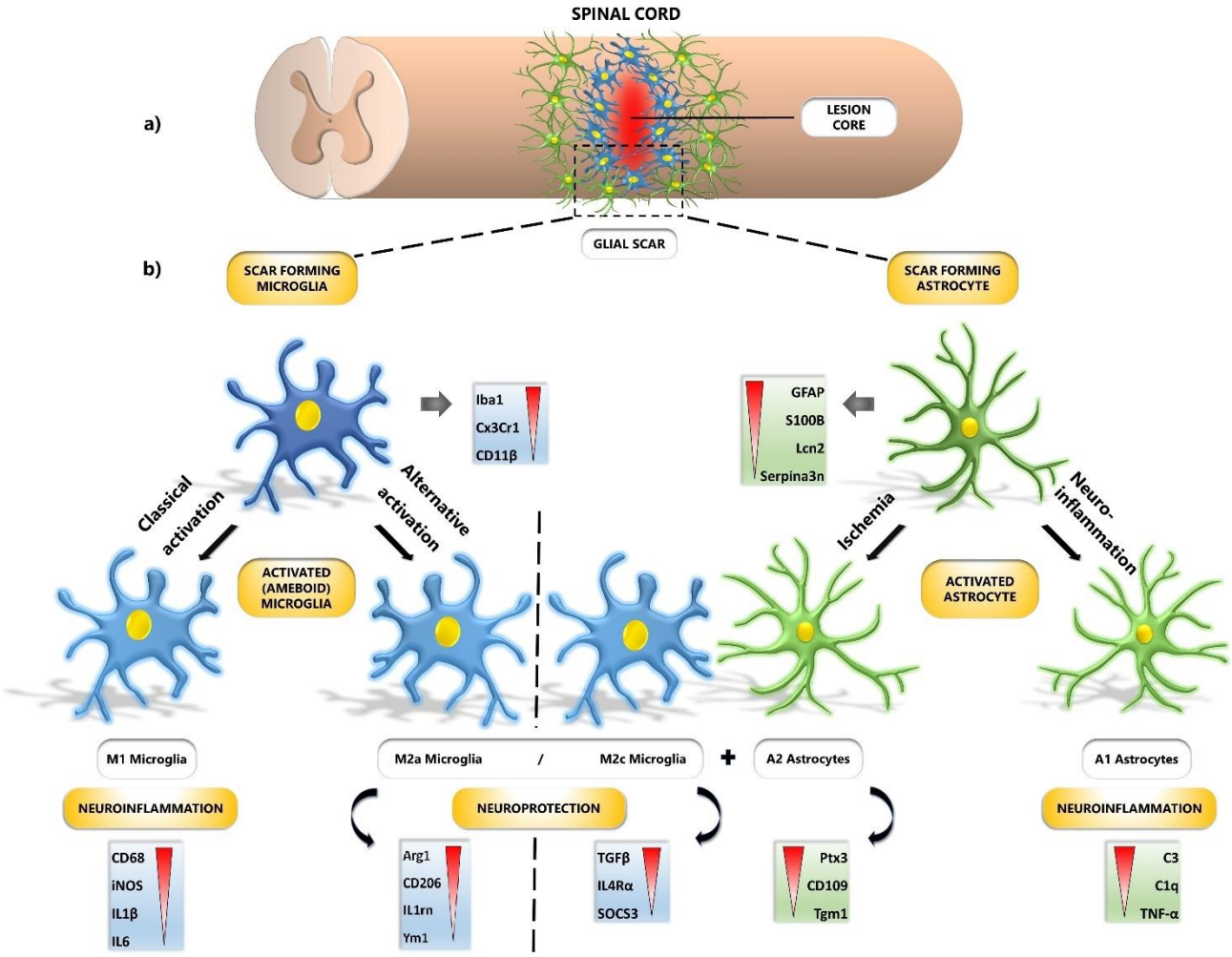

Figure 1. Formation of glial scar after SCI. a) Spinal cord with illustrated lesion core surrounded by microglia and astrocytes, forming a glial scar. **b)** Focusing on specific types of glial cells: microglia and astrocytes with their corresponding genes ranked according to expression level. Resting microglia and astrocytes acquire scar-forming phenotypes through their activation under certain conditions; they differentiate into several subtypes involved either in neuroinflammation (M1, A1) or neuroprotection (M2a, M2c, A2). The M1 phenotype of microglia is acquired by classical activation, whereas M2a and M2c phenotypes are acquired by alternative activation pathways. Astrocytes, which are differentiated into A2 phenotype under ischemic conditions, promote neuronal survival and tissue repair. A1 phenotype is acquired *via* secretion of neuroinflammatory markers. Iba1 - ionized calcium-binding adaptor molecule 1; Cx3Cr1—fractalkine receptor; Cd11β—beta-integrin marker of microglia; GFAP—Glial fibrillary acidic protein; S100B—Calcium-binding protein B; Lcn2—Lipocalin 2; Serpina3n—Serine (or cysteine) peptidase inhibitor; CD68—cluster of differentiation 68; iNOS—inducible nitric oxide synthase; IL-1β—interleukin-1β; IL-6—interleukin-6; Arg-1—arginase-1; CD206—mannose receptor and C-type lectin; IL1rn—interleukin 1 receptor antagonist; Ym1—chitinase-like protein-1; TGF-β—transforming growth factor beta; IL4Rα—interleukin 4 receptor alpha; SOCS3—suppressor of cytokine signaling 3; Ptx3—Pentraxin 3; CD109—cluster of differentiation 109; Tgm1—Transglutaminase 1; C3—Complement 3; C1q—Complement component 1q; TNF-α—tumor necrosis factor-alpha; SCI—spinal cord injury.

M2b and M2c microglia are largely phagocytic. M2b microglia involves T-cells recruitment and are activated by Toll-like receptors (TLRs), playing a key role in the innate immune system and immune complexes, resulting in the expression of high levels of anti-inflammatory cytokines (Il-1 and Il-10) and low levels of Il-12. M2c subtype is also involved in inflammation dampening and healing and is activated by a potent anti-inflammatory cytokine Il-10 and glucocorticoids, resulting in high transforming growth factor beta (TGF-β) expression [45,80]. M2d microglia, unlike the above mentioned subsets of alternatively-activated microglia are induced by "switching" from a classically-activated

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inflammatory phenotype to an alternatively-activated anti-inflammatory/pro-angiogenic phenotype. The M2d subtype originates from the M1 pro-inflammatory phenotype through the activation of adenosine A2A receptors [81].

Spinal cord lesions produce an inhibitory microenvironment which is not in favour of the M2 phenotypes, so the M1 phenotype dominates [82]. Recent evidence suggests that gene expression of anti-inflammatory M2a microglia (CD206, CHICHI, Il1rn, Arg-1), M2c microglia (TGF-β, SOCS3, IL4R α) and A2 astrocytes (Tgm1, Ptx3, CD109) is significantly overactivated at the lesion site one week after SCI [9]. The authors also found positive correlation between neurological outcome and the expression of neuroprotective microglia and astrocytes phenotypes. These results provided evidence for the first time that modulation of reactive microglia/astrocytes into their neuroprotective phenotypes contributes to spontaneous locomotor recovery after SCI. Molecular changes leading to functional remodeling could be identified by the use of a set of microglia and astrocyte-specific markers (Fig. 1).

8. Early modulation of inflammatory response after SCI

The timing of the modulation of inflammatory response after SCI has been of great interest to many researchers over the last few years. Despite advancements in understanding of the pathophysiological mechanisms of secondary inflammation in the spinal cord, treatment options have remained limited in this area. The rationale for modulation of the inflammatory response includes the potential for decreasing the massive spread of the injury which occurs after this traumatic event.

Several recent studies have pointed out the importance of early post-SCI alleviation of the inflammatory response. Zhao et al. [83] found that XIST- (a cancer-related gene which participates in the development of SCI) was upregulated after spinal trauma in rats (*in vivo*) and LPS-activated microglia (*in vitro*). Knockdown of XIST with lentivirus vectors containing sh-XIST immediately after SCI suppressed cell apoptosis and inflammatory response probably through sponging of miR-27a and downregulating Smurf1 in vivo and *in vitro*. Papa et al. [84] demonstrated that inhibitory treatment of microglia with minocycline-loaded nanoparticles, applied immediately after SCI, induced a major long-lasting effect up to 63 days post injury, confirming the relevant pro-inflammatory effect of activated microglial cells in the earliest stages of degeneration after spinal trauma. It is well known that IL-1β is a main pro-inflammatory cytokine in the spinal cord, producing a harmful microenviroment in injured tissue and amplifying the extent of the injury. An antagonist to IL-1β receptor has also been shown to alleviate the actions of IL-1β by decreasing the severity of neuronal damage, reducing cell death and improving motor function [85]. Our study also pointed out the importance of early modulation of the inflammatory response after traumatic SCI. A single dose of 3-hydroxyl-3-methylglutaryl-coenzyme A reductase (HMG-CoA) inhibitor- Atorvastatin (ATR, 5mg/kg, i.p.) applied immediately after spinal trauma significantly reduced IL-1β levels (almost to control level), decreased microglial activation in the dorsolateral area, inhibited macrophage infiltration into the white and gray matter and significantly decreased the expression of apoptotic markers 24 hours after Th9 compression [14]. The therapeutic benefit of ATR has been presented in several other studies addressing SCI. These studies monitored long-term administration *vs* single dose of ATR *via* per-oral and intraperitoneal application [86-92]. However, the most effective method for ATR administration proved to be early, intraperitoneal injection.

As mentioned above, macrophages/microglia may initiate pathological secondary mechanisms, and on the other hand they can promote regeneration of traumatized spinal cord based on their phenotype (destructive M1 vs beneficial M2 status) [93]. One day after SCI we observed significant increase in gene expression of both phenotypes, however the expression of M1 prevailed over the M2 phenotype. ATR significantly reduced both M1 and M2 phenotypes at the epicenter of injury and in the adjacent cranial segment. Since ATR modulated the M1 phenotype more markedly than the M2 antigenic marker, we

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assume that the neuroprotective effect of ATR could lie in their polarization. In addition, marked activity of a pro-apoptotic protein- caspase-3 was noticed throughout the whole injured area in neurons and glial cells (astrocytes and oligodendrocytes). Atorvastatin treatment visibly reduced the cleavage of caspase-3 and acted as a neuroprotective agent in neuronal and glial cells [14]. Sohn et al. [94] showed that another HMG-CoA- inhibitor called simvastatin effectively decreased cytotoxicity and spinal cord neuronal death due to ischemia–reperfusion injury, probably via moderation of oxidative stress. In this study, simvastatin was applied from the beginning of oxygen and glucose deprivation *in vitro* and was maintained during the following 24-h reoxygenation period. Liang et al. [95] used simvastatin (10mg/kg) in combination with ezetimibe (cholesterol-reducing drug) in three doses during the first 72 hours after weight- drop spinal cord injury. They pointed out that the combination of these agents could improve the neurological score and attenuate the endothelial inflammatory response after SCI in rats.

Essentially, the acute inflammatory response following spinal trauma is a crucial element for amplification, spreading and chronicity of the injury [96]. As mentioned above, immediate modulation of the inflammatory response could be an important step towards more successful treatment of traumatic SCI.

9. Spinal cord injury and gut microbiota

Novel data show that SCI sets in a motion a systematic breakdown of communication between the nervous system, immune system and gastrointestinal system [97]. When the spinal cord is injured, axons which normally descend from brain/brainstem regions to control spinal sympathetic neurons are lost or damaged. The subsequent loss of normal sympathetic tone throughout the body leads to chronic immune dysfunction and gut dysbiosis which can contribute to the development of intraspinal and systemic pathology [97-103].

Changes in gut permeability induced by trauma can liberate commensal bacteria from the gut lumen, allowing microbes and their metabolites to enter the circulation and trigger inflammation throughout the body [101]. Various genes encoding transcription factors or epithelial tight-junction proteins which regulate paracellular permeability or the proliferation and differentiation of epithelia are dramatically affected by SCI (e.g. Tcf712, Cdx1, Cdx2, Jam2, etc.). In the neurogenic bowel, impaired intestinal transit limits the delivery of important nutrients to the microbiota in the distal colon, and altered mucin production impairs production of the mucus layer which is colonized by gut microorganisms creating a biofilm [97]. Changes in relative abundance of certain gut bacteria induced by SCI correlate with locomotor and immune functions as well [101]. In mice with SCI-induced dysbiosis, exacerbated lesion pathology and intraspinal inflammation (enhanced CD11b+ CNS macrophage response at the lesion epicenter and total number of infiltrating CD3+ T cells and CD45R+ B cells) has been observed. Similarly, changes in GALT (gut-associated lymphoid tissue)- immune cell composition (e.g. B220+ cells, CD8+ T cells, CD11c+ cells, CD11b+ macrophages found in mesenteric lymph nodes by 3dpi) occured in parallel with increased expression levels of pro- and anti-inflammatory cytokines (TNF-α, IL-1β, TGF-β, IL-10) seven days post-injury. O'Connor et al. [102] found significantly elevated pro-inflammatory cytokines (IL-12, MIP-2, TNF-α) in the rat intestine four weeks post-SCI. They also found a correlation between cytokine levels (IL-1β, IL-12, MIP-2) and differences in gut microbiota diversity at eight week post-SCI.

One of the therapeutic tools for managing SCI-induced inflammatory events in the gut could be the application of health-promoting probiotic bacteria with the potential to modulate the gut microflora and thus contribute to restoration of intestinal immune homeostasis. In generally, recognition of probiotic bacteria via TLRs in intestinal dendritic cells leads to their maturation and to release of cytokines, which coordinate the differentiation of naive T-helper cells (Th0) into mature Th1, Th2 or Th3/Treg subpopulations [104-105]. Probiotic bacteria do not cause inflammation, because they can regulate the immune response *via* a complex of mechanisms including reduction of some TLRs, inhibition of

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NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways, and induction of TLR-negative regulators [106-108]. Application of probiotic Lactobacilli to SCI mice triggered a protective immune response in GALT and improved locomotor recovery [101]. Since the number of immunoregulatory Treg lymphocytes (CD4+CD25+FoxP3+ T cells) and CD11c+ dendritic cells in mesenteric lymph nodes was increased and the lesion volume and axon/myelin pathology at the injury epicenter was reduced, it is reasonable to assume that the gut-CNS-immune axis could play a crucial role in regulation of functional post-SCI recovery.

In conclusion, the composition of gut microflora significantly affects many physiological processes in the body and the overall health of the host. Currently, there are several studies focusing on analyses of the direct impact of SCI-induced gut dysbiosis on immune and neural functions in rodent models, and also on finding possible therapeutic approaches for regulating inflammation induced by SCI *via* remodelling of the gut microbiome. Nevertheless, the precise molecular mechanisms participating in the gut/CNS/immune system axis including receptors, their down-stream molecules or transcription factors are still not fully understood.

10. The effect of weak long-term electrostimulation on spinal cord functional recovery

One of the major limiting factors for functional regeneration after traumatic SCI is the inability of damaged axons to re-establish their interconnections with target fibers on the opposite side of the lesion. Application of a weak electric field over the injury site is one of the methods enabling the regrowth and proper alignment of damaged nerve fibers. Extracellular electric fields produced by weak electrostimulation presenting the voltage gradient within tissue might provide the necessary stimulus directing astrocyte behavior after CNS injury and gradually dissolving glial scar integrity. It has been shown previously, that electric fields affect directly-induced cellular behaviors, e.g. migration [109-110], proliferation [111-112], differentiation [113-114] and morphology [115-116] among the variety of ectodermally and mesodermally-derived cell types [117-118].

In the past, Borgens et al. [119] demonstrated that glial cells, and astrocytes in particular, are able to respond to weak electric fields. These authors showed that rat cortical astrocytes oriented themselves along the applied voltage gradient in experiments *in vitro*. Moriarty and Borgens [120] also reported that applied voltage reduced the number of astrocytes accumulating at the site of SCI and suppressed the extension of astrocytic processes within the lesion site. On the other hand, the other major components of the inhibitory glial scar, macrophages, do not seem to be affected by exogenous electric gradients [121]. To enhance the regeneration of both ascendent and descendent neural pathways simultaneously, an oscillating electric field stimulation (OFS) technique which periodically (every 15 minutes) changes the polarity of the electric field has been developed. The application of a weak oscillating field current over the lesion site of SCI mimicking the polarity guidance in the developmental stages in CNS, has been shown to promote regeneration of injured axons, stimulating them to grow across the injury site [122].

We designed a miniature electric stimulator (50 μA) with oscillating electric field (OSF) and used it in SCI experiments *in vivo* [123-124]. Spinal cord trauma caused considerable increase in activated forms of astrocytes, a typical feature of the ongoing inflammatory reaction four weeks post-SCI. The greatest accumulation of reactive astrocytes was observed in the areas of the dorsal and lateral spinal funiculi. This observation correlated with histopathological findings indicating the greatest tissue and myelin loss in these white matter regions. Stimulated animals (SCI+OFS) showed a significantly lower number of activated astrocytes and larger area of preserved spinal cord tissue compared to their state after SCI, with higher locomotor activity of the hind limbs and earlier onset of spontaneous urination [123].

Zhang et al. [125] and Jing et al. [126] proposed that electrical stimulation might promote spinal tissue integrity and contribute to remyelination after SCI *via* improved differentiation of oligodendrocyte precursor cells. A similar beneficial outcome was reported in

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a study of epidural stimulation after SCI, where the electrostimulation upregulated myelin basic protein mRNA levels and reduced oligodendrocyte loss by promoting their differentiation and inhibiting apoptosis [109]. Long-term (eight weeks) epidural stimulation with OFS applied immediately after spinal trauma significantly reduced oligodendrocytes loss and promoted their density in the areas of the greatest tissue damage [124]. Similarly, by reducing reactive astrogliosis and glial scarring, where the oligodendrocytes are greatly affected by inhibitory components of the glial scar [127], they were able to migrate towards the lesion site and initiate remyelination. According to these data, we suppose that OF stimulation applied after SCI could provide a more hospitable microenviroment either for neurons or glial cells by triggering the regenerative processes in the acute phase of injury.

The exact mechanism responsible for axonal and glial regeneration in response to applied electrical stimulus is not yet fully understood. Axonal growth after electrical stimulation has been presumed to be mediated by membrane receptors and secondary messengers such as adenylcyclase and interaction with other physiological neurotrophins presented in the CNS [128-129]. Electrostimulation has also been shown to enhance the expression of regeneration-associated genes – RAGs [130-131], which are functionally required for neural recovery [132-133].

11. Rehabiltation - comprehensive and effective therapeutic strategy after SCI

Changes at molecular and cellular levels could provide new insights into mechanisms by which exercise has a positive impact on functional deficits occuring after SCI. Since positive effects of physical activity and exercise have been clearly demonstrated in patients after traumatic SCI [134], rehabilitation and exercise appear to be the most effective non-invasive post-SCI therapeutic strategy. In addition to strengthening muscle mass, rehabilitation is effective in endogenous stimulation of growth factors [135-137]. Previous experimental studies have shown that various forms of rehabilitation (treadmill, swimming, physiotherapy) significantly supported functional spinal cord regeneration [138], and that physical training is important for regaining motor and sensory function after SCI.

Exercise is no longer strictly a tool for rehabilitation and there are many exciting aspects of this therapy which remain to be explored, such as the time post-injury when exercise is best initiated, the most appropriate intensity, duration and frequency, and the best use of task-specific and non-task specific training for recovery of multiple functional modalities [137]. Novel data show that treadmill training (six weeks) prior to SCI markedly increased the activity of PLCγ-PKC signaling at both transcript and protein levels at and around the lesion site. Similar effects were seen in expression of PI3k/Akt and Ras/Erk1/2 signaling responsible for cell survival and regeneration [139]. Molecular analysis of the signaling pathways responsible for survival, plasticity and neuroregeneration after assisted long-term post-SCI training could be very useful, and could be used in further experimental post-SCI rehabilitation strategies.

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