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EFFECT OF VITAMIN B COMPLEX THERAPY ON PROCESS OF NEUROINFLAMMATION AND REGENERATION OF RAT PERIPHERAL NERVE AFTER INJURY

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УНИВЕРЗИТЕТ У БЕОГРАДУ

БИОЛОШКИ ФАКУЛТЕТ

Адил О. Ехмедах

УТИЦАЈ КОМПЛЕКСА Бе ВИТАМИНА НА ПРОЦЕС НЕУРОИНФЛАМАЦИЈЕ И РЕГЕНЕРАЦИЈЕ ПЕРИФЕРНОГ НЕРВА ПАЦОВА НАКОН ПОВРЕДЕ

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DEDICATION

For my Mother and my Father

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In the name of Allah the most beneficent most merciful.

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Effect of Vitamin B Complex Therapy on Process of Neuroinflammation and Regeneration of Rat Peripheral Nerve after Injury

Abstract

Peripheral nerve injury (PNI) often results in substantial neurological deficits with slow and usually incomplete functional recovery. PNI leads to a series of cellular and molecular events necessary for axon regeneration and reinnervation of target tissues, among which, inflammation is crucial for the orchestration of all these processes. PNI triggers a complex multi-cellular response involving injured neurons, Schwann cells and immune cells. Macrophages activation underlies the pathogenesis of PNI and is characterized by morphological/phenotype transformation from proinflammatory (M1) to an anti-inflammatory (M2) type, displaying different functions during the inflammatory and reparative process. Moreover, Schwann cells, together with macrophages that infiltrate damaged tissue, are considered the key to the regeneration of the peripheral nerve. The aim of this study was to investigate the effects of the treatment with the vitamin B (B1, B2, B3, B5, B6, and B12) complex on the process of neuroinflammation and on the crosstalk between macrophages and Schwann cells during the recovery period after PNI and to evaluate the influence of L-type CaV1.2 calcium channels on cells implicated in the process of neuroregeneration.

Controlled transection of the motor branch of the femoral peripheral nerve was used as an experimental model. Experimental procedures were performed on male Albino Oxford rats. The animals were divided into experimental and control groups. Unilateral transection of the motor branch of the femoral nerve was performed in the group of experimental animals, after which immediate reconstruction was done by end-to-end anastomosis using 9-0 suture (Ethicon Inc, Norderstedt, Germany). The group of control animals underwent the same experimental procedure, but without nerve transection. Animals were sacrificed after 1, 3, 7, and 14 days of the vitamin B complex therapy, after surgery. Femoral nerves were isolated. Isolated femoral nerves (operated, reconstructed, as well as contralateral, intact) from the sham (S), operated (O), and operated groups treated with the B vitamins (OT group) were used for immunofluorescence analysis.

The treatment with the vitamin B complex decreases the expression of proinflammatory and increases the expression of anti-inflammatory cytokines by macrophages and Schwann cells, thus contributing to the resolution of neuroinflammation. Comparative analysis of the time-dependent changes in macrophages/Schwann cells interaction within the femoral nerve cross sections obtained from the S, O, and OT groups during the investigated postoperative period (1^{st}, 3^{rd}, 7^{th}, and 14^{th} day

post operation) revealed close association only between macrophages with the M2-like morphology and non-myelinating Schwann cells, as well as between macrophages with the M2-like morphology and Schwann cells, that degenerate or remove myelin undergoing dedifferentiation. The effect of B vitamins complex on both cell types was accompanied with an increase in macrophage/Schwann cells interactions, all of which correlated with the regeneration of the injured nerve. Furthermore, no interactions between mature, myelinating Schwann cells and M2- or M1- type macrophages were detected in the both O and OT groups. At the end of the observation (14 day post operation), using serial transversal sections tight interactions were visualized between M2 macrophages and Schwann cells, that were aligned to form the bands of Büngner, and also intensively stained with the antibody specific for anti-inflammatory cytokine IL(interleukin)-10. B vitamins down-regulate the expression of $Ca_V1.2$ channel in M1 macrophages, and up-regulate the expression of the same channel in M2 macrophages, Schwann cells and axons, suggesting their role in improving recovery of the injured nerve.

The data presented indicate that PNI modulates interactions between macrophages and Schwann cells in a time-dependent manner. The treatment with the B vitamins complex accelerates the transition from the non-myelin to myelin-forming Schwann cells, thus regulating the Schwann cells maturation, promoting the M1 to M2 macrophage polarization and consequently altering macrophages/Schwann cells interactions, thereby enhancing the regeneration of the injured nerve. Clearly, the capacity of B vitamins to modulate macrophages-Schwann cells interaction may be considered promising for the treatment of PNI. B vitamins clearly displayed the potential for the treatment of neuroinflammation and neuroregeneration and therefore could serve as an effective therapy of PNI in humans.

Key words: Peripheral Nerve Injury, Vitamin B Complex Treatment, Neuroinflammation, Neuroregeneration; Macrophages, Schwann cells, L-type $Ca_V1.2$ calcium channels

Scientific field: Biology

Scientific subfield: Immunology - Neuroimmunology

Утицај комплекса Бе витамина на процес неуроинфламације и регенерације периферног нерва пацова након повреде

Сажетак

Повреда периферног нерва (енгл. *Peripheral Nerve Injury*, ПНИ) често доводи до значајних неуролошких дефицита са спорим и обично непотпуним функционалним опоравком. ПНИ покреће низ ћелијских и молекулских сигнала неопходних за регенерацију аксона и реинервацију циљних ткива, при чему кључну улогу у оркестрацији ових догађаја има инфламација. Покренут одговор је сложен и вишећелијски, укључује повређене неуроне, Шванове ћелије и имунске ћелије. Активација макрофага лежи у основи патогенезе ПНИ и карактерише се морфолошким и фенотипском трансформацијом из проинфламаторног (М1) у анти-инфламаторни (М2) тип макрофага, који показују различите функције током инфламаторног и репаративног процеса. Заправо, Шванове ћелије, заједно са макрофагима који се инфилтрирају у оштећено ткиво, сматрају се кључним молекулима за регенерацију периферног нерва. Циљ ове дисертације био је да се испитају ефекти суплементације комплексом витамина Бе (Бе1, Бе2, Бе3, Бе5, Бе6 и Бе12) на процес неуроинфламације и на комуникацију између макрофага и Шванових ћелија након ПНИ (током периода опоравка), као и процена утицаја Л типа волтажно-зависних калцијумових канала ($Ca_V1.2$) на ћелије које учествују у процесу неурорегенерације.

Као експериментални модел коришћена је контролисана трансекција моторне гране феморалног периферног нерва. Експерименти су изведени на мужјацима пацова Албино Оксфорд. Животиње су подељене у експерименталне и контролне групе. Унилатерална трансекција моторне гране феморалног нерва извршена је у експерименталној групи, након чега је извршена тренутна реконструкција „end-to-end“ анастомозом помоћу конца 9-0 (Ethicon Inc, Norderstedt, Немачка). Група контролних животиња подвргнута је истом експерименталном поступку, али без трансекције нерва. Животиње су жртвоване 1, 3, 7 и 14 дана након операције и третмана комплексом витамина Б. Изоловани су феморални нерви. За имунофлуоресцентну анализу коришћени су изоловани феморални нерви (оперисани, реконструисани, као и контралатерални, нетакнути) из лажно оперисаних (С), оперисаних (О) и оперисаних и витамина Бе третираних (ОТ) животиња.

Третман комплексом витамина Бе смањује експресију проинфламаторних, а повећава експресију анти-инфламаторних цитокина од стране макрофага и Шванових ћелија, и на тај

начин доприноси резолуцији неуроинфламације. Анализом временски зависних промена у интеракцији макрофага и Шванових ћелија унутар пресека феморалног нерва добијених из животиња С, О и ОТ групе током испитиваног постоперативног периода (1., 3., 7. и 14. дан после операције, дпо) откривена је повезаност само између макрофага М2-сличне морфологије и немијелинизујућих Шванових ћелија, као и између макрофага М2-сличне морфологије и Шванових ћелија које уклањају мијелин. Ефекат комплекса витамина Бе на оба типа ћелија био је праћен повећањем интеракције макрофага и Шванових ћелија, што је у корелацији са регенерацијом повређеног нерва. Такође, у групама О и ОТ нису откривене интеракције између зрелих, мијелинизујућих Шванових ћелија и макрофага М2 или М1 типа. На крају посматрања (14. дпо) на попречном пресеку нерва уочене су блиске интеракције између М2 макрофага и Шванових ћелија, које су поређане тако да формирају Büngner-ове траке, и интензивно обојене антителом специфичним за анти-инфламаторни цитокин интерлеукин-10. Витамини групе Бе смањују експресију $Ca_V1.2$ канала у М1 макрофагима, а експресију истих канала повећавају у М2 макрофагима, Швановим ћелијама и аксонима, сугеришући њихову улогу у подстицању опоравка повређеног нерва.

Приказани подаци указују да ПНИ модулира интеракције између макрофага и Шванових ћелија на временски зависан начин. Третман комплексом витамина Бе убрзава прелазак са немијелинизујућих на Шванове ћелије које формирају мијелин, регулишући на тај начин сазревање Шванових ћелија, промовишући поларизацију М1 до М2 макрофага и последично мењајући интеракције између макрофага и Шванових ћелија, што подстиче регенерацију повређеног нерва. Способност витамина Бе да модулирају интеракцију између макрофага и Шванових ћелија је веома обећавајућа у третману ПНИ. Витамини Бе групе су јасно показали потенцијал за третман неуроинфламације и неурорегенерације и стога би могли да послуже као ефикасна терапија ПНИ код људи.

Кључне речи: повреда периферног нерва, третман витаминима Бе комплекса, неуроинфламација, неурорегенерација, макрофаги, Шванове ћелије, Л-тип $Ca_V1.2$ калијумови канали

Научна област: Биологија
Ужа научна област: Имунологија - Неуроимунологија

List of abbreviations

AO ─ Albino Oxford

BDNF ─ brain-derived neurotrophic factor

CNTF ─ ciliary neurotrophic factor

dpo ─ days post-operation

ECM ─ extracellular matrix

GAP43─ growth associated protein

HIF-1α ─ hypoxia-inducible factor 1-alpha

HVA ─ high voltage-activated VGCC

IF ─ immunofluorescence

IL ─ interleukin

iNOS — inducible nitric oxide synthase

LVA ─ low voltage-activated VGCC

LVGCC ─ L-type voltage-gated calcium channels

MCP-1─ monocyte chemoattractant protein-1

NDS ─ normal donkey serum

NGF ─ nerve growth factor

PBS ─ phosphate buffer saline

PNI ─ peripheral nerve injury

RT ─ room temperature

TGF-β ─ tumor growth factor-beta

Th1─ T helper type 1

Th2─ T helper type 2

TNF-α ─ as tumor necrosis factor-alpha

VEGF-A ─ vascular endothelial growth factor A

VGCC ─ voltage-gated calcium channels

Table of Contents

1 INTRODUCTION

1.1 Peripheral nerve

1.1.1 Peripheral nerve anatomy

The peripheral nervous system is composed of neurons which transmit signals from the brain and spinal cord to the rest of the body (Menorca et al., 2013). These neurons are constituted of arrays of axons enclosed in structural units called fascicles, which are surrounded by collagenous matrix *endoneurium*. Individual fascicles are separated by a layer called *perineurium* and wrapped by a connective tissue layer *epineurium*, which plays an important role in nourishing and protecting the fascicles (Lee and Wolfe, 2000) (Figure 1). The blood supply of peripheral nerves is a complex network of blood vessels and capillaries. Other integral parts which are crucial for function and homeostasis of peripheral nerves include Schwann cells, macrophages, fibroblasts, and endothelial-like cells (Flores et al., 2000).

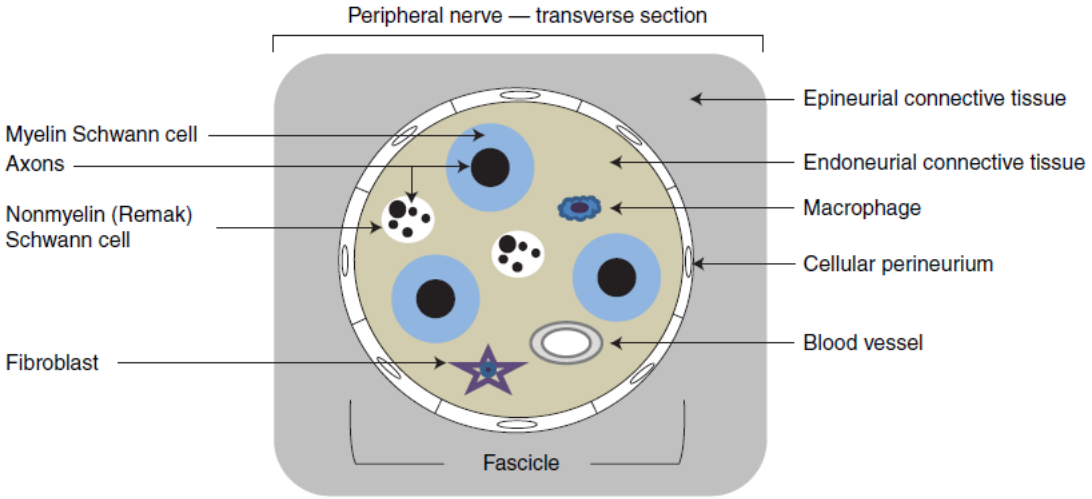

Figure 1. Peripheral nerve illustration - the arrangement of tissue compartments (endoneurium, epineurium, perineurium) and cellular components including Schwann cells, macrophages, and fibroblasts (Jessen et al., 2015).

1.1.2 Peripheral nerve injury and recovery

Peripheral nerve injuries are classified in different types depending on their pathophysiology (Menorca et al., 2013).

There are three main groups including:

1. Neuropraxia (local myelin damage with the intact nerve),
2. Axonotmesis (loss of axon continuity),
3. Neurotmesis (complete disruption of the nerve trunk).

Numerous studies have shown that damaged axons have great ability of regrowth and regeneration. After a peripheral nerve injury (PNI), a series of molecular and cellular changes take place. Firstly, the cell body of the neuron becomes larger, the Nissl bodies disperse, while the nucleus is placed peripherally (Gaudet et al., 2011).

The distal part of the axon goes through progressive fragmentation, known as Wallerian degeneration, which includes the breakdown of both axons and myelin, as well as disruption of the blood-nerve barrier. The Wallerian process goes along with the migration of Schwann cells and immune cells such as macrophages, as well as secretion of signaling mediators including extracellular matrix (ECM) proteins, growth factors, chemokines and cytokines, which are responsible for maintaining the suitable conditions for the successful axonal recovery regeneration (Gaudet et al., 2011). These cells are recruited within hours to days after nerve injury which trigger neuroinflammatory response and help the clearing of debris, as well as the establishment of microenvironment required for axonal regeneration (Chen et al., 2015).

1.1.3 L-type voltage-dependent calcium channels

Voltage-dependent channels are a type of transmembrane proteins that form ion channels, which open due to changes in the electrical potential of the membrane. The shift in membrane potential leads to a conformational change of the protein, causing the channel opening or closing. As the cell membrane is not permeable to ions, the movement of ions is enabled through these channels (Simms and Zamponi, 2014).Voltage-dependent channels are especially important for excitable cells such as nerve cells and muscle tissue cells, because they allow coordinated depolarization of the cell in response to a change in potential. These channels are most represented along the axons and within the synapse and usually pass one type of ion. So far,

voltage-dependent channels for sodium (Na^+), potassium (K^+), chlorine (Cl^-), and calcium (Ca^{2+}) have been identified.

There are two types of voltage-gated calcium channels (VGCC):

1) High voltage-activated (HVA) VGCCs that open due to high membrane depolarization

2) VGCC low voltage-activated (LVA) which open due to low voltage change (Armstrong, 1985)

Based on biochemical analyzes (Curtis and Catterall, 1984), it is known that HVA channels consist of heteromultimeric protein complexes consisting of the $Ca_V\alpha1$ subunit and the two accompanying subunits of $Ca_V\beta$ and $Ca_V\alpha2\delta$, while LVA consists of only the $Ca_V\alpha1$ subunit (Catterall et al., 2005).

The $Ca_V\alpha1$ subunit is crucial in determining the VGCC subtype. There are three main families $Ca_V\alpha1$: Ca_V1, Ca_V2 and Ca_V3 within which a larger number of members differ. The Ca_V1 family includes L-type voltage-gated calcium channels (LVGCC), channels present in neurons ($Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$), and one isoform specific for skeletal muscle, $Ca_V1.1$ (Bech-Hansen et al., 1998). These channels have the characteristics of HVA channels, meaning that they are activated by high voltage, and also are sensitive to various dihydropyridine antagonists and agonists (Randall and Tsien, 1995).

L-type VGCCs control the passage of positive calcium current that affects membrane excitability and intracellular signal transduction within the nervous system. Positive ions passing through the channels lead to depolarization of the neuronal membrane (Chan et al., 2007) or to a decrease in irritability by binding to Ca^{2+} activating K^+ channels (Marrion and Tavalin, 1998). Ca^{2+} signals mediated by Ca_V1 VGCC activate an intracellular transduction cascade that controls gene transcription in the brain (West et al., 2001) and the release of neurotransmitters in the retina and inner ear (Brandt et al., 2003).

These signals affect the formation of synapses and synaptic plasticity, processes that are necessary for the adequate development and functioning of the nervous system. As mentioned before, the Ca_V1 family of channels includes $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$ channels. $Ca_V1.1$ and $Ca_V1.4$ channels are present in skeletal muscle and retinal cells, while $Ca_V1.2$ and

$Ca_V1.3$ are expressed in the nervous system. $Ca_V1.2$ LVGCC consists of four subunits: α1 forms a Ca^{2+} selective pore and contains a voltage sensor as well as a binding site for specific channel modulators, while α2δ, β and γ provide anchoring, ion passage, and have a regulatory function (Hofmann et al., 2014).

The change in $Ca_V1.2$ expression is associated with PNI. Namely, after nerve injury, there is a change in transcription factors that induce the processes of regeneration, axon elongation and nerve recovery. However, during these processes, the expression of Ca^{2+} channels changes. In the following study (Tedeschi and He, 2010) it was postulated that during the recovery process there is a decrease in the expression of LVGCC within the axon. One study (Westenbroek et al., 2004) monitored the expression of $Ca_V1.2$ in dental pulp i.e. in odontoblasts and fibroblasts. It was shown that the expression of Ca^{2+} channels is lost even 10 minutes after the injury. However, one hour after injury, there was a re-increase in $Ca_V1.2$ channel expression in odontoblasts and especially in fibroblasts. $Ca_V1.2$ expression is also associated with pain. A study (Wilson et al., 2012) has proposed the use of synthetic proteins that modulate signal transduction of these channels lead to a reduction in pain sensation. Nonetheless, it was emphasized that the influence of synthetic proteins on the further regulation of $Ca_V1.2$ channels is unknown.

Processes underlying macrophage activation, such as alterations in morphology, proliferation, and production of proinflammatory mediators, are associated with calcium entry via the L-type VDCCs (Espinosa-Parrilla et al., 2015; Hegg et al., 2000; Silei et al., 1999). PNI induces changes in the expression pattern of LVDCC isoform $Ca_V1.2$, primarily through membrane potential regulation (Westenbroek et al., 2004). This process is also substantially modulated by soluble molecules — hormones, cytokines, and neurotransmitters (Hofmann et al., 2014). Also, it has been shown that Ca^{2+} influx mediated by L-type VDCCs is necessary for normal myelination and facilitates axon – glial interaction during the first steps of myelin formation (Cheli et al., 2015; Cheli et al., 2016). Their expression has been shown in Schwann cells as well (Cheli et al., 2015; Westenbroek et al., 2004). Wide expression of L-type VDCCs and their involvement in the processes of inflammation and regeneration make them a promising target for numerous therapeutic modalities.

1.2 Schwann cells in neuroinflammation and neuroregeneration

1.2.1 Schwann cells–classification and function

Schwann cells represent one of the most significant glial cells of the peripheral nervous system which are required for myelin formation and delivering nutrients to the neurons (Jessen et al., 2015). Aside from their role in the function and development of the neurons, Schwann cells are also important during the processes regarding nerve injury and regeneration. Their ability of dedifferentiation, phagocytosis and secretory capacity contributes in debris removal from the injury site, as well as recruitment of essential immune cells (Jessen et al., 2015).

Schwann cells are in close contact with axons and can be classified as myelinating and non-myelinating cells (Jessen et al., 2015). They are covered by a basal lamina which has been shown that also plays an important role in nerve regeneration (IDE, 1983). External lamina is encompassed by a layer of connective tissue, while the internal surface of the lamina is in close contact with SC plasma membrane. Extracellular matrix of the basal lamina contains laminin, fibronectin, and collagen which control several processes involved in Schwann cells development (Bunge et al., 1986).

1.2.2 Schwann cells response in peripheral nerve injury and regeneration

PNI pathophysiology revealed that Schwann cells, along with inflammatory cells are crucial components of regulatory mechanisms involved in degeneration and regeneration of the peripheral nerve (Lee and Wolfe, 2000).

During PNI, axonal disorganization promotes the activation of Schwann cells which results in releasing of extracellular matrix molecules, cytokines, and growth factors that are needed for the axon regrowth (Rodrigues et al., 2012). The exact mechanisms through which Schwann cells and the injured axons communicate are not well understood. One study suggested that it may be mediated by Toll-like receptors. Injured axons secrete Toll-like receptor ligands which induce Schwann cells activity and immune cells to begin the regeneration process (Lee et al., 2006).The activated local Schwann cells proliferate and participate in phagocytosis of the initial clearing of axonal and myelin debris (Jessen et al., 2015).

The main reason responsible for regenerative potential of the peripheral nervous system is the phenotypic plasticity of the Schwann cells. In response to nerve damage, denervated

Schwann cells shift to a phenotype which support regeneration (Jessen et al., 2015). These repair Schwann cells, also called *Büngner* cells, provide essential support for injured neurons (Jessen et al., 2015). Down-regulation of the myelin cells to repair cells is controlled by the transcription factor c-Jun that is activated in Schwann cells after injury (Menorca et al., 2013). Activated Schwann cells start proliferating, phagocytosing debris, and releasing cytokines that recruit monocytes/macrophages for myelin clearance. Schwann cells regulate the secretion of specific chemokines and cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) which attract macrophages into the injury site (Jessen et al., 2015). Moreover, Schwann cells also release high levels of different growth factors, such as neurotrophins (nerve growth factor-NGF and ciliary neurotrophic factor-CNTF) which promote and stimulate neuronal development, branching and innervations (Gulati, 1998).

Unlike the axons, Schwann cells basal lamina and connective sheaths remain as tubular scaffolds between the proximal and distal segment. This permits axons to reach the distal stump in their original basal lamina tubes. The axonal growth is guided by a basal lamina and Schwann cells that fill the empty endoneurial tubes and form regeneration tracks, called *Büngner Bands* (Jessen et al., 2015) (Figure 2). In order to support the regeneration process, extracellular matrix molecules, such as laminin and collagen are being produced. SC also express cell adhesion molecules and receptors, including IL-1, N-cadherin, integrins, and the neural cell adhesion molecule (Rodrigues et al., 2012).

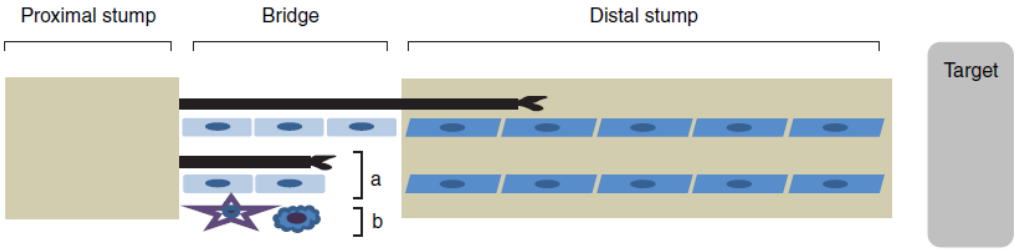

Figure 2. Illustration of peripheral nerve regeneration - (a) Regeneration segment, and (b) fibroblasts and immune cells placed between proximal and distal segment. Schwann cells form *Büngner* repair bands in the distal segment guide growing axons and provide trophic support for damaged neurons (Jessen et al., 2015).

1.3 Macrophages in neuroinflammation and neuroregeneration

1.3.1 Macrophages– function, origin and polarization

Macrophages are specialized immune cells which play a fundamental role in promoting the inflammation process and repairing the injured tissue (Liu et al., 2019). Their function is reflected in phagocytic function and secretion of cytokines and chemokines. In this way, macrophages remove foreign bodies and apoptotic cells, but also promote the cell proliferation and tissue remodeling after injury (Liu et al., 2019).

Tissue-resident and circulating macrophages originate from bone marrow hematopoietic stem cells. Macrophages are highly plastic and exist in several different phenotypic states. According to the activation state and functions, they are classified into two distinct polarized phenotypes: proinflammatory (classically activated macrophage - M1) involved in the protection of pathogens through secretion of pro-inflammatory cytokines and chemokines (such as IL-6, IL-12 and TNF-α), presentation of antigens and production of free radical and pathogen protection; and anti-inflammatory (alternatively activated macrophage - M2), which have the immunosuppressive function through secretion of anti-inflammatory cytokines (arginase-1, IL-10 and tumor growth factor-beta - TGF-β) and also have an important role in wound healing and tissue repair (Das et al., 2015).

The macrophage polarization depends on their current microenvironment and can be altered by different modulating signals. M1-polarized macrophages are activated by bacterial lipopolysaccharide and T helper type 1 (Th1) cytokines (such as interferon gamma, IFN-γ). On the other hand, M2-polarized macrophages are stimulated by T helper type 2 (Th2) cytokines (such as IL-4 and IL-13). A switch from M1 polarized macrophages into M2 phenotype is mediated by IL-4 produced by Th2 cells (Cassetta et al., 2011). Additionally, studies have also shown that an inhibitor of histone deacetylase (MS-275), as well as IL-13, induces the transformation of M1 to M2 phenotype (Liu et al., 2019). The balance between M1 and M2 phenotype is crucial in the regulation of inflammation and tissue homeostasis.

1.3.2 Macrophages response to peripheral nerve injury and regeneration

In response to injury, macrophages are polarized into different subtypes with an increased phagocytic ability (Chen et al., 2015). M2 polarized macrophages play a crucial role in the

regulation of neuroinflammation and axonal regeneration. Along with their anti-inflammatory effects, M2 macrophage subtypes (M2a, M2b, M2c and M2d) also promote cell proliferation, cell maturation, migration, production of growth factors, removal of apoptotic cells, extracellular matrix synthesis, angiogenesis, and tissue remodeling (Chen et al., 2015).

Wallerian degeneration induces a release of cytokines such as TNF-α and IFN-γ, as well as an increase of inducible nitric oxide synthase (iNOS) level which contribute to pro-inflammatory response (Ydens et al., 2012). As the first stage of the inflammatory response neutrophils are being recruited (Stratton and Shah, 2016). Furthermore, mast cells accumulate and release mediators that contribute to the recruitment of neutrophils and macrophages. Following axonal degradation, resident macrophages and local Schwann cells start the myelin phagocytosis (Stratton and Shah, 2016). Infiltration of blood-borne monocytes into distal injured nerves starts from two to three days after damage (Perry et al., 1987). Subsequently, they differentiate into phagocytic macrophages, which start the clearing process essential for axonal regeneration. An important step in nerve repair is the clearance of the injured tissue, as it was shown that myelin debris contains inhibitors of axonal growth. During this process, macrophages are recruited to the injury site where they start the phagocytosis of the dead cells (Figure 3) (Chen et al., 2015).

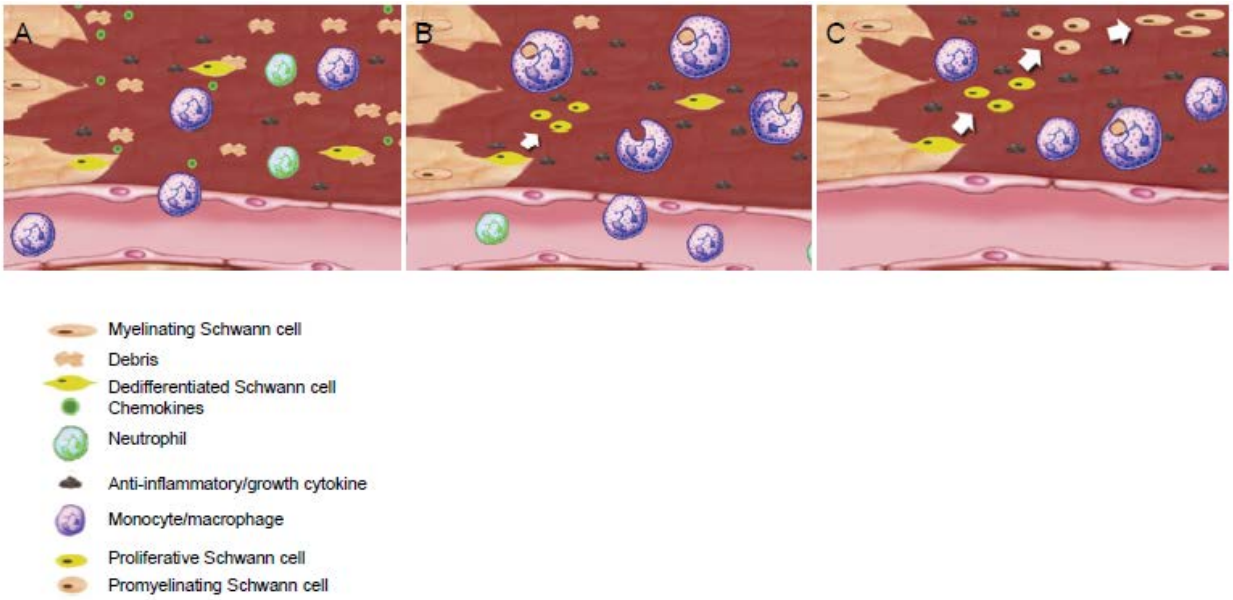

Figure 3. Illustration of the neuroinflammatory cascade – activation of Schwann cells and recruitment of neutrophils and monocytes/macrophage into the injury site (Stratton and Shah, 2016).

Macrophages have several roles in PNI including the promotion of angiogenesis, regulation of Schwann cells differentiation and remyelination (Liu et al., 2019). Studies have shown that upon damage to peripheral nerves, macrophages can sense and respond to hypoxia. This process is accompanied through activation of the transcription factor, hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor A (VEGF-A) expression which stimulates endothelial cell proliferation and the migration to the injury site (Liu et al., 2019). In this way, macrophages promote angiogenesis - vascularization of the injured site which provides nutrition for the nerve reparation (Gaudet et al., 2011). Furthermore, it has been demonstrated that recruited macrophages can modulate the activities of Schwann cells, affecting their proliferation and migration (Stratton and Shah, 2016).

1.3.3 Schwann cells and macrophages interactions

The axonal regeneration process is promoted by coordinated cross-talk between signaling mediators released by macrophages and Schwann cells (Figure 4). This process includes different cytokines and trophins, which enhance neuronal repair and myelin cell maturation and differentiation. Schwann cells are the main source of MCP-1 which enables recruitment of macrophages. After activation of Schwann cells and recruitment of immune cells, macrophages start to release molecules which further promote Schwann cells proliferation (Menorca et al., 2013).

Infiltrated macrophages secrete inflammatory cytokines such as TNF-α, chemokines, such as chemokine ligands (CCL2, CCL3, and CCL4), interferons and interleukins (IL-1, IL-12) (Liu et al., 2019). It has been demonstrated that IL-1 is of special significance because of its potential to induce synthesis of NGF and the expression of NGF-receptor on Schwann cells (Gulati, 1998). Neurotrophins such as NGF are important in enhancing neuronal regeneration after tissue injury (Jessen and Mirsky, 2019). NGF has been shown to induce proliferation of Schwann cells in growing axon and also has an immunomodulatory role in regard to the phagocytic function of macrophages. In the damaged area, macrophages also secrete mediators such as growth factors and IL-10 or TGF-β which stimulate Schwann cells and fibroblast proliferation (Liu et al., 2019). Moreover, by promoting angiogenesis, macrophages allow endothelial cells to guide the Schwann cells migration and at the same time provide support for nerve repair and regeneration (Jessen et al., 2015).

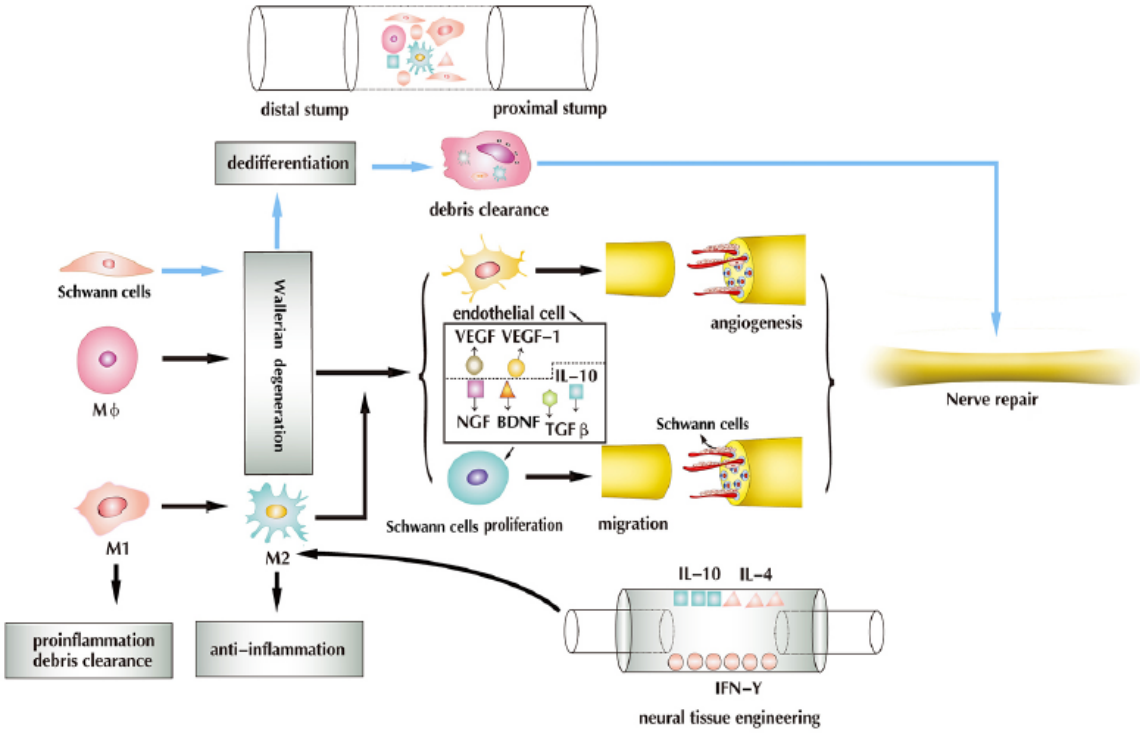

Figure 4. Schematic demonstration of molecular and cellular mechanisms underlying Wallerian degradation and nerve reparation (Liu et al., 2019).

1.4 Treatment of peripheral nerve injuries

The treatment of peripheral nerve injury depends on the location, type, and degree of injury. Although there are several therapeutic approaches in peripheral nerve regeneration, complete functional recovery is still a major challenge. There is an increasing need for the discovery of compounds that would ensure full recovery and improve patient's quality of life (Hussain et al., 2020). Peripheral nerves have an innate ability to regrow, yet this process is limited and surgical intervention is usually necessary. Therefore, surgery is the treatment of choice for the majority of peripheral nerve injuries. Currently accepted gold standard treatments are either direct nerve repair or autologous nerve grafts, depending on the presence of nerve gap (Grinsell and Keating, 2014). There are several alternative therapeutic strategies such as nerve transfers, nerve conduits, cell-based therapy, as well as adjuvant non-surgical approaches like medications, electrical nerve stimulation, and treatment with vitamin B complex (Hussain et al., 2020; Nedeljković et al., 2018).Which treatment strategy will be selected depends on multiple

factors such as patient's age, injury location and degree, presence of additional disorders, and others.

1.5 Direct nerve repair as surgical therapeutic approaches for peripheral nerve recovery

Nerve repairs are divided on primary and secondary based on the repair time after the damage. Primary nerve repair refers to a direct reattachment of the transected nerve right after the disruption. Some opinions were that it is better to wait about three weeks before the repair so that the Wallerian degeneration process could be completed. However, during the time of three weeks, a nerve may lose up to 8% of its length (Siemionow and Sonmez, 2007). Therefore, it was concluded that prompt repair gives better outcome (Mackinnon, 1989). When a primary repair cannot be performed, postponed or secondary repair takes place.

Direct end-to-end nerve repair is the most frequently used nerve repair technique (Griffin et al., 2014). It remains the surgical treatment of choice when a tension-free coaptation of the proximal and distal stumps can be performed. Generally, this is possible in cases of clean-cut nerve transections with a slight gap and minimal neuronal tissue damage, in areas with proper blood supply and soft tissue coverage. Various factors may influence the nerve repair outcome. The precise connection between two ends of a transected nerve with minimum stitches is one of the most important above-mentioned factors. Moreover, nerve endings should be cut to the extent necessary for proper alignment, with minimal tension (Hussain et al., 2020). The greatest results are obtained when nerves are either totally sensor or totally motor and when the intraneural connective tissue component is small (Siemionow and Sonmez, 2007). Functional recovery after nerve repair is not guaranteed and this is the main disadvantage of nerve repair technique.

Nerve repair techniques are generally classified into three categories: epineurial repair, perineurial repair, and group fascicular repair. Epineurial repair implies that nerve repair is supported by stitches into the epineurium, the outer sheath of the nerve. Group fascicular repair is generally favored for larger nerves where motor and sensory fibers can be fixed separately (Griffin et al., 2014; Hussain et al., 2020).

Fibrin glue is used as an alternative to either complement or replace sutures, utilizing adhesive material called fibrin sealant (Hussain et al., 2020). It is considered as an efficient

repair strategy to minimize trauma to the nerve ends and avoid suturing, as well as to decrease inflammatory reactions and fibrosis. This technique is fast and easy to use, though it cannot be used in cases of severe injuries due to its inferior holding strength (Panagopoulos et al., 2017).

1.6 Non-surgical therapeutic approaches for peripheral nerve recovery

Non-surgical therapeutic approaches have been developed to maximize the recovery process after surgical interventions. The need for this adjuvant therapy is constantly increasing, which is why it represents a very important area of research.

1.6.1 Cell-based therapy

Different strategies with the aim to enhance nerve conduit efficacy and to extend the length over which a conduit usage would be suitable have been considered. Incorporation of various luminal additives, including supportive cells (Schwann cells, stem cells), structural components (fibrin, laminin, and collagen), and neurotrophic factors proved to be effective (Evans et al., 2002; Keilhoff et al., 2006; Mosahebi et al., 2003).Cell-based therapy is aimed to provide supportive cells to the injured site and facilitate the nerve regeneration process. This way, the limitations such as slow nerve regeneration and inadequate filling of wide gaps could be surpassed.

It has been shown that the most suitable supporting cells for conduit incorporation are Schwann cells. The primary source of Schwann cells is neural crest cells. These cells migrate to the injured site where they can secret numerous signaling molecules, neurotrophic factors, and ECM molecules, which are all necessary for axonal regeneration. Moreover, Schwann cells have the potential for self-proliferation, immune system modulation, and remyelination. However, some of the major drawbacks regarding Schwann cells are the difficulty of harvest, slow growth in culture, and high immunogenicity (Fathi and Zaminy, 2017; Hussain et al., 2020). Different types of stem cells (differentiated and undifferentiated) are further alternatives to Schwann cells. Stem cells have the capacity to differentiate into a variety of specialized cell types and great proliferation ability. Some of the commonly used stem cells are embryonic stem cells, neural stem cells, bone marrow-derived stem cells, fetal stem cells, adipose stem cells, and many others. All of these cells demonstrate different benefits and limitations. For instance, they have the ability to differentiate into Schwann cells directly at the site of injury, regulate the activity of

native Schwann cells, and to increase myelination. Stem cells should be easily accessible, expand quickly, able to integrate into the host tissue, non-immunogenic, and non-tumorigenic (Fathi and Zaminy, 2017; Panagopoulos et al., 2017). Although cell-based therapy is promising, it is quite expensive and takes considerable time. Furthermore, there are still not enough preclinical trials for this procedure.

1.6.2 Growth factors and extracellular matrix molecules

Several studies have shown the significance of growth factors in peripheral nerve repair (Lee et al., 2003; Sterne et al., 1997; Zhang et al., 2019). Growth factors are proteins or hormones that promote cell survival, proliferation, and differentiation. They can be incorporated straight into the nerve conduits to increase the speed and accuracy of the nerve regeneration. Some of these growth factors are NGF, brain-derived neurotrophic factor (BDNF), CNTF, and IGF-1, which are all secreted by Schwann cells. Although their expression is upregulated in injured nerves, the level of endogenous growth factors is not sufficient for axonal myelination and nerve outgrowth. The administration of exogenous growth factors consistently during a long-time period ensures trophic support for axon regeneration (Li et al., 2020). The extracellular matrix molecules have a significant role in axonal extension and leading the nerve regeneration. Extracellular matrix molecules include fibronectin, collagen, and laminin. When incorporated into the conduit, they act as guidance channels (Griffin et al., 2014).

1.6.3 Medications

Medications that are currently in use as adjuvant therapy in the treatment of peripheral nerve injury are analgesics, corticosteroids, gels, and opioids. Although useful for relieving the pain, medications cannot accelerate the recovery and nerve regeneration, especially when the injury is serious (Hussain et al., 2020).

1.6.4 Electrical nerve/muscle stimulation

Peripheral nerve injuries of any kind may cause a disturbed communication between the nerve and the muscle. This leads to denervation of the target muscle and, consequently, to its atrophy. Electrical stimulation has been proved as beneficial in reducing muscle’s atrophy and speeding up axonal regeneration (Houschyar et al., 2016). It is conducted by utilizing electrical current straight to the skin and underlying muscle so that the muscle could contract and recover

its function. Several animal and human studies have shown that nerve electrical stimulation promotes the target muscle's reinnervation (Gordon et al., 2010; Khuong and Midha, 2013).

1.6.5 Phytochemicals

Phytochemicals are compounds that are produced in plants through primary or secondary metabolism. They are useful in the treatment of a large number of diseases due to numerous biological activities and minimum side effects they exhibit. Compounds which have been proved as successful in the nerve recovery process are quercetin, ursolic acid, curcumin, and 7,8-dihydroxycoumarin, as well as plants and extracts like red propolis, extract of *Lyciumbarbarum*, *Centellaasiatica* and *Lumbricus* (Du et al., 2012; Liu et al., 2016; Soumyanath et al., 2005; Türedi et al., 2018; Wei et al., 2009; Zhao et al., 2016). They exhibit several activities including anti-inflammatory, antioxidant, neuroprotective, immunomodulatory, improvement of neurite outgrowth, axonal regeneration, functional recovery, and nerve conduction velocity. Even though quite promising as supportive treatment of peripheral nerve injuries, plants and plant-derived compounds need to be further investigated for the exact mechanisms of their action.

1.6.6 Treatment with vitamin B complex

Vitamins are organic compounds essential for the proper functioning of the human body. They cannot be endogenously synthesized and therefore have to be obtained through the diet. Vitamins from the B group are water-soluble compounds with different chemical structures and biological activities. This group includes thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine(B6), biotin (B7), folate (B9), and cobalamin (B12) (Ang et al., 2008; Kennedy, 2016). Vitamins of the B complex serve as coenzymes in the significant number of enzymatic reactions and play key roles in multiple cellular functions. They are traditionally used, individually or in various combinations, for the treatment of both central and peripheral nervous system injuries.

Numerous studies have shown that B complex vitamins represent an effective adjuvant therapy for peripheral nerve recovery (Altun and Kurutaş, 2016; Ehmedah et al., 2019; Nedeljković et al., 2018; Nedeljković et al., 2017). It has been reported that the sciatic nerve injury is followed by decreased levels of vitamin B complex and vitamin B12 (Altun and Kurutaş, 2016), which implies that the administration of these vitamins may enhance the nerve

regeneration process. Moreover, it was demonstrated that treatment with B complex vitamins right after the injury and reconstruction of peripheral motor nerve increase regenerative capacity and improve recovery in rats (Nedeljković et al., 2017). Vitamin B12 exhibits a positive influence on Schwann cells proliferation and migration as well as on myelination of axons after end-to-side neurorrhaphy in rats (Liao et al., 2013). Vitamins B1, B6, and B12 express analgesic effects in experimental animal models with acute and chronic pain after the neuronal injury (Wang et al., 2005). Additionally, these vitamins promote neurite outgrowth and increase nerve conduction velocity in rat acrylamide-induced neuropathy (Fujii et al., 1996). The presented findings suggest that B complex vitamins may be considered as an effective adjuvant therapy of peripheral nerve injury in humans, although the exact mechanism of their action remains to be further explored.

2 AIMS

The goal of this study is to investigate the influence of vitamin B complex therapy on the process of nerve damage progression and regeneration of a damaged nerve after transection and reconstruction of the motor branch of the rat femoral nerve.

Based on literature data, the working hypothesis has been established:

Vitamin B complex therapy affects the expression of L-type $Ca_V1.2$ voltage-dependent calcium channels and improves recovery after transection and reconstruction of the motor branch of the femoral nerve (*n. femoralis*) using end-to-end neurorrhaphy.

According to the working hypothesis, the main goals of the research were determined:

To examine the impact of vitamin B complex therapy (B1, B2, B3, B5, B6, B12) on (1) presence and macrophage phenotype, (2) type of Schwann cells and macrophage/Schwann cells interaction, (3) degree of axonal regrowth, as well as (4) expression of $Ca_V1.2$ channels in examined cells during the early recovery period (first 14 days) after injury of the motor branch of rat femoral nerve.

To evaluate the effects of peripheral nerve injury and vitamin B complex therapy the specific aims were defined to:

1. Examine the expression of ED1 marker as the indicator of macrophage presence, expression of proinflammatory cytokines TNF-α and iNOS, and expression of anti-inflammatory cytokines IL-4 and IL-10 as differential markers of M1 and M2 macrophages;

2. Determine the expression of Schwann cells marker, protein S100;

3. Investigate the expression of protein GAP-43, which indicates the degree of axonal regrowth;

4. Characterize the expression of L-type $Ca_V1.2$ channels in all examined cells.

The proposed research of this dissertation could contribute to a better understanding of the molecular and cellular mechanisms underlying recovery after peripheral nerve injury and to elucidate the role of vitamin B in these processes not previously shown in the literature. Therefore, the results of this study could indicate whether vitamin B complex therapy could be an adjuvant therapy to standard surgical treatment of peripheral nerve injury.

3 MATERIAL AND METHODS

3.1 Materials

3.1.1 Ethics Committee Consent

The research was approved by the Ministry of Agriculture and Environmental Protection, Republic of Serbia, Veterinary Directorate No. 323-07-7363/2014-05/5.

3.1.2 Experimental animals

In the present study, adult male Albino Oxford (AO) rats, weighing 250 g to 300 g were used. The animals were raised under the same conditions in the vivarium of the Institute for Medical Research of the Military Medical Academy in Belgrade, where animal surgeries were performed. During the experiment, animals were kept in the same environmental conditions (temperature 23 ± 2 ℃, humidity 50 - 60% and light conditions 12 h/12 h light/dark cycle, with free access to food and water).

3.1.3 Reagents and solutions

The list of reagents and solutions used to perform the experimental tests and methods is shown in Tables 1 and 2.

Table 1. Reagents

Name	Manfacturer
DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride)	Invitrogen, SAD
Vitamin B Complex (Beviplex)	Hemofarm, Vršac
Triton X-100 (t-Octylphenoxypolyethoxyethanol)	Sigma, Germany

Table 2. Solutions

Name	Manufacturer
0,01 M PBS (Phosphate buffer saline), Na-phosphate buffer	50 ml 0.2 M PB; 8.7 g NaCl i do 1000 ml mqH2O
Citrate buffer, pH 6	1 g citric acid; 6,10 g Tri-Na-citrate; 500 ml dH2O
Mowiol	9,6 g Mowiol-a (Calbiochem, EMD Millipore, SAD); 24 ml glycerol (Lach Ner, Czech Republic)
Normal donkey serum (NDS)	Sigma, Germany

The list of primary and secondary antibodies used in the present study is given in Tables 3 and 4.

Table 3. Primary antibodies

Specificity	Description	Manufacturer	Dilution
Anti - ED1	mouse, monoclonal	Abcam	1:100
Anti - GAP-43	mouse, monoclonal	Chemicon	1:100
Anti - IL-10	goat, polyclonal	Santa Cruz	1:100
Anti - $Ca_V1.2$	rabbit, polyclonal	Sigma-Aldrich	1:200
Anti - S100β	mouse, monoclonal	Santa Cruz	1:200
Anti - TNF-α	goat, polyclonal	Santa Cruz	1:100

Table 4. Secondary antibodies

Reactivity	Conjugate	Description	Manufacturer	Dilution
against rabbit	Alexa Fluor 488	donkey	Invitrogen	1:200
	Alexa Fluor 350			1:200
against mouse	Alexa Fluor 568			1:200
	Alexa Fluor 350			1:200
against goat	Alexa Fluor 555			1:200

3.2 Methods

3.2.1 The experimental model of motor branch injury of rat femoral nerve

Controlled peripheral nerve transaction represents a well-characterized model for studying peripheral nerve regeneration after injury. The anesthetic procedure used for experimental animals (aged 2.5 – 3 months), implied intraperitoneal administration of ketamine (50 mg/kg; Ketalar, Eczacibasi, Turkey) and xylazine (5 mg/kg; Rompun, Bayer, Turkey). Afterwards, experimental animals were properly placed and fixed. Identification of the motor branch of the femoral nerve on the left hind leg of the rat was performed by skin incision in the left limb. Using a surgical microscope, the motor branch of the femoral nerve was isolated from the surrounding tissue and transected. Reconstruction of the proximal and distal nerve was executed with 9.0 surgical nylon sutures using the termino-terminal anastomosis technique, while the skin was sutured with 3.0 nylon sutures.

3.2.2 Experimental groups and animal treatment

After transection of the motor branch of the femoral nerve and its reconstruction using the technique of termino-terminal anastomosis, experimental animals were divided into 2 groups. The first group included operated animals (O) that were treated intraperitoneally with physiological solution. The second group consists of animals that were surgically treated in the same way but additionally treated with vitamin B complex (OT). The third control group consisted of "sham operated" animals (S) who underwent surgery as well as the first two groups of animals, but nerve transection was not performed. Animals from all three groups (O, OT, S) were examined 1, 3, 7, and 14 days post-operation (dpo). Based on this, the control and experimental groups were divided into 4 subgroups (Table 5). The first subgroup consists of animals examined in 1st dpo; the second subgroup consists of animals examined in 3rd dpo; the third subgroup consists of animals examined in 7th dpo; the fourth subgroup consists of animals examined in the 14 dpo. The animals of the OT group were treated with the vitamin B complex from the day of the surgery until the day of sacrifice. One dose contained a complex of vitamin B in the following concentrations: B1 (37 mg/kg/day), B2 (3.7 mg/kg/day), B3 (93 mg/kg/day), B5 (9.3 mg/kg/day), B6 (7.4 mg/kg/day), B12 (3.7 μg/kg/day). The vitamin B complex was applied intraperitoneally 15 minutes after the operation, and every 24 hours from the day of the operation (day 0) until the day of sacrifice (day 1, 3, 7, or 14). Control animals from O group were treated in the same intervals with physiological saline (PS). The effect of vitamin B complex was monitored until the 14th postoperative day. A schematic representation of the experiment is shown in Figure 5 and Table 5.

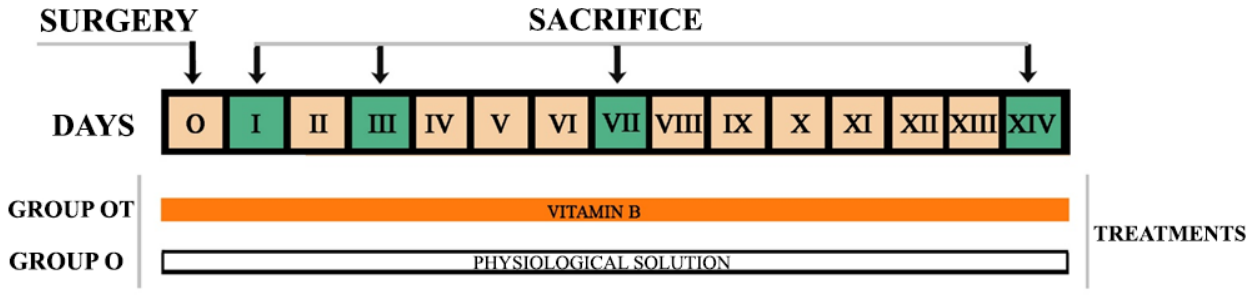

Table 5. Animal groups used in the experiment

Experimental group	Treatment	Number of treatments	Sacrifice day	Number of animals
"Sham operated" animals (S)	/	/	1. dpo	4
			3. dpo	4
			7. dpo	4
			14. dpo	4
Operated and treated animals (OT)	Vitamin B Complex	one	1. dpo	4
		three	3. dpo	4
		seven	7. dpo	4
		fourteen	14. dpo	4
Operated animals (O)	Physiological Saline	one	1. dpo	4
		three	3. dpo	4
		seven	7. dpo	4
		fourteen	14. dpo	4

3.2.3 Paraffin Tissue Preparation

The immunohistochemical procedure was carried out in the Laboratory for Pathohistology and Cytology HistoLab, Belgrade. Firstly, the nerve samples were collected and fixed in the 10% formaldehyde solution to maintain the tissue morphology and antigenicity of target molecules. Subsequently, the isolated tissue went through dehydration steps at room temperature (RT): (1) 3 × 30 min in 70% ethanol; (2) 3 × 30 min in 90% ethanol; (3) 3 × 30 min in 100% ethanol; and (4) 3 × 30 min in xylene. After dehydration, the tissue was incubated with the melted paraffin wax heated at 58 °C. Afterwards, the embedded tissue was cut using a

microtome (at a thickness of 5 μm). Sections were then dried at 56 °C in a water bath, padded onto microscope slides pre-coated with gelatin and dried overnight at room temperature (RT).

3.2.4 Immunofluorescence Staining

Detection of the target proteins on the nerve slides was performed using immunofluorescence (IF) staining method. For this procedure, the fluorescent-dye conjugated secondary antibody which binds to the unlabeled primary antibody was used. The IF staining procedure was done at RT, except for the incubation with the primary antibody, which was performed at a temperature of 4 °C. The solutions were prepared in 0.01 M PBS, pH 7.4, which was also used as a washing medium. Double IF staining was performed according to the following steps:

(1) Deparaffinization and rehydration: Microscope slides with paraffin-embedded sections were deparaffinized and washed in xylene 1, xylene 2, absolute alcohol, 95% alcohol, 70% alcohol, and distilled water, for 5 min in each solution. (2) Antigen retrieval: Antigenic epitope unmasking- microscope slides were incubated in 0.01 M sodium citrate buffer, pH=6, for 8 min at 99% – 100 °C, and then dried at RT for 30 min and 3 × 5 min and washed with PBS. (3) Blocking: Washed microscope slides were kept for 60 min in 5% blocking serum (originating from the same species as the secondary antibody) to inhibit nonspecific binding of the secondary antibody. In blocking serum, 0.5% Triton X-100 detergent was added, to enable membrane permeabilization. A primary antibody diluted in PBS was then administrated onto slides and incubated overnight at 4 °C temperature. Following day, slides were washed out 3 × 5 min in PBS and incubated with secondary antibody (diluted in PBS it specifically binds to the present primary antibody. Slides were next washed for 3 × 5 min in PBS. Regarding double or triple IF staining, the steps starting from the incubation in the blocking serum were repeated for the next markers. The primary and secondary antibodies used for IF labeling is demonstrated in Table 6. After incubation with the secondary antibodies, slides were washed 6 × 5 min in PBS, mounted with Mowiol (Calbiochem, Millipore, Germany) and allowed to dry overnight. Microscope slides that underwent the same IF procedure, but without the primary antibody administration, were used as a staining control.

Table 6. List of primary and secondary antibodies used for immunofluorescence labeling

Antibody	Source	Dilution	Company
Anti - $Ca_V1.2$	rabbit	1:200	Sigma-Aldrich, Munich, Germany
Anti - CD68 (ED1)	mouse	1:100	Abcam, Cambridge, MA, USA,
Anti - TNF-α	goat	1:100	Santa Cruz Biotechnology, CA, USA
Anti - iNOS	rabbit	1:100	Santa Cruz Biotechnology, CA, USA
Anti - IL-4	rabbit	1:100	Santa Cruz Biotechnology, CA, USA
Anti - IL-10	goat	1:100	Santa Cruz Biotechnology, CA, USA
Anti - Arg-1	rabbit	1:200	Sigma-Aldrich, Munich, Germany
anti-rabbit anti - IgG Alexa Fluor 488	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-mouse anti - IgG Alexa Fluor 555	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-goat anti - IgG Alexa Fluor 350	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-goat anti - IgG Alexa Fluor 488	donkey	1:200	Invitrogen,Carlsbad, CA, USA

3.2.5 Digital Image Processing

The images of the prepared nerve sections were obtained using Carl Zeiss Axiovert fluorescent microscope, equipped with the AxioCam monochromatic camera (Axio Observer Microscope Z1, ZEISS, Gottingen, Germany), at the magnifications of 20×, 40×, 63×, and 100× and saved in .tiff format. ApoTome software was used to compose images at 63× and 100× magnification. Co-localization on the obtained fluorescent images was performed using AxioVision Rel. 4.6 program, which represents a standard part of the Zeiss Axiovert microscope equipment. Afterwards, images were assembled and labeled in Photoshop CS6 (Adobe Systems). The quantification of single, double, or triple-positive cells from experimental groups (F, O, OT) was performed for each time point (1, 3, 7, 14 dpo), and gather from three independent experiments. High resolution digital images (600 pixels/inch) captured at 40× magnification (three images/group/independent experiment) were used for cells counting. The total number of

single, double, or triple-positive cells was counted manually by two independent observers using ImageJ open-source platform (National Institutes of Health, USA; http://imagej.nih.gov/ij/download.html) with ImageJ cell-counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) and Adobe Photoshop Creative Cloud (Version 14.0). Also, the percentage of double or triple-positive cells in some examined cells populations was calculated and presented.

3.2.6 Statistical Analysis

For statistical comparison between two experimental groups, a two-sided Student's t-test was used and a value of $p < 0.05$ or less was considered significant. Results were presented as mean values with standard error.

4 RESULTS

4.1 The effect of vitamin B complex treatment on abundance and phenotype of macrophages after PNI

4.1.1 Vitamin B complex treatment affects the morphology of macrophages after PNI

Macrophages play an important role in the pathogenesis of PNI as well as in tissue remodeling and repair following the nerve injury. These cells possess the ability to switch phenotype from proinflammatory ("classically activated" M1 macrophages) to anti-inflammatory ("alternatively activated" M2 macrophages) depending on the current microenvironment. This remarkable plasticity allows macrophages to participate sequentially in both the induction and the resolution of inflammation after the nerve injury (Porcheray et al., 2005).

Therefore, during the post-injury period (1, 3, 7, and 14 days post operation (dpo)) we examined the alteration in macrophage morphology in order to determine the impact of PNI and vitamin B complex treatment on the activation of macrophages (Figure 6A). ED1 antibody has been used as a marker of activated macrophages. Figure 6B represents the total number of $ED1^+$ cells, expressed as a number of $ED1^+/mm^2$.

The results demonstrated only a few $ED1^+$ cells in the sham-operated (S) group at all investigated time points (Figure 6A, B). On the other hand, in the operated (O) group, the number of $ED1^+$ cells dramatically increased after PNI, peaking at day 7 post operation. Most of these $ED1^+$ cells at 1 and 3 dpo exhibited morphology similar to the M1 phenotype, whereas at 7 and 14 dpo the majority of the cells acquired the M2 phenotype (Figure 6A, B).

Following the treatment with complex of B vitamins (OT group), the number of proinflammatory M1 macrophages at 1 dpo was reduced for approximately 45% (Figure 6B), while the number of M2 macrophages was increasing, starting from day 3 until 14 dpo (Figure 6A). This increase caused by B complex vitamin treatment was the most pronounced at 7 dpo (compared to the O group, the increase was 33%) (Figure 6B). These data demonstrated that there is a time-dependent change in the macrophage morphology after the PNI, which was manifested as a subsequent transition from M1 proinflammatory to M2 anti-inflammatory type, a process that may be accelerated via the vitamin B complex treatment.

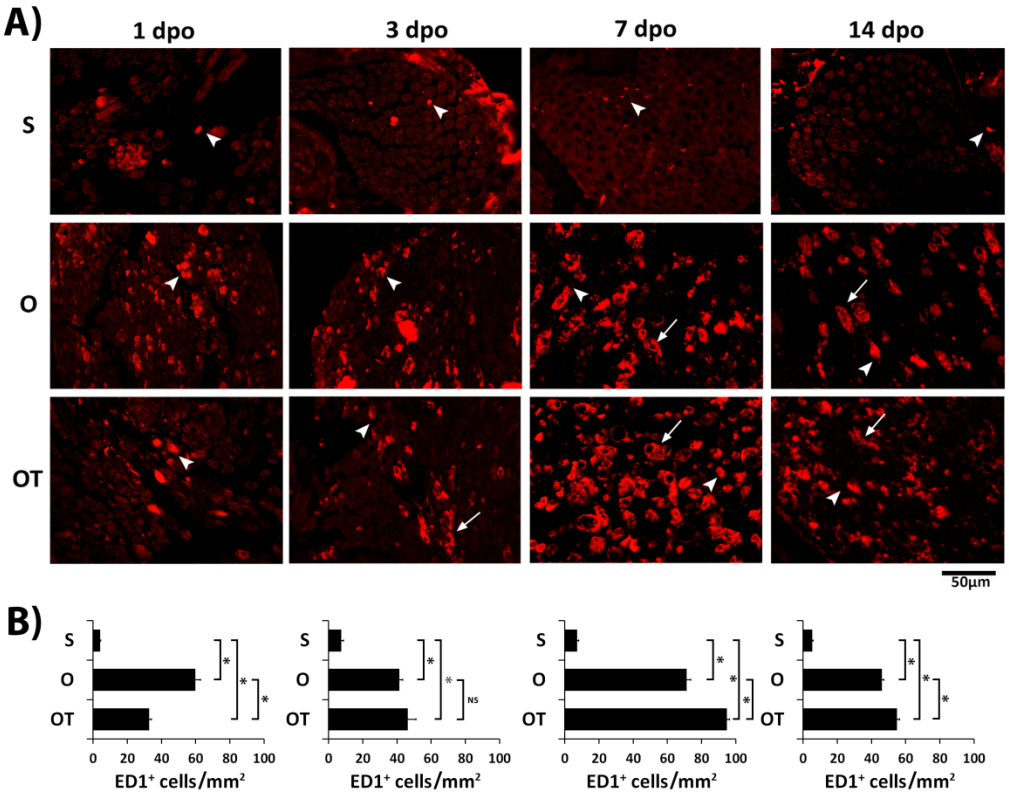

Figure 6. Effect of peripheral nerve injury (PNI) and treatment with B vitamins on macrophage morphology. Cross sections of femoral nerve obtained from the sham (S), operated (O, transection of motor branch and immediate reconstruction using termino–terminal anastomosis), and operated and treated with vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (red), which is a common marker of activated macrophages. **(A)** The representative images showed morphological changes of $ED1^+$ macrophages during the postoperative period (1, 3, 7, and 14 days) and after 1, 3, 7, and 14 injections of complex of B vitamins. Transition from the M1 (arrow heads) to M2 (arrows) morphology type in the O group is seen at day 7 and 14 post-injury. After treatment with B vitamins the appearance of M2 macrophages began after the third injection. Scale bar: 50 µm. **(B)** Total quantification of ED1-positive cells/mm^2 from experimental groups is depicted in the graphs (black bars). The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's t-test (* $p < 0.05$ *vs*. control, or *vs*. O group, as indicated at the graphs).

4.1.2 Treatment with vitamin B complex after PNI affects the expression of pro-inflammatory cytokines in macrophages

It has been demonstrated that two main pro-inflammatory marker proteins expressed in M1 macrophages are TNF-α and iNOS (Martinez and Gordon, 2014; Mills, 2012; Shapouri-Moghaddam et al., 2018).

Hence, the aim of our investigation was to determine if treatment with B complex vitamins would modulate the expression profile of these marker proteins in macrophages after the PNI.

The results indicate that there is a large number of $ED1^+/TNF\text{-}\alpha^+$ (Figure 7A insert) and $ED1^+/iNOS^+$ cells (Figure 7C, insert) (yellow fluorescence) at the 1st dpo. These cells had round and oval shapes and were spread over the nerve slices of O group.

After the treatment with B vitamins, the number of TNF-α and iNOS expressing $ED1^+$ macrophages was significantly reduced in OT group (Figures 7A and 7C, respectively). Figures 7B and 7D illustrate the total number of $ED1^+/TNF\text{-}\alpha^+$ cells and $ED1^+/iNOS^+$ cells expressed as number of double-positive cells/mm^2 and the percentage of double positive cells in the $ED1^+$ cell population. Similar staining pattern was observed at 3 dpo and after three treatments with B vitamins. At day 7 post-injury in both the O and OT group most of the $ED1^+$ macrophages had a morphological characteristic of M2 phenotype, but were not TNF-α and iNOS co-stained (Figures 7A and 7C, respectively). However, those $ED1^+$ macrophages that retained the oval/round shape were co-labeled with TNF-α and iNOS. Additionally, some $ED1^-$ cells were also co-labeled with both TNF-α and iNOS (Figure 7A and C, inserts, yellow arrows).

Altogether, these results indicate that following PNI there is an increased expression of proinflammatory mediators in M1 macrophages, whereas as time post-injury elapsed they were replaced with the M2 macrophages. Vitamin B complex treatment significantly decreased the number of $ED1^+$ macrophages expressing TNF-α and iNOS, thus exhibiting an anti-inflammatory effect, which was the most noticeable at the 1st and 3rd dpo.

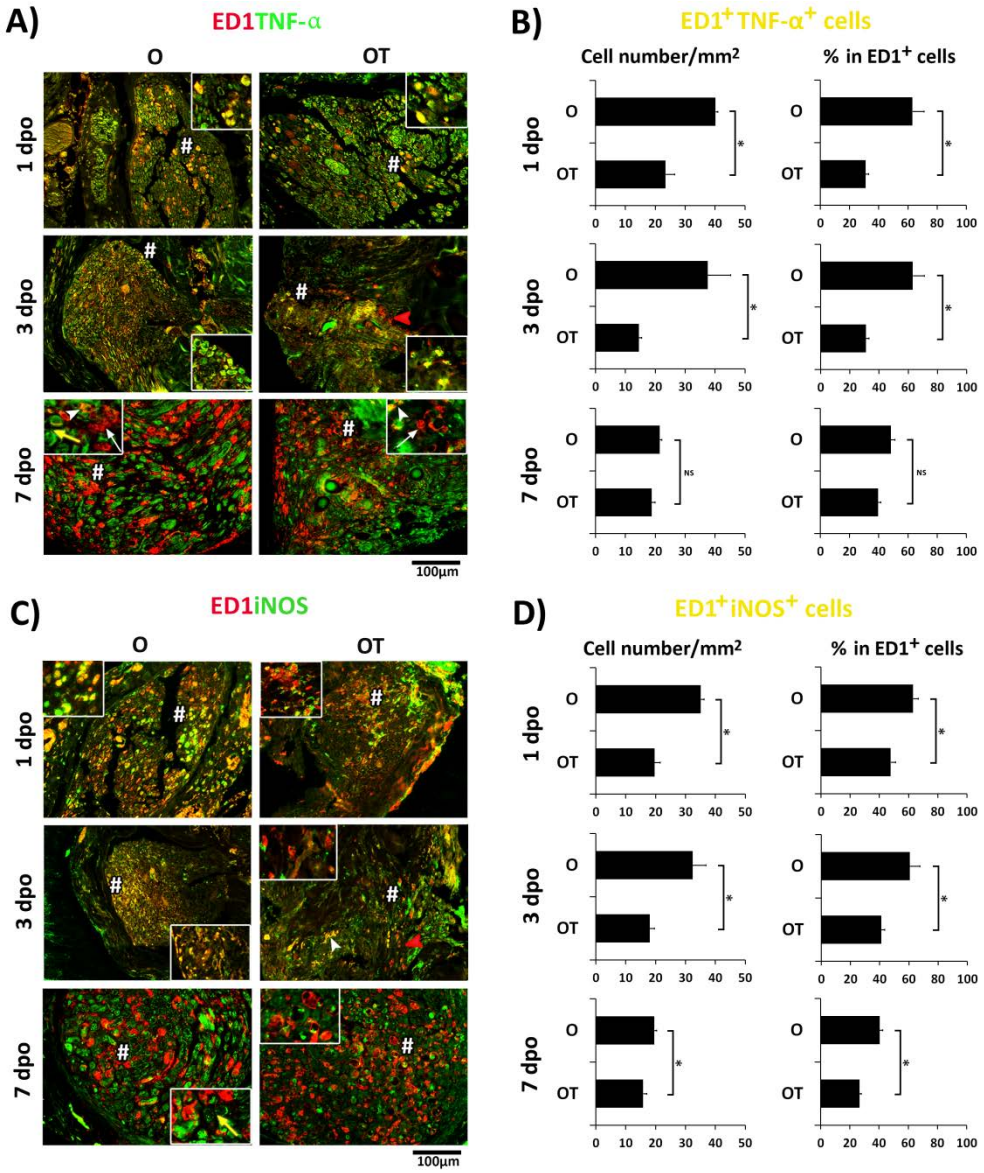

Figure 7. Treatment with B vitamins reduced PNI-induced expression of proinflammatory mediators in M1 macrophages. (**A**, **C**) Cross sections of femoral nerves obtained from the operated (O) and operated and treated with vitamin B complex (OT) groups were counterstained with (**A**) anti-TNF-α (M1 marker, green) and anti-ED1 (red) or with (**C**) anti-iNOS (M1 marker, green) and anti-ED1 (red) antibodies. The quantification of double-positive ED1$^+$/TNF-α$^+$ cells (**B**) and ED1$^+$/iNOS $^+$ cells (**D**) is presented as number of double-positive cells/mm^2 and the percentage of double-positive cells in the ED1$^+$ cell population. The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p< 0.05$ OT vs. O group, as indicated at the graphs). At day 1 and 3 post operation (dpo) ED1$^+$/TNF-α$^+$, as well as ED1$^+$/iNOS$^+$ macrophages in both O and OT groups, had oval

and round morphology and showed complete overlapping (yellow fluorescence)(inserts). Treatment with B vitamins reduced TNF-α and iNOS staining and the majority of macrophages were only $ED1^+$ (red arrow head). At day 7 post-injury most of macrophages were only $ED1^+$ and were polarized toward M2 type (white arrows, insert), while only a few $ED1^+$/TNF-α^+ (white arrow head, insert) macrophages were noticed. Some $ED1^-$ cells (yellow arrows) that were both TNF-α^+ and $iNOS^+$ were also noticed. # indicates where the high magnification images in inserts are taken from. Scale bar: 100 µm.

4.1.3 Vitamin B complex treatment applied after the PNI affects the expression of anti-inflammatory cytokines in macrophages

The potential of vitamin B complex to modulate the expression of anti-inflammatory cytokines, IL-4 and IL-10, in $ED1^+$ macrophages after PNI was examined as well (Figure 8A and C, respectively).

Cytokine IL-4 was highly expressed in M2a, while IL-10 was expressed in M2c subtype of M2 macrophages, as previously demonstrated (Chen et al., 2011; Mantovani et al., 2004; Rőszer, 2015; Wang et al., 2014b).

In the O group, there was a complete overlapping (yellow) of IL-4 (green) and ED1 (red) staining in round and oval-shaped macrophages at day 3 post-operation (Figure 8A, insert).

Following the treatment with B vitamins, macrophages exhibited morphology described as “foamy” and were entirely labeled with anti-IL-4 antibody (Figure 8A). There was a large number of round and oval-shaped $ED1^+$/$IL\text{-}10^+$ macrophages in the O group. In the OT group only $ED1^+$ macrophages with small, round, and oval cell bodies (Figure 8C) were labeled with anti-IL-10 antibody, while large $ED1^+$ macrophages were not labeled. At day 7 post-operation the majority of $ED1^+$ cells exhibited a “foamy” morphology and these cells were IL-4 negative in both O and OT groups (Figure 8C). Furthermore, there were several $ED1^+$/$IL\text{-}4^+$ labeled macrophages with round and oval cell bodies, as well as some $ED1^-$/$IL\text{-}4^+$ labeled cells. Additionally, at 7 dpo, IL-10 labeled $ED1^-$ cells in both O and OT groups were abundant. $ED1^+$ macrophages with “foamy” morphology in the O group did not express IL-10 while those with an oval morphology expressed IL-10 in the same group. The latter were less abundant when comparing these two macrophage types. On the other hand, following the vitamin B treatment foamy $ED1^+$ also expressed $IL\text{-}10^+$, while round and oval-shaped macrophages were only $ED1^+$.

The number of ED1$^+$/IL-4$^+$ cells and ED1$^+$/IL-10$^+$ was reduced after the treatment with vitamin B complex at 14 dpo. On the contrary, the contribution of these cells in total ED1$^+$ cell population in both O and OT group were equivalent. Figures 8B and 8D illustrate the total number of ED1$^+$/IL-4$^+$ cells and ED1$^+$/IL-10$^+$ cells, respectively, which are expressed as the number of double-positive cells/mm^2 and the percentage of double-positive cells in the ED1$^+$ cell population. Based on the previous data, it can be concluded that PNI as well as vitamin B treatment may induce the manifestation of different subtypes of M2 macrophages.

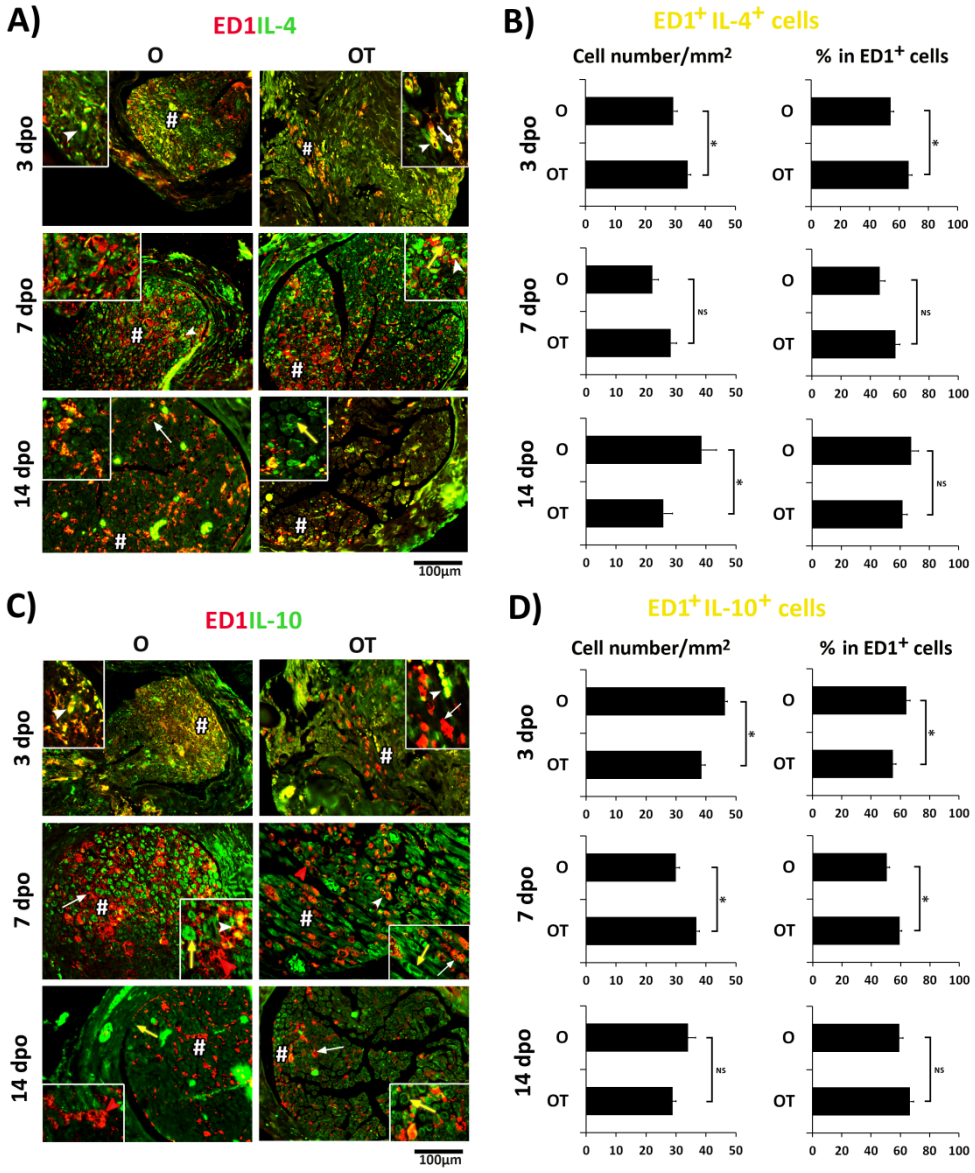

Figure 8. Effects of PNI and B vitamin treatment on expression of anti-inflammatory cytokines in M2 macrophages. (A, C) Cross sections of femoral nerve obtained from the operated (O) and operated

and treated with vitamin B complex (OT) groups were counterstained with (A) anti-IL-4 (M2a marker, green) and anti-ED1 (red) or with (C) anti-IL-10 (M2c marker, green) and anti-ED1 (red) antibodies. The quantification of double-positive $ED1^+/IL\text{-}4^+$ cells (B) and $ED1^+/IL\text{-}10^+$ cells (D) is presented as number of double-positive cells/mm^2 and the percentage of double positive cells in the $ED1^+$ cell population. The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's t-test (* $p < 0.05$ OT *vs.* O group, as indicated at the graphs). At day 3 post-injury $ED1^+/IL\text{-}4^+$ as well as $ED1^+/IL\text{-}10^+$ macrophages with oval and round morphology in both the O and OT groups showed complete overlapping (yellow fluorescence, white arrow head) (inserts). Treatment with B vitamins increased IL-4 immunoreactivity in "foamy" M2 macrophages (white arrow), while IL-10 staining was reduced and the majority of M2 macrophages were only $ED1^+$ (white arrow). At 7 and 14 dpo IL-4 and IL-10 staining was seen in $ED1^-$ cells in both groups (yellow arrows). M2 macrophages were void of IL-4 at day 7, but IL-4 was abundantly present at day 14 (white arrow). $ED1^+/IL\text{-}10^+$ M2 macrophages were seen at 7 dpo (white arrows), but were sparsely present at day 14. # indicates where the high magnification images in inserts are taken from. Scale bar: 100 μm.

4.2 The effect of vitamin B complex treatment on interaction between macrophages and Schwann cells after PNI and on PNI – induced changes in Schwann cells type

4.2.1 Vitamin B complex treatment after PNI affects the interaction between macrophages and Schwann cells

Femoral nerve motor branch was used to examine the effects of vitamin B complex treatment on the interaction between macrophages and Schwann cells during the recovery period after PNI. The control group (sham control group, S) was compared to the operated (O) group and operated group after the administration of 1, 3, 7, and 14 intraperitoneal injections of vitamin B complex (OT group). Various investigation time points were used – 1, 3, 7, and 14 days post operation. ED1 antibody (anti-CD68) has been used as a marker of activated macrophages, whereas S100 antibody was utilized as a marker of Schwann cells (Liu et al., 2015; Mata et al., 1990; Shearman and Franks, 1987).

The overlapping between CD68 and S100 was anticipated via double immunofluorescence staining. As previously mentioned only a small number of $ED1^+$ cells were discovered in the sham operated (S) group, and this number did not significantly change during

the investigated time intervals (Figures 9(I)–13(I) A, D, G, white arrow heads and 13 (I) A, D, G, J). The majority of Schwann cells was stained with S100 and had the morphology of myelin-forming Schwann cells, while no overlapping of ED1 and S100 immunoreactivity was observed.

Total number of $ED1^+$ cells in the O group was elevated at day 1 post-operation. These macrophages were round and oval-shaped (Figure 9(I) B, E, H, white arrow heads) similar to the M1-type and were organized in clusters around the dark spots (Figure 9(I) B, E, K, red arrow heads) composed of Schwann cells with low or no S100 immunoreactivity. Besides these Schwann cells, there were also intensively stained $S100^+$ cells (Figure 9(I) K, yellow arrow heads) which had myelinating Schwann cells morphology, but without overlapping of ED1 and S100 immunoreactivity.

After the administration of one injection of vitamin B complex, the number of $ED1^+$ cells declined (Figure 9(I) I, white arrow heads), whereas the morphology of $S100^+$ Schwann cells resembled to those detected in the S group (Figure 9(I) F and L, yellow arrow heads). Figure 9(II) illustrates the total number of $ED1^+/S100^+$ cells expressed as a number of double positive cells/mm^2, while in Figure 9(III) are expressed as the percentage of double positive cells in the whole cell population. In the O group, the number of $ED1^+/S100^+$ cells was insignificant (10.04 ± 0.93/mm^2 , which was 9.86 ± 0.76% of total $ED1^+$ cells), while in the OT group the number of double positive cells was slightly higher (1.5-fold) but still represents a minor part (17.72 ± 0.62%) of total $ED1^+$ cell number.

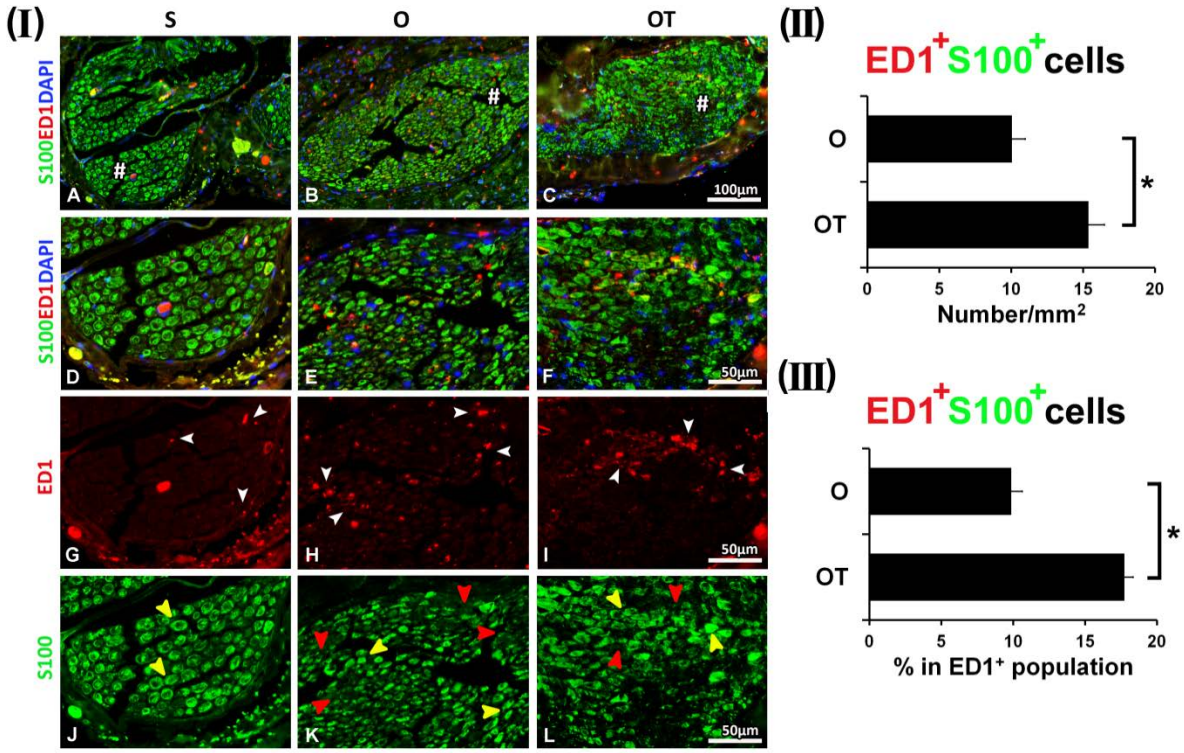

Figure 9. Effect of peripheral nerve injury (PNI) and treatment with B vitamins on Schwann cells-macrophages co-localization at the first day post operation (dpo). (**I**) Cross sections of femoral nerve obtained from sham (S), operated (O, the transection of the motor branch and immediate reconstruction using termino-terminal anastomosis) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (anti-CD68, red) antibody, a marker of activated macrophages. Anti-S100 antibody (green) was used as a marker of Schwann cells. The sections were counterstained with DAPI (blue) to visualize cell nuclei. The representative images demonstrated that in the S group only a few $ED1^+$ cells were detected (**G**, white arrow heads), most of the Schwann cells had morphology of myelin-forming Schwann cells and were intensively stained with S100 (**J**, yellow arrow heads). There was negligible overlapping of ED1 and S100 immunoreactivity (**A**–**D**). In the O group, $ED1^+$ macrophages with round and oval morphology of the M1 type were abundantly present (**H**, white arrow heads), particularly around the dark spots (**E**, **K**, red arrow heads), consisting of Schwann cells with low S100 immunoreactivity. $S100^+$ cells (**K**, yellow arrow heads) of myelinating Schwann cells morphology were also seen, but without ED1 and S100 immunoreactivity overlapping (**B**, **E**). After one vitamin B complex injection, the number of $ED1^+$ cells was decreased (**I**, white arrow heads). $S100^+$ cells of the myelinating Schwann cells morphology were also present (**F** and **L**, yellow arrow heads). # indicates where the high

magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**L** = 50 µm.(**II**) The quantification of double positive ED1$^+$/S100$^+$ cells from the O and OT experimental groups is depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the ED1$^+$ cells population. The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's t-test (*p< 0.05 vs. O group, as indicated at the graphs).

In both O and the OT group at day 3 post-operation there were ED1$^+$/S100$^+$ cells (yellow fluorescence) (Figure 10(I) E and F, yellow arrows). Figure 10(I) H and Figure 10(I) I represent ED1$^+$ macrophages closely related to Schwann cells, which have a "foamy" morphology resembling to M2 type, and were more present in the OT group.

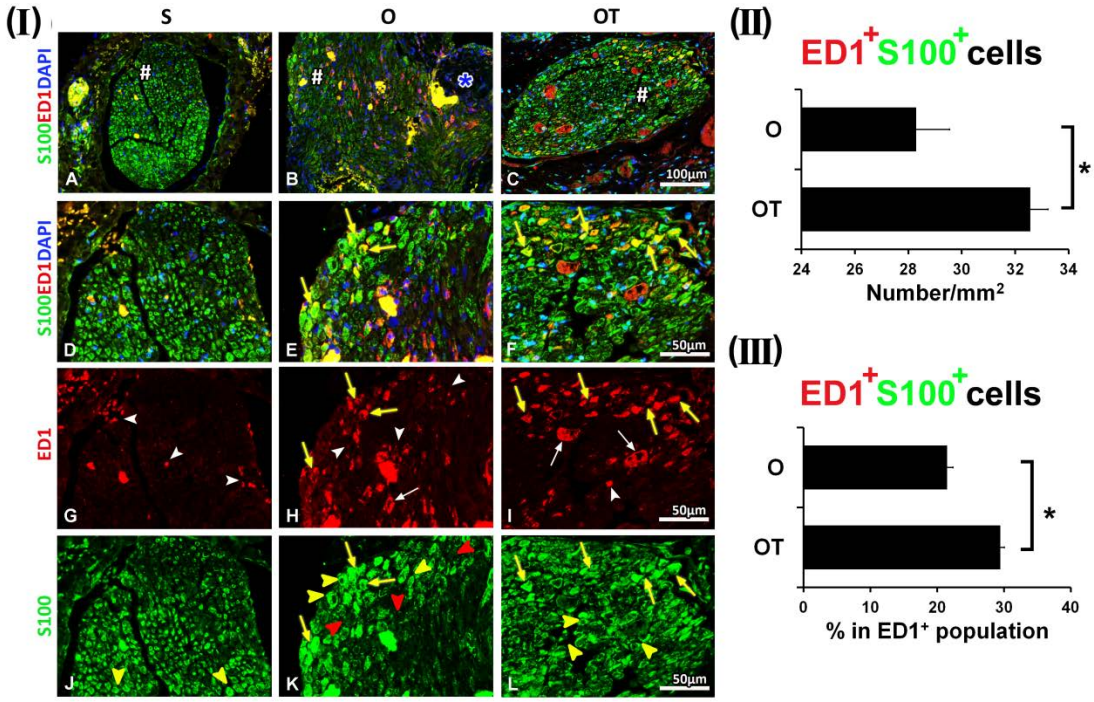

Figure 10. Effect of PNI and the treatment with B vitamins on Schwann cells-macrophages co-localization at the third dpo. (**I**) Cross sections of the femoral nerve obtained from the sham (S), operated (O, the transection of the motor branch) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (red), a marker of activated macrophages, anti-S100 antibody (green) as a marker of Schwann cells, and

counterstained with DAPI (blue) for visualizing cell nuclei. In the S group, a paucity of $ED1^+$ cells was detected (**G**, white arrow heads), myelinating, mature Schwann cells were the predominant cell type (**J**, yellow arrow heads) and there was no overlapping of ED1 and S100 immunoreactivity (**A**, **D**). In the O and OT groups $ED1^+/S100^+$ cells (**E** and **F**, yellow arrows) were noticed, whereby $ED1^+$ macrophages, closely associated to Schwann cells, had "foamy" morphology of M2 type (**H** and **I**, yellow arrows). In both groups, $ED1^+$ macrophages with the "foamy" morphology (**H** and **I**, white arrows), and Schwann cells that were only $S100^+$ (**K** and **L**, yellow arrow heads) were noticed as well. In the O group some $ED1^+$ macrophages of the M1 type morphology (**H**, white arrow heads), were detected around faintly stained Schwann cells (**K**, red arrow heads). Blue asterisk marks the site of transection and immediate reconstruction by termino-terminal anastomosis, while # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 μm, **D**–**L** = 50 μm.(**II**) The quantification of double positive $ED1^+/S100^+$ cells from the O and OT experimental groups is depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the $ED1^+$ cells population. The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's t-test (*p< 0.05 vs. O group, as indicated at the graphs).

Figure 10(II) illustrates the total number of $ED1^+/S100^+$ cells expressed as a number of double positive cells/mm^2, whereas Figure 10(III) represents the percentage of double positive cells in the total population of $ED1^+$ cells. In the O group, the number of $ED1^+$ macrophages that co-localized with $S100^+$ Schwann cells was 28.29 ± 1.26/mm^2, which corresponds to 21.44 ± 0.91% of total $ED1^+$ cells. Next, in the OT group, the total number of $ED1^+/S100^+$ cells was 32.56 ± 0.67/mm^2, or in percentage 29.42 ± 0.66% of total $ED1^+$ cells. Additionally, in both O and OT group, there were macrophages with "foamy" morphology that were only $ED1^+$ (Figure 10(I) H and I, white arrows), and Schwann cells that were only $S100^+$ (Figure 10(I) K and L, yellow arrow heads). In the O group there were also some $ED1^+$ macrophages resembling M1-type morphology (Figure 10(I) H, white arrow heads), which were observed around Schwann cells with low S100 immunoreactivity (Figure 10(I) K, red arrow heads).

During the recovery period, the most notable pattern of interaction between macrophages and Schwann cells was noticed at day 7 dpo (Figure 11). In both groups (O and OT) there were great amounts of $ED1^+$ macrophages, which mainly had M2-like morphology, while only a small

number of M1-likeED1^{+} macrophages were detected (Figure 11(I) H and I, white arrows and white arrow heads, respectively).There was a wide spread distribution of the dark spots (Figure 11(I) B, E, K), that contained Schwann cells with low S100 immunoreactivity (Figure 11(I) K, red arrow heads) in the O group. These dark spots were enclosed with plenty of ED1^{+}/S100^{+} macrophages (yellow fluorescence) (Figure 11(I) E and H, yellow arrows) which had the morphology between the M1- and M2-type. Figure 11(II) represents the number of ED1^{+}/S100^{+}cells per mm^{2}, while in the Figure 11(III) these double positive cells were expressed as the percentage in the total ED1^{+} cells population. In the O group, the total number of ED1^{+} macrophages that co-localized with S100^{+} Schwann cells was 24.94 ± 0.72/mm^{2}, or in percentage– 21.22 ± 0.76% of total ED1^{+} cells. On the other hand, in the OT group there was a statistically significant higher number of co-localized cells –37.41 ± 1.21/mm^{2} representing 29.23 ± 0.54% of the total ED1^{+} cell population. Furthermore, there were some macrophages that were only ED1^{+}and some of them have shown “foamy”, M2-like morphology, (Figure 11(I) H, white arrows), while others displayed the M1-like morphology (Figure 11(I) H, white arrow heads). After the administration of seven consecutive injections of B vitamin complex, the number of ED1^{+}/S100^{+} cells increased (Figure 11(I) F, yellow arrows), ED1^{+} macrophages resembled to M2-type (Figure 11(I) I, yellow arrows), and were closely related to S100^{+} Schwann cells with the non-myelinating morphology (Figure 11(I) L, red arrows). There was no co-localization of the ED1 immunoreactivity with the S100^{+}myelinating Schwann cells (Figure 11(I) L, yellow arrow heads).

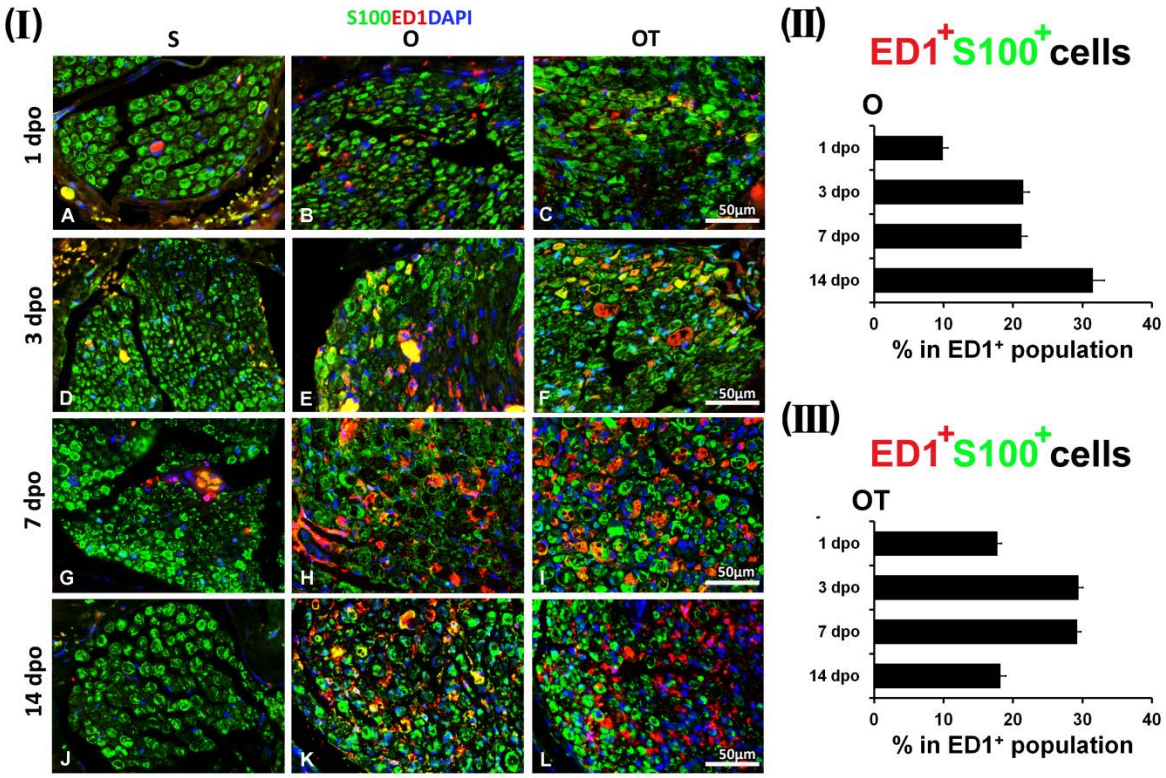

Figure 11. Time course immunohistochemical analysis of macrophages/Schwann cells co-localization after PNI and the treatment with B vitamins. (**I**) Comparative analysis of macrophages/Schwann cells crosstalk in the cross sections of the femoral nerve obtained from the sham (S) group at different time points (**A**—1 dpo; **D**—3 dpo; **G**—7 dpo; **J**—14 dpo). Time course of changes in macrophages/Schwann cells co-localization was analyzed in the operated (O) femoral nerve during the 1, 3, 7, and 14 days of the postoperative period (**B**—1 dpo; **E**—3 dpo; **H**—7 dpo; **K**—14 dpo) and after 1, 3, 7, and 14 injections of the complex of B (B1, B2, B3, B5, B6, and B12) vitamins (OT group) (**C**—1 dpo; **F**—3 dpo; **I**—7 dpo; **L**—14 dpo). ED1 (red) was used as a common marker of activated macrophages, anti-S100 antibody (green) as a marker of Schwann cells and DAPI (blue) for visualizing cell nuclei. Scale bar: 50 µm. Time-dependent changes in the percentage of double positive $ED1^{+}/S100^{+}$cells in $ED1^{+}$cells population from the (**II**) O and (**III**) OT experimental groups was depicted in the graphs (black bars). The data were shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured).

In both O and OT groups, M2-like $ED1^{+}$ macrophages seemed to be predominant by the fourteenth day (Figure 12(I) H and I, white arrows). Regarding Schwann cells, the most prevalent phenotype was the myelin-forming phenotype (Figure 12(I) K and L, yellow arrow

heads). ED1^{+}/S100^{+} cells (Figure 12(I) E, yellow arrows) were detected mainly in the O group. These macrophages expressed M2-like morphology and were related to the S100^{+} non-myelinating Schwann cells (Figure 12(I) K, red arrow) and some myelinating Schwann cells (Figure 12(I) K, yellow arrows). However, in the OT group, the co-localization of the ED1 and S100 immunoreactivity decreased (Figure 12(I) F), showing that after 14 injections of complex B vitamins, Schwann cells completely transformed to myelin-forming phenotype. Figure 12(II) represents the number of counted ED1^{+}/S100^{+} cells expressed as a number of these double positive cells per mm^{2}. Figure 12(III) represents the number of counted ED1^{+}/S100^{+} cells expressed as the percentage of double positive cells in the total population of ED1^{+} cells. In the O group, the number of double positive cells was 21.87 ± 1.38/mm^{2} which represented 31.49 ± 1.66% of total ED1^{+} cells. On the contrary, the number of these cells in the OT group was statistically lower 15.44 ± 0.69/mm^{2} and the corresponding percentage was only 18.17 ± 0.78%.

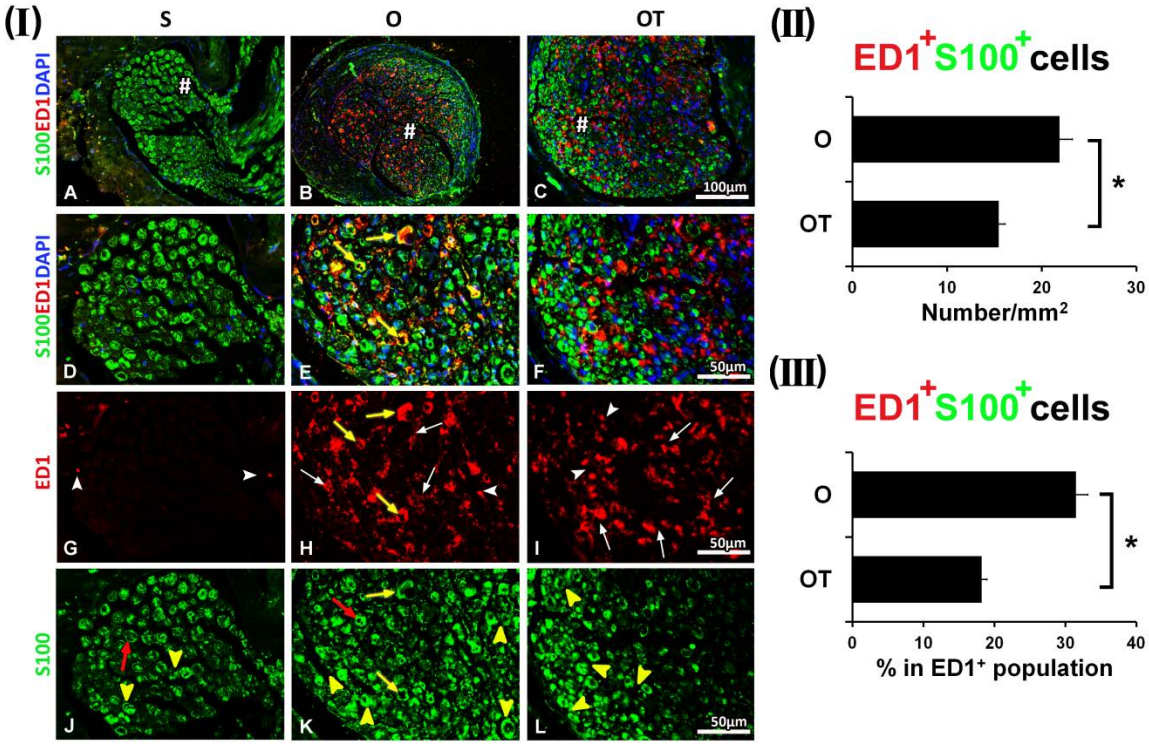

Figure 12. Effect of PNI and the treatment with B vitamins on Schwann cells-macrophages co-localization at the fourteenth dpo. (**I**) ED1 (red), a common marker of activated macrophages, anti-S100 antibody (green), a marker of Schwann cells and DAPI (blue) for visualizing cell nuclei, were used for immunostaining of the femoral nerve cross sections of the sham (S), operated (O, the transection of the motor branch) and operated and treated with the vitamin B

complex (B1, B2, B3, B5, B6, and B12) (OT) groups. Almost no $ED1^+$ cells were detected (**G**, white arrow heads) in the S group. Apart from myelinating Schwann cells (**J**, yellow arrow heads), some $S100^+$ Schwann cells of the non-myelinating morphology (**J**, red arrows) were seen as well. There was no overlapping of ED1 and S100 immunoreactivity (A, D). $ED1^+$ macrophages with the M2-type morphology were predominant in the both O and OT groups (**H** and **I**, white arrows), while $S100^+$ Schwann cells mostly belong to myelin-forming Schwann cells (**K** and **L**, yellow arrow heads). $ED1^+/S100^+$ cells (**E**, yellow arrows) were detected only in the O group. $ED1^+$ macrophages of the M2-type morphology were associated (**E**, yellow arrows) to the $S100^+$ non-myelinating Schwann cells (**K**, red arrow) and some myelinating Schwann cells (**K**, yellow arrows). Minor overlapping of ED1 and S100 immunoreactivity (**C**, **F**) was obtained in the OT group. # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**L** = 50 µm. (**II**) The quantification of double positive $ED1^+/S100^+$ cells from the O and OT experimental groups was depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the $ED1^+$ cells population. The data were shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's t-test (*$p< 0.05$ *vs.* O group, as indicated at the graphs).

Figure 13(I) represents the comparison of the femoral nerve cross sections between S, O, and OT groups during the investigation period (1, 3, 7, and 14 dpo) and after the administration of vitamin B complex injections (1, 3, 7, and 14 doses). As previously mentioned, within the cross nerve sections of the S group, $ED1^+$ cells were detected in a small quantity and were not changed during the investigated time intervals. The most prevalent cell type was myelinating mature Schwann cells. Also, there was no overlapping of the ED1 and S100 immunoreactivity (Figure 13(I)A, D, G and J).

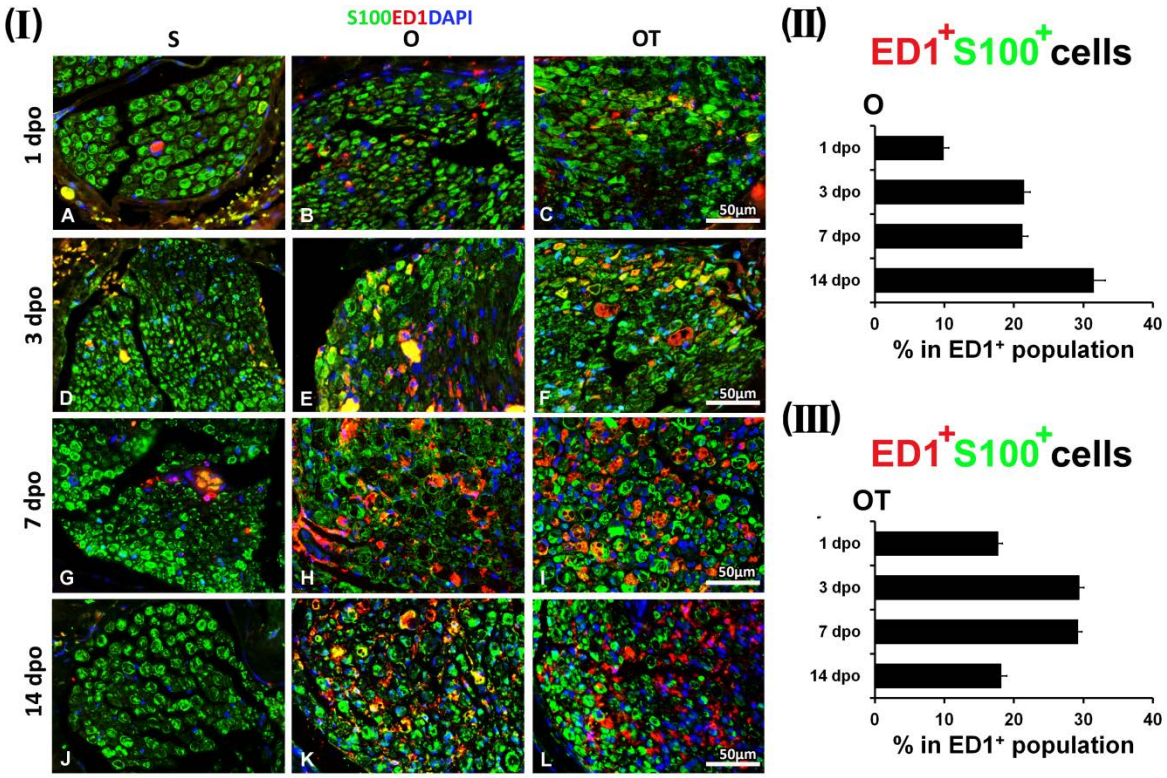

Figure 13. Time course immunohistochemical analysis of macrophages/Schwann cells co-localization after PNI and the treatment with B vitamins. (**I**) Comparative analysis of macrophages/Schwann cells crosstalk in the cross sections of the femoral nerve obtained from the sham (S) group at different time points (**A**—1 dpo; **D**—3 dpo; **G**—7 dpo; **J**—14 dpo). Time course of changes in macrophages/Schwann cells co-localization was analyzed in the operated (O) femoral nerve during the 1, 3, 7, and 14 days of the postoperative period (**B**—1 dpo; **E**—3 dpo; **H**—7 dpo; **K**—14 dpo) and after 1, 3, 7, and 14 injections of the complex of B (B1, B2, B3, B5, B6, and B12) vitamins (OT group) (**C**—1 dpo; **F**—3 dpo; **I**—7 dpo; **L**—14 dpo). ED1 (red) was used as a common marker of activated macrophages, anti-S100 antibody (green) as a marker of Schwann cells and DAPI (blue) for visualizing cell nuclei. Scale bar: 50 µm. Time-dependent changes in the percentage of double positive $ED1^+/S100^+$cells in $ED1^+$ cells population from the (**II**) O and (**III**) OT experimental groups were depicted in the graphs (black bars). The data were shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured).

As presented in the illustration (Figure 13(I) E, H and K), an intensive overlapping of the ED1/S100 immunoreactivity was seen at the third, seventh, and fourteenth dpo in the O group, while the same was detected after the treatment of three and seven vitamin B complex injections

in the OT group (Figure 13(I) F, I). Close ED1/S100 interactions were shown only between macrophages with the M2-like morphology and non-myelinating Schwann cells, as well as between the macrophages with the M2-like morphology and Schwann cells with low S100 immunoreactivity. However, no interactions between mature, myelinating Schwann cells and M2- or M1- type macrophages were noticed in the both O and OT group. The temporal pattern of $ED1^+/S100^+$ participation in the total $ED1^+$ cells population in the femoral nerve cross sections from O (Figure 13(II)) and OT experimental groups (Figure 8(III)) is demonstrated in the graphs (black bars). It was revealed that the fraction of $ED1^+/S100^+$ cells in the total $ED1^+$ population fluctuated over the recovery period. At the fourteenth dpo, in the O group, the participation of $ED1^+/S100^+$ cells in the $ED1^+$ cells population was elevated over the time post-injury reaching 31.49 ± 1.66%, and when compared to the first dpo a three-fold increase was noted.

It is important to note that in the OT group, treatments with vitamin B complex caused an increase in the fraction of $ED1^+/S100^+$cells, reaching 30% even after three injections and it didn't change for seven days. On the other hand, after 14 days, administration of the vitamin B complex decreased the number of $ED1^+/S100^+$ cells and the percentage in the total $ED1^+$ cell population was only 18.17 ± 0.78%. As only minor interaction between macrophages and mature, myelin-forming Schwann cells was detected, obtained data imply that administration of the B vitamin complex after 14 days induced the conversion to mature, myelin-forming Schwann cells. According to all above- mentioned data, it can be suggested that PNI modulates interactions between macrophages and Schwann cells in a time-dependent manner, while the treatment with the B vitamins complex stimulates the progression from the non-myelin to myelin-forming Schwann cells type and from M1- to M2-like macrophage morphology.

4.2.2 Vitamin B complex treatment applied after PNI affects the expression of proinflammatory cytokine TNF-α in Schwann cells

Furthermore, we investigated the PNI impact on the expression profile of proinflammatory cytokine TNF-α in Schwann cells and whether the treatment with B vitamins could alter this TNF-α expression pattern after the PNI.

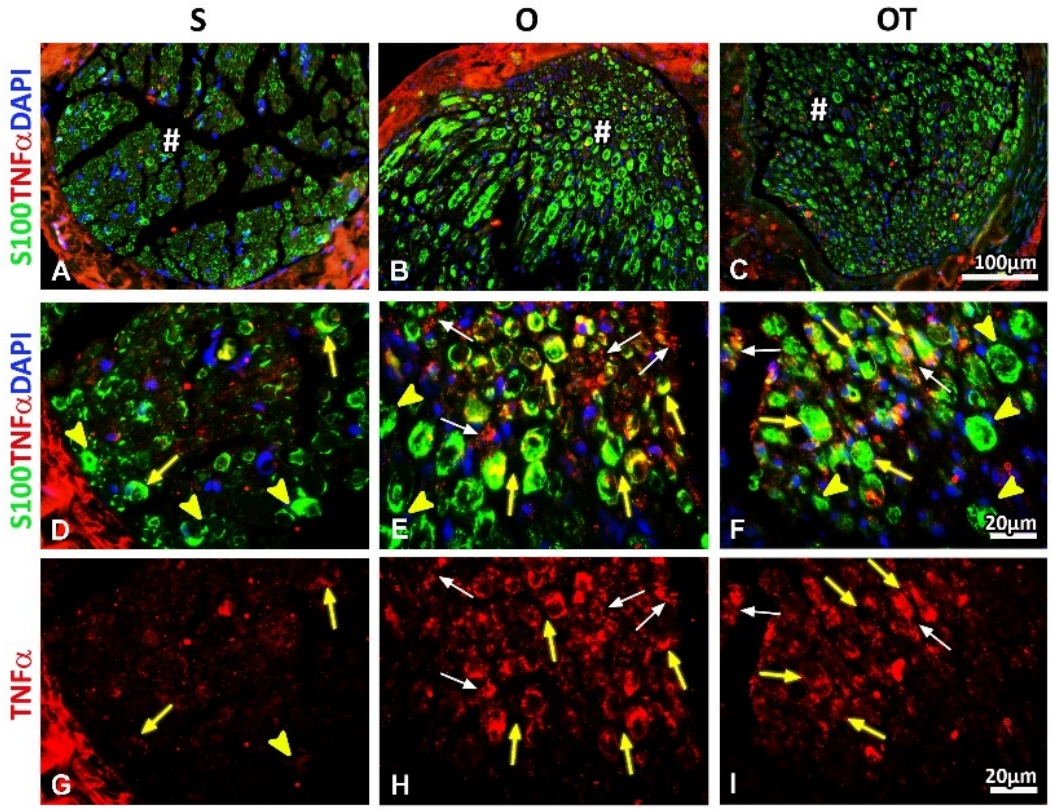

Figure 14. Effects of PNI and the B vitamins treatment on expression of proinflammatory cytokine tumor necrosis factor alpha (TNF-α) in Schwann cells. Femoral nerve cross sections obtained from the: sham (S: **A, D, G**), operated (O: **B, E, H**) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT: **C, F, I**) group immunostained for TNF-α (red) demonstrated strong increase of immunofluorescence intensity in the O group (H) compared to the S group (**G**) at the fourteenth dpo. Immunofluorescence staining for TNF-α protein was observed in some (**E**, **F**. **H**, **I**, yellow arrows), but not all Schwann cells (**E**, **F**. **H**, **I**, yellow arrow heads) as detected by co-localization with S100 immunostaining (green). DAPI (blue) was used for visualizing cell nuclei. Administration of 14 injections of the vitamin B complex (OT group) reduced TNF-α expression (**I**). TNF-α immunoreactivity was detected in some $S100^{+}$ myelinated Schwann cells (**F**, yellow arrows). In addition, TNF-α immunoreactivity was demonstrated in macrophages with the M2-like morphology (**E**, **F**, **H**, **I**, white arrows). # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**I** = 20 µm.

At the 14 dpo, in the present model of the femoral nerve transection, an increased expression of TNF-α within cross sections of the operated nerve (O) (Figure 14H) compared to

the sham-operated controls (S) (Figure 14G) was detected. The treatment with 14 injections of the vitamin B complex (OT group) decreased the TNF-α expression (Figure 14I), which was, however, still higher compared to the S group (Figure 14G). Interestingly, besides in macrophages with the M2-like morphology (Figure 14E, F, H, I, white arrows), the TNF-α immunoreactivity was observed in some (Figure 14E, F, H, I, yellow arrows), but not all Schwann cells (Figure 14E, F, H, I, yellow arrow heads).

4.2.3 Vitamin B complex treatment applied after PNI affects the expression of anti-inflammatory cytokine IL-10 in Schwann cells

The expression of anti-inflammatory cytokine IL-10 in Schwann cells in all of the investigated groups (S, O, and OT) was also determined. IL-10 immunoreactivity was mostly represented in the O group (Figure 15H), but it was shown in all other groups as well (Figure 15G, H and I). Interestingly, an enormous portion of IL-10 immunoreactivity was detected in IL-10^+ cells resembling M2-macrophages, although a huge number of IL-10^+/$S100^+$ cells was also present (Figure 15E and F, yellow arrows). Regarding OT group, although the largest part of $S100^+$ Schwann cells were IL-10^- (Figure 15F, yellow arrow heads), the overlapping of IL-10/S100 immunoreactivity (Figure 15F and I, yellow arrows) was observed in mature, myelinating Schwann cells. In addition, nearly identical pattern of IL-10 immunoreactivity was shown in the S group, nevertheless IL-10^+/$S100^+$ cells were displayed in minor quantity (Figure 15D and G, yellow arrows, and yellow arrow heads).

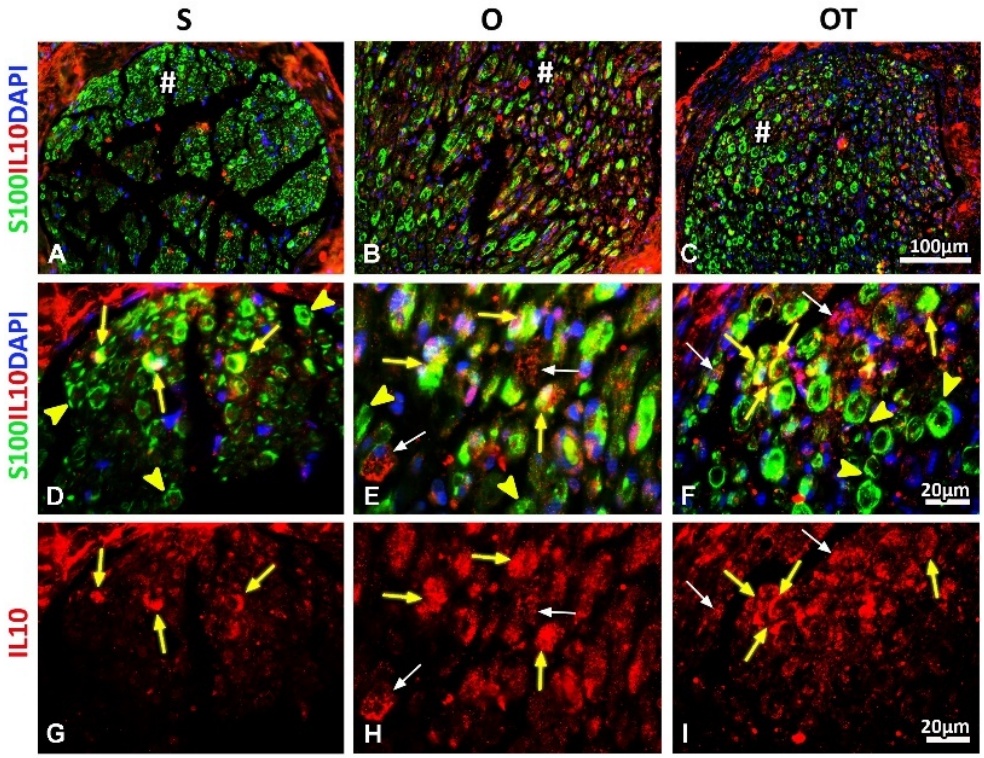

Figure 15. Effects of PNI and the B vitamins treatment on expression of anti-inflammatory cytokine interleukin 10 (IL-10) in Schwann cells. Cross sections of the femoral nerve obtained from the: sham (S), operated (O) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT) group immunostained for IL-10 (red) showed strong IL-10 immunoreactivity in all of the groups (**G**, **H**, and **I**) at the fourteenth dpo, being the most pronounced in the O group (**H**). Increased IL-10 immunoreactivity was found in $S100^{+}$ (green) myelinating Schwann cells (**E** and **F**, yellow arrows) and in IL-10^{+} macrophages with the M2-like morphology (**E**, **F**, **H**, and **I**, white arrows) that were closely associated to them. In the OT group, the larger part of $S100^{+}$ Schwann cells was IL-10^{-} (**F**, yellow arrow heads). The similar pattern of IL-10 immunoreactivity was seen in the S group, although IL-10^{+}/$S100^{+}$ cells were less represented (**D** and **G**, yellow arrows and yellow arrow heads). DAPI (blue) was used to visualize cell nuclei. # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**I** = 20 µm.

With the use of serial transversal sections we have visualized tight interactions between M2-macrophages and Schwann cells, that were aligned to form bands of Büngner (Figure 16A–C) and were intensively stained with IL-10 (Figure 16D–F).

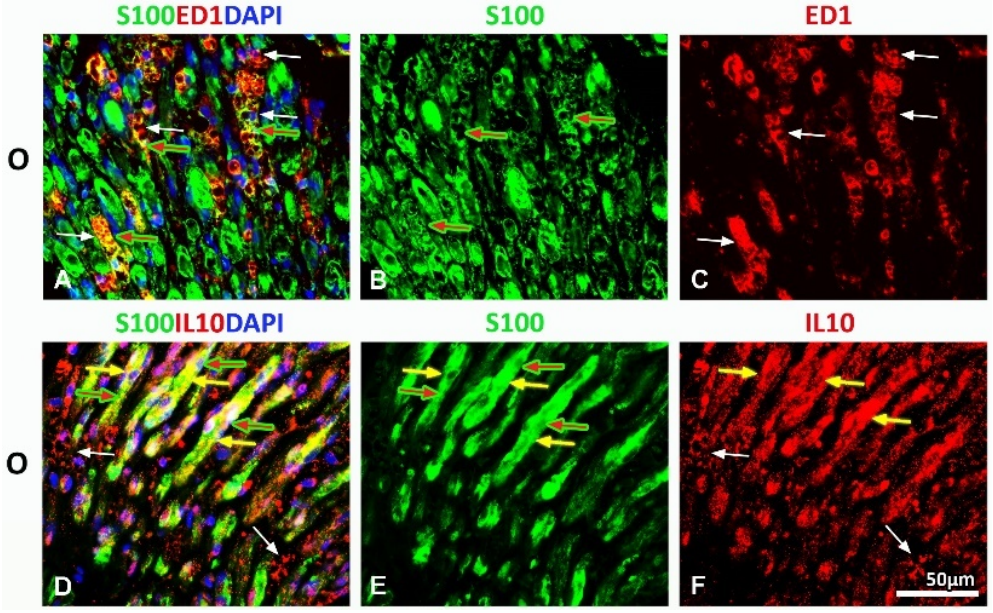

Figure 16. Interactions between M2 macrophages and Schwann cells in injured femoral nerve. (**A**–**C**) We used double immunofluorescence to visualize the close contact between M2 macrophages and Schwann cells at the fourteenth dpo. Serial transversal sections obtained from the operated (O) femoral nerve were immunostained for ED1 (anti-CD68, red) antibody, as a marker of activated macrophages (**C**, white arrows), anti-S100 (green) antibody as a marker of Schwann cells (**B**, red arrows) and DAPI (blue) for visualizing cell nuclei. Complete overlapping (**A**, yellow fluorescence) of ED1 (white arrows) and S100 (red arrows) immunoreactivity confirmed tight interactions between M2 macrophages and Schwann cells that were aligned to form bands of Büngner. (**D**–**F**) Transversal sections of the operated (O) femoral nerve immunostained with S100 (green), IL10 (red) and DAPI (blue) demonstrated that M2 macrophages (**D**, **F**, white arrows) and $S100^{+}$ Schwann cells (**E**, red arrows) in bands of Büngner were intensively stained with IL-10 (**D**, yellow arrows).

4.3 Effect of Vitamin B Complex on expression of GAP43 in Schwann cells and axon regrowth after PNI

4.3.1 Vitamin B complex treatment applied after PNI alters the Schwann Cells type during the recovery period

In order to determine that the $S100^{+}$ Schwann cells, which are closely related to $ED1^{+}$ macrophages, were the non-myelinating Schwann cells, we carried out a double immunofluorescence staining with growth associated protein 43 (GAP43), which is a well-

known marker of growing axons (Donnelly et al., 2013), as well as a marker of non-myelinating Schwann cells (Curtis et al., 1992; Liu et al., 2015; Sensenbrenner et al., 1997). Regarding sham controls, GAP43 immunostaining was mainly noticed in large-diameter myelinated axons, at both-time points (7 and 14 dpo) (Figure 17A and D, red asterisk). The most of the myelinating $S100^+$ Schwann cells, wrapping these axons, were $GAP43^-$ (Figure 17A and D, yellow arrow heads), but some of them were also $S100^+/GAP43^+$ (Figure 17A and D, yellow arrows). Furthermore, rare non-myelinating $GAP43^+$ Schwann cells were detected as well (Figure 17D, red arrows). At the seventh dpo, the nerve tissue was impaired, the largest part of myelin sheaths and axons was degraded, and the majority of the Schwann cells underwent degeneration (Figure 17B, red arrow heads). However, in the OT group, in addition to myelin-forming Schwann cells (Figure 17C, yellow arrow heads), seven consecutive injections of B vitamins increased the number of non-myelinating $S100^+/GAP43^+$ Schwann cells (Figure 17C, red arrows) that wrapped multiple, small-diameter, non-myelinated axons (Figure 17C, white asterisk).Likewise, at the fourteenth dpo, in the O group (Figure 17E, arrow head), huge number of $S100^+/GAP43^+$ non-myelinating Schwann cells ensheathing multiple small-caliber axons (Figure 17E, red arrows), along with a small number of myelin-forming $S100^+/GAP43^+$ (Figure 17E, yellow arrows) and $S100^+/GAP43^-$ (Figure 17E, yellow arrow heads) Schwann cells, was observed. On the contrary, in the OT, as well as in the S group, myelinating, mature $S100^+/GAP43^-$ Schwann cells were detected as a major cell type (Figure 17F, yellow arrow heads), with only a few $S100^+/GAP43^+$myelinating Schwann cells (Figure 17F, yellow arrows). Moreover, strong GAP43 immunostaining was revealed in large-diameter myelinated axons (Figure 17F, red asterisk).

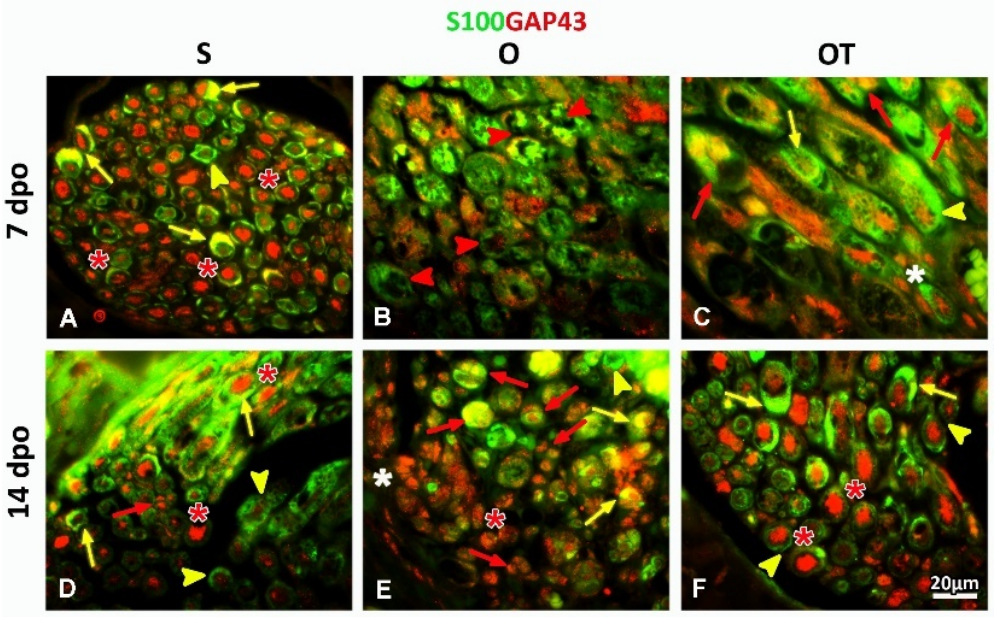

Figure 17. GAP43 (growth associated protein 43) expression in Schwann cells and axons after PNI and the treatment with B vitamins. Expression of GAP43 (red) in Schwann cells was determined in the femoral nerve cross sections obtained from the: sham (S), operated (O) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT) group at the seventh and fourteenth dpo. Anti-S100 antibody (green) was used as a marker of Schwann cells. In the S group, both at the seventh (**A**) and the fourteenth (**D**) dpo GAP43 immunostaining was mostly detected in large-diameter myelinated axons (red asterisk), and in a few myelinated $S100^{+}/GAP43^{+}$ (yellow arrows) and non-myelinated Schwann cells (red arrows), while $S100^{+}/GAP43^{-}$ (yellow arrow heads) Schwann cells were predominant. (**B**) In the O group, at the seventh dpo most of the axons were destroyed and the majority of Schwann cells degenerated (red arrow heads). (**C**) 7 consecutive injections of B vitamins (OT) increased the number of non-myelinating $S100^{+}/GAP43^{+}$ Schwann cells (red arrows) wrapping multiple small-diameter $GAP43^{+}$ non-myelinated axons (white asterisk). Only a few $S100^{+}/GAP43^{+}$ myelinated Schwann cells (yellow arrows) and $S100^{+}/GAP43^{-}$ (yellow arrow heads) were seen. (**E**) At the fourteenth dpo, in the O group, a huge number of $S100^{+}/GAP43^{+}$ non-myelinated Schwann cells (red arrows) unsheathing multiple small-caliber axons (white asterisk), and a paucity of myelin-forming $S100^{+}/GAP43^{+}$ (yellow arrows) and $S100^{+}/GAP43^{-}$ (yellow arrow heads) Schwann cells was detected. (**F**) After 14 treatments with B vitamins myelinating, mature $S100^{+}/GAP43^{-}$ Schwann cells emerged as the principal cell type (yellow arrow heads), while $S100^{+}/GAP43^{+}$ myelinating Schwann cells were rarely present (yellow arrows). Strong

GAP43 immunostaining was detected in large-diameter myelinated axons (red asterisk). Scale bar: 20 μm.

4.4 Effect of Vitamin B Complex on expression of the $Ca_V1.2$ Channel in Macrophages and Schwann cells after PNI

4.4.1 Vitamin B complex treatment applied after PNI alters the number of M1 macrophages expressing the $Ca_V1.2$ channel

As Ca^{2+} influx mediated by L-type VDCCs is essential for macrophage activation, as well as changes in morphology, and generation of proinflammatory mediators (Espinosa-Parrilla et al., 2015), the following step was to assess the effects of PNI and treatment with B vitamins on expression of the $Ca_V1.2$ isoform of L-type VDCCs in the injured peripheral femoral nerve. In the O group (Figure 18A), expression of $Ca_V1.2$ starts to gradually decline after PNI. The largest number of $Ca_V1.2^+/ED1^+/TNF\text{-}\alpha^+$ was detected at day 1 post-injury (Figure 18C), while at 7 dpo the majority of $ED1^+$ macrophages obtained a "foamy" morphology, which is characteristic for the M2 phenotype, and were $Ca_V1.2^-/TNF\alpha^-$. Interestingly, treatment with complex of B vitamins (Figures 18B and 13C) demonstrated a notable reduce in number of proinflammatory M1 macrophages ($ED1^+/TNF\text{-}\alpha^+$) which co-express the $Ca_V1.2$ isoform. Nevertheless, the fractions of $Ca_V1.2^+/ED1^+/TNF\text{-}\alpha^+$ cells in total macrophages ($ED1^+$ cells) and M1 macrophages ($ED1^+/TNF\text{-}\alpha^+$ cells) were the same for the O and OT group. However, along with down-regulation of $Ca_V1.2$ expression in M1 macrophages, its expression was remarkably up-regulated in axons and in some $ED1^-$ cells (Figures 18A and 18B, higher magnification, green asterisks and yellow arrows, respectively). The obtained data imply that treatment with B vitamins diminished the number of M1 macrophages which express $Ca_V1.2$ channel.

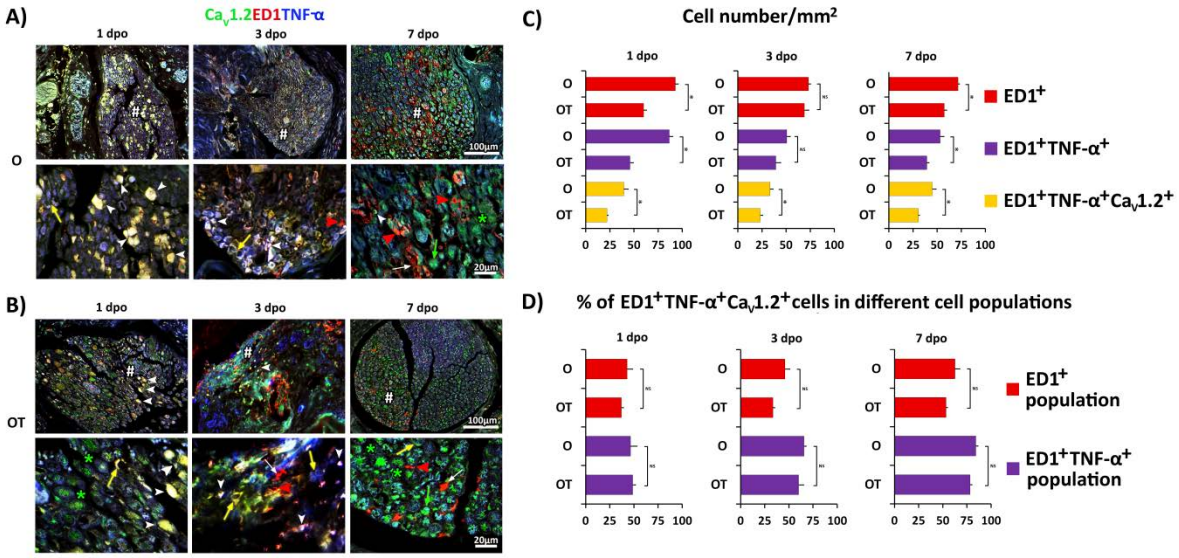

Figure 18. Treatment with vitamin B complex induces time-dependent changes of $Ca_V1.2$ channel expression in M1 macrophages after PNI. To evaluate cellular distribution of the $Ca_V1.2$ isoform of L-type VDCCs (green), cross sections of femoral nerve obtained from the: (**A**) operated (O); and (**B**) operated and treated with vitamin B complex (OT) groups were counterstained with anti-TNF-α (M1 marker, blue) and anti-ED1 (red) antibodies. The quantification of single-, double-, and triple-positive cells is presented as number of $ED1^+$ cells/mm^2, $ED1^+$/TNF-α^+ cells/mm^2, and $ED1^+$/TNF-α^+/$Ca_V1.2^+$ cells/mm^2 (**C**), and as the percentage of triple -positive cells ($ED1^+$/TNF-α^+/$Ca_V1.2^+$ cells) in $ED1^+$ and $ED1^+$/TNF-α^+ cell populations (**D**). The data are shown as the mean ± standard error of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (*p< 0.05 OT *vs*. O group, as indicated at the graphs). Intensive $Ca_V1.2$ staining, besides in M1 macrophages, was observed in axons (green asterisks) and in some $ED1^-$ cells (yellow and green arrows) as well. $ED1^+$/$Ca_V1.2^+$/TNF-α^+ M1 macrophages are marked with a white arrowhead, $ED1^+$ macrophages with oval/round morphology (M1 type) are marked with a red arrowhead, and "foamy" $ED1^+$ macrophages (M2) are indicated with white arrows. # indicates where the high magnification micrographs are taken from. Scale bars: 20 μm and 100 μm.

4.4.2 Vitamin B complex treatment applied after PNI affects the expression of $Ca_V1.2$ channel in M2 macrophages

The effects of PNI and treatment with B vitamins on $Ca_V1.2$ expression in various subtypes of M2 macrophages were examined in this study. Two time-points, at day 7 and 14 post-injury (Figures 19A and 19B, respectively) were selected for further investigation as it was shown that M2 type of macrophages were the main type. Regarding O group (at both time points), most of the $ED1^+$ macrophages had a "foamy" morphology of the M2 type and were not co-stained with anti-$Ca_V1.2$ antibody (Figures 19A and 19B). The sum number of $ED1^+$/IL-10^+/$Ca_V1.2^+$ cells is given in Figure 19C. In O and OT group at 7 dpo, the fractions of $Ca_V1.2^+$/$ED1^+$/IL-10^+ cells in total macrophages ($ED1^+$ cells) and M2 macrophages ($ED1^+$/IL-10^+ cells) were the same. However, in the group treated with B vitamins a difference in macrophage morphology, as well as in $Ca_V1.2$ expression was observed. Macrophages with "foamy" morphology were $ED1^+$/$Ca_V1.2^+$/IL-10^+. The $Ca_V1.2$ expression was shown in different types of macrophages at 14 dpo. As presented in Figure 19B some of the macrophages had a more oval morphology and were $ED1^+$/$Ca_V1.2^+$. In macrophages with "foamy" morphology, a different IL-10 expression was detected (Figure 19B): some of them were $ED1^+$/$Ca_V1.2^+$, but IL-10^- (yellow arrowhead), while others were $ED1^+$/$Ca_V1.2^+$/IL-10^+ (blue arrow). These "foamy" $ED1^+$/IL-10^- cells were positive for arginase-1 (Arg-1, Figure 19B), which is a considered as a classic M2a marker (Jablonski et al., 2015; Rőszer, 2015). As discussed before, the most potent $Ca_V1.2$ immunoreactivity was noticed in axons (green asterisks) and some $ED1^-$IL-10^+ cells (yellow arrows). This was especially considerable at 14 dpo in the OT group (Figure 19B). The fractions of $Ca_V1.2^+$/$ED1^+$/IL-10^+ cells in total macrophages ($ED1^+$ cells) and M2 macrophages ($ED1^+$/IL-10^+ cells) were statistically elevated in OT group at 14 dpo. These findings may suggest that $Ca_V1.2$ has a time-dependent pattern of expression in different types of M2 macrophages.

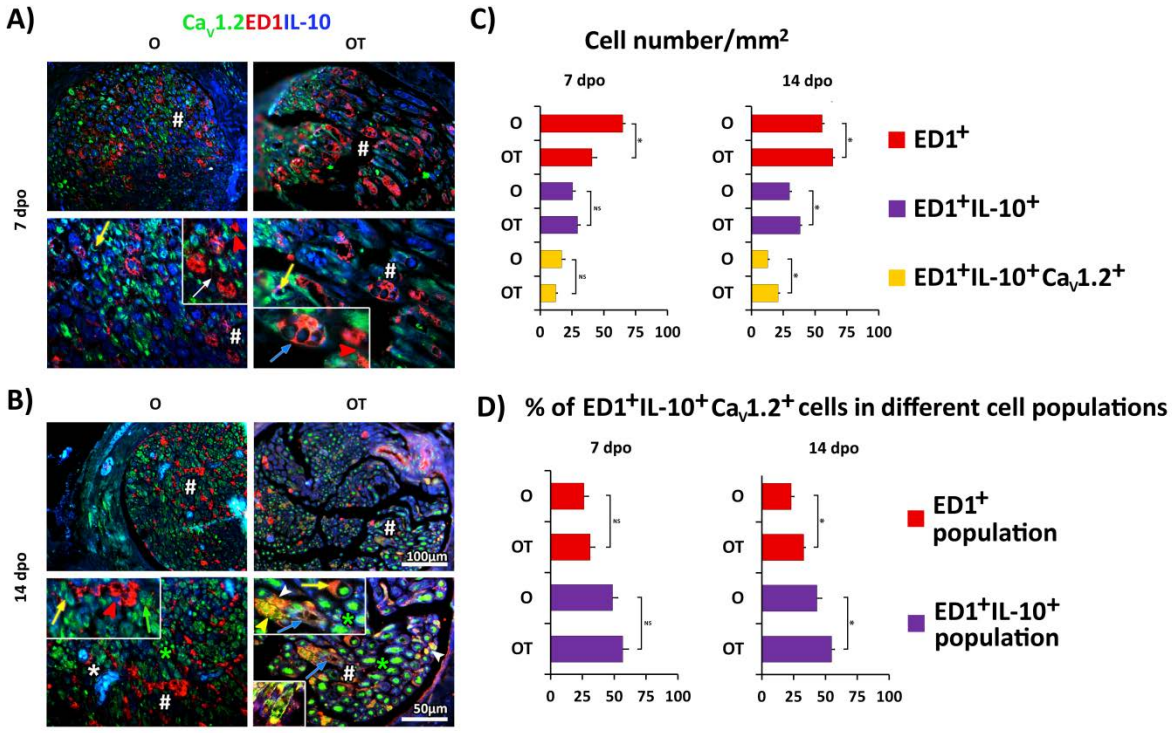

Figure 19. Treatment with vitamin B complex induces time-dependent changes in $Ca_V1.2$ channel expression in M2 macrophages after PNI. To evaluate cellular distribution of the $Ca_V1.2$ isoform of L-type VDCCs (green), triple immunofluorescence staining of femoral nerve cross sections obtained from the operated (O) and operated and treated with vitamin B complex (OT) groups at (**A**) 7 dpo and (**B**) 14 dpo was performed. Anti-$Ca_V1.2$ (green), anti-IL-10 (M2 marker, blue), and anti-ED1 (red) antibodies were used. The quantification of single, double, and triple-positive cells is presented as the number of $ED1^+$ cells/mm^2, $ED1^+$/IL-10^+ cells/mm^2, and $ED1^+$/IL-10^+/$Ca_V1.2^+$ cells/mm^2 (**C**) and as the percentage of triple-positive cells ($ED1^+$/IL-10^+/$Ca_V1.2^+$ cells) in $ED1^+$ and $ED1^+$/IL-10^+ cell populations (**D**). The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (*p< 0.05 OT *vs*. O group, as indicated at the graphs). In the O group at both time-points (7 and 14 dpo) $ED1^+$ macrophages with either oval or "foamy" morphology (red arrowhead and white arrows, respectively) were not co-stained with $Ca_V1.2$ and IL-10. After treatment with 7 and particularly after 14 injections of B vitamins they were mostly $ED1^+$/$Ca_V1.2^+$/IL-10^+ (white arrowheads and blue arrows, respectively). At 14 dpo in the OT group some of the "foamy" macrophages were $ED1^+$/$Ca_V1.2^+$/IL-10^- (yellow arrowheads), and these IL-10^- cells were $ED1^+$/Arg-1^+ (yellow,

insert in the left lower corner). Intensive $Ca_V1.2$ staining was seen in axons (green asterisks) and in some $ED1^-/IL\text{-}10^+$ cells (yellow and green arrows). # indicates where the high magnification micrographs are taken from. Scale bars: 50 µm and 100 µm.

4.4.3 Vitamin B complex treatment applied after PNI increased the expression of the $Ca_V1.2$ channel in myelin-forming Schwann cells

As mentioned previously, cells with Schwann cells-like morphology were intensively stained with $Ca_V1.2$ antibody. To confirm this fact we thereby performed double-immunofluorescence staining of cross sections from sham, operated and operated and treated with complex of B vitamins femoral nerves with S100 antibody which is well-known marker of Schwann cells (Liu et al., 2015). In sham controls, $Ca_V1.2$ immunostaining was detected in mature, myelinating S100+ Schwann cells and their nuclei as well (Figures 20A, and B, S, yellow arrows) at both-time points (7^{th} and 14^{th} dpo). Robust $Ca_V1.2$ immunoreactivity was seen in axons wrapped with Schwann cells in all investigated groups (S, O, and OT) (Figure 20, green asterisks). At the 7^{th} dpo nerve tissue was damaged, major part of myelin sheaths was degraded and axons were stripped (Figure 20A, O, green asterisks), while most of the Schwann cells degenerate. However, remaining Schwann cells were $Ca_V1.2^+/S100^+$. Interestingly, in OT group beside myelin-forming Schwann cells (Figure 20A, OT, arrows), seven consecutive injections of B vitamins significantly increased the number of non-myelinating Schwann cells that wrapped multiple small-diameter unmyelinated axons (Figure 20A, OT, asterisks) which were faintly stained with $Ca_V1.2$ antibody (Figure 20A, OT, arrow head). Huge number of $Ca_V1.2^+/S100^+$ non-myelinating Schwann cells ensheathing multiple small-caliber axons and a paucity of myelin-forming Schwann cells was seen 14 days post-injury in O group (Figure 20B, O, arrow head). In contrast, in OT group only a few $Ca_V1.2^+/S100^+$ non-myelinating Schwann cells were found (Figure 20B, OT, arrow head), while myelinating, mature Schwann cells were predominant cell type (Figure 20B, OT, arrows). Notably, cellular profile of $Ca_V1.2$ expression was similar to this detected in S group (Figure 20B). Based on these results it can be concluded that PNI alters expression of L-type of VDCCs in Schwann cells in time-dependent manner, while treatment with B vitamins accelerates transition from non-myelin to myelin-forming Schwann cell type.

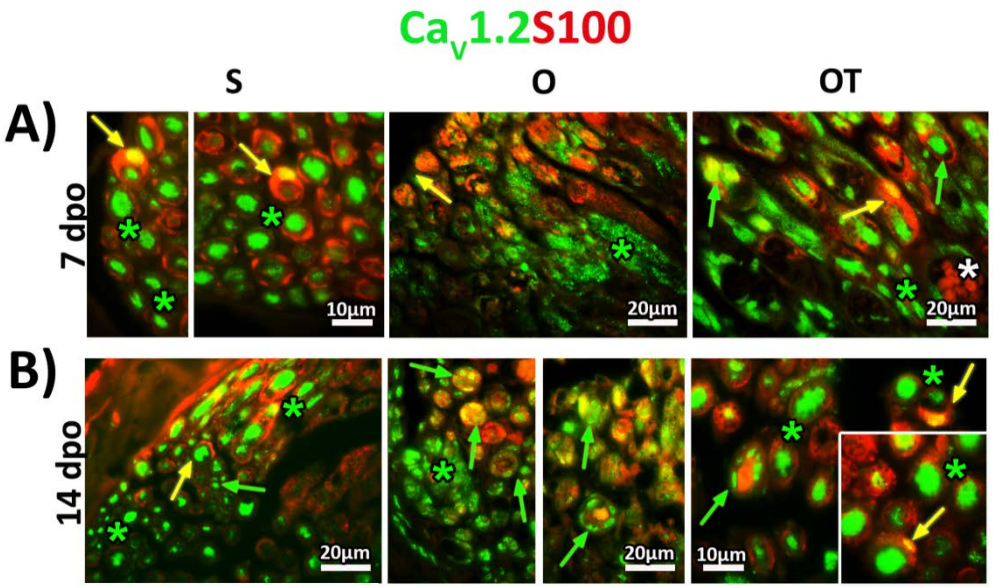

Figure 20. Effects of PNI and B vitamins treatment on expression of $Ca_V1.2$ in Schwann cells. Distribution of $Ca_V1.2$ isoform of L-type VDCCs (green) in Schwann cells was determined in femoral nerve cross sections obtained from: sham (S), operated (O) and operated and treated with vitamin B complex (OT) group at (A) the 7th dpo and (B) the 14th dpo. Anti-S100 antibody (red) was used as a marker of Schwann cells. Myelinated Schwann cells double stained with $Ca_V1.2$ and S100 (yellow arrows) were predominant in Sham-operated (S) group, and a paucity of $S100^+/Ca_V1.2^+$ non-myelinated Schwann cells (green arrows) was noted. In O group, at the 7th dpo most of the axons were stripped and were $Ca_V1.2^+$ (green asterisk), and only a few $S100^+/Ca_V1.2^+$ myelinated Schwann cells (yellow arrows) were seen. Alternatively, at the 14th dpo a huge number of $S100^+/Ca_V1.2^+$ non-myelinated Schwann cells (green arrows) unsheathing multiple small-caliber axons (green asterisk) were observed. Treatment with B vitamins increased the number of $S100^+/Ca_V1.2^+$ non-myelinated Schwann cells (green arrows) at the 7th dpo, while at the 14th dpo myelinated $S100^+/Ca_V1.2^+$ Schwann cells (yellow arrows) were predominant like in S group. White asterisk (*) marks blood vessels. Scale bars: 10 µm and 20 µm.

5 DISCUSSION

The first therapeutic method of choice to treat most types of PNI is surgery, where direct microsurgical nerve repair or autologous nerve grafts are currently considered as gold standard treatments. Upon undergoing surgical reconstruction, peripheral nerve has an innate capacity to induce the process of repair; however, regeneration of motor and sensory functions often remains incomplete. Additionally, it is important to emphasize that the clinical outcome of motor function recovery in adults is often disappointing (unlike sensory, which usually achieves a good degree of recovery) (Scholz et al., 2009) so this was the reason to investigate only the motor branch of the mixed peripheral nerve. The femoral nerve is a mixed nerve containing motor fibers for innervation of *m. quadriceps femoris* and sensitive fibers for innervation of the skin. Directly below the inguinal ligament, the femoral nerve divides into two, nearly identical branches: the sensory (cutaneous branch) of *n. saphenus* and the motor branch for innervation of *m. quadriceps femoris*. Transection of the nerve proximal to the bifurcation induces isolated failure of the *m. quadriceps femoris* function. After nerve repair (suture), there is an equal chance that the motor axons for the quadriceps will sprout the original path toward the quadriceps, or go the wrong way, towards a purely sensitive saphenous nerve. The mentioned fact enables the analysis of cellular and molecular mechanisms specific to preferential motor reinnervation. The model is interesting because the injury causes a failure of one muscle, the quadriceps, which has a unique function on the large extremity joint that is knee extension, as well. In this study, the transection of the motor branch of the femoral nerve was performed. This procedure is leaving the sensitive (saphenous) branch intact in order to exclude the possibility of misalignment of the axons from the motor to the sensitive part and vice versa. In this way, this is focused exclusively on monitoring and examining motor regeneration of the nerve and muscle, excluding the possibility of the effect of misdirected axons on the results. The simplicity of the motor deficit allows for easy access to functional analysis. Complications such as automation, limb contracture and skin ulceration have not been observed so far (Irintchev, 2011). Thus, the development of adjuvant repair therapeutic strategies to complement well established surgical procedures currently is recognized as highly needed and represents very attractive area of research (Wiberg and Terenghi, 2003). In this study, the complex of B vitamins (B1, B2, B3, B5, B6, B12) was examined in the context of a possible adjuvant therapeutic for the treatment of PNI owing to their

numerous positive features, both from the applicable and also underlying mechanistic aspects. Firstly, B vitamins are very convenient when it comes to molecular manipulation and their turnover can be considered unlimited. B vitamins participate as coenzymes in numerous enzymatic processes, which are crucial for exerting many of the cellular functions, leaving strong impact on proper functioning of both the peripheral and central nervous system also (Kennedy, 2016). These well-acknowledged positive effects on nervous system functioning repurposed B vitamins as therapeutics for treating a range of different pathologies (Hoane et al., 2005; Kuypers and Hoane, 2010; Maiese et al., 2009), where the vitamin B12 emerged as most potent *in vivo* for the regeneration of peripheral nerves. Namely, B12 acts through many different pathways, including the activation of kinases Erk1/2 and Akt after trauma resulting in improved axonal outgrowth and survival of the neurons (Liao et al., 2013; Okada et al., 2010); enhanced proliferation of Schwann cells during regeneration and axon myelination, assuming both increase in myelin sheath diameter and axonal maturation. Besides improved axonal outgrowth, vitamin B12, together with vitamins B1 and B6 also accelerate impulse delivery through the nerve (by reduction of axon degeneration in rat acrylamide-induced neuropathy) (Fujii et al., 1996). In line with this, there is data demonstrating that the acceleration of nerve regeneration during the acute period after PNI can be achieved with the supplementation of B12, and the remaining B vitamins (Altun and Kurutaş, 2016). Nevertheless, for B12 it has also been shown that the combination with dexamethasone contributes positively to the repair of injured peripheral nerves (Sun et al., 2012). Altogether, this significant role of B vitamins in proper functioning and also repair/regeneration of injured peripheral nervous system provides rationale for further exploring them in this context, both in combinations or separately, to formulate novel adjuvant treatments for the peripheral nerve injury.

When occurs, PNI is followed by a heavy proinflammatory immune response, orchestrated principally by Schwann cells, resident macrophages and fibroblasts. This happens as a reaction to the infiltrating monocytes from the blood to the injury site in only two to three days after the injury (Mueller et al., 2003). To facilitate the upcoming process of neuroregeneration, to the strong proinflammatory reaction needs to be attenuated by a matching anti-inflammatory process, which is orchestrated at the injury site by the same cellular players, invoking prompt functional and phenotypic transformation.

Macrophages stand for potent immune effector cells effectively switching the roles between the promotion of tissue injury, from one side, and wound healing and tissue repair, from the other, across different pathological conditions in the body (Duffield et al., 2005; Mantovani et al., 2013). Numerous compelling evidence from versatile *in vivo* and *in vitro* studies point to the activated macrophage states identification, along with targeting of the M1-to-M2 (and *vice versa*) macrophage polarization, can deliver innovative therapeutic strategies to combat a wide spectrum of different pathologies (Waidmann et al., 2013; Wan et al., 2014; Wei et al., 2013), including PNI. The aim of this thesis was to provide evidence for attenuation of inflammation mediated by macrophages in injured peripheral nerve, in response to vitamin B complex treatment.

Results of this study identified a clear time-course of changing macrophage morphology during the post-PNI recovery, from the round-shaped, smaller M1 cells, functionally corresponding to the proinflammatory phenotype, to the "foamy"-shaped, larger M2 cells, functionally corresponding to the anti-inflammatory. Importantly, the treatment with the vitamin B complex accelerates the observed changes in macrophage morphology which aligns with enhanced recovery of the motor nerve branch together with locomotor performances in rats (Nedeljković et al., 2017). Noticed changes in macrophage morphology, which accelerate in to the treatment with B vitamins, was further demonstrated by the profile of macrophage cytokine production indicating elevated expression of TNF-α and iNOS, in response to PNI, corresponding therefore to the proinflammatory M1 phenotype, that is, to the well-acknowledged role of this macrophage subset at the injury site during Wallerian degeneration (Mueller et al., 2003). Next on, the phenotypic switch from M1 to M2 macrophages was demonstrated by the expression of IL-10 and IL-4. Macrophage phenotypic transition can be influenced by different factors, primarily by the mixture of local cytokines arising as a consequence of the neuronal and Schwann cell activity at the injury site (Stratton and Shah, 2016). B vitamins greatly reduced the number of M1 macrophages, hindering their proinflammatory activity up to the 3rd dpo, thus strongly limiting further deleterious effects in the injured nerve. The accelerated transition from the proinflammatory to the repair-promoting macrophage phenotype, mediated by the vitamins B complex treatment, fine-tunes the balance between the indispensable inflammation, right after the injury, and subsequent M2-macrophages-induced neuroreparation. In this context, it is important to emphasize that the up-regulation of the IL-10, which represents an anti-

inflammatory cytokine, was demonstrated from 7 days up to 28 days upon the injury in the distal nerve segment (George et al., 2004; Sawada et al., 2007). This cytokine effectively modulates the expression of proinflammatory counterparts and also contributes to the axonal plasticity (Vidal et al., 2013). Since macrophages act as the main producers for the IL-10 (George et al., 2004), its enhanced expression M2 macrophages, that we successfully detected after the administration of the B vitamins complex, it can be safely concluded the applied treatment effectively promotes the resolution of inflammation and, therefore, nerve repair, in response to the femoral nerve injury.

Manifold effects of the applied treatment of the vitamins B complex after PNI, observed in this study, stand as a finding of paramount significance, since most compounds that attenuate neuroinflammation act *via* M1 macrophage subset solely (Kalkman and Feuerbach, 2016). What is most important, the obtained results speak in favor of a rapidly achieved balance in M1/M2 phenotypic switch in response to the vitamin B complex treatment (which can also be completely reverted) right after PNI. The exact signaling pathways, underlying macrophage subsets differentiation in response to the B vitamins, remain to be further resolved in the upcoming studies. The observed plasticity in macrophage polarization gives a rational for developing innovative therapeutic strategies centered on macrophage activity to combat more effectively PNI, together with the indispensable immediate surgical treatment. Schwann cells are considered to have a very prominent role in orchestrating distinct functional modalities between macrophage subsets. Firstly, the resident Schwann cells, experiencing hyperproliferation, reinforce the initial macrophage activity by secreting cytokines that further recruit monocytes to the injured site (Pan et al., 2017). By this, Schwann cells change the phenotype to the pre-myelinating state. However, re-established interactions between Schwann cells with the tissue macrophages restore the re-myelinating Schwann cell phenotype which behaves indispensable during the course of axonal regeneration (Lutz and Barres, 2014). Nevertheless, the layers of novel myelin may stimulate macrophages to finalize the on-going inflammation at the injured nerve, so that the cross-talk between macrophages and Schwann cells clearly enables fine tuning of the reparatory processes, demanding clarification to a greater extent. Herewith, the cross-talk between macrophages and Schwann cells, occurring upon the controlled transection of the peripheral nerve, which represents a widely used, highly valuable and reproducible model of the peripheral nerve regeneration in rats (Irintchev, 2011; Nedeljković et al., 2017), emerged as an important target of

the applied vitamin B complex treatment. It should be noted that these macrophage/ Schwann cells interactions were modulated in a time-dependent manner either after PNI alone, or upon the application of B vitamins. However, following the treatment with the complex of B vitamins, the transition from the non-myelin to myelin-forming Schwann cells phenotype was accelerated, thus contributing to Schwann cells maturation. Furthermore, due to the stimulation of the M1-to-M2 macrophage polarization, macrophage/Schwann cells interactions were consequently altered.

Cross-talk between macrophages and Schwan cells represents the most crucial type of interactions between different cells that during the course of PNI-triggered neuroinflammation. These inflammatory processes involve also the unaffected nerve stumps distal to the lesion site. Damaged axons get eliminated by Schwann cells, which undergo demyelination, which is subsequently followed by the bone-marrow-derived macrophage and activated resident Schwann cells infiltration to cleanse the remaining tissue debris (Rotshenker, 2011). Results obtained in this study reveal extensive presence of the areas containing destructed axons in the injured femoral nerve of the O animal group, day 1 post-injury, analogously with the Schwann cells dedifferentiation/demyelination. Observed changes match the fact that in young rats, like the ones used throughout this study, the lag period separating the injury and axon degeneration refers to the first 24-48 hours (Lubińska, 1977). What is more important, the detached axonal segments remain intact and able to transmit action potentials in response to stimulation several days after PNI (Luttges et al., 1976; Tsao et al., 1999). In accordance with this, the observed drop in M1-macrophage numbers along with the Schwann cells morphology/myelination preservation in the injured nerve of OT animals, allows the hypothesis that the B vitamins treatment prolongs the lag period for reduced axonal degeneration.

Results of this study clearly showed that upon the completion of the severe axonal destruction, triggered by injury, in both OT and O animals $ED1^+$ macrophages characterized with the M2-like, “foamy” morphology appear closely associated to Schwann cells at day 3 after the injury. This phenomenon was more prominent in the group of OT animals, i.e. the ones that had received the vitamin B complex treatment, while in the group of only operated animals association between the M1-like $ED1^+$ macrophages and Schwann cells displaying low S100 immunoreactivity was observed in parallel. According to Stratton and Shah (Stratton and Shah, 2016) these interaction between macrophages and Schwann cells are crucial for proper

neuroregeneration after PNI, since Schwann cells presumably promote the macrophage functioning during the recovery post-PNI period, by expressing different ligands for receptors found on macrophages, putatively regulating M1-to-M2 phenotypic switch. Namely, Schwann cells behave as strong inducers of the reparatory M2 type macrophages via the production of well-acknowledged M2-associated cytokines. M2 macrophages, in turn, orchestrate the neuroregeneration after PNI by supporting remyelination through the activation of endogenous Schwann cells. Nevertheless, a well-known role of macrophages in the regulation of Schwann cells maturation after nerve injury suggests that this process could also be bidirectional, having in mind the close interaction between these two cell types after PNI (Stratton et al., 2018). However, the arrangement of interactions between macrophages and Schwann cells during the post-PNI recovery appeared most versatile at day 7 post-injury. At this time-point, under all conditions, a broad repertoire of $ED1^+$ macrophages and Schwann cells were detectable in the injured nerve, with the M2-type macrophages comprising the most prevalent group, and M1-like macrophages being found in a very small proportion. On top of this, in the group of only operated animals a diffuse distribution of the dark spots was noticeable, corresponding to damaged axons, which are found marginal to Schwann cells displaying low S100 immunoreactivity, most probably due to on-going degeneration/dedifferentiation. These dark spots were, however, not observed in the injured nerves of animals treated with B vitamins. Areas of degenerating axons and Schwann cells degeneration appeared marginal to many $ED1^+/S100^+$ macrophages, characterized by undifferentiated morphology, i.e. corresponding to the ongoing transition between the M1 and M2 type. Besides these transitional macrophages, cells displaying clear M1 or M2 morphology could also be detected. Else ways, a series of seven B vitamins injections significantly increased tight $S100^+$ Schwann cells to $ED1^+$ cells association, whereby this $ED1^+$ cellular fraction corresponded to the “foamy” M2-macrophages, while the closely associated $S100^+$ Schwann cells, appeared only as the ones with the non-myelinating morphology. Noticeably, the non-myelinating $S100^+$ cells were found to wrap multiple non-myelinated axons of small diameter, and were also $GAP43^+$.

Finally, at day14 after the injury, analogous pattern of interactions between M2-like macrophages and non-myelinating $GAP43^+$ Schwann cells could be detected in the injured nerve of only operated animals. GAP43 represents an established marker of growing axons (Donnelly et al., 2013) which is also considered a marker of the non-myelinating Schwann cells (Curtis et

al., 1992; Rotshenker, 2011; Sensenbrenner et al., 1997), confirming that detected Schwann cells display the non-myelinating morphology due to the S100–GAP43 immunoreactivity overlap. In parallel, paucity scarcity of the Schwann cells displaying myelin-forming morphology ($S100^{+}/GAP43^{+}$ and $S100^{+}/GAP43^{-}$) was observed also in the injured nerves of only operated (O) animals. At the same time-point, the treatment with B vitamins results with the predominant observation of $GAP43^{-}$, mature and myelinating, Schwann cells in the injured nerves of OT animals, with concomitant, strong GAP43 immunostaining in the myelinated axons of large diameter.

This study clearly showed that one of the hallmark consequences of PNI is the destruction of the large pool of Schwann cells seven days after the injury, invoking consequent reprogramming from the myelin to non-myelin-forming (Remak) Schwann cells cell type. These aligns well with the PNI-induced large-scale changes in gene expression patterns of both the myelin-forming and non-myelin-forming Schwann cells, which ultimately leads to the restoration of the repair-promoting Schwann cells phenotype (Jessen and Mirsky, 2016). Notably, since the non-myelin-forming (Remak) Schwann cells wrap uninjured nerve fibers, sensing injury/disease in their vicinity (Armati and Mathey, 2013; Griffin and Thompson, 2008), the appearance of this cell type as the most prevalent Schwann cells fraction at days 7 and 14 post-injury can be seen as an expected result.

In view of all the obtained results from the study, it can be concluded that the B vitamins treatment acts protective for the fraction of myelin-forming Schwann cells and, at the same time, accelerates the emergence of non-myelin-forming Schwann cells, which largely preserves the functional capacity of the injured femoral nerve. This was clearly demonstrated as the improvement of the locomotor performances recovery in rats (Nedeljković et al., 2017). In line with this, data from a number of recent publications reveal positive effects of applying doses of B vitamins individually (B12 > B1 > B6) on the damaged sciatic nerve repair. The underlying mechanism is also *via* the modulation of myelination process and Schwann cells functionality and morphology. What is more important, the use of B vitamins in combinations was proposed for boosting regenerative effects (Al-saaeed and Al-khalisy, 2017). Returning to the application of individual B vitamins, it should be mentioned that B12 displays positive effects in a focal demyelination rat model, contributing to the more expeditious process of remyelination,

Schwann cell differentiation and overall improved recovery of both the motor and sensory function of the demyelinated sciatic nerve (Nishimoto et al., 2015). Also, for folic acid it has been demonstrated in one recent publication the capacity of promoting the post-PNI reparatory processes via stimulation of the proliferation and migration of Schwann cells, and also the nerve growth factor secretion (Kang et al., 2019).

To restate, results of this study revealed a time-course of clear phenotypic changes in macrophages and Schwann cells in response to PNI, corresponding to the switch from the round-shaped, smaller, proinflammatory M1 to the "foamy", repair-promoting M2 macrophages of the larger size, and the transition from the non-myelinating to the myelinating and mature Schwann cells. Therefore, the treatment with B vitamins fine-tunes the interaction between macrophages and Schwann cells, effectively limiting the damage of the injured nerve *via* accelerating the transition from the immediate and indispensable inflammation to neuroreparatory processes.

Accordingly, in this study the expression of proinflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines by Schwann cells, in response to PNI, was determined, implying that the vitamins B complex treatment for 14 days post-injury modulates this expression pattern. There is data showing that after the sciatic nerve transection, the expression of the TNF-α mRNA is characterized with three distinct peaks corresponding to the following time-points: immediately (14 h) upon the transection, five days upon and, finally two weeks after in the distal part (Okamoto et al., 2001; Taskinen et al., 2000). This aligns with the results obtained in this study, showing that at the day14 post-injury, increased expression of TNF-α within femoral nerve cross sections in the O animal group can be observed, compared to the S control group. Notably, a series of the B vitamins B injections attenuated the TNF-α immunoreactivity, which could be, to some extent, still be observed in a fraction of Schwann cells. Reduced TNF-α expression in response to the B vitamins treatment can be considered as protective for the myelin-forming Schwann cells, which secrete IL-10. This is manifested as an increase in GAP43 expression and, overall, improved recovery of the locomotor performances.

IL-10 is well-recognized as an anti-inflammatory cytokine, whose increased expression occurs in the period from 7 days up to 28 days after the nerve injury, in the distal segment of the injured nerve (George et al., 2004; Sawada et al., 2007). This cytokine was to assumed to have modulatory effect on the levels of proinflammatory cytokines, but also on the extent of axonal

plasticity (Vidal et al., 2013). Gradual increase in IL-10 mRNA expression during Wallerian degeneration was also shown in the model of the transected rat sciatic nerve (Taskinen et al., 2000) and the model of rat sciatic nerve chronic constriction injury (Okamoto et al., 2001). In line with this, predominant abundance of IL-10-secreting macrophages was observed at day 14 after the injury, where behaves as a marker of the specific M2c anti-inflammatory macrophage subtype (Chen et al., 2015; Mantovani et al., 2013; Rőszer, 2015; Wang et al., 2014a),. Interestingly, in this study equal fractions of $ED1^+/IL\text{-}10^+$ cells in the total $ED1^+$ cell pool were observed in the group of animals only operated and operated and consequently treated with B vitamins. Importantly, a fraction of $IL\text{-}10^+$ cells, displaying no ED1 immunoreactivity, characterized by the Schwann cells-like morphology, were noticed also in the previous part of thesis. In the current study, at 14 dpo, the bulk of IL-10 immunoreactivity was detected in M2-like $ED1^+$ macrophages, and also in broadly found $IL\text{-}10^+/S100^+$ cells. The underlying assumption is that increased IL-10 secretion by M2c macrophages jointly with the $IL\text{-}10^+/S100^+$ Schwann cells promote the resolution of immediate post-PNI inflammation, and subsequently nerve repair. Conversely, vitamins B treatment greatly reduced the overlap between IL-10 and S100 immunoreactivity in myelin-forming, mature Schwann cells, so that the predominantly observed $S100^+$ Schwann cells were IL-10 negative, just like in the group of sham-operated controls. Interestingly, analogous pattern of PNI-induced Schwann cells-phenotypes was observed by Dubový et al. (Dubový et al., 2014), who proposed that concomitant up-regulation of proinflammatory and anti-inflammatory cytokines fine-tunes the inflammatory response of Schwann cells to PNI for supported axonal outgrowth.

In line with all the previous, one of the main objectives behind this thesis was to explore the close interplay between different macrophage and Schwann cells phenotypes during post-PNI recovery and infer if the administration of the vitamin B complex is able to affect positively these cellular interaction for improved overall nerve recovery. Hereby, only M2 anti-inflammatory macrophages were found closely associated (predominantly) to non-myelinating Schwann cells. Quantification of $ED1^+/S100^+$ cells with respect to the total pool of $ED1^+$ cell provided valuable insights into the extent of interaction between $ED1^+$ macrophage and $S100^+$ Schwann cells during the post-PNI recovery. Likewise, the effects of the vitamin B complex treatment on the time-course of these interactions could be inferred, providing clues regarding the improvements in the recovery process. In the group of only operated animals $ED1^+/S100^+$

cells dominated the pool of $ED1^+$ cells 14^{th} dpo, speaking in favor of tight and intense interactions between the, overwhelming at a given time-point, M2 macrophages and non-myelinating Schwann cells. These cells jointly contribute to the formation of Büngner bands, which serve regeneration tracks for the growing axons for navigating them to proper targets (Jessen and Mirsky, 2016; Nedeljković et al., 2017). Contrarily to this, after 14 days of the B vitamins treatment a notable reduction of the $ED1^+/S100^+$ cells, along with their fraction in the total $ED1^+$ pool occurred. Axons displaying renewed myelin sheath could be largely observed, together with the mature, myelin-forming Schwann cells as the most prevalent Schwann cells phenotype, just like in the S control group, while only minor macrophage-Schwann cells interactions could be detected. These results indicate that a 14 days series of B vitamins administration promotes the complete Schwann cells transition to mature, myelin-forming phenotype coating large-diameter axons, intensively GAP43-stained, marking the process of intense axonal outgrowth. The time-line for completing reparatory processes in response to vitamins B treatment, observed in this study, had previously also been confirmed in our group through behavioral and EMG testing (Nedeljković et al., 2017).

The process of axonal regeneration progresses with the speed of 1-3 mm/day, depending on multiple factors, such as the location with respect to the neuron, as availability of building blocks, i.e. different cytoskeletal components, etc. The subsequent elongation goes along the remaining endoneurial tube for proper orientation of the outgrowing axons to their targets, where Schwann cells behave crucial since they form Büngner repair bands, which safeguard and insulate the navigating endoneurial channel. Besides this, Schwann cells, just like as macrophages, secrete different neurotrophic factors, which promote regrowth of the nerve. Upon approaching the endoneurial tube, the growth cone more likely reaches target tissues to finish the complex process of maturation, involving remyelination, expansion of the axon together with functional re-innervation (Menorca et al., 2013). In this study, close association between IL-10 immunoreactive M2 macrophages and Schwann cells were spotted in transversal sections of the injured femoral nerve, Notably, IL-10 immunoreactivity was also associated to the columns of Schwann cells that form the Büngner bands. Just like the Stratton and colleagues (Stratton et al., 2018), in this study we recognized macrophages as important regulators of the Schwann cells maturation triggered by the nerve injury.

Just like in the case of the effects on different macrophage phenotypes, versatile effects of the B vitamins treatment stand as an important finding since almost all compounds that enable the reduction of neuroinflammation only affect myelin-forming Schwann cells. Results from this study show that the vitamin B complex treatment is able of both protecting the myelin-forming Schwann cells, and also of stimulating the transition to the non-myelin-forming phenotype and *vice-versa* during the early post-PNI recovery, whereby the mechanistic basis for this subtle B vitamins effects remain to be further explored in upcoming studies. All in all, observed B vitamins effects on the macrophage and Schwann cells plasticity in response to PNI opens new avenues for designing innovative macrophage- and SC-centered complementary therapeutic strategies to support the recovery after the surgical treatment, which demands clarification of the exact molecular interactions between macrophage and Schwann cells interactions in future studies.

Data coming from a number of recent publication imply that Schwann cells likely act supportive for macrophage functioning in neuroreparation after PNI, presumably by producing different ligands for macrophage receptors thus regulating the transition between M1and M2 phenotypes (Stratton and Shah, 2016). In relation to this, Schwann cells also produce hallmark M2 cytokines and strongly induce M2macrophage phenotypes for better axonal outgrowth (Stratton and Shah, 2016). In this study up-regulation of anti-inflammatory cytokines, IL-4 and IL-10, was observed in Schwann cells at 7^{th} dpo predominantly, and to a certain extent at day 14 after the injury, whereby the noticed up-regulation was much higher in response to the complex vitamins treatment. Concomitantly, more notable presence of M2-like macrophages, which also co-produce IL-4 and IL-10, could be noticed. At the same time-point, i.e. at day 7 after the injury, an increase in the production of proinflammatory cytokine TNF-α and iNOS immunostaining in Schwann cells could be observed, particularly at the immediate site of nerve transection. analogous profile of cytokine production in response to PNI was also reported by Dubový et al. (2014) (Dubový et al., 2014), who proposed that concomitant induction of proinflammatory and anti-inflammatory cytokines enables fine-tuning of the inflammatory Schwann cells response to support axonal outgrowth. The interaction going other way around, from macrophages to Schwann cell maturation, should also be considered as a possibility since macrophages were already shown as modulators of Schwann cell maturation upon the nerve injury (Stratton et al., 2018).

Entry of the calcium through LVDCCs correlates with multiple functional and phenotypic changes in macrophages, such as: changing morphology, proliferation status and also the expression profile of pro- and anti-inflammatory mediators (Espinosa-Parrilla et al., 2015; Hegg et al., 2000; Silei et al., 1999). Calcium entry through LVDCCs is indispensable for proper myelination, whereby it was found to facilitate the interactions between axons and glia during the process of myelin formation (Cheli et al., 2015; Cheli et al., 2016). Therefore, in the present study we investigated possible involvement of LVDCCs in the processes occurring in response to PNI solely, or in combination to the B vitamins treatment. The attention was limited to Ca^{2+} signaling through the $Ca_V1.2$ LVDCC isoform, since this particular isoform appeared recently as an important regulator of immune and Schwann cell functioning, including: cell activation, cell proliferation and survival, and also the profiling of cytokine productions (Badou et al., 2013; Chen et al., 2011; Davenport et al., 2015; Suzuki et al., 2010; Westenbroek et al., 2004). More precisely, in this study we wanted to infer if the observed phenotypic transition between M1 and M2 macrophages during the post-PNI recovery in femoral nerve – shown to be supported by the B vitamins treatment – is accompanied by the changes in the expression profile of the $Ca_V1.2$ isoform. This assumption was confirmed by observing the peak in $Ca_V1.2$ immunoreactivity in $ED1^+/TNF\text{-}\alpha^+$ M1 macrophages at 1^{st} dpo post-injury, followed by a gradual decrease occurring in parallel with the M1 to M2 macrophage transition. Analogous role of the $Ca_V1.2$ isoform in the regulation of microglial proinflammatory activity was reported by Espinosa-Parrilla et al. for the rat model of N-methyl-D-Aspartate-induced hippocampal neurodegeneration (Espinosa-Parrilla et al., 2015), while the results on possible roles in PNI-induced neuroinflammatory processes are still lacking. Importantly, the B vitamins treatment attenuates the $Ca_V1.2$ expression in proinflammatory M1 ($ED1^+/TNF\text{-}\alpha^+$) macrophage phenotype. Conversely, B vitamins elevate the expression of $Ca_V1.2$ in reparatory M2 macrophages, which may represent the route for proposed roles in nerve protection and repair. Nonetheless, a substantial up-regulation of $Ca_V1.2$ in myelinated Schwann cells, and to a certain extent in non-myelinated Schwann cells phenotype suggests the involvement of calcium currents *via* $Ca_V1.2$ in the Schwann cells activity in response to PNI. Remarkably, the most intensive $Ca_V1.2$ expression of was detected in axons across of all the investigated groups of animals. Analogously, high immunostaining signals for the L-type $Ca2^+$ channels were detected in odontoblast cell bodies

and associated processes, but also in fibroblast cell bodies and Schwann cells just like in unmyelinated and myelinated axons in root nerves and proximal branches in coronal pulp (Westenbroek et al., 2004). Injury, however, triggers short-term down-regulation of immunostaining signals for these LVDCCs. Data obtained in this study imply that the treatment with B vitamins certainly accelerates the M1-to-M2 transition, whereby L-type $Ca_V1.2$ calcium channels might affect the underlying process of functional alterations corresponding to the profiles of cytokine secretion.

To summarize, the results of this thesis indicate for the first time that the treatment with the complex of B (B1, B2, B3, B5, B6, and B12) vitamins accelerates the PNI-induced M1 to M2 macrophage transition and also limit the extent of immediate inflammation by decreasing the expression of proinflammatory and up-regulating the expression of anti-inflammatory cytokines. Also, the investigated B vitamins treatment was shown to effectively support the switch between the non-myelinating to myelin-forming-Schwann cells, contributing also to the suppression of neurodegeneration by inducing analogous expression profile of proinflammatory and anti-inflammatory cytokines, seen already in macrophages. Induced changes concomitantly alter the interactions patterns between macrophage and Schwann cells, for promoted regeneration of the injured nerve. Finally, this thesis results imply putative role for the $Ca_V1.2$ subunit of L-type of VDCCs in PNI-induced neuroinflammation, where the B vitamins treatment up-regulates $Ca_V1.2$ in M2 reparatory macrophages, Schwann cells and axons, assuming the role of $Ca_V1.2$ in the promotion of injured nerve recovery. Overall, the results of this thesis strongly that thanks to the manifold and versatile effects of the vitamins B complex treatment it should be considered as an alternative therapeutic strategy in the treatment of neuroinflammation and neuroregeneration. The capacity of B vitamins to affect crucial cell to cell interactions between macrophages and Schwann cells leading to improved nerve regeneration strongly suggests their application for treating peripheral nerve injury in humans, which, however, requires more detailed examination in upcoming studies and further confirmation in rigorous clinical trials.

6 CONCLUSIONS

Based on the results of this study, the following conclusions can be drawn:

1) This study confirms that the treatment with complex of B vitamins (B1, B2, B3, B5, B6, and B12) suppresses neurodegeneration by reducing the expression of proinflammatory and up-regulating the expression of anti-inflammatory cytokines, produced by macrophages and Schwann cells;

2) The treatment with the complex of B vitamins (B1, B2, B3, B5, B6, and B12) can effectively promote PNI-induced M1 to M2 macrophage polarization;

3) The treatment with complex of B vitamins (B1, B2, B3, B5, B6, and B12) effectively promotes the PNI-induced transition of the Schwann cells phenotype from non-myelinating to myelin-forming-Schwann cells;

4) Previously described processes consequently alter interactions between macrophages and Schwann cells, where these novel interactions, supported by the vitamin B complex (B1, B2, B3, B5, B6, and B12) treatment after PNI, contribute to the regeneration of the injured nerve;

5) The ability of B vitamins complex (B1, B2, B3, B5, B6, and B12) to modulate macrophages to Schwann cells interaction reveals their potential as an additional tool in peripheral nerve regeneration therapies in humans;

6) This study confirms the potential involvement of the $Ca_V1.2$ subunit of the L type VDCCs in the processes of inflammation after PNI, *via* detected increased $Ca_V1.2$ abundance in M2 macrophages, Schwann cells and axons, suggesting their mediating role in improving recovery of the injured peripheral nerve;

7) Finally, complex of B vitamins (B1, B2, B3, B5, B6, and B12), owing to their pleiotropic effects, displayed the significant potential in the treatment of neuroinflammation and neuroregeneration, and can be considered as a promising adjuvant therapy of PNI in humans, which remains to be further explored.

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BIOGRAPHY

Adil Othman Ehmedah was born on 11th, January 1976 in Souq Alkhamees, Libya. He received a bachelor's degree in Medical technology (Medical laboratory) from Alfateh University for Medical Science, Libya in 1996. In January 1999, he started working as a medical technician in Tripoli Medical Center in departments of Hematology, Biochemistry, and Blood Bank until June 2007. Then he started Master studies in Transfusion Science at University Science Malaysia in Malaysia. He carried out experimental part of his Master studies in National Blood Center in Kuala Lumpur. He received Master's degree in 2008. Then he returned back working in Tripoli Medical Center until 2012. Then he started working in Almergib University at the faculty of medical technology as an Assistant Lecturer in Medical Laboratory until he has got a scholarship for PhD study and started his PhD study in 2015 at the faculty of Biology (module: Immunobiology), University of Belgrade. Adil has authored two and co-authored one reviewed papers in international journals during his PhD study.

PRILOZI

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2. **Prilog 2** – Izjava o istovetnosti štampane i elektronske verzije

3. **Prilog 3** – Izjava o korišćenju

4. **Prilog 4** – Publikacija: Ehmedah A, Nedeljkovic P, Dacic S, Repac J, Draskovic Pavlovic B, Vucevic D, Pekovic S, Bozic Nedeljkovic B. **(**2019) Vitamin B Complex Treatment Attenuates Local Inflammation after Peripheral Nerve Injury. Molecules 24(24). pii: E4615. doi: 10.3390/molecules24244615

Линк ка публикацији: https://www.mdpi.com/1420-3049/24/24/4615

5. **Prilog 5** – Publikacija: Ehmedah A, Nedeljkovic P, Dacic S, Repac J, Draskovic Pavlovic B, Vucevic D, Pekovic S, Bozic Nedeljkovic B. **(**2020) Effect of Vitamin B Complex Treatment on Macrophages to Schwann Cells Association during Neuroinflammation after Peripheral Nerve Injury. Molecules 25(22). pii: E5426. doi.org/10.3390/molecules25225426

Линк ка публикацији: https://www.mdpi.com/1420-3049/25/22/5426

Прилог 1.

Изјава о ауторству

Потписани **Адил О. Ехмедах**

број индекса **Б3006/2015**

Изјављујем

да је докторска дисертација под насловом

"Утицај комплекса витамина Бе на процес неуроинфламације и регенерације периферног нерва пацова након повреде: улога L типа волтажно-зависних калцијумових канала" (енгл. „Effect of vitamin B complex therapy on process of neuroinflammation and regeneration of rat peripheral nerve after injury: role of L type of voltage-dependent calcium channels")

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Име и презиме аутора **Адил О. Ехмедах**

Број индекса **Б3006/2015**

Студијски програм **БИОЛОГИЈА**

Наслов рада **"Утицај комплекса витамина Бе на процес неуроинфламације и регенерације периферног нерва пацова након повреде: улога L типа волтажно-зависних калцијумових канала" (енгл. „Effect of vitamin B complex therapy on process of neuroinflammation and regeneration of rat peripheral nerve after injury: role of L type of voltage-dependent calcium channels")**

Ментор **проф. др Биљана Божић Недељковић**

Потписани **Адил О. Ехмедах**

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

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Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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Vitamin B Complex Treatment Attenuates Local Inflammation after Peripheral Nerve Injury

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Abstract: Peripheral nerve injury (PNI) leads to a series of cellular and molecular events necessary for axon regeneration and reinnervation of target tissues, among which inflammation is crucial for the orchestration of all these processes. Macrophage activation underlies the pathogenesis of PNI and is characterized by morphological/phenotype transformation from proinflammatory (M1) to an anti-inflammatory (M2) type with different functions in the inflammatory and reparative process. The aim of this study was to evaluate influence of the vitamin B (B1, B2, B3, B5, B6, and B12) complex on the process of neuroinflammation that is in part regulated by L-type $Ca_V1.2$ calcium channels. A controlled transection of the motor branch of the femoral peripheral nerve was used as an experimental model. Animals were sacrificed after 1, 3, 7, and 14 injections of vitamin B complex. Isolated nerves were used for immunofluorescence analysis. Treatment with vitamin B complex decreased expression of proinflammatory and increased expression of anti-inflammatory cytokines, thus contributing to the resolution of neuroinflammation. In parallel, B vitamins decreased the number of M1 macrophages that expressed the $Ca_V1.2$ channel, and increased the number of M2 macrophages that expressed this channel, suggesting their role in M1/M2 transition after PNI. In conclusion, B vitamins had the potential for treatment of neuroinflammation and neuroregeneration and thereby might be an effective therapy for PNI in humans.

Keywords: peripheral nerve injury; neuroinflammation; regeneration; M1/M2 macrophages; calcium channels; vitamin B complex therapy

1. Introduction

Injury to the peripheral nervous system (PNI) of the upper and lower limbs represents a serious issue in developed countries due to the increasing incidence associated with the modern lifestyle, characterized by high rate of traffic injuries, industrial traumatism, and injuries in the workplace. In Europe, the incidence of PNI is estimated at ~300,000 cases per year [1,2]. The major problem from socio-economic perspective is the fact that PNI commonly occurs in young male subjects at their most productive age, where actively serving military officers comprise a significant fraction of

affected individuals. Concerning the low rate of recovery, this consequently leads to reduced working capabilities and overall life quality.

PNI triggers Wallerian degeneration, where neuroinflammation—mediated by series of cellular and molecular events—represents the most important stage for axon regeneration and reinnervation of target tissues [3]. Investigation of PNI pathophysiology revealed that Schwann cells and not the neurons are mainly affected by PNI. They are also suggested to be the primary mediators in triggering many of the events in Wallerian degeneration, while changes in their protein expression at the site of injury are supposed to be the key to axon regeneration [4]. Moreover, Schwann cells, together with inflammatory cells that infiltrate damaged tissue, represent crucial components of a series of neuroinflammatory events involved in injury and regeneration of the peripheral nerve. Among them, neutrophils are the first cell type that penetrates the injured nerve, followed by monocytes that differentiate to tissue macrophages, responsible for phagocytosis of myelin and tissue debris [5–8]. On the top of this, haematogenic macrophages, together with resident macrophages, enable the production of cytokines and neurotrophic factors necessary for activation of Schwann cells and extracellular matrix (ECM) remodeling, which is crucial for axon regeneration [4,9].

In general, macrophages are classified into two main subtypes: (1) M1 macrophages, which are active at inflammatory sites as primary phagocytic cells; and (2) M2 macrophages, which take over the tissue remodeling process after the inflammatory activity of M1 cells. According to this, M1 cells are known as classically activated or proinflammatory macrophages, while M2 cells are designated as alternatively activated or anti-inflammatory macrophages [10–12]. M1 activity assumes production of proinflammatory cytokines that can be neurotoxic to the regenerating axon, thus potentially amplifying the on-going process of neurodegeneration. To impede this potentially neurotoxic effect of M1 cells, the timely activation of reparatory M2 cells is crucial for initiation and execution of the cascade of neuroreparative events. M2 macrophages encompass a functionally diverse group of macrophages that are further divided into the M2a, M2b, M2c, and M2d subtypes [13–15].

The pro-healing activity of macrophages is further supported by hyper-proliferative, resident Schwann cells [16], which undergo a process of de-differentiation to the stage of pre-myelinating cells, releasing cytokines important for monocyte infiltration to the injury site [17]. Upon this, Schwann cells make novel contact with tissue macrophages, causing them to re-differentiate to the re-myelinating phenotype crucial for axonal regeneration. Further on, the interaction of macrophages with the newly-synthesized myelin can lead to resolution of inflammatory processes at the injured nerve. Thus, this bidirectional interaction between Schwann cells and macrophages is crucial for the tight control of peripheral nerve regeneration [18,19].

Processes underlying macrophage activation, such as alterations in morphology, proliferation, and production of proinflammatory mediators, are associated with calcium entry via the L type of voltage-dependent calcium channels (LVDCCs) [20–22]. PNI induces changes in the expression pattern of LVDCC isoform $Ca_V1.2$, primarily through membrane potential regulation [23]. This process is also substantially modulated by soluble molecules—hormones, cytokines, and neurotransmitters [24]. Also, it has been shown that Ca^{2+} influx mediated by L-type VDCCs is necessary for normal myelination and facilitates axon–glial interaction during the first steps of myelin formation [25,26]. Their expression has been shown in Schwann cells as well [23,27]. Broad expression of L-type VDCCs and their involvement in the processes of inflammation and regeneration make them an interesting target for different therapeutic modalities.

For mechanical damage to the injured nerve, surgical treatment is the first therapeutic choice for most types of PNI. While being indispensable, surgical treatment requires additional adjuvant therapeutic modalities to maximize the recovery process [28]. Vitamins from the B group, separately or in different combinations, are used for the treatment of central and peripheral nervous system injuries, giving the best results in neuropathic pain reduction [29] and improvement of regenerative capacity [30]. Additionally, Altun and Kurutas [31] showed that injury to the sciatic nerve is accompanied by a lower level of vitamin B complex and vitamin B12 in homogenates of injured nerve after seven days,

suggesting that supplementation of these vitamins would be beneficial for acceleration of nerve regeneration. Therefore, the aim of this study was to evaluate the influence of vitamin B (B1, B2, B3, B5, B6, and B12) complex therapy on the processes of neuroinflammation and neuroregeneration in the rat model of the femoral nerve motor branch injury. This assumes examining the effects of vitamin B complex therapy on pro- and anti-inflammatory cytokine production and phenotype profiles of macrophages (M1 and M2). Since these processes are partly regulated by $Ca_V1.2$ subunit of LVDCCs, the potential role of this channel have been investigated.

2. Results

2.1. Treatment with Vitamin B Complex Alters Macrophage Morphology after PNI

Macrophage activation underlies the pathogenesis of PNI and is characterized by morphological transformation. Also, depending on extracellular milieu they have capability to switch from proinflammatory to anti-inflammatory activation phenotype participating sequentially in both the induction and the resolution of inflammation [32]. Therefore, to estimate the impact of PNI and the effects of vitamin B complex treatment on macrophage activation, we first followed changes of macrophages morphology during the post-injury period (1, 3, 7, and 14 days post operation (dpo), Figure 1A). As a commonly utilized marker of activated macrophages we used the ED1 antibody (Table 1). The total number of $ED1^+$ cells is presented in Figure 1B as a number of $ED1^+/mm^2$. In the sham-operated (S) group only a few $ED1^+$ cells were detected and this number was not significantly changed at all investigated time points (Figure 1A,B). The number of $ED1^+$ cells dramatically increased after PNI in the operated (O) group, peaking at the 7 dpo. Interestingly, at days 1 and 3 post-injury these $ED1^+$ cells had a morphology resembling the M1 phenotype, while at 7 and 14 dpo most of them acquired the M2 phenotype. Treatment with B vitamins reduced the number of proinflammatory M1 macrophages at day 1 post-injury for 45% (Figure 1B), while starting from day 3 until 14 dpo enlarged the number of $ED1^+$ macrophages with the "foamy" morphology of the M2 phenotype (Figure 1A). The effect of B vitamins was particularly pronounced at 7 dpo (the increase was 33% compared to the O group). These results indicated that PNI induced time-dependent changes in the macrophages morphology and transition from M1 proinflammatory to M2 regenerative type during the recovery period. Treatment with vitamin B complex accelerates this transition.

Table 1. List of primary and secondary antibodies used for immunofluorescence labeling.

Antibody	Source	Dilution	Company
anti-$Ca_V1.2$	rabbit	1:200	Sigma-Aldrich, Munich, Germany
anti-CD68 (ED1)	mouse	1:100	Abcam, Cambridge, MA, USA,
anti-TNF-α	goat	1:100	Santa Cruz Biotechnology, CA, USA
anti-iNOS	rabbit	1:100	Santa Cruz Biotechnology, CA, USA
anti-IL-4	rabbit	1:100	Santa Cruz Biotechnology, CA, USA
anti-IL-10	goat	1:100	Santa Cruz Biotechnology, CA, USA
anti-Arg-1	rabbit	1:200	Sigma-Aldrich, Munich, Germany
anti-rabbit anti-IgG Alexa Fluor 488	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-mouse anti-IgG Alexa Fluor 555	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-goat anti-IgG Alexa Fluor 350	donkey	1:200	Invitrogen, Carlsbad, CA, USA
anti-goat anti-IgG Alexa Fluor 488	donkey	1:200	Invitrogen, Carlsbad, CA, USA

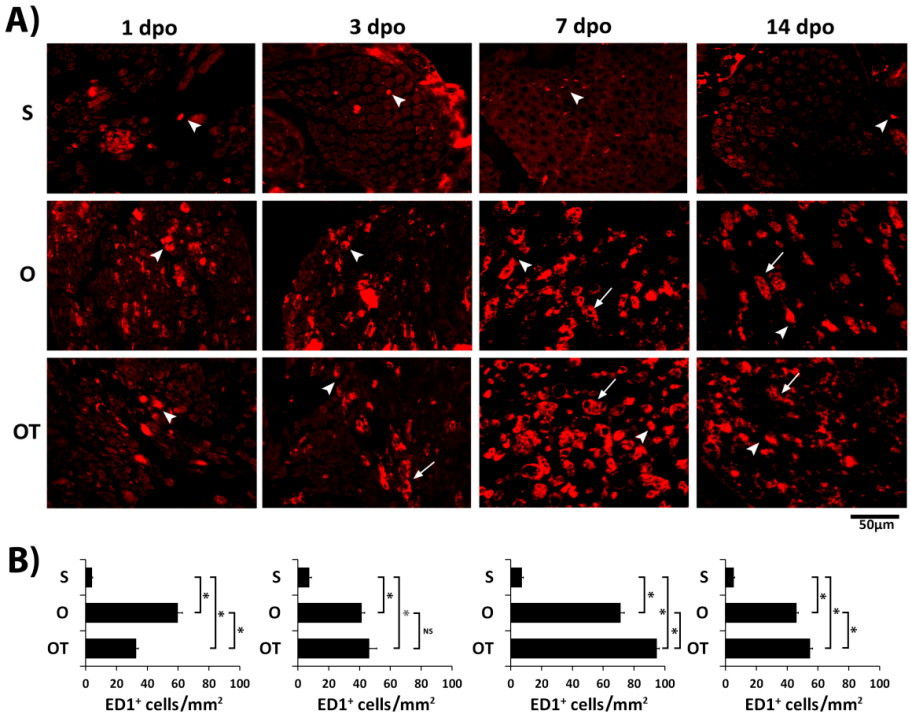

Figure 1. Effect of peripheral nerve injury (PNI) and treatment with B vitamins on macrophage morphology. Cross sections of femoral nerve obtained from the sham (S), operated (O, transection of motor branch and immediate reconstruction using termino–terminal anastomosis), and operated and treated with vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (red) which is a common marker of activated macrophages. (**A**) The representative images showed morphological changes of ED1$^+$ macrophages during the postoperative period (1, 3, 7, and 14 days) and after 1, 3, 7, and 14 injections of complex of B vitamins. Transition from the M1 (arrow heads) to M2 (arrows) morphology type in the O group is seen at day 7 and 14 post-injury. After treatment with B vitamins the appearance of M2 macrophages began after the third injection. Scale bar: 50 µm. (**B**) Total quantification of ED1-positive cells/mm^2 from experimental groups is depicted in the graphs (black bars). The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p < 0.05$ vs. control, or vs. O group, as indicated at the graphs).

2.2. The Effect of Vitamin B Complex on Expression of Pro-Inflammatory Mediators after PNI

Given that tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) are mainly expressed marker proteins in M1 macrophages [10–12], we then investigated whether treatment with B vitamins would modulate expression profile of these cytokines in macrophages after PNI. At day 1 post-injury a huge number of ED1$^+$/TNF-α$^+$ (Figure 2A, insert) and ED1$^+$/iNOS$^+$ cells (Figure 2C, insert) (yellow) with round and oval shapes were widespread through the nerve slices of O group. Treatment with the cocktail of B vitamins reduced TNF-α and iNOS staining in ED1$^+$ macrophages (Figure 2A,C, OT, inserts). The total number of ED1$^+$/TNF-α$^+$ cells and ED1$^+$/iNOS$^+$ cells is presented in Figure 2B,D, respectively, as a number of double-positive cells/mm^2 and the percentage of double positive cells in the ED1$^+$ cell population. A similar pattern of staining was detected at 3 dpo and after three treatments with B vitamins. At 7 dpo most of the ED1$^+$ macrophages in both the O and OT group acquired a "foamy" morphology characteristic of M2 phenotype. However, they were not TNF-α and iNOS co-stained (Figure 2A,C (inserts)). Only those ED1$^+$ cells which still had oval/round morphology were co-labeled with TNF-α and iNOS. Importantly, both TNF-α and iNOS staining was detected in some ED1$^-$ cells (Figure 2, yellow arrows). Obtained results confirmed our previous observation that PNI induces expression of proinflammatory mediators in M1 macrophages, and that over time

post-injury this M1 proinflammatory type of macrophages was replaced with the M2 type. Treatment with B vitamins significantly reduces the number of ED1$^+$ macrophages expressing proinflammatory mediators, TNF-α, and iNOS, and in that way has an anti-inflammatory effect. These effects were the most pronounced at days 1 and 3 post-injury.

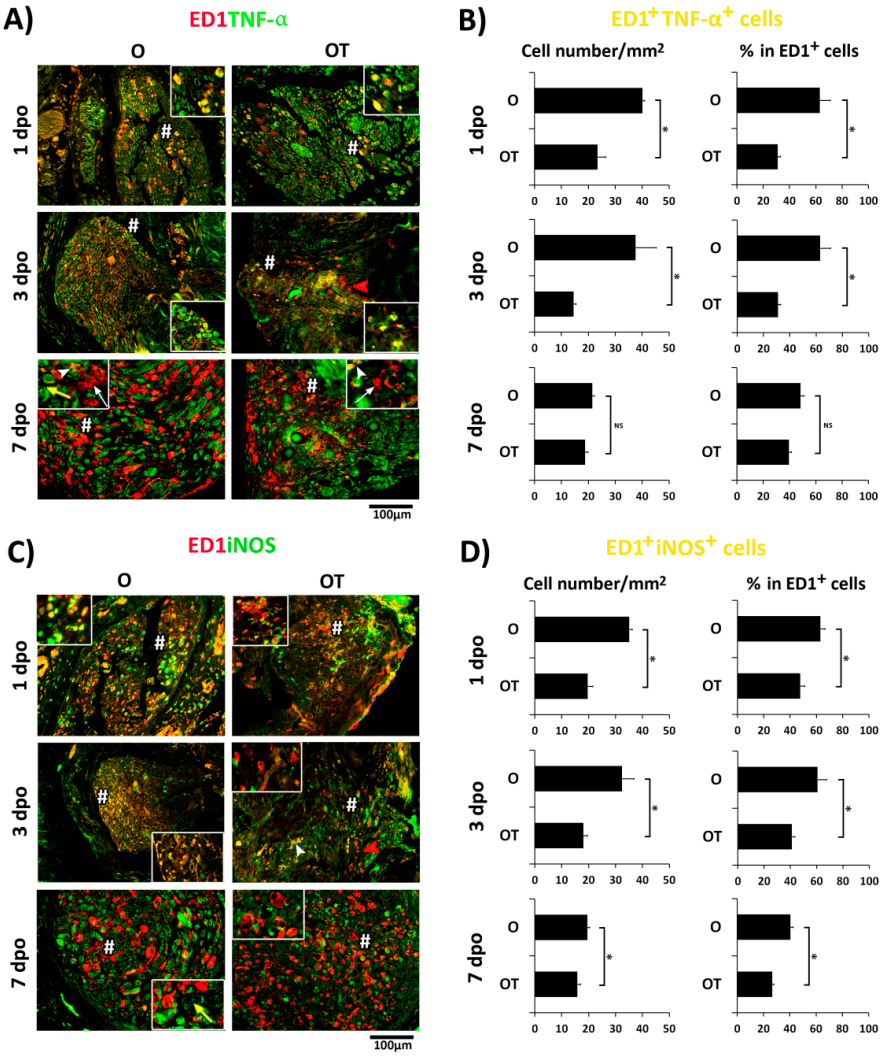

Figure 2. Treatment with B vitamins reduced PNI-induced expression of proinflammatory mediators in M1 macrophages. (**A**,**C**) Cross sections of femoral nerves obtained from the operated (O) and operated and treated with vitamin B complex (OT) groups were counterstained with (**A**) anti-TNF-α (M1 marker, green) and anti-ED1 (red) or with (**C**) anti-iNOS (M1 marker, green) and anti-ED1 (red) antibodies. The quantification of double-positive ED1$^+$/TNF-α$^+$ cells (**B**) and ED1$^+$/iNOS $^+$ cells (**D**) is presented as number of double-positive cells/mm^2 and the percentage of double-positive cells in the ED1$^+$ cell population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p < 0.05$ OT vs. O group, as indicated at the graphs). At day 1 and 3 days post operation (dpo) ED1$^+$/TNF-α$^+$, as well as ED1$^+$/iNOS$^+$ macrophages in both the O and OT groups, had oval and round morphology and showed complete overlapping (yellow fluorescence)(inserts). Treatment with B vitamins reduced TNF-α and iNOS staining and the majority of macrophages were only ED1$^+$ (red arrow head). At day 7 post-injury most of macrophages were only ED1$^+$ and were polarized toward M2 type (white arrows, insert), while only a few ED1$^+$/TNF-α$^+$ (white arrow head, insert) macrophages were noticed. Some ED1$^-$ cells (yellow arrows) that were both TNF-α$^+$ and iNOS$^+$ were also noticed. # indicates where the high magnification images in inserts are taken from. Scale bar: 100 μm. PNI: peripheral nerve injury; TNF: tumor necrosis factor; iNOS: inducible nitric oxide synthase.

2.3. The Effect of Vitamin B Complex on Expression of Anti-Inflammatory Cytokines after PNI

Further, we wanted to determine whether treatment with vitamin B complex would enhance expression of anti-inflammatory cytokines, interleukin (IL)-4 and IL-10, in ED1$^+$ macrophages (Figure 3). It is shown that the IL-4 cytokine is expressed in M2a, while the IL-10 is a marker of the M2c subtype of M2 macrophages [13,15,27,33]. At 3 dpo in the O group of animals complete overlapping (yellow) of IL-4 (green) and ED1 (red) staining was detected in macrophages with round and oval cell body (Figure 3A, insert). After treatment with B vitamins, macrophages in the injured nerve acquired a "foamy" morphology and were intensively labeled with anti-IL-4 antibody (Figure 3A, insert). Similarly, a huge number of ED1$^+$/IL-10$^+$ macrophages with a round and oval cell body (Figure 3C, insert) were detected in the O group. However, in the OT group large ED1$^+$ macrophages were not labeled with anti-IL-10 antibody, and only those with small, round, and oval cell bodies (Figure 3C, insert) were ED1$^+$/IL-10$^+$ (Figure 3C and 3C(insert)). At 7 dpo in both the O and OT groups (Figure 3A and 3A(inserts)), most of the ED1$^+$ cells had a "foamy" morphology and were IL-4 negative, although a few ED1$^+$/IL-4$^+$ macrophages with round and oval cell bodies were found. Interestingly, IL-4 expression was seen in some ED1$^-$ cells as well. Similarly, at the same time point post-injury, abundant IL-10 expression was seen in some ED1$^-$ cells in both O and OT groups. In the O group, ED1$^+$ macrophages with "foamy" morphology did not express IL-10 and were pre-dominant compared to those with an oval morphology that were ED1$^+$/IL-10$^+$ co-stained (Figure 3C and 3C(insert)). In contrast, after treatment with complex of B vitamins the foamy macrophages were ED1$^+$/IL-10$^+$ (Figure 3C and 3C(insert)), while those with round and oval morphology were only ED1$^+$. At 14 dpo the number of ED1$^+$/IL-4$^+$ cells, as well as ED1$^+$/IL-10$^+$ cells, was decreased after treatment with vitamin B complex, while the fractions of these cells in total ED1$^+$ cell population were the same in the O and OT group. The total number of ED1$^+$/IL-4$^+$ cells and ED1$^+$/IL-10$^+$ cells is presented in Figure 3B,D, respectively, in terms of the number of double-positive cells/mm^2 and the percentage of double-positive cells in the ED1$^+$ cell population. Taken together, these results suggest that PNI and treatment with B vitamins cause the appearance of different subtypes of M2 macrophages.

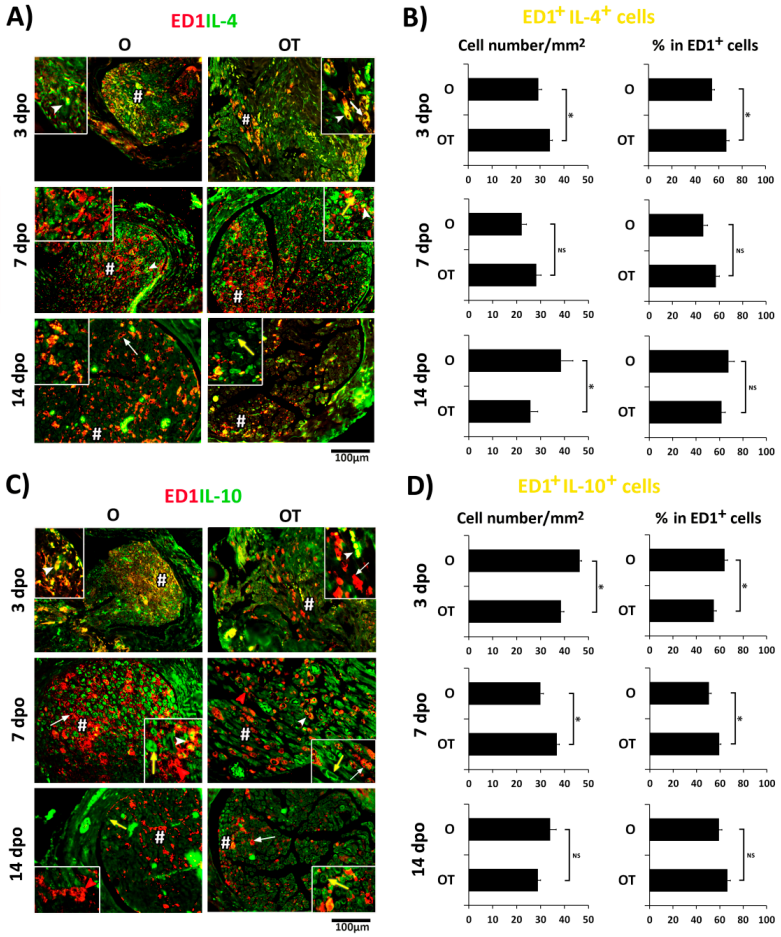

Figure 3. Effects of PNI and B vitamin treatment on expression of anti-inflammatory cytokines in M2 macrophages. (**A**,**C**) Cross sections of femoral nerve obtained from the operated (O) and operated and treated with vitamin B complex (OT) groups were counterstained with (**A**) anti-IL4 (M2a marker, green) and anti-ED1 (red) or with (**C**) anti-IL10 (M2c marker, green) and anti-ED1 (red) antibodies. The quantification of double-positive ED1$^+$/IL-4$^+$ cells (**B**) and ED1$^+$/IL-10$^+$ cells (**D**) is presented as number of double-positive cells/mm^2 and the percentage of double positive cells in the ED1$^+$ cell population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p < 0.05$ OT vs. O group, as indicated at the graphs). At day 3 post-injury ED1$^+$/IL-4$^+$ as well as ED1$^+$/IL-10$^+$ macrophages with oval and round morphology in both the O and OT groups showed complete overlapping (yellow fluorescence, white arrow head) (inserts). Treatment with B vitamins increased IL-4 immunoreactivity in "foamy" M2 macrophages (white arrow), while IL-10 staining was reduced and the majority of M2 macrophages were only ED1$^+$ (white arrow). At 7 and 14 dpo IL-4 and IL-10 staining was seen in ED1$^-$ cells in both groups (yellow arrows). M2 macrophages were void of IL-4 at day 7, but IL-4 was abundantly present at day 14 (white arrow). ED1$^+$/IL-10$^+$ M2 macrophages were seen at 7 dpo (white arrows), but were sparsely present at day 14. # indicates where the high magnification images in inserts are taken from. Scale bar: 100 µm. PNI: peripheral nerve injury; IL-4: interleukin -4; IL-10: interleukin-10.

2.4. Treatment with Vitamin B Complex Reduces Number M1 Macrophages Expressing the $Ca_V1.2$ Channel after PNI

Having in mind that Ca^{2+} influx mediated by L-type VDCCs is necessary for macrophage activation, alterations in morphology, and production of proinflammatory mediators [22], we next evaluated the effects of PNI and treatment with B vitamins on expression of the $Ca_V1.2$ isoform of L-VDCCs in the injured peripheral femoral nerve. As shown in Figure 4A in the O group, expression of $Ca_V1.2$

gradually decreases with time elapsed from PNI. The highest number of $Ca_V1.2^+/ED1^+/TNF\text{-}\alpha^+$ was observed at day 1 post-injury (Figure 4C). At 7 dpo most of the $ED1^+$ macrophages acquired a "foamy" morphology characteristic for the M2 phenotype and were $Ca_V1.2^-/TNF\alpha^-$. Treatment with complex of B vitamins (Figure 4B,C) significantly diminished the number of proinflammatory M1 macrophages ($ED1^+/TNF\text{-}\alpha^+$) that co-express the $Ca_V1.2$ isoform. However, the fractions of $Ca_V1.2^+/ED1^+/TNF\text{-}\alpha^+$ cells in total macrophages ($ED1^+$ cells) and M1 macrophages ($ED1^+/TNF\text{-}\alpha^+$ cells) were the same in the O and OT group. Interestingly, concomitantly with down-regulation of $Ca_V1.2$ expression in M1 macrophages, its expression was significantly up-regulated in axons and in some $ED1^-$ cells (Figure 4A,B, higher magnification, green asterisks and yellow arrows, respectively). In summary, these results indicate that treatment with B vitamins reduced the number of M1 macrophages that express $Ca_V1.2$ channel.

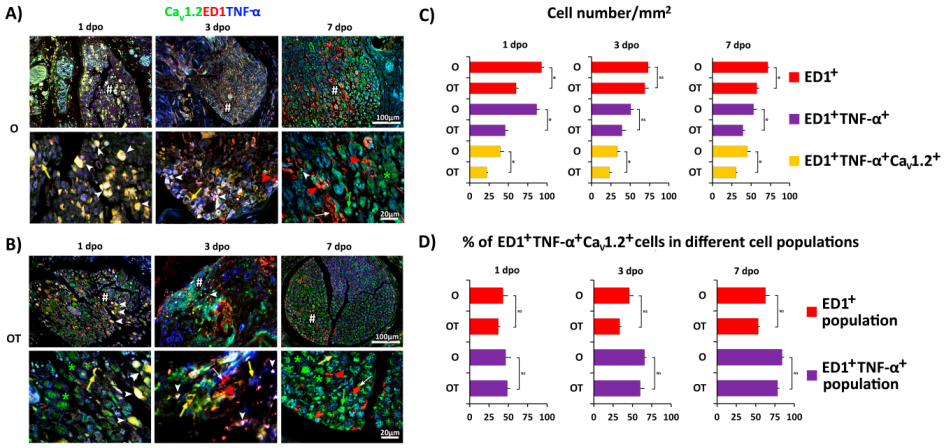

Figure 4. Treatment with vitamin B complex induces time-dependent changes of $Ca_V1.2$ channel expression in M1 macrophages after PNI. To evaluate cellular distribution of the $Ca_V1.2$ isoform of L-VDCCs (green), cross sections of femoral nerve obtained from the: (**A**) operated (O); and (**B**) operated and treated with vitamin B complex (OT) groups were counterstained with anti-TNF-α (M1 marker, blue) and anti-ED1 (red) antibodies. The quantification of single-, double-, and triple-positive cells is presented as number of $ED1^+$ cells/mm^2, $ED1^+/TNF\text{-}\alpha^+$ cells/mm^2, and $ED1^+/TNF\text{-}\alpha^+/Ca_V1.2^+$ cells/mm^2 (**C**), and as the percentage of triple -positive cells ($ED1^+/TNF\text{-}\alpha^+/Ca_V1.2^+$ cells) in $ED1^+$ and $ED1^+/TNF\text{-}\alpha^+$ cell populations (**D**). The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p < 0.05$ OT vs. O group, as indicated at the graphs). Intensive $Ca_V1.2$ staining, besides in M1 macrophages, was observed in axons (green asterisks) and in some $ED1^-$ cells (yellow and green arrows) as well. $ED1^+/Ca_V1.2^+/TNF\text{-}\alpha^+$ M1 macrophages are marked with a white arrowhead, $ED1^+$ macrophages with oval/round morphology (M1 type) are marked with a red arrowhead, and "foamy" $ED1^+$ macrophages (M2) are indicated with white arrows. # indicates where the high magnification micrographs are taken from. Scale bars: 20 μm and 100 μm. PNI: peripheral nerve injury; TNF: tumor necrosis factor.

2.5. Treatment with Vitamin B Complex Alters Expression of $Ca_V1.2$ Channel in M2 Macrophages after PNI

Next, we examine the effects of PNI and treatment with B vitamins on $Ca_V1.2$ expression in different subtypes of M2 macrophages. As shown in Figure 3, M2 type of macrophages were predominant type at day 7 and 14 post-injury and therefore we focused our attention on these two time-points (Figure 5A,B, respectively). At both time points, in the O group most of the $ED1^+$ macrophages had a "foamy" morphology of the M2 type and were not co-stained with anti-$Ca_V1.2$ antibody (Figure 5A and B(inserts)). The total number of $ED1^+/IL\text{-}10^+/Ca_V1.2^+$ cells is presented in Figure 5C. The fractions of $Ca_V1.2^+/ED1^+/IL\text{-}10^+$ cells in total macrophages ($ED1^+$ cells) and M2 macrophages ($ED1^+/IL\text{-}10^+$ cells) were the same in O and OT group at 7 dpo. In contrast, in the group treated with B vitamins

these macrophages with "foamy" morphology were ED1$^+$/Ca$_V$1.2$^+$/IL-10$^+$. At 14 dpo we detected Ca$_V$1.2 expression in different types of macrophages. As shown in Figure 5B and B(insert) some of the macrophages had a more oval morphology and were ED1$^+$/Ca$_V$1.2$^+$. Macrophages that had "foamy" morphology showed different IL-10 expression (Figure 5B insert in the left upper corner): some of them were ED1$^+$/Ca$_V$1.2$^+$, but IL-10$^-$ (yellow arrowhead), whereas others were ED1$^+$/Ca$_V$1.2$^+$/IL-10$^+$ (blue arrow). Interestingly, these "foamy" ED1$^+$/IL-10$^-$ cells were positive for arginase-1 (Arg-1, Figure 5B insert in the left lower corner), which is a considered a classic M2a marker [15,34]. As previously mentioned, the most intensive Ca$_V$1.2 immunoreactivity was detected in axons (green asterisks) and some ED1$^-$ IL-10$^+$ cells (yellow arrows), and this was particularly extensive at 14 dpo in the OT group (Figure 5B and B(upper insert)). The fractions of Ca$_V$1.2$^+$/ED1$^+$/IL-10$^+$ cells in total macrophages (ED1$^+$ cells) and M2 macrophages (ED1$^+$/IL-10$^+$ cells) were statistically higher in OT group at 14 dpo. These results indicate that Ca$_V$1.2 has a time-dependent pattern of expression in different types of M2 macrophages.

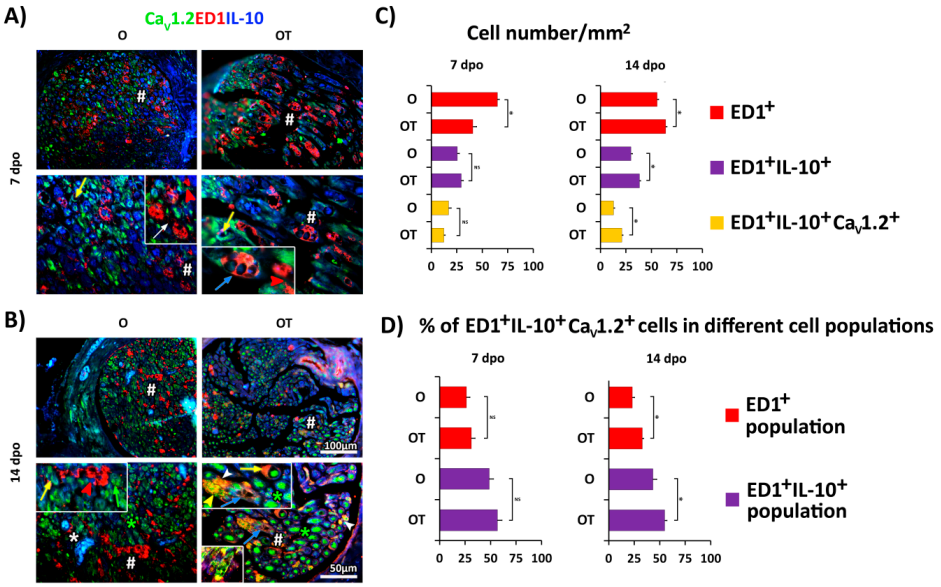

Figure 5. Treatment with vitamin B complex induces time-dependent changes in Ca$_V$1.2 channel expression in M2 macrophages after PNI. To evaluate cellular distribution of the Ca$_V$1.2 isoform of the L type of voltage-dependent calcium channels (L-VDCCs (green)), triple immunofluorescence staining of femoral nerve cross sections obtained from the operated (O) and operated and treated with vitamin B complex (OT) groups at (**A**) 7 dpo and (**B**) 14 dpo was performed. Anti-Ca$_V$1.2 (green), anti-IL-10 (M2 marker, blue), and anti-ED1 (red) antibodies were used. The quantification of single, double, and triple-positive cells is presented as the number of ED1$^+$ cells/mm^2, ED1$^+$/IL-10$^+$ cells/mm^2, and ED1$^+$/IL-10$^+$/Ca$_V$1.2$^+$ cells/mm^2 (**C**) and as the percentage of triple-positive cells (ED1$^+$/ IL-10$^+$/Ca$_V$1.2$^+$ cells) in ED1$^+$ and ED1$^+$/IL-10$^+$ cell populations (**D**). The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was performed using a two-sided Student's *t*-test (* $p < 0.05$ OT vs. O group, as indicated at the graphs). In the O group at both time-points (7 and 14 dpo) ED1$^+$ macrophages with either oval or "foamy" morphology (inserts, red arrowhead and white arrows, respectively) were not co-stained with Ca$_V$1.2 and IL-10. After treatment with 7 and particularly after 14 injections of B vitamins they were mostly ED1$^+$/Ca$_V$1.2$^+$/IL-10$^+$ (inserts, white arrowheads and blue arrows, respectively). At 14 dpo in the OT group some of the "foamy" macrophages were ED1$^+$/Ca$_V$1.2$^+$/IL-10$^-$ (yellow arrowheads), and these IL-10$^-$ cells were ED1$^+$/Arg-1$^+$ (yellow, insert in the left lower corner). Intensive Ca$_V$1.2 staining was seen in axons (green asterisks) and in some ED1$^-$/IL-10$^+$ cells (yellow and green arrows). # indicates where the high magnification micrographs are taken from. Scale bars: 50 µm and 100 µm. PNI: peripheral nerve injury; IL-10: interleukin-10.

3. Discussion

Surgery represents the first therapeutic method of choice to treat most types of PNI, where direct microsurgical nerve repair or autologous nerve grafts are currently considered as gold standard treatments. Upon undergoing surgical reconstruction, the peripheral nerve has an innate capacity to induce the process of repair; however, the regeneration of motor and sensory functions often remains incomplete. Thus, the development of alternative repair strategies and treatments to complement well established surgical procedures is recognized as highly needed, and therefore represents a very attractive area of research [35].

Among variety of proposed adjuvant therapeutic modalities related to peripheral nerve regeneration, in this work we focused our attention on vitamins of the B complex (B1, B2, B3, B5, B6, B12) as possible candidates to treat PNI, due to their infinite renewability and amenability to molecular manipulation. Namely, vitamins of the B group act as coenzymes in a substantial fraction of enzymatic processes and play key interacting roles in a majority of cellular functions, thus being important for normal functioning of the nervous system as well [36]. Importantly, due to their well-recognized positive effects on both the central and peripheral nervous system, they are already often used in the treatment of various pathological conditions [37–39]. Vitamin B12 has been shown in vivo to be the most effective of all B vitamins in peripheral nerve regeneration. The positive effect is manifested in several ways: B12 enhances neuronal survival and axonal outgrowth after trauma by activation of Erk1/2 and Akt protein kinases [40], facilitates axonal sprouting from the proximal to the distal part of the injured nerve [41], and has a positive effect on Schwann cell proliferation during regeneration [41]. It also improves myelination of axons, i.e., by increasing the diameter of the myelin sheath, and accelerating and enhancing axonal maturation. These positive effects of vitamin B12 are due to its metabolic activity. In addition to these effects, Fujii et al. [42] demonstrated that vitamins B1, B6 as well as B 12 increase the rate of impulse delivery through the nerve (by reduction of axon degeneration in rat acrylamide-induced neuropathy) and enhance the outgrowth of regenerating axons. Some data indicate that supplementation of B12 vitamin and other B vitamins in the acute period after peripheral nerve injury may be beneficial for the acceleration of nerve regeneration [31]. Additionally, there is evidence that vitamin B12 in combination with dexamethasone promotes peripheral nerve repair [43]. All presented findings provide new insight into the role of vitamins of the B complex and support the investigation of each B vitamin or their combinations for further investigation of their effects as a potential treatment of peripheral nerve injury.

As a reaction to PNI, a strong proinflammatory immune response, mediated by Schwann cells, resident macrophages, and fibroblasts, is triggered as a consequence of blood-borne monocytes infiltrating the damaged nerve only two to three days upon injury [44]. To enable adequate propagation of neuroreparation/neuroregeneration, this first response to PNI needs to be modulated by the anti-inflammatory process, mediated by the same type of cells at the injury site. Clearly, these antagonistic immune activities invoke mediator cells to undergo functional and phenotypic transformation.

Macrophages have long been held as potent immune effector cells with well-established roles in both, the tissue homeostasis and injuries, e.g., in the promotion of the tissue injury initiation and progression and also wound healing improvement and tissue remodeling in various pathological conditions [45,46]. Noticeably, mounting evidence from a number of different in vivo and in vitro studies has generally demonstrated that identification of the activated macrophage states together with macrophage M1 to M2 polarization targeting (or vice versa) might serve as novel therapeutic strategies to treat different pathological conditions [47–49], such as PNI.

Time-dependent changes in macrophage morphology, characterized by the transition from round shaped, smaller M1 (proinflammatory) to a “foamy” shaped, larger M2 (anti-inflammatory) phenotype was confirmed by our results during the recovery period after PNI. Interestingly, our results also indicate that the vitamin B complex treatment accelerates this M1 to M2 transition, and, as we recently published [30], was accompanied with improved recovery of the motor nerve and locomotor

performances in rats. The observed change in macrophage morphology during recovery from PNI, along with its acceleration in response to the vitamin B complex treatment, was confirmed by the cytokine production of the involved macrophages. In this context, our results demonstrated that PNI induces the expression of TNF-α and iNOS in macrophages, which then represent the proinflammatory M1 phenotype, being in line with the well-established role of activated M1 macrophages at the injury site during Wallerian degeneration [44]. Further on, the replacement of the type M1 with M2 macrophages during the post-injury period was evidenced by the production of IL-4 and IL-10. A number of factors can affect the process of macrophage type/phenotype transition, including local cytokine milieu at the injury site, molded by the neuronal and Schwann cell activity [18]. Treatment with B vitamin complex reduced the number of M1 macrophages, limiting their effects up to 3 dpo. Concerning this, we conclude that the treatment with the vitamin B complex expresses an anti-inflammatory effect, thus limiting the damage of the injured nerve by shortening the transition period from the indispensable inflammation immediately after PNI to the process of neuroreparation, mediated by M2 macrophages. IL-10 is an anti-inflammatory cytokine whose up-regulation is shown from 7 days up to 28 days after injury in the distal segment of the nerve [50,51]. Its well-known role is in modulation of proinflammatory cytokine expression and axonal plasticity [52]. Given that macrophages are the main cell type that expresses this cytokine [50], increased expression of IL-10 in "foamy" M2c macrophages that we detected after treatment with B vitamins, therefore, may contribute to resolution of inflammation induced by femoral nerve injury and promotion of nerve repair.

The versatile effects of the investigated vitamin B complex treatment should be considered as an important result, keeping in mind that most of the compounds that reduce neuroinflammation act only as M1 macrophage inhibitors [53]. Most importantly, our data show that M1/M2 polarization balance after PNI can be rapidly induced and completely reversed by the vitamin B complex treatment during early period of the recovery, whereas underlying cellular signaling pathways need to be elucidated in upcoming studies. Overall, macrophage polarization plasticity provides a basis for macrophage-centered therapeutic strategies as an alternative repair approach to complement the surgery after PNI. Concerning this, in our future work, the exact cellular pathways underlying the macrophage subset differentiation, induced by the combination of B vitamins, which we used in this study, will be thoroughly investigated and discussed.

Recently, results of several studies indicated that the Schwann cells most likely interact with macrophages to support their function in peripheral nerve injury, probably via expressing several ligands that are known to interact with receptors expressed by macrophages, and that Schwann cells may regulate M1/M2 transition [18]. Moreover, it was shown that Schwann cells secrete classic M2-associated cytokines and are potent inducers of M2-phenotypes in macrophages, and that these macrophages promote axonal outgrowth [18]. Accordingly, in our study we have noted up-regulation of anti-inflammatory cytokines, IL-4 and IL-10, in ED1$^-$ cells with Schwann-cell-like morphology at day 7 post-injury, and to lesser extent at 14 dpo, which was significantly potentiated after treatment with vitamin B complex. This was accompanied with increased appearance of "foamy" M2 macrophages co-expressing IL-4 and/or IL-10. Interestingly, at 7 dpo we also noted a PNI-induced increase of the proinflammatory mediators TNF-α and iNOS, with immunostaining in some ED1$^-$ cells resembling Schwann-cell-like morphology, particularly at the site of nerve injury. Similarly, Dubový et al. [54] suggested that such a simultaneous induction of proinflammatory and anti-inflammatory cytokines in Schwann cells after PNI is responsible for maintaining a balance in the inflammatory reaction of Schwann cells and in promoting axonal growth. However, one could not exclude that the macrophage–Schwann cell interaction operates vice versa since macrophages are shown to regulate Schwann cell maturation after nerve injury [19].

Given that calcium entry via LVDCCs is associated with changes in macrophage morphology, proliferation, and production of pro- and anti-inflammatory mediators [20–22], is necessary for normal myelination, and facilitates axon–glial interaction during the myelin formation [25,26], we further characterized their involvement in the observed effects of PNI and treatment with B

vitamins. Specifically, we focused our attention to Ca^{2+} signaling via $Ca_V1.2$ LVDCC isoform that appeared as "a new player" in the regulation of cell activation, proliferation, survival, and cytokine production of immune cells [55–57] and Schwann cells as well [23,27]. Since our data point to M1 toward M2 polarization during recovery period after femoral nerve injury, which is facilitated after administration of B vitamins, we investigated whether this phenotypic switch includes changes in the expression of the $Ca_V1.2$. Indeed, the most intensive $Ca_V1.2$ immunoreactivity was noted in $ED1^+$/TNF-α^+ M1 macrophages at day 1 post-injury and gradually declined with time elapsed from PNI and was concomitant with transition from M1 toward M2 type of macrophages. Similar involvement of $Ca_V1.2$ in controlling microglial proinflammatory activity was detected in a rat model of *N*-methyl-D-aspartate-induced hippocampal neurodegeneration [22]. However, data concerning their role in neuroinflammatory processes after PNI are still obscure. Considering the effect of B vitamins on $Ca_V1.2$ expression in macrophages, it is important to note that applied treatment reduces the number of proinflammatory M1 ($Ca_V1.2^+$/$ED1^+$/TNF-α^+) macrophages, but increases $Ca_V1.2$ abundance in regenerative M2 (M2a and M2c) macrophages. These results suggest that investigated channel may have implications in nerve protection and repair. In addition, we demonstrated remarkable $Ca_V1.2$ up-regulation in myelinated and to less extent in non-myelinated Schwann cells implying their potential role in activity of these cells after the PNI. The most pronounced expression of $Ca_V1.2$ was detected in axons of all investigated groups. Similarly, high levels of immunostaining of L-type Ca^{2+} channels were found in odontoblast cell bodies and their processes, in fibroblast cell bodies, and in Schwann cells, as well as in unmyelinated and myelinated axons in root nerves and proximal branches in coronal pulp [23]. However, immunostaining of these LVDCCs was shown to be transiently down-regulated in response to injury.

4. Materials and Methods

4.1. Experimental Protocol

In total, 48 adult male Albino Oxford (AO) rats, weighing between 250 and 300 g, were used throughout the study. Experimental animals were randomly divided into three groups (containing 16 animals per group). The first group comprised "operated animals" (O), in which transection of the femoral nerve motor branch was performed with immediate reconstruction using a technique of termino-terminal anastomosis. The second group (OT) included animals that were surgically treated in the same way but were additionally receiving vitamin B complex therapy. The third group included "sham operated" animals (S), which underwent the same procedure (dissection of the motor branch of the femoral nerve), but without transection of the nerve. All groups were additionally divided into sub-groups (four per group) that were sacrificed 1, 3, 7, and 14 days post-operation (dpo). Before and during the experiment, all animals were kept in the same environmental conditions (laboratory temperature 23 ± 2 °C, humidity between 50% and 60%, 12 h/12 h light/dark cycle with lights on at 07:00 h, free availability of water and food). All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of Military Medical Academy and Ministry of Agriculture and Environmental Protection Republic of Serbia, Veterinary Directorate No. 323-07-7363/2014-05/5.

Surgery

Controlled transection of the peripheral nerve is a well-described model for the examination of peripheral nerve regeneration [58]. Animals were anesthetized by intraperitoneal application of ketamine (50 mg/kg; Ketalar, Eczacibasi, Turkey) and xylazine (5 mg/kg; Rompun, Bayer, Turkey). Following anesthesia, the animals from all investigated groups (S, O, and OT) were appropriately positioned for identification of the femoral nerve motor branch on the rat left hind paw by skin incision in the left groin and femoral region, under aseptic conditions (as described in [30]). In all groups of animals (S, O, and OT), the motor branch was identified just before entry into the quadricep muscle. Further, in animals from O and OT group, using microscope magnification, transection of the branch

was done and immediate reconstruction performed using a 10.0 non-absorbable suture in the form of termino-terminal anastomosis. The skin was sutured using a 4.0 absorbable suture (Peters Surgical, Paris, France). All animals used in the experiments survived the surgical procedure and were subjected to the same set of analyses. At the appropriate time point the rats were sacrificed by intravenous injection of ketamine/xylazine at a lethal dose. All procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals. Motor branches of the femoral nerve (both reconstructed and intact contralateral) were isolated and further used for immunofluorescence analysis.

4.2. Treatment Protocol

For the investigation of vitamin B complex treatment, ampoules (2 mL) of Beviplex (Beviplex®, Galenika a.d. Belgrade, Serbia), each containing B1 (40 mg), B2 (4 mg), B3 (100 mg), B5 (10 mg), B6 (8 mg), and B12 (4 μg), were used. The given dose was 1.85 mL/kg/day. The vitamin B complex was injected intraperitoneally immediately (15 min) after the operation and then every 24 h from the day of the operation until the day of sacrifice. Operated but untreated animals (O), were intraperitoneally injected with the same volume of physiological solution.

4.3. Paraffin Tissue Preparation

The isolated nerves were prepared for immunohistochemistry in the Laboratory for Pathohistology and Cytology HistoLab, Belgrade, by the following the procedure:

The isolated nerve samples underwent the fixation procedure in the 10% formaldehyde solution to preserve the tissue morphology and antigenicity of target molecules on the dissected nerve. Prior to the addition of melted paraffin wax, the isolated tissue underwent a series of dehydration steps at room temperature (RT): (1) 3 × 30 min in 70% ethanol; (2) 3 × 30 min in 90% ethanol; (3) 3 × 30 min in 100% ethanol; and (4) 3 × 30 min in xylene. Following dehydration, the tissue was immerged into the melted paraffin wax at 58 °C. Microtome sectioning of the paraffin-embedded tissue was next done at a thickness of 5 μm. Sections were then incubated at 56 °C in water bath, mounted onto histological slides pre-coated with gelatin for better tissue adhesion, and dried overnight at RT.

4.4. Immunofluorescence Staining

Immunofluorescence (IF) staining was used for protein localization on tissue slides. For immunofluorescent staining, the fluorescent-dye conjugated secondary antibody which binds to the unlabeled primary antibody was used. Except for the incubation with primary antibody, which was performed at a temperature of 4 °C, the IF staining procedure was done at RT. All the solutions were prepared in 0.01 M PBS, pH 7.4, which was also used for washing agent after certain steps. Double IF staining proceeded according to the following steps:

Deparaffinization and rehydration: Microscope slides with paraffin-embedded sections were deparaffinized and rinsed in xylene 1, xylene 2, absolute alcohol, 95% alcohol, 70% alcohol, and distilled water, for 5 min in each solution. Antigen retrieval: Antigenic epitope unmasking was done by boiling microscope slides in 0.01 M sodium citrate buffer, pH 6, for 8 min at 99%–100 °C, followed by cooling at RT for 30 min and 3 × 5 min PBS washing. Blocking solution: After the washing step, microscope slides were incubated for 60 min in 5% blocking serum (originating from the same species as the secondary antibody) to prevent nonspecific binding of the secondary antibody. To enable membrane permeabilization, 0.5% Triton X-100 detergent was added to the blocking serum. Primary antibody, diluted in PBS, was applied onto slides and incubated overnight at 4 °C temperature. Next day, slides were washed out 3 × 5 min in PBS. Secondary antibody, diluted in PBS, was applied onto slides, where it specifically binds to the present primary antibody. Slides were next washed for 3 × 5 min in PBS. In the case of double or triple IF staining, the steps starting from the incubation in the blocking serum were repeated for the next markers. The primary and secondary antibodies used for IF labeling are indicated in the Table 1. After incubation with the last secondary antibody slides were washed 6 × 5 min in PBS and mounted with Mowiol (Calbiochem, Millipore, Germany). After drying overnight,

slides were ready for viewing under the microscope. As a staining control, microscope slides that underwent the same IF procedure, but without the primary antibody application, were used.

4.5. Digital Image Processing

The images of the prepared nerve sections were acquired using Carl Zeiss Axiovert fluorescent microscope, equipped with the AxioCam monochromatic camera (Axio Observer Microscope Z1, ZEISS, Gottingen, Germany), at the magnifications of 20×, 40×, 63×, and 100× and saved in .tiff format. To capture images at 63× and 100× magnification ApoTome software was used. Co-localization on the obtained fluorescent images was done using AxioVision Rel. 4.6 program, which represents a standard part of the Zeiss Axiovert microscope equipment, and then assembled and labeled in Photoshop CS6 (Adobe Systems). The quantification of single, double, or triple-positive cells from experimental groups (S, O, OT) was performed for each time point (1, 3, 7, 14 dpo), and obtained from three independent experiments. High resolution digital images (600 pixels/inch) captured at 40× magnification (three images/group/independent experiment) were used for cells counting. The total number of single, double, or triple-positive cells was counted manually by two independent observers using ImageJ open-source platform (National Institutes of Health, USA; http://imagej.nih.gov/ij/download.html) with ImageJ cell-counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) and Adobe Photoshop Creative Cloud (Version 14.0). Additionally, the percentage of double or triple-positive cells in some investigated cells populations was calculated and presented.

4.6. Statistical Analysis

For statistical comparison between two experimental groups a two-sided Student's *t*-test was performed and a value of $p < 0.05$ or less was considered significant. Values were shown as mean values with standard error (SEM).

5. Conclusions

In conclusion, we report for the first time that treatment with a complex of B (B1, B2, B3, B5, B6, and B12) vitamins could effectively promote PNI-induced M1 to M2 macrophage polarization and suppress inflammatory response by reducing expression of proinflammatory and up-regulation of anti-inflammatory cytokines. Moreover, our findings point to the potential involvement of the $Ca_V1.2$ subunit of the L type of VDCCs in the processes of inflammation after PNI. Treatment with B vitamins increases $Ca_V1.2$ abundance in M2 macrophages, suggesting their mediating role in improving recovery of the injured nerve. Thus, our study exhibited that B vitamins, owing to their pleiotropic effects, had the potential for treatment of neuroinflammation and neuroregeneration, and can be considered as a promising adjuvant therapy of PNI in humans, which remains to be further explored.

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Sample Availability: Samples of the compounds are available from the authors. It is commercial product Beviplex (Beviplex®, Galenika a.d. Belgrade, Serbia) and it is written in the Section 4.2. Treatment protocol.

molecules

MDPI

Article

Effect of Vitamin B Complex Treatment on Macrophages to Schwann Cells Association during Neuroinflammation after Peripheral Nerve Injury

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Abstract: Peripheral nerve injury (PNI) triggers a complex multi-cellular response involving the injured neurons, Schwann cells (SCs), and immune cells, often resulting in poor functional recovery. The aim of this study was to investigate the effects of the treatment with vitamin B (B1, B2, B3, B5, B6, and B12) complex on the interaction between macrophages and SCs during the recovery period after PNI. Transection of the motor branch of the femoral nerve followed by reconstruction by termino-terminal anastomosis was used as an experimental model. Isolated nerves from the sham (S), operated (O), and operated groups treated with the B vitamins (OT group) were used for immunofluorescence analysis. The obtained data indicated that PNI modulates interactions between macrophages and SCs in a time-dependent manner. The treatment with B vitamins complex promoted the M1-to M2-macrophage polarization and accelerated the transition from the non-myelin to myelin-forming SCs, an indicative of SCs maturation. The effect of B vitamins complex on both cell types was accompanied with an increase in macrophage/SC interactions, all of which correlated with the regeneration of the injured nerve. Clearly, the capacity of B vitamins to modulate macrophages-SCs interaction may be promising for the treatment of PNI.

Keywords: peripheral nerve injury; vitamin B complex; neuroinflammation; macrophages; Schwann cells

1. Introduction

Peripheral nerve injuries (PNI) represent a considerable health burden and a far-reaching issue of the modern lifestyle, with an estimated incidence of even ~300,000 cases per year in Europe, mostly caused by increasing rates of traffic, industrial and workplace-associated traumatism [1]. Neuroinflammation induced by PNI assumes precise orchestration of interactions between different cells, primarily Schwann cells (SCs) and phagocytic-macrophages. While latter get recruited to the injury site by cytokines released from the denervated-SCs [2–5], the recruited hematogenous macrophages, together with the resident-population, release cytokines necessary for the subsequent SCs' activation and extracellular matrix remodeling [6].

The inflammatory and reparatory roles of macrophages were proven essential for all tissues. Accordingly, it has been shown that the macrophage depletion, following PNI, triggers an impaired process of neuroregeneration, coupled with a very poor outcome [7]. Despite this, unambiguous data about the precise macrophage contribution to neuroregeneration is still missing [8], although the importance of recruited monocytes and resident macrophages in PNI-triggered neuroinflammation has been well acknowledged. Moreover, despite how distinct resident macrophage subsets have been associated to peripheral nerves in various tissues, and linked to protective and deleterious effects on neuroregeneration, a systematic analysis of macrophage type association with peripheral nerves is still lacking [9]. Macrophages exhibit remarkable plasticity and are highly heterogeneous. According to the activation state and functions, they are classified into two polarized phenotypes: "classically activated" pro-inflammatory macrophages (M1) and "alternatively activated" anti-inflammatory macrophages (M2) [10]. So far, M1-macrophages have been recognized as primary phagocytes at the PNI site, whilst M2-macrophages subsequently take over neuroreparation, whose timely activation becomes vital to circumvent the putative M1-subset neurotoxicity [11].

SCs are considered crucial in orchestrating distinct functional modalities between macrophage subsets. Firstly, the resident-SCs, experiencing hyperproliferation, reinforce the initial macrophage activity by secreting cytokines that further recruit monocytes to the injured site [12]. As a result, SCs change the phenotype to the pre-myelinating state. Re-established interactions between SCs with the tissue-macrophages restore the remyelinating-SC phenotype, which behaves indispensable during the axonal regeneration [13]. The layers of novel myelin may stimulate macrophages to finalize the on-going inflammation at the injured nerve, so that the macrophage-SCs interaction enables fine tuning of the reparatory processes, which demands clarification to a greater extent.

Injuries of the peripheral nerves result in long-term disability and conditions characterized by wide-ranging symptoms, depending on the severity and involved nerve types. The principal PNI target is axon where microsurgery represents the first therapeutic method of choice [14]. In the context of enhanced potential for neuroregeneration [15], the development of adjuvant strategies was nowadays recognized as highly needed, transforming quickly into a novel and attractive research field [16]. Hereby, B vitamins might serve as a prominent choice, due to their broad usage in regenerative medicine, as well as in treating injuries of both central and peripheral nervous system [17].

Vitamins of the B complex act as coenzymes in a plethora of enzymatic reactions, critically affecting vital cellular functions. Numerous studies clearly revealed therapeutic potential of the B complex vitamins in peripheral nerve recovery [18–20]. For example, the sciatic nerve injury has been associated to decreased levels of the vitamin B complex and vitamin B12 [18], indicating that the administration of these vitamins may improve the nerve regeneration process. Moreover, a positive effect of the vitamin B12 on SC proliferation and migration has been shown, including also the myelination of axons after end-to-side neurorrhaphy in rats [21]. Further on, vitamins B1, B6, and B12, display analgesic effects in experimental animal models for acute and chronic pain, upon the neuronal injury [22]. In addition, the same vitamins have been found to promote neurite outgrowth and enhance the velocity of nerve conduction in rat acrylamide-induced neuropathy [23]. In our previously published papers we have reported that treatment with vitamin B (B1, B2, B3, B5, B6, and B12) complex could improve the recovery of the motor nerve [19], and this progress was caused by effective transition from M1-proinflammatory to M2-anti-inflammatory/reparatory macrophage phenotype, followed by the inflammatory response suppression [20]. Consistently, the aim of the present study was to demonstrate whether the same B vitamins cocktail (B1, B2, B3, B5, B6, and B12) could affect the relationship between macrophages and SCs, the essential constituents of PNI-triggered processes of neuroinflammation/neuroregeneration; thus, ultimately promoting enhanced nerve repair.

2. Results

2.1. Treatment with Vitamin B Complex Altered Macrophages/Schwann Cells Interaction during the Recovery Period after PNI

To explore the effects of vitamin B complex on the spatiotemporal relationships of macrophages and SCs during the recovery period after PNI, immunohistochemical analyses were performed on the cross sections of the femoral nerve motor branch. Results of the sham control groups (S) were compared to the profiles obtained at different investigated time points (1st, 3rd, 7th, and 14th day post operation (dpo)) in the operated (O) and in the operated groups after the administration of 1, 3, 7, and 14 intraperitoneal (i.p.) injections of vitamin B complex (OT group). We used ED1 (anti-CD68) antibody as a commonly utilized marker of activated macrophages, and S100 antibody as a well-established marker of SCs [24–26]. Double immunofluorescence (IF) staining was performed to visualize the CD68/S100 overlapping. As we have also noticed in our recently published paper [18], only a few ED1$^+$ cells were detected in the sham operated (S) group, and this number was not significantly changed during all of the investigated time points (Figure 1(I)–Figure 4(I) A, D, G, white arrow heads and Figure 6(I) A, D, G, J). Remarkably, most of the SCs, intensively stained with S100 (Figure 1(I)–Figure 4(I) A, D, yellow arrow heads), displayed morphology of myelin-forming SCs and no overlapping of ED1 and S100 immunoreactivity was detected. In the O group, the number of ED1$^+$ cells was increased at the 1st dpo, where these macrophages had round and oval morphology (Figure 1(I) B, E, H, white arrow heads) resembling the M1-type. The observed macrophages were concentrated in clusters surrounding the dark spots (Figure 1(I) B, E, K, red arrow heads), consisting of SCs with very low or without S100 immunoreactivity. Additionally, strongly stained S100$^+$ cells (Figure 1(I) K, yellow arrow heads) with myelinating SCs morphology were also detected, but with no overlapping of ED1 and S100 immunoreactivity. The administration of one vitamin B complex injection was not sufficient to overcome all of the effects of PNI, although the number of ED1$^+$ cells was decreased (Figure 1(I) I, white arrow heads), while the morphology of S100$^+$ SCs was similar to those observed in the S group (Figure 1(I) F and L, yellow arrow heads). The total number of ED1$^+$/S100$^+$ cells was presented in Figure 1(II), as a number of double positive cells/mm^2 and in Figure 1(III) as the percentage of double positive cells in ED1$^+$ cells population. The number of ED1$^+$ macrophages that co-localized with S100$^+$ SCs was negligible in the O group (10.04 ± 0.93/mm^2 and represented only 9.86 ± 0.76% of total ED1$^+$ cells), while in the OT group it was 1.5-fold higher (15.36 ± 1.14/mm^2), but still represented a small fraction (17.72 ± 0.62%) of total ED1$^+$ cells.

Interestingly, at the 3rd dpo in the O, as well as in the OT group, we have noticed ED1$^+$/S100$^+$ cells (yellow fluorescence) (Figure 2(I) E and F, yellow arrows). As depicted in Figure 2(I) H and I, these ED1$^+$ macrophages, found in close association with SCs, had more "foamy" morphology of the M2 type. Anyhow, these ED1$^+$/S100$^+$ cells were more represented in the OT group.

The quantification of ED1$^+$/S100$^+$ cells was presented in Figure 2(II), as a number of double positive cells/mm^2 and in Figure 2(III) as the percentage of double positive cells in the total population of ED1$^+$ cells. The number of ED1$^+$ macrophages that co-localized with S100$^+$ SCs in the O group was 28.29 ± 1.26/mm^2 and represented 21.44 ± 0.91% of total ED1$^+$ cells, while in the OT group, it was 32.56 ± 0.67/mm^2, representing 29.42 ± 0.66% of total ED1$^+$ cells. Besides these ED1$^+$/S100$^+$ cells, in both O and OT group we have noticed macrophages with "foamy" morphology that were only ED1$^+$ (Figure 2(I) H and I, white arrows), and SCs that were only S100$^+$ (Figure 2(I) K and L, yellow arrow heads). In addition, in the O group, some ED1$^+$ macrophages of the M1-type morphology (Figure 2(I) H, white arrow heads), were still detected around SCs with weak S100 immunoreactivity (Figure 2(I) K, red arrow heads).

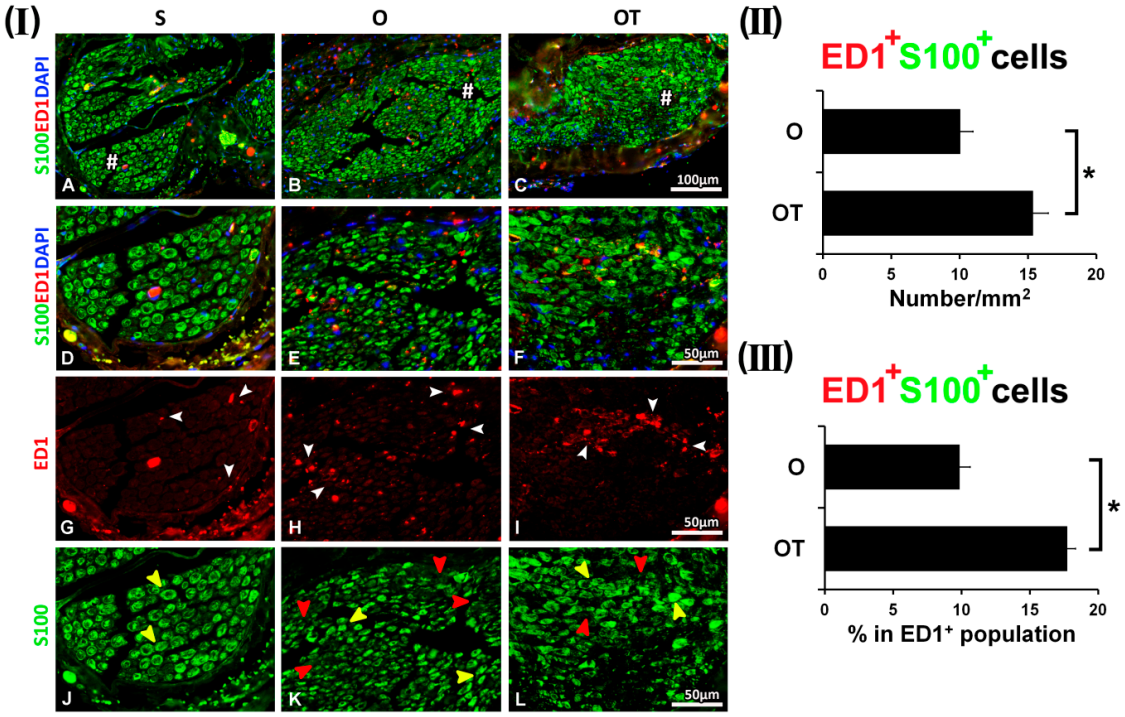

Figure 1. Effect of peripheral nerve injury (PNI) and treatment with B vitamins on Schwann cells (SCs)-macrophages co-localization at the 1st day post operation (dpo). (**I**) Cross sections of femoral nerve obtained from sham (S), operated (O, the transection of the motor branch and immediate reconstruction using termino-terminal anastomosis) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (anti-CD68, red) antibody, a marker of activated macrophages. Anti-S100 antibody (green) was used as a marker of SCs. The sections were counterstained with DAPI (blue) to visualize cell nuclei. The representative images demonstrated that in the S group only a few ED1$^+$ cells were detected (**G**, white arrow heads), most of the SCs had morphology of myelin-forming SCs and were intensively stained with S100 (**J**, yellow arrow heads). There was negligible overlapping of ED1 and S100 immunoreactivity (**A**–**D**). In the O group, ED1$^+$ macrophages with round and oval morphology of the M1 type were abundantly present (**H**, white arrow heads), particularly around the dark spots (**E**, **K**, red arrow heads), consisting of SCs with low S100 immunoreactivity. S100$^+$ cells (**K**, yellow arrow heads) of myelinating SCs morphology were also seen, but without ED1 and S100 immunoreactivity overlapping (**B**, **E**). After one vitamin B complex injection, the number of ED1$^+$ cells was decreased (**I**, white arrow heads). S100$^+$ cells of the myelinating SCs morphology were also present (**F** and **L**, yellow arrow heads). # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**L** = 50 µm. (**II**) The quantification of double positive ED1$^+$/S100$^+$ cells from the O and OT experimental groups is depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the ED1$^+$ cells population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's *t*-test (* $p < 0.05$ vs. O group, as indicated at the graphs).

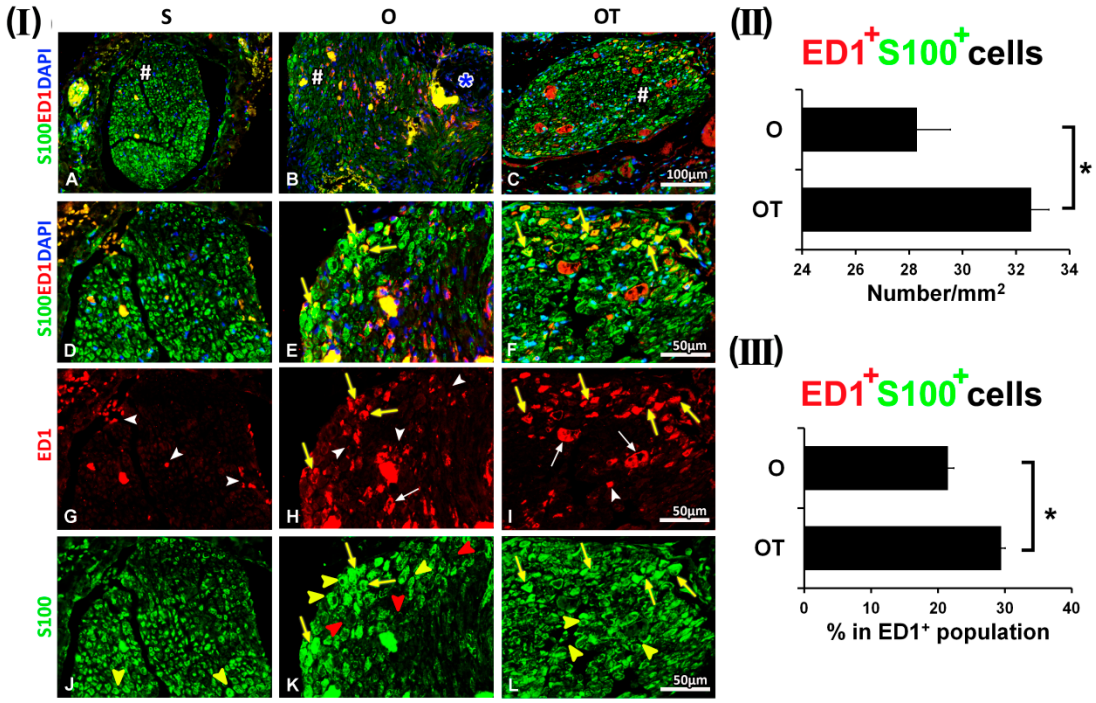

Figure 2. Effect of PNI and the treatment with B vitamins on SCs-macrophages co-localization at the 3rd dpo. (**I**) Cross sections of the femoral nerve obtained from the sham (S), operated (O, the transection of the motor branch) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (red), a marker of activated macrophages, anti-S100 antibody (green) as a marker of SCs, and counterstained with DAPI (blue) for visualizing cell nuclei. In the S group, a paucity of ED1$^+$ cells was detected (**G**, white arrow heads), myelinating, mature SCs were the predominant cell type (**J**, yellow arrow heads) and there was no overlapping of ED1 and S100 immunoreactivity (**A**, **D**). In the O and OT groups ED1$^+$/S100$^+$ cells (**E** and **F**, yellow arrows) were noticed, whereby ED1$^+$ macrophages, closely associated to SCs, had "foamy" morphology of M2 type (**H** and **I**, yellow arrows). In both groups, ED1$^+$ macrophages with the "foamy" morphology (**H** and **I**, white arrows), and SCs that were only S100$^+$ (**K** and **L**, yellow arrow heads) were noticed as well. In the O group some ED1$^+$ macrophages of the M1 type morphology (**H**, white arrow heads), were detected around faintly stained SCs (**K**, red arrow heads). Blue asterisk marks the site of transection and immediate reconstruction by termino-terminal anastomosis, while # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**L** = 50 µm. (**II**) The quantification of double positive ED1$^+$/S100$^+$ cells from the O and OT experimental groups is depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the ED1$^+$ cells population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's *t*-test (* $p < 0.05$ vs. O group, as indicated at the graphs).

During the recovery period after the PNI, the most interesting interaction pattern between macrophages and SCs was observed at the 7th dpo (Figure 3). Equally, in both the O and OT group, a huge number of ED1$^+$ macrophages, predominantly with the M2-like morphology and only a paucity of ED1$^+$ macrophages with the M1-like morphology were detected (Figure 3(I) H and I, white arrows and white arrow heads, respectively). In the O group we found a wide spread distribution of the dark spots (Figure 3(I) B, E, K), consisting of SCs with low S100 immunoreactivity (Figure 3(I) K, red arrow heads). These spots were surrounded with many ED1$^+$/S100$^+$ macrophages (yellow fluorescence) (Figure 3(I) E and H, yellow arrows), characterized by the transitional morphology (between the M1- and M2-type). These ED1$^+$/S100$^+$ cells were counted and the corresponding quantification was presented in Figure 3(II), as a number of double positive cells/mm^2 and in Figure 3(III) as the

percentage of double positive cells in the ED1$^+$ cells population. The number of ED1$^+$ macrophages that co-localized with S100$^+$ SCs in the O group was 24.94 ± 0.72/mm^2 and represented 21.22 ± 0.76% of total ED1$^+$ cells, while in the OT group their number was statistically higher 37.41 ± 1.21/mm^2, comprising 29.23 ± 0.54% of the total ED1$^+$ cell population. In addition, besides these ED1$^+$/S100$^+$ cells, we have also noticed macrophages that were only ED1$^+$, some of them showing the "foamy", M2-type-like morphology (Figure 3(I) H, white arrows), while others displaying the M1-type-like morphology (Figure 3(I) H, white arrow heads). After the administration of seven consecutive injections of the vitamin B complex, we detected increased number of ED1$^+$/S100$^+$ cells (Figure 3(I) F, yellow arrows), with ED1$^+$ macrophages, closely associated to S100$^+$ SCs, exhibiting the "foamy" M2-type-like morphology (Figure 3(I) I, yellow arrows). Importantly, these ED1$^+$ macrophages were closely associated only to S100$^+$ SCs with the non-myelinating morphology (Figure 3(I) L, red arrows), while no co-localization of the ED1 immunoreactivity with the S100$^+$ myelinating SCs was noted (Figure 3(I) L, yellow arrow heads).

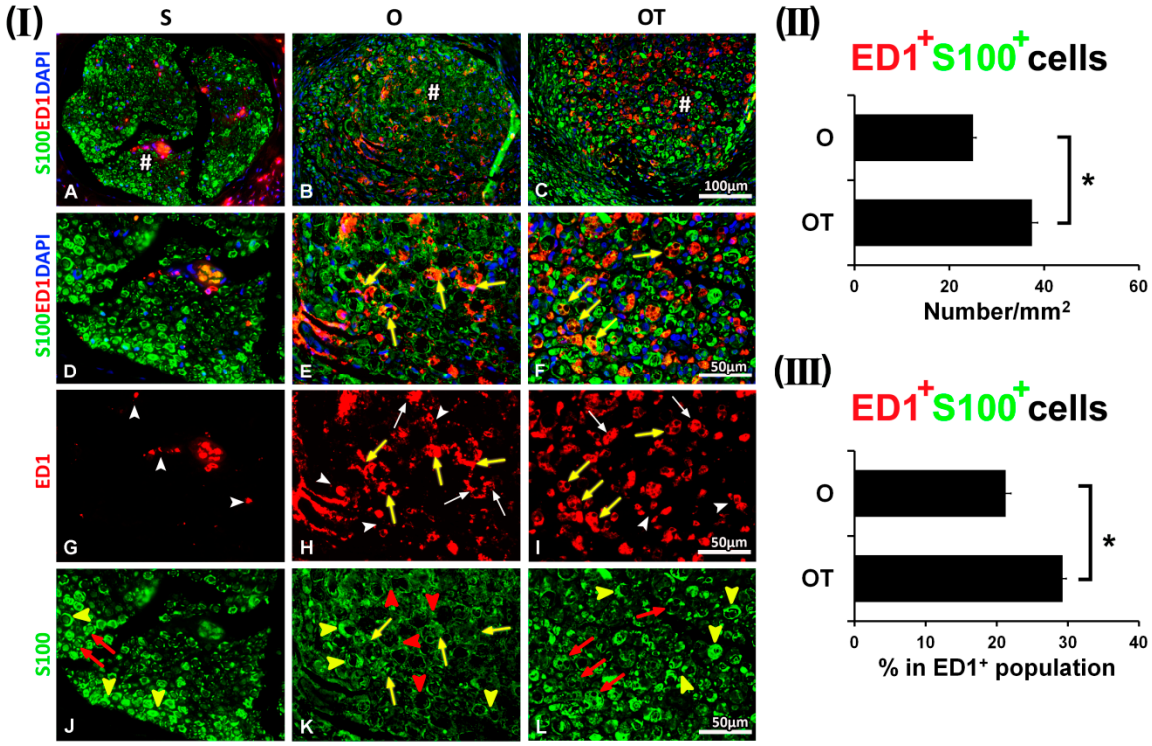

Figure 3. Effect of PNI and the treatment with B vitamins on SCs-macrophages co-localization at the 7th dpo. (**I**) Cross sections of femoral nerve obtained from the sham (S), operated (O, the transection of the motor branch) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups were stained for ED1 (red), a marker of activated macrophages, anti-S100 antibody (green) as a marker of SCs, and counterstained with DAPI (blue) for visualizing cell nuclei. In the S group rare ED1$^+$ cells were detected (**G**, white arrow heads), while besides myelinating, mature SCs (**J**, yellow arrow heads) some S100$^+$ SCs of the non-myelinating morphology (**J**, red arrows) were seen. No overlapping of ED1 and S100 immunoreactivity was noted (**A**, **D**). The representative images of the O and in the OT group revealed a huge number of ED1$^+$ macrophages with the predominantly M2-like morphology and a few ED1$^+$ macrophages with the M1-like morphology (**H** and **I**, white arrows and white arrow heads, respectively). In the O group a widespread distribution of dark spots (**B**, **E**, **K**), consisting of faintly-stained SCs (**K**, red arrow heads) and surrounded with many ED1$^+$/S100$^+$ macrophages (**E** and **H**, yellow arrows), was observed. Some of the macrophages that were only ED1$^+$ had "foamy" morphology of the M2 type (**H**, white arrows), while others were of the M1 type morphology (**H**, white arrow heads). Seven consecutive injections of the vitamin B complex increased the number of ED1$^+$/S100$^+$ cells (**F**, yellow arrows), whereby ED1$^+$ macrophages of the "foamy" morphology resembling the M2

type (**I**, yellow arrows) were tightly associated to S100$^+$ SCs of the non-myelinating morphology (**L**, red arrows). No co-localization of the ED1 immunoreactivity with S100$^+$ myelinating SCs was noted (**F**, **L**, yellow arrow heads). # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 μm, **D**–**L** = 50 μm. (**II**) The quantification of double positive ED1$^+$/S100$^+$ cells from the O and OT experimental groups is given in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the ED1$^+$ cells population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's *t*-test (* $p < 0.05$ vs. O group, as indicated at the graphs).

By day 14, ED1$^+$ macrophages with the M2-type morphology appeared to be prevalent in the both O and OT groups (Figure 4(I) H and I, white arrows), while S100$^+$ SCs were predominantly of the myelin-forming phenotype (Figure 4(I) K and L, yellow arrow heads). Interestingly, ED1$^+$/S100$^+$ cells (Figure 4(I) E, yellow arrows) were mostly detected in the O group, and these ED1$^+$ macrophages with the M2-type morphology were associated (Figure 4(I) E, yellow arrows) to the S100$^+$ non-myelinating SCs (Figure 4(I) K, red arrow) and some myelinating SCs (Figure 4(I) K, yellow arrows). In contrast, in the OT group, reduced co-localization of the ED1 and S100 immunoreactivity was detected (Figure 4(I) F), indicating that following 14 injections of the B vitamin complex the complete transition to mature, myelin-forming SCs occurred. ED1$^+$/S100$^+$ cells were counted and the values obtained are given in Figure 4(II), as a number of double positive cells/mm^2 and in Figure 4(III) as the percentage of double positive cells in the total ED1$^+$ cell population. The number of ED1$^+$ macrophages that co-localized with S100$^+$ SCs in the O group was 21.87 ± 1.38/mm^2 and represented 31.49 ± 1.66% of total ED1$^+$ cells, while in the OT group their number was statistically lower (15.44 ± 0.69/mm^2), and the corresponding fraction in the total ED1$^+$ cell population was only 18.17 ± 0.78%.

To confirm that the S100$^+$ SCs, closely associated to ED1$^+$ macrophages, were the non-myelinating SCs, we performed double immunofluorescence staining with growth associated protein 43 (GAP43), a well-known marker of growing axons [27], but also a marker of non-myelinating SCs [26,28,29]. In sham controls, GAP43 immunostaining was predominantly detected in large-diameter myelinated axons, at both-time points (7th and 14th dpo) (Figure 5A,D, red asterisk). Although the majority of the myelinating S100$^+$ SCs, wrapping these axons, were GAP43$^-$ (Figure 5A,D, yellow arrow heads), some of them were also S100$^+$/GAP43$^+$ (Figure 5A,D, yellow arrows). In addition, rare non-myelinating GAP43$^+$ SCs were observed as well (Figure 5D, red arrows). At the 7th dpo, the nerve tissue was damaged, the major portion of myelin sheaths was degraded and axons destroyed, while most of the SCs underwent degeneration (Figure 5B, red arrow heads). Interestingly, in the OT group, beside myelin-forming SCs (Figure 5C, yellow arrow heads), seven consecutive injections of B vitamins significantly increased the number of non-myelinating S100$^+$/GAP43$^+$ SCs (Figure 5C, red arrows) that wrapped multiple, small-diameter, non-myelinated axons (Figure 5C, white asterisk). Similarly, a vast number of S100$^+$/GAP43$^+$ non-myelinating SCs ensheathing multiple small-caliber axons (Figure 5E, red arrows) together with a paucity of myelin-forming S100$^+$/GAP43$^+$ (Figure 5E, yellow arrows) and S100$^+$/GAP43$^-$ (Figure 5E, yellow arrow heads) SCs, was detected at the 14th dpo in the O group (Figure 5E, arrow head). In contrast, in the OT, as well as in the S group, myelinating, mature S100$^+$/GAP43$^-$ SCs were detected as a predominant cell type (Figure 5F, yellow arrow heads), with only a few S100$^+$/GAP43$^+$ myelinating SCs found (Figure 5F, yellow arrows). Furthermore, strong GAP43 immunostaining was detected in large-diameter myelinated axons (Figure 5F, red asterisk).

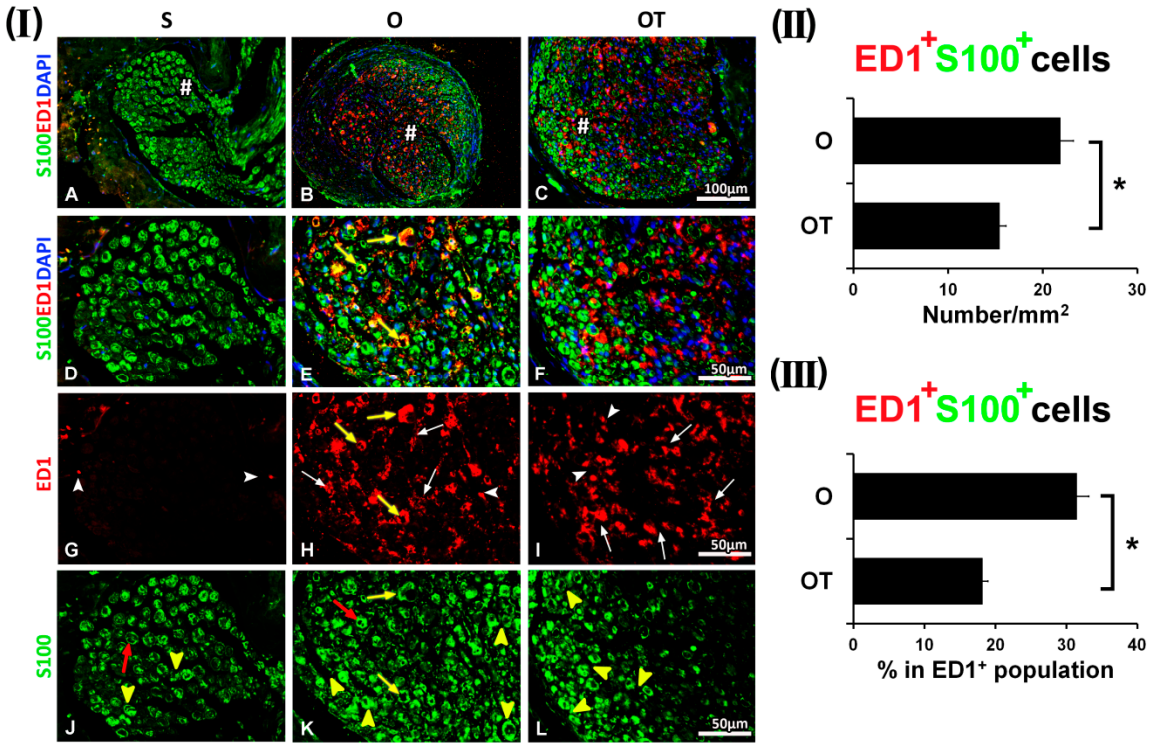

Figure 4. Effect of PNI and the treatment with B vitamins on SCs-macrophages co-localization at the 14th dpo. (**I**) ED1 (red), a common marker of activated macrophages, anti-S100 antibody (green), a marker of SCs and DAPI (blue) for visualizing cell nuclei, were used for immunostaining of the femoral nerve cross sections of the sham (S), operated (O, the transection of the motor branch) and operated and treated with the vitamin B complex (B1, B2, B3, B5, B6, and B12) (OT) groups. Almost no ED1$^+$ cells were detected (**G**, white arrow heads) in the S group. Apart from myelinating SCs (**J**, yellow arrow heads), some S100$^+$ SCs of the non-myelinating morphology (**J**, red arrows) were seen as well. There was no overlapping of ED1 and S100 immunoreactivity (**A**, **D**). ED1$^+$ macrophages with the M2-type morphology were predominant in the both O and OT groups (**H** and **I**, white arrows), while S100$^+$ SCs mostly belong to myelin-forming SCs (**K** and **L**, yellow arrow heads). ED1$^+$/S100$^+$ cells (**E**, yellow arrows) were detected only in the O group. ED1$^+$ macrophages of the M2-type morphology were associated (**E**, yellow arrows) to the S100$^+$ non-myelinating SCs (**K**, red arrow) and some myelinating SCs (**K**, yellow arrows). Minor overlapping of ED1 and S100 immunoreactivity (**C**, **F**) was obtained in the OT group. # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**L** = 50 µm. (**II**) The quantification of double positive ED1$^+$/S100$^+$ cells from the O and OT experimental groups was depicted in the graphs (black bars) as a number of double positive cells/mm^2 and (**III**) the percentage of double positive cells in the ED1$^+$ cells population. The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured). Statistical analysis was done using a two-sided Student's *t*-test (* $p < 0.05$ vs. O group, as indicated at the graphs).

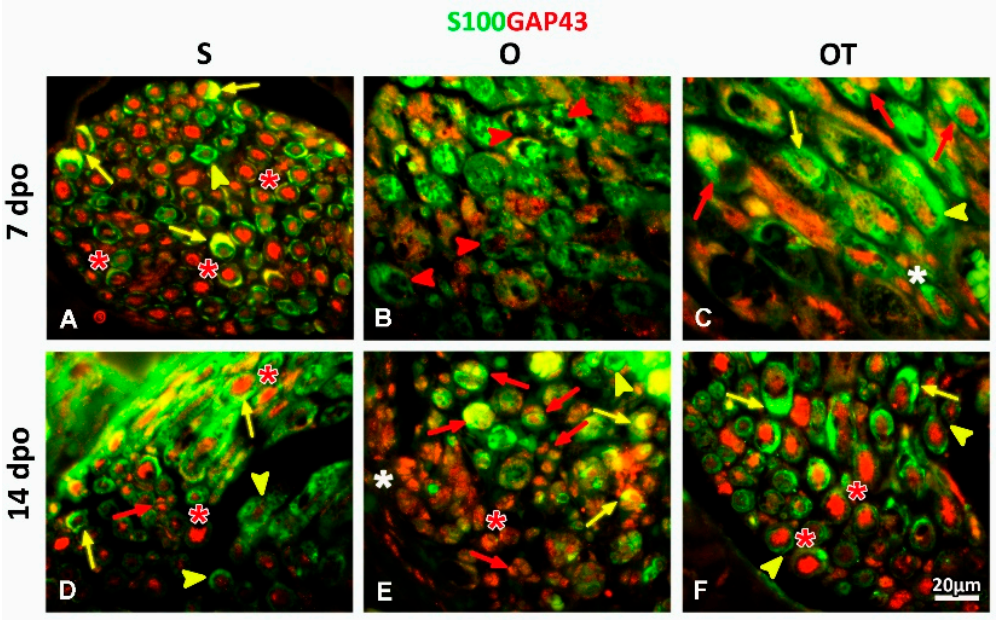

Figure 5. GAP43 (growth associated protein 43) expression in SCs and axons after PNI and the treatment with B vitamins. Expression of GAP43 (red) in SCs was determined in the femoral nerve cross sections obtained from the: sham (S), operated (O) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT) group at the 7th and 14th dpo. Anti-S100 antibody (green) was used as a marker of SCs. In the S group, both at the 7th (**A**) and the 14th (**D**) dpo GAP43 immunostaining was mostly detected in large-diameter myelinated axons (red asterisk), and in a few myelinated $S100^+/GAP43^+$ (yellow arrows) and non-myelinated SCs (red arrows), while $S100^+/GAP43^-$ (yellow arrow heads) SCs were predominant. (**B**) In the O group, at the 7th dpo most of the axons were destroyed and the majority of SCs degenerated (red arrow heads). (**C**) 7 consecutive injections of B vitamins (OT) increased the number of non-myelinating $S100^+/GAP43^+$ SCs (red arrows) wrapping multiple small-diameter $GAP43^+$ non-myelinated axons (white asterisk). Only a few $S100^+/GAP43^+$ myelinated SCs (yellow arrows) and $S100^+/GAP43^-$ (yellow arrow heads) were seen. (**E**) At the 14th dpo, in the O group, a huge number of $S100^+/GAP43^+$ non-myelinated SCs (red arrows) unsheathing multiple small-caliber axons (white asterisk), and a paucity of myelin-forming $S100^+/GAP43^+$ (yellow arrows) and $S100^+/GAP43^-$ (yellow arrow heads) SCs was detected. (**F**) After 14 treatments with B vitamins myelinating, mature $S100^+/GAP43^-$ SCs emerged as the principal cell type (yellow arrow heads), while $S100^+/GAP43^+$ myelinating SCs were rarely present (yellow arrows). Strong GAP43 immunostaining was detected in large-diameter myelinated axons (red asterisk). Scale bar: 20 μm.

The comparative presentation of time-dependent changes in macrophages-SCs co-localization within the cross sections of the femoral nerve obtained from the S, O, and OT groups, during the investigated postoperative period (1, 3, 7, and 14 days) and upon the administration of 1, 3, 7, and 14 injections of the B vitamin complex, was depicted in Figure 6(I). As mentioned above, within the cross nerve sections of the S group, only a paucity of $ED1^+$ cells was detected; their number did not undergo significant changes during all of the investigated time points; myelinating, mature SCs were detected as a predominant cell type and no overlapping of the ED1 and S100 immunoreactivity was noted (Figure 6(I) A, D, G and J).

In the O group (Figure 6(I) E, H and K), an intensive overlapping of the ED1/S100 immunoreactivity was seen at the 3rd, 7th, and 14th dpo, whereas in the OT group, the same was noticed after the administration of three and seven vitamin B complex injections (Figure 6(I) F, I). However, close ED1/S100 interactions were detected only between macrophages with the M2-like morphology and non-myelinating SCs, as well as between the macrophages with the M2-like morphology and SCs with low S100 immunoreactivity. In the both O and OT group, no interactions between mature, myelinating SCs and M2- or M1- type macrophages was detected. The temporal pattern of $ED1^+/S100^+$

participation in the ED1$^+$ cells population in the femoral nerve cross sections obtained from Figure 6(II) O and Figure 6(III) OT experimental groups is given in the graphs (black bars). The fraction of ED1$^+$/S100$^+$ cells in the total ED1$^+$ population varied over the recovery period. In the O group, the participation of ED1$^+$/S100$^+$ cells in the ED1$^+$ cells population increased over the time post-injury reaching 31.49 ± 1.66% at the 14th dpo. At this post-injury time-point, a 3-fold increase was detected in the O group when compared to the 1st dpo. In the OT group, treatments with vitamin B complex increased the percentage of ED1$^+$/S100$^+$ cells, reaching 30% even after 3 injections and remained at the same level for seven days. However, the vitamin B complex treatment after 14 days reduced the number of ED1$^+$/S100$^+$ cells and their fraction in the total ED1$^+$ cell population was only 18.17 ± 0.78%. Given that interaction between macrophages and mature, myelin-forming SCs was negligible, obtained results indicated that treatment with the B vitamin complex after 14 days triggered the complete transition to mature, myelin-forming SCs. Based on these results, it can be concluded that PNI alters interactions between macrophages and SCs in a time-dependent manner, while the treatment with the B vitamins complex accelerates the transition from the non-myelin to myelin-forming SCs type and from M1- to M2-like macrophage morphology.

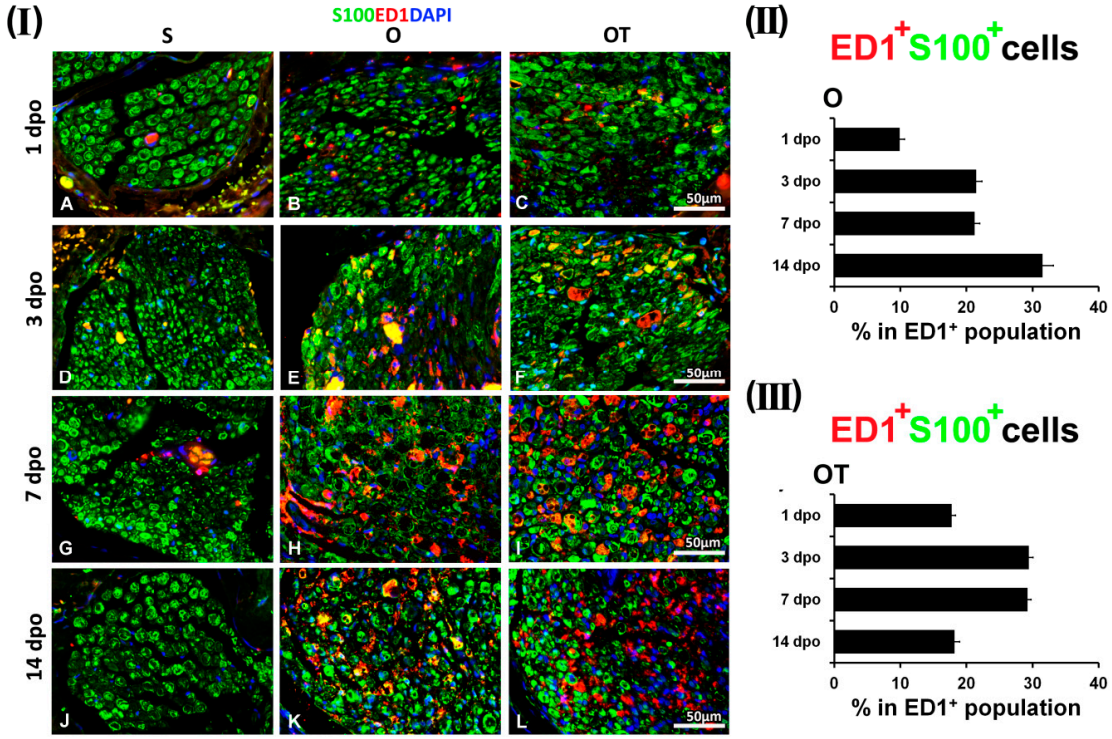

Figure 6. Time course immunohistochemical analysis of macrophages/SCs co-localization after PNI and the treatment with B vitamins. (**I**) Comparative analysis of macrophages/SCs crosstalk in the cross sections of the femoral nerve obtained from the sham (S) group at different time points (**A**—1 dpo; **D**—3 dpo; **G**—7 dpo; **J**—14 dpo). Time course of changes in macrophages/SCs co-localization was analyzed in the operated (O) femoral nerve during the 1, 3, 7, and 14 days of the postoperative period (**B**—1 dpo; **E**—3 dpo; **H**—7 dpo; **K**—14 dpo) and after 1, 3, 7, and 14 injections of the complex of B (B1, B2, B3, B5, B6, and B12) vitamins (OT group) (**C**—1 dpo; **F**—3 dpo; **I**—7 dpo; **L**—14 dpo). ED1 (red) was used as a common marker of activated macrophages, anti-S100 antibody (green) as a marker of SCs and DAPI (blue) for visualizing cell nuclei. Scale bar: 50 µm. Time-dependent changes in the percentage of double positive ED1$^+$/S100$^+$ cells in ED1$^+$ cells population from the (**II**) O and (**III**) OT experimental groups was depicted in the graphs (black bars). The data are shown as the mean ± SEM of three independent experiments (three images/group/independent experiment were captured).

2.2. Administration of the Vitamin B Complex Reduced the Expression of Proinflammatory Cytokine TNF-α in SCs after the PNI

Next, we wanted to investigate how the PNI affects the expression profile of proinflammatory cytokine tumor necrosis factor alpha (TNF-α) in SCs and whether the treatment with B vitamins could modulate this TNF-α expression pattern after the PNI.

In our model of the femoral nerve transection at the 14th dpo we have noted increased expression of TNF-α within cross sections of the operated nerve (O) (Figure 7H) compared to the sham-operated controls (S) (Figure 7G). The administration of 14 injections of the vitamin B complex (OT group) reduced the TNF-α expression (Figure 7I), which was, however, still higher compared to the S group (Figure 7G). Interestingly, besides in macrophages with the M2-like morphology (Figure 7E,F,H,I, white arrows), the TNF-α immunoreactivity was detected in some (Figure 7E,F,H,I, yellow arrows), but not all SCs (Figure 6E,F,H,I, yellow arrow heads).

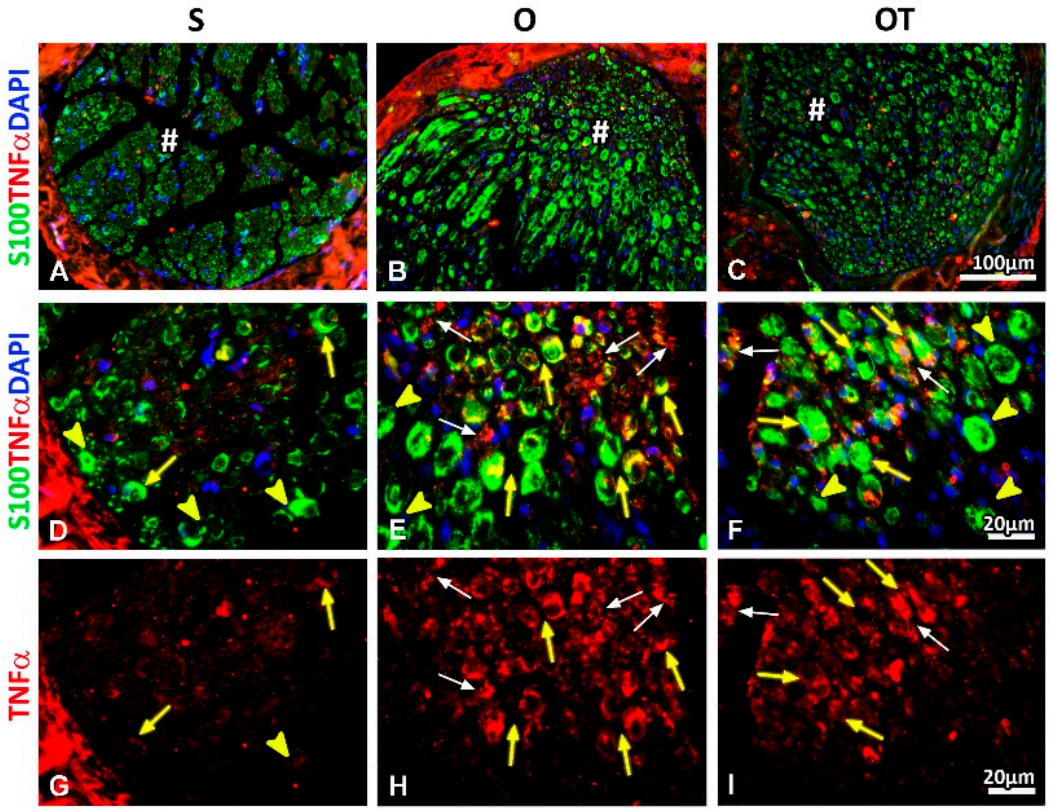

Figure 7. Effects of PNI and the B vitamins treatment on expression of proinflammatory cytokine tumor necrosis factor alpha (TNF-α) in SCs. Femoral nerve cross sections obtained from the: sham (S: **A**, **D**, **G**), operated (O: **B**, **E**, **H**) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT: **C**, **F**, **I**) group immunostained for TNF-α (red) demonstrated strong increase of immunofluorescence intensity in the O group (H) compared to the S group (**G**) at the 14th dpo. Immunofluorescence staining for TNF-α protein was observed in some (**E**, **F**, **H**, **I**, yellow arrows), but not all SCs (**E**, **F**, **H**, **I**, yellow arrow heads) as detected by co-localization with S100 immunostaining (green). DAPI (blue) was used for visualizing cell nuclei. Administration of 14 injections of the vitamin B complex (OT group) reduced TNF-α expression (**I**). TNF-α immunoreactivity was detected in some $S100^{+}$ myelinated SCs (**F**, yellow arrows). In addition, TNF-α immunoreactivity was demonstrated in macrophages with the M2-like morphology (**E**, **F**, **H**, **I**, white arrows). # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 μm, **D**–**I** = 20 μm.

2.3. Effect of PNI and the Vitamin B Complex Treatment on the Expression of Anti-Inflammatory Cytokine IL-10 in SCs

Further, we investigated the expression of anti-inflammatory cytokine interleukin 10 (IL-10) in SCs in all of the examined (S, O, and OT) groups. IL-10 immunoreactivity was detected in all of these groups (Figure 8G–I), being mostly pronounced in the O group (Figure 7H). Strikingly, the bulk of IL-10 immunoreactivity was noticed in IL-10$^+$ cells resembling M2-macrophages, although IL-10$^+$/S100$^+$ cells were abundantly present as well (Figure 8E,F, yellow arrows). In the OT group, the overlapping of IL-10/S100 immunoreactivity (Figure 8F,I, yellow arrows) was detected in mature, myelinating SCs, albeit the larger part of S100$^+$ SCs were IL-10$^-$ (Figure 8F, yellow arrow heads). The similar pattern of IL-10 immunoreactivity was seen in the S group, but IL-10$^+$/S100$^+$ cells were less represented (Figure 8D,G, yellow arrows, and yellow arrow heads).

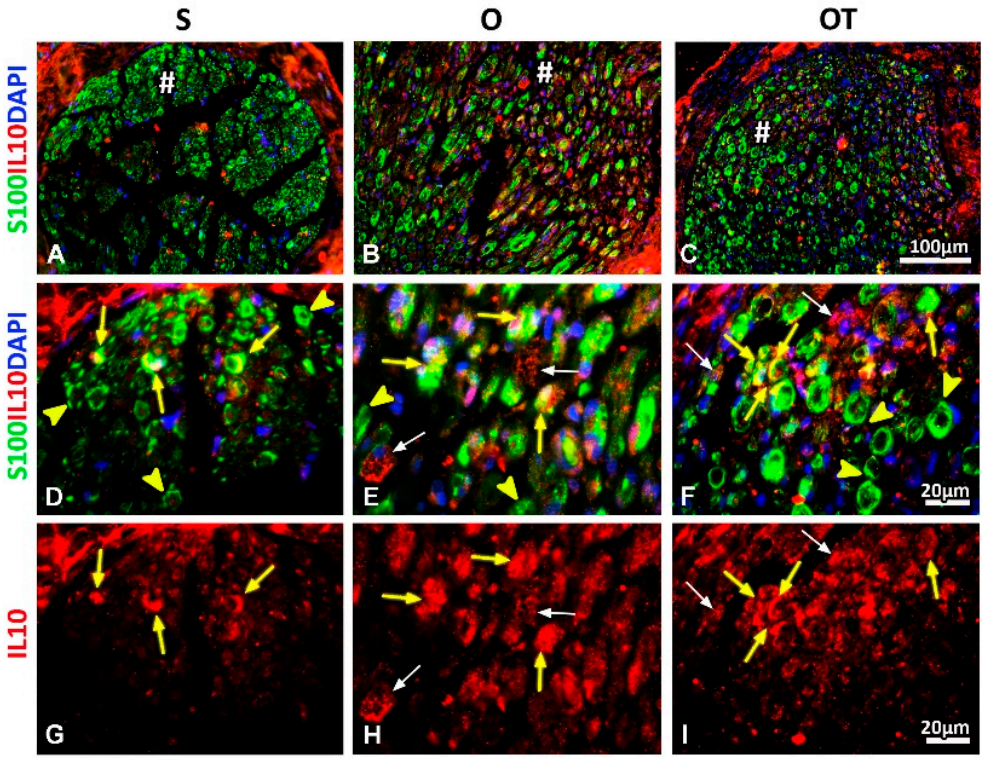

Figure 8. Effects of PNI and the B vitamins treatment on expression of anti-inflammatory cytokine interleukin 10 (IL-10) in SCs. Cross sections of the femoral nerve obtained from the: sham (S), operated (O) and operated and treated with the vitamin B (B1, B2, B3, B5, B6, and B12) complex (OT) group immunostained for IL-10 (red) showed strong IL-10 immunoreactivity in all of the groups (**G**, **H**, and **I**) at the 14th dpo, being the most pronounced in the O group (**H**). Increased IL-10 immunoreactivity was found in S100$^+$ (green) myelinating SCs (**E** and **F**, yellow arrows) and in IL-10$^+$ macrophages with the M2-like morphology (**E**, **F**, **H**, and **I**, white arrows) that were closely associated to them. In the OT group, the larger part of S100$^+$ SCs was IL-10$^-$ (**F**, yellow arrow heads). The similar pattern of IL-10 immunoreactivity was seen in the S group, although IL-10$^+$/S100$^+$ cells were less represented (**D** and **G**, yellow arrows and yellow arrow heads). DAPI (blue) was used to visualize cell nuclei. # indicates where the high magnification micrographs were taken from. Scale bars: **A**–**C** = 100 µm, **D**–**I** = 20 µm.

Using serial transversal sections we were able to visualize tight interactions between M2-macrophages and SCs, that were aligned to form bands of Büngner (Figure 9A–C) and were intensively stained with IL-10 (Figure 9D–F).

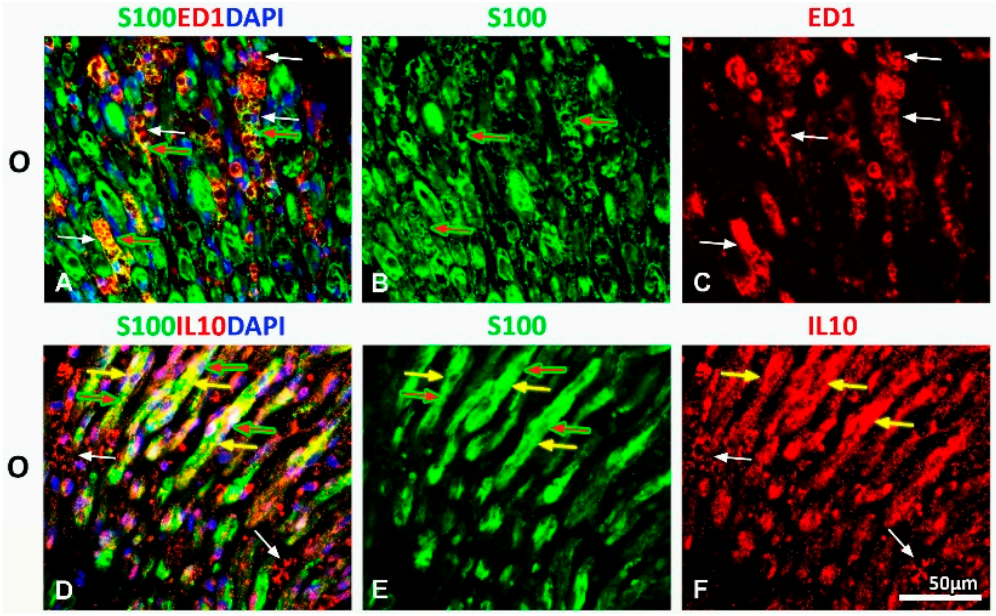

Figure 9. Interactions between M2 macrophages and SCs in injured femoral nerve. (**A**–**C**) We used double immunofluorescence to visualize the close contact between M2 macrophages and SCs at the 14th dpo. Serial transversal sections obtained from the operated (O) femoral nerve were immunostained for ED1 (anti-CD68, red) antibody, as a marker of activated macrophages (**C**, white arrows), anti-S100 (green) antibody as a marker of SCs (**B**, red arrows) and DAPI (blue) for visualizing cell nuclei. Complete overlapping (**A**, yellow fluorescence) of ED1 (white arrows) and S100 (red arrows) immunoreactivity confirmed tight interactions between M2 macrophages and SCs that were aligned to form bands of Büngner. (**D**–**F**) Transversal sections of the operated (O) femoral nerve immunostained with S100 (green), IL10 (red) and DAPI (blue) demonstrated that M2 macrophages (**D**, **F**, white arrows) and $S100^{+}$ SCs (**E**, red arrows) in bands of Büngner were intensively stained with IL-10 (**D**, yellow arrows).

3. Discussion

The aim of this study was to highlight the molecular mechanism underlying previously detected B vitamins-induced locomotor activity improvement after PNI. Herewith, the macrophages-SCs interaction, following the peripheral nerve controlled transection emerged as an important target of the applied treatment [19,30]. Noteworthy, these macrophages-SCs interactions were modulated in a time-dependent manner either post-PNI alone, or upon B vitamins application. However, the treatment accelerated the transition from the non-myelin- to myelin-forming-SCs phenotype. Furthermore, the stimulation of the M1-to-M2 macrophage phenotype switching consequently altered macrophages-SCs interactions.

Previously, we have shown that the vitamin B complex treatment effectively promotes PNI-induced M1-to-M2 macrophage polarization and suppresses inflammation, by reducing the expression of proinflammatory and up-regulating the expression of anti-inflammatory cytokines [20]. Herewith, we address the relationship between macrophages and SCs, the most fundamental cell-to-cell interaction during the PNI-triggered neuroinflammation. Hereby, Wallerian degeneration affects the nerve stumps distal to the lesion, which are not directly physically traumatized. SCs initiate the elimination of damaged axons by rejecting the myelin and, subsequently, recruit the bone-marrow-derived macrophages together with activated-resident-SCs for tissue debris removal [31]. Our results indicate the copious presence of destructed axon areas in the injured nerve of O animals, at the 1st dpo, concurrently with the dedifferentiation of demyelinated-SCs. Consistent to this, in young rats, such as our animals, the lag period separating the injury and axon degeneration involves the first 24–48 h [32]. The detached axon segments remain intact for days post-PNI, and can still transmit action potentials

when stimulated [33,34]. Accordingly, the noted decrease in the number of M1-like-macrophages along with the preserved SCs morphology and myelination in the injured OT animals nerve, led us to hypothesize that the B vitamins treatment may prolong the lag period and reduce the extent of axon degeneration.

After the first period of intensive PNI-induced axon destruction, at the 3rd dpo in both O and OT animals, we noticed ED1$^+$ macrophages closely associated to SCs, displaying more "foamy"-M2-type morphology, particularly in the OT group. Conversely, in the O group, ED1$^+$ macrophages of M1-type-morphology and SCs with low S100 immunoreactivity were still detected. According to a study [8], it was proposed that SCs most likely support the macrophage PNI-functioning via expressing several ligands known to interact with macrophage receptors, thus regulating the M1-to-M2 transition. SCs secrete classical M2-associated cytokines and behave as potent inducers of M2-macrophages. These, in turn, stimulate tissue repair, via promoting remyelination by activating endogenous SCs. Moreover, since macrophages were shown to regulate PNI-triggered SCs maturation, one could not exclude that macrophages-SCs interaction operates vice-versa as well [35].

The most interesting pattern of post-PNI macrophage-SC interaction was noted in our study at the 7th dpo. Regardless of the treatment conditions, we detected an extensive repertoire of ED1$^+$ macrophages and SCs in the injured nerve. M2-like-macrophages appeared predominant, with only a small fraction of M1-like-cells observed. Additionally, in the O group, a widespread distribution of the dark spots representing damaged axons was noted marginal to SCs, with weak S100 immunoreactivity, probably undergoing degeneration/dedifferentiation. The analogous was not observed after the B vitamins treatment. Interestingly, these areas of axonal/SC-degeneration appeared borderline to many ED1$^+$/S100$^+$ macrophages displaying transitional M1-to-M2 morphology. Macrophages with the M1- or M2-morphology were also present. On the other hand, we noticed that B vitamins significantly increased S100$^+$ SCs closely associated to ED1$^+$ cells. Importantly, these ED1$^+$ macrophages displayed the M2-like-morphology and were closely associated to only S100$^+$ SCs of non-myelinating morphology, wrapping multiple small-diameter non-myelinated axons and being GAP43$^+$.

A similar profile of M2-macrophages to non-myelinating-SCs interaction was detected in the O nerve at the 14th dpo. These S100$^+$ SCs were also GAP43$^+$. Considering that GAP43, a marker of growing axons [27], may also represent a marker of the non-myelinating-SCs [26,28,29], the overlapping between S100 and GAP43 immunoreactivity classifies implicated SCs to the non-myelinating class. Contrary, only a paucity of the myelin-forming (S100$^+$/GAP43$^+$ and S100$^+$/GAP43$^-$) SCs was detected in the O group. However, after 14 days of exposure to B vitamins the myelinating, mature SCs, which were GAP43$^-$, appeared as the predominant SC-type in the OT group, while a strong GAP43 immunostaining was detected in the large-diameter myelinated-axons.

As evidenced in our study, PNI causes the destruction of the majority of SCs at the 7th dpo, as well as reprogramming from the myelin to non-myelin-forming (Remak) SCs. This aligns with both SC classes undergoing large-scale gene expression post-PNI, leading to the specialized, repair-promoting phenotype [36]. Given that the Remak SCs unsheathed uninjured fibers and are capable of acting as "sentinels" of injury/disease in proximity [37,38], it is not surprising that exactly this SCs phenotype appears most abundant at the 7th and 14th dpo.

Considering all, we can safely conclude that the B vitamins treatment protects myelin-forming SCs and accelerates the appearance of non-myelin-forming SCs. This preserves the functionality of the injured femoral nerve, as manifested by enhanced recovery of the locomotor performances in rats, which was demonstrated previously by our group [19]. Consistently, some recent publications report positive effects of individual B vitamins application (B12 > B1 > B6) on the damaged sciatic nerve repair by affecting myelination and SCs. Importantly, to obtain an optimal regenerative effect, the usage of B vitamins cocktail was proposed [39]. Moreover, beneficial effects of vitamin B12 were acknowledged in a focal demyelination rat model, in terms of accelerated re-myelination, improved recovery of motor/sensory functions, and stimulation of SCs differentiation [40]. Likewise, folic acid

may stimulate the post-PNI repair by promoting SCs proliferation and migration, and secretion of nerve growth factors [41].

As outlined above, during the post-PNI recovery, we observed time-dependent changes in macrophage and SC morphology in terms of transition from the round-shaped, smaller M1-, to the "foamy"-shaped, larger M2-macrophages, and non-myelinating-SCs to the myelinating-mature-SCs. Concerning this, we conclude that the B vitamins treatment balances the macrophages-to-SCs interaction to limit the injured nerve damage by accelerating the transition from indispensable inflammation to neuroreparation right after PNI.

Consistently, we demonstrated that PNI affects the expression profile of TNF-α and IL-10 in SCs, this being modulated through the administration of B vitamins for 14 dpo. Following the sciatic nerve transection a phasic TNF-α mRNA expression pattern was observed, peaking immediately (14 h), after 5 days and also two weeks [42,43]. In our model of the femoral nerve transection, we also noted increased expression of TNF-α at the 14th dpo within O, compared to S animals, while the administration of B vitamins reduced the TNF-α immunoreactivity. Apart in M2-macrophages, the TNF-α immunoreactivity was detected in some, but not all SCs. Interestingly, the B vitamins treatment reduces the TNF-α expression, thus, protecting myelin-forming-SCs that produce IL-10. Hence, the preserved femoral nerve functionality is manifested as increase in GAP-43 expression and improved locomotor recovery.

IL-10, whose up-regulation was shown from 7 up to 28 days post-injury [44,45], was proposed to modulate the proinflammatory cytokines expression and axonal plasticity [46]. In two different PNI models [42,43], the expression of IL-10 mRNA underwent gradual increase during Wallerian degeneration, while the recent results [20] imply the prevalence of macrophages expressing IL-10, which represents the M2-(anti-inflammatory)-phenotype marker [47–50] at the 14th dpo. Remarkably, fractions of ED1$^+$/IL-10$^+$ cells in total ED1$^+$ population were equivalent between O and OT animals. Moreover, some IL-10$^+$ cells lacking ED1 immunoreactivity, with SCs-like morphology were observed as well [20]. In the present study, at the 14th dpo bulk of IL-10 immunoreactivity was detected in ED1$^+$ M2-like-morphology macrophages, and in IL-10$^+$/S100$^+$ cells, which were also abundantly present. We assumed that increased IL-10 expression in "foamy"-M2-macrophages [20] together with the IL-10$^+$/S100$^+$ SCs presence may contribute to the resolution of PNI-triggered inflammation/nerve repair. In contrast, B vitamins treatment diminishes the overlapping of IL-10 and S100 immunoreactivity in myelinating, mature SCs, with larger fraction of S100$^+$ SCs being IL-10$^-$, as seen in the S group. Analogous inflammatory profile of post-PNI SCs was obtained by Dubový et al. [51], who suggested that such a simultaneous induction of proinflammatory and anti-inflammatory cytokines balances PNI-induced inflammation to promote axonal growth.

Accordingly, the main aim of our study was to investigate the association between different types of macrophages and SCs after PNI and to explore whether the treatment with the vitamin B complex could influence this relationship. We have clearly demonstrated that only M2-repair-promoting macrophages were in close-association with SCs, particularly of the non-myelinating type, while no co-localization between macrophages and myelin-forming SCs was observed. Regarding the quantification of ED1$^+$/S100$^+$ cells and their fraction in the total ED1$^+$ cell population, these results gave us the information about the extent of ED1$^+$ macrophages and S100$^+$ SCs interaction during the recovery period after PNI and also how the B vitamin treatment affected the temporal profile of the corresponding interactions, all telling us about the success/extent of the recovery. Thus, in the O group, we have noticed that at the 14th dpo the prevalence of ED1$^+$/S100$^+$ cells in the total population of ED1$^+$ cells was the highest. This suggests that interactions between macrophages and SCs are intense. Most likely, the majority of these SCs belong to the non-myelinating SCs class, while ED1$^+$ macrophages belong to the M2 phenotype, being involved, together with aforementioned non-myelinating S100$^+$ SCs, in the formation of Büngner bands, which are shown to represent the regeneration tracks for directing axons to their targets [19,36]. In contrast, after 14 days of the treatment with the vitamin B complex we have noted significant reduction in the number of ED1$^+$/S100$^+$ cells and their fraction in the total ED1$^+$

cell population. Most of the axons had a renewed myelin sheath and myelin-forming SCs were the predominant type of SCs, as we have also seen in the sham-control nerve sections. Given that the interaction between macrophages and myelin-forming SCs was negligible, obtained results indicated that 14 days of the consecutive B vitamin complex treatment triggered the complete transition to mature, myelin-forming SCs that wrapped large-caliber axons intensively labeled with GAP43, a marker of axonal outgrowth. These results suggested that, by the 14th dpo, the regeneration of injured nerve and the recovery of muscle function gets completed after the treatment with B vitamins, which we have confirmed with behavioral and electromyography testing in our previously published paper [19].

Axon regeneration proceeds at a rate of 1–3 mm/day and depends on the location along the neuron, as well as cytoskeletal materials and proteins, such as actin and tubulin. Further elongation happens through the remaining endoneurial tube, which directs axons back to their original target organs. SCs are essential at this stage of regeneration, as they form Büngner repair bands, which protect and preserve the endoneurial channel. Moreover, together with macrophages, SCs release various neurotrophic factors to stimulate nerve regrowth. After reaching the endoneurial tube, the growth cone has a higher probability of reaching the target organ and triggers the maturation process. This process involves remyelination, axon expansion, and ultimately, functional re-innervation [52]. Related to this, in this study, tight M2-macrophages-to-SCs interactions were confirmed in transversal sections of the injured nerve. Moreover, we clearly demonstrated that the IL-10 immunoreactivity was associated with M2-macrophages, but also with SCs forming the Büngner's bands. Consistent with the literature data [35], our results confirm the role of macrophages as regulators of SCs maturation after PNI.

Versatile effects of the investigated B vitamins treatment stand as an important result, since most compounds that reduce neuroinflammation safeguard the myelin-forming SCs. Importantly, our data show that following PNI, a balance in myelin-forming SCs protection, transition to non-myelin-forming SCs, and vice-versa, can be rapidly established by applying B vitamins during the early recovery period. The underlying molecular and intracellular signaling pathways pave for more thorough clarification. Overall, macrophage/SCs plasticity induced by applying adjuvant to surgery after PNI provides a basis for macrophage/SCs-centered therapeutic strategy, as an alternative repair approach. Concerning this, in the upcoming research, the exact molecular basis of macrophage-SCs interactions in response to the B vitamins, applied hereby, remain to be thoroughly examined.

4. Materials and Methods

4.1. Ethical Approval and Consent to Participate

The study was approved by the Ethics Review Committee for Animal Experimentation of the Military Medical Academy and Ministry of Agriculture and Environmental Protection Republic of Serbia, Veterinary Directorate No. 323-07-7363/2014-05/5.

4.2. Femoral Nerve Injury Rat Model

Irintchev and colleagues described the controlled transection of the peripheral nerve as a widely used model for the examination of peripheral nerve regeneration [30]. In this study, we used adult male Albino Oxford (AO) rats (15 in total), weighing between 250 and 300 g, that were randomly divided into three groups (5 per group). Animals that underwent transection of the femoral nerve motor branch with immediate reconstruction, using a technique of termino-terminal anastomosis, form the first group of so called "operated animals" (O). The second group (OT) included animals that passed the same surgical procedure but were additionally receiving vitamin B complex therapy. The "sham operated" animals (S), which also underwent the dissection of the femoral nerve motor branch but without transection, represent the third experimental group. All of the groups were additionally divided into sub-groups (four per group), based on the post-operation day that (dpo) the animals were sacrificed on (1, 3, 7, and 14 dpo). During the entire period of the study, the animals were kept under

the same environmental conditions (laboratory temperature 23 ± 2 °C, humidity between 50% and 60%, 12 h/12 h light/dark cycle with lights on at 07:00 a.m., free availability of water and food).

As anesthesia, intraperitoneal application of ketamine (50 mg/kg; Ketalar, Eczacibasi, Turkey) and xylazine (5 mg/kg; Rompun, Bayer, Turkey) was used on all animals. Following anesthesia, the animals from all investigated groups (S, O, and OT) were appropriately positioned for identification of the femoral nerve motor branch on the rat left hind paw by skin incision in the left groin and femoral region, under aseptic conditions (as previously described [19]). In all experimental groups (S, O, and OT), the motor branch was identified just before entry into the quadriceps muscle. Subsequently, animals from the O and OT groups underwent the transection of the branch, and immediate reconstruction using a 10.0 non-absorbable suture in the form of termino-terminal anastomosis, under the microscope magnification. The skin was sutured using a 4.0 absorbable suture (Peters Surgical, Paris, France). At selected time points, the animals were sacrificed by intravenous injection of a lethal dose of ketamine/xylazine. The motor branches of the femoral nerves (both reconstructed and intact contralateral) were isolated for subsequent immunofluorescence staining. All of the procedures performed in this study were based on the rules and guidelines of the EU Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes.

4.3. Protocol for Vitamin B Complex Treatment

For the investigation of vitamin B complex treatment, ampoules (2 mL) of Beviplex (Beviplex®, Galenika a.d. Belgrade, Serbia), each containing B1 (40 mg), B2 (4 mg), B3 (100 mg), B5 (10 mg), B6 (8 mg), and B12 (4 μg), were used. The given dose was 1.85 mL/kg/day. The complex of B vitamins was injected intraperitoneally immediately (15 min) after the operation and then every 24 h from the day of the operation until the day of sacrifice. Operated, but untreated animals (O) were intraperitoneally injected with the same volume of physiological solution.

4.4. Femoral Nerve Processing Procedure for Immunofluorescence Staining

In the Laboratory for Pathohistology and Cytology HistoLab, Belgrade, all of the isolated motor branches of femoral nerves were prepared for immunohistochemistry in this study. The isolated nerve samples underwent the fixation procedure in the 10% formaldehyde solution to preserve the tissue morphology and antigenicity of target molecules on the dissected nerve. Prior to the addition of melted paraffin wax, the isolated nerves underwent a series of dehydration steps at room temperature (RT): (1) 3 × 30 min in 70% ethanol; (2) 3 × 30 min in 90% ethanol; (3) 3 × 30 min in 100% ethanol; and (4) 3 × 30 min in xylene. Following dehydration, the tissue was immerged into the melted paraffin wax at 58 °C. Microtome sectioning of the paraffin-embedded tissue was next done at a thickness of 5 μm. Sections were then incubated at 56 °C in water bath, mounted onto histological slides, pre-coated with gelatin for better tissue adhesion, and dried overnight at RT.

4.5. Procedure of Immunofluorescence Staining and Digital Image Processing

Immunofluorescence (IF) staining was used for protein localization on nerves slides. For indirect immunofluorescence staining, the fluorescent-dye conjugated secondary antibody, which binds to the unlabeled primary antibody, was used. The all IF staining procedures were done at RT. Only incubation with primary antibody was performed at a temperature of 4 °C. All of the solutions were prepared in 0.01 M Phosphate-Buffered Saline (PBS), pH 7.4, which was also used for washing after certain steps. Double IF staining proceeded according to the following steps:

Deparaffinization and rehydration: Microscope slides with paraffin-embedded sections were deparaffinized and rinsed in xylene 1, xylene 2, absolute alcohol, 95% alcohol, 70% alcohol, and distilled water, for 5 min in each solution. Antigen retrieval: Antigenic epitope unmasking was done by boiling microscope slides in 0.01 M sodium citrate buffer, pH 6, for 8 min at 99 °C–100 °C, followed by cooling at RT for 30 min and 3 × 5 min PBS washing. Blocking solution: after the washing step, microscope slides were incubated for 60 min in 5% blocking serum (originating from the same species as the

secondary antibody) to prevent nonspecific binding of the secondary antibody. To enable membrane permeabilization, 0.5% Triton X-100 detergent was added to the blocking serum. Primary antibody, diluted in PBS, was applied onto slides and incubated overnight at 4 °C temperature. Next day, slides were washed out 3 × 5 min in PBS. Secondary antibody, diluted in PBS, was applied onto slides, where it specifically binds to the present primary antibody. Slides were next washed for 3 × 5 min in PBS. In the case of double or triple IF staining, the steps starting from the incubation in the blocking serum were repeated for the following markers. The primary and secondary antibodies used for IF labeling are indicated in the Table 1.

Table 1. List of primary and secondary antibodies used for immunofluorescence labeling.

Antibodies	Dilution	Company
Mouse monoclonal anti-CD68 (Clone ED1)	1:100	Abcam, Cambridge, MA, USA
Goat monoclonal anti-TNF-α	1:100	Santa Cruz Biotechnology, CA, USA
Goat monoclonal anti-IL-10	1:100	Santa Cruz Biotechnology, CA, USA
Mouse monoclonal anti-S100	1:200	Chemicon International, CA, USA
Rabbit monoclonal anti-S100	1:200	Bio-Rad Laboratories, CA, USA
Rabbit monoclonal anti-GAP43	1:200	Millipore, Darmstadt, Germany
Donkey anti-goat IgG (Alexa Fluor 555)	1:200	Invitrogen, Carlsbad, CA, USA
Donkey anti-rabbit IgG (Alexa Fluor 488)	1:200	Invitrogen, Carlsbad, CA, USA
Donkey anti-rabbit IgG (Alexa Flour 555)	1:200	Invitrogen, Carlsbad, CA, USA
Donkey anti-mouse IgG (Alexa Fluor 488)	1:200	Invitrogen, Carlsbad, CA, USA
Donkey anti-mouse IgG (Alexa Fluor 555)	1:200	Invitrogen, Carlsbad, CA, USA

After incubation with the last secondary antibody, slides were incubated in 4′,6-diamidin-2-fenilindolom (DAPI; Invitrogen, Grand Island, NY, USA) for 10 min to counterstain the nuclei and then washed 6 × 5 min in PBS and mounted with Mowiol (Calbiochem, Millipore, Germany). After drying overnight, slides were ready for viewing under the microscope. As a staining control, microscope slides that underwent the same IF procedure, but without the primary antibody application, were used.

The Carl Zeiss Axiovert fluorescent microscope, equipped with the Axiocam monochromatic camera (Axio Observer Microscope Z1, ZEISS, Gottingen, Germany), at the magnifications of 20×, 40×, and 63× was used for image processing of the prepared motor branch of femoral nerve sections and saved in .tiff format. To capture images at 63× magnification ApoTome software was used. Co-localization on the obtained fluorescent images was done using AxioVision Rel. 4.6 program, which represents a standard part of the Zeiss Axiovert microscope equipment, and then assembled and labeled in Photoshop CS6 (Adobe Systems). The quantification of single- and double-stained cells from experimental groups (S, O, OT) was performed for each time point (1, 3, 7, 14 dpo), and obtained from three independent experiments. High resolution digital images (600 pixels/inch) captured at 40× magnification (1388 μm × 1040 μm) (three images/group/independent experiment) were used for cells counting. The total number of single- or double-positive cells was counted manually using Adobe Photoshop Creative Cloud (Version 14.0). Additionally, the percentage of double-positive cells in some investigated cells populations was calculated and presented.

4.6. Statistical Analysis

Statistical comparison between two experimental groups was performed using a two-sided Student's t test and a value of $p < 0.05$ or less was considered significant. Values were shown as mean values with standard error (SEM).

5. Conclusions

In this study we report for the first time that the treatment with the complex of B vitamins (B1, B2, B3, B5, B6, and B12) could effectively promote PNI-induced transition of the non-myelinating

to myelin-forming-SCs phenotype and suppress neurodegeneration by reducing the expression of proinflammatory and up-regulating the expression of anti-inflammatory cytokines, produced by macrophages and SCs. This consequently changes interactions between these cells, thereby contributing to the regeneration of the injured nerve. In conclusion, the ability of B vitamins to modulate macrophages-SCs interaction reveals their potential as an additional tool in peripheral nerve regeneration therapies in humans, which requires extensive further research and confirmation in clinical trials.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors. It is commercial product Beviplex (Beviplex®, Galenika a.d. Belgrade, Serbia) and it is written in the Section 4.3. Protocol for Vitamin B Complex Treatment.